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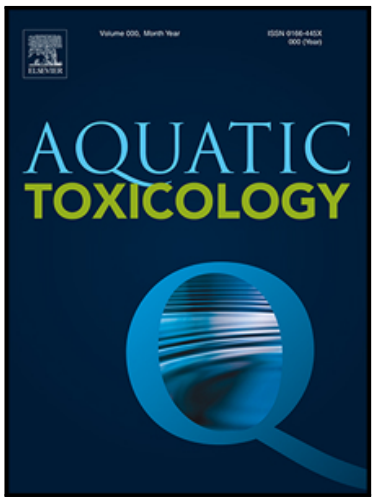

Toxicity investigation of CeO_2 nanoparticles coated with glucose and exopolysaccharides levan and pullulan on the bacterium Vibrio fischeri and aquatic organisms Daphnia magna and Danio rerio

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Highlights

- Levan and pullulan coated CeO_2 nanoparticles decrease toxicity to *V. fischeri*
- Coating increased nanoparticles' bioaccumulation in *Daphnia magna*
- All coated CeO_2 decrease the toxicity of uncoated ones at 100 mg L^{-1} in *D. magna*
- Glucose-coated CeO_2 increased the CO_2 production in *Daphnia magna* at 200 mg L^{-1}
- Uncoated and coated CeO_2 did not affect early zebrafish development and mortality

Toxicity investigation of CeO_2 nanoparticles coated with glucose and exopolysaccharides levan and pullulan on the bacterium *Vibrio fischeri* and aquatic organisms *Daphnia magna* and *Danio rerio*

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Abstract

Cerium oxide nanoparticles ($nCeO_2$) have widespread applications, but they can be hazardous to the environment. Some reports indicate the toxic effect of $nCeO_2$ on tested animals, but literature data are mainly contradictory. Coating of $nCeO_2$ can improve their suspension stability and change their interaction with the environment, which can consequently decrease their toxic effects. Herein, the exopolysaccharides levan and pullulan, due to their high water solubility, biocompatibility, and ability to form film, were used to coat $nCeO_2$. Additionally, the monosaccharide glucose was used, since it is a common material for nanoparticle coating. This is the first study investigating the impact of carbohydrate-coated $nCeO_2$ in comparison to uncoated $nCeO_2$ using different model organisms. The aim of this study was to test the acute toxicity of

carbohydrate-coated $nCeO_2$ on the bacterium *Vibrio fischeri* NRRL B-11177, the crustacean *Daphnia magna*, and zebrafish *Danio rerio*. The second aim was to investigate the effects of $nCeO_2$ on respiration in *Daphnia magna* which was performed for the first time. Finally, it was important to see the relation between Ce bioaccumulation in *Daphnia magna* and *Danio rerio* and other investigated parameters. Our results revealed that the coating decreased the toxicity of $nCeO_2$ on *Vibrio fischeri*. The coating of $nCeO_2$ did not affect the nanoparticles' accumulation/adsorption or mortality in *Daphnia magna* or *Danio rerio*. Monitoring of respiration in *Daphnia magna* revealed changes in CO_2 production after exposure to coated $nCeO_2$, while the crustacean's O_2 consumption was not affected by any of the coated $nCeO_2$. In summary, this study revealed that, at 200 mg L^{-1}, uncoated and carbohydrate-coated $nCeO_2$ are not toxic for the tested organisms, however, the CO_2 production in *Daphnia magna* is different when they are treated with coated and uncoated $nCeO_2$. The highest production was in glucose and levan-coated $nCeO_2$ according to their highest suspension stability.

Daphnia magna (*D. magna*), *Danio rerio* (*D. rerio*), *Vibrio fischeri* (*V. fischeri*)

Keywords: *Vibrio fischeri*; CeO_2; coating; *Daphnia magna*; *Danio rerio*; nanoparticles.

1. INTRODUCTION

Given the rapid growth of nanotechnology, increased application of nanoparticles in medicine (Xu et al., 2013), electronics (Turković and Orel, 1997), cosmetics (Masui et al., 2000), fuel (Park et al., 2008), paints (Fedel et al., 2012), and agriculture (Chen et al., 2014) can lead to enlarged environmental risks due to their accumulation in the environment. The potential ecotoxicity of nanoparticles has not been sufficiently investigated, and therefore, examination of their environmental impacts has become important in recent years.

Cerium oxide nanoparticles ($nCeO_2$), with the production of around 10,000 metric tons per year (Majumdar et al., 2014), have been widely used due to the redox properties that enable them an easy transition between Ce^{3+} and Ce^{4+} oxidation states and, consequently, to act as an antioxidant (Lee et al., 2013) or a reactive oxygen species producer (Auffan et al., 2009). The great tendency of $nCeO_2$ to agglomerate in water is a big challenge in their investigation which has led many researchers to coat the nanoparticle surfaces with different polymers (such as dextran, heparin, chitosan, polyethylene-glycol, and citric acid) (Kim and Chung, 2016; Lord et al., 2013; Qia et al.; Siriwardane, 2012; Yazici et al., 2015; Zhai et al., 2013), to improve their aqueous stability. The type and amount of polymer coating over the nanoparticles, as well as the synthesis procedure, are of great importance in the polymer selection process because they affect suspension stability and their final size. The focus of this research is $nCeO_2$ coated with glucose, levan, and pullulan, which we have structurally characterized in detail and showed that those carbohydrates increase suspension stability of $nCeO_2$ (Milenković et al., 2019; Milenković et al., 2018). Initially, glucose was selected as a common coating material (Karakoti et al., 2007; Liu et al., 2006; Milenković et al., 2018), while levan (fructan with β-(2→6) linkages in core chain and β-(2→1) linked branching points) and pullulan (glucan composed of α-D-(1→6) linked repeating maltotriose units) were used as non-toxic, biocompatible and water-soluble microbial exopolysaccharides (Jakovljevic et al., 2001; Kojić et al., 2016; Rekha and Sharma, 2007).

Among aquatic organisms, the crustacean *D. magna* and zebrafish *D. rerio* are commonly used and are internationally accepted model organisms for indication the potential hazard of chemicals as well as for the evaluation of the toxicity of water and wastewater (Sharma, 2009). Also, the *Vibrio fischeri* luminescence inhibition bioassay has been widely applied for the monitoring of toxicity because of multiple advantages such as high sensitivity, shorter test

duration, applicability to all types of matrices, and good correlativity with other tests (Abbas et al., 2018; Parvez et al., 2006). However, literature data show contradictory results about the effect of $nCeO_2$ on these organisms.

