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Fate and Effects of CeO$_2$ Nanoparticles in Aquatic Ecotoxicity Tests

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Cerium dioxide nanoparticles (CeO$_2$ NPs) are increasingly being used as a catalyst in the automotive industry. Consequently, increasing amounts of CeO$_2$ NPs are expected to enter the environment where their fate in and potential impacts are unknown. In this paper we describe the fate and effects of CeO$_2$ NPs of three different sizes (14, 20, and 29 nm) in aquatic toxicity tests. In each standard test medium (pH 7.4) the CeO$_2$ nanoparticles aggregated (mean aggregate size approximately 400 nm). Four test organisms covering three different trophic levels were investigated, i.e., the unicellular green alga Pseudokirchneriella subcapitata, two crustaceans: Daphnia magna and Thamnocephalus platyurus, and embryos of Danio rerio. No acute toxicity was observed for the two crustaceans and D. rerio embryos, up to test concentrations of 1000, 5000, and 200 mg/L, respectively. In contrast, significant chronic toxicity to P. subcapitata with 10% effect concentrations (EC10s) between 2.6 and 5.4 mg/L was observed. Food shortage resulted in chronic toxicity to D. magna, for which EC10s of $\geq 8.8$ and $\leq 20.0$ mg/L were established. Chronic toxicity was found to increase with decreasing nominal particle diameter and the difference in toxicity could be explained by the difference in surface area. Using the data set, PNEC$_{aquatic}$ $\geq 0.052$ and $< 0.108$ mg/L were derived. Further experiments were performed to explain the observed toxicity to the most sensitive organism, i.e., P. subcapitata. Toxicity could not be related to a direct effect of dissolved Ce or CeO$_2$ NP uptake or adsorption, nor to an indirect effect of nutrient depletion (by sorption to NPs) or physical light restriction (through shading by the NPs). However, observed clustering of NPs around algal cells may locally cause a direct or indirect effect.

Introduction

The production and the number of applications of engineered nanoparticles (NPs) is increasing rapidly worldwide. Current applications include the use of nanoparticles in consumer products, construction materials, medical and pharmaceutical industries, agriculture, and information technology (1, 2). Nanoparticles are of interest because of their unique properties, such as an increased reactivity due to the high surface to volume ratio, light absorbing potential, or magnetic characteristics.

Currently, the lanthanide oxide cerium dioxide (CeO$_2$) is used in many new nanotechnology applications in which its high oxygen storage capacity (3), the low redox potential between Ce$^{4+}$ and Ce$^{3+}$ (4), and its UV absorbing potential (5) are exploited.

Recently, CeO$_2$ is increasingly being used in the automotive industry, both as diesel fuel additive to reduce the exhaust content of particulates after combustion and as constituent of catalytic converters (3, 6). However, the environmental release of CeO$_2$ NPs from various applications, and the subsequent behavior and effects of the released NPs are currently unclear. Because of the increasing use of CeO$_2$ NPs the assessment of the potential ecotoxicological effects of CeO$_2$ NPs must be considered an urgent need. Indeed, CeO$_2$ nanoparticles are on the OECD list of priority nanomaterials for immediate testing (7). However, at present little or no ecotoxicity data are available and hence, no risk assessment can be performed.

In the present study, the behavior and toxic effects of CeO$_2$ nanoparticles of three different primary nanoparticle sizes (14, 20, and 29 nm) in standard aquatic toxicity tests were investigated. In order to determine the fate of the NPs in the various test media, particle size distributions and zeta potentials were measured. Four test organisms of three different trophic levels were included in the effects assessment. Acute tests, which assessed immobility, mortality, and developmental malformations, were performed with the freshwater crustaceans Daphnia magna and Thamnocephalus platyurus and with embryos of the fish Danio rerio,
respectively. Chronic toxicity was investigated using the unicellular alga *Pseudokirchneriella subcapitata* and *Daphnia magna*. Where possible, ecotoxicological effect estimators, including x% effect concentrations (ECx), no observed effect concentrations (NOECs), and lowest observed effect concentrations (LOECs), were determined and the possible occurrence of a NP size effect was investigated. A size effect appears when nominally smaller NPs are more toxic than larger NPs when concentration is expressed as mass. Predicted no effect concentrations for the aquatic environment (PNEAguaqunt) were derived according to the Guidance document for the implementation of REACH (8). Furthermore, an explanation for the toxicity toward *P. subcapitata* was sought. Five hypotheses were tested: (1) toxicity was an artifact resulting from the clustering of algal cells with CeO2 aggregates, reducing the amount of detected cells; (2) toxicity was due to a direct effect related to either CeO2 NP uptake and/or strong adsorption to the algal cell wall, or (3) either to the presence of ionic cerium through partial dissolution of the CeO2 NPs in the test medium; finally, toxicity was suggested to be caused by an indirect effect of (4) nutrient deficiency originated from adsorption of ammonium and/or phosphate to the NP surface or an indirect effect of (5) the appearance of shading, i.e., the CeO2 NP (aggregates) shielded the algal cells and physically restrained photons, thereby inhibiting photosynthesis.

