Toxicity, bioaccumulation and trophic transfer of engineered nanoparticles in the aquatic environment

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Chapter 2

Effects of humic substances on the aqueous stability of cerium dioxide nanoparticles and their toxicity to aquatic organisms

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Published in *Science of the Total Environment* 2021, 781: 146583

Abstract

The impacts of humic substances (HS) on the aquatic stability and toxicity of cerium dioxide nanoparticles (CeO_2NPs) to three organisms with different exposure characteristics were investigated. Addition of HS to suspensions of CeO_2NPs lowered the surface zeta potential of the particles, reduced their hydrodynamic size, and increased the energy barrier as indicated by the total potential energy profile. This resulted in a more stable suspension compared to suspensions without HS added. Moreover, a higher concentration of HS further stabilized CeO_2NPs in the suspension. Acute toxicity of the suspensions to the unicellular green alga *Raphidocelis subcapitata* and to the crustacean *Chydorus sphaericus* was lower as compared to exposure without HS added. The acute toxicity of CeO_2NPs suspensions to the zebrafish (*Danio rerio*) eleutheroembryo was on the other hand significantly enhanced (additive and synergistic) upon increasing HS concentration. Our findings emphasize that HS is important to stabilize the nano-suspensions and that its impact on CeO_2NPs toxicity differs across different aquatic organisms. Emphasizing the exposure characteristics of each of the organisms selected from the trophic levels can explain how particle stability impacts particle toxicity.

Keywords: CeO_2NPs; Stability; Toxicity; Humic substances; Aquatic test species

2.1 Introduction

The prospects of engineered nanoparticles (ENPs) of different sizes, shapes, and material properties in a number of applications have progressed rapidly (Guinée et al., 2017; Martínez et al., 2020), although the market benefits brought about by ENPs have also created some concerns of their possible effects on human health and environmental safety (Savolainen et al., 2013; Baun et al., 2017; Deng et al., 2017). Many studies emphasized the challenging relationship between typical ENPs features such as shape, size, modifications and the abiotic factors of the environment such as pH, divalent cation ions, dissolved organic carbon (Lu et al., 2017; Liu et al., 2018; Yu et al., 2018; Singh et al., 2021). Understanding how ENPs interact with the environment can assist in predicting the fate and effect of ENPs and may provide a basis for their ecological risk assessment.

Cerium dioxide nanoparticles (CeO_2NPs) is increasingly being applied in fuel additives and polishing agents (Collin et al., 2014). To date, the estimated production of CeO_2NPs is around 1000 tons/year and it has become one of the most produced ENPs in the world (Piccino et al., 2012). Upon their release into the aquatic environment, CeO_2NPs may provoke adverse effects to various organisms (Rundle et al., 2016; Taylor et al., 2016; Kosak et al., 2018; Wang and Nowack 2018; Correia et al., 2020).

Natural organic matter (NOM) is ubiquitous in every water and is known to affect the fate and toxicity of ENPs by modifying the surface properties (e.g., charge and hydrophobicity), subsequently affecting their stability as well as nanoparticle-cellular interactions (Van Hoecke et al., 2011; Loosli et al., 2013; Baalousha et al., 2018; Liu et al., 2020). Evidence was found that the presence of Suwannee River

and Bihain NOM can stabilize CeO_2NPs in an algae growth medium (Quik et al., 2010). Moreover, Suwannee River NOM can alleviate the adverse effects of NPs on algal growth (Cerrillo et al., 2016). However, how the CeO_2NPs-NOM interaction alters the toxicity of CeO_2NPs to aquatic organisms is not completely understood.

This work aimed to explore the impact of humic substances (HS) on the aqueous stability and subsequent toxicity of CeO_2NPs to different organisms. The aqueous stability of CeO_2NPs in the presence of HS was determined by measuring the zeta potential of suspensions and the sizes of the agglomerates formed, whereas suspension concentrations were measured in simulated natural environmental conditions. Short-term experiments were performed with aquatic organisms with different exposure characteristics, namely the microalga *Raphidocelis subcapitata*, the microcrustacean *Chydorus sphaericus*, and the fish *Danio rerio*. The different exposure characteristics of the organisms assist in understanding the actual exposure of aquatic organisms to nanoparticles and the effective interactions with nanoparticles. The starting hypotheses were:

$H0_1$ = Algal cells and HS will compete for binding with the nanoparticles. Thus, it is expected that the addition of HS to CeO_2NPs suspensions will diminish the toxicity of the suspensions to algae.

