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Evaluation of Alpha and Gamma Aluminum Oxide Nanoparticle Accumulation, Toxicity and Depuration in *Artemia Salina* Larvae

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Abstract

In this study, *Artemia salina* (crustacean filter feeders) larvae were used as a test model to investigate the toxicity of aluminum oxide nanoparticles (Al₂O₃ NPs) on marine microorganisms. The uptake, toxicity and elimination of α-Al₂O₃ (50 nm and 3.5 μm) and γ-Al₂O₃ (5 nm and 0.4 μm) NPs were studied. Twenty-four and ninety-six hour exposures of different concentrations of Al₂O₃ NPs to *Artemia* larvae were conducted in a seawater medium. When suspended in water, Al₂O₃ NPs aggregated substantially with the sizes ranging from 6.3 nm to > 0.3 μm for spherical NPs, and from 250 to 756 nm for rod-shaped NPs. The phase contrast microscope images revealed that NPs deposited inside the guts as aggregates. ICP-MS analysis showed that large particles (3.5 μm α-Al₂O₃) were not taken up by *Artemia*, while fine NPs (0.4 μm γ-Al₂O₃) and ultra-fine NPs (5 nm γ-Al₂O₃ and 50 nm α-Al₂O₃) accumulated substantially. Differences in toxicity were detected as changing with NP size and morphology. The malondialdehyde (MDA) levels indicated that smaller γ-Al₂O₃ (5 nm) NPs were more toxic than larger γ-Al₂O₃ (0.4 μm) particulates in 96 h. The highest mortality was measured as 34% in 96 h for γ-Al₂O₃ NPs (5 nm) at 100 mg/L (LC₅₀ > 100 mg/L). γ-Al₂O₃ NPs were more toxic than α-Al₂O₃ NPs at in all conditions.

Keywords

Aluminum oxide; nanoparticles; accumulation; toxicity; oxidative stress; *Artemia*

INTRODUCTION

Nanoscience is becoming a prominent area of research in today's society. For years, nanoparticles (NPs), natural and manmade, have been entering the environment while their effects are unknown. The large increase in production, species and utilization of

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manufactured NPs has raised concerns that the release of these materials into the environment may pose a serious threat, leading to calls for an environmental risk assessment of NPs (Moore, 2006; Nowack and Bucheli, 2007; Handy et al., 2008; Klaine et al., 2008). As nanomaterials are being widely used in modern technology, there is an increasing need for information regarding their human health and environmental implications. To date, the human health impacts of NPs have received the greatest attention, which has been demonstrated through both *in vivo* and *in vitro* studies on mammalian test systems. The properties that make NPs so attractive for commercial applications (e.g. NPs' size and increased surface area) also can be potentially responsible for undesirable health effects (Oberdörster et al., 2005; Meng et al., 2007; Papageorgiou et al., 2007; Singh et al., 2007; Poland et al., 2008).

Metal and metal oxide NPs are receiving increasing attention for a variety of applications. NPs have unique physicochemical properties, such as their chemistry, tiny size, large surface area, surface reactivity, charge, shape and media interactions. As a result, the properties of NPs differ substantially from their respective bulk materials of the same composition. However, certain novel properties of NPs could lead to adverse biological effects, with the potential for toxicity (Oberdörster et al., 2005; Nel et al., 2006). Aluminum oxide (Al_2O_3) NPs have been widely used as abrasives, as wear-resistant coatings on propeller shafts of ships, as a way to increase the specific impulse per weight of composite propellants used in solid rocket fuel and as drug delivery systems to increase solubility (Miziolek, 2002; Tyner et al., 2004; Wagner et al., 2007). However, the risks posed by the increased use of Al_2O_3 NPs are little known. Recently, several researchers studied the effects of Al_2O_3 NPs on organisms such as *E. coli* (Sadiq et al., 2009), bacterial cells (Jiang et al., 2009) and microalgae species like *Scenedesmus* sp. and *Chlorella* sp. (Sadiq et al., 2011).

The use of aquatic models to address ecotoxicological issues has become prevalent in recent years (Rozita et al., 2010). *Artemia salina*, a zooplanktonic crustacean found in a variety of seawater systems from swamps to lakes – and the most popular live food for marine and aquarium fishes – was studied because the species filters a large amount of water per hour. This means that they have significant interaction with their aquatic environment, causing them to face a higher risk of exposure to pollutants than other aquatic species. *Artemia* larvae are sensitive to toxic substances (Milhem et al., 2008) and are commonly used for toxicity assays in pharmacology and ecotoxicity in a variety of nanoscale materials in marine ecosystems through laboratory experiments. An advantage of the *Artemia* test is the homogeneity in eggs and freshly-born individuals. Freshly-hatched individuals, called nauplii (larvae), were used for testing. Though the freshly-natal individuals are immediately applied in testing, difficult cultivation is not demanded.