Previous reports showed toxic effects of $nCeO_2$ on *D. magna* at concentrations between 5 and 64 mg L^{-1} and 5-90 nm sizes in acute 96 h test (Alam et al., 2016), at 8.8-20.0 mg L^{-1} and 14-29 nm sizes in chronic 21 days test (Hoecke et al., 2009), at 0.012 mg mL^{-1} and 6,5 nm size in acute 48 h test (García et al., 2011) and genotoxic effects at 1 mg L^{-1} and 15-30 nm sizes in acute 96 h test (Lee et al., 2009). On the other hand, no acute toxicity was observed at 0-10 μg mL^{-1} and 25 nm size in acute 96 h test (Gaiser et al., 2009), at 100-200 mg L^{-1} in acute 48 h test (Jemec et al., 2012), and at 200-5000 mL^{-1} and 14-29 nm sizes in acute 48 h test (Hoecke et al., 2009). Various effects on the zebrafish, *D. rerio*, were reported in several papers. Lin et al. (Lin et al., 2014) demonstrated the dependence of $nCeO_2$ toxicity (at 25 mg L^{-1} and 7-9,5 nm sizes) in zebrafish on nanoparticles' shape after pulse-exposure during 6 h. Özel et al. (Özel et al., 2013) showed alteration of the neurological system after long-term exposure to $nCeO_2$ (at 20 and 50 ppm and 20-40 nm sizes). No acute toxicity on *D. rerio* was demonstrated at concentrations: up to 50 mg L^{-1} of 2.8 nm $nCeO_2$ after acute five days' treatment (Wehmas et al., 2015); up to 100 mg L^{-1} and 10-15 nm sizes in acute 4 days test (Jemec et al., 2015); over 200 mg L^{-1} and 14-29 nm sizes in acute 72 h test (Hoecke et al., 2009), and after injection of 0.3 pg or 0.75 pg of $nCeO_2$ hydrodynamic size 53.34 nm directly into the yolk (Arnold et al., 2013). A few reports investigated the impact of $nCeO_2$ on *V. fischeri* in acute tests: $nCeO_2$ (2-4 nm) in citrate did not affect the enzymatic activity of *V. fischeri* at 0.63-20 mmol L^{-1} (Shekunova et al., 2012), but showed low toxicity (IC_{50} 21.76 mg L^{-1}) at 320 mg L^{-1} and size 12 nm with hexamethylenetetramine (Recillas et al., 2010) and extreme toxicity at 0.064 mg mL^{-1} and size 6.5 nm (García et al., 2011). Inconsistency in the toxicity of $nCeO_2$ could be caused by differences in nanoparticles' diameters, shapes, surface charges, and other physicochemical characteristics, as a consequence of various starting materials and methods used for their synthesis.

Ce concentration in the environment is in the range from 2 to 150 mg kg^{-1} in soil (Emsley, 2011) and around 80 μg L^{-1} in wastewater (Collin et al., 2014), but taking into account an increase of global production of $nCeO_2$, concentration of Ce in the environment is rapidly

increasing. Given that the effects of glucose-, levan- and pullulan-coated $nCeO_2$ (G-, L-, and P-CeO_2, respectively) were tested on plants (Milenković et al., 2019; Milenković et al., 2021), the current research examined the impact of these nanoparticles on bacteria and aquatic organisms, so contributing to environmental toxicity data. In this study, the effects of uncoated ($nCeO_2$) and glucose-, levan-, and pullulan-coated (G-CeO_2, L-CeO_2, P-CeO_2, respectively) nanoparticles were tested on bacteria (*V. fischeri*) and two aquatic organisms (*D. magna* and *D. rerio*). The initial aim of this research was to assess the potential environmental impact of carbohydrate-coated $nCeO_2$ compared to the uncoated nanoparticles. In this regard, we tested the acute toxicity of the various $nCeO_2$ on the crustacean, *D. magna*, and the zebrafish, *D. rerio*, by monitoring mortality after 48 h and 72 h, respectively, as well as on *V. fischeri* by measuring bioluminescence inhibition. Since data on the bioaccumulation and effects of coated $nCeO_2$ on edible organisms are scarce, we intended to find out if coating with natural carbohydrates changes the Ce concentration in chosen organisms, using inductively coupled plasma optical emission spectrometry (ICP-OES). Finally, we wanted to examine if the coating of $nCeO_2$ creates changes in the respiration of *D. magna*, in comparison with the uncoated nanoparticles. This research, for the first time, demonstrated the effects of carbohydrate coated CeO_2 on the bacterium *V. fischeri* and aqueous organisms *D. magna* and *D. rerio*, as well as the impact of all used $nCeO_2$ on *D. magna* respiration.

2. MATERIALS AND METHODS

2.1. Materials

Chemical reagents used in this work were: $Ce(NO_3)_3 \cdot 6H_2O$ (Acros Organics, USA), NaOH (Carlo Erba Reagents, France), glucose (Sigma-Aldrich, USA), NaCl (Merck, Germany), sea salt (Aqua Medic, Germany), $CaCl_2 \cdot H_2O$ (Kemika, Croatia), $NaHCO_3$ (Kemika, Croatia), Na_2SeO_3 (Riedel-de Haën, Germany), HCl (Zorka, Serbia), $K_2Cr_2O_7$ (Alkaloid, Macedonia), HNO_3 (J. T. Baker, USA), H_2O_2 (AppliChem, Germany), methylene blue (Sigma-Aldrich, USA), and Tricaine-S (Western Chemical, USA). All the chemicals were of analytical grade.

2.2. Polysaccharide production

Levan was obtained by fermentation of *Bacillus licheniformis* NS032 (Genbank accession number JF826527) on a modified sucrose medium with ammonium chloride as a nitrogen source

(pH 7), outlined in Kekez *et al*. (2015). After cultivation, polysaccharide was isolated by ethanol precipitation and purified by dialysis and lyophilization, and its structure was characterized as described in detail previously Kekez *et al*. (2015).

Pullulan was prepared using *Aureobasidium pullulans* CH-1 (Collection of Microorganisms at the Center for Chemistry - Institute of Chemistry, Technology, and Metallurgy, University of Belgrade). The polysaccharide was isolated by ethanol precipitation and purified by gel filtration on a Sephadex G-200 column, as described previously in Jakovljević *et al*. (2001) and Radulović *et al*. (2008).

