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Fate and Effects of CeO₂ Nanoparticles in Aquatic Ecotoxicity Tests

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Cerium dioxide nanoparticles (CeO₂ NPs) are increasingly being used as a catalyst in the automotive industry. Consequently, increasing amounts of CeO₂ 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 CeO₂ NPs of three different sizes (14, 20, and 29 nm) in aquatic toxicity tests. In each standard test medium (pH 7.4) the CeO₂ 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 wich EC10s of ≥8.8 and ≤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$_{aquatic}$s ≥ 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 CeO₂ 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 (CeO₂) is used in many new nanotechnology applications in which its high oxygen storage capacity (*3*), the low redox potential between Ce³⁺ and Ce⁴⁺ (*4*), and its UV absorbing potential (*5*) are exploited.

Recently, CeO₂ 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 CeO₂ NPs from various applications, and the subsequent behavior and effects of the released NPs are currently unclear. Because of the increasing use of CeO₂ NPs the assessment of the potential ecotoxicological effects of CeO₂ NPs must be considered an urgent need. Indeed, CeO₂ 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 CeO₂ 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*,

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respectively. Chronic toxicity was investigated using the unicellular alga *Pseudokirchneriella subcapitata* and *Daphnia magna*. Where possible, ecotoxicological effect estimators, including *x*% effect concentrations (EC*x*), 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 (PNEC$_{aquatic}$) 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 CeO$_2$ aggregates, reducing the amount of detected cells; (2) toxicity was due to a direct effect related to either CeO$_2$ 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 CeO$_2$ 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 CeO$_2$ NP (aggregates) shielded the algal cells and physically restrained photons, thereby inhibiting photosynthesis.

Materials and Methods

CeO$_2$ 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 Faria and Trasatti (1994) (*9*). Use of the same batches across the ecotoxicological research laboratories was assured.

Experimental test concentrations were prepared by dropwise addition of the CeO$_2$ nanoparticle stock suspensions to the test media adjusted to pH 4 using a 1 M HCl solution, while stirring. Subsequently, the pH of the test suspensions was adjusted to 7.4. Prior to pH adjustment, 750 mg/L MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer was added to the media used for the 72 h chronic algal growth inhibition and the 21d chronic *Daphnia magna* reproduction test.

Characterization of CeO$_2$ Nanoparticle Suspensions. The particle size distributions of 14, 20, and 29 nm CeO$_2$ nanoparticles in test media at 50 mg/L and pH 7.4 were determined 24 h after preparation of the suspensions following the procedure outlined above, using Nanoparticle Tracking Analysis (NTA) with a Nanosight LM20 system (Nanosight Ltd., Wiltshire, UK). A 1 mL sample was introduced into the LM20 system using a syringe. Particle size distributions were derived from a video recording using the nanoparticle tracking analysis (NTA) 1.5 software. Twenty-four hours after preparation, CeO$_2$ nanoparticle suspensions (10 mg/L) in test medium were introduced into a Zetasizer 3000 HSA (Malvern Instruments, Worcestershire, UK) using a syringe. The zetapotential of each sample was measured three times.

The Ce L$_3$ X-ray absorption near edge structure (XANES) measurements were performed at the beamline L of the HASYLAB synchrotron laboratory (Hamburg). Pure dry CeF$_3$

and CeO$_2$ powders were used as Ce$^{(III)}$ and Ce$^{(IV)}$ reference samples. The XANES spectrum of these references was used to analyze the oxidation state of a 100 g/L 14 nm stock in deionized water and 1 g/L 14 nm CeO$_2$ sample suspended in the algae medium for six days. A detailed description of the instrument parameters is given in the SI.

Experimental Setup of Ecotoxicity Tests and Measured End Points. The unicellular freshwater green alga *Pseudokirchneriella subcapitata* 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 CeO$_2$ 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 10^4 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$_r$C10 was equal in manually and continuously shaken replicates. The 14, 20, and 29 nm CeO$_2$ 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 CeO$_2$ 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 CeO$_2$ 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 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 CeO$_2$/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 (*14*). The culturing procedure is described in the SI. All test concentrations (0, 13, 25, 50, 100, and 200 mg CeO$_2$/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 end points were assessed. Zebrafish embryos were considered dead in the case of coagulation of 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. After 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 E$_r$Cx 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 CeO$_2$ NP sizes are described separately. A *p*-value was derived from the χ^2 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 (PNEC$_{aquatic}$), 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 CeO$_2$ 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 CeO$_2$ NPs and algal cells in order to test the second hypothesis (i.e., CeO$_2$ NPs

