

20 **ABSTRACT**

21 The influence of pH (6.0-9.0), natural organic matter (NOM) (0-10 mgC/L) and ionic
22 strength (IS) (1.7-40 mM) on 14 nm CeO₂ NP aggregation and ecotoxicity towards the alga
23 *Pseudokirchneriella subcapitata* was assessed following a central composite design. Mean
24 NP aggregate sizes ranged between 200 and 10000 nm. Increasing pH and IS enhanced
25 aggregation, while increasing NOM decreased mean aggregate sizes. The 48 h-E_rC₂₀s
26 ranged between 4.7 and 395.8 mgCeO₂/L. An equation for predicting the 48 h-E_rC₂₀ (48 h-
27 E_rC₂₀ = -1626.4x(pH) + 109.45x(pH)² + 116.49x([NOM]) - 14.317x(pH)x([NOM]) +
28 6007.2) was developed. In a validation study with natural waters the predicted 48 h-E_rC₂₀
29 was a factor 1.08-2.57 lower compared to the experimental values.

30

31 **KEYWORDS**

32 CeO₂ nanoparticles – alga – natural organic matter – natural waters

33

34 **CAPSULE**

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

37 INTRODUCTION

38 The lanthanide oxide CeO₂ in its nanoparticulate form is currently being used as a
39 diesel fuel additive, as a constituent of catalytic converters and as a UV blocking agent.
40 Therefore, CeO₂ NPs are expected to enter the environment, where they may pose a
41 potential risk to aquatic and sediment organisms. The need for an environmental effects
42 assessment of these nanomaterials was expressed by international organisations such as the
43 Organisation for Economic Cooperation and Development (OECD) (OECD, 2008). A
44 previous study generated acute and chronic ecotoxicity data for three sizes of CeO₂ NPs
45 (14, 20 and 29 nm) according to the standard OECD test methods (Van Hoecke et al.,
46 2009). For 14 nm CeO₂ NPs, a 48 h 20 % effect concentration on *P. subcapitata* growth
47 rate (48 h-E_rC₂₀) (SD) of 3.5 (0.8) mg/L was obtained in the standard OECD algal test
48 medium at pH 7.4. Despite the usefulness of the standard test medium in comparing
49 experimental results, concerns have been raised regarding its environmental relevance for
50 ecotoxicity tests with manufactured nanomaterials (Crane et al., 2008; Handy et al., 2008).
51 Indeed, since physico-chemical water characteristics such as pH, ionic strength (IS) and
52 natural organic matter (NOM) concentration can vary greatly among natural surface waters,
53 the use of just one test medium at pH 7.4, low IS (1.7 mM) and in absence of NOM may
54 not be representative for realistic waters. Moreover, the above mentioned abiotic factors are
55 also known to be important parameters determining nanoparticle surface characteristics and
56 aggregation behaviour.

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

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

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

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

74

75 **MATERIALS AND METHODS**

76 *CeO₂ nanoparticles and natural organic matter*

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

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

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

88 *NOM adsorption studies*

89 The amount of NOM adsorbed to CeO₂ NPs in 20 ml OECD medium (OECD,
90 2006) containing 8.0 mg C/L NOM was measured under various conditions of pH (6.0, 7.5
91 and 9.0) and NaCl concentrations (0, 20 and 40 mM). A high concentration of CeO₂ NPs
92 was necessary to be able to detect a significant adsorption of NOM to the particle surface.
93 Therefore, suspensions containing 220 mg CeO₂/L were incubated at 25 °C under
94 continuous illumination for 48 h. Twice a day, pH values were checked and adjusted when
95 ≥ 0.2 pH units deviation was observed. The increase in IS due to pH adjustments was ≤ 0.2
96 mM. At the end of the incubation period, two 10 ml samples of each medium were
97 centrifuged for 15 minutes at 2000g. Finally, 7 ml of the supernatant was used for NOM
98 analysis, for which a TOC-500 (Shimadzu, Duisburg, Germany) carbon analyzer was used.
99 The experiment was performed twice. One-way ANOVA ($\alpha = 0.05$) was performed to
100 establish the significance ($p < 0.05$) of pH and/or IS on NOM adsorption.