**Materials and Methods**

**CeO2 Nanoparticles: Origin, Dialysis and Dispersion in Test Media.** Ceria nanoparticles of three different nominal or primary particle sizes (14, 20, and 29 nm diameter) were supplied by one of the NanoInteract industry partners (Umicore). The ceria particles have a face centered cubic crystal structure. Scanning electron microscopy images of the powders can be found in Supporting Information (SI) Figure S1. Specific surface areas of 61, 42, and 29 m2/g, respectively, were measured by the manufacturer using the BET (Brunauer Emmet Teller) method. The particles were redispersed by milling into Milli-Q water at pH 4 containing nitric acid. This resulted in 10 wt % dispersions. An isoelectric point of 7.9 was established (SI Figure S2), which is in very good agreement with the isoelectric point determined by De Printz was used in 72 h growth inhibition experiments, conducted in accordance with OECD guideline No. 201 (10). The culturing procedure is described in the SI. Prior to the start of a test, all test concentrations (3.2, 5.6, 10, 18, and 32 mg/L CeO2 NPs) were equilibrated at 25 °C for 24 h. For each test concentration three replicates and one background correction (no algae added) were included. The replicates were inoculated with 104 algal cells/mL. During the 72 h test, all flasks were incubated at a temperature of 25 °C under continuous illumination (70 µE/(m2·s)) and were shaken manually three times a day. A preliminary experiment indicated that the E.C10 was equal in manually and continuously shaken replicates. The 14, 20, and 29 nm CeO2 NPs were tested simultaneously using the same algae batch. Every 24 h, the cell density was measured using a cell counter (Beckman Coulter Counter, Gent, Belgium). The average specific growth rate µ (d−1) was calculated as the slope of a linear regression of the natural logarithm of the measured cell density (corrected for background) versus time. This entire set of tests was repeated four times. The toxicity of CeO2 bulk powder (Sigma Aldrich, no. 22390) toward *P. subcapitata* was assessed in a separate experiment.

The freshwater cladoceran *Daphnia magna* was used in 48 h acute immobility tests and 21 day chronic reproduction tests performed in accordance with the OECD guidelines No. 202 and 211, respectively (11, 12). All test suspensions were prepared in Elendt M4 medium (13). In the 48 h acute immobility test, 30 juveniles (<24 h old) were transferred to three polyethylene cups (10 per cup) each containing 25 mL of a control solution or test suspension with 10 to 1000 mg/L CeO2 NPs. The juveniles were not fed during the experiment and after 48 h the number of immobile organisms was counted. At the start of a 21 day chronic reproduction test, 10 juveniles were transferred individually to polyethylene cups containing 50 mL of a control solution or test suspension of each concentration. The concentration range tested was 10–100 mg/L. Every day the daphnids were fed a mixture of the green freshwater algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio. As the organisms grew, increasing amounts of food were supplied: from day 1 to 7, from day 8 to 14, and from day 15 to 21 each daphnid was fed 250, 500, and 750 µg dry weight of algae mixture per day, respectively. Three times a week, the medium was renewed and parent mortality and the number of offspring was noted. At the end of the test, net reproduction (mean number of juveniles produced per parent animal alive) was calculated. After 7 days, the size of the organisms was measured. In both the acute and chronic tests, the organisms were kept at 20 ± 1 °C with a 12 h photoperiod.