are taken up or adsorb to the algal cell wall). Therefore, after 72 h of exposure to 5.6 mg/L 14 nm CeO$_2$ 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% CaCl$_2$ 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 samples were imaged with an FEI Tecnai G^2 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 CeO$_2$ NPs in algal test medium was investigated. For three days, 0, 3.2, and 32 mgCeO$_2$/L (14, 20, and 29 nm) suspensions in OECD algae medium were incubated in triplicate at 25 °C under continuous illumination. To remove CeO$_2$ 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 HNO$_3$ and Ce concentration was measured using an Element 2 high resolution inductively coupled plasma mass spectrometer (Thermo, Bremen, Germany). As internal standard 10 μg/L rhodium was used and Ce quantification was carried out by external five-point-calibration.

Two experiments were performed to test the fourth hypothesis (i.e., toxicity is due to nutrient depletion). In the first experiment CeO$_2$ NPs and bulk material at concentrations used in the algal growth inhibition tests (including a control) were prepared in the OECD algae medium in duplicate. After 24 h and 72 h of illumination at 25 °C, the particles were spun down by centrifugation (10 min, 2000g) and the supernatant was used for colorimetric analysis of NH$_4^+$ and PO$_4^{3-}$ (measured against a standard curve of these analytes in the OECD algae medium) using commercial analysis kits (no. 1.14848.0001 and no. 1.14752.0001) from Merck KGaA (Darmstadt, Germany). In the second experiment, an algal growth inhibition test with a gradual reduction of the phosphate concentration in comparison with the standard test medium was performed.

The final hypothesis (toxicity due to shading) was tested using the approach described by Hund–Rinke et al. (*21*). Briefly, the chlorophyll contents upon spatially separating particles and algal cells in two different 96 well plates is compared to the chlorophyll contents when algal cells and NPs are added to the same well and allowed to stand for 3 days at 25 °C under continuous illumination. The initial cell density in the wells was 10 000cells/mL.

Results and Discussion

Characterization of CeO$_2$ Nanoparticle Dispersions. Investigation of the initial CeO$_2$ 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 ($\pm$2%) is present as nonaggregated primary CeO$_2$ NPs.

Following dilution into the various test media and adjustment of the pH to 7.4, the suspensions became turbid

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 $\pm$ Standard Deviation ($n=3$) is Given

	DIW		OECD (*P. subcapitata*)			M4 (*D. magna*)			EPA (*T. platyurus*)		ZFM (*D. rerio*)	
nominal diameter (nm)	*d* (nm)	mode (nm)	*d* (nm)	mode (nm)	ζ (mV)	*d* (nm)	mode (nm)	ζ (mV)	*d* (nm)	mode (nm)	*d* (nm)	mode (nm)
14	145	115	479	375	-19.6 ± 0.3	549	375	-6.6 ± 1.9	746	275	441	425
20	122	95	462	325	-16.1 ± 0.6	500	525	-9.1 ± 2.6	758	425	543	425
29	140	95	552	475	-15.0 ± 0.7	425	425	-3.9 ± 0.8	467	375	464	275

