2014

Comparison of TiO₂ nanoparticle and graphene–TiO₂ nanoparticle composite phototoxicity to *Daphnia magna* and *Oryzias latipes*

Shibin Li

U.S. Environmental Protection Agency, li.shibin@epa.gov

Xuan Pan

Texas Tech University

Lindsay K. Wallis

U.S. Environmental Protection Agency

Zhaoyang Fan

Texas Tech University

Zuliang Chen

Fujian Normal University, Fujian Province, China

See next page for additional authors

Follow this and additional works at: <http://digitalcommons.unl.edu/usepapapers>

Li, Shibin; Pan, Xuan; Wallis, Lindsay K.; Fan, Zhaoyang; Chen, Zuliang; and Diamond, Stephen A., "Comparison of TiO₂ nanoparticle and graphene–TiO₂ nanoparticle composite phototoxicity to *Daphnia magna* and *Oryzias latipes*" (2014). *U.S. Environmental Protection Agency Papers*. 215.
<http://digitalcommons.unl.edu/usepapapers/215>

This Article is brought to you for free and open access by the U.S. Environmental Protection Agency at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in U.S. Environmental Protection Agency Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Shibin Li, Xuan Pan, Lindsay K. Wallis, Zhaoyang Fan, Zuliang Chen, and Stephen A. Diamond

Comparison of TiO₂ nanoparticle and graphene–TiO₂ nanoparticle composite phototoxicity to *Daphnia magna* and *Oryzias latipes*

Shibin Li ^{a,*}, Xuan Pan ^b, Lindsay K. Wallis ^a, Zhaoyang Fan ^b, ZuLiang Chen ^c, Stephen A. Diamond ^d

^a U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, MN, USA

^b Department of Electrical and Computer Engineering and Nano Tech Center, Texas Tech University, Lubbock, TX, USA

^c School of Chemistry and Materials Science, Fujian Normal University, Fuzhou 350007, Fujian Province, China

^d Nanosafe Inc., Blacksburg, VA, USA

HIGHLIGHTS

- Graphene–TiO₂ nanocomposite (GNP) was synthesized by a hydrothermal method.
- GNP has increased photoactivity, especially in the visible light region.
- Nano-TiO₂ and GNP exhibited similar parts-per-billion level phototoxicity.
- Both primary particle reactivity and environmental factors determine toxicity.
- Graphene exhibited no toxicity with or without illumination.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:

Received 5 November 2013

Received in revised form 13 March 2014

Accepted 16 March 2014

Handling Editor: S. Jobling

Keywords:

Nano-TiO₂
Graphene
Nanocomposite
Phototoxicity
Aquatic organism

ABSTRACT

With a dramatic rise in complexity, needs of nanotoxicology research go beyond simple forms of nanomaterials. This study compared the phototoxicity of nano-TiO₂ and graphene–TiO₂ nanocomposite (GNP). GNP was synthesized based on a hydrothermal method, which simultaneously performed the reduction of graphene oxide and nano-TiO₂ loading. A series of acute toxicity tests of nano-TiO₂, graphene and GNP was performed on two aquatic organisms, *Daphnia magna* and *Oryzias latipes*. Fast and substantial agglomeration and sedimentation of nanoparticles in test media and surface attachment of nano-TiO₂ and GNP on *D. magna* surface was observed. Similar phototoxicity of nano-TiO₂ and GNP for both species existed, though compared with nano-TiO₂, GNP had a 2.3-fold increase in visible light photocatalytic ROS generation. In summary, this study demonstrated the significance of illumination spectrum, particle behavior, and species sensitivity on nanophototoxicity, and the needs for research on increasingly sophisticated functional materials.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

One of the consistent goals of nanotechnology development has been the production of ever more photoreactive nanomaterials. A broad array of such materials is currently being considered and investigated (e.g. fullerenes, carbon nanotubes, nano-zinc oxide,

titanium) as potential photocatalysts in surface coatings, solar voltaics, water and air purification, and other applications. Among these materials, TiO₂ appears to be the most promising, due in part to its abundance, stability, and low cost. Equally importantly, the photoreactivity of TiO₂ has been shown to be remarkably labile; manipulation of crystal structure, combination with other mineral and organic materials, and a variety of coatings can limit or extend activation energies and lifetimes (Mohamed et al., 2007; Woan et al., 2009; Zhang et al., 2009; Leary and westwood, 2011; Pan et al., 2012).

