1 Aggregation and ecotoxicity of CeO₂ nanoparticles in
2 synthetic and natural waters with variable pH, organic matter
3 concentration and ionic strength

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ABSTRACT

The influence of pH (6.0-9.0), natural organic matter (NOM) (0-10 mgC/L) and ionic strength (IS) (1.7-40 mM) on 14 nm CeO₂ NP aggregation and ecotoxicity towards the alga *Pseudokirchneriella subcapitata* was assessed following a central composite design. Mean NP aggregate sizes ranged between 200 and 10000 nm. Increasing pH and IS enhanced aggregation, while increasing NOM decreased mean aggregate sizes. The 48 h-E_rC20s ranged between 4.7 and 395.8 mgCeO₂/L. An equation for predicting the 48 h-E_rC20 (48 h-E_rC20 = -1626.4x(pH) + 109.45x(pH)² + 116.49x([NOM]) - 14.317x(pH)x([NOM]) + 6007.2) was developed. In a validation study with natural waters the predicted 48 h-E_rC20 was a factor 1.08-2.57 lower compared to the experimental values.

KEYWORDS

CeO₂ nanoparticles – alga – natural organic matter – natural waters

CAPSULE

CeO₂ nanoparticle aggregation and toxicity depend on abiotic factors such as pH, NOM and IS. Effect concentrations can be predicted as a function of pH and NOM.

INTRODUCTION

The lanthanide oxide CeO₂ in its nanoparticulate form is currently being used as a diesel fuel additive, as a constituent of catalytic converters and as a UV blocking agent. Therefore, CeO₂ NPs are expected to enter the environment, where they may pose a potential risk to aquatic and sediment organisms. The need for an environmental effects assessment of these nanomaterials was expressed by international organisations such as the Organisation for Economic Cooperation and Development (OECD) (OECD, 2008). A previous study generated acute and chronic ecotoxicity data for three sizes of CeO₂ NPs (14, 20 and 29 nm) according to the standard OECD test methods (Van Hoecke et al., 2009). For 14 nm CeO₂ NPs, a 48 h 20 % effect concentration on *P. subcapitata* growth rate (48 h-E_rC₂₀) (SD) of 3.5 (0.8) mg/L was obtained in the standard OECD algal test medium at pH 7.4. Despite the usefulness of the standard test medium in comparing experimental results, concerns have been raised regarding its environmental relevance for ecotoxicity tests with manufactured nanomaterials (Crane et al., 2008; Handy et al., 2008). Indeed, since physico-chemical water characteristics such as pH, ionic strength (IS) and natural organic matter (NOM) concentration can vary greatly among natural surface waters, the use of just one test medium at pH 7.4, low IS (1.7 mM) and in absence of NOM may not be representative for realistic waters. Moreover, the above mentioned abiotic factors are also known to be important parameters determining nanoparticle surface characteristics and aggregation behaviour.

The goals of the present study were (1) to investigate the influence of the abiotic factors pH, NOM concentration and IS on CeO₂ NP aggregation and toxicity towards the unicellular alga *Pseudokirchneriella subcapitata*, (2) to develop an empirical model which predicts the 48 h-E_rC₂₀ of the alga as a function of pH, NOM and IS of the medium and

finally, (3) to validate the empirical model using four natural surface waters with various characteristics.

In a first experiment, CeO₂ toxicity and stability was investigated in standard algal test medium supplemented with NOM. In a second part of the study, 17 synthetic test media with different characteristics were prepared following a central composite experimental design (CCD). Subsequently, 48 h-E_rC20 for *P. subcapitata* was determined in each of these media. Non linear regression analysis allowed to mathematically express the relations between the abiotic factors and the established 48 h-E_rC20. Finally, the predictive value of the developed model was assessed using 4 natural surface waters with various characteristics.

The empirical model can be used for predicting effect concentrations of CeO₂ NPs in natural waters when basic water characteristics, i.e. pH and NOM concentration, are known.

MATERIALS AND METHODS

CeO₂ nanoparticles and natural organic matter

Crystalline 14 nm CeO₂ NPs were supplied by an industrial NanoInteract project partner (cfr. supporting information). The particles were dried after synthesis and were redispersed into Milli-Q water by ball milling. Prior to the redispersion, the pH of Milli-Q water was set to 4 using diluted nitric acid. A final stock of 100 g/L CeO₂ NPs was obtained. A detailed characterization of the particles, including the particle size distribution, the isoelectric point and the cerium oxidation state in the stock suspension is described in Van Hoecke et al. (2009). More details are given in the supporting information.