within an hour of suspension preparation, indicating particle aggregation to diameters around 400 nm. Hence, the organisms are exposed to CeO_2 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 overlap. The zeta potential of 14, 20, and 29 nm CeO_2 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 CeO_2 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 CeO_2 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 CeO_2 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 L_3 spectrum for $Ce^{(IV)}$ compounds 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 CeO_2 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 $\geq 99.7\%$ 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 CeO_2 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 CeO_2 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 CeO_2 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
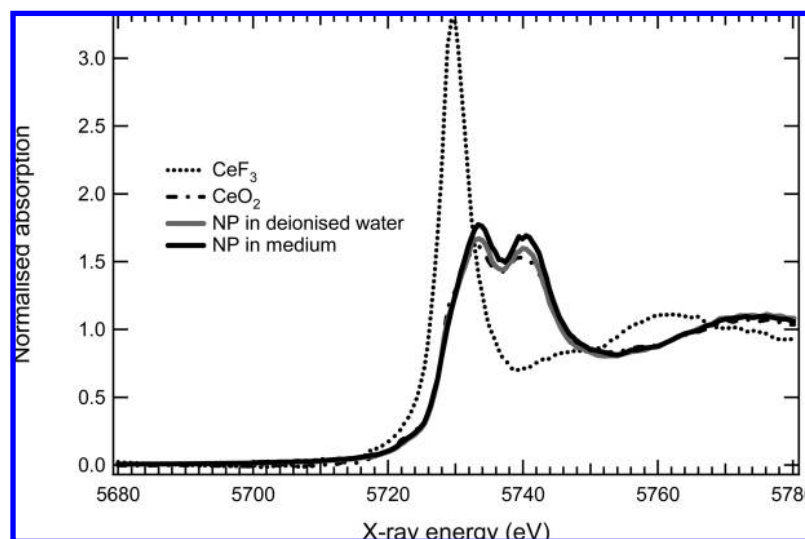

FIGURE 1. Fluorescence Ce L_3 XANES spectra for CeO_2 NPs in deionized water and in OECD algae medium, corrected for self-absorption, compared to the normalized transmission CeO_2 and CeF_3 reference spectra.

CeO₂ NPs. However, according to Fischer's exact test this was not statistically significant.

Chronic Aquatic Toxicity Tests. The concentration−response curves of all 72 h algal growth inhibition experiments with 14, 20, and 29 nm CeO_2 NPs are shown in Figure 2. Onset of toxicity was found at 5.6 mg/L for all NP sizes. The concentration which resulted in 50% decrease in average specific growth rate depended on the primary particle size and was observed between 7.6 and 28.8 mg/L. When concentration is expressed as surface area (right panels), the curves collapse onto a single curve. The CeO_2 bulk material did not cause any reduction in algal growth inhibition at the maximum test concentration of 1000 mg/L. Ecotoxicological effect estimators, i.e., NOEC, LOEC, and mean E_rC10, E_rC20, and E_rC50 values 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_rC10 and E_rC20, respectively. The Scheffé posthoc test indicated that the 14 and 20 nm particles were more toxic than the 29 nm particles. No significant difference between the 14 and 20 nm particles was determined. However, when concentration was expressed as surface area, no size effect was observed (p-values 0.521 and 0.327). Consequently, toxicity is related to surface area and not exclusively to mass, despite the NP aggregation. While we have no final explanation for the central role of surface rather than mass, the experimental observations are unambiguous. Additional experiments supporting this observation are described below.

In the 21 day chronic *D. magna* reproduction test significant adverse effects were found in the 10−100 mg/L concentration range. Within nine days of the start of the experiment, all test organisms died when exposed to 56 and 100 mg/L of 14 and 20 nm CeO_2 NPs. The 29 nm NPs caused 100% mortality at 100 mg/L. Organisms exposed to 100 mg/L CeO_2 bulk material all survived. However, it is very likely that a secondary effect of lack of food contributed to the observed chronic effects. This statement is supported by three visual observations. First, the CeO_2 aggregates clustered together with algal cells, resulting in pale (during the first week) to green (during the third week) clumps with diameters exceeding 1 mm. Second, the daphnids exposed to CeO_2 NPs remained smaller than those in the control treatments. Mean length (±standard deviation, SD) of the test organisms exposed to 100 mg/L of 14, 20, and 29 nm CeO_2 NPs after 7 days was 1.06 (±0.18), 1.17 (±0.27), and 1.27 (±0.04) mm, respectively, while daphnids in the control experiments measured 2.48 (±0.02) mm. Third, algae were absent in the gut of exposed organisms. These observations, illustrated in SI Figure S6, suggest that decreased reproduction and eventual mortality was due to the inability to take up sufficient food. The decrease in food concentration was experimentally assessed, as described in the SI. In conclusion, the decrease in food concentration was highly affected by the presence of CeO_2 aggregates in suspension and hence depended on the details of experimental work, such as whether or not the CeO_2 agglomerates were resuspended upon food addition.