Aluminium oxide exists in various metastable polymorphs (transition aluminas) including γ -phases of Al_2O_3 in addition to the thermodynamically stable α - Al_2O_3 form (corundum). The transition aluminas (especially the γ -form) have fine particle sizes and high surface areas with enhanced catalytic surface activity of their surfaces, and find industrial applications as adsorbents, catalysts or catalysts carriers, coatings and soft abrasives (Levin and Brandon 1998, Temujin et al., 2000). The aim of the current investigation was to study the difference (if any) in toxic response and characterization of uncoated α - Al_2O_3 and γ -

Al₂O₃ NPs. The uptake, toxicity and depuration of these NPs also were compared to those of different sizes and crystal structures. To analyze the possible dose-dependent toxic effects of NPs *in vivo*, *Artemia* under acute exposure for 24 and 96 h were used.

MATERIALS AND METHODS

Reagent and Preparation of Nanoparticle Suspension

Dry α -Al₂O₃ (50 nm and 3.5 μ m) and γ -Al₂O₃ (5 nm and 0.4 μ m) NPs were obtained from Skyspring Nanomaterials Inc., in Houston, TX. The NPs were stored at room temperature in the laboratory until the implementation of the experimental studies. The NP stock solutions were prepared as described by Wang et al. (2009). The stock suspensions of α -Al₂O₃ and γ -Al₂O₃ were prepared separately by direct addition into MilliQ water (Barnstead E-pure system with resistivity of 18.0 M Ω cm⁻¹) at a stock concentration of 20% w/v. The freshly-prepared stock solutions were vortexed (20 s, 2000 rpm) and ultrasonicated for maximum dispersion using a bath sonicator (10 min). Small magnetic bars were placed into the suspensions for stirring during dilution to avoid particle aggregation and deposition. Appropriate volumes of the stock suspension were immediately transferred into the exposure flask that already contained *Artemia* larvae in the seawater medium. All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Characterization of Nanoparticles

Powdered X-Ray Diffraction Analysis (XRD)—The powder samples were stored in a desiccator until they were needed. The crystal structures of the NPs were characterized by powdered X-ray powder diffraction (XRD) (D8 Advanced X-ray diffractometer, Bruker, Germany) and scanned with 2.2 kW Cu anode radiation at wavelength 1.54 Å produced by a Ceramic X-ray tube. About 250 mg of α -Al₂O₃ and γ -Al₂O₃ NPs were deposited separately on the sample holder for scanning in the range of 10° to 100°. The crystalline formation was determined from the diffraction pattern and the crystallite size was calculated with the Scherrer formula.

Fourier Transform Infrared Spectroscopic Analysis (FT-IR)—All dry α -Al₂O₃ and γ -Al₂O₃ NP samples were prepared by an identical procedure for Fourier transform infrared spectroscopy (FT-IR). The FT-IR spectra were recorded using a spectrometer Nicolet Nexus 6700 FT-IR (Thermo Scientific Instruments Groups, Madison, WI) in the range of 4000 to 400 cm⁻¹ and samples were prepared in potassium bromide pellets. Spectra were collected in single beam mode with 32 scans at 4 cm⁻¹ resolution. The instrument detector was set on DTGS (deuterated triglycine sulfate – IR detector material), and the Beam Splitter was set on KBr. The gain parameter used to amplify the signal of the detector was set to Autogain, the velocity of the mirror was set at 0.6329 and the aperture was set at value 60. KBr (150 mg) and the sample (2 to 4 mg) were ground together in a mortar using a pestle, and pellets with good transparency were prepared. First, the background spectrum was recorded, then the sample spectrum was collected in the same conditions. Background was automatically subtracted from sample spectra, and final spectra were recorded in transmission mode. The management of spectra was realized with instrument software (Omnic User's Guide, 2006; Štengl et al., 2010; Palaniappan and Pramod, 2011).