The natural organic matter (NOM) was sampled from a creek in Bihain, Belgium, using a portable reverse-osmosis based device (RealSoft PROS/2S) in order to concentrate

NOM as described by Serkiz and Perdue (1990) and Sun et al. (1995). The sampling procedure is described in more detail in De Schamphelaere et al. (2003).

NOM adsorption studies

The amount of NOM adsorbed to CeO₂ NPs in 20 ml OECD medium (OECD, 2006) containing 8.0 mg C/L NOM was measured under various conditions of pH (6.0, 7.5 and 9.0) and NaCl concentrations (0, 20 and 40 mM). A high concentration of CeO₂ NPs was necessary to be able to detect a significant adsorption of NOM to the particle surface. Therefore, suspensions containing 220 mg CeO₂/L were incubated at 25 °C under continuous illumination for 48 h. Twice a day, pH values were checked and adjusted when ≥ 0.2 pH units deviation was observed. The increase in IS due to pH adjustments was ≤ 0.2 mM. At the end of the incubation period, two 10 ml samples of each medium were centrifuged for 15 minutes at 2000g. Finally, 7 ml of the supernatant was used for NOM analysis, for which a TOC-500 (Shimadzu, Duisburg, Germany) carbon analyzer was used. The experiment was performed twice. One-way ANOVA ($\alpha = 0.05$) was performed to establish the significance ($p < 0.05$) of pH and/or IS on NOM adsorption.

One factor experiment

In the one factor experiment, only the concentration of NOM was varied. Two environmentally relevant concentrations for river water in cool and warm temperate climates, in which NOM ranges between 2 and 15 mg C/L with means of 3 and 7 mg C/L, respectively (Thurman, 1985). Test suspensions were prepared by dropwise addition of the 14 nm CeO₂ NP stock suspension in stirred standard OECD algal medium to which 3.6 mM 3-(N-Morpholino)propanesulfonic acid (MOPS) buffer (pH 7.4) and the desired amount of Bihain NOM (2.2 and 7.4 mg C/L) were added. Test medium containing NOM without addition of CeO₂ was used as control medium. Concentrations of CeO₂ NPs ranged

between 3.2 and 180 mg CeO₂/L. The NOM concentration was monitored daily in a control medium replicate without addition of buffer.

Multivariate experiment

The three factor (pH, NOM and IS) experiment, was performed according to a complete second-order three factor central composite design (CCD) in three experimental blocks, which consisted of 17 algal growth inhibition tests (Box and Draper, 1987). The pH of the algal test medium was varied between 6.0 and 9.0, NOM concentrations were varied between 0 and 10 mg C/L and NaCl was added in concentrations ranging between 0 and 40 mM. The nominal values of the three factors in each of the 17 test media are presented in **Table 1**.

The 17 test media were prepared by addition of 3.6 mM buffer and the desired amount of NOM to the standard OECD algal test medium and subsequent pH adjustment using HCl or NaOH. To maintain a pH value of 6.0 2-(N-Morpholino)ethanesulfonic acid (MES) buffer was used, while MOPS buffer adequately stabilized the media pH at values 6.6 and 7.5. The 8.4 and 9.0 pH values required addition of 2-(Cyclohexylamino)ethanesulfonic acid (CHES) buffer. In order to assure an equal minimum conductivity across the whole pH range, the test media were equilibrated at 25 °C and conductivity of all media was set to 375 µS/cm using NaCl before addition of the amount of NaCl as specified in the CCD. Finally, CeO₂ NP stock suspension was added by dropwise addition to the stirred media, until desired concentrations between 2.2 and 460 mg CeO₂/L were reached.

During the algal growth inhibition tests, pH, NOM and conductivity were monitored daily. A pH electrode (P407, Consort, Turnhout, Belgium) was used to measure pH. In case ≥ 0.2 pH units deviation from the theoretical value was observed, pH was adjusted using 1 M NaOH or HCl solutions. The media at pH values 8.4 and 9.0 were checked twice a day.

Natural organic matter concentrations were monitored in a buffer free replicate in OECD algal test medium incubated under the same conditions as the algal growth inhibition tests. Finally, conductivity measurements using a cond 315i portable device equipped with a TetraCon 325 electrode (WTW, Weilheim, Germany) were performed to monitor the IS in all test suspensions, according to the method described by Simon and Garcia (1999). More details on the relation between electrical conductivity and IS can be found in the supporting information.