Concentration−response curves presenting the effects on *D. magna* reproduction are given in Figure 3, with concentration expressed as mass (panel A) and as surface area (panel B). Panel A also presents the effects of CeO_2 bulk material on *D. magna* reproduction. Bulk CeO_2 caused a 40% decrease in reproduction at the maximum test concentration. In analogy with the chronic algal growth inhibition data, a clear NP size effect can be observed. The outcome of the MLE analysis showed that when the NP concentration is expressed

as mass, the response of the particles should be described using three curves, one for each particle ($p = 6.8 \times 10^{-15}$). When the concentration is expressed as surface area, however, the three separate curves can be replaced by one common concentration−response curve ($p = 0.063$). The resulting fits are shown in Figure 3. It is not clear if the size effect on reproduction was also caused by the lack of food. The decrease in algal cell density measured after 24 and 48 h (SI Tables S2 and S3) did not depend on the nominal particle diameter. The occurrence of another mode of toxic action can not be ruled out on the basis of these observations.

The calculated ECx values for reproduction are given in Table 2. The EC50s range from 20.5 to 42.7 mg/L, depending on the NP size. The EC10 and EC20 of 14 and 20 nm NPs are extrapolated values, making them less reliable. This issue could be overcome by using the common concentration−response curve with concentration expressed as surface area (Panel B, Figure 3). Non extrapolated EC10 and EC20 values expressed as surface area of 0.54 and 0.72 m²/L, respectively, could be derived from the common fit. These were subsequently converted to ECx expressed as mass and are reported in Table 2. The latter values, however, differ only slightly from the extrapolated values, with relative differences ranging between 0.5 and 12%.

Preliminary Effects Assessment. Since two long-term EC10 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 EC10 values, i.e. the 72 h E_rC10 values for *P. subcapitata*. As a result, PNEC$_{aquatic}$ for 14, 20, and 29 nm CeO_2 NPs are 0.052, 0.068, and 0.108 mg/L, respectively. When the mean EC10 values of the particles are expressed on a surface area basis, a mean EC10 of 0.153 m²/L is obtained, which in turn results in a PNEC$_{aquatic}$ of 0.0031 m²/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 CeO₂ NP Toxicity toward *P. subcapitata*. For the first hypothesis that toxicity is an artifact due to clustering of CeO_2 NPs with algal 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 mgCeO₂/L a large discrepancy exists; i.e., only little or no chlorophyll was detected. Consequently, E_rC10s and E_rC20s are equal, but the E_rC50 values determined using chlorophyll extraction are 50% lower. Illustrations of concentration−response curves and E_rCx values based on chlorophyll extraction are shown in SI Figure S7. In conclusion,
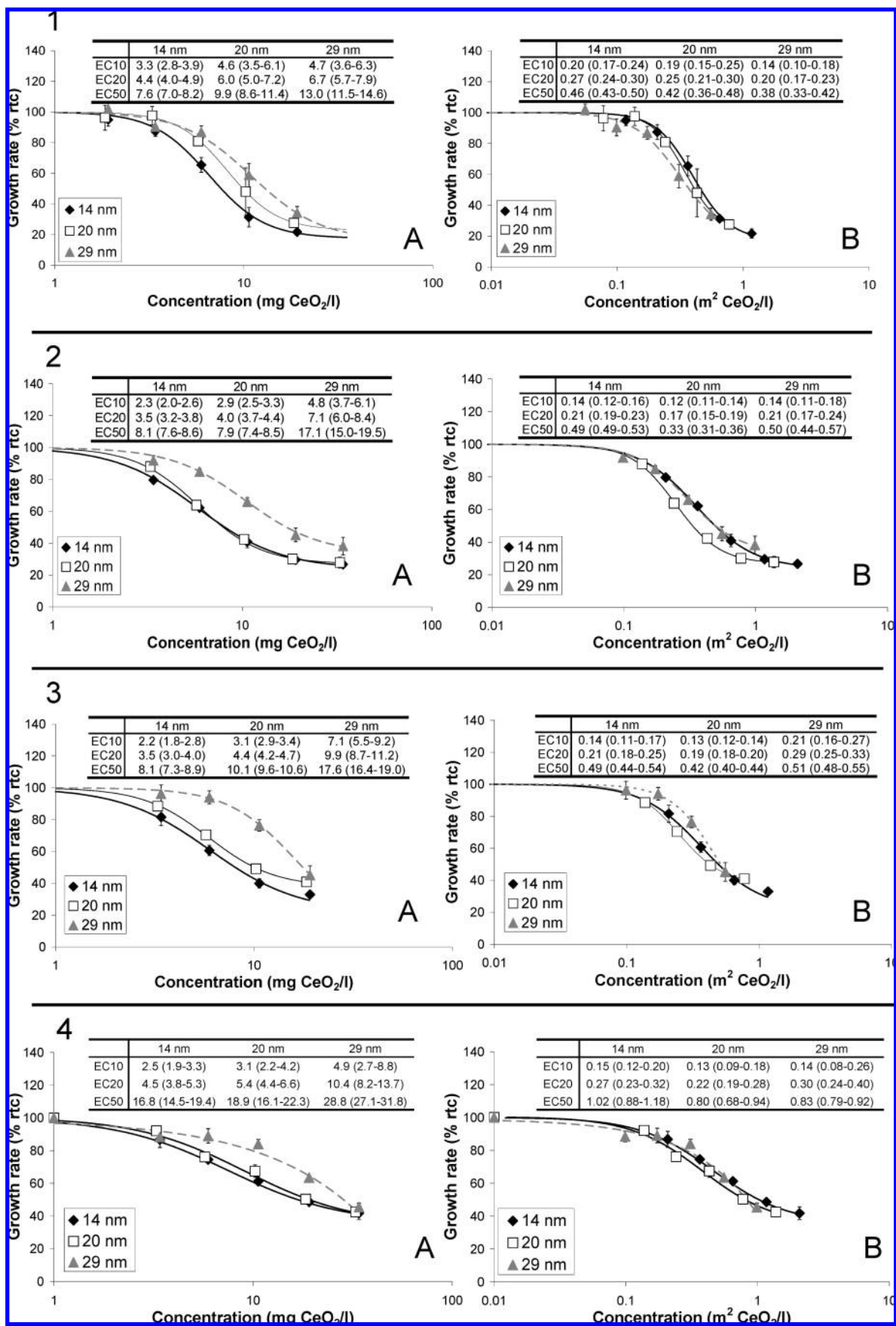
FIGURE 2. Concentration−response curves from four (panels 1−4) independent 72 h growth inhibition tests with *P. subcapitata* obtained for 14, 20, and 29 nm CeO₂ NPs. Concentration is expressed as mass (panels A) and as surface area (panels B). Average specific growth rate for each concentration is shown in percentage relative to the control value (% rtc). In each panel, E$_r$Cx values and their 95% confidence interval are given.