Transmission Electron Microscopic Analysis (TEM)—The particle size and shape of α -Al₂O₃ and γ -Al₂O₃ particles were characterized by transmission electron microscopy (TEM) using a JOEL-1011 TEM instrument providing a resolution of JEM-1011 of 0.2 nm lattice with magnification of 50 to 1x10⁶ under the accelerating voltage of 40 to 100 kV. For TEM measurements, a drop of the colloidal solution of NPs was placed onto 50 Å thick carbon-coated copper grids and allowed to dry to record the TEM images. The average particle diameter and size distribution were determined by using ImageJ software. The diameters of approximately 100 particles of α -Al₂O₃ and γ -Al₂O₃ were measured in random fields of view of three images to estimate their average size.

Hydrodynamic Light Scattering Analysis (DLS)—The actual size distribution of the α -Al₂O₃ and γ -Al₂O₃ NPs in the stock solution and exposure medium was determined by DLS measurements (Malvern Zetasizer Nano ZS Instrument). The actual size distributions of the NPs were examined after suspension in deionized water at the stock concentration. However, the concentration was too high; hence, it was further diluted and adjusted to a lower concentration, so that the device could acquire enough counts per second. A portion of each particular suspension was sonicated for a few minutes to break down visible clumps, then it was placed in a clean 1.5 ml square cuvette for DLS measurements. Five DLS measurements were taken successively for each solution, and an average size was obtained by the software from the intensity, volume and number distributions measured.

Test Organism

The acute toxicity of these NPs was also evaluated using the *Artemia salina* nauplii (larvae). The *Artemia* cysts were purchased from Artemia International LLC, Houston, TX and were kept at a temperature of 4°C in a moisture-free container in a refrigerator. The cysts were hatched in artificial seawater (3% m/v). The seawater was prepared by dissolving an appropriate amount of Instant Ocean® salt in deionized water, stirring it for 24 h under aeration and then filtering it through 30- μ m Millipore cellulose filters. *Artemia* were hatched by using the procedure described by Persoone et al. (1989). Briefly, encysted *Artemia* were first hydrated in distilled water at 4 °C for 12 h and washed to separate the floating cysts from those that sink. The sinking cysts were collected on a Buchner funnel and washed with cold deionized water. Approximately 3 g of the pre-cleaned cysts were incubated in 1.5 L seawater in a conical plastic container with graduations at 30 $\pm$ 1 °C. A 1500 lux day-light was provided continuously by a fluorescent lamp. Aeration was maintained by a small line extending to the bottom of the hatching device from an aquarium air pump. Under these conditions, *Artemia* larvae hatched within 24 h.

Acute Toxicity Bioassays

A preliminary experiment was conducted to evaluate the *Artemia* larvae's response to different concentrations (5, 10, 50 and 100 mg/L) of the α -Al₂O₃ (50 nm and 3.5 μ m) and γ -Al₂O₃ (5 nm and 0.4 μ m) NPs separately for 24 and 96 h exposure. Acute toxicity immobilization tests were performed on each of the NPs according to the Organization for Economic Cooperation and Development (OECD part 202) testing guidelines (OECD 2004). A control group also was setup without the target compound. Studies were carried out in triplicate measurements in conical plastic containers (1 L inner volume). Exposures were

conducted in 500 mL of seawater for *Artemia* larvae. Slight aeration was provided by a line extending to the bottom of the conical flask to prevent the settling of NPs from suspension during the course of exposure. The number of *Artemia* larvae in each group visually counted under magnification lens after sequential dilutions before exposure. The number of larvae were found approximately in the range of 12.9 to 16.1 ($\times 10^3$). A salinity range of 2.9 to 3‰ m/v, a light regime ratio of 16:8 h light:dark, a water temperature of 24 ± 2 °C, and pH range of 7.6 to 8.8 were the conditions maintained during the experiment. There was no feeding during the tests.

Phase Contrast Microscopic Analysis

The process of quantifying different kinds of samples in terms of their phase contrast values was carried out in grayscale using a phase contrast microscope (Micromaster, Model 12-575-252, Fisher Scientific) equipped with a digital camera to elucidate the deposition of NPs. *Artemia* larvae from each group were examined at the end of the exposure period. Images were captured by Micron Imaging software from live *Artemia* in petri dishes.