$H0_2$ = The microcrustacean are bottom-feeders that graze on the bottom of the vessels. Stabilization of CeO_2NPs suspensions by HS decreases the toxicity to Chydoridae compared to relative instable CeO_2NPs suspensions without HS addition.

$H0_3$ = Nanoparticle sedimentation increases the particle concentration at the bottom of the test well and that is where the zebrafish embryos reside. Accordingly, the embryos are exposed to CeO_2NPs at a higher extent when the CeO_2NPs is not stabilized. When

hatched, the larvae swim around and mix the CeO_2NPs and thus higher toxicity is expected without HS added.

2.2 Materials and Methods

2.2.1 Test materials and medium

CeO_2 nanoparticles were supplied by Umicore Ltd as powders. The particles were redispersed and stocked by milling into Milli-Q water as 10 wt% suspensions at pH 4 containing nitric acid, as well as stored at 4 °C for later use. Moreover, the test suspensions were characterized immediately after their preparation. The primary particle size of CeO_2NPs was 20 nm and the specific surface area was 42 m^2/g as determined by the manufacturer. The model HS purchased from J&K Chemical, a humic acid sodium salt (50–60% as humic acid, CAS: 68131-04-4), was selected to mimic natural organic matter (NOM). The humic acid sodium salt acts as a proxy for humic-like substances that were also identified as a major component of NOM. The exposure medium was defined to be the Dutch standard (DSW) (Hermsen et al., 2011) consisting of $NaHCO_3$ (1.19 mM), $KHCO_3$ (0.20 mM), $CaCl_2$ (1.36 mM), and $MgSO_4$ (0.73 mM) with a pH adjustment of 8.0 ± 0.2.

2.2.2 Treatments and concentrations tested

Drop-wise addition of the CeO_2NPs stock suspensions into the DSW was carried out to prepare the different test dosages of the nanoparticles, which were 0.5, 1, 5, 10, 25, 50, 100 mg/L. Moreover, the test suspensions of the CeO_2NPs were stirred for 24 h in the dark.

A HS stock solution was prepared by dissolving 100 mg/L by stirring for 24 h at room temperature and filtering over a 0.20 μm nylon membrane filter prior to use. The actual HS concentrations of suspensions of nominal 0.5, 10, and 40 mg C/L in the DSW were measured by using a Thermo Hiper TOC analyzer. The CeO_2NPs suspensions in the presence of HS were prepared by adding the CeO_2NPs stock suspensions into the HS solutions. The suspensions of CeO_2NPs with HS were further stirred for 24 h in the dark. The pH values of all samples were adjusted to 8.0 ± 0.2 using a 1 mol/L HCl or NaOH solution.

2.2.3 Physical and chemical analysis

The physical and chemical measurements were performed on the 10 mg/L CeO_2NPs suspension in glass flasks (5 cm in diameter and 10 cm in height) with 100 mL under the same conditions as the toxicity testing for 0, 24, 48, 72, and 96 h. Zeta potentials, mean (the average of all particles) particle diameters, and particle concentrations of suspensions in the absence and presence of HS were determined at the end of different intervals. The zeta potential was measured by a ZetaSizer (Nano series, Malvern Instruments) in triplicate. The particle size distribution of CeO_2NPs in the DSW was measured by a Nanosight LM20 system (NanoSight Ltd., Salisbury, UK) using Nanoparticle Tracking Analysis (NTA) (software version 1.5). From the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Cosgrove 2005), the stability of a particle in the test medium was determined by simulating the total potential energy for interactions between the CeO_2 particles in the absence and presence of HS. The detailed process and parameter settings of the DLVO calculations are

described in the Supplementary Information. The total Ce concentration was determined by high resolution inductively coupled plasma mass spectroscopy (Element 2 HR-ICP-MS, Thermo, Bremen, Germany) after a 2 h hot open destruction treatment with 1 mL hydrogen peroxide (9.8 mol/L) and 7 mL nitric acid (14.4 mol/L) at 103°C.

2.2.4 Bioassays

Toxicity of the CeO_2NPs in the absence and presence of HS to the test organisms was assessed by concentration–response experiments. For each batch of toxicity tests, a control consisting of the test medium was included to ensure that the observed effects were associated with exposure to the test compounds.