2.3. Synthesis of uncoated $nCeO_2$ and $nCeO_2$ coated with carbohydrates

Synthesis of $nCeO_2$ was performed using the self-propagating room temperature method according to the procedure previously given (Milenković et al., 2018). This method was chosen for the synthesis of uncoated $nCeO_2$ because no harmful effects have been reported for nanoparticles synthesized by room temperature methods (Karakoti et al., 2012). Briefly, starting materials $Ce(NO_3)_3{\cdot}6H_2O$ and NaOH were hand mixed in a mortar with a pestle for about 5-10 min. The obtained product was rinsed three times with deionized water and twice with ethanol in a centrifuge (Centurion 1020D) for 10 min at 4200 rpm. The powders were dried overnight at 70 °C. Next, $nCeO_2$ were coated with three different carbohydrates (glucose, levan, or pullulan) to obtain G-, L-, and P-CeO_2, respectively. The mass ratio of carbohydrates to $nCeO_2$ was 1:7, or 2.523 g of carbohydrates and 0.360 g of $nCeO_2$ in Teflon™-lined stainless steel autoclave. All nanoparticle suspensions were prepared in 50 mL of deionized water and were ultrasonicated in an ultrasound bath Ultrasons HD (J. P. Selecta s.a., Spain) for 60 min at 120 W before each treatment to prevent aggregation unless stated otherwise. The characterization of synthesized nanoparticles was performed previously using different characterization methods, such as transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared spectroscopy (FT-IR), and dynamic light scattering (DLS). XPS method verified the formation of carbohydrate layer on the nanoparticle surface due to the hydrogen bonding between oxygen atoms of CeO_2 structure and the organic coating which cause an increase in the contributions of at. % C showing the increase of carbon atomic %, as well as partial reduction of Ce^{4+} after all coatings. TEM analysis showed the round shape and differences in the size of uncoated and coated $nCeO_2$. Measuring the hydrodynamic size (385,

235, 216, and 314 nm for CeO_2, G-, L-, and P-CeO_2, respectively), zeta potential values (32.2, 31.3, 20.8, and 19.8 nm for CeO_2, G-, L-, and P-CeO_2, respectively), and sizes of dry $nCeO_2$ (4-5 nm for uncoated and 8-13 nm for coated $nCeO_2$) also confirmed the coating of their surface. Turbidity measurements confirmed improved suspension stability of $nCeO_2$ was after coating. These results was reported in Milenković et al. (Milenković et al., 2019; Milenković et al., 2018).

2.4. Animal models used in the study

The freshwater crustacean, *D. magna* (MicroBioTests Inc.), was used as a model organism to indicate the impact of $nCeO_2$ on the environment. Animals were prepared for the experiments according to the supplier's instructions (Klüttgen et al., 1994). All tests were performed in Aachener Daphnien Medium (ADaM) (Klüttgen et al., 1994) containing 0,333 g L^{-1} sea salt, 117,6 g L^{-1} $CaCl_2{\cdot}H_2O$, 25,2 g L^{-1} $NaHCO_3$, and 21,6 mg L^{-1} Na_2SeO_3. Neonates were held at a constant temperature (20 ± 1 ºC) and light conditions (16/8 h (light/dark) photoperiod). Experiments were performed according to the OECD procedure (OECD 202, 2004) described in Lončarević et al. (Lončarević et al., 2019). Details of experimental conditions for the animals' acute exposure are outlined in Sections 2.5., 2.8., and 2.9.

The bacterium, *V. fischeri* NRRL B-11177 (Macherey-Nagel GmbH & Co. KG, Duren, Germany), was also used to investigate the effect of $nCeO_2$ by measuring the inhibition of bacterial bioluminescence. The experiments were performed according to the ISO 11348 standard method (ISO, 2007) and summarized in Section 2.7.

Zebrafish wild-type adults (*D. rerio*, Tübingen strain) were housed and maintained in a zebrafish facility (Institute of Molecular Genetics and Genetic Engineering, IMGGE). All experiments involving zebrafish were performed in compliance with the ethical guidelines in the Guide for Care and Use of Laboratory Animals of the IMGGE, University of Belgrade. Zebrafish were kept at 28 ± 1 °C on a 14/10 h (light/dark) photoperiod. Healthy adult fish were maintained in aged filtered tap water, while zebrafish embryos were maintained in egg water with 0.0002% methylene blue solution for embryo disinfection (Sigma-Aldrich, USA). Zebrafish were spawned once per week. Spawn was discarded if more than 10% of eggs were unfertilized. Male and female fish were kept in separate tanks. One day before harvesting the eggs, three male and two

female fish were put into a breeding tank. Spawning was triggered by light and was completed within 30 minutes. Afterward, the eggs were rinsed several times with egg water. Collected viable embryos were staged as documented previously (Kimmel et al., 1995) under a binocular stereomicroscope (PXS-VI, Optica, China) and were further used for toxicity testing. Details of experimental conditions for the animals' acute exposure are outlined in Sections 2.6. and 2.8.

2.5. Acute toxicity screening with *Daphnia magna*

The acute toxicity tests with *D. magna* were previously reported only for uncoated $nCeO_2$ and no harmful effects were not noticed at 100-200 mg L^{-1} (Jemec et al., 2012) and 200-5000 mg mL^{-1} (Hoecke et al., 2009). In our research, the concentrations of uncoated and coated $nCeO_2$ were in the range 50-400 mg L^{-1}, which is significantly higher than expected in wastewater (80 μg L^{-1}). *D. magna* were exposed to nanoparticles according to the previously reported procedure (Kekez et al., 2015; Lončarević et al., 2019). Before experiments, the beakers used in all experiments were cleaned with 10% HNO_3 and thoroughly rinsed with MilliQ water (18Ω, 25 °C). All test suspensions were prepared shortly before experiments in AdaM test media (previously aerated, 24 h). The assay was performed by treating one-day-old *D. magna* with uncoated ($nCeO_2$) and three types of carbohydrate-coated nanoparticles (G-CeO_2, L-CeO_2, and P-CeO_2) at 50, 100, 200, and 400 mg L^{-1} for 48 h. Per five *D. magna* neonates were used in each test treatment and control in triplicate i.e. 75 neonates per concentration. Neonates were not fed during the test (48 h) and were held at a constant temperature (20 ± 1 ºC) and light conditions (16/8 h (light/dark) photoperiod). The endpoint was the mortality of neonates, and the number of dead organisms was determined after 24 h and 48 h. Results were expressed as percent mortality after 48 h. Tests were considered valid if the mortality rate in the control did not exceed 10%.