101 *One factor experiment*

102 In the one factor experiment, only the concentration of NOM was varied. Two
103 environmentally relevant concentrations for river water in cool and warm temperate
104 climates, in which NOM ranges between 2 and 15 mg C/L with means of 3 and 7 mg C/L,
105 respectively (Thurman, 1985). Test suspensions were prepared by dropwise addition of the
106 14 nm CeO₂ NP stock suspension in stirred standard OECD algal medium to which 3.6 mM
107 3-(N-Morpholino)propanesulfonic acid (MOPS) buffer (pH 7.4) and the desired amount of
108 Bihain NOM (2.2 and 7.4 mg C/L) were added. Test medium containing NOM without
109 addition of CeO₂ was used as control medium. Concentrations of CeO₂ NPs ranged

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

112 *Multivariate experiment*

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

120 The 17 test media were prepared by addition of 3.6 mM buffer and the desired
121 amount of NOM to the standard OECD algal test medium and subsequent pH adjustment
122 using HCl or NaOH. To maintain a pH value of 6.0 2-(N-Morpholino)ethanesulfonic acid
123 (MES) buffer was used, while MOPS buffer adequately stabilized the media pH at values
124 6.6 and 7.5. The 8.4 and 9.0 pH values required addition of 2-
125 (Cyclohexylamino)ethanesulfonic acid (CHES) buffer. In order to assure an equal
126 minimum conductivity across the whole pH range, the test media were equilibrated at 25 °C
127 and conductivity of all media was set to 375 µS/cm using NaCl before addition of the
128 amount of NaCl as specified in the CCD. Finally, CeO₂ NP stock suspension was added by
129 dropwise addition to the stirred media, until desired concentrations between 2.2 and 460 mg
130 CeO₂/L were reached.

131 During the algal growth inhibition tests, pH, NOM and conductivity were monitored
132 daily. A pH electrode (P407, Consort, Turnhout, Belgium) was used to measure pH. In case
133 ≥ 0.2 pH units deviation from the theoretical value was observed, pH was adjusted using
134 1 M NaOH or HCl solutions. The media at pH values 8.4 and 9.0 were checked twice a day.

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

142 *Particle size distribution analysis*

143 Particle size distributions of the 22.0 mg/L 14 nm CeO₂ NP suspensions were
144 analyzed using dynamic light scattering (DLS) three days after the start of the algal growth
145 inhibition test. In case of severe aggregation resulting in aggregates with a Z-average
146 hydrodynamic diameter (as determined by DLS) > 1000 nm, laser diffraction was
147 performed with a Mastersizer (Malvern Instruments, Worcestershire, UK) equipped with a
148 300 RF fourier lens to analyze particle size distributions. More details on instrument
149 settings can be found in the supporting information.

150 *Algal toxicity testing*

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

159 Each of the test media from both the one factor and the multivariate experiment was
160 used to prepare one control medium and five different test concentrations of CeO₂ NPs.
161 Equilibration took place overnight at 25 °C in the dark. Subsequently, four 50 ml portions
162 of each concentration were transferred to 100 ml erlenmeyer flasks, three of which were
163 inoculated with 10,000 cells/ml of the grown culture and one was used as a background
164 correction. One 50 ml portion of a 22.0 mg CeO₂/L suspensions was sealed with parafilm®
165 to serve for aggregation behaviour analysis. All flasks were incubated at 25 °C under
166 illumination and were shaken manually three times a day. After 24, 48 and 72 h, the algal
167 cell density was measured using a cell counter (Beckman Coulter Counter, Ghent,
168 Belgium). The average specific growth rate μ (1/d) in each concentration was calculated
169 after 48 h exposure, because the test validity criteria were not met in 4 experiments of the
170 CCD when data was analyzed after 72 h. More specifically, the mean coefficient of
171 variation (CV) on sectional growth rate, which checks for exponential growth in the control
172 throughout the test, was > 35 %. In this case the OECD test protocol prescribes the use of
173 48 h toxicity data.

174 *Data treatment and analysis*

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

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

188 *Validation study with natural waters*

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

200 **RESULTS**

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

202 Addition of NOM to the OECD algal test medium prior to spiking CeO₂ NPs highly
203 affected both CeO₂ suspension stability and toxicity in comparison with the NOM free
204 standard OECD algal test medium at pH 7.4. The particle size distributions and
205 concentration-response curves are presented in **Figures 1 and 2**, respectively. While severe
206 aggregation in absence of NOM resulted in a mean particle size of 4500 nm, the NOM
207 stabilized small particle aggregates of 100-200 nm. Pictures are given in the supporting
208 information. At the low NOM concentration of 2.2 mg C/L, 48 h-E₁₀C10 and E₁₀C20 values

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

213 *NOM adsorption to CeO₂ NPs*

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

220 *Central composite design to test the influence of pH, NOM and IS on aggregation* 221 *behaviour and 48 h-E_rC20 of CeO₂ NPs*

