Fate of engineered cerium oxide nanoparticles in an aquatic environment and their toxicity toward 14 ciliated protist species

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ABSTRACT

The potential environmental impacts of engineered cerium oxide nanoparticles (CeO₂ NPs) on aquatic organisms have remained largely unknown. Therefore, the laboratory study featured herein was performed to determine the fate of CeO₂ NPs in an aquatic environment and their toxicity towards 14 different ciliated protist species at a specified population level. An investigation of 48-hour aggregation kinetics in the Dryl's solution showed the CeO₂ NPs to be relatively stable. The pH values in three test medium were too far away from PZC, which explained the stability of CeO₂ NPs. CeO₂ NPs generally elicited more toxicity with increasing NP concentration, following certain dose-response relationships. Nano-CeO₂ resulted in greater toxicity in a particle state than when added as bulk material. LC₅₀ values showed a negative correlation with the surface-to-volume ratio for these protists, suggesting that surface adsorption of CeO₂ NPs might contribute to the observed toxicity. Additionally, acute toxic responses of 14 ciliated protist species to CeO₂ NPs were not significantly phylogenetically conserved. The results of these observations provide a better insight into the

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potential risks of CeO₂ NPs in an aquatic environment.

Keywords: Acute toxicity; CeO₂ NPs; Ciliated protist; Environmental fate; Phylogenetic conservatism.

Capsule: This study is the first to demonstrate the fate of CeO₂ NPs in an aquatic environment and their acute toxicity toward 14 ciliated protozoans.

1. Introduction

Nanotechnology utilizes nanoparticles (NPs) that are unique not only due to their minute size (smaller than 100 nanometers) but also because of their size-dependent characteristics. NPs are being spotlighted as a center stage phenomenon for future industry (Arnaldi, 2014). The production and number of applications of engineered NPs are increasing rapidly worldwide. Current applications include the use of NPs in consumer products, construction materials, medical and pharmaceutical industries, agriculture, and information technology (Karen et al., 2009). Metal oxide NPs are an important category of manufactured NPs, accounting for about one-third of the consumer products nanotechnology market. For instance, cerium oxide (CeO₂) NPs are increasingly used as a catalyst in the automotive industry (Zhao et al., 2012; Zhang et al., 2014). Consequently, CeO₂ NPs are expected to enter environmental water samples via waste streams from industries that synthesize or use CeO₂ (Hu et al., 2012; Auffan et al., 2013). CeO₂ NPs are on the Organization for Economic Co-operation and Development (OECD) list of priority nanomaterials for immediate testing and it is imperative to perform the risk assessment of their potential eco-toxicological effects (OECD, 2010ab). To date, the studies on the CeO₂ NPs toxicity conducted with aquatic organisms are rather limited and their environmental fate and impacts have remained largely unknown.

NP dispersion in aqueous media for toxicity studies remains a challenge because of the tendency for NPs to aggregate. Aggregation will cause the decrease of exposed surface area (Dhawan et al., 2009), changing oxidative damage induced by the self-quench of reactive oxygen species (Hotze et al., 2010). Previous studies have shown that the problems are exacerbated in culture media due to the presence of salts and other compounds (Fubini et al., 2010). Solution pH is an essential factor governing NP aggregation. Aggregation can be prevented by adjusting the solution pH away from the NP point of zero charge (PZC), thereby conferring a

net negative or positive charge to cerium oxide surfaces. This results in an increased interparticle repulsion, decreasing the rate of aggregation (Hotze et al., 2010). The extent to which aggregation of CeO₂ NPs in different types of solutions influences NP dispersion needs to be addressed before conducting the toxicity study. Additionally, CeO₂ NPs dissolution could play an important role in toxicity evaluation. During the incubation the core metal Ce could be released by oxidative and photolytic conditions from the NPs, which might be a key factor in determining its toxicity to aquatic organisms (Dahle et al., 2015; Zhao et al., 2012).

Previous tests on environmental toxicity of NPs in freshwater usually focused on conventional model organisms such as bacteria, algae (single-celled with cell walls), *Daphnia* (multicellular organisms), and zebrafish (Zhang et al., 2012). Additionally, the factors (and related underlying mechanism) that influence environmental toxicity among different species remain largely unknown. A variety of other organisms in the aquatic environment are important to maintain the balance of ecological systems, and the toxicity of NPs against these organisms has not been extensively investigated.