A 24 h mortality test with instar II–III larvae of the crustacean *Thamnocephalus platyurus* hatched from cysts was conducted using the toxicity test-kit Thamnotoxkit F (MicroBioTests, Mariakerke, Belgium). The 24 h mortality test was performed according to the standard operational procedure (http://www.microbiotests.be/). All test suspensions (0, 100, 500, 1000, 3000, and 5000 mg CeO2/L) were prepared in EPA medium, and each suspension was transferred to three wells of a 24-well plate. At the start of the test, 10 larvae were introduced to each well (30 larvae per concentration) and after 24 h of incubation at 25 °C in the
dark mortality was determined. This assay was performed twice (20 and 29 nm particles) or three times (14 nm particles).

An early life stage test with zebrafish embryos (Danio rerio) was conducted in accordance with the draft OECD guideline on fish embryo toxicity testing (J4). The culturing procedure is described in the SI. All test concentrations (0, 13, 25, 50, 100, and 200 mg CeO₂/L) were prepared in zebrafish medium (SI Table S1). Twenty fish embryos were exposed to each concentration. One embryo was transferred to each well of a 24 well plate containing 2 mL of control solution or test suspension. The well plates were incubated at 28 °C for 72 h. The fish embryos were microscopically examined every day and after 24, 48, and 72 h lethal and sublethal endpoint scores were assessed. Zebrafish embryos were considered dead if the ciliation and motility of the embryos, irregular somite formation, nondetachment of the tail or lack of a heartbeat. At the same time the appearance of edema and scoliosis was assessed. At 72 h the number of hatched embryos was determined.

Statistical Data Treatment. The trimmed Spearman-Karber method was used to determine 21d chronic EC50 values of D. magna mortality (15).

Statistica 6.0 statistical software (Statsoft, Tulsa, OK) was used to fit a log-logistic or modified log-logistic model to concentration-response curves obtained in chronic tests and to calculate ECx values (see SI).

The determination of NOECs and LOECs was performed according to the OECD series on testing and assessment No. 54 (16). For continuous data the Jonckheere–Terpstra step down trend test was used at the 95% confidence level (α = 0.05). For quantal (mortality) data, a Fisher’s exact test (α = 0.05) with Bonferroni–Holm correction was used instead.

For the alga P. subcapitata, the appearance of a size effect was examined using one-way ANOVA on log transformed ECx values, both expressed as mass and as surface area (of the primary NPs, determined using BET). The Shapiro–Wilk test and Levene’s test (α = 0.05) were used to check for normality and homogeneity of variances, respectively. Scheffé was used as a posthoc test (17). In the chronic D. magna test the size effect was investigated using maximum likelihood estimation (MLE) (18). Briefly, this statistical method tests the null hypothesis that the sum of squared errors (SSE) when one common concentration-response curve is drawn through all data of the three particle sizes, equals the SSE when the three concentration-response curves of the 14, 20, and 29 nm CeO₂ NP sizes are described separately. A ρ value was derived from the χ² distribution using the MLE statistic, calculated on the basis of the natural logarithm of the ratio of SSEs. This was done for concentration expressed as both mass and surface area.

Preliminary Effects Assessment. Predicted no effect concentrations for the aquatic environment (PNECaquatic), with concentration expressed both as mass and as primary particle surface area were calculated according to the effects assessment methodology prescribed by the European Chemicals Agency (8).

Hypothesis Testing to Explain CeO₂ NP Toxicity toward P. subcapitata. To test the first hypothesis (i.e., toxicity is due to a measurement artifact), a separate algal growth inhibition test was performed using both cell number measurements and fluorescence spectroscopy of extracted chlorophyll to determine algal cell densities. Thereby, it is reasonable to assume that extraction of chlorophyll is not affected by clustering. Chlorophyll extraction was performed according to the method described by Mayer et al. (19). Fluorescence was measured by a LS 50B Luminescence spectrometer (Perkin-Elmer, Waltham, MA).

Transmission electron microscopy (TEM) was used to visualize the interaction between the CeO₂ NPs and algal cells in order to test the second hypothesis (i.e., CeO₂ NPs are taken up or adsorb to the algal cell wall). Therefore, after 72 h of exposure to 5.6 mg/L 14 nm CeO₂ NPs, algal cells were collected by centrifugation (15 min, 2000g) and fixed overnight using Karnovsky’s fixative composed of 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.5% CaCl₂ in 0.134 M sodium cacodylate buffer. After fixation, the procedure described in Van Hoecke et al. (20) was followed.