Analytical Methods to Determine Aluminum Content

Trace metal grade nitric acid (HNO_3 , Fisher Scientific) was used for the dissolution of the *Artemia* samples collected after exposure to determine their total uptake and depuration levels. Stock Aluminum (Al) standard solution ($100 \mu\text{g mL}^{-1}$) was purchased from SCP Science (Champlain, NY). Calibration standards for inductively coupled plasma mass spectrometry (ICP-MS) determinations were prepared within a range of 0 to $100 \mu\text{g/L}$ from the stock Al solution in 5% HNO_3 . At the end of the exposure and depuration experiments, *Artemia* were sampled and thoroughly washed with deionized water through a $40 \mu\text{m}$ plankton net. The cleaned samples were then filtered with $0.45 \mu\text{m}$ Whatman filter paper. For instrumental analysis, about 0.1 g of wet *Artemia* were weighed and digested in teflon vessels in 2 mL of concentrated HNO_3 for 2 h using a digestion block (DigiPrep MS, SCP Science) at 160°C , according to the protocols described previously (Arslan et al., 2011). Once completely mineralized, the contents were diluted to 50 mL with deionized water and visually inspected for complete dissolution of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs (e.g., clear solution without any turbidity). The sample solutions were analyzed by ICP-MS using a Varian 820 MS ICP-MS instrument (Varian, Australia). The aluminum (Al) content of the solutions were measured to determine the accumulation pattern of the NPs across the dose of exposure. The total Al concentration detected was then translated into corresponding $\alpha\text{-Al}_2\text{O}_3$ (50 nm and $3.5 \mu\text{m}$) and $\gamma\text{-Al}_2\text{O}_3$ (5 nm and $0.4 \mu\text{m}$) NP concentrations.

Malondialdehyde Assay

The malondialdehyde (MDA) assay is the most prominent assay being used as an index for lipid peroxidation in biological systems by the measurement of the thiobarbituric acid-reactive substances (TBARS) through the thiobarbituric acid (TBA) test (Gutteridge and Halliwell, 1990). TBARS were measured to determine the lipid peroxidation products as a measure of oxidative stress. The composition of lipid hydroperoxides leads to a variety of end products, one of them being MDA, which is now accepted as a reliable marker of lipid peroxidation (Ohkawa and Ohishi, 1979). The MDA concentration was measured as

described by Van Ye et al. (1993). At the end of the exposure period, 0.1 g of *Artemia* were washed with cold water and assayed using the MDA assay kit obtained from Northwest Life Science Specialties, LLC, Vancouver, WA. Samples were homogenized in 2 mL of phosphate buffer solution (pH 7.2) by an ultrasonic homogenizer and centrifuged at 6,000 rpm for 10 min. The resulting supernatant was then immediately used for a biochemical assay. Briefly, 10 μ L of butylated hydroxytoluene reagents (BHT), 0.25 mL of sample supernatant, 0.25 mL of phosphoric acid reagent and 0.25 mL of TBA reagent were added to a vial. A set of MDA assay standards was freshly prepared, also from tetramethoxypropane. All samples and standards were incubated at 90 °C for one hour and centrifuged after cooling at 13,000 rpm for 10 min to precipitate suspending tissue. The reaction mixture was then transferred to UV-vis cuvettes and the absorbance was measured at 532 nm. Measurements were performed in triplicate for all experimental groups. The values were expressed as total MDA concentration per gram of *Artemia*.

Statistics

All experiments were repeated three times independently, and data were recorded as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons was used to detect significant differences in mortality, uptake and elimination/depuration rates among the controls and treatments. In all data analyses, a p-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of Nano α -Al₂O₃ and γ -Al₂O₃

The characterization of aluminum oxide NPs was carried out by X-ray diffraction, FT-IR and TEM analysis before introducing them to test conditions. The X-ray diffractograms of nano aluminum oxide data are shown in Figure 1. For nano α -Al₂O₃ (50 nm and 3.5 μ m) six diffraction peaks (at 57.0°, 53.8°, 44.5°, 37.5°, 35.5° and 25.0°) were observed (see Figure 1A, 1B). Sharp peaks of α -alumina proved its good crystallization (Sun et al., 2008; Shek et al., 1997). The data obtained were matched with the database of the Joint Committee on Powder Diffraction Standards (JCPDS) card file No. 46-1215, which confirmed the crystalline structure of alumina. The Debye-Scherrer equation was used to derive the crystallite size from the XRD data by determining the width of the 57.01° peak. The crystallite size was determined to be 17 ± 3 nm. Broad diffraction peaks were recorded for γ -Al₂O₃ 5 nm and 0.4 μ m (Figure 1C, 1D). They are similar to those previously reported and showed an amorphous structure with a small crystallization degree (Hosseini et al., 2012; Sun et al., 2008).