Particle size distribution analysis

Particle size distributions of the 22.0 mg/L 14 nm CeO₂ NP suspensions were analyzed using dynamic light scattering (DLS) three days after the start of the algal growth inhibition test. In case of severe aggregation resulting in aggregates with a Z-average hydrodynamic diameter (as determined by DLS) > 1000 nm, laser diffraction was performed with a Mastersizer (Malvern Instruments, Worcestershire, UK) equipped with a 300 RF fourier lens to analyze particle size distributions. More details on instrument settings can be found in the supporting information.

Algal toxicity testing

The green alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak was obtained from the Canadian Phycological Culture Centre (CPCC, Waterloo, Canada). Algae were continuously cultured in two fold diluted ES-medium (Provasoli, 1966) prepared in carbon filtered aerated Ghent city tap water. The resulting medium was supplemented with 1.4 mg/L FeSO₄·7H₂O, 15 mg/L NaH₂PO₄·2H₂O, 150 mg/L NaNO₃ and 2.35 mg/L MnCl₂·4H₂O. Four days prior to the start of a series of algal growth inhibition experiments, a new algal culture was prepared and allowed to grow on a shaking table at 20 ± 1 °C under continuous illumination (70 µE/(m²·s¹)).

Each of the test media from both the one factor and the multivariate experiment was used to prepare one control medium and five different test concentrations of CeO₂ NPs. Equilibration took place overnight at 25 °C in the dark. Subsequently, four 50 ml portions of each concentration were transferred to 100 ml erlenmeyer flasks, three of which were inoculated with 10,000 cells/ml of the grown culture and one was used as a background correction. One 50 ml portion of a 22.0 mg CeO₂/L suspensions was sealed with parafilm[®] to serve for aggregation behaviour analysis. All flasks were incubated at 25 °C under illumination and were shaken manually three times a day. After 24, 48 and 72 h, the algal cell density was measured using a cell counter (Beckman Coulter Counter, Ghent, Belgium). The average specific growth rate μ (1/d) in each concentration was calculated after 48 h exposure, because the test validity criteria were not met in 4 experiments of the CCD when data was analyzed after 72 h. More specifically, the mean coefficient of variation (CV) on sectional growth rate, which checks for exponential growth in the control throughout the test, was > 35 %. In this case the OECD test protocol prescribes the use of 48 h toxicity data.

Data treatment and analysis

Either a log-logistic or a modified log-logistic model (Van Hoecke et al., 2009), was chosen to fit the concentration-response curves, depending on the highest correlation coefficient. Effect concentrations and their 95 % confidence intervals were calculated with Statistica[®] (Statsoft, Tulsa, OK, USA). First, the effect of nominal pH, NOM and IS (independent variables) on the control growth rates was analyzed using Statistica's CCD feature. Subsequently, the CCD analysis was applied to the 48 h-E_rC20s in order to identify any significant linear, quadratic and interaction effects. Subsequently, non linear least squares regression analysis using measured factor levels was applied to develop an empirical model and to assess the variability explained by the different linear and quadratic

effects. The Levenberg-Marquardt algorithm was used to estimate regression coefficients (Moré, 1977). Additional factors were added according to a minimisation of the Akaike information criterion (Akaike, 1974). Throughout the study, a significance level (α) of 0.05 was adopted.

Validation study with natural waters

Four natural waters, Ankeveensche Plas (a lake system in lowland peat area, Nederhorst den Berg, NL), Markermeer (part of a large lake cut off from the North Sea by a dam, Marken, NL), Le Veyon (a small stream in a forested area, Trélon, Fr) and Ourthe orientale (a river in mixed forest, Brisy, BE) were used in a validation study. **Table 1** contains some characteristics of these waters. One day prior to the start of an algal growth inhibition test, all natural waters were filtered through a 1.2 μm Whatman glass microfiber filter and through 0.8 and 0.45 μm Pall Supor[®] membrane filters. In order to meet the OECD validity criteria for algal testing (OECD, 2006), natural waters had to be supplied with all nutrients in the same concentration of the standard OECD algal test medium prior to buffer addition and spiking of 14 nm CeO₂ NP stock suspensions. Subsequently, tests and measurements were performed as described above.

RESULTS

Preliminary one factor study of influence of NOM on CeO₂ NP toxicity and stability

Addition of NOM to the OECD algal test medium prior to spiking CeO₂ NPs highly affected both CeO₂ suspension stability and toxicity in comparison with the NOM free standard OECD algal test medium at pH 7.4. The particle size distributions and concentration-response curves are presented in **Figures 1 and 2**, respectively. While severe aggregation in absence of NOM resulted in a mean particle size of 4500 nm, the NOM stabilized small particle aggregates of 100-200 nm. Pictures are given in the supporting information. At the low NOM concentration of 2.2 mg C/L, 48 h-E_rC₁₀ and E_rC₂₀ values

increased with 7.7 and 7.4 fold up to 16.2 and 26.0 mg CeO₂/L compared to the NOM free medium, respectively. At 7.4 mg C/L, the effect concentrations increased 31.5 and 23.3 fold up to 66.4 and 81.6 mg CeO₂/L, respectively. A linear relationship between the NOM concentration and the effect concentrations was found (Figure 1 panel B).

