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The effects of engineered nanoparticles on survival, reproduction, and behaviour of freshwater snail, *Physa acuta* (Draparnaud, 1805)

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ABSTRACT

Increasing uses of engineered nanoparticles (ENPs) in commercial products and industrial applications has eventually resulted to their releases into atmospheric, terrestrial, and aquatic environments. However, knowledge gaps in ENPs toxicity, fate, and behaviour currently limit our ability to quantify risk assessment of materials with nanoscale dimensions, and therefore, the extent of the resultant environmental impacts remains unknown. In the present study, we evaluated the effects of γ-alumina, α-alumina, modified TiO_2 (M-TiO_2), and commercial TiO_2 (C-TiO_2) ENPs on the survival, behaviour, and early life stages of the freshwater snail *Physa acuta* (Draparnaud). The toxicity evaluation was carried out after spiking commercial sand with ENPs concentrations of 0.005, 0.05, or 0.5 g kg^{-1}. Our findings suggest that increases of γ-alumina and α-alumina concentrations at sub-lethal level concentrations caused significant reduction in the embryo growth rate and embryo hatchability. In addition, these ENPs induced observable developmental deformities of the embryos. In addition, toxicity evaluations using acute 96-h and chronic 28-d tests showed exposure duration may be a significant factor in ENPs-induced toxicity. Therefore, long-term exposure of aquatic organisms to ENPs – potentially can alter certain ecological populations at different trophic levels – and may compromise the entire aquatic ecological functionality. The percentage hatchlings in test chambers containing 0.5 g kg^{-1} γ-alumina and α-alumina concentration was 50% less to those observed in the controls. Our results suggest the embryonic growth and hatchability tests are useful endpoints in chronic sediment toxicity tests for determining the toxic thresholds of ENPs in sediment environment. Although no snail mortalities were observed during the static 96-h test containing sediment spiked with different concentrations of M-TiO_2, C-TiO_2, γ-alumina and α-alumina – the antioxidant enzymatic assay results indicated a significant change in antioxidant levels which altered peroxidation at 0.05 or 0.5 g kg^{-1}concentrations for both γ-alumina and α-alumina.

1. Introduction

Rapidly increasing quantities of engineered nanoparticles (ENPs) in commercial products and industrial applications poses yet to be fully determined health hazards and environmental impacts (Oberdörster et al., 2005, 2006; Maynard et al., 2006; Moore, 2006; Klaine et al., 2008). For instance, Fernandes et al. (2007) suggested that ENPs may affect organism behaviour, organism survivorship, community structure, function, or the biogeochemical processes. Moreover, toxicity assessments of ENPs in sediments are very limited because of limitations similar to those encountered in dealing with macroscale chemicals in the same environmental compartment such as the complexity of sediment–water column and sediment–biota interactions (White, 1988). Additional limitations of ENPs in sediments include the abiotic factors (temperature, natural organic matter, pH, etc.), biotic factors (surface chemistry, surface area, applied surface treatment (functionalization, etc.), and the application conditions (Klaine et al., 2008).

Sediments are an ecologically important component of the aquatic habitat because it concentrates toxic substances, and act as a sink for pollutants (Gerhardt and Schmidt, 2002). Therefore, toxicity in the water column, often monitored using planktonic or pelagic organisms is an insufficient indicator of toxicity for the sediment compartment. Sediment toxicity tests are often performed with pore water, and seldom using real sediment – partly due to lack of suitable methods to directly observe the test organ-

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isms, and secondly, inability to quantitatively measure parameters such as mortality, physiology, and behaviour in real time (Giesy et al., 1990; Burton and MacPherson, 1995). At present, there are no factual measured data on the concentrations and quantities of ENPs in the soil and sediment environments – apart from estimations derived using computer simulation models (Blaser et al., 2008; Mueller and Nowack, 2008; Gottschalk et al., 2009), and certainly none on their physicochemical forms or distribution (Klaine et al., 2008). ENPs of TiO_2 are widely used in applications such as solar energy cells, paints, coatings (e.g. self-cleaning coatings) (Pitkethly, 2004), cosmetics, toothpaste, sunscreens (Nowack and Bucheli, 2007; Serpone et al., 2007), and textiles (Yuranova et al., 2007). On the other hand, alumina-based ENPs are used in paints, abrasive agents, or insulators because of their good dielectric and abrasive properties (Diebold, 2003; Baraton and Merhari, 2004).

Reported toxicological assessments of ENPs are for fish or mammalian organisms (Borm et al., 2006) with limited studies in aquatic organisms (Kahru and Dubourguier, 2010). For instance, recent studies have investigated the potential toxicity of TiO_2 in mammalian (Lam et al., 2004, 2006; Lin et al., 2006; Long et al., 2006; Warheit et al., 2007a) biological systems. For the alumina-based ENPs, they were shown to cause significant lung inflammation when instilled into rat lungs because of their large surface activity (Lu et al., 2009). In addition, available ecotoxicological studies of TiO_2 ENPs in aquatic organisms are for species such as *Daphnia magna* (Hund-Rinke and Simon, 2006; Lovern and Klaper, 2006; Lovern et al., 2007; Warheit et al., 2007b; Heinlaan et al., 2008), algae (Hund-Rinke and Simon, 2006), fish (Kashiwada, 2006; Warheit et al., 2007b; Zhu et al., 2008), as well as nematodes *Caenorhabditis elegans* (Wang et al., 2009). However, to the authors' knowledge, the effects of TiO_2 and aluminas (γ-alumina or α-alumina) on the epic-benthic and sediment dweller freshwater snails *Physa acuta* (Dillon et al., 2002) remain unexplored. Yet the metal oxide ENPs are likely to pose increasing environmental concerns because of their chemistry, size, and non-biodegradability – and the potential to rapidly distribute throughout the environment with unknown consequences particularly in the sediment compartments.