FT-IR spectroscopy is widely used for the analysis of nano-size metal oxides (Prasad et al., 2006; Gondal et al., 2012; Guo et al., 2008; Nassar and Ahmed, 2011). Specific bands due to metal-oxygen bonds were recorded below 1000 cm⁻¹ for these compounds (Alizadeh-Gheshlaghi et al., 2012). For α -Al₂O₃ 50 nm, both a sharp band with a maximum of 454.2 cm⁻¹ and a broad band with multiple peaks at 602.2, 644.3 and 729.1 cm⁻¹ assigned to stretching and bending vibrations of Al-O were recorded. These were the most intense bands of the α -Al₂O₃ FT-IR spectrum, with transmittance values between 22.7 and 37.2% (Table

1). The peak of 1114.4 cm^{-1} may be assigned to Al-OH bending vibration (Manivasakan et al., 2011; Crisan et al., 2000). Also, a few small bands were observed: the first with maxima at 1436.8 and 1384.7 cm^{-1} , the second with peaks at 1592.6 and 1630.6 cm^{-1} and the third with a large band at 3438.5 cm^{-1} . The transmittance values of these were small, which ranged from 69.1 to 79.8%. The band centered at 3438.5 cm^{-1} was assigned to O-H stretching vibration of adsorbed water, and those from 1630.6 to 1592.6 cm^{-1} were assigned to bending vibrations of H-O-H (Gondal et al., 2012; Nassar and Ahmed, 2011). Bands with peaks at 1436.8 and 1384.7 cm^{-1} were assigned to carbon dioxide $\nu(\text{O-C-O})$ symmetric vibration in the forms of carbonate and bicarbonate, respectively. Because alumina is an amphoteric oxide it contains both basic and acidic sites: by carbon dioxide adsorption both carbonate and bicarbonate species, respectively, may be formed (Galhotra, 2010; Crisan et al., 2000).

The spectra of $\alpha\text{-Al}_2\text{O}_3$ 50 nm and $3.5\text{ }\mu\text{m}$ are similar, with minor differences between wavelengths of some absorption maxima: e.g., the peak recorded at 602.2 cm^{-1} for $\alpha\text{-Al}_2\text{O}_3$ 50nm was determined at 594.1 cm^{-1} for $3.5\text{ }\mu\text{m}$ (Table 1). The absorption maxima observed for $\alpha\text{-Al}_2\text{O}_3$ were matched with those previously reported, and the presence of multiple peaks was associated with the crystalline form of α -alumina (Boumaza et al., 2010; Sun et al., 2008; Shek et al., 1997). For $\gamma\text{-Al}_2\text{O}_3$ 5 nm, a broad and intense band with small peaks at 572.6 , 624 , 701.6 , 750.7 and 807.9 cm^{-1} and transmittance values between 20.3 and 23.8 % were recorded. The band due carbon dioxide absorption had maxima at 1436.6 and 1384.5 cm^{-1} and those corresponding to water at 3440.3 , 1631.2 and 1588.5 cm^{-1} . The small peak at 1118.5 cm^{-1} was assigned to $\delta\text{Al-OH}$ vibration. In the case of $\gamma\text{-Al}_2\text{O}_3$ $0.4\text{ }\mu\text{m}$, a similar spectra with a fine structure between 822.5 and 570 cm^{-1} was recorded. The broad bands of $\gamma\text{-Al}_2\text{O}_3$ with some fine structures are characteristic of amorphous powders with poor crystallization (Sun et al., 2008; Shek et al., 1997). Thus, it was determined that for the same polymorph phase, like $\alpha\text{-Al}_2\text{O}_3$ (50 nm, $3.5\text{ }\mu\text{m}$) and $\gamma\text{-Al}_2\text{O}_3$ (5 nm, $0.4\text{ }\mu\text{m}$), the influence of particle size on FT-IR spectra was not significant. Both phases presented specific transmittance values ranging from 731.2 to 449 cm^{-1} and from 822.5 to 570 cm^{-1} , respectively. The observations from FT-IR spectra analysis of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ are in agreement with XRD results.

