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Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* to screen the ecological risks of nanoparticle exposure

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ABSTRACT

Genotoxic and ecotoxic assessments of widely used nanoparticles, cerium dioxide (CeO_2), silicon dioxide (SiO_2) and titanium dioxide (TiO_2), were conducted on two aquatic sentinel species, the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius*. CeO_2 may have genotoxic effects on *D. magna* and *C. riparius*, given that the DNA strand breaks increased in both species when exposed to this nanoparticle; whereas, neither exposure to SiO_2 nor TiO_2 had a genotoxic effect on either species. A statistically significant correlation was observed between DNA damage and mortality in the CeO_2-exposed *C. riparius*, which suggests that CeO_2-induced DNA damage might provoke higher-level consequences. SiO_2 did not seem to affect the DNA integrity; whereas, the mortality of both the SiO_2-exposed *D. magna* and *C. riparius* increased. The TiO_2 nanoparticle did not lead to significant alterations in geno- or ecotoxic parameters of both species. Overall, these results suggest that CeO_2 nanoparticles may be genotoxic toward aquatic organisms, which may contribute to the knowledge relating to the aquatic toxicity of the most widely used nanomaterials on aquatic ecosystems, for which little data are available.

1. Introduction

Numerous new industrial nanomaterials have been synthesized for commercial and industrial purposes. Cerium dioxide (CeO_2), silicon dioxide (SiO_2) and titanium dioxide (TiO_2) nanoparticles are widely used manufactured nanomaterials due to their potential broad applications. As lanthanide element oxides, CeO_2 nanoparticles are among the most important nanomaterials, and are used in a wide range of applications, including catalysis, solar, fuel cells, phosphor/luminescence, abrasives for chemical/mechanical planarizations gas sensors, oxygen pumps, and metallurgical and glass/ceramic applications (Murray et al., 1999; Corma et al., 2004a,b; Izu et al., 2004; Zheng et al., 2005). As a non-metal oxide, SiO_2 nanoparticles have been extensively applied to chemical/mechanical polishing and as additives to drugs, cosmetics, printer toners, varnishes and foods, as well as in biomedical fields (Hirsch et al., 2003; Zhang et al., 2004; Gemeinhart et al., 2005; Venkatesan et al., 2005). TiO_2 is a potent photocatalyst, which can break down almost any organic compound when exposed to sunlight, with potential wide applications in self-cleaning fabrics, auto-body finishes and ceramic tiles (Gratzel, 1999; Fujishima et al., 2000; Caruso et al., 2001).

Despite the dramatic increase in the use of such nanomaterials, little information is available on their potential harmful effects on the environment. Most current literature on the toxicity of nanoparticles; however, comes from mammalian studies that have focused on respiratory exposure, or from *in vitro* assays using mammalian cells (Lam et al., 2004; Braydich-Stolle et al., 2005; Hussain et al., 2005; Monteiro-Riviere et al., 2005; Limbach et al., 2007; Eom and Choi, 2009). Ecotoxicological studies on nanoparticles are even more limited, with only a few reports on the acute toxic effects of nanoparticles on aquatic organisms (Kerstin and Markus, 2006; Lovern and Klaper, 2006; Handy and Shaw, 2007; Sarah et al., 2007). Few ecotoxicity studies on aquatic organisms have been performed that include genotoxic endpoints. However, the presence of genotoxic and potentially carcinogenic compounds in aquatic environments is of major concern with respect to the health of aquatic media biota (Houk and Waters, 1996; Ohe et al., 2004; Nehls and Segner, 2005). The potential genotoxic effects of emerging nanomaterials on aquatic systems should be identified to allow for their safe use.

Genotoxic assessments of nanoparticles were conducted on two aquatic sentinel species widely used in biomonitoring, the freshwater crustacean *Daphnia magna*, and the larva of the aquatic midge *Chironomus riparius*. The small-sized freshwater crustacean, *D. magna,* and the aquatic larvae of the non-biting midge, *C. riparius,* hold an important position in the aquatic food chain, respond to many pollutants, easy to culture and have short life cycles; thus,

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are considered suitable species for aquatic biomonitoring (Giesy et al., 1988; Cranston, 1995; Choi et al., 2000; Atienzar et al., 2001; Lee and Choi, 2006). Conventional ecotoxicity tests were also conducted on the *Daphnia* and *Chironomus* systems, as they may provide insights to the potential toxic effects of nanoparticles on aquatic environments. Given the importance of *D. magna* and *C. riparius* in aquatic ecosystems, information concerning geno- and ecotoxicity of widely used nanomaterials on these species could be valuable in relation to aquatic nanoecotoxicology. Moreover, to investigate the relationship between physico-chemical properties and ecotoxicities, primary characterization of the studied nanoparticles was performed using Branauer, Emmett & Teller (BET) and transmission electron microscopy (TEM) methods, which provided information on the surface area, and morphological shape and size of nanoparticles, respectively.