Therefore, in this study, the *P. acuta* species were considered ideal organisms for evaluating the toxicity of ENPs in the sediment compartment. First, it is because of their benthic locomotory and feeding habits which makes them vulnerable to elevated concentration of ENPs in the sediments. In addition, the *P. acuta* were used as test organism because of their important role in freshwater systems as they represent a significant part of many fish and waterfowl diet (Gomot, 1998), and particularly in the South African aquatic environments. And finally, the aquatic gastropods represents between 20% to 60% of the global macroinvertebrate abundance and biomass in freshwater ecosystems (Habdija et al., 1995) which makes them ideal organisms for toxicity testing in sediment environment.

Generally bioassays use mortality as an endpoint; however, sub-lethal endpoints in the recent years have been established to be more sensitive because the first reaction of an organism to stress is physiological in nature (Gerhardt, 1996). Additionally, acute tests results cannot provide substantive information about the sub-lethal or cumulative effects of test substances as they cannot predict potential chronic toxicity (Macek et al., 1978). The endpoints measured in this study were; percentage survival after a 96-h static acute test, peroxidation enzyme activity as biomarker for adverse effects on ROS species as well as embryo development and hatching over a 4 week period.

In summary, the study's objectives were twofold, viz.: (i) to determine the toxicological impacts of TiO_2- and alumina-based ENPs on aquatic organisms; and (ii) to examine the effect of exposure duration on the toxicity of TiO_2- and alumina-based ENPs on aquatic organisms using *P. acuta* as the test organism under controlled laboratory conditions, and different concentrations. The study was carried out using four ENPs, namely: alumina (γ-alumina and α-alumina) and, TiO_2 (modified TiO_2 (M-TiO_2), and commercial TiO_2 (C-TiO_2)) chosen because of their high use in consumer products and industrial applications, and therefore, poses potential exposure to aquatic organisms. A review of the scientific literature suggest that numerous acute toxicity tests of ENPs have been reported, however, very limited chronic results have been published – though the latter holds the promise of elucidating the long-term effects of nanoscale materials in the aquatic environments.

2. Materials and methods

2.1. Culturing of test specimens

P. acuta is a common pulmonate snail mostly found in streams, ponds, and lakes throughout South Africa. The *P. acuta* snails used in this bioassay were obtained from batch cultures of offspring collected from the Rietvlei nature reserve wetland area, South Africa, since 2004. The adult snails used in the experiments had an average wet weight with shell of 0.7 g (range 0.47–0.91 g), and an average shell length of 10–11 mm. *P. acuta* snails were cultured in 10 L water-sized aquariums containing 9 Ls of AFNOR artificial fresh water (200 mg L^{-1} $NaHCO_3$, 297 mg L^{-1} $CaCl_2 \cdots 2H_2O$, 167 mg L^{-1} $MgCl_2 \cdots 6H_2O$, 26 mg L^{-1} anhydrous K_2SO_4) at a constant temperature (21 ± 1 °C), and a 12:12-h light: dark photoperiod (Gomot, 1998). The water from the aquariums was used in the controls during the entire experimental duration. The *P. acuta* snails were fed with 0.5 g of Tetramin fish food (TetraWerk, Germany) throughout the experiment period.

2.2. Nanoparticles characterization

Before the initiation of the 96-h toxicity and reproduction experiments, each NM (TiO_2, commercial TiO_2, γ-alumina and α-alumina) was characterized to provide a basis for understanding their observed toxic effects. The ENPs were characterized using a range of instrumental techniques. Physicochemical properties that were determined included zeta potential, particle size, shape, density, solubility, surface area, and morphology. The X-ray powder diffraction (XRD, a Phillips PW 1830) generator was used to determine whether the compounds were in crystalline or amorphous forms. The morphology and size estimation of nano powders were assessed in a JEOL JSM 1560 LV scanning electron microscope (SEM) (Jeol, Peabody, MA) under low magnification. Elemental composition was determined using energy dispersive X-ray fluorescence (XRF) analysis. Particle size and size distributions were determined on a Malvern Zetasizer 3000 HSA (Malvern Instruments). Braunner, Emmett, and Teller (BET) surface areas were measured on a Micromeritics Flowsorb II 2300 instrument.

2.3. Sediment characterization and spiking

The sediment used in the acute and chronic tests had a relative dry weight of 80% acid washed sand, and 20% kaolin clay (BDH Chemicals Ltd., Poole, England, supplied both products). Physical characterization of the sediment entailed analysing the grain size with mean size of 0.25 mm (Plumb, 1981), and had total organic carbon of 0.01% (TOC) (ASTM, 1985, modified for sediment). The sediments were spiked with three different concentrations of 0.005, 0.05, and 0.5 g kg^{-1} for each ENP excluding the control samples (sediment without ENMs but containing *P. acuta* test specimens). The sediment was spiked mechanically through

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mixing of the appropriate concentration of each ENP under investigation.