Further characterization of the size and shape of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs was measured in different media and carried out by TEM images (Figure 2). The sizes measurements in dried suspension of small and large spherical NPs ranged from 45 to 100 nm, from 6 to 45 nm and $> 0.3\text{ }\mu\text{m}$ for $\alpha\text{-Al}_2\text{O}_3$ (50 nm), $\gamma\text{-Al}_2\text{O}_3$ (5 nm) and $\gamma\text{-Al}_2\text{O}_3$ ($0.4\text{ }\mu\text{m}$) NPs, excluding $\alpha\text{-Al}_2\text{O}_3$ ($3.5\text{ }\mu\text{m}$), respectively. Nanorod lengths of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ were also measured from 240 to 750 nm with diameters of 10 nm. DLS results showed a wide distribution of particle size for all types of NPs, excluding $\alpha\text{-Al}_2\text{O}_3$ ($3.5\text{ }\mu\text{m}$), which typically showed two distinctive populations in both the several hundred nanometers region and the several microns domain, a result that is consistent with previous reports (Franklin et al., 2007; Peng et al., 2011; Ates et al., 2013). DLS provided the size distribution for the hydrated forms the NPs as 412.1, 561.9 and 1096 nm for $\alpha\text{-Al}_2\text{O}_3$ (50 nm), $\gamma\text{-Al}_2\text{O}_3$ (5 nm), $\gamma\text{-Al}_2\text{O}_3$ ($0.4\text{ }\mu\text{m}$) NPs, respectively. Particle size increased by a factor of above 40 for spherical NPs, indicative of the considerable degree of aggregation within NPs as a consequence of the high surface-area-to-volume ratio of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs. Zeta potential were

measured negatively charged for ultrafine α -Al₂O₃ (50 nm) and γ -Al₂O₃ (5 nm) NPs, and positively charged for larger particles. In fact, it is not uncommon for particles to appear as agglomerates in solution, since there was no surfactant capping on their surface (Adams et al., 2006; Miller et al., 2010). The colloidal suspensions were found to be enhanced with smaller α -Al₂O₃ and γ -Al₂O₃ NPs compared to larger particles, even though the NPs were present in solution as aggregates with hydrodynamic diameters ranging from 1 to 5 μ m. It is clear from these results that the aggregation of α -Al₂O₃ and γ -Al₂O₃ NPs in water is inevitable, unless a suitable surface modification is made to render the NPs water-soluble.

Uptake and Depuration of Nano α -Al₂O₃ and γ -Al₂O₃ in *Artemia*

Artemia, especially large taxa like *Daphnia*, are considered non-selective filter feeders; thus they can ingest readily fine particles smaller than 50 μ m (Hund-Rinke and Simon, 2006). Accumulation was clearly visible under the microscope, manifested by a dark line inside the guts of the larvae (Figure 3). The images demonstrated that the guts for the control were empty (transparent), whereas they were entirely filled with NPs in the treatment, especially for those in Groups C and D which were exposed to 50 and 100 mg/L levels, respectively. The results indicate that *Artemia* larvae did not show any discrimination to particle size variation among the NP suspensions, and that they readily accumulated the aggregates of α -Al₂O₃ and γ -Al₂O₃ NPs to elevated levels (e.g., mg/g) in their guts (see Figure 3). But the largest particulates of α -Al₂O₃ (3.5 μ m) were not taken up by *Artemia* in either exposure period and its image was similar to that of the control (Figure was not shown).

The total α -Al₂O₃ and γ -Al₂O₃ NP contents of *Artemia* samples were determined by ICPMS. The concentration values were based on the wet weight of *Artemia*, reflecting the total body burden across the concentration gradient from 10 to 100 mg NP L⁻¹. ICP-MS analysis showed that accumulation was dependent on NP size, and that no aluminum uptake was detected in the controls and groups exposed to the largest particulate of α -Al₂O₃ (3.5 μ m) in either exposure period. The accumulation profiles of all experimental groups were illustrated in Figure 4. Total NP uptake by *Artemia* increased with increasing initial NP concentration in the medium and with exposure time. The accumulations of both NPs in *Artemia* were significantly higher in 96 h than in 24 h ($p < 0.05$). Although larger particles (α -Al₂O₃ 3.5 μ m) were not taken up by *Artemia*, fine NPs (0.4 μ m γ -Al₂O₃) and ultra-fine NPs (5 nm γ -Al₂O₃ and 50 nm α -Al₂O₃) accumulated substantially. Average Al concentration resulting from exposure to the suspension of 50 nm α -Al₂O₃ NPs ranged from 6.33 to 105 ppb and from 22.15 to 337.18 ppb for 24 and 96 h, respectively, with increasing initial NP concentration in the medium. For 5 nm γ -Al₂O₃ NPs, the uptake ranged from 48.46 to 384.11 ppb and from 198.68 to 1113.6 ppb for 24 and 96 h, respectively. For 0.4 μ m γ -Al₂O₃ particulate, the uptake ranged from 61.95 to 520.5 ppb and from 179.65 to 1445.12 ppb for 24 and 96 h, respectively. Average Al uptake was higher for larger γ -Al₂O₃ particulates than it was for all smaller NP groups within the same exposure regimes.