Microalga *R. subcapitata* from a continuous culture were suspended in DSW to a total volume of 3 mL in glass vials, to obtain a final density of 1×10^6 cells/mL. The inhibition of 4.5 h photosynthetic efficiency was determined for the algae using a pulse-amplitude modulation fluorometer (Wang et al., 2012). The algae were exposed to various initial concentrations (0.5, 1, 5, 10, 25, 50, and 100 mg/L) of the CeO_2NPs suspensions in the absence and presence of HS (0.5, 10, and 40 mg C/L), as well as the HS alone (0.5, 10, and 40 mg C/L).

The 48 h acute immobilization tests with *C. sphaericus* followed the protocol of the Chydotox toxicity test developed in the National Institute of Public Health and the Environment (Netherlands) (Verweij et al., 2009). Twenty neonates (< 24 h) at each exposure concentration, divided into four batches of five animals each, were added to a single droplet of DSW with a diameter of approximately 5 mm in the glass vial. 250 µL test solution was added into each vial.

The vials were covered with a crimp cap to prevent evaporation and incubated for 48 h in a climate room at 20 °C and a light : dark regime of 16 : 8 h.

Crustaceans *C. sphaericus* were exposed to various initial concentrations (0.5, 1, 5, 10, 25, 50, and 100 mg/L) of the CeO_2NPs suspensions in the absence and presence of HS (0.5, 10, and 40 mg C/L), as well as the HS alone (0.5, 10, and 40 mg C/L). After 48 h, the vials were placed under a reverse dissecting microscope and the immobility was determined by activation of *C. sphaericus* by slightly shaking the vial and monitoring them for 30 sec.

An early life stage test with zebrafish (*D. rerio*) embryos was performed in accordance with the procedure described by Lammer et al. (2009). Each exposure concentration and control in 3 independent experiments was tested with 10 eggs/embryos (4-64 cell stage) from 20 vital and fertilized eggs, and the selected eggs were transferred into 24-well cell culture plates with 2 mL freshly prepared control medium or test suspensions. Embryos were incubated at 26 ± 1 °C for 96 h and were exposed to three CeO_2NPs exposure concentrations (1, 10, and 100 mg/L) in the absence and presence of HS (10 and 40 mg C/L), as well as the HS alone (10 and 40 mg C/L). The development of zebrafish embryos was microscopically screened daily. Lethality as well as sub-lethal developmental morphology and teratogenicity toxicological endpoints (Hermsen et al., 2011) were scored after 96 h (Supplementary Information, Table S2.1). Embryos were scored according to the classifications given by King-Heiden et al. (2009) as (0) no toxic response, (1) one or two toxic endpoints, (2) two or three toxic endpoints, (3) more than three toxic endpoints, and (4) dead. The choice of the scores depends on the severity of the toxicity endpoint. In order to avoid overestimating the toxicity of the test

materials, a lower score was selected if a certain toxicity endpoint of an embryo was slightly damaged.

2.2.5 Toxicity evaluating of CeO_2NPs in the presence of HS

CeO_2NPs with the addition of HS can be regarded as a binary mixture system. The observed mixture toxicity effect, ME_{obs}, determined in the toxicity testing was compared with the theoretically predicted mixture toxicity effect, ME_{pre}, calculated using a probability theory based model (Hadjispyrou et al. 2001):

$$ME_{pre} = E_{nCeO2} + E_{HS} - (E_{nCeO2} \cdot E_{HS}/100) \quad (1)$$

where E_{nCeO2} is the single toxicity effect of CeO_2NPs and E_{HS} is the single toxicity effect of HS.

The result was applied to reflect a synergistic or antagonistic effect if ME_{obs} was significantly higher or lower than ME_{pre}, respectively. On the contrary, the interaction of the binary mixture was considered as an additive effect only if there was no significant difference between ME_{obs} and ME_{pre}. The presented method was applied to predict the toxicity of the studied systems to *D. rerio*, which is due to the fact that there was no observed toxicity to the algae and to the cladoceran induced by the HS studied.

All values were reported as mean ± standard deviation (SD). SD of the mean was calculated from parallel experiments. Statistically significant differences between test treatments in the present study were determined by the Student's *t*-test. The significance levels $p < 0.05$, $p < 0.01$, and $p < 0.001$ were used.