2.4. Acute toxicity tests

The exposure of the *P. acuta* snails to different panel of M-TiO_2, C-TiO_2, γ-alumina and α-alumina ENPs were conducted as 96-h static acute tests (without renewal of water) using snails (shell length, 10–11 mm) from the batch cultures. Because the *P. acuta* are epic-benthic grazers – sediment was used as the exposure media for each ENP under investigation. The toxicity tests were conducted in 200 mL beakers in three replicate chambers, and also three controls were set up. The sediment volume for each test was 50 mL (200 g dry^{-1} weight), and 100 mL of overlying snail cultured water. In this experiment, only commercially washed sediment was used. Sediments were spiked with three different ENPs concentrations of 0.005, 0.05, and 0.5 g $kg^{-1}$for each ENP type. The values were selected in accordance to the recent findings of Boxall et al. (2007) which suggested the concentrations of most common ENPs expected to be present in natural waters will be in the range of 1–10 μg L^{-1} and total ENP concentrations may reach up to 100 μg L^{-1} while values in sediment compartment may be much higher. Recently Klaine et al. (2008) suggested that sediments, and therefore, benthic organisms are expected to be the main sinks and receptors of ENPs in surface waters as metals normally sorbs into small colloids that aggregate and settle out from the water column onto the sediments (Sigg, 1994).

The ENPs were mixed with the sediment manually to ensure the homogeneity of the sample to avoid inconsistencies of the data from the ecotoxicological analysis. In addition, to reflect the likely possible natural settling of ENPs in real environment-settings as opposed to dispersion through use of dispersants and sonication techniques. Overlying water (snail culture water) was added to the test chambers 3 d (72-h) before test initiation to allow the system to equilibrate in accordance to the USEPA (2000) guidelines. Ten snails were placed in each test chambers, with three replicates for each of the three treatment concentrations of the ENPs under investigation. On the other hand, a negative control in triplicate was set up containing only culture water and ten snails. Adverse effects were expressed as percentage lethality. The number of living and dead *P. acuta* snails in each chamber was recorded after 96-h. Snails that did not respond to a gentle prodding with forceps were considered to be dead. Each bioassay was conduced at approximately 21–22 °C, and a 12:12-h light:dark photoperiod. The toxicological endpoints measured were; the survival percentage and antioxidant enzyme activity after 96-h of exposure.

The following water quality parameters were measured, namely; pH, alkalinity and conductivity at test initiation and termination times. In addition, temperature and dissolved oxygen (DO) of the overlaying water were monitored daily for 96-h. After 96-h, the mean survival snails exposed to the sediment at different concentrations per each ENP type were counted, and compared to the number of test specimens in the negative control. Different ENPs concentrations were considered to be toxic if a given test endpoint namely survival and reproduction were statistically different from those of the control test organisms ($P < 0.05$), and at least 20% lower than the mean test organism response in the negative control sample (USEPA, 1994; Thursby et al., 1997).

2.5. Chronic toxicity tests

In the chronic toxicity testing, similar concentrations identical to those used for the acute toxicity were used. Three replicates were used for each treatment as well as the control group. The preparation of the test organism, spiking of the sediment with ENPs, and the culturing of the snails followed the same procedures as described for the acute toxicity. After the 28-d exposure similar endpoints as for the acute toxicity were evaluated. The snails were fed throughout the experimental period as per the culturing conditions to ensure there was no shock that could affect their survival or reproduction capabilities. During the 28 d chronic exposure tests to the ENPs materials, the tested snail specimens were fed on Tetramin fish food (TetraWerk, Germany).

2.6. Antioxidant enzyme assay

For the enzyme assays, snails exposed to different concentrations of ENPs after the 96-h static test were placed individually in Eppendorf tubes, snap frozen in liquid nitrogen, and stored at −80 °C before peroxidation enzyme activity were determined. The peroxidation enzyme was used as it is generic and representative of all cellular peroxidation reactions. Only alive snails were considered for enzyme assays. The soft parts of the tested snails (head, foot, visceral mass, and the mantle) were homogenized after the removal of the shells (10% w/v) in 0.1 M phosphate buffer (pH 7.5). The crude homogenates were centrifuged at 12 000*g* for 10 min. The resultant supernatant of the snails were used as the enzyme source to estimate the enzymatic activities (antioxidants). All the enzyme preparations were carried out at 4 °C and the total peroxidation activity in snails were determined using a guaiacol test (George, 1953). The ratio between the peroxidase activities to the protein content were expressed as optical density (O.D.)/min per milligram protein. The protein content was calculated using the Bradford method (Bradford, 1979).

2.7. Alterations of reproduction and development test

For the snail reproduction and embryo development tests, two separate sets of test chambers each containing different concentrations of sediment spiked ENPs were used. Both the sets contained three replicate chambers for each concentration and three negative controls without ENPs but test specimens. The tests were carried out over 28-d. The first set was used to make observations regarding the abnormal development at various stages of embryogenesis while the second set was used to establish the duration of incubation, and the number of hatchlings. The volume of the bottom sediment containing ENPs for each 200 mL test chamber was 50 mL (200 g dry^{-1} weight), while 100 mL of overlying snail cultured water was added. After a 1-w acclimation period, 10 adult snails per test chamber were added.