2.6. Acute toxicity screening with zebrafish *D. rerio*

Hoecke et al. (Hoecke et al., 2009) have showed that 200 mg L^{-1} of uncoated $nCeO_2$ were not toxic to the zebrafish in acute toxicity tests. For this reason, we used the same concentration of coated $nCeO_2$ to investigate the toxicity of coated nanoparticles to early zebrafish development.

Before the treatment, uncoated ($nCeO_2$) and three types of carbohydrate-coated $nCeO_2$ (G-CeO_2, L-CeO_2, and P-CeO_2) were dissolved in egg water and dispersed by a Soniprep 150 (MSE,

UK) sonicator for 15 min with an amplitude of 5 µm. Zebrafish embryos were treated with 200 mg L^{-1} of uncoated or coated $nCeO_2$ at 6 h post-fertilization (hpf) in 24-well plates, N = 12 embryos per well, in a volume of 1 mL. The influence of uncoated and carbohydrate-coated $nCeO_2$ exposure on the development of zebrafish embryos was investigated during the first 72 hpf. In all experiments, untreated embryos were used as controls. A total of 48 embryos were used per treatment. All treatments were repeated three times (144 embryos in total) using embryos obtained from independent spawns.

The embryos were microscopically observed at 24, 48, and 72 hpf under a light microscope (CKX41, Olympus, Germany) at 40× magnification. At 72 hpf, before the imaging, zebrafish embryos were anesthetized in 0.003% Tricaine-S solution. Zebrafish embryos were considered dead in cases of coagulation of embryos or lack of heartbeat. Embryo hatching rates were observed at 48 and 72 hpf. Mortality and morphological abnormalities such as pericardial edema, yolk deformation/edema, non-detachment of the tail, scoliosis, tail circulation, somite formation, eye and body pigmentation, and growth retardation were recorded if present.

2.7. *V. fischeri* toxicity tests

The inhibitory effect of uncoated and carbohydrate-coated $nCeO_2$ on the light emission of *V. fischeri* was measured according to the standard method ISO 11348 (ISO, 2007) using commercial lyophilized bacteria (*V. fischeri* NRRL B-11177). Before experiments, freeze-dried bacteria were suspended in the reconstitution solution (Macherey-Nagel GmbH & Co. KG, Duren, Germany) (Lončarević et al., 2019). Bacteria were incubated at 15 °C with 50 mL of bacterial medium for freeze-dried luminous bacteria (Macherey-Nagel GmbH & Co. KG, Duren, Germany) to obtain a bacterial suspension. Then, 500 µL of bacterial suspension was mixed with suspensions of $nCeO_2$, G-CeO_2, L-CeO_2, or P-CeO_2 prepared in 2% NaCl and incubated at 15 °C for 15 min. Serial dilutions were prepared by diluting each starting concentration by 50% (the range of concentrations was 100-6.25 mg L^{-1}). The concentrations of different nanoparticles for the *V. fischeri* toxicity test were chosen according to the other treatments and available literature data (García et al., 2011; Hoecke et al., 2009). Prior to bacteria addition, the suspensions were sonicated in an ultrasound bath for 30 min, the concentration of oxygen in the suspensions was > 3 mg L^{-1}, and pH was adjusted to between 6 and 8 with 1M HCl or 1M NaOH solutions. The bioluminescence of the sonicated suspensions was measured after 15 min. The measurements

were performed in duplicate, and $K_2Cr_2O_7$ (105.8 mg L^{-1}) was used as a reference substance. Results were expressed as effective concentrations causing 10% (EC_{10}) and 20% (EC_{20}) inhibition of bioluminescence.

2.8. Acute exposure and sample preparation for ICP-OES analysis

To determine the Ce concentration in *D. magna* after acute exposure, fifty neonates were treated with 200 mg L^{-1} uncoated ($nCeO_2$) or coated (G-CeO_2, L-CeO_2, or P-CeO_2) nanoparticles for 48 h. After exposure, neonates from each treatment were washed with MilliQ water three times, filtered, and prepared for ICP-OES analysis according to the procedure described below. The wet mass of animals was measured on an analytical balance. Experiments were performed in triplicate.

For the same purpose, 48 individual *D. rerio* were treated with 200 mg L^{-1} uncoated ($nCeO_2$) or coated (G-CeO_2, L-CeO_2, or P-CeO_2) nanoparticles for 72 h. Experiments were performed in triplicate. After the treatment, the hatched organisms were washed with egg water three times and prepared for ICP-OES analysis.

The concentration of nanoparticles used for the exposure (200 mg L^{-1}) was chosen as a concentration which is in the lower range of reported concentrations in the literature for estimation of acute toxicity in tested animals (10 µg L^{-1} to 5,000 mg L^{-1} for *D. rerio* and 20-5,000 mg L^{-1} for *D. magna*) (Hoecke et al., 2009; Lee et al., 2009). The chosen concentration (200 mg L^{-1}) was in accordance with increasing Ce concentration in the environment (2 to 150 mg kg^{-1} in soil (Emsley, 2011) and 80 µg L^{-1} in wastewater (Collin et al., 2014)).

After overnight lyophilization, *D. magna* and *D. rerio* were digested with 96-98% HNO_3 and 30% H_2O_2 (1:4) at 120 ºC in the Tecator digestion system until the solutions were transparent (Gaiser et al., 2009). After cooling to room temperature, the digests were filtered using Whatman filter paper No 4, and volumes were adjusted with MilliQ water to 25 mL. Measurements were performed in triplicate. Total Ce concentrations in the diluted digests were analyzed using ICP/OES Perkin Elmer Optima 4300 DV. Results were expressed as ng of Ce per dry weight for *D. rerio* and as mg of Ce per wet weight for *D. magna*.

2.9. Respiration monitoring of O_2 and CO_2

Respiration monitoring was performed according to the procedure described in Lončarević et al. (Lončarević et al., 2019). *D. magna* neonates were placed in Micro-Oxymax light-proof 500 mL bottles (twenty juveniles per treatment) containing 100 mL ADaM (control) and ADaM with 200 mg L^{-1} nanoparticles ($nCeO_2$, G-CeO_2, L-CeO_2, and P-CeO_2). For the reasons described above, the same concentration (200 mg L^{-1}) was chosen for respiration tests. Juveniles were exposed to nanoparticles at 25 ± 2 °C and 16/8 h light/dark photoperiod for 42 h. Oxygen (O_2) consumption and carbon dioxide (CO_2) production were continuously recorded using the closed-circuit 12-channel Micro-Oxymax respirometer (Columbus Instruments, USA) supplied with O_2 paramagnetic sensor, CO_2 infrared sensor, and PC. The O_2 consumption and CO_2 production in the headspace of the test bottles were measured periodically (at 2.5 h intervals) by pumping air from the headspace through the sensor. The amount of consumed/produced gases (mL) was determined using MicroOxymax software. Neonates were not fed during the experiment.