Freshwater protist species provide a good model for study on how environmental toxicity differs among species because of their ubiquitous global distribution and special sensitivity to environmental contaminants. Protozoa are single cell organisms that do not possess a protective cell wall. As a consequence, NPs could enter protozoan cells more easily than bacterial and algal cells and interact directly with the cellular structures and organelles (Mortimer et al., 2014; Zou et al., 2013). The ciliated protists have been widely studied by eco-toxicologists not only because of their role in the regulation of microbial populations through the ingestion and digestion of bacteria but also because of their high sensitivity to chemical materials; hence, they are often used as indicator species of environmental pollution (Bick, 1972).

Species phylogenetic histories often place a constraint on their trait evolution, such that more closely related species tend to exhibit more similar traits, a phenomenon termed phylogenetic trait conservatism (Harvey and Pagel, 1991; Bello et al., 2009). On the other hand, convergent evolution could result in distantly related species that are similar in their traits; this could serve to decouple the linkage between species phylogenetic relatedness and trait similarity (Losos et al., 2003).

The objectives of this research were 1) to assess the environmental fate of CeO₂ NPs and their impacts on 14 freshwater ciliated protist species by conducting acute toxicity tests 2) to further evaluate whether acute

toxicological responses of these ciliated protist species to CeO₂ NPs are phylogenetically conserved. Towards these goals, the fate of CeO₂ NPs in test media was first determined via the kinetic study of hydrodynamic size and zeta potentials. LC₅₀ values of CeO₂ NPs derived from acute toxicity tests for 14 different protist species were then estimated, and the phylogenetic signal of LC₅₀ for each tested protist species was analyzed. Our findings establish a useful scientific basis for CeO₂ NPs ecological risk assessment.

2. Materials and methods

2.1. Chemicals

Aqueous cerium (IV) oxide NPs and bulk were purchased from Sigma-Aldrich (St. Louis, MO, USA), and CeO₂ NPs stock suspension was made with the concentration of 100 g/L. All the solvents used in the experiments were of analytical grade.

2.2. Ciliated protozoans

A total of 14 common ciliated protist species in an aquatic environment were used in the experiment. These species include *Blepharisma americanum* (*B. americanum*), *Colpidium kleini* (*C. kleini*), *Colpidium striatum* (*C. striatum*), *Glaucoma scintillans* (*G. scintillans*), *Loxocephalus sp.* (*L. sp.*), *Paramecium aurelia* (*P. aurelia*), *Paramecium bursaria* (*P. bursaria*), *Paramecium multimicronucleatum* (*P. multimicronucleatu*), *Paramecium tetraurelia* (*P. tetraurelia*), *Spirostomum teres* (*S. teres*), *Spirostomum ambiguum* (*S. ambiguum*), *Tetrahymena pyriformis* (*T. pyriformis*), *Tetrahymena thermophila* (*T. thermophila*), and *Tetrahymena vorax* (*T. vorax*).

These species were isolated from local ponds or obtained from the Carolina Biological Supply Company (Burlington, NC, USA).

2.3. Dryl's solution

The Dryl's solution was prepared and autoclaved according to the following recipe: 1 mM NaH₂PO₄-monobasic (Fisher Biotech, Pittsburgh, PA), 1 mM Na₂HPO₄-dibasic (Fisher Chemical, Pittsburgh, PA), 2 mM trisodium citrate dehydrate (Fisher Scientific, Pittsburgh, PA), and 1.5 mM CaCl₂ (Fisher Scientific, Pittsburgh, PA). The Dryl's solution was autoclaved before use, with CaCl₂ solution autoclaved separately.

2.4. Protozoan pellet medium (PPM)

The growth medium was made by dissolving protozoan pellets (concentration: 0.55 g pellet/L) in deionized (DI) water. To provide bacterial food for the protists, we inoculated three bacterial species (*Bacillus cereus*, *Bacillus subtilis*, and *Serratia marcescens*) into the autoclave-sterilized medium. After 24 hours of bacterial incubation, we dispensed the medium into individual microcosms. Each microcosm also received two autoclaved wheat seeds that provided additional carbon. All the microcosms and stock cultures were stored in an incubator maintained at room temperature (22 °C).