To investigate the abnormal development at various stages of embryogenesis – egg masses laid on the surface walls of the test chamber were collected weekly, and analysed under a dissecting microscope. The duration of incubation and the number of hatchlings were also noted for each ENP type and concentration. If an egg did not hatch after 28-d it was considered damaged. For example, Fig. 1 presents the embryo's exposed to different concentrations of γ-alumina and α-alumina indicating typical development abnormalities and malformations detected at various developmental stages. In the contrast, for the embryos exposed to M-TiO_2 and C-TiO_2 ENPs no abnormalities and malformations were observed although ENPs were observed pitting on the egg cells as shown in Fig. 2. Embryos were designated as abnormal if they satisfied at least one or more of the following criteria: (i) aberrant gross morphology involving abnormal growth, or gross morphological deformities compared with control embryos at the same development stage; (ii) abnormal pigmentation of embryo tissues with white or transparent regions in contrast to the normal brown colour; and (iii) obvious decomposition and/or arrested development for more than 2 weeks (Cheung and Lam, 1998; Hunter, 1988).

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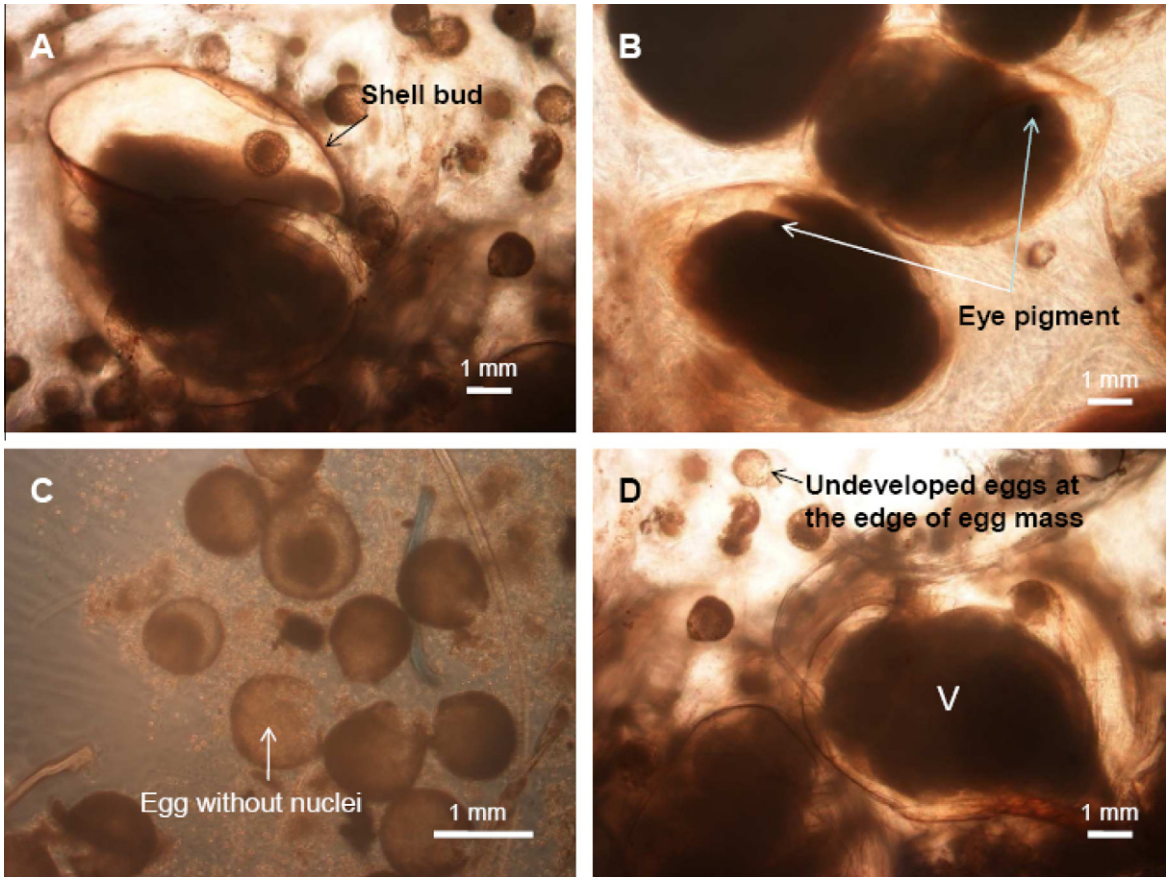

Fig. 1. Appearance of the main anomalies of embryogenesis induced at 0.5 g kg^{-1} for γ-alumina and α-alumina. (a) In the egg mass subjected to 0.5 g kg^{-1} γ-alumina and α-alumina, underdeveloped eggs and almost entirely formed embryos (the isthmus which separating the food from the visceral mass and the shell bud is visible) to hatch with a ±10 d delay can be distinguished as well as hatching delayed eggs. (b) In the control egg mass almost all the eggs reached hatching stage at the same time, without any anomalies of embryogenesis. (c) Eggs without nuclei visible from exposure to 0.5 g kg^{-1} of γ-alumina and α-alumina did not develop over the 4 week period. (d) Veliger stages are visible at the center of the egg mass, while a clearly distinguishable inhibition of eggs is seen on the edge of the egg mass, indicating great delays in hatching.