* Corresponding author. Tel.: +1 218 529 5214; fax: +1 218 529 5003.

E-mail address: li.shibin@epa.gov (S. Li).

The mechanism of nano-TiO₂ photoactivity involves interaction of photons in the ultraviolet radiation (UV) wavelength range (wavelengths shorter than approximately 380 nm) with valence bands electrons. The photon energy moves electrons into the conduction band, generating an electron–hole pair. This pair is able to strongly react with oxygen or water to form reactive oxygen species (ROS, OH[•], and O₂^{•−}), that can damage or degrade organic and biomolecules and potentially cause toxic effects in organisms (Miller et al., 2012; Ma et al., 2012b). There is also some potential for direct production of organic free radicals when target molecules interact with the strongly positively charged “hole” side of the electron–hole pair. This mechanism might be important in degradation of contaminants (Linsebigler et al., 1995).

There are two primary approaches to developing more reactive TiO₂-based photocatalysts. One involves sequestering electrons in the conduction band to reduce the probability of electron–hole recombinations. The other involves reduction of the energy required to move electrons from valence to conduction bands. In the former case, the hole side of the electron–hole pair is longer lived, providing more opportunity for ROS (or free radical) production; in the latter case the material can be activated by photons with lower energy (longer wavelengths) thus increasing levels of reactivity under natural lighting conditions. One promising material for achieving these higher levels of reactivity is graphene.

Graphene has a honeycomb structure composed of an atomic sheet of sp²-bonded carbon atoms, and has large surface area, high transparency, and high electric charge carrier mobility. These electronic and photonic properties (Ishigami et al., 2007) make it an ideal candidate material for enhancement of TiO₂ photoreactivity (Sant and Kamat, 2002). Recently, Zhang et al. (2009), Pan et al. (2012) demonstrated that graphene–TiO₂ nanoparticle composites (GNP) do exhibit enhanced photoactivity derived from increased quantum efficiency, extended visible light absorption, and strong affinity for other organic materials (Zhang et al., 2009; Pan et al., 2012). As with other nanomaterials, the increased development and use of graphene nanomaterials suggests the need for assessment of their risk to human and environmental health (Jastrzebska et al., 2012).

An obvious concern with photoreactive materials is their potential phototoxicity if released into natural environments. Their hazard has been clearly demonstrated in several studies (Ma et al., 2012a, b, c; Miller et al., 2012; Li et al., 2013a) and it is expected that hazard will increase in direct proportion to the level of enhancement of photoreactivity. We evaluated this potential by comparing toxicity between simple (the well-tested Evonik p25 Aeroxide) and a composite comprised of p25 TiO₂ and a graphene material. *Daphnia magna* and *Oryzias latipes*, two commonly used aquatic species in toxicological studies, were chosen to study whether or not species susceptibility existed for nano-TiO₂ phototoxicity. Acute bioassays were conducted to evaluate the toxicity of nano-TiO₂ and GNP in *D. magna* and *O. latipes* with or without the presence of SSR (simulated solar radiation). These outcomes were compared with results of photochemical ROS production assays conducted under similar conditions. Graphene with no composited TiO₂ was tested simultaneously to isolate its toxicity from that of composited material. Fate of materials in assay chambers was also carefully characterized to evaluate the importance of exposure variation (primarily in concentration and agglomeration) in determining toxicity and photoreactivity.

2. Materials and methods

2.1. Nanoparticle source, synthesis, and characterization

Nano-TiO₂ (Aeroxide TiO₂ P25) was obtained from Evonik Degussa Corporation. Graphene oxide was synthesized by oxidizing expanded flake graphite (3805, Asbury Graphite Mills, Inc.,

NJ, USA) via modified Hummers' method (Pan et al., 2012). Specifically, 3 g of graphite was added into a mixture of 2.5 g of K₂S₂O₈, 2.5 g of P₂O₅, and 12 mL of concentrated H₂SO₄. After being heated to 80 °C and kept stirring for 5 h, 500 mL of deionized water was slowly added into the mixture for dilution. Re-oxidization was implemented by the addition of a large amount of deionized water (500 mL) and 30% H₂O₂ solution (10 mL), causing violent effervescence and temperature increase. Graphite oxide was recovered by repetitive washing of the reaction media through 0.2 μm Whatman Nylon film filters with deionized water until the pH of the filtrate was neutral, followed by drying the product in an oven at 60 °C for 2 h. Exfoliation was performed by sonicating 0.1 mg mL^{−1} of graphite oxide dispersion for 1 h. The graphene oxide (GO) was recovered by filtration again and vacuum drying. Graphene was then obtained by reducing the graphene oxide. GNP was synthesized by employing the one-step hydrothermal method, which simultaneously reduced the graphene oxide and loaded nano-TiO₂ on the platform of graphene nanosheets. The synthesis and characterization details were listed in our previous work (Pan et al., 2012).