2.3 Results and Discussion

2.3.1 Effects of HS on aqueous stability of CeO_2NPs

The variation of the zeta potential values of CeO_2NPs with the HS concentrations and with the time of incubation is shown in Figure 2.1A. The zeta potential values of the CeO_2NPs were found to be negative and changed slightly over time. Furthermore, the zeta potential values were more negative when the HS were present compared with the zeta potential values when the HS were absent, implying that the HS can influence electrokinetic properties, causing an increased nanoparticle surface charge. The HS effect on the particle zeta potential depended on the HS concentrations, irrespectively of the time of incubation.

Observed changes in the mean particle diameters with the HS concentrations over the experimental duration in the absence and presence of HS are presented in Figure 2.1B. The CeO_2NPs particles immediately agglomerated in DSW-medium at an initial mean particle diameters size of 218 nm, which is a tenfold higher than the primary size (20 nm). This implies that the CeO_2NPs particles were agglomerated. Upon increasing HS concentrations, the mean particle diameters of the CeO_2NPs agglomerates decreased. This may be explained because the HS acts as a polyelectrolyte or surfactant, thus providing electrostatic repulsive forces of the particles (Baalousha et al., 2008; Domingos et al., 2009). This stabilization of the particles by HS and subsequent decrease of the mean particle diameters of the agglomerates, was detectable regardless of exposure time.

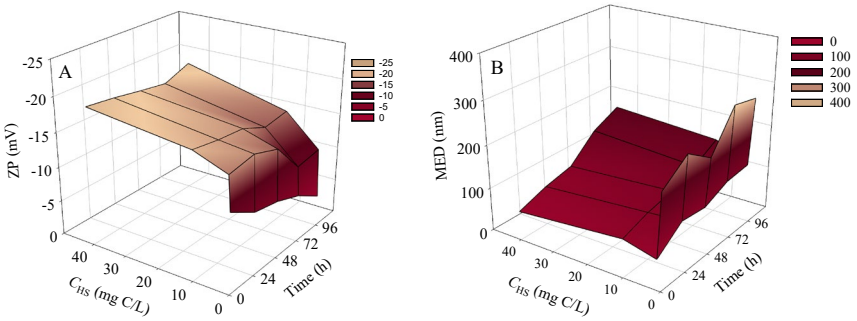

Figure 2.1 Zeta potential (ZP) (A) and mean particle diameter (MED) (B) of the CeO_2NPs suspensions with particle concentration of 10 mg/L in the absence and presence of different concentrations of HS (C_{HS}) (0.5, 10, and 40 mg C/L) (pH = 8.0). Results are expressed as mean (n = 3)

On the basis of the DLVO theory (Cosgrove 2005), the total potential energy profiles for the CeO_2NPs colloids with nanoparticle concentrations of 10 mg/L were calculated (Figure 2.2). The magnitudes of the primary maximum of the CeO_2NPs colloids in the presence of the different concentrations of the HS follow the order: CeO_2NPs + 10 mg C/L HS (19.1 K_BT) is approximately equal to CeO_2NPs + 40 mg C/L HS (18.9 K_BT) > CeO_2NPs + 0.5 mg C/L HS (9.8 K_BT) > CeO_2NPs (6.6 K_BT), implying that the stability of the colloids studied follows the order: CeO_2NPs + 10 mg C/L HS is approximately equal to CeO_2NPs + 40 mg C/L HS > CeO_2NPs + 0.5 mg C/L HS > CeO_2NPs. It is the primary maximum in the total energy which provides the mechanism for the stability of the charged colloidal particles. As two particles approach each other, they must collide with sufficient energy to overcome a barrier provided by the primary maximum. Thus, the larger the barrier, the longer the system will remain stable. In the present study, we also observed that the

stability of the binary systems of CeO_2NPs + 10 mg C/L HS is approximately equal to the stability of the combination of CeO_2NPs + 40 mg C/L HS. This also implies that only a part of the HS at the highest concentrations (40 mg C/L HS) could be actually adsorbed.