Ultrathin sections (150 nm) were cut using an ultramicrotome (RMC, PowerTome XL) with a diamond knife (Drukker). These sections were imaged with an FEI Tecnai G² Spirit Biotwin TEM (Hillsboro, OR) at an operating voltage of 120 kV.

To test the third hypothesis (toxicity due to dissolved Ce), the dissolution behavior of CeO₂ NPs in algal test medium was investigated. For three days, 0, 3.2, and 32 mg CeO₂/L (14, 20, and 29 nm) suspensions in OECD algae medium were incubated in triplicate at 25 °C under continuous illumination. To remove CeO₂ aggregates, samples were first centrifuged for 20 min at 2000g. Subsequently, 10 mL of the supernatants was filtered through a 0.2 μm Acrodisc syringe filter and a 10 k MWCO filter (Sartorius AG, Goettingen, Germany) with pores <5 nm. The filtrate was acidified with concentrated HNO₃ and Ce concentration was measured using an Element 2 high resolution inductively coupled plasma mass spectrometer (Thermo, Bremen, Germany).

Results and Discussion

Characterization of CeO₂ Nanoparticle Dispersions. Investigation of the initial CeO₂ dispersions upon dilution at pH 4 illustrated that the dispersions contained small aggregates of the primary particles (14, 20, and 29 nm), with a mean size of approximately 100 nm (Table 1). This is not surprising, given that the particles were dried down during their synthesis and resuspended by milling. Aggregation of particles during drying is inevitable and mechanical processes, such as milling, are unable to overcome the strong forces holding nanoparticle clusters together. Still, this sample preparation is relevant for ecotoxicological assessment, since this processing is widely used in industrial applications of nanoparticles.

From the cumulative distribution curve, it could be derived that only a very small fraction of the particles (±2%) is present as nonaggregated primary CeO₂ NPs.

Following dilution into the various test media and adjustment of the pH to 7.4, the suspensions became turbid.
within an hour of suspension preparation, indicating particle aggregation to diameters around 400 nm. Hence, the organisms are exposed to CeO2 NP aggregates of which the mean diameters and modes (values appearing most often) are given in Table 1. Differential and cumulative particle size distributions determined using NTA are illustrated in SI Figure S3 and Figure S4. The particle size distributions analyzed in the different test media overlapped. The zeta potential of 14, 20, and 29 nm CeO2 NP aggregates was determined in OECD algae medium and in M4 Daphnia medium (lowest and highest ionic strength media used). Table 1 summarizes the mean values and standard deviations (n = 3). The particles bore a negative surface charge in the test media at pH 7.4, while in 10 mM KCl solutions the surface charge was positive at this pH (SI Figure S2). The shift in sign of the surface charge can be explained by the high tendency of the CeO2 NPs to adsorb phosphate ions (further experimental details are provided below). Second, the particles clearly bear a higher charge in the lower ionic strength medium. The differences in zetapotential between particles dispersed in OECD medium and in M4 medium were significant for each CeO2 NP size (as determined in three t-tests at the 95% confidence level). The p-values obtained were 0.000, 0.010, and 0.001, for 14, 20, and 29 nm particles, respectively. The latter observation is in accordance with the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory (22, 23). Our results clearly show that differences in NP characteristics can arise due to changes in medium composition even when using the same NP batch. In this context, the use of different test media for different test organisms, can be seen as a drawback of the standard test protocols, since the media introduce an additional variable into the test design, making comparisons between tests less certain.

A comparison between the XANES spectra of the 14 nm CeO2 NPs dispersed in deionized water and in OECD algae medium and the Ce(IV) and Ce(III) reference substances is given in Figure 1. The Ce L3 spectrum for Ce(IV) consists of two peaks (called “white line”) above the absorption edge, while the Ce(III) compounds contain only one white line (24). There is a very good agreement between the CeO2 reference spectrum and the nanoparticles in deionized water as well as in OECD algae medium (1 g/L). If a significant amount of Ce(III) was present in the NP samples, a clear shoulder would be observed at the absorption edge in the XANES spectra. Calculations suggest that ≥99.7% of Ce in NPs is present as Ce(IV) in OECD medium and that the majority of the surface atoms in the NPs are in the Ce(IV) form and are not reduced to Ce(III) (more details in the SI). It should be mentioned that XANES analysis at a lower CeO2 concentration would have been more representative for the ecotoxicity tests performed. However, no fluorescence emission could be obtained for 32 and 100 mg/L of the 14 nm CeO2 nanoparticle suspensions in OECD algae medium and hence no XANES spectra could be recorded for these samples.