The morphology of nanoparticles was characterized using field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM). Chemical bond formation and skeletal vibration of the graphene sheets in the hybrid nanostructures were identified by Fourier transform infrared spectroscopy (FTIR) with a Mattson Instruments RS/1 FTIR spectrometer operating at 2 cm^{−1} resolution using a liquid nitrogen cooled MCT detector. Crystalline State was evaluated by X-ray diffraction (XRD) experiments using a Siemens/Bruker AXS D5005 X-ray diffractometer.

2.2. Nano-TiO₂, graphene and GNP assay suspensions

Stock suspension (500 mg L^{−1}) of nano-TiO₂, GNP, and graphene were produced by adding materials (in powder form) to moderately hard reconstituted water (MHRW) for 0.5 h in a bath-type sonicator (35 kHz frequency; Fisher Scientific). Stock solutions were stored in the dark and sonicated for 30 min prior to making working suspensions (Weber, 1993). Working suspensions were diluted with MHRW to target concentrations and stirred for 15 min before the initiation of the bioassays.

2.3. Test organism husbandry

Test organism cultures were all maintained at U.S. EPA's Mid-Continent Ecology Laboratory, Duluth, MN, USA. Specifically for *D. magna*, neonates (≤48-h old) were collected from *D. magna* cultures which were maintained in a 1-L beaker at 21 ± 1 °C. Before the initiation of the bioassays, these neonates were maintained for 2–3 d in 1 L of MHRW. Daily food was a 1:1:1 mixture of yeast: Ceropyl: Tetramin (YCT) and *Pseudokirchneriella subcapitata*. For *O. latipes*, fertilized eggs collected from the brood stock (23 ± 1 °C) were raised into hatch. Then, larval fish (24- to 48-h old) were collected for the bioassays. Daily food was live brine shrimp (5 mL, 5–5.5 mg mL^{−1}).

2.4. Nanoparticle monitoring

2.4.1. Nanoparticles in exposure media

Agglomerate size (average hydrodynamic diameter) was measured using dynamic light scattering (DLS, 90 Plus/Bi-MAS, Zeta PALS; Brookhaven Instruments) and was calculated from the autocorrelation function of the intensity of light scattered from the agglomerates. Note that there are limitations of current DLS techniques. DLS data typically is interpreted with an assumption that analyte behavior can be treated as that of a sphere having some measured diameter. In contrast to this assumption, graphene has

a large aspect ratio (is in the form of thin sheets or plates), and forms relatively large nano-TiO₂ agglomerates with irregular shape. DLS diameter estimates are also skewed toward larger sizes. Despite these limitations, in this study the particle agglomerate size was monitored for the following reasons: (1) DLS-related particle/agglomerate size can be used to compare (with appropriate caveats) results among studies, (2) DLS-determined particle/agglomerate size might be adjusted based on results of more accurate and reliable, but less available techniques, and (3) as indicated in Stokes' law, agglomeration is related to the settling rate of nano-TiO₂. Hence, DLS-determined particle size indirectly indicates the sedimentation behavior of nanoparticles.

Particle sedimentation was investigated using J-Y Ultima 2 Inductively coupled argon plasma emission spectrometer (ICP-AES) (HORIBA Scientific, Inc., NA, USA). Specifically, nano-TiO₂ concentration in the water column was quantified by ICP-AES, and from this, nano-TiO₂ on the bottom was calculated by subtracting the total amount of nano-TiO₂ with that in the water column. For ICP analysis, nano-TiO₂ was digested in 50-mL polypropylene digestion tubes with a mixture of nitric acid and hydrochloric acid following EPA Method 200.2, and quantified based on EPA Method 200.7 (EPA, 1996). Recoveries were consistently greater than 90%. Limit of detection and quantification were 3 and 8 µg L⁻¹ respectively.