All 14 protozoans are filter feeders, which feed on bacteria and other small particles. These ciliates are small to medium sized, with generation times ranging from a few hours to no more than two days. These species were separately cultivated in stock cultures and these cultures were periodically renewed by a spot of solution transfer. When inoculating the protists into microcosms, we always used 2-week-old stock cultures to minimize the differences in the physiological conditions of species.

2.5. Particle size distribution (PSD) and zeta potential determination

PSD was determined on a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) by filling 1.5 mL CeO₂ NPs solution into a clean cuvette. The light scattering detector was positioned at a scattering angle of 173° from the incident laser beam, and the autocorrelation function automatically accumulated for at least 10 runs for each sample.

Zeta potential was also measured by a Zetasizer Nano ZS instrument. Four measurements were repeated for each test condition.

2.6. Aggregation kinetics test

To determine the stability of spiked CeO₂ NPs in the Dryl's solution, which was used for the set-up of the short-term toxicity test, two different concentrations (10 and 1000 mg/L) of CeO₂ NPs were prepared by adding corresponding amounts of CeO₂ stock solution into the Dryl's solution. Samples were collected at 0, 1, 2, 3, 4, 5, 7, 24, 32 and 48 hours with four parallel replicates measured for each sampling event. Prepared

solutions of 1.5 mL were inserted into a clean cuvette and placed into the ZS Zetasizer machine. Number averaged hydrodynamic radii and the zeta potential of CeO₂ NPs in the Dryl's solution were obtained using dynamic light scattering (DLS) on the Zetasizer Nano ZS instrument.

2.7. Effect of pH on zeta potential of CeO₂ NPs

To determine the PZC values of CeO₂ NPs in three different aqueous solutions, including the DI water, Dryl's solution and PPM solution, we investigated the relationship between pH and zeta potential. CeO₂ NPs were added to pH adjusted test aqueous solutions, and each solution was vortexed briefly. The pH was adjusted by adding either 0.1 M HNO₃ or 1 M KOH with a pH ranging from 2 to 12.5. Zeta potential measurements were replicated four times for each pH level. PZC was estimated from the zeta potential vs pH plot.

2.8. Acute toxicity test

Acute toxicity of CeO₂ NPs to the tested 14 common ciliated protist species was assessed. These species were cultured in the lab on multiple bacterial species, including *Serratia marcescens*, *Bacillus cereus*, and *Bacillus subtilis*. The population size in the stock culture of each protist species was determined by taking a small sample from the stock culture and counting the number of individuals in the sample under a stereomicroscope.

The experiment was conducted in the Dryl's solution. In order to determine exposure concentrations, based on previous toxicity information of CeO₂ NPs, we carried out some preliminary experiments, and then prepared CeO₂ NPs at seven different concentration levels (200, 1000, 2000, 3000, 3600, 4000 and 5000 mg/L). In addition, due to the higher toxicity of CeO₂ NPs on *L. sp.*, we conducted the supplementary experiments, including the additional concentration levels (100, 350, 600 and 1700 mg/L) to better estimate the LC₅₀ of *L. sp.*.

For nine species (*G. scintillans*, *C. striatum*, *L. sp.*, *T. vorax*, *C. kleini*, *T. pyriformis*, *T. thermophila*, *P. tetraurelia*, and *S. teres*) that were too small to be transferred individually, we created two sets of four replicate slots for each concentration in the microwells. However, for the other five species (*P. aurelia*, *P.*

multimicronucleatum, *P. bursaria*, *B. americanum*, and *S. ambiguum*) that were large enough to be transferred individually, we created two sets of 12 replicate slots for each concentration in the microwells.

For large species, each microwell contained 200 μ L of Dryl's solution and 200 μ L of either CeO₂ bulk or NPs solution. One individual of these species was transferred into each microwell, whereas for small species, the microwells contained 200 μ L of either CeO₂ bulk or NPs solution and 200 μ L of the specie's stock culture (population density in the stock culture was determined immediately by counting before the experiment). For all of the species, the control experiments were performed without CeO₂ addition. The protists were not fed with bacteria during the entire test period of 48 hours.