NOM adsorption to CeO₂ NPs

The amount of NOM adsorbed to CeO₂ NPs varied between 0.006 and 0.062 mg C in a 220 mg CeO₂/L suspension, which corresponds to 0.0014 and 0.014 mg C/mg CeO₂. **Table 2** shows the amount of NOM adsorbed as a function of pH and IS. One-way ANOVA indicated that pH significantly influenced NOM adsorption ($p = 1.8 \times 10^{-5}$), with higher adsorption observed at pH 6 compared to pH 9. No significant influence of IS was observed.

Central composite design to test the influence of pH, NOM and IS on aggregation behaviour and 48 h-E_rC20 of CeO₂ NPs

In all test media particle aggregation occurred. Mean particle aggregate sizes as determined by DLS (media no. 3, 4, 5, 8 and 16 as listed in Table 1) or laser diffraction are given in **Table 1**. Predominantly in media with low pH and in presence of NOM severe aggregation was prevented, resulting in Z-average hydrodynamic diameters of a few hundred nanometer in media 4, 5, 8 and 16. Also in medium 3 with high pH and NOM concentration, but low IS, the mean particle size was < 1000 nm. In all other media severe aggregation to particles > 1000 nm occurred. In the CCD analysis, all linear main effects were significant with p-values of 2.0×10^{-4} , 0.013 and 0.022 for pH, NOM concentration and IS, respectively. Increasing pH and IS enhanced aggregation, while increasing NOM concentration decreased the tendency towards aggregation. Furthermore, a significant quadratic pH effect was identified with a p-value of 0.020. A scatterplot of mean aggregate sizes as determined by DLS or laser diffraction as a function of pH, NOM and IS is given

in **Figure 3**. The pH was found to be the dominant factor, as derived from the ratio of the pH's sum of squares (SS) to the total SS. The ratio was 0.682 for linear and quadratic pH effect, while it was only 0.091 and 0.070 for NOM concentration and IS, respectively.

The CCD analysis first revealed a significant linear and quadratic pH effect on the growth rate of algae in the control. The p-values for these effects were 0.032, and 0.0036, respectively. **Table 1** contains all control growth rates. The control growth rate at pH 9 (experiment 17) was only 0.97/d. Furthermore, the validity conditions of this algae test were not met. More specifically, the algal cell density in the control did not increase with a factor of 16 within 48 h and the mean sectional CV was > 35 %, indicating the deviation from exponential growth in the control replicates. Therefore, data point 17 in Table 1 was deleted from subsequent non linear regression analysis.

The 48 h- E_rC_{20} values and their 95 % confidence interval are given in **Table 1**. A factor of 84 separates the lowest E_rC_{20} value of 4.7 from the highest of 395.8 mg CeO_2/L . Significant linear pH and NOM effects ($p = 0.0041$ and 0.017 , respectively) were found in the CCD analysis, while pH also showed a significant quadratic effect ($p = 1.8 \times 10^{-4}$). Furthermore, an interaction effect between pH and NOM was identified ($p = 0.030$). Except for pH values of 8.4, increasing the NOM concentration lead to a decrease in toxicity, which is in agreement with the first experiment. **Figure 4** plots the 48 h- E_rC_{20} s as a function of the three factors. The significant effects identified by the CCD were subsequently used in the non linear regression analysis in order to derive an empirical model. As a result, a mathematical expression of the form: $48 \text{ h-}E_rC_{20} = Ax(pH) + Bx(pH)^2 + Cx([NOM]) + Dx(pH)x([NOM]) + E$ was derived. In this equation, pH and [NOM] are expressed numerically, with [NOM] expressed as mg C/L. **Table 3** summarizes the parameter coefficients, standard errors, p-values and percentage of total variability explained by each effect. In total, 93.7 % of the variability in 48 h E_rC_{20} values could be

explained by the variation in pH and NOM concentration. The overall p-value of the least squares regression was 2.9×10^{-7} . A graphical illustration and description of the model outcome as a function of pH and NOM concentration can be found in the [Supporting information section](#).