2.10. Statistical analysis

Statistical significances of the effects of uncoated and carbohydrate-coated $nCeO_2$ acute toxicity in *D. magna* were evaluated using the non-parametric Scheirer Ray Hare test. Input values were transformed by ranks. This test was following by post-hoc Duncan's test. The non-parametric Kruskal-Wallis and Mann-Whitney tests were performed (n=3) for testing of statistical significances of the effects of uncoated and carbohydrate-coated $nCeO_2$ acute toxicity in *D. rerio* and Ce concentration in *D. magna* and *D. rerio*. Results were considered to be statistically significant at $p < 0.05$. Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analyses. Data are presented as the mean ± standard error of the mean (SEM).

3. RESULTS AND DISCUSSION

In this study, we investigated the effect of coating on the bioaccumulation/adsorption of nanoparticles by the *D. magna* and *D. rerio* by measuring the Ce concentration in aqueous organisms after exposure to different $nCeO_2$. Also, we tested the acute toxicity of uncoated and carbohydrate-coated $nCeO_2$ on the crustacean *D. magna* and the zebrafish *D. rerio* by monitoring the mortality and on *V. fischeri* by measuring the bioluminescence inhibition. Finally, we

examined the effect of $nCeO_2$ coating on *D. magna* respiration and compared it with *D. magna* respiration in the presence of uncoated $nCeO_2$. The size 5 nm of uncoated $nCeO_2$ was chosen as the smallest of those we have synthesized for the treatments in this research due to their easier entrance to tested organisms as well as the greater ability of transformation between Ce^{4+} and Ce^{3+} compared to bigger ones (Karakoti et al., 2019).

3.1. Nanoparticle accumulation/adsorption in *D. magna* and *D. rerio*

Cerium concentrations in *D. magna* (**Figure 1**) after acute exposure to coated $nCeO_2$ (2489.65 mg kg^{-1}, 2344.65 mg kg^{-1}, and 3429.89 mg kg^{-1} for G-, L-, and P-CeO_2, respectively) and uncoated $nCeO_2$ (1577.01 mg kg^{-1}) were significantly different compared to control (< 0.05) in *D. magna*. Also, L- and P-CeO_2 significantly increased nanoparticles' concentration compared to uncoated ones, which indicates that coating of $nCeO_2$ with carbohydrates improve their accumulation/adsorption. In *D. magna* cerium accumulation was also reported earlier at concentrations 0-10 μg/ml and 25 nm size (Gaiser et al., 2009; Gaiser et al., 2012) and at 500-5000 μg/L, size 10.2 nm (Johnston et al., 2010). The higher accumulation/adsorption of coated nanoparticles than uncoated ones was due to their smaller hydrodynamic size shown in Milenković et al., 2018. On the other side, the size of uncoated $nCeO_2$ in the powder state is lower than the sizes of coated ones, which was shown in Milenković et al., 2019. It can be concluded that coating $nCeO_2$ with used carbohydrates enhanced the entrance of nanoparticles to *D. magna* as a consequence of their size and higher suspension stability shown in Milenković et al., 2018. This effect was the most pronounced after the treatment with P-CeO_2 which may be due to the lowest zeta potential values of these nanoparticles (Milenković et al., 2018).

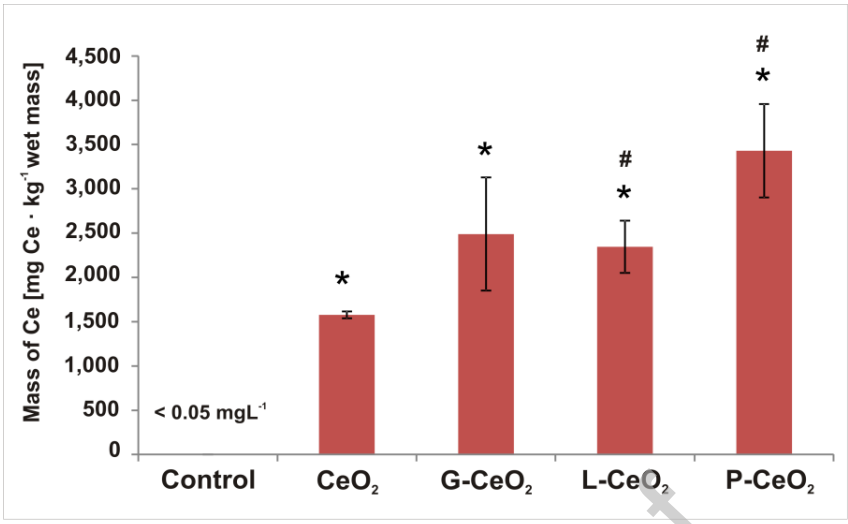

Figure 1. Cerium concentration (mean ± SEM) in *D. magna* after exposure to uncoated ($nCeO_2$) and coated $nCeO_2$ (G-CeO_2, L-CeO_2, and P-CeO_2) at 200 mg L^{-1}. The detection limit was 0.05 mg L^{-1}. The significant difference between mean values in the treatments: control vs. nanoparticles treatments (*) and uncoated vs. coated nanoparticles (#) ($p<0.05$).

Cerium accumulation/adsorption in *D. rerio* after exposure (72 h) to $nCeO_2$ is shown in **Figure 2**. Similar to results in *D. magna*, the accumulation/adsorption of coated nanoparticles in *D. rerio* (449.49 mg kg^{-1}, 356.73 mg kg^{-1}, and 175.89 mg kg^{-1} for G-, L-, and P-CeO_2, respectively) did not significantly differ from that of uncoated nanoparticles (267.09 mg kg^{-1}). But, comparing all $nCeO_2$ treatments with control it was obtained a significant increase. Although turbidity data (Milenković et al., 2018) showed the greater stability of nanoparticles' aqueous suspensions after coating, Ce concentrations in the tested organisms did not significantly differ between the exposure to coated and uncoated $nCeO_2$. Although it was expected that coating will significantly improve the entrance of nanoparticles into the tested organisms, this was not the case.