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

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

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

245 The 48 h- E_rC_{20} values and their 95 % confidence interval are given in **Table 1**. A
246 factor of 84 separates the lowest E_rC_{20} value of 4.7 from the highest of 395.8 mg CeO_2/L .
247 Significant linear pH and NOM effects ($p = 0.0041$ and 0.017 , respectively) were found in
248 the CCD analysis, while pH also showed a significant quadratic effect ($p = 1.8 \times 10^{-4}$).
249 Furthermore, an interaction effect between pH and NOM was identified ($p = 0.030$). Except
250 for pH values of 8.4, increasing the NOM concentration lead to a decrease in toxicity,
251 which is in agreement with the first experiment. **Figure 4** plots the 48 h- E_rC_{20} s as a
252 function of the three factors. The significant effects identified by the CCD were
253 subsequently used in the non linear regression analysis in order to derive an empirical
254 model. As a result, a mathematical expression of the form: $48 \text{ h-}E_rC_{20} = Ax(pH) +$
255 $Bx(pH)^2 + Cx([NOM]) + Dx(pH)x([NOM]) + E$ was derived. In this equation, pH and
256 [NOM] are expressed numerically, with [NOM] expressed as mg C/L. **Table 3** summarizes
257 the parameter coefficients, standard errors, p-values and percentage of total variability
258 explained by each effect. In total, 93.7 % of the variability in 48 h E_rC_{20} values could be

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

263 *Validation study*

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

276 **DISCUSSION**

277 *Effect of NOM only*

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

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

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

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

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

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

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

316 When combining data on mean aggregate size and 48 h-E_rC₂₀, it became clear that
317 the more stable suspensions gave rise to the lowest observed toxicities (data points 4, 8 and
318 16 listed in Table 1). In Figure S5 in supporting information, the logarithms of 48 h-E_rC₂₀
319 of each medium are plotted against average aggregate sizes as determined by DLS and laser
320 diffraction. Interestingly, a negative linear relationship was established ($p = 1.6 \times 10^{-10}$).

321 A clear relation between CeO₂ toxicity and aggregation behaviour and the affinity
322 towards NOM adsorption follows from **Figures 3 and 4** and **Table 2**. For CeO₂ toxicity and
323 stability as well as its affinity towards NOM, the pH was the dominant factor. Adsorption
324 of NOM was favoured at low pH values (6, 6.6), which resulted in the lowest toxicity and
325 highest stability observed. On the other hand, NOM adsorption decreased as pH increased,
326 which adversely affected stability and toxicity. From these observations, it was clear that
327 adsorption of NOM to the CeO₂ NP surface prevented the particles from directly interacting
328 with each other and with algal cells. The decrease in toxicity therefore was due to a
329 reduction in bioavailability of the particles. A similar observation was made by Li et al.
330 (2010), who reported a decrease in the toxicity of zerovalent iron NPs towards *E. coli*,
331 which the authors attributed to the loss of adhesion of NOM coated particles to the bacterial
332 cell wall.

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

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

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

363 *CeO₂ NP toxicity and behaviour in natural waters*

364 The observed E_rC₂₀ values in the four natural waters corresponded to the predicted
365 values within a factor of 2.57. In previous experiments with the 14 nm CeO₂ NPs the
366 individual 48 h-E_rC₂₀ measurements differed by a factor of 2.1. As a consequence, the
367 discrepancy between measured and model predicted 48 h-E_rC₂₀s cannot be explained by
368 experimental variability only. The composition of NOM is known to differ between natural
369 waters and could have influenced the adsorption affinity and stabilizing potency towards
370 NPs. Secondly, the higher concentration of divalent ions such as Ca²⁺ and Mg²⁺ in the
371 natural waters compared to the synthetic waters may help explain the observed discrepancy.
372 The test media used in the CCD analysis all have a hardness of 24 mg CaCO₃/L. With
373 exception of Le Vuyon natural water, the river waters all have a much higher hardness of
374 53-267 mg CaCO₃/L. The divalent ions Ca²⁺ and Mg²⁺ are also more effective in
375 destabilising particle suspensions, which is known as the Schulze-Hardy rule. Furthermore,
376 Sander et al. (2004) reported the ability of Ca²⁺ at a concentration of 10 mM to bridge
377 adsorbed NOM layers on several particles, thereby enhancing the aggregation process.
378 Therefore, the large mean aggregate sizes observed in natural waters (Table 1) may be
379 partly explained by the higher hardness.

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

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

403 **CONCLUSION**

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

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

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

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503 mobility and reproduction of the freshwater invertebrate *Daphnia magna*. *Chemosphere* 76,
504 1356–1365.

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

520 **Figure legends**

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

523

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

528

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

533

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