The number of remaining individuals of each species was quantified under a stereomicroscope every 24 and 48 hours after inoculation. Swimming protists were counted as live cells, and cells that were ruptured or could not be found were considered dead (Hu et al., 2012). Linear regressions were used to relate species survival rate, calculated as the proportion of the original population size remaining at each sampling point, to CeO₂ concentration. LC₅₀ was calculated as the CeO₂ concentration corresponding to 50% survival along the regression line. All statistical analyses were conducted in SPSS version 13.0.

2.9. Phylogeny construction and phylogenetic conservatism determination of LC₅₀ among 14 protists after exposure to CeO₂ NPs

Small subunit ribosomal RNA (SSU rRNA) gene sequences were used to construct the phylogeny of these 14 protist species. The SSU rRNA gene has been frequently used to indicate the evolutionary relationship among protist species (Baroin-Tourancheau et al., 1992; Van de Peer et al., 1996) because of its highly conserved sequences. SSU rRNA sequences of the 14 protist species used in the experiment and two outgroup species, *Prorocentrum gracile* and *Sarcocystis lacerate*, were obtained from the SILVA rRNA database (Pruesse et al., 2007). We used the sequence of the evolutionarily closest related congener to *L. sp.* in the phylogeny construction. The phylogeny had no qualitative change if sequences from other congeners with similar phylogenetic relatedness were used or the species was omitted. We manually eliminated the divergent regions and poorly aligned positions and checked the selection of sequence blocks in Gblocks (Castresana, 2000). A model test (Darriba et al., 2012) suggested a HKY substitution model with a gamma-distributed rate

variation (HKY+G, $k=2.794$, $G=0.259$) as the best-fit model for nucleotide substitution. We constructed a maximum likelihood (ML) phylogenetic tree with the suggested model by using PhyML (Guindon et al., 2010) with a BIONJ starting tree. The ML phylogram was then transformed into an ultrametric chronogram by using a nonparametric rate smoothing method in r8s (Sanderson, 2003).

3. Results and discussion

Laboratory microcosms contain less ecological complexity than most natural communities and are highly amenable to experimental manipulation, which means they allow the examination of ecological hypotheses that may be difficult to assess in natural environments (Jessup et al., 2004; Benton et al., 2007). The short generation time of protist species allows the observation of long-term community dynamics in a relatively short time span (Connell and Sousa, 1983). Therefore, laboratory microcosms were performed to determine the fate of CeO_2 NPs in an aquatic environment and acute toxic effects on ciliated protist species.

3.1. Dispersion characterization and aggregation kinetics of CeO_2 NPs

The PSD of CeO_2 NPs suspension at 500 mg/L is illustrated in Fig. 1, which shows a narrow size distribution as indicated by the low polydispersity index (PDI) value and a mean size of approximately 115 nm.

The aggregation kinetics of CeO_2 NPs in the Dryl's solution was also investigated with an acute toxicity test during a 48-hour period. The aggregation curves of CeO_2 NPs in the Dryl's solution at 10 and 1000 mg/L, representative of the low and high test concentrations, respectively, are presented in Fig. 2. The measured hydrodynamic size of CeO_2 NPs ranged from 250 to 310 nm (mean PSD=245 nm) in the Dryl's solution, which was significantly higher than its initial size in the stock suspension (mean PSD=115 nm). The results indicate the immediate occurrence of aggregation of CeO_2 NPs upon their dispersion in the test solutions, but NPs generally remained relatively stable (i.e., shape, size and concentration, etc.) in the subsequent stage, suggesting that the observed toxicity during the entire exposure period was indeed caused by CeO_2 NPs. The zeta potential of CeO_2 NPs was determined to be -17, -22, and -29 mV in the DI water, Dryl's solution and PPM solution, respectively.

We also found that in comparison to the lower concentration level of CeO₂ NPs suspension, the higher concentration of NPs in the Dryl's solution exhibited a higher aggregation rate in the first eight hours as reflected by the steeper initial slope in the aggregation curve (Fig. 2). This is not surprising considering that the concentration level is an essential factor governing the aggregation kinetics of NP suspension as reported by previous studies (Li et al., 2012; Bouhaik et al., 2013; Son et al., 2015).