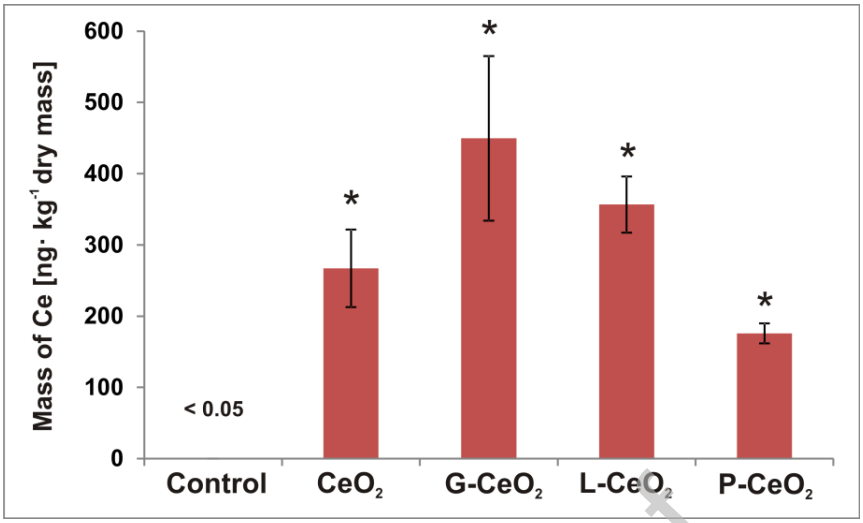

Figure 2. Cerium concentration (mean ± SEM) in *D. rerio* after exposure to uncoated ($nCeO_2$) and coated $nCeO_2$ (G-CeO_2, L-CeO_2, and P-CeO_2) at 200 mg L^{-1}. The detection limit was 0.05 mg L^{-1}.

3.2. Ecotoxicity tests

3.2.1. Effect of uncoated and carbohydrate-coated $nCeO_2$ on acute toxicity in *D. magna*

To determine if the coatings change the acute effects of $nCeO_2$, *D. magna* neonates were exposed to uncoated and coated $nCeO_2$ (G-CeO_2, L-CeO_2, and P-CeO_2) using concentrations of 50, 100, 200, and 400 mg L^{-1} over 48 h. The results obtained are shown in **Table 1**. Applied non-parametric Scheirer Ray Hare Test showed that the concentration of nanoparticles was a significant factor ($H=11.15$, $p=0.011$), while the nanoparticles treatment has a tendency to be significant ($H=9.47$, $p=0.05025$), and there is no interaction between these two factors. Thus, nanoparticles' concentration had an effect on mortality percent, but a significant difference between coated and uncoated $nCeO_2$ was not proved, except at 100 mg L^{-1}. Compared to CeO_2 at 100 mg L^{-1}, G-CeO_2, L-CeO_2, and P-CeO_2 showed a significant decrease of acute toxicity to *D. magna*. A similar trend was shown at 50 mg L^{-1} but was not statistically significant. Also, after exposing animals to concentrations of 200 and 400 mg L^{-1}, coated nanoparticles did not show statistically significant differences compared to uncoated ones, which suggests that coating with glucose, levan, and pullulan did not stimulate toxicity of nanoparticles. Obviously, the higher suspension stability of coated nanoparticles (Milenković et al., 2018) impacts their

accumulation/adsorption or, consequently, the mortality of *D. magna* (**Figure 1**). These results suggest that coating of $nCeO_2$ can decrease their toxicity to *D. magna* at lower concentrations.

Table 1. Mortality (mean % ± SEM) of *D. magna* after the 48 h-exposure to uncoated ($nCeO_2$) and coated $nCeO_2$ (G-CeO_2, L-CeO_2, and P-CeO_2) at four different concentrations.

	Mortality (%)				
Concentration (mg L^{-1})	Control	CeO_2	G-CeO_2	L-CeO_2	P-CeO_2
50	0.00 ± 0.00	6.67 ± 6.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
100	0.00 ± 0.00 [a]	13.33 ± 6.67 [b]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
200	0.00 ± 0.00	13.33 ± 6.67	13.33 ± 6.67	6.67 ± 6.67	6.67 ± 6.67
400	0.00 ± 0.00	13.33 ± 6.67	20.00 ± 11.55	20.00 ± 11.55	13.33 ± 6.67

3.2.2. Effect of uncoated and carbohydrate-coated $nCeO_2$ on early life stages of zebrafish *D. rerio*

Zebrafish embryos were microscopically observed during their exposure to uncoated $nCeO_2$ and carbohydrate-coated $nCeO_2$. As can be seen from **Figure 3**, zebrafish embryos did not show any signs of developmental malformations, such as short yolk extension, pericardial or yolk edema, deformed tail and somite, weak pigmentation, hemagglutination, in any of the treated groups in comparison to control embryos that were not exposed to nanoparticles. Hatching rates and mortality (the total number of coagulated embryos and unhatched embryos at 72 hpf) are expressed as percentages and shown in **Figure 4**.

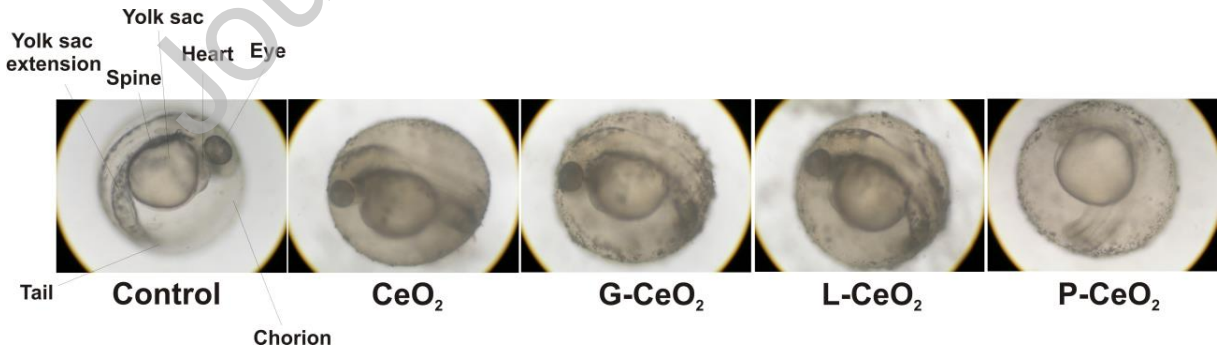

Figure 3. Zebrafish embryos at 48 hpf, exposed to uncoated (CeO_2) and coated (G-, L-, and P-CeO_2) nanoparticles, and viewed under a light microscope at 40× magnification.