2. Materials and methods

2.1. Organism culture and exposure to nanoparticles

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), *D. magna* and *C. riparius* larvae were obtained from adults reared in our laboratory, as described previously (Park and Choi, 2007; Lee et al., 2008). 7 and 10 nm SiO_2 and 7 and 20 nm TiO_2 nanoparticles were purchased from Sigma (Sigma Corp., St. Louis, MO, USA); whereas, 15 and 30 nm CeO_2 nanoparticles were synthesized, as described previously (Park et al., 2008). Test solutions of CeO_2, SiO_2 and TiO_2 nanoparticles were prepared in culture media and dispersed for 15 min using a sonicator (Branson Inc., Danbury, CT, USA) to prevent aggregation. During the tests, the nanoparticle suspensions were stable and uniform in the culture media. The concentration used in this study to prevent the aggregation and/or precipitation of the particles was 1 mg/L. To measure the surface area of the nanoparticles, the BET method was used, employing a volumetric adsorption apparatus, BELSORP-mini II (BEL Japan Inc., Osaka, Japan). To investigate the size and shape of the nanoparticles, 20 μL of a particle suspension from the test medium was dried on a 400 mesh carbon-coated copper grid and imaged using a JEM 1010 TEM (JEOL, Tokyo, Japan) at 40–100 kV.

2.2. Comet assay

To prepare *Daphnia*, a total of 150 neonates, aged less than 24 h, were collected 24 h from the control and experimental tanks after exposure to nanoparticles and pooled for a Comet assay; whereas, for *Chironomus*, 10 larvae of 4th instar *Chironomus* were pooled. Organisms were placed in 1 mL of phosphate-buffered saline (PBS), containing 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO), and disintegrated mechanically by mincing. An alkaline Comet assay was performed based on the method by Singh et al. (1988), with adaptation for *Daphnia* and *Chironomus*, as described previously (Park and Choi, 2007). Briefly, about 50 cells per slide (3 slides per treatment) were analyzed using a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter with a BP 546/12 nm and 590 nm barrier filter at 400× magnification. DNA damage was expressed as the tail and olive tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.3. Ecotoxicity assays

Daphnia mortality and reproduction tests were conducted according to the OECD guidelines (OECD, 1984, 1998). For the Daphnia growth test, 20 individuals were incubated with nanoparticles for 96 h, with the fresh weight measured immediately after exposure. The body dry weight was evaluated after drying *Daphnia* at 105 °C for 24 h. *Chironomus* mortality and growth tests were performed as described previously (Lee et al., 2008) using 4th instar larvae. Three replicates were conducted for each experiment.

2.4. Data analysis

The genotoxic and ecotoxic assays results were tested for significance using an analysis of variance (ANOVA) test, with the Dunnett's multiple comparison test. The correlation tests were conducted using the Pearson test. All statistical tests were performed using SPSS® 12.0 KO (SPSS Incorporated, Chicago, IL, USA).

3. Results

Prior to the toxicity study, characterization of the nanoparticles was performed using BET and TEM methods (Fig. 1). The BET surface areas results showed that smaller-sized nanoparticles have larger surface area, with the exception of SiO_2 nanoparticles. The TEM images of the nanoparticles from the test medium showed the size difference of the nanoparticles tested.

DNA damage, particularly DNA strand breaks, was measured using a Comet assay to evaluate whether the nanoparticles exerted genotoxicity on *D. magna* (Fig. 2) and *C. riparius* (Fig. 3). Tail and olive tail moments increased in both *D. magna* and *C. riparius* exposed to CeO_2 nanoparticles. The smaller-sized CeO_2 nanoparticles seemed to cause more DNA strand breaks. Neither SiO_2 nor TiO_2 exposure had a genotoxic effect on either species, as no significant increase in the tail/olive tail moments was observed in these species when exposed to the said nanoparticles.