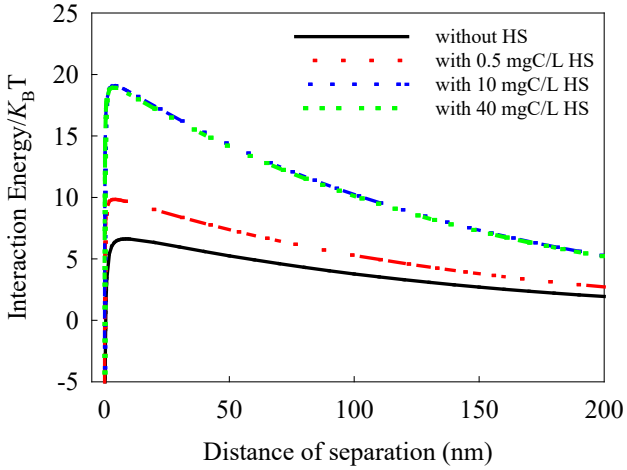

Figure 2.2 Total potential energy curves for the CeO_2NPs with particle concentration of 10 mg/L in the presence of HS of 0.5, 10, and 40 mg C/L

The stability of CeO_2NPs in suspensions depends on the total potential energy of interaction between colloidal particles according to the DLVO theory. In the present study, the steric stabilization induced by HS was not considered in calculating the interaction energy between the colloidal particles as the thickness of the surface coating layer was found to be very small (≤ 0.8 nm) (Baalousha et al., 2008). Moreover, the studied HS analogue is a small molecule (MW: 226.14 g/mol) without any significant spatial extent and CeO_2NPs is thus considered to be a hard sphere. Although these assumptions and DLVO calculations are simplistic, they are indicative of the interaction

forces between the nanoparticles and therefore the aggregation mechanisms (Baalousha 2009).

Figure 2.3A shows the variation of the ratios of the particle concentrations of suspensions to the initial particle concentration of CeO_2NPs in the absence and presence of HS over time. In the first 24 h this decline was steep and similar for all treatments. In the time span of 24 h to 96 h this steep decline could only be observed in the treatment without HS addition. The treatments with HS addition showed a dose-dependent stabilization of the CeO_2NPs suspensions. As shown in Figure 2.3B, the CeO_2NPs-HS interaction resulted in a co-sedimentation behavior of HS and CeO_2NPs. This evidence supports the adsorption of HS upon the surface of the particles. Over 96 h, the amount of HS adsorbed on the particles was estimated to be approximately 0.1, 1.2, and 1.8 mg C/L corresponding to the initial HS concentration of 0.5, 10, and 40 mg C/L, respectively.

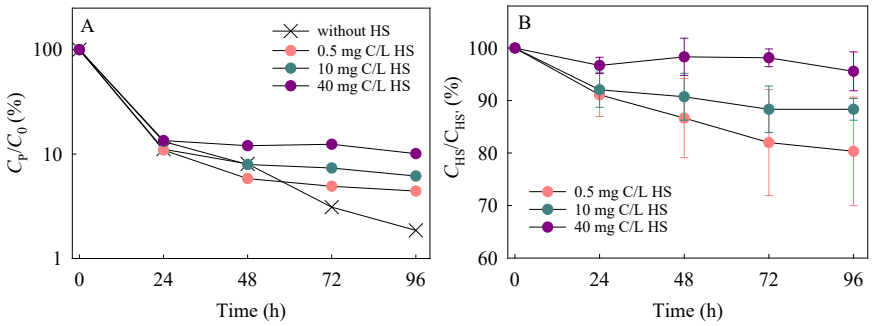

Figure 2.3 Ratios of the suspended particle concentrations (C_P) to the initial particle concentration (C_0, 10 mg/L) of CeO_2NPs in the absence and presence of HS (A, results are expressed as a single value) and ratios of the concentration of HS in the suspensions of CeO_2NPs in the presence of HS (C_{HS}) to the concentration of only HS (C_{HS}') (B, results are expressed as mean ± SD (n = 3))

2.3.2 Effects of HS on aquatic toxicity of CeO_2NPs

Concentration-dependent effects obtained for CeO_2NPs in the presence of different initial HS concentrations for the algae and cladoceran are given in Figure 2.4, and for the zebrafish embryos in Figure 2.5A. Throughout the test duration the *R. subcapitata* cells are gentle shaken continuously on a shaking table, hence the algal exposure was mainly within the water column where the nanoparticles were as well, representing relative high chances for exposure. As shown in Figure 2.4A, the addition of 10 and 40 mg C/L HS to the CeO_2NPs suspensions significantly inhibited the acute toxicity measured as photosynthetic activity to *R. subcapitata* ($p < 0.05$). Note that no toxic effects on the algae were observed for the HS. This means that the presence of 10 or 40 mg C/L HS interfered with the interaction of CeO_2NPs with the algal cells and weakened the toxic effect. This interference might also be explained by the fact that the physicochemical analysis indicated adsorption of HS to the CeO_2NPs particles, which subsequently hinders the particles from directly interacting with algal cells. Van Hoecke et al. (2011) also concluded that decrease in algal toxicity might be due to a reduction in bioavailability of the particles when NOM is present. Cerrillo et al. (2016) found that NOM alleviated the adverse effects of CeO_2NPs on algal growth to some extent and suggested a 'camouflage' effect of CeO_2NPs.