Acute Aquatic Toxicity Tests. No acute toxicity was observed for any of the three species tested. After a 24 h exposure of T. platyurus to 5000 mg/L 14, 20, and 29 nm CeO2 particles, only 12, 10, and 7% mortality was noted, respectively. These mortality values are not significant. Likewise, no immobility was observed in the acute D. magna test at 1000 mg/L. Finally, despite the adherence of NP aggregates to the outer surface of the chorion, no acute toxicity to zebrafish embryos was observed at 200 mg/L. The only minor effect was a 15% decrease in the number of embryos hatching following exposure to 200 mg/L 20 nm

**TABLE 1.** Mean Diameter (d) and Mode Determined Using Nanoparticle Tracking Analysis (Nta) (Mean: Average of All Particles, Mode: Value Appearing Most Often) in Stock Dilutions (pH 4) in Deionized Water (DIW) and in Various Test Media (pH 7.4), Measured 24 h after Preparation. For Particles Dispersed in OECD and M4 Medium the Zetapotential ± Standard Deviation (n=3) is Given

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>DIW</th>
<th>OECD (P. subcapitata)</th>
<th>M4 (D. magna)</th>
<th>EPA (T. platyurus)</th>
<th>ZFM (D. rerio)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d</td>
<td>mode</td>
<td>d</td>
<td>mode</td>
<td>d</td>
</tr>
<tr>
<td>14</td>
<td>145</td>
<td>115</td>
<td>479</td>
<td>375</td>
<td>549</td>
</tr>
<tr>
<td>20</td>
<td>122</td>
<td>95</td>
<td>462</td>
<td>325</td>
<td>500</td>
</tr>
<tr>
<td>29</td>
<td>140</td>
<td>95</td>
<td>552</td>
<td>475</td>
<td>425</td>
</tr>
</tbody>
</table>

FIGURE 1. Fluorescence Ce L3 XANES spectra for CeO2 NPs in deionized water and in OECD algae medium, corrected for self-absorption, compared to the normalized transmission CeO2 and CeF3 reference spectra.
of CeO$_2$ aggregates in suspension and hence depended on in food concentration was highly affected by the presence food. The decrease in food concentration was experimentally SI Figure S6, suggest that decreased reproduction and gut of exposed organisms. These observations, illustrated in concentration expressed as mass (panel A) and as surface area (panel D). The EC$_{10}$ and EC$_{20}$ of the four experiments are given in Table 2.

After log transformation, data sets containing 10 and 20% effect concentrations were normally distributed and variances were homogeneous. When concentration was expressed as mass, a significant difference in toxicity for various CeO$_2$ NP sizes was found with p-values of 0.004 and 0.000 on the basis of E$_{10}$, E$_{12}$, and E$_{50}$ values of the four experiments are equal, but the E$_{50}$ values determined using chlorophyll extraction are 50% lower. Illustrations of concentration—response curves and E$_{50}$ values from species of two different trophic levels, i.e. algae and daphnids, are available, the ECHA technical guidance document (8) prescribes the use of an extrapolation factor of 50 to the lowest EC$_10$ values, i.e. the 72 h E$_{10}$ values for P. subcapitata. As a result, PNEC$_{	ext{aqueous}}$ for 14, 20, and 29 nm CeO$_2$ NPs are 0.052, 0.068, and 0.108 mg/L, respectively. When the EC$_{10}$ values of the particles are expressed as surface area basis, a mean EC$_{10}$ of 0.153 m$^2$/L is obtained, which in turn results in a PNEC$_{	ext{aqueous}}$ of 0.006 m$^2$/L.

It should be emphasized that in this study standard test conditions were applied, despite the recent concerns raised about the relevance of these methods for assessing the risks of nanoparticles (25). It has been suggested that test organisms should be exposed to nanoparticles in an environmentally relevant way in order for the PEC and the PNEC to be based on the same nanoparticulate form (26). However, measurement or prediction of environmental concentrations of engineered NPs is still hampered by many difficulties, including detection, differentiation between natural forms of the material and engineered or nanoscale formats etc. (27), which currently impedes a relevant exposure design. Furthermore, no data is available on the appropriateness of the extrapolation factors used for calculating PNECs. A discussion concerning the latter issue is added in the SI. In conclusion, current knowledge on NP specific interactions with the aqueous environment is limited and therefore the currently derived PNEC values are preliminary. A crucial step in order to make standard ecotoxicity test methods more reliable for NPs, is the collection of knowledge on NP specific direct and indirect effects. Hypothesis testing and the assessment of non standard end points may play an important role in this process.