2.4.2. Nanoparticles on organism surfaces

Material accumulation on *D. magna* and *O. latipes* exterior surfaces was monitored using a Tescan Vega 3 LM Scanning Electron Microscope (SEM) equipped with a cold sample stage (−20 °C), and a QUANTAX Energy-dispersive detector (EDS). Detailed instrumental parameters and experimental procedures were listed in our previous work (Li et al., 2013a).

2.5. Test system

Tests under SSR were completed in a solar simulator (Q-Sun 3000 Xenon Test Chamber; Q-Lab) that accurately simulates the full wavelength spectrum of solar radiation from 280 to 800 nm. Test chambers were 30-mL glass beakers, covered with three neutral-density screen filters (L × W: 55 × cm × 30 cm), and contained in a 69 × 40 cm temperature-controlled water bath (22 ± 1 °C). SSR intensity at the surface of the water bath was 1700 µW cm⁻², as measured by a photodiode array spectrometer (model S2000; Ocean Optics).

2.6. Photocatalytic ROS generation

ROS generation (especially OH· for nano-TiO₂), and ROS-related toxic effect on biomolecules is the primary mechanism of photo-induced activity and toxicity. Various biomarkers have been used to distinguish this mechanism, including lipid peroxidation, formation of DNA adducts, activities of superoxide dismutase, catalase, glutathione S-transferase, and those of acid phosphatase and Na, K-ATPase (Ma et al., 2012c; Clemente et al., 2013). In this study, photocatalytic ROS generation was measured using a ROS indicator, 3' [p-aminophenyl] fluorescein (APF; Invitrogen), which responds specifically to hydroxyl radicals, hence a direct measurement of photoactivity of nano-TiO₂-based materials. ROS production was measured in 96-well microplates with each well containing 250 µL of the reaction mixture containing 10 µM APF stock in dimethylsulfoxide [DMSO]) with 2 mL of each nanoparticle suspension at a concentration of 20 mg L⁻¹ (separately). Fluorescence was measured using a BioTek H4 microplate reader (ex: 485 nm; em: 535 nm). ROS production was standardized to molar units of hypochlorite (OCl⁻) (Setsukinai et al., 2003). Reactions were completed under SSR for 0, 10, and 20 min, Irradiance

treatments included unfiltered full spectrum, 360-nm optical cut-off filters, and 400-nm optical cut-off filters (Newport Corporation, CA, USA) (Ma et al., 2012a). The purpose of these spectral treatments was to determine if GNP ROS production occurs at longer, lower-energy wavelengths, compared with Aeroxide; our previous work indicated that photoreactivity of Aeroxide was undetectable under wavelengths above approximately 380 nm (Ma et al., 2012a).

2.7. Toxicity assays

2.7.1. *D. magna* toxicity assay

Nano-TiO₂, graphene, and GNP, were tested separately using the following procedures. Three day old *D. magna* were exposed for 48 h to each nanomaterial in separate assays, and under dark (on the laboratory bench top and covered with aluminum foil) or SSR conditions. Assays were conducted with three replicates in 30-mL beakers containing 15-mL exposure media and 10 *D. magna*. Organisms were fed 2 h prior to starting assays; no additional food was added during the exposure. All test solutions were kept at 22 ± 1 °C during the exposure process. SSR treatment chambers were placed in the solar simulator for 4 h early in each 24 h test period. Mortality of aquatic organisms was recorded at 4, 24, 28, and 48 h. Media were not renewed during the 48-h test. Based on preliminary studies, nano-TiO₂ and GNP concentrations tested were 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mg L⁻¹ under SSR exposure and 0, 1, 10, 50, 100, 167, 200, 400, and 500 mg L⁻¹ in the dark condition. Graphene concentrations were employed as 0, 0.5, 1, 10, 50, and 100 mg L⁻¹. Temperature, dissolved oxygen (DO), pH, and conductivity were measured once per day in representative chambers throughout the bioassays.

2.7.2. *O. latipes* toxicity assay

A similar procedure as that described for *D. magna* was employed for *O. latipes* with the following exceptions: (1) each beaker had 8 *O. latipes* (24- to 48-h posthatch), and (2) nano-TiO₂ and GNP concentrations of exposure media were 0, 2, 5, 8, 10, 14, 17, and 20 mg L⁻¹ under SSR exposure, and 0, 167, and 500 mg L⁻¹ under dark conditions.