The hatching rates (**Figure 4, A**) after 72 hpf of acute exposure to nanoparticles were almost 100% after all treatments and were not significantly different compared to that of the control. Also, there were no differences in *D. rerio* mortality between exposure to uncoated and coated nanoparticles, nor between all the treatments and the control (**Figure 4, B**). As in *D. magna* acute exposure, the higher suspension stability of coated nanoparticles (Milenković et al., 2018) did not impact the accumulation/adsorption of these nanoparticles (**Figure 2**) or, consequently, the mortality and hatchability of *D. rerio*. Therefore, the coating of $nCeO_2$ using glucose, levan, or pullulan does not affect the nanoparticles' toxicity toward *D. rerio*, and the examined nanoparticles are safe for the tested water organisms at the applied concentration. No effect of uncoated $nCeO_2$ on the early-life stage of zebrafish was also shown by Jemec et al. (2012) at concentrations up to 500 mg L^{-1} and size 8.2 nm. These results also suggest that the concentrations up to 200 mg L^{-1} of tested $nCeO_2$ will not endanger *D. rerio* organisms in the environment.

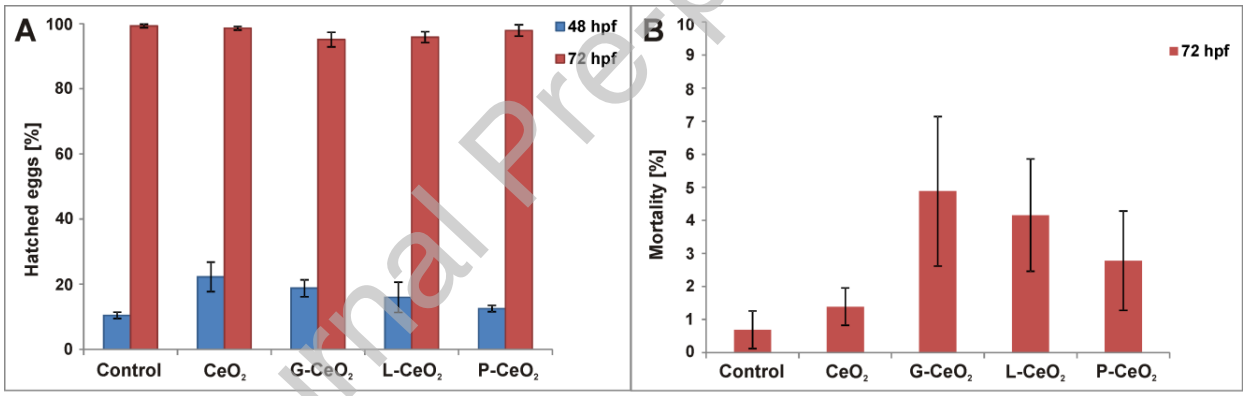

Figure 4. Hatchability (A) and mortality (B) expressed in mean % ± SEM at 48 and 72 hpf of exposure to uncoated ($nCeO_2$) and coated $nCeO_2$ ($G\text{-}CeO_2$, $L\text{-}CeO_2$, and $P\text{-}CeO_2$) at 200 mg L^{-1}.

3.2.3. Impact of uncoated and carbohydrate-coated $nCeO_2$ on the inhibition of bioluminescence in *V. fischeri*

V. fischeri is a bioluminescent, Gram-negative marine bacterium that has been routinely used for toxicity testing. Bioluminescence is directly proportional to the metabolic activity of the bacterial population, and any inhibition of enzymatic activity due to toxicity causes a decrease in bioluminescence (Abbas et al., 2018). The bacterial strain *V. fischeri* was previously used to better understand the effect of levan and pullulan on copper toxicity (Lončarević et al., 2019). In

Table 2, EC_{10} and EC_{20} values represent the concentrations of $nCeO_2$ that cause 10% and 20% bioluminescence inhibition, respectively. According to the EC_{20} values, $nCeO_2$ and G-CeO_2 affect the bioluminescence similarly and exhibit the highest inhibition. The lowest inhibition of luminescence was observed in *V. fischeri* treated with exopolysaccharide-coated $nCeO_2$, especially L-CeO_2. Thus, it can be concluded that the coating of $nCeO_2$ with microbial polysaccharides can reduce the toxic effect of $nCeO_2$ on *V. fischeri*. These results promote increasing application of exopolysaccharide-coated $nCeO_2$, in particular levan polysaccharide, for a different purpose without the fear to be harmful to the environment.

Table 2. EC_{10} and EC_{20} values and 95% confidence intervals after 15 min exposure of *V. fischeri* to uncoated (CeO_2) and coated (G-CeO_2, L-CeO_2, and P-CeO_2) nanoparticles.

Nanoparticle type	EC_{10}*	Confidence interval	EC_{20}*	Confidence interval
$nCeO_2$	< 1.56	n/a	3.42	± 2.65
G-CeO_2	< 1.56	n/a	2.75	± 1.80
L-CeO_2	20.58	± 9.27	60.16	± 5.36
P-CeO_2	9.91	± 10.48	16.62	± 8.85

*the values are expressed as mg L^{-1} of nanoparticles, n/a- could not be calculated

3.2.4. Effects of uncoated and carbohydrate-coated $nCeO_2$ on respiration activity in *D. magna*

The cumulative O_2 consumption and CO_2 production by *D. magna* neonates, during acute exposure (42 h) to 200 mg L^{-1} of different $nCeO_2$ are shown in **Figure 5**. This figure presents the respiration of 20 neonates per treatment without standardization regarding their mass.

The cumulative O_2 consumption and CO_2 production of animals after 42 h of exposure are shown in **Figure 5.** The criterion of test validity was fulfilled because mortality in the control did not exceed 10%. Based on the results obtained, it can be concluded that differences in cumulative O_2 consumption between the treatments did not exist (**Figure 5, A**), while cumulative CO_2 production (**Figure 5, B**) was the highest after 42 h of acute exposure to G-CeO_2 (1.79 mL) compared to exposure to other $nCeO_2$ (1.23, 1.31, and 1.11 mL for CeO_2, L-CeO_2, and P-CeO_2, respectively), and was two-fold greater compared to the control (0.98 mL). Higher CO_2 production during exposure to G-CeO_2 and L-CeO_2 nanoparticles could be due to the higher

turbidity of their aqueous suspensions (Milenković et al., 2018), which potentially impacts *Daphnia* respiration. Except for two studies (Knops et al., 2001; Lončarević et al., 2019) that monitored O_2 consumption and CO_2 production in neonates during stress, the impact of $nCeO_2$ on *D. magna* respiration was monitored for the first time in this research.