Hypothesis Testing to Explain CeO$_2$ NP Toxicity toward P. subcapitata. For the first hypothesis that toxicity is an artifact due to clustering of CeO$_2$ NPs with algae cells, identical results were obtained using both algal cell density measurement methods at low CeO$_2$ concentrations. However, at the highest test concentrations of 18 and 32 mgCeO$_2$/L a large discrepancy exists; i.e., only little or no chlorophyll was detected. Consequently, E$_{10}$ and E$_{20}$ are equal, but the E$_{50}$ values determined using chlorophyll extraction are 50% lower. Illustrations of concentration—response curves and E$_{50}$ values based on chlorophyll extraction are shown in SI Figure S7. In conclusion,
FIGURE 2. Concentration–response curves from four (panels 1–4) independent 72 h growth inhibition tests with *P. subcapitata* obtained for 14, 20, and 29 nm CeO$_2$ NPs. Concentration is expressed as mass (panels A) and as surface area (panels B). Average specific growth rate for each concentration is shown in percentage relative to the control value (% rtc). In each panel, EC$_{x}$ values and their 95% confidence interval are given.
<table>
<thead>
<tr>
<th>organism</th>
<th>P. subcapitata</th>
<th>T. platyurus</th>
<th>D. magna</th>
<th>D. rerio</th>
</tr>
</thead>
<tbody>
<tr>
<td>test duration</td>
<td>72 h chronic</td>
<td>24 h acute</td>
<td>48 h acute</td>
<td>21 day chronic</td>
</tr>
<tr>
<td>end point</td>
<td>growth inhibition</td>
<td>mortality</td>
<td>immobility</td>
<td>survival</td>
</tr>
<tr>
<td>units</td>
<td>mg/L</td>
<td>m^2/L</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>14 nm CeO₂</td>
<td>NOEC</td>
<td>3.2</td>
<td>0.20</td>
<td>&gt;5,000</td>
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<tr>
<td></td>
<td>LOEC</td>
<td>5.6</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC10</td>
<td>2.8 ± 0.5</td>
<td>0.16 ± 0.03</td>
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<tr>
<td></td>
<td>EC20</td>
<td>4.0 ± 0.6</td>
<td>0.24 ± 0.03</td>
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<tr>
<td></td>
<td>EC50</td>
<td>10.2 ± 4.4</td>
<td>0.62 ± 0.27</td>
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</tr>
<tr>
<td>20 nm CeO₂</td>
<td>NOEC</td>
<td>3.2</td>
<td>0.13</td>
<td>&gt;5000</td>
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<tr>
<td></td>
<td>LOEC</td>
<td>5.6</td>
<td>0.24</td>
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</tr>
<tr>
<td></td>
<td>EC10</td>
<td>3.4 ± 0.8</td>
<td>0.14 ± 0.03</td>
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<tr>
<td></td>
<td>EC20</td>
<td>5.0 ± 0.9</td>
<td>0.21 ± 0.04</td>
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<tr>
<td></td>
<td>EC50</td>
<td>11.7 ± 4.9</td>
<td>0.49 ± 0.21</td>
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</tr>
<tr>
<td>29 nm CeO₂</td>
<td>NOEC</td>
<td>3.2</td>
<td>0.09</td>
<td>&gt;5000</td>
</tr>
<tr>
<td></td>
<td>LOEC</td>
<td>5.6</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC10</td>
<td>5.4 ± 1.2</td>
<td>0.16 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC20</td>
<td>8.5 ± 1.9</td>
<td>0.25 ± 0.05</td>
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<tr>
<td></td>
<td>EC50</td>
<td>19.1 ± 6.8</td>
<td>0.56 ± 0.19</td>
<td></td>
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<tr>
<td>CeO₂ bulk</td>
<td>NOEC</td>
<td>&gt;1000</td>
<td>nd</td>
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<td></td>
<td>LOEC</td>
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<td>EC50</td>
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</table>

*a* For *P. subcapitata* ECₙ values and standard deviations (SD) are given (*n* = 4). For *D. magna* 95% confidence intervals are listed in between brackets. nd = not determined.
decreased algal cell density was not due to clustering. In case of severe clustering of algal cells and CeO₂ in an algal growth inhibition experiment, one would expect lower toxicity when the algal cell density is analyzed using chlorophyll extraction. However, based on the experiments performed, we have no explanation for the absence of chlorophyll at higher test concentrations.