3.2. Effect of pH on zeta potential of CeO₂ NPs

The zeta potential of CeO₂ NPs as a function of pH in different aqueous solutions is presented in Fig. 3. As the pH of the solution shifted from acidic to basic, zeta potentials changed from positive to negative. The pH at the PZC was approximately 9.3, 3.9, and 5.8 for the CeO₂ NPs suspensions in the DI water, Dryl's solution and PPM solution, respectively. Under these pH conditions, CeO₂ NPs were highly unstable. Dryl's solution was composed of phosphate. According to Hoecke et al. (2009), phosphate ions may adsorb to the surface of CeO₂ NPs and then affects the surface charge, which could explain the differences in zeta potential for the three solutions.

The measured pH in the Dryl's solution and PPM solution were approximately 7.0 and 7.4, respectively, which were away from the PZC. CeO₂ NPs were thus expected to be negatively charged, and they were supposed to be resistant to aggregation due to electrostatic repulsion. This could be the reason why CeO₂ NPs were observed to be stable in these solutions. Additionally, there was a discrepancy between the estimated PZC for CeO₂ NPs in the DI water in this study from that reported previously by Li et al. (2011), who determined a PZC value of approximately 7.6. Such discrepancy might be attributed to the presence of additional dissolved organic matter and/or microorganisms in the DI water of our experiment.

3.3. Acute toxicity of CeO₂ NPs to ciliated protists

King-Heiden and Zhang observed that zebrafish larvae showed signs of Cd²⁺ toxicity, while the nanoscale properties of CdSe QDs were even more potent and produced end points of toxicity distinct from that of released Cd²⁺ from QDs (King-Heiden et al., 2009; Zhang et al., 2012). For CeO₂ NPs test runs, Ce ion concentrations were determined by ICP-MS in the filtered samples after the incubating of 200~5,000 mg/L

CeO₂ NPs in the Dryl's solution for 48 h. We observed minimal Ce release (less than 10% of total Ce, 1.2~8.4%) from CeO₂ NPs core under the test conditions, and these released ions barely resulted in obvious adverse impacts on the organisms (survival rate was similar to the control), indicating that scale effects of CeO₂ NPs should act as a more important function for the observed acute toxicity.

Two acute toxicity endpoints of CeO₂ on nano and bulk powder toward ciliated protozoans were investigated and the estimated 24 and 48 h LC₅₀ values were determined along with the calculated 95% confidence intervals as shown in Table 1. The original data are summarized in Support information 1 and 2.

Generally, higher exposure concentration or longer exposure time led to increased observed mortality, indicating a distinct dose-response and time-effect trend. The highest 48 h LC₅₀ of CeO₂ NPs was observed for *C. striatum* with the estimated value of 1267.65 mg/L, while the lowest value (115.88 mg/L) was observed in *L. sp.* for which all test concentrations were lethal. Moreover, compared with the 24 h exposure, lower 48 h LC₅₀ values were observed, indicating the accumulative toxicity of nano-CeO₂ in test organisms. After a 48 hour exposure to CeO₂ NPs, significant mortality was observed for almost all protist species at higher exposure levels. Additionally, the calculated 48 h LC₅₀ values for CeO₂ NPs were used in the subsequent analyses, because all the regressions were significant ($P < 0.05$) for all 14 species, suggesting that 48 h data may be more reliable. Based on 48 h LC₅₀ of these species, we concluded that the toxicity of CeO₂ NPs toward the tested ciliated protozoans follows the order: *L. sp.* > *G. scintillans* > *P. aurelia* > *T. pyriformis* > *P. bursaria* > *T. vorax* > *T. thermophila* > *S. teres* > *P. tetraurelia* > *S. ambiguum* > *P. multimicronucleatu* > *C. kleini* > *B. americanum* > *C. striatum*. This order indicated that 14 ciliated protist species showed different response sensitivities towards CeO₂ NPs stress.

Potential mechanisms of CeO₂ NPs toxicity to ciliated protists are that it may induce cell death, oxidative stress or DNA damage due to the leaching of toxic heavy metals from the colloidal form and/or the size and surface chemistry of NPs (Zhang et al., 2012). In the present study, the observed different toxicological responses among the 14 tested ciliated protists might be attributed to their varying size and specific surface area. The acute toxicity results also suggest that larger species such as *P. aurelia* showed a slower but rather sensitive response to the exposed CeO₂ NPs, as reflected by the abruptly increasing mortality toward the end of the 48 hours.