The ability of *Artemia* to depurate the ingested α -Al₂O₃ and γ -Al₂O₃ NPs was also investigated. After exposure, *Artemia* were transferred to a freshly-prepared seawater culture medium. The entire transfer process for each test was completed within 24 h without rinsing with clean seawater to minimize the damage to *Artemia* larvae. After *Artemia* were being

collected in a mesh, they were filtered, washed and digested in acid to determine the loss in Al_2O_3 NP content. The elimination profiles for the accumulated Al are illustrated in Figure 4. No mortality was observed during the depuration process. These results demonstrated that the excretion of the ingested NPs appears to be very slow, and that *Artemia* were unable to eliminate the NPs as rapidly as they accumulated, most likely due to the formation of larger aggregates inside the guts. As expected, the highest loss occurred for the groups exposed to 100 mg L^{-1} levels for which the average concentration of NPs eliminated in 24 h was about 5.0 and 34.65 ppb for $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs, respectively. These values increased to about 9.8 and 103.2 ppb, respectively, in 96 h. The elimination of the $0.4 \mu\text{m}$ $\gamma\text{-Al}_2\text{O}_3$ in 24 and 96 h increased from 48.1 to 130.5 ppb. The observed effects may not reflect those of NPs but aggregates of NPs, which points to the fact that the physicochemical properties (size, shape and reactivity) of ingested particles/aggregates are important factors that should be considered when evaluating the toxicity of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs and other similar metal oxide NPs.

Toxicity and Lipid Peroxidation Detection by Melondialdehyde Levels

The toxic effects of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs were different on *Artemia* for 24 and 96 h exposure. The controls and $3.5 \mu\text{m}$ $\alpha\text{-Al}_2\text{O}_3$ showed about 1 to 4% mortality in 24 and 96 h, which were not statistically significant ($p > 0.05$). As aforementioned, the exposures were conducted in the absence of food. The experimental mortalities for the controls clearly demonstrated that deprivation from food did not induce any lethal effect on *Artemia* larvae, even up to 96 h. The mortality ranges were about 4 to 11%, 5 to 13%, 2 to 9% in 24 h, but they were 8 to 30%, 9 to 34% and 7 to 28% in 96 h for $\alpha\text{-Al}_2\text{O}_3$ (50 nm), $\gamma\text{-Al}_2\text{O}_3$ (5 nm) NPs, and $\gamma\text{-Al}_2\text{O}_3$ (0.4 μm) particulates, respectively (Figure 5). In the treatments, the mortalities significantly increased with increasing NP concentration and time ($p < 0.05$). These results demonstrated that Al_2O_3 NPs were relatively benign to *Artemia* larvae during 24 h exposure, even at the highest test concentration. The lethal effects recorded for 96 h exposure were more prominent. Results also indicated that $\gamma\text{-Al}_2\text{O}_3$ NPs were more toxic than $\alpha\text{-Al}_2\text{O}_3$ NPs at all conditions, and the highest mortality was measured from $\gamma\text{-Al}_2\text{O}_3$ NPs (5 nm) at 100 mg/L ($\text{LC}_{50} > 100 \text{ mg/L}$). Moreover, smaller NPs exhibited a more toxic effect than larger particles.

Oxidative deterioration of cell membrane lipids has been used extensively as a marker of oxidative stress in lipid peroxidation detection measured as total MDA concentration. The MDA results of this study are displayed in Figure 6. The data were consistent with the mortality rates for 24 and 96 h exposure in different concentrations of all Al_2O_3 particles. MDA values detected in treatments were not statistically different than the control after exposure of both NPs for 24 h ($p > 0.05$); however, a significant increase in MDA values was found after exposure of NPs for 96 h ($p < 0.05$). This indicated that *Artemia* were undergoing oxidative stress, which was consistent with our results of a higher concentration of $\alpha\text{-Al}_2\text{O}_3$ and $\gamma\text{-Al}_2\text{O}_3$ NPs exhibiting more potent effects on disturbance to the antioxidant defense systems of *Artemia* in 96 h (Figure 6). Increasing NP concentration from 5 to 100 mg/L also caused a significant increase in 96 h MDA levels ($p < 0.05$). MDA levels indicated that $\gamma\text{-Al}_2\text{O}_3$ (5 nm) NPs (MDA = 45.1 nmol/g) were more toxic than $\alpha\text{-Al}_2\text{O}_3$ (50 nm) NPs (MDA = 41.1 nmol/g) in 96 h. It was found that larvae were more vulnerable and

always experienced relatively higher mortalities in 96 h, and that the toxic effects of α - Al_2O_3 and γ - Al_2O_3 NPs were mediated by oxidative stress. The 96 h mortality percentage showed a high correlation with 96 h MDA values ($r^2 = 0.951$), suggesting that lethal effects were due to the elevated oxidative stress induced by α - Al_2O_3 and γ - Al_2O_3 NPs.