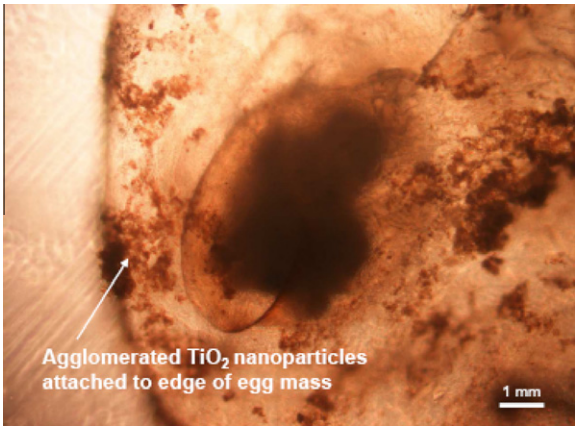

Fig. 2. Agglomerated C-TiO_2 nanoparticles attached to the egg mass, but the eggs of the exposed egg mass did not show any anomalies of embryogenesis during the 4 weeks of exposure.

2.8. Statistical analysis

All data were analysed statistically using the software package PRISM® Version 3.01 (Graph-Pad Software, San Diego, CA, USA). We calculated mean and standard error for each treatment and performed one-way-ANOVAs (analyses of variance), followed by Tukey multiple comparisons of the means to check for differences between the treatments and the controls. A *P*-value ($P < 0.05$) was considered statistically significant.

3. Results

3.1. ENPs synthesis and characterizations

The textural data for the ENPs (M-TiO_2, C-TiO_2, γ-alumina and α-alumina) are summarised in Table 1. The specific BET surface area of the tubular M-TiO_2 (302 m^2 g^{-1}) was six times larger than that of the commercial sample (C-TiO^2, 50 m^2 g^{-1}). The larger specific surface area of M-TiO_2 is due to the tubular nature of the material. Interestingly, the specific surface area of γ-alumina (72 m^2 g^{-1}) was found to be six times larger than that of α-alumina (13 m^2 g^{-1}). The difference in surface area of alumina samples is attributed to the difference in particle size and intra-particle porosity.

The SEM micrographs of all samples (M-TiO_2, C-TiO_2, γ-alumina and α-alumina ENPs) are shown in Fig. 3. M-TiO_2 is made up of tubular structures with the diameter range of 7–11 nm (see Fig. 3a). The tubes are randomly distributed and tended to form bundles. The tubes are open-ended on either side and have thin walls. Furthermore, C-TiO_2, γ-alumina and α-alumina showed a spherical but irregular morphology (Fig. 3b, c, and d). M-TiO_2, C-TiO_2 and α-alumina showed narrow particle size distribution

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Table 1
Measured physicochemical properties of the ENPs.

Test	Property/units	C-TiO_2	M-TiO_2	α-Alumina	γ-Alumina
Zeta potential	mV	–	–	19.7	−18.7
BET	Surface area ($m^2\ g^{-1}$)	50.3	301.7	13	72
XRD	Morphology	Crystalline	Crystalline	Amorphous	Crystalline
SEM	Particle shape	Spherical	Tubular	Spherical	Spherical
Particle size	Size (nm)	40–60 nm	7–11 nm	20–50	80–400
Solubility	Degree	Insoluble	Insoluble	Insoluble	Slightly soluble
Degree of dispersion	No units	Mono	Mono	Mono	Poly

Notes: BET: Braunner, Emmett, and Teller; XRD: X-ray diffraction; SEM; scanning electron microscope.

of 7–11, 40–60 and 20–50 nm, respectively. On the contrary γ-alumina showed a wide particle size distribution (80–400 nm).

3.2. Acute toxicity, chronic toxicity, and reproduction

No mortalities of adult *P. acuta* snails exposed to different treatment concentrations of M-TiO_2, C-TiO_2, γ-alumina and α-alumina ENPs were observed during the 96-h static acute tests. From all the test chambers containing different concentrations of M-TiO_2, C-TiO_2, γ-alumina and α-alumina ENPs – only 1 or 2 individuals per snail group of 10 died in the test chambers containing 0.5 g kg^{-1} of γ-alumina and α-alumina concentration. Furthermore, mortalities of adult snails were very low during the 4-w reproduction and embryo development test. For instance, over the 28-d chronical exposure period, only five snail deaths were observed in a population of 60 used for test chambers containing 0.5 g kg^{-1} of γ-alumina and α-alumina concentration (about 8% mortality). In the control test chambers a 98% survival was observed over the 28-d chronical exposure test period. The deaths occurred near the end of the 4 week experimental period.