TABLE 2. Overview of Ecotoxicological Effect Estimators[a]

organism		P. subcapitata		T. platyurus	D. magna	D. magna			D. rerio		
test duration		72 h chronic		24 h acute	48 h acute	21 day chronic			72 h acute		
end point		growth inhibition		mortality	immobility	survival	reproduction		mortality	hatching	scoliosis,oedeme
units		mg/L	m²/L	mg/L	mg/L	mg/L	mg/L	m²/L	mg/L	mg/L	mg/L
14 nm CeO₂	NOEC	3.2	0.20	>5,000	>1,000	32	<18.0	<1.10	>200	>200	>200
	LOEC	5.6	0.34			56	18.0	1.10			
	EC10	2.6 ± 0.5	0.16 ± 0.03			nd	8.8 (7.2−10.9)	0.54 (0.44−0.66)			
	EC20	4.0 ± 0.6	0.24 ± 0.03			nd	11.8 (10.1−13.8)	0.72 (0.62−0.84)			
	EC50	10.2 ± 4.4	0.62 ± 0.27			40.7 (33.6−49.3)	20.5 (17.7−23.7)	1.25 (1.08−1.45)			
20 nm CeO₂	NOEC	3.2	0.13	>5000	>1000	32	<18.0	<0.76	>200	>200	>200
	LOEC	5.6	0.24			56	18.0	0.76			
	EC10	3.4 ± 0.8	0.14 ± 0.03			nd	12.8 (10.4−15.8)	0.54 (0.44−0.66)			
	EC20	5.0 ± 0.9	0.21 ± 0.04			nd	17.2 (14.7−20.0)	0.72 (0.62−0.84)			
	EC50	11.7 ± 4.9	0.49 ± 0.21			36.9 (27.6−49.3)	25.4 (21.4−30.3)	1.07 (0.90−1.27)			
29 nm CeO₂	NOEC	3.2	0.09	>5000	>1000	56	18.0	0.52	>200	>200	>200
	LOEC	5.6	0.16			100	32.0	0.93			
	EC10	5.4 ± 1.2	0.16 ± 0.04			nd	20.0 (14.2−28.3)	0.58 (0.41−0.82)			
	EC20	8.5 ± 1.9	0.25 ± 0.05			nd	26.5 (20.4−34.4)	0.77 (0.59−1.00)			
	EC50	19.1 ± 6.8	0.56 ± 0.19			71.1 (61.0−82.8)	42.7 (36.7−49.8)	1.24 (1.06−1.44)			
CeO₂ bulk	NOEC	>1000	nd	nd	>1000	>100	32.0	nd		nd	
	LOEC						56.0				
	EC10						nd				
	EC20						32.0 (9.8−104.6)				
	EC50						182.4 (64.0−519.8)				