To investigate the physiological- and organism-level effects of the tested nanoparticles, conventional ecotoxicity tests were conducted using mortality, growth and reproduction as the endpoints (Tables 1 and 2). A slight increase in the mortality rate of *Daphnia* was observed after treatment with 15 nm CeO_2 and 7 and 10 nm SiO_2 nanoparticles, but no significant changes were observed in either the growth or reproduction parameters. In *C. riparius*, an increase in mortality was observed after exposure to 15 and 30 nm CeO_2 and 10 nm SiO_2 nanoparticles, but the growth indicators were not significantly changed after nanoparticle exposure. The TiO_2 nanoparticles did not significantly alter the mortality, growth or reproduction of either species.

The correlation between geno- and ecotoxicity parameters was analyzed to validate the ecotoxicological relevance of the response of DNA damage due to nanoparticle exposure in *D. magna* and *C. riparius* (Table 3). Statistically significant correlations were observed between the DNA damage and reproduction (for *D. magna*) and mortality (for *C. riparius*) on exposure to CeO_2 nanoparticles.

4. Discussion

In relation to nanotoxicity, it is often expected that the smaller the size, the stronger the exerted toxicity (Oberdörster et al., 2005). In our study, the characterization of the nanoparticles in test media was investigated to gain an understanding of their influence on

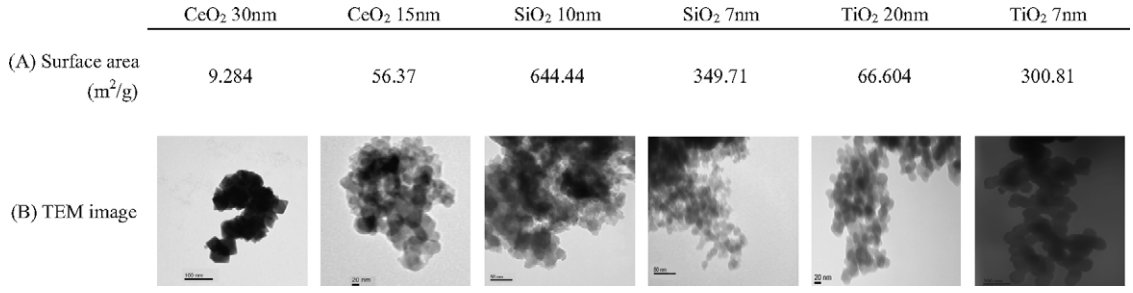

Fig. 1. Characterization of different sized CeO_2, SiO_2 and TiO_2 nanoparticles using BET and TEM methods. Surface areas of nanoparticles were measured using the BET method (A) and particle shapes were analyzed by TEM (B).