Egg laying frequency gradually increased during the first 2-w of the experiment, and the laying activities (number of egg masses and eggs per mass) remained relatively close in numbers in test chambers containing different concentrations for all the ENPs types in comparison to the controls as shown in Table 2. However, the egg masses and number of eggs per mass in test chamber with exposure concentrations of 0.05 g kg^{-1} γ-alumina and α-alumina were irregular with normal masses of 50–75 eggs and smaller

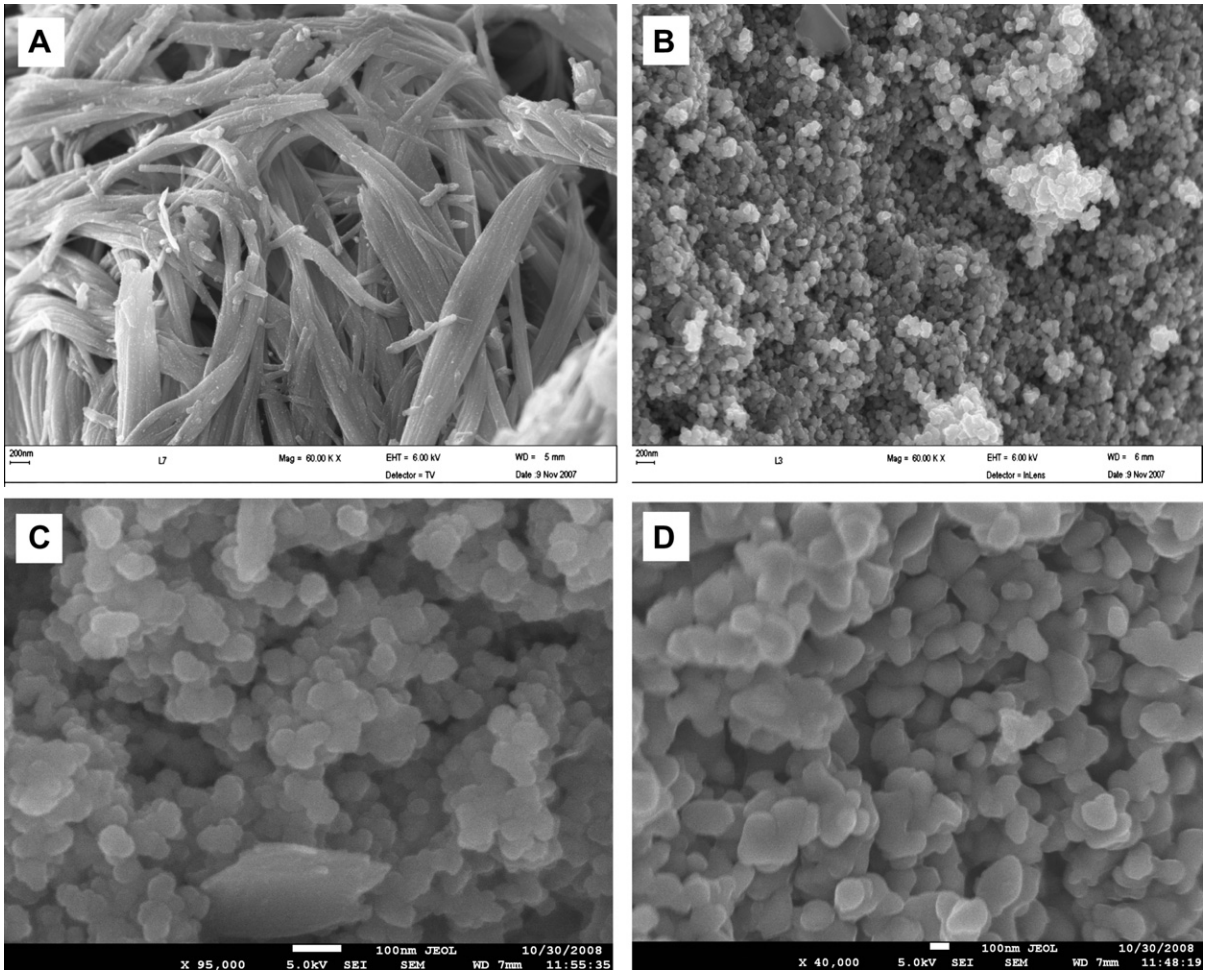

Fig. 3. SEM images of; (a) M-TiO_2, (b) C-TiO_2, (c) α-alumina, and (d) γ-alumina ENPs, respectively.

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Table 2
Egg production and hatching success for *Physa acuta* during 28-d period.

Concentration (g kg^{-1})	Number of egg masses from week 1 to 4	Number of eggs week 1 to 4	Mean (±SD) eggs per egg mass	Total (±SD) hatchlings in 4 weeks	Mean% per week
M-TiO$_2$					
0 (control)	11	201	18 (±2)	156 (±4)	19
0.005	10	200	20 (±3)	161 (±3)	20
0.05	11	198	18 (±1)	163 (±6)	20
0.5	11	194	17 (±2)	167 (±4)	21
C-TiO$_2$					
0 (control)	10	200	20 (±2)	147 (±4)	18
0.005	11	205	18 (±1)	162 (±4)	19
0.05	11	202	18 (±3)	157 (±5)	19
0.5	10	199	19 (±2)	164 (±6)	20
α-Alumina					
0 (control)	10	197	19 (±3)	161 (±4)	20
0.005	10	140	14 (±3)	112 (±5)	20
0.05	6	71	11 (±2)	40 (±3)	14
0.5	3	25*	8 (±1)*	8 (±1)*	8*
γ-Alumina					
0 (control)	11	198	18 (±2)	161 (±5)	20
0.005	9	131	14 (±3)	92 (±6)	17
0.05	5	49	9 (±2)	18 (±1)*	9*
0.5	3	22*	7 (±1)*	7 (±1)*	7*

* Significant difference compared with control ($P < 0.05$).

masses with about 5–8 eggs, while in the test chambers containing concentrations of 0.5 g kg^{-1} γ-alumina had only a few egg masses – each containing an average of four eggs.