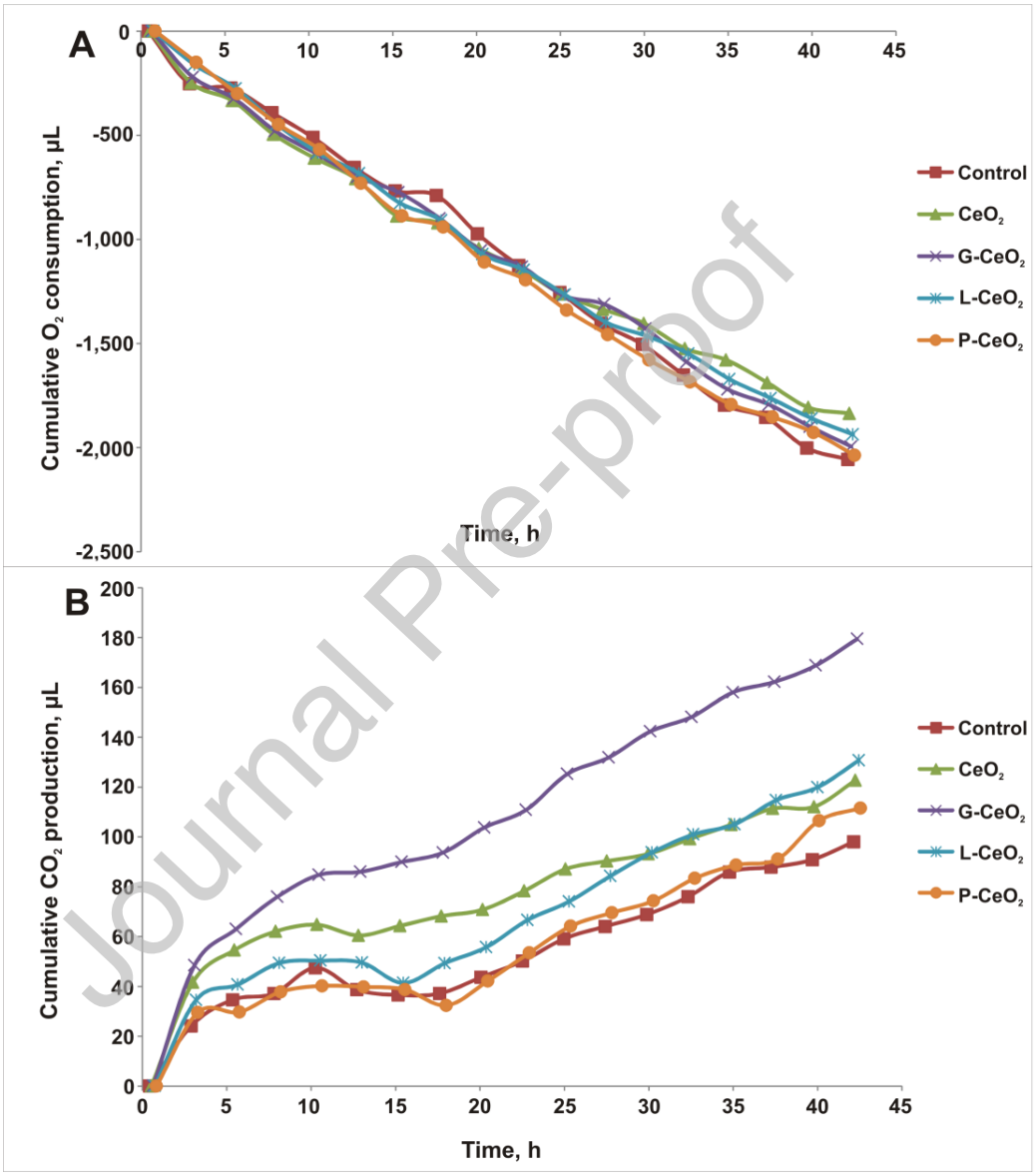

Figure 5. Effect of 200 mg L^{-1} uncoated ($nCeO_2$) and coated ($G\text{-}CeO_2$, $L\text{-}CeO_2$, and $P\text{-}CeO_2$) nanoparticles on cumulative: O_2 consumption (A), and; CO_2 production (B) in twenty *D. magna* neonates

4. CONCLUSIONS

This study, for the first time, demonstrated the effects of G-, L-, and P-CeO_2 on the bacterium *V. fischeri* and aqueous organisms *D. magna* and *D. rerio*. Results indicate that Ce concentrations in these aqueous organisms do not significantly change after exposure to the various nanoparticles. Acute toxicity testing in *D. magna* showed no mortality in most treatments at concentrations lower than 100 mg L^{-1} and the absence of differences between exposure to uncoated and coated nanoparticles, as well as between all the treatments and the control. In *D. rerio*, we did not observe any developmental malformations, and there was no statistically significant difference in hatching rate and mortality of embryos exposed to uncoated or coated nCeO_2 in comparison to control embryos, meaning uncoated and coated nCeO_2 did not affect early zebrafish development. In *V. fischeri*, uncoated nCeO_2 and G-CeO_2 inhibited bioluminescence the most, while the least inhibition occurred after exposure to L-CeO_2. *D. magna* respiration produced the highest CO_2 amounts after the crustaceans were exposed to G-CeO_2, while O_2 consumption did not differ between the treatments. This report demonstrates that uncoated and carbohydrate-coated nCeO_2 at 200 mg L^{-1} are not toxic to the tested organisms for the parameters examined, which contributes to investigations of the environmental risks of nanoparticle coatings used to improve nanoparticle stability in aqueous media. Future studies should focus on the examination of chronic exposure effects of nCeO_2 and the mechanism of their actions on suitable test organisms.

AUTHORS STATEMENT

Ivana Milenković: Investigation, Writing, Visualization. **Ksenija Radotić**: Conceptualization, Methodology, Writing - Review & Editing, Resources. **Jovana Despotović**: Investigation, Writing - Review & Editing, Validation. **Branka Lončarević**: Investigation, Writing - Review & Editing, Validation. **Marija Lješević**: Investigation, Writing - Review & Editing, Validation. **Slađana Z. Spasić**: Formal Analysis. **Aleksandra Nikolić**: Methodology, Resources. **Vladimir P. Beškoski**: Conceptualization, Methodology, Writing - Review & Editing, Resources, Supervision.

Conflict of interest

The authors declare no competing financial interest.

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