During the experiment, CeO₂ NPs solution were highly stable in terms of NP properties (i.e., shape and size); hence, we can compare the toxicity difference between bulk and nano CeO₂. Generally, higher LC₅₀ values of bulk powder were reported for all 14 tested species, indicating that CeO₂ NPs resulted in greater toxicity for test organisms in their separated NPs solution than in their bulk state where they were subjected to a heavier powder mixture resulting in a denser solution. A possible mechanism behind the NP interaction might be that NPs extremely small particle size enters protozoan cells more easily than bulk powder and interact directly with the cellular structures and organelles. Rossetto suggested that size is one of the key factors influencing the toxic effects of NPs (Rossetto et al., 2014). Kasemets observed that NPs are more toxic than bulk particles (Kasemets et al., 2009). Abdel-Khalek also reported that the LC₅₀/96 h of bulk and nano copper oxide particles were 2,205 and 150 mg/L, respectively (Abdel-Khalek et al., 2015). NPs possess unique physicochemical properties that are different from their bulk counterparts, and have a stronger tendency to exhibit toxic effects (Harman et al., 2002). Hou et al. (2015) reported that Nano CeO₂ could cause damages to cell membranes and viability by measuring the intracellular ROS production and extracellular LDH release in the biofilms. The LDH value is regarded as an indicator of membrane integrity (Hou et al., 2014), whereas the ROS value is used to measure oxidative stress (Applerot et al., 2012). Experiments to observe LDH release and ROS production can assess chemical impacts on cell viability and growth of the organisms. Therefore, the two parameters should be surveyed to determine possible toxicity difference mechanisms of nano and bulk CeO₂ toward 14 ciliated protists. Additionally, in order to explore how CeO₂ NPs interact with protist cells, we can try to measure intracellular CeO₂ concentrations or use SEM and TEM images to show whether protists take up NPs or whether particles cluster near protist cells to determine CeO₂ action at protists surface.

The surface areas and volumes of protist cell were determined by camera microscope. Log₁₀-48 h LC₅₀ was negatively correlated with protist cell surface-to-volume ratio (linear regression, R=0.577 and P=0.018 as per Fig. 4) and positively correlated with protist cell volume and surface area (linear regression, cell volume: R=0.538, P=0.027; surface area: R=0.517 and P=0.034 as per Fig. 4). The results indicate that the higher surface-to-volume of a ciliated protist might uptake or adsorb more CeO₂ NPs, thus resulting in greater toxicity to species, while the bigger cell volumes or surface areas meant higher resistance to contaminants. This effect can originate from an increasing reactive surface area between NPs and ciliated protists. As the

surface-to-volume ratio increases, it can allow a greater proportion of protist cells to be exposed to the exterior. A similar observation was reported in previous research (Zhou et al., 2004; Nel et al., 2006; Rivera-Gil et al., 2013).

3.4. Phylogenetic niche conservatism

Phylogenetic signals of 48 h LC₅₀ values among the 14 protists were tested by using Blomberg's K (Fig. 5) (Blomberg et al., 2003). The results indicated that LC₅₀ was not significantly phylogenetically conserved (Blomberg's K=0.0508 and P=0.322), suggesting that the phylogenetic relationship did not have much influence on toxic effects compared to the surface-to-volume ratio of protist cells. Fig. 4 data reflects this finding by showing the LC₅₀ value to be largely driven and determined by this same surface-to-volume ratio of protist cells. According to our observation and previous reports, this ratio can't be phylogenetically conserved in protist species, which might be the reason why LC₅₀ value is barely phylogenetically conserved with no relevant significance.

4. Conclusions

This study creatively contributed to future CeO₂ NPs research in three main aspects. First, our experiment demonstrated that CeO₂ NPs show toxicological effects on 14 common ciliated protist species in relatively short-term scales (24 and 48 hours), pointing to the possibility that many other protists may also be affected by the increasing use of these CeO₂ NPs. Future investigations should consider their effects on more complex organisms (e.g., multicellular organism), and their chronic toxicity. Second, although the aggregation and dissolution took place immediately in the test media employed for acute (Dryl's solution) assessment, CeO₂ NPs remained relatively stable throughout the test period, which provided enough evidence that the observed toxicity were indeed caused by the CeO₂ NPs. Third, LC₅₀ were not significantly phylogenetically conserved across our study species, and the surface-to-volume ratio might be more important in driving the observed toxicity of these species. In summary, the toxicity determination of CeO₂ NPs will help to establish water-quality standards to protect aquatic life. However, further studies are needed to identify potential mechanisms of the toxicity to aquatic organisms, especially the impacts and contribution of the phylogenetic

relationship as well as the surface-to-volume interactions of ciliated protist species.