The Chydoridae species is commonly grazing on the bottom of a vessel and sedimentation would increase exposure in case of particle agglomeration and subsequently sedimentation. These processes were largely affected by the addition of HS, with treatments without HS addition yielding relative instable nanoparticle suspensions. The

acute toxicity of the CeO_2NPs to *C. sphaericus* (Figure 2.4B) was mitigated when HS was present, particular for the concentration of 40 mg C/L HS. The physicochemical analysis here also showed that HS stabilized the CeO_2NPs suspensions. Taking into account that *C. sphaericus* is a benthic cladoceran species and is likely to ingest sediment particles into the gut and adsorb them on to carapaces, the reduced toxicity to *C. sphaericus* might be explained by the fact that a lower amount of NPs were allowed to sediment and subsequently, a lower amount of particles was accumulated in the cladoceran when HS was present as compared to the situation in which HS were absent. The analysis for the variation of the initial particle concentration of CeO_2NPs in the absence and presence of HS over time (Figure 2.3A) also supports the conclusion that relatively more particles were stabilized when HS were present during the exposure.

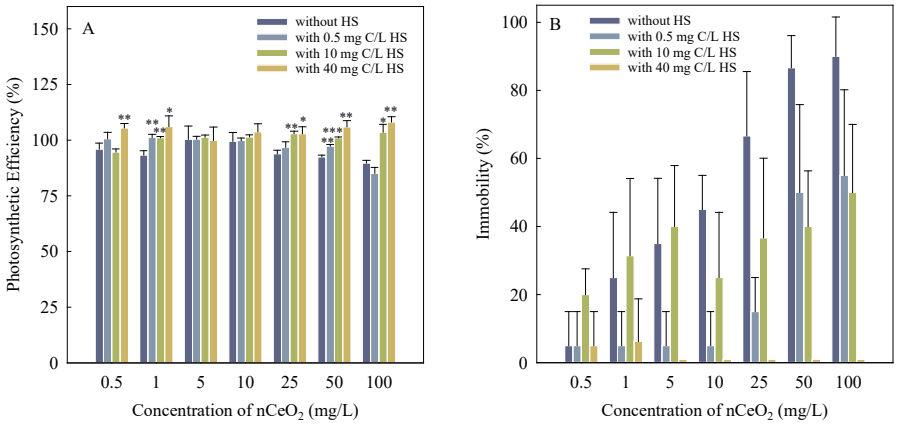

Figure 2.4 Concentration-dependent effects for *Raphidocelis subcapitata* (A) and *Chydorus sphaericus* (B) exposed to CeO_2NPs suspensions in the presence and absence of humic substances (HS) (*t*-test $*p < 0.05$ when comparing the treatments of CeO_2NPs without HS to CeO_2NPs with HS). Results are expressed as mean ± SD ($n = 3$

for *R. subcapitata* and $n = 4$ for *C. sphaericus*)

As shown in Figure 2.5A, the toxicity of CeO_2NPs to the larvae of *D. rerio* in the presence of 40 mg C/L HS was significantly enhanced in comparison with the toxicity of CeO_2NPs to the larvae in the absence of HS. In addition, the toxicity of 10 mg C/L HS alone treatment was equivalent to the toxicity of 1 and 10 mg/L CeO_2NPs and significantly lower than 100 mg/L CeO_2NPs. Thus, it can be concluded that concomitant exposure of the HS and CeO_2NPs caused the toxicity to *D. rerio*. The observed toxicity effects of the binary mixtures of the HS and CeO_2NPs were compared with their predicted toxicity (Figure 2.5B), calculated using the result of individual toxic effects based on the equation 1. No significant differences were observed for the cases 1 and 10 mg/L CeO_2 + 10 mg C/L HS between the results of the observation and prediction ($p < 0.05$), implying that these combinations showed additive action (observed toxicity similar to expected toxicity). The predicted mixture toxicity of the treatments 100 mg/L CeO_2 + 10 mg C/L HS and 1 and 10 mg/L CeO_2 + 40 mg C/L HS was significantly greater than their observed mixture toxicity, indicating that these combinations had synergistic effects. In addition, the average value of the predicted toxicity of the binary mixture of 100 mg/L CeO_2 and 40 mg C/L HS was higher than the observed toxicity, although there was no significant difference between the observation and prediction.