Validation study

Also in natural surface waters the CeO₂ NPs aggregated up to particles > 1000 nm. The mean aggregate sizes determined by laser diffraction are shown in [Table 1](#). The experimentally determined 48 h-E_rC₂₀ values in the 4 natural waters ranged between 46.3 and 103.2 mg CeO₂/L. [Table 1](#) contains both observed and predicted 48 h-E_rC₂₀ and their ratios. The concentration-response curve obtained in Markermeer natural water was not monotonous, giving rise to the large confidence interval of the measured 48 h-E_rC₂₀ value (Table 1). For all natural waters, the predicted E_rC₂₀ was lower than the experimentally determined value and the difference between observed and predicted value differed with a factor of 1.08 to 2.57. When considering the experimentally determined 95 % confidence intervals, the model predicted effect concentration of 55.2 mg/L for Le Voon and of 31.5 mg/L for Ourthe Orientale are both values not contained within the experimentally determined 95 % confidence intervals (see Table 1).

DISCUSSION

Effect of NOM only

The preliminary experiment in NOM containing medium demonstrated the stabilizing effect of NOM on CeO₂ NPs. Similarly, Keller et al. (2010) and Quik et al. (2010) demonstrated the stabilization of small CeO₂ NP aggregates of a few hundred nm by NOM. Also other types of NPs can be stabilized by NOM, e.g. carbon NPs (fullerene as well as carbon nanotubes (CNT)) (Chen and Elimelech, 2007; Hyung et al., 2007), TiO₂ and ZnO NPs (Keller et al., 2010).

The Z-average hydrodynamic diameter around 100 nm corresponded to small aggregates which were already present in the stock suspensions in deionized water at pH 4 (Van Hoecke et al., 2009). As a result, the NOM was able to prevent further aggregation in the test medium but was unable to overcome the strong van der Waals forces holding small NP aggregates together. A similar conclusion was drawn by Walker and Bob (2001).

All effect concentrations for CeO₂ NPs were found within the mg/L concentration range, which is very high in comparison with predicted concentrations in the environment (O'Brien and Cummins, 2010). Therefore, a low risk quotient is expected, especially in presence of NOM.

Influence of multiple abiotic factors on CeO₂ NP toxicity and aggregation behaviour

A more complex situation arose when in addition to NOM concentration pH and IS of the test media were varied. Concerning the E_rC₂₀ values in **Figure 4**, three groups can be identified. First, media containing ≥ 5 mg C/L NOM at low pH resulted in low toxicity (48 h-E_rC₂₀ ≥ 183 mg/L) as represented by data points 4, 8 and 16. The highest toxicity (48 h-E_rC₂₀ ≤ 11.0 mg/L) was found for media 1 and 13, i.e. at the highest pH of 8.4 in presence of only 2 mg C/L NOM and at pH 7.5 in absence of NOM, respectively. Finally, the largest group of media caused an intermediate toxicity. In general, this group contained media with low NOM concentration at low pH and media with high NOM concentration (≥ 5 mg C/L) at high pH. The CCD's central point also resulted in an intermediate toxicity. These 48 h-E_rC₂₀s can be used to evaluate the reproducibility of the algal growth inhibition tests. With a relative standard deviation of 13.6 % on data points 2, 6 and 11 the reproducibility was good.

For all synthetic waters, effect concentrations for CeO₂ NPs were similar or higher compared to previously reported low mg/L values obtained in standard algal tests (Van Hoecke et al., 2009; Rogers et al., 2010). This indicates that the standard algal tests can be

considered a worst case scenario for the CeO₂ NPs. On the other hand, if toxicity is highly overestimated for certain waters, this might lead to overly conservative environmental quality standards. Using the developed model, the discrepancy between the toxicity in standard test medium and in a more realistic test medium containing NOM can be minimized up to a factor of 3.2, according to the ratio of observed towards predicted E_rC₂₀ values given in **Table 1**. When no NOM was added, medium 13, a negative value was predicted. Therefore, the model is of no use for media without NOM around pH 7-8.

When combining data on mean aggregate size and 48 h-E_rC₂₀, it became clear that the more stable suspensions gave rise to the lowest observed toxicities (data points 4, 8 and 16 listed in Table 1). In Figure S5 in supporting information, the logarithms of 48 h-E_rC₂₀ of each medium are plotted against average aggregate sizes as determined by DLS and laser diffraction. Interestingly, a negative linear relationship was established ($p = 1.6 \times 10^{-10}$).