Aberrant embryo development was also observed in these test chambers containing 0.5 g kg^{-1} γ-alumina and α-alumina concentrations. Some eggs in the egg masses at this concentration of γ-alumina and α-alumina halted development at the four blastomere and morula stages, while the other eggs in the egg masses underwent an embryonic development that was morphologically normal but slower in comparison to the negative controls. The EC_{50} (concentration of the substance causing a 50% decrease of performance compared to the controls) established for hatchlings were 0.5 g kg^{-1} γ-alumina and α-alumina, respectively using the Spearman–Karber method. No EC_{50} was established for M-TiO_2 or C-TiO_2 since no adverse effects were observed in comparison to the controls. One-way ANOVA indicated that an increase in γ-alumina and α-alumina concentrations caused a significant decrease ($P < 0.01$) in the percentage of embryo hatched.

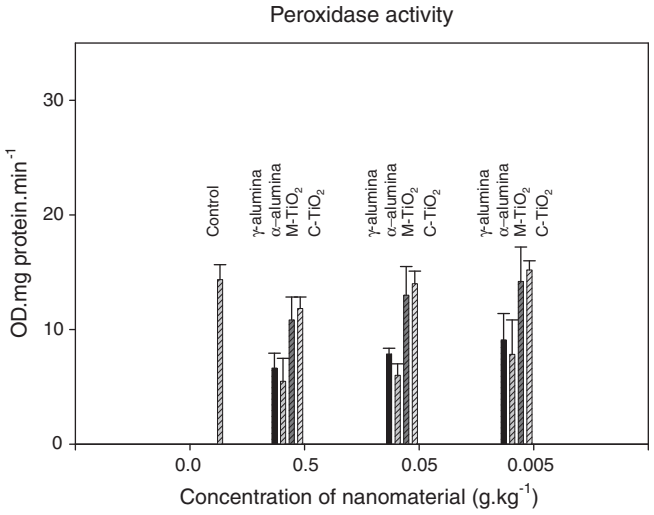

Fig. 4. Peroxidase activities (O.D. mg protein min^{-1}) in the freshwater snail *Physa acuta* (Draparnaud), after a 96 h exposure to spiked sediment with different concentrations of nanomaterials (means of three replicates ±SD error bars). Similar letters are indicative of significant differences between treatments ($P < 0.05$).

3.3. Antioxidant enzymes assays

The peroxidation experiment was to determine whether the toxicity and reproduction effects were due to oxidative stress induced by the ENPs exposed to the snails. The lowest peroxidase enzyme activities were observed in specimens exposed to γ-alumina and α-alumina ENPs as shown in Fig. 4. The decrease of peroxidase activity (average 8 OD mg protein min^{-1}) correlated negatively ($P \leqslant 0.05$) with the snails exposed to the highest concentration (0.5 g kg^{-1}) for γ-alumina and α-alumina. Our results suggest that the peroxidase activities decreased as γ-alumina and α-alumina concentrations increased, particularly when compared with the control. These findings suggest that the ENPs could have inhibited the anti-oxidant stress response. The enzyme activity of specimens exposed to sediment spiked with concentrations of M-TiO_2 and M-TiO_2 ENPs were comparable to the average enzyme activity in the test specimens in the negative controls (see Fig. 3).

4. Discussion

Analytical confirmation of the concentrations of the tested ENPs in the sediment and water column after 96-h were not attempted because of the present limitations of measuring ENPs against a high background of natural colloids in sediment environments (Klaine et al., 2008). Although preliminary work using the flow field-flow fractionation technique coupled to an inductively coupled plasma mass spectrometer has the potential to provide concentrations, only preliminary data are available and further research is required (Klaine et al., 2008). This implies detecting and quantifying ENPs in statistically significant numbers in environmental matrices using imaging techniques (e.g., scanning electron microscopy and atomic force microscopy) cannot be used for in situ measurements of colloidal behaviour of ENPs in aqueous matrices as they rely on per-particle analysis – which are highly labour-intensive and only view a comparably small statistical sample set. However, in future experiments it would be advisable to measure the actual sediment concentrations of tested ENPs when the appropriate analytical techniques are available. Considering the above, it is likely that the sediment concentrations of the different tested ENPs decreased with time due to uptake and possible metabolism by the snails. The possibility that the tested ENPs desorbed from the sediment into the water column during the experiment cannot be over-ruled, and should be considered when evaluating the results.

Although no snail mortalities were observed during the static 96-h test containing sediment spiked with different concentrations of M-TiO_2, C-TiO_2, γ-alumina and α-alumina, the antioxidant enzyme assay indicated significant change in the antioxidant activity. The peroxidation was altered at concentrations of 0.05 g kg^{-1} and above for the γ-alumina and α-alumina ENPs. This was probably triggered by the release of excessive free radicals generation beyond the normal levels possible to quench (Vutukuru et al., 2006). The toxicological interactions between the ENPs and proteins are either due to the ENP physically interacting with proteins, or the ENP producing ROS or other damaging radicals. Our results suggest that the peroxidase enzyme activity decreased with increasing dose implying that the ENPs may have been responsible for inhibiting the anti-oxidant response. This phenomenon was observed as being more dominant for the γ-alumina and α-alumina

ENPs, and was less evident for the M-TiO_2 and C-TiO_2 ENPs in comparison to the controls (Fig. 4).