The images of zebrafish larvae exposed to different concentrations of CeO_2NPs in the absence and presence of HS at 96 h depict the concentration-dependent malformations during development. Compared to the control (Figure 2.5C), to the HS alone (Figure 2.5D, no obvious anomalies), and to the individual CeO_2NPs (Figure 2.5E,

only scoliosis), the combination of CeO_2NPs and 40 mg C/L HS induced more morphology and teratogenicity toxicity endpoints including pericardial oedema, yolk sac oedema, and scoliosis (Figure 2.5F). Here again, the presence of HS stabilized the particles of suspensions by decreasing their agglomerated MED size, implying that the zebrafish embryo would be exposed to the particles with lower size. This also means that the particles in suspensions with lower size induced more toxicity to the zebrafish larvae.

Thereupon, the zebrafish eggs with a chorion lie at the bottom of the test well. The chorion is considered as an effective barrier to protect the embryo from uptake of nanoparticles (Brun et al., 2018; Brinkmann et al., 2020). Due to the sedimentation of CeO_2NPs, the zebrafish embryos were exposed to a higher extent to the nanoparticles. However, the chorion protected the zebrafish larvae against CeO_2NPs toxicity. After the hatching, the zebrafish larvae actively swam around in the water column and mixed with the nanoparticles. As aforementioned, the HS stabilized more nanoparticles than when the HS was absent. Consequently, the direct exposure of the zebrafish larvae to CeO_2NPs in the presence of HS was relatively higher than the direct exposure of the zebrafish larvae to CeO_2NPs in the absence of HS, which could lead to a higher toxicity induced by CeO_2NPs when the HS was present. Furthermore, increasing the amount of the HS increased the severity of toxicity. Taken together, comparison analysis on the toxicity testing results suggests that the studied HS had a dual impact on the aquatic toxicity of CeO_2NPs, depending on the exposure characteristics of the test species. Moreover, the HS concentration modulates its degree of influence on the toxicity.

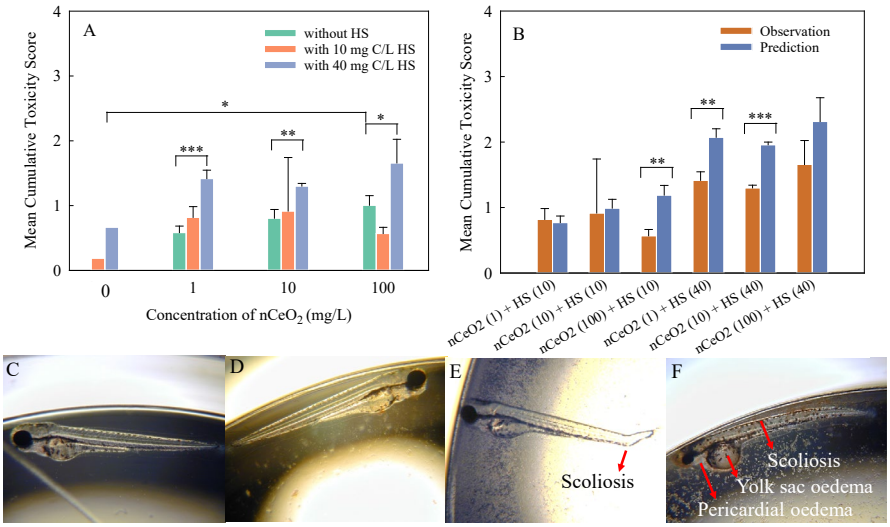

Figure 2.5 Concentration-dependent effects (A) for *Danio rerio* exposed to the CeO_2NPs suspensions in the presence and absence of HS, summary of the observed and expected toxic effects (B) of the binary mixtures of the CeO_2NPs (1, 10, 100 mg/L) and HS (10 and 40 mg C/L). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when comparing the treatments, results are expressed as mean ± SD ($n = 3$). Photos of zebrafish larvae exposed to different concentrations of CeO_2NPs in the absence and presence of HS at 96 h (C: control; D: 40 mg C/L HS; E: 10 mg/L CeO_2; F: 10 mg/L CeO_2 + 40 mg C/L HS)