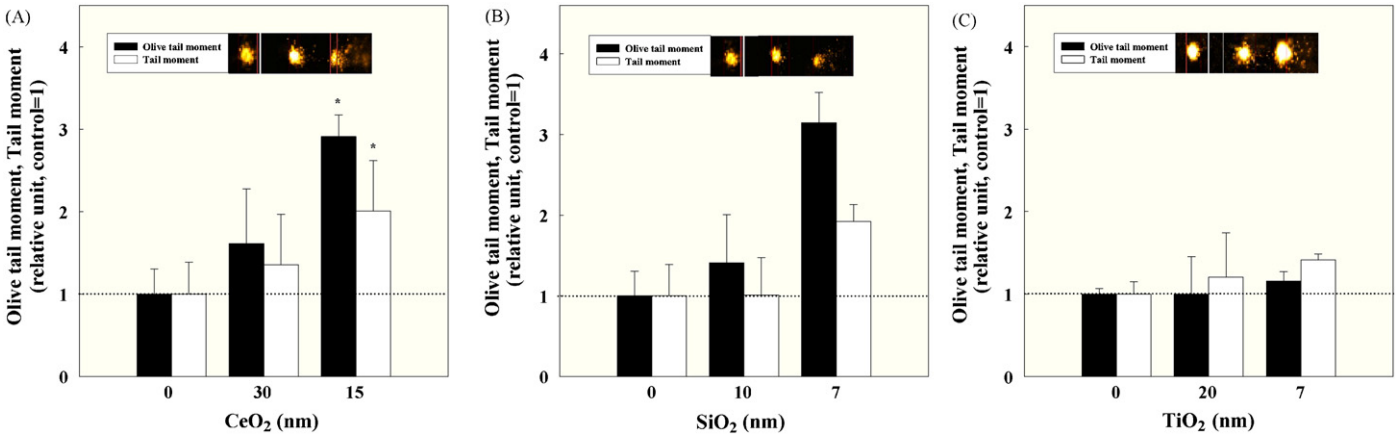

Fig. 2. DNA damage measured in CeO_2 (A), SiO_2 (B) and TiO_2 nanoparticles (C) exposed *D. magna*. The results were expressed as olive tail moment and tail moment obtained by Comet assay (number = 3, mean ± standard error of mean). Asterisks (*) indicate statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

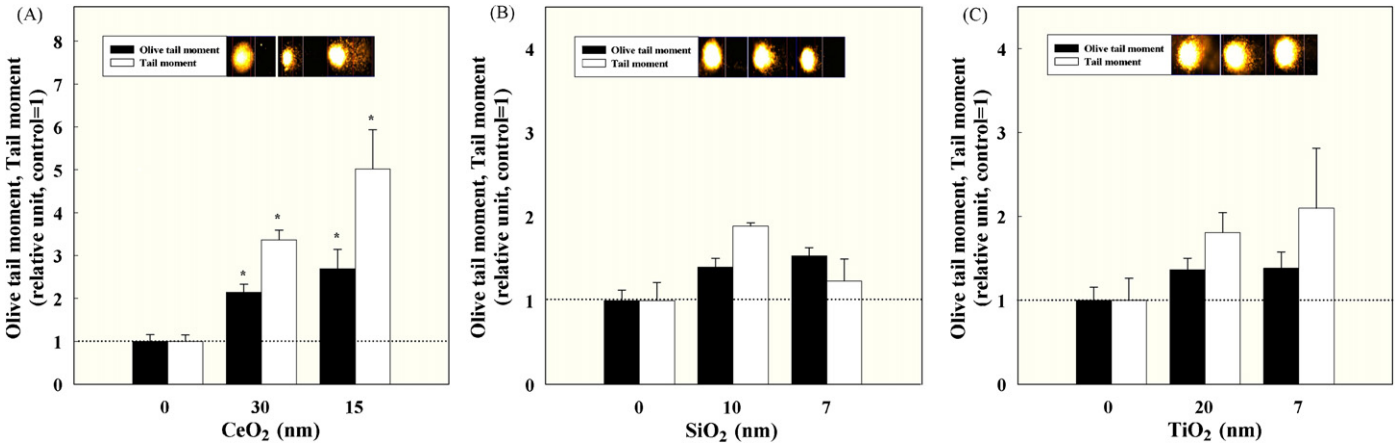

Fig. 3. DNA damage measured in CeO_2 (A), SiO_2 (B) and TiO_2 nanoparticles (C) exposed *C. riparius*. The results were expressed as olive tail moment and tail moment obtained by Comet assay (number = 3, mean ± standard error of mean). Asterisks (*) indicate statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

ecotoxicity (Fig. 1). The BET surface areas showed that smaller-sized CeO_2 and TiO_2 nanoparticles have larger surface areas; whereas, no such tendency was observed with SiO_2 nanoparticles, which may have been due to the too smaller size difference in SiO_2 and their different physical properties (fumed *vs.* porous type for 7 and 10 nm of SiO_2, respectively). Indeed, the relationship between the physico-chemical properties of nanoparticle and their toxicities seems to be much more complicated than just being related to their size and surface area (i.e. shape, charge, concentration, etc.); much debate is still on going (Hussain et al., 2005; Sayes et al., 2006; Fujiwara et al., 2008). Many studies have failed to show any clear relationship between toxicity and the size of nanoparticles (Hussain et al., 2005; Yin et al., 2005). The TEM images of the nanoparticles from the test medium showed the size difference of the nanoparticles tested.

Table 1
Mortality, growth and reproduction parameters investigated in CeO_2, SiO_2 and TiO_2 nanoparticles exposed *D. magna* (number = 3, mean ± standard error of mean).