On the other hand, the M-TiO_2 caused lower peroxidase activity than the C-TiO_2. This can be attributed to the functionalization of the M-TiO_2 where the active surfaces were significantly reduced through coating and inhibited the particles from inducing any significant biological effects. Our experimental results are in agreement with earlier findings of Klaper and co-workers (2009) where certain functionalized fullerene treatments as well as TiO_2 exposures were less toxic than unmodified fullerene preparation supporting the hypothesis that functionalization affects toxicity and core ENP chemistry also impacts toxicity (Klaper et al., 2009).

ENPs can indirectly cause membrane damage through the generation of reactive oxygen species (ROS), which can oxidize double bonds on fatty acid tails of membrane phospholipids in a process known as lipid peroxidation. This increases membrane permeability and fluidity, making cells more susceptible to osmotic stress or hindering nutrient uptake (Cabiscol et al., 2000). It is difficult to compare our results of exposure to the different concentrations of tested ENPs via sediment with other results because of lack of sediment studies in the scientific literature. Previous studies by Warheit et al. (2007a,b) reported that exposure of daphnids to dilution water control and fine or ultrafine TiO_2 particle concentrations of 0.1, 1.0, 10 and 100 mg L^{-1} over 48-h period – the static acute test showed low concern for aquatic hazard which is concurrent with results obtained from the exposure of snails to spiked sediments in our study in the 96-h study.

However, Lovern et al. (2007) reported behavioural and physiological changes in *D. magna* when exposed to nanoparticle suspensions of TiO_2, Nano-C_{60} and $C_{60}HxC_{70}Hx$. From our study, though the results showed no acute toxicity, however, sub-lethal responses were observed which could cause ecological consequences considering the possible release of ENPs into the environment, and resulting to the sediment compartment as the final sink. The observations of abnormal *P. acuta* embryos at low level concentrations of γ-alumina and α-alumina, suggested that these ENPs are teratogenic. The adverse effects of these ENPs on growth and development rates would mean fewer offspring produced per unit time. As a result, even at low concentrations, a significant impact on the snail population may occur and such disruptions could initiate knock-off effects on the functioning of the aquatic ecosystem. This is because snails are important species in the freshwater food webs as they graze on algae, and themselves are prey to fish, and other invertebrate predators (Osenberg and Mittelbach, 1989; Underwood, 1991; Bernot and Turner, 2001).

In addition, the pulmonate snails are important detritivores in standing and running water. Cummins (1974) devised four main categories of invertebrate consumers in streams, and freshwater snails like *P. acuta* were classified as 'grazer-scrapers' that have mouth parts appropriate for scraping off and consuming the organic layer (composed of attached algae, fungi and dead organic matter) attached to rocks and stones. Although concentrations of 0.5 g kg^{-1} of γ-alumina and α-alumina inhibits hatching or induce slow development, it did not prevent egg laying, which indicate that the eggs and embryos were much more sensitive than the adult snails based on the comparison of results derived from the 96-h static tests.

The question raised by these results is how does γ-alumina and α-alumina reach the eggs and embryos, since egg cells benefits from two levels of protection namely the gelatinous matter surrounding it – and from the envelope in which the embryo can survive (Cheung and Lam, 1998). In the present observation, it seems that in *P. acuta*, the oocytes in the ovotestis and the eggs after fertilization during the deposition of the substances secreted by the genital tract were protected from the γ-alumina and α-alumina concentrations up to 0.05 g kg^{-1}. However, the egg membranes were compromised when exposed to concentrations of γ-alumina and α-alumina beyond this threshold-value. Impairment of behaviour such as avoiding the sediment surface by snails in the highest concentration (0.5 g kg^{-1}) for γ-alumina and α-alumina as seen in our study are likely to reduce the fitness of aquatic organisms (Connell et al., 1999). From our observations none of the different concentrations of TiO_2, commercial TiO_2, γ-alumina and α-alumina prevented egg lying.

Results of from this study facilitated to undertake a comparison of the relative sensitivity and usefulness of different endpoints towards the four tested ENPs. Reproduction is obviously the most important function in the life cycle of an organism, since successful reproduction is essential for the continuation of the snail species. Reduction of the snails' fertility due to contaminants affects the eggs viability, egg development, or hatchability. The observations made during the experiment provide information on the survival, snail behaviour, egg laying activity, and development of embryos up to the hatching stage.

5. Conclusion

The present study represents, to our knowledge, the first ecotoxicological tests of ENPs on the *P. acuta* snails as a epic-benthic grazer on the sediments. From our study findings, the dose–response relationship characterizes the toxicity of tested ENPs in sediment, and permits the quantification of the adverse effects threshold concentration for certain specific ENPs. The embryonic growth and hatchability were significantly affected by γ-alumina and α-alumina ENPs. However, M-TiO_2, and C-TiO_2, did not show any adverse affects on the tested snail specimens. Overall, our results suggest that the embryos of *P. acuta* are useful in NM ecotoxicological assessment. It is also evident that toxicity level is not always dependent on the size effects and surface properties of the ENPs, but on the nature of the materials and probably the method of synthesis.

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