2.4 Conclusions

Our results reveal how and to what extend the stability of CeO_2NPs was enhanced when HS was present in the aquatic medium at concentrations exceeding 0.5 mg C/L. Like expected by means of hypothesis H01, the addition of HS mitigated the CeO_2NPs suspensions toxicity to algae *R. subcapitata*. The $H0_2$ for the *C.*

sphaericus species was accepted; CeO_2NPs suspensions stabilized by HS were less toxic as compared to relative instable suspensions of CeO_2NPs without HS added. Suspension of CeO_2NPs without HS added were more toxic to zebrafish larvae, which made us reject the Ho_3.

The extent and the effects of HS on the toxicity were associated with the concentration (ranging from 0.5 to 40 mg C/L) of HS added. Furthermore, the aqueous stability of CeO_2NPs and the aquatic species influenced the toxicity of the particles in the presence of HS. Our results are stepping stones towards improving the understanding the processes that determine the actual exposure of a suite of aquatic organisms to exposure media of different composition, mimicking to an increasing extend natural aquatic systems. Understanding the exposure characteristics of the organisms selected – explicitly considering where in the water column of the experimental test the organisms is most present will explain if stabilization of nanomaterials with HS or any other type of NOM will affect the toxicity of the nanomaterials.

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Supplementary Information

The classical DLVO calculation

Based on the classical DLVO theory (Cosgrove et al., 2005), the stability of a particle in solution is dependent upon the total potential energy of interaction between colloidal particles (V_T). V_T is the balance of two competing contributions:

$$V_T = V_A + V_R \quad \text{(S1)}$$

where V_A is the attractive potential energy; V_R stands for the repulsive potential energy. The V_A between two identical spherical particles can be written as:

$$V_A = -\frac{A}{12}\left[\frac{1}{x(x+2)} + \frac{1}{(x+1)^2} + 2\ln\frac{x(x+2)}{(x+1)^2}\right], x = \frac{h}{2r} \quad \text{(S2)}$$

where r is a particle radius; h is the distance of separation; A is the Hamaker constant. In this study, A was estimated as the geometric mean of the Hamaker constants of the particle (A_{CeO_2}) and of the medium (A_{water}) with respect to their values in vacuum, i.e.

$$A = (\sqrt{A_{CeO_2}} - \sqrt{A_{water}})^2 \quad \text{(S3)}$$

Where the values of A_{CeO_2} (5.56×10^{-20} J) (Song et al., 2011) and A_{water} (3.7×10^{-20} J) **(**Karimian and Babaluo, 2007**)** were adopted. The electrostatic repulsion is expressed as:

$$V_R = 2\pi\varepsilon r\zeta^2 \exp(-\kappa h) \quad \text{(S4)}$$

where ε is the dielectric constant for water (81 F/m); ζ represents the zeta potential; κ stands for the Debye constant. The value of κ for CeO_2NPs in the test media can be estimated by the expression:

$$\kappa = \sqrt{\frac{2e^2 \, \mathrm{N_A} \, I}{\varepsilon \varepsilon_0 \, \mathrm{K_B} \, T}} \tag{S5}$$

where e is the formal charge on an electron (1.60×10^{-19} C); N_A is Avogadro's constant (6.02×10^{23} mol^{-1}); I is the ionic strength of the test media (8.39 mM); ε_o is the vacuum permittivity (8.85×10^{-12} $C^2\cdot N^{-1}\cdot m^{-2}$); K_B is the Boltzman's constant (1.38×10^{-23} $C^2\cdot J\cdot K^{-1}$); T is temperature (K).

Table S2.1 Developmental morphology and teratogenicity endpoints in the zebrafish (*Danio rerio*) test (*adopted from* Hermsen et al. (2011))

Toxicological endpoints
Morphology
Tail detachment
Formation of somite
Eye development
Spontaneous movement
Beating heart
Reductions in blood circulation or loss of circulation
Pigmentation head-body
Pigmentation tail
Pectoral fin
Protruding mouth
Teratogenicity
Pericardial oedema
Yolk sac oedema
Eye oedema
Head malformation
Absence/malformation of sacculi/otoliths
Malformation of tail
Malformation of heart
Modified chorda structure
Scoliosis
Rachischisis
Yolk deformation

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