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444 **Table captions:**

445 **Table 1** Estimated LC_{50} (mg/L) of CeO_2 NPs and bulk particles for the 14 ciliated protozoans at 24 and 48 h.

446 **Table 1**

447 Estimated LC₅₀ (mg/L) of CeO₂ NPs and bulk particles for the 14 ciliated protozoans at 24 and 48 h.

Ciliated protist species	CeO ₂ NPs				CeO ₂ bulk			
	24 h		48 h		24 h		48 h	
	LC ₅₀	P-value	LC ₅₀	P-value	LC ₅₀	P-value	LC ₅₀	P-value
<i>B. americanum</i>	3639.15	0.311	851.14	0.003	9908.32	0.258	3411.93	0.261
<i>S. teres</i>	669.88	0.001	400.87	0.001	1352.07	0.007	1042.32	0.027
<i>S. ambiguum</i>	1256.03	0.001	698.23	0.001	698.23	0.438	805.38	0.399
<i>T. thermophila</i>	939.72	0.007	370.68	0.001	974.99	0.834	1016.25	0.002
<i>T. vorax</i>	225.94	0.001	334.97	0.001	746.45	0.013	1061.70	0.129
<i>T. pyriformis</i>	729.46	0.001	251.77	0.008	1389.95	0.008	924.70	0.067
<i>G. scintillans</i>	183.65	0.001	128.53	0.001	704.69	0.001	497.74	0.001
<i>C. kleini</i>	1396.37	0.001	781.63	0.001	1303.17	0.095	1324.34	0.504
<i>C. striatum</i>	954.99	0.001	1267.65	0.006	1753.88	0.001	1655.77	0.141
<i>L. sp.</i>	136.46	0.001	115.88	0.001	283.14	0.001	500.03	0.001
<i>P. tetraurelia</i>	29.17	0.001	495.45	0.006	330.37	0.001	968.28	0.054
<i>P. aurelia</i>	183.23	0.001	229.09	0.001	920.45	0.616	1757.92	0.133
<i>P. multimicronucleatu*</i>	NA	NA	727.78	0.001	NA	NA	24490.63	0.016
<i>P. bursaria</i>	141.58	0.001	275.42	0.021	669.88	0.007	722.77	0.169

448 **P. multimicronucleatu* had NA data at 24 h because the species couldn't be easily observed from the
 449 background containing CeO₂ NPs or bulk particles (The observed ones were alive, of course). We used a
 450 destructive method at 48 h by adding a large amount of clear medium to dilute the samples.

Figure captions:

Fig. 1 Particle size distribution in 500 mg/L CeO₂ NPs suspension.

Fig. 2 Representative aggregation kinetics profiles of CeO₂ NPs in the Dryl's solution.

Fig. 3 Zeta potential of 50 mg/L CeO₂ NPs in three different solutions as function of pH.

Fig. 4 Regression analysis of LC₅₀ against the specific surface area.

Fig. 5 A maximum likelihood (ML) phylogenetic tree of the 14 species based on the subunit ribosomal RNA (SSU rRNA) gene sequences.

Fig. 1 Particle size distribution in 500 mg/L CeO₂ NPs suspension.

Fig. 2 Representative aggregation kinetics profiles of CeO₂ NPs in the Dryl's solution. (A) 10 mg/L CeO₂ NPs; (B) 1000 mg/L CeO₂ NPs.

Fig. 3 Zeta potential of 50 mg/L CeO₂ NPs in three different solutions as function of pH.

Fig. 4 Regression analysis of LC_{50} against the specific surface area.

Fig. 5 A maximum likelihood (ML) phylogenetic tree of the 14 species based on the subunit ribosomal RNA (SSU rRNA) gene sequences. Scores on the nodes represent the support of divergences from approximate likelihood ratio tests (Guindon et al., 2010).