The TEM study to test the second hypothesis (direct effect through NP uptake and/or adsorption) revealed only weak physical interaction between single NPs or aggregates and algal cells (Figure 4). No convincing images of either uptake or strong adsorption to the cell wall were found. The absence of NP uptake is consistent with findings reported in Van Hoecke et al. (20). Furthermore, Navarro...
et al. (28) hypothesized that NPs or NP aggregates larger than the pores present in the cell wall cannot be taken up by algae. However, it is clear that NP aggregates seem to cluster around cells instead of being adsorbed to the cells. This situation clearly differs from the strong adsorption of CeO₂ NPs to the entire cell wall of *E. coli* described by Thill et al. (29). Also Limbach et al. (30) observed adsorption and clustering of CeO₂ NPs to and with biological sludge. No definitive evidence to support the hypothesis of uptake and/or strong adsorption was found in this study.

The concentration of dissolved cerium in all samples in the OECD algae medium was beneath the detection level of the ICP-MS analysis. This indicates that dissolution of the CeO₂ NPs in the test medium is negligible. For CeO₂ long-term (4 months and 96 h) NOEC values on population growth of *Chlorella vulgaris* and *Scenedesmus quadricauda* of 2 and 0.15 mg/L were reported in De Jong (31 and 32). Consequently, it can be ruled out that dissolved Ce was the cause of toxicity in our experiments.

The first experiment of the fourth hypothesis testing (the observed toxicity is due to an indirect effect of nutrient deficiency caused by the adsorption of nutrients to the CeO₂ NP surface) showed no adsorption of ammonium, but adsorption of phosphate to the particle surface was observed to a large extent; e.g. around 50% in the 32 mg/L 14 nm CeO₂ suspensions. The adsorption measured after 24 h and 72 h was identical and depended on CeO₂ concentration and nominal particle diameter, as shown in Figure 5. Again, MLE analysis showed that data from all three particle sizes can be described by one common curve when the CeO₂ concentration is expressed as surface area (p = 3.71 × 10⁻¹⁵). In the second experiment it was shown that a 50% reduction of phosphate in the OECD algae medium had no significant effect on the algal growth rate. The outcome of this experiment is shown in SI Figure S8. Reduction in algal growth due to phosphate deficiency is observed only when 60% of the PO₄³⁻ is depleted (corresponding to 0.00366 mM PO₄³⁻ remaining in solution). A 50% reduction in growth rate was observed in phosphate free medium. As a consequence, the fourth hypothesis is not valid. However, it cannot be ruled out that physicochemical changes occurred in the liquid interface between CeO₂ NPs and algal cells.

In the final hypothesis testing, no evidence of shading was found. When 14 nm CeO₂ NPs and algal cells were spatially separated in such a way that light had to cross a suspension of CeO₂ NPs before reaching the algal cells, no decrease in algal cell density was observed. See SI Figure S9. Consequently, the final hypothesis is unvalid. Two other studies already showed that the shading effect is negligible for TiO₂ NPs (21, 33). Arujoa et al. furthermore reported the same conclusion for CuO and ZnO NPs.

In summary, none of the hypotheses studied can unambiguously be considered as valid. The clustering of the particle aggregates around the algal cells, however, might have caused toxicity through a local direct effect or local nutrient depletion and/or shading at the cellular level.

Considering the observations made in this study, the current EU effects assessment methodology should be applied to NPs with caution, and consideration of the effects of NPs on the test medium, and effects of the test medium on the NP physicochemical properties must be included. Therefore, it is advisable to analyze different end points and to consider the appearance of measurement artifacts and indirect effects.

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**Supporting Information Available**

More details on experimental setup, particle characterization and toxicity studies. This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**


(32) Bringmann, G.; Kuhn, R. Comparative water-toxicological investigations on bacteria, algae and Daphnia. *Gesundheitsingenieur* 1959, 80, 115–120.


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