Nanoparticles (nm)	Mortality	Growth		Reproduction
	Dead individual (%)	BFW (mg/individual)	AFDBW (mg/individual)	Neonates/individual (number)
Control	5 ± 4.08	1.520 ± 0.609	0.022 ± 0.001	36.78 ± 2.41
CeO_2				
30	0	1.421 ± 0.401	0.032 ± 0.002	38.76 ± 2.27
15	10 ± 0*	1.040 ± 0.348	0.020 ± 0.003	46.31 ± 4.57
SiO_2				
10	15 ± 4.080*	1.538 ± 0.299	0.024 ± 0.001	41.64 ± 1.36
7	10 ± 8.160*	1.179 ± 0.221	0.020 ± 0.002	38.50 ± 2.39
TiO_2				
20	0	1.389 ± 0.412	0.024 ± 0.009	37.13 ± 2.05
7	5 ± 4.080	1.480 ± 0.301	0.025 ± 0.010	43.26 ± 4.46

BFW: body fresh weight; AFDBW: ash free dry weight.
* Statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

Table 2
Mortality and growth parameters investigated in CeO_2, SiO_2 and TiO_2 nanoparticles exposed *C. riparius* (number = 3, mean ± standard error of mean).

Nanoparticles (nm)	Mortality	Growth	
	Dead individual (%)	BFW (mg/individual)	AFDBW (mg/individual)
Control	0	4.817 ± 0.368	0.068 ± 0.018
CeO_2			
30	10 ± 0*	4.917 ± 0.170	0.061 ± 0.007
15	15 ± 4.080*	5.023 ± 0.169	0.079 ± 0.019
SiO_2			
10	20 ± 0.000*	4.717 ± 0.033	0.050 ± 0.005
7	5 ± 4.080	5.081 ± 0.048	0.081 ± 0.019
TiO_2			
20	0	5.014 ± 0.229	0.113 ± 0.026
7	0	4.602 ± 0.400	0.066 ± 0.006

BFW: body fresh weight; AFDBW: ash free dry body weight.
* Statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

However, the line of evidence provided from the present study is rather limited; therefore, to identify key properties of nanoparticles for causing ecotoxicity, toxic responses of a broad range of physico-chemical properties to various classes of nanoparticles may be investigated in various environmental relevant species.

Even though genotoxicity tests with the Comet assay are widely used in aquatic environmental monitoring, most Comet assays have been performed on *in vitro* systems of aquatic species, mostly using fish-driven cell lines (Cotelle and Ferard, 1999; Nehls and Segner, 2005). In this study; however, *D. magna* and *C. riparius* were exposed to widely used nanoparticles (CeO_2, SiO_2 and TiO_2) *in vivo*, with the DNA damage assessed in subsequently isolated cells. The measurement of genotoxic effects of emerging nanomaterials, using *in vivo* genotoxicity biomarker in aquatic invertebrates, could be a useful tool for monitoring aquatic toxicity due to nanoparticles. CeO_2 may have genotoxic effects on *D. magna* and *C. riparius*, given that DNA strand breaks increased in both species when exposed to this nanoparticle (Figs. 2 and 3). Nanomaterials may influence the genetic constitution of populations by directly damaging DNA molecules within the individual cell nucleus, but the ecological relevance of changes in single cells within some tissues of certain individual organisms is extremely difficult to assess. The response of *Daphnia* and *Chironomus* to the tested nanoparticles in terms of their mortality, growth and reproduction (Tables 1 and 2) may explain higher biological-level consequences of the DNA damage shown in Figs. 2 and 3. These aquatic toxicity tests may provide insights to the relative sensitivity of these species to the tested nanoparticles, which may also provide information on the impact of nanoparticles on water systems, as these species hold important positions in aquatic ecosystems (OECD, 1984; Okamura et al., 1999; Kikuchi et al., 2000; Lee and Choi, 2006). Even though a statistical correlation analysis cannot provide information on the existence of a causal relationship between these two correlated parameters, the fact that DNA damage occurred concomitantly with a decrease in the organism-level toxicity indicator (mortality) after CeO_2 nanoparticle exposure may suggest that the DNA damage induced by this nanoparticle might provoke higher-level consequences. The impairment of survival due to CeO_2 nanoparticle exposure may be considered a consequence of a serious progression of sub-organism-level toxicities, such as the increased DNA damage in *Chironomus*. However, it is difficult to explain the biological meaning of positive correlation between DNA damage and reproduction potential in *D. magna*, to analyze this, more detailed experiments with more replicates would be needed. The experiments with the SiO_2-exposed *D. magna* and *C. riparius* showed that the DNA integrity had no effect on the increased mortality, which may be an example of a false negative result from the biomarker's perspective. It is clear that this type of error can occur; however, this result could be interpreted as there being an alternative mechanism other than genetic alteration involved in the SiO_2-induced mortality of *D. magna* and *C. riparius*. As mortality is the most obvious sign of progression of serious toxicity at the organism level, further studies on the mechanism behind SiO_2 nanoparticle-induced mortality are needed to better explain the ecotoxicity of the SiO_2 nanoparticle. In this study, TiO_2 nanoparticle provoked neither DNA damage nor alteration in the mortality, growth or reproduction of either species. In our experimental design, ultra violet (UV), which is generally considered critical for TiO_2 toxicity (Williams et al., 2008; Aita et al., 2009; Kim and Kwak, 2009), was not applied. This may be a partial explanation for the negative results with TiO_2; however, if more toxicity indicators had been tested, with/without UV conditions, the toxicity due to TiO_2 could probably have been better evaluated and explained.