CONCLUSIONS

In this study, the uptake, toxicity and elimination of α - Al_2O_3 (50 nm and 3.5 μm) and γ - Al_2O_3 (5 nm and 0.4 μm) NPs were studied. The responses observed from *Artemia* exposed to NPs are consistent with those of marine organisms. Thus, *Artemia* could be used as a robust test model for studying the ecological risks of nanomaterials on marine ecosystems. Both α - Al_2O_3 and γ - Al_2O_3 NPs aggregate in saltwater to form micrometer-size particles. However, this phenomenon does not have any effect on the uptake of particles from water. Larvae could readily accumulate α - Al_2O_3 and γ - Al_2O_3 NPs to elevated levels, excluding larger ones like α - Al_2O_3 (3.5 μm). While no acute toxicity was detected in 24 h, mortality and MDA levels increased in 96 h. The results demonstrate that α - Al_2O_3 and γ - Al_2O_3 NPs are not totally benign to *Artemia*, but that they exhibit marginal acute toxicity. The toxic effects are mediated by oxidative stress, which induces lethal effects after prolonged exposure. An important conclusion from this study is that the sensitivity of each assay is dependent on the physico-chemical characteristics of the particle, emphasizing the importance of a multi-trophic approach. The principal concern related to the release of NPs and their smaller particles may change the materials' transport and potential toxicity to aquatic organisms compared to larger particles.

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Figure 1.
Powdered XRD spectrum for Al_2O_3 nanoparticles (A: $\alpha\text{-Al}_2\text{O}_3$ 50 nm; B: $\alpha\text{-Al}_2\text{O}_3$ 3.5 μm ;
C: $\gamma\text{-Al}_2\text{O}_3$ 5nm; D: $\gamma\text{-Al}_2\text{O}_3$ 0.4 μm) sample.

Figure 2.
The images of α - Al_2O_3 and γ - Al_2O_3 nanoparticles (A: α - Al_2O_3 50 nm; B: α - Al_2O_3 3.5 μm ; C: γ - Al_2O_3 5nm; D: γ - Al_2O_3 0.4 μm).

Figure 3. Phase contrast microscope images of the nanoparticles inside *Artemia* larvae. (A: The guts are empty in controls. B: Nanoparticles are visible as a dark line inside the guts of treatments).

Figure 4.

Accumulation and elimination (in 24 h) profiles of α -Al₂O₃ (a) and γ -Al₂O₃ (b) nanoparticles by *Artemia* larvae for 24 h and 96 h exposure ($\mu\text{g/g}$).

Figure 5.

Percent mortality rates for *Artemia salina* larvae from exposure to α -Al₂O₃ and γ -Al₂O₃ particles for 24 h and 96 h.

Figure 6. Malondialdehyde levels measured in *Artemia* larvae exposed to α -Al₂O₃ and γ -Al₂O₃ nanoparticles for 24 h and 96 h exposure (nmol/g)

Table 1

Specific wavelengths and their corresponding transmittance values for FT-IR spectra of nano-size α -Al₂O₃ and γ - Al₂O₃.

α -50 nm		α -3.5 μ m		γ - 5 nm		γ -0.4 μ m	
cm ⁻¹	% T	cm ⁻¹	% T	cm ⁻¹	% T	cm ⁻¹	% T
3438.5	75.7	3430.6	78.1	3440.3	39.4	3450.3	69.2
1630.6	77.1	1636.1	79.0	1631.2	65.1	1631	86.6
1592.6	79.8	1597	-	1588.5	71.5	-	-
1436.8	69.1	1436.8	71.8	1436.6	53.5	1437.5	80.4
1384.7	76.7	1380.7	82.4	1384.5	55.4	1383.8	89.8
1114.4	86.8	1111	86.5	1118.5	80.2	1116.4	94.3
729.1	27.1	731.2	27.4	807.9	23.8	822.5	29.6
644.3	25.2	641.1	25.2	750.7	22.1	752.9	28.9
602.2	22.7	594.1	16.5	701.6	22.7	692.8	38.9
452.2	37.2	449	28.1	624	20.5	630.4	31.3
-	-	-	-	572.6	20.3	570	30.8