A clear relation between CeO₂ toxicity and aggregation behaviour and the affinity towards NOM adsorption follows from **Figures 3 and 4** and **Table 2**. For CeO₂ toxicity and stability as well as its affinity towards NOM, the pH was the dominant factor. Adsorption of NOM was favoured at low pH values (6, 6.6), which resulted in the lowest toxicity and highest stability observed. On the other hand, NOM adsorption decreased as pH increased, which adversely affected stability and toxicity. From these observations, it was clear that adsorption of NOM to the CeO₂ NP surface prevented the particles from directly interacting with each other and with algal cells. The decrease in toxicity therefore was due to a reduction in bioavailability of the particles. A similar observation was made by Li et al. (2010), who reported a decrease in the toxicity of zerovalent iron NPs towards *E. coli*, which the authors attributed to the loss of adhesion of NOM coated particles to the bacterial cell wall.

The observations concerning NOM adsorption as a function of pH are in agreement with previous research on NOM adsorption to oxide surfaces by Au et al. (1999). In this study, a decrease in NOM adsorption density was observed with increasing pH. At high pH, lateral repulsion of charged NOM fragments bound to the particle surface prevented additional NOM molecules from adsorbing to the surface. On the other hand, the adsorbed NOM layer protruded further from the NP surface into the solution at higher pH. At low pH, the NOM bore less negative charges, which resulted in lower repulsion and hence higher adsorption density. In our experiments, decreased NOM adsorption at pH 9 could also be due to an increased concentration of CO_3^{-2} , which could have competed with NOM for adsorption to the CeO_2 surface. At lower pH values, the species H_2CO_3 and HCO_3^- dominated.

The relation between NOM adsorption and pH might as well explain the observed quadratic pH effect in the toxicity model. Since at intermediate pH values NOM adsorption is lower compared to pH 6 and the NOM layer does not extend far into the solution, as expected at the highest pH values, a maximum direct interaction between the CeO_2 NPs and algal cells can be expected around pH 7.5, which would explain the maximum toxicity observed at pH 7.5. According to Au et al. (1999), increasing the IS enhanced NOM adsorption, since NOM fragments were able to coil when its negative charges were partly shielded by the positive counterions of the electrolyte, thereby reducing steric and electrostatic repulsion. However, increasing the IS lead to a compression of the electrostatic double layer, which adversely affected the stabilising potency of the NOM and thereby the stability of the particle suspensions. A decrease in stabilising potency of the NOM at higher IS was also observed in the present study, however, no enhancement of the NOM adsorption was noted within the range of IS studied. Several studies with various NP types reported similar behaviour as a function of pH and IS in presence of NOM. For example,

Domingos et al. (2009) found increased aggregation of 5 nm TiO₂ NPs with increasing IS and in general, the tendency towards aggregation increased under conditions that were unfavorable for NOM adsorption. Furthermore, increased stability of Au NPs was observed in presence of NOM by Diegoli et al. (2008). Once again, high IS was capable of eliminating the enhanced stability of NOM coated Au NPs.

CeO₂ NP toxicity and behaviour in natural waters

The observed E_rC₂₀ values in the four natural waters corresponded to the predicted values within a factor of 2.57. In previous experiments with the 14 nm CeO₂ NPs the individual 48 h-E_rC₂₀ measurements differed by a factor of 2.1. As a consequence, the discrepancy between measured and model predicted 48 h-E_rC₂₀s cannot be explained by experimental variability only. The composition of NOM is known to differ between natural waters and could have influenced the adsorption affinity and stabilizing potency towards NPs. Secondly, the higher concentration of divalent ions such as Ca²⁺ and Mg²⁺ in the natural waters compared to the synthetic waters may help explain the observed discrepancy. The test media used in the CCD analysis all have a hardness of 24 mg CaCO₃/L. With exception of Le Vayon natural water, the river waters all have a much higher hardness of 53-267 mg CaCO₃/L. The divalent ions Ca²⁺ and Mg²⁺ are also more effective in destabilising particle suspensions, which is known as the Schulze-Hardy rule. Furthermore, Sander et al. (2004) reported the ability of Ca²⁺ at a concentration of 10 mM to bridge adsorbed NOM layers on several particles, thereby enhancing the aggregation process. Therefore, the large mean aggregate sizes observed in natural waters (Table 1) may be partly explained by the higher hardness.