The relationships between the responses of the genotoxic biomarker and the physiological/individual/population effects are

Table 3
Coefficients of correlation among observed parameters measured in CeO_2, SiO_2 and TiO_2 nanoparticles exposed *D. magna* and *C. riparius*.

	Growth	Reproduction	Mortality
D. magna			
CeO_2			
DNA damage	−0.321(0.679)	0.987(0.013)*	0.637(0.363)
Growth		−0.428(0.572)	−0.821(0.179)
Reproduction			0.750(0.250)
SiO_2			
DNA damage	−0.097(0.903)	−0.197(0.803)	0.131(0.869)
Growth		0.054(0.946)	0.645(0.355)
Reproduction			0.722(0.278)
TiO_2			
DNA damage	0.978(0.021)*	0.858(0.142)	0.023(0.977)
Growth		0.735(0.265)	−0.132(0.868)
Reproduction			0.353(0.647)
C. riparius			
CeO_2			
DNA damage	0.054(0.946)		0.977(0.024)*
Growth			0.141(0.859)
SiO_2			
DNA damage	−0.736(0.264)		0.838(0.162)
Growth			−0.756(0.244)
TiO_2			
DNA damage	−0.255(0.745)		0.558(0.442)
Growth			−0.698(0.302)

Pearson correlation analysis was conducted using SPSS® 12.0.
* Indicate statistically significant differences between treatments and the corresponding control group, $^*p < 0.05$.

complicated due to the compensatory mechanisms regulating the physiological/individual fitness and population dynamics in a natural system. As the mere presence of genotoxic compounds, which are potentially carcinogenic, is of major concern in human and ecosystem health, the sensitive and rapid detection of the genotoxic properties of aquatic systems themselves is considered important, although does not necessarily include alteration at a higher level of biological organization. Especially for the nanomaterials concerned, despite the dramatic increase in the use of nanomaterials and; hence, their ubiquitous distribution in aquatic environments, little information is available on their potential genotoxicity on aquatic organisms. Considering the potential of *D. magna* and *C. riparius* as bioindicator species, and the importance of the genotoxicity of nanoparticles in ecotoxicity monitoring, the measurement of the DNA damage in these species after exposure to nanoparticles could provide useful information for freshwater monitoring.

In this study, the geno- and ecotoxicity of CeO_2, SiO_2 and TiO_2 nanoparticles on *D. magna* and *C. riparius* were evaluated. The results suggest that CeO_2 nanoparticles may have genotoxic potential toward aquatic organisms, and CeO_2-induced DNA damage might provoke higher-level consequences. SiO_2 did not seem to affect the DNA integrity; whereas, the mortality of the SiO_2-exposed *D. magna* and *C. riparius* increased. TiO_2 nanoparticles did not lead to significant alteration of the geno- and ecotoxic parameters. The above could comprise a contribution to knowledge on the aquatic toxicity of the most widely used nanomaterials, CeO_2, SiO_2 and TiO_2, on aquatic ecosystems, for which little data are available.

Conflict of interest

None declared.

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