[a] For P. subcapitata E,Cx values and standard deviations (SD) are given (n = 4). For D. magna 95% confidence intervals are listed in between brackets. nd = not determined.
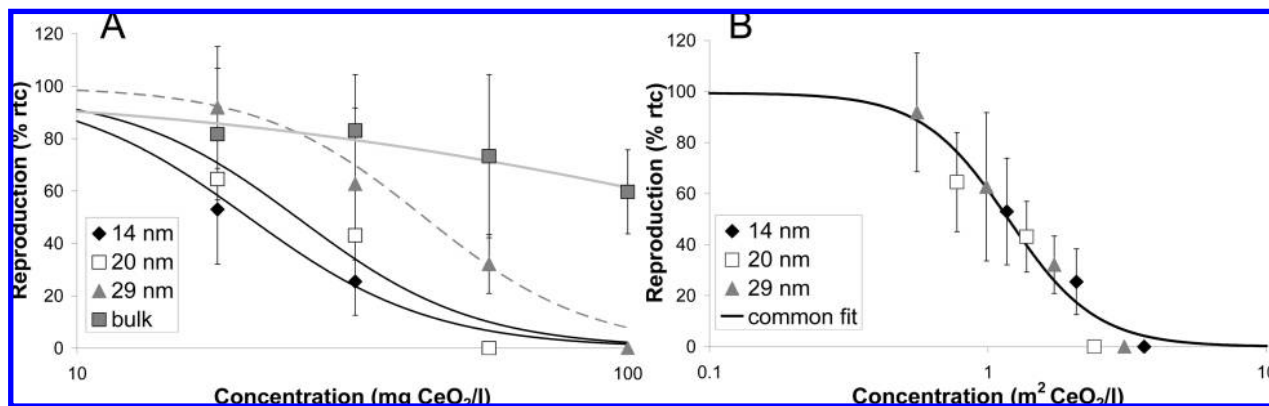
FIGURE 3. Concentration−response curves and calculated log−logistic fits of a 21 days reproduction test with _D. magna_ obtained for 14, 20, and 29 nm CeO_2 NPs and CeO_2 bulk material. Concentration is expressed as mass (A) and as surface area (B).
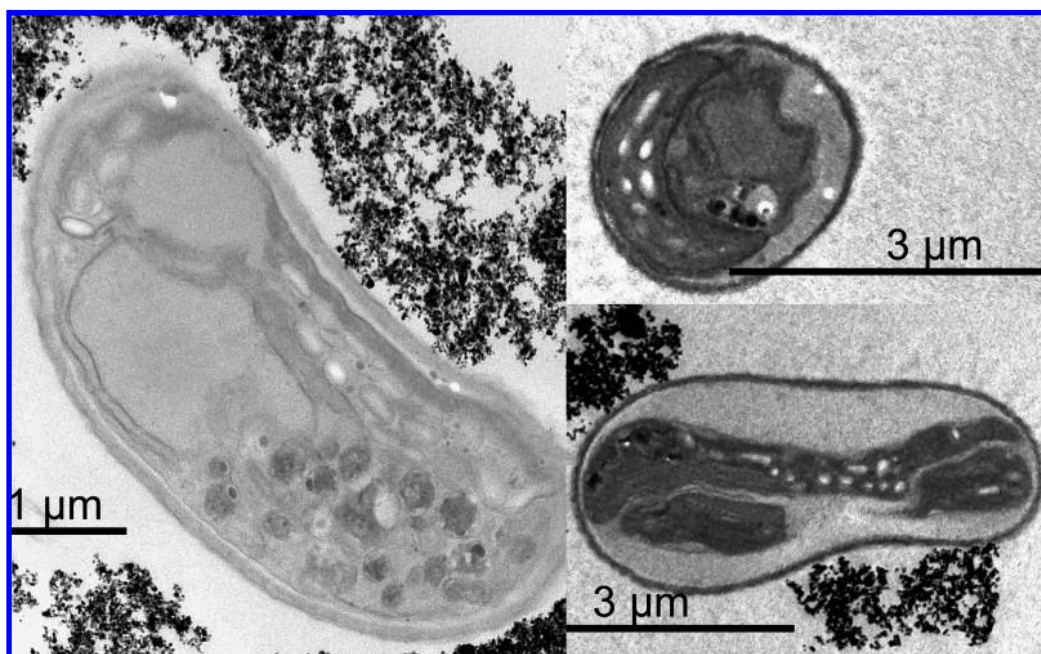