To the best of our knowledge, no ecotoxicity studies on CeO₂ NPs in natural waters have been published. However, several studies on metal and metal oxide NPs experimentally confirmed that water characteristics can have important implications for

either or both NP toxicity and stability. Wiench et al. (2009) reported lower sedimentation of TiO₂ NPs in NOM containing pond water, while Gao et al. (2009) limited aggregation of Ag and Cu NPs up to 100 nm in natural waters with high NOM and low salt contents. They furthermore found that the stabilising effect was counteracted when IS increased, which agrees with our conclusions and those of Keller et al. (2010). Concerning ecotoxicity test results on cladocerans, the conclusions of three studies are not entirely in agreement with each other. Wiench et al. (2009) reported a decrease in acute *D. magna* EC10 values for coated ZnO NPs in natural waters, while Blinova et al. (2010) did not. On the other hand, the latter study indicated an NOM dependent increase in acute *D. magna* EC50 values (up to 140 fold) for CuO NPs when dispersed in natural waters. Gao et al. (2009) described a decrease in acute toxicity towards *Ceriodaphnia dubia* when exposed to Ag and Cu NPs dispersed in water with increasing NOM and IS levels, although the effect of IS was not so clear in case of Ag NPs. All papers note that dissolution of Ag, Cu, CuO and ZnO most likely contributed to the observed effects. Hence, NOM could act on the dissolution and bioavailability of the formed ions. This situation differs from the current study with CeO₂ NPs, which did not dissolve (Van Hoecke et al., 2009). This difference in physico-chemical properties of the NPs might be the reason why no effect of IS on toxicity of CeO₂ NPs was observed in this study in contrast with the two other studies using TiO₂ and ZnO NPs. In addition, it is expected that the exposure pathways of daphnids used in these studies differ from the exposure pathway of the alga.

CONCLUSION

This study demonstrates that test media characteristics highly affect CeO₂ NP toxicity and stability. In fact, the standard OECD test medium without NOM should be considered a worst case as far as CeO₂ NP toxicity is concerned. In the tested synthetic and natural waters containing NOM, the observed 48 h-E_rC₂₀ values were a factor 3 to 113

higher as compared to the 3.5 mg CeO₂/L 48 h-E_rC₂₀ value obtained in standard OECD test medium at pH 7.4. Furthermore, the NOM was found to adsorb to the CeO₂ NP surface, which could explain the reduction in toxicity through a decrease in bioavailability of the particles.

An empirical model taking into account medium pH and NOM concentration could predict 48 h-E_rC₂₀ values for CeO₂ NPs in natural surface waters within a factor of 2.57.

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505 **Table 1** – Nominal & measured factor levels, mean aggregate size, control growth rates, measured & predicted 48h-E_rC₂₀ and their ratios

No.	Point ^a	Block ^b	pH ^c	NOM ^c	IS ^c	pH ^d	NOM ^d	IS ^d	Mean aggregate size ^e	Control growth rate	Experimental 48 h E _r C ₂₀	95% CI ^f	Model predicted 48 h-E _r C ₂₀	E _r C ₂₀ (obs)/E _r C ₂₀ (pred) (95 % CI)
				mg C/L	mM		mg C/l	mM	nm	1/d	mg CeO ₂ /l	mg CeO ₂ /l	mg CeO ₂ /l	
1	cube	1	8.4	2	32	8.25	2.19	33.4	7800	1.32	11.0	4.4-27.7	35.1	0.31 (0.13-0.79)
2	center	1	7.5	5	20	7.49	4.91	21.6	1710	1.53	25.0	23.0-27.1	10.9	2.29 (2.11-2.49)
3	cube	1	8.4	8	8	8.28	7.82	11.4	517	1.44	41.6	37.2-46.6	28.1	1.48 (1.33-1.66)
4	cube	1	6.6	8	32	6.68	7.82	30.7	832	1.55	183.0	164.5-203.5	189.7	0.96 (0.87-1.07)
5	cube	1	6.6	2	8	6.65	2.19	9.2	367	1.48	30.8	25.1-37.8	78.2	0.39 (0.32-0.48)
6	center	2	7.5	5	20	7.55	4.97	22.3	2650	1.58	25.0	22.5-27.7	8.3	3.00 (2.71-3.34)
7	cube	2	6.6	2	32	6.6	1.85	34.2	1410	1.41	51.7	35.5-75.1	81.1	0.64 (0.44-0.93)
8	cube	2	6.6	8	8	6.6	8.09	11.5	185	1.50	204.6	186.3-224.8	218.4	0.94 (0.85-1.03)
9	cube	2	8.4	8	32	8.34	8.09	34.1	7320	1.40	38.4	31.6-46.8	32.1	1.20 (0.98-1.46)
10	cube	2	8.4	2	8	8.36	1.85	11.8	6150	1.54	30.5	26.7-34.8	53.8	0.57 (0.50-0.65)
11	center	3	7.5	5	20	7.49	5.43	25.8	1850	1.52	31.4	26.3-37.5	15.7	2.00 (1.68-2.39)
12	star	3	7.5	10	20	7.48	11.44	24.9	1010	1.58	41.7	36.8-47.2	72.9	0.57 (0.50-0.65)
13	star	3	7.5	0	20	7.47	0.00	23.9	4880	1.60	4.7	3.8-5.7	-34.8	n.a.
14	star	3	7.5	5	40	7.44	5.43	43.7	3080	1.44	31.8	28.7-35.1	19.2	1.65 (1.49-1.83)
15	star	3	7.5	5	0	7.47	5.43	3.7	2160	1.70	34.0	30.8-37.6	17.0	2.00 (1.81-2.21)
16	star	3	6.0	5	20	6.00	5.43	23.4	537	1.37	395.8	339.8-461.0	354.9	1.11 (0.96-1.30)
17	star	3	9.0	5	20	8.91	5.43	26.1	9790	0.97	257.5	106.1-624.8	144.7	1.78 (0.73-4.32)
Ankeveensche plas			n.a.			7.36	11.74	10.3	4860	1.73	103.2	90.4-117.7	95.7	1.08 (0.95-1.23)
Markermeer			n.a.			8.11	6.61	10.6	14930	1.64	46.3	5.4-398.1	18.0	2.57 (0.30-22.12)
Le Vuyon			n.a.			6.77	2.18	2.8	1550	1.75	80.3	74.7-86.3	55.2	1.45 (1.35-1.56)
Ourthe orientale			n.a.			6.78	1.05	3.4	2780	1.75	69.6	64.1-75.6	31.5	2.21 (1.95-2.40)

- 506 ^a: Identification of data point in central composite design. Either cube, center or star points are used in the design (Box and Draper, 1987).
- 507 ^b: Refers to the experimental block/week in which the experiment was performed.
- 508 ^c: Nominal factor levels as specified in the central composite design.
- 509 ^d: Measured factor levels.
- 510 ^e: as determined by DLS (media no. 3, 4, 5, 8 and 16) or laser diffraction.
- 511 ^f: CI = confidence interval.

512 **Table 2** – Adsorption of NOM to CeO₂ NPs in OECD medium (220 mg CeO₂/L) with
513 various pH and IS. Mean (standard deviation) of 2 replicated experiments are given.

pH	IS	NOM adsorbed
	mM	mg C
6.0	0	0.038 (0.009)
7.5	0	0.034 (0.010)
9.0	0	0.017 (0.002)
6.0	20	0.053 (0.014)
7.5	20	0.045 (0.002)
9.0	20	0.006 (0.008)
6.0	40	0.062 (0.007)
7.5	40	0.045 (<0.001)
9.0	40	0.017 (0.004)

514

515 **Table 3** – Summary of model coefficients, standard errors, p-values and percentage of
 516 variance explained by each factor as determined by non linear estimation. The
 517 mathematical expression of the model is: $48h-E_rC20 = AxpH + BxpH^2 + CxNOM +$
 518 $DxpHxNOM + E$.

	A	B	C	D	E
E_rC20	-1626.4	109.45	116.49	-14.317	6007.2
Std. error	222.5	15.06	31.95	4.263	824.7
p-value	1.5×10^{-5}	1.6×10^{-5}	3.8×10^{-3}	6.4×10^{-3}	1.6×10^{-5}
% of variance explained	48.4	31.0	7.8	6.5	/
Cumulative % variance explained: 93.7					

519

Figure legends

Figure 1 – Particle size distributions of 22 mg/L 14 nm CeO₂ NPs in OECD algal medium without and with 2.2 and 7.4 mg C/L NOM.

Figure 2 – Concentration-response curves of 14 nm CeO₂ NPs in OECD algal medium at pH 7.4 without and with 2.2 and 7.4 mg C/L natural organic matter (NOM) (A) and the derived 48 h-E_rC₁₀ and E_rC₂₀ values as a function of the NOM concentration (B). Error bars in panel B present 95 % confidence interval on E_rC_x values.

Figure 3 –Scatterplot of average aggregate sizes as determined by DLS or laser diffraction. The label near each data point refers to the same number listed in Table 2. As given in the figure legend, the grey scale of the label refers to NOM content label magnitude refers to IS.

Figure 4 – Scatterplot of 48h-E_rC₂₀ values as a function of pH, NOM and IS in synthetic waters. The label near each data point refers to the same number listed in Table 2. The grey scale of the label refers to pH and label magnitude refers to IS. Error bars represent 95 % confidence interval on 48h-E_rC₂₀ value.