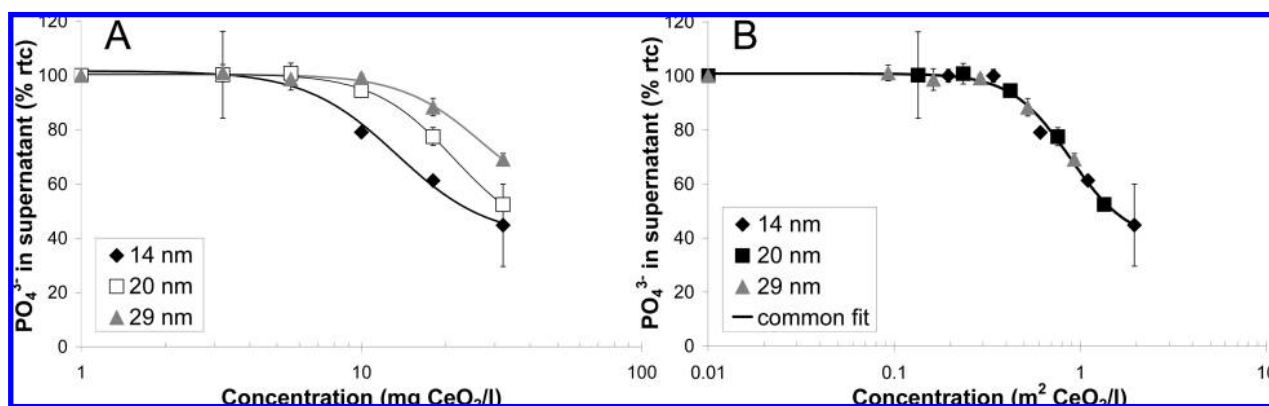**FIGURE 4. Transmission electron microscopy (TEM) images of algal cells exposed to 14 nm CeO_2 NPs (5.6 mg/L).**

FIGURE 5. Phosphate concentration in OECD algae medium exposed to CeO_2 NPs after centrifugation (2000g, 10 min), relative to CeO_2-free medium. In panel A the CeO_2 NP concentration is expressed as mass, in panel B as surface area.

decreased algal cell density was not due to clustering. In case of severe clustering of algal cells and CeO_2 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 CeO_2 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 CeO_2 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 CeO_2 NPs in the test medium is negligible. For Ce^{3+} long-term (3−4 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 in Bringmann and Kuhn (32), respectively. A 96 h NOEC on growth inhibition in *S. quadricauda* exposed to Ce^{4+} was 0.14 mg/L (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 CeO_2 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 CeO_2 suspensions. The adsorption measured after 24 h and 72 h was identical and depended on CeO_2 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 CeO_2 concentration is expressed as surface area ($p = 0.46$). However, when the CeO_2 concentration is expressed as mass, the adsorption of phosphate should be described for each particle size separately ($p = 3.71 \times 10^{-11}$). 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 PO_4^{3-} is depleted (corresponding to 0.00366 mM PO_4^{3-} 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 CeO_2 NPs and algal cells.

In the final hypothesis testing, no evidence of shading was found. When 14 nm CeO_2 NPs and algal cells were spatially separated in such a way that light had to cross a suspension of CeO_2 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 TiO_2 NPs (21, 33). Aruoja 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.

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.

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