2.8. Data analysis

Median lethal doses (LD50s) and associated 95% confidence intervals were estimated from a tolerance distribution analysis using a three-parameter probit model (TRAP; Toxicity Relationship Analysis Program, v. 2.21, U.S. EPA). Means were compared with analysis of variance (ANOVA) (SPSS16.0 for Windows, SPSS Inc.). Multiple comparisons were conducted with the Tukey's honestly significant difference (HSD) test. Significant differences were analyzed by applying the least significant difference of means at a 5% confidence level ($p \leq 0.05$).

3. Results and discussion

3.1. Nanoparticle characterization

As shown in Fig. 1a (SEM) and b (TEM), nano-TiO₂ had a similar primary-particle morphology (aspect, spheroid; primary particle size, 21 nm) as those reported by the manufacturer and our previous work (Ma et al., 2012a, b; Li et al., 2013a; Ma and Diamond, 2013). Typical morphological information of the as-synthesized GNP was reported in Fig. 1c (SEM) and d (TEM). Nano-TiO₂ was loaded on graphene sheets, with apparent accumulation along the wrinkles and edges caused by the presence of carboxylic acid groups on graphene oxide (Zhang et al., 2009). The Fourier trans-

Fig. 1. Electron microscopy characterizations of nano-TiO₂ ((a) FE-SEM; (b) TEM), and GNP ((c) FE-SEM; (d) TEM). Black arrows in TEM images indicate graphene.

form infrared spectroscopy (FTIR) transmission spectra proved the formation of Ti–O–C bonds (798 cm^{-1}), indicating the firm connection between graphene and nano-TiO₂ (Fig. S1). No obvious impact of the synthesis process on nano-TiO₂ crystal structure was observed, as indicated by the similar XRD pattern in Fig. S2.

3.2. Nanomaterial behavior

Nanoparticle behavior in exposure media is a key determinant of toxicity (Ma et al., 2012c; Li et al., 2013b, c, d). Fig. 2a depicts nanoparticle agglomeration at representative concentrations. Agglomeration size was consistently over 900 nm, irrespective of nanoparticle type, and was independent of illumination condition for all nanoparticles. For example, at 4 h, similar agglomeration of 100 mg L^{-1} graphene was observed, with $914 \pm 23\text{ nm}$ in the dark condition and $1085 \pm 115\text{ nm}$ under SSR. No apparent dependence of agglomeration on concentration was observed, although theoretically, higher initial concentrations should lead to increased collision frequency and more rapid agglomeration. In general, no difference in agglomeration between nano-TiO₂ and GNP was observed, but both were greater than that of graphene alone. Nano-TiO₂ and GNP agglomerates ranged from 1600 to 3400 nm, whereas agglomerates of graphene were approximately 1000 nm. Large agglomeration of nanoparticles in this study was expected based on the presence of moderate levels of electrolytes (Elimelech, 1998). It should be noted that in the natural environment, agglomeration is more complex. Not only ionic strength, pH and conductivity, but also the presence of naturally occurring organic matter change the surface chemistry and agglomeration state of nanoparticles (Sharma, 2009; Yang et al., 2009; Hotze et al., 2010b; Jassby et al., 2012).

For water-column species, nanoparticle exposure, and toxicity, is determined by settling rates of particles and agglomerates (Ma et al., 2012c; Li et al., 2013a). Fig. 2b (dark) and c (SSR) show nanoparticle sedimentation at concentrations representative of those

used in the present study. Rapid and substantial sedimentation was consistently observed, and illumination conditions did not affect sedimentation. Under the same exposure scenario, no apparent differences in sedimentation were observed between nano-TiO₂ and GNP. For example, for *O. latipes* bioassays, 79% of nano-TiO₂ at 438 mg L^{-1} , and 80% of GNP at 456 mg L^{-1} settled on the bottom after 4-h exposure. These levels of sedimentation are in accordance with the observed agglomeration, as predicted by Stokes' law.

The surface attachment of nano-TiO₂ is likely to be a strong determinant of phototoxicity. ROS are extremely short-lived (on the order of ns to ms), suggesting a requirement for close contact between materials and biological targets (Ma et al., 2012a, b). Interaction of nanoparticles with organism surfaces were qualitatively examined using SEM as shown in Fig. 3. For *O. latipes*, minimal surface attachment was observed regardless of nanoparticle type, initial concentration, exposure duration, and illumination conditions (Fig. 3a). For *D. magna*, surface attachment of nano-TiO₂ (Fig. 3c) and GNP (d) were readily apparent, whereas minimal attachment was observed for graphene, regardless of initial concentration, exposure duration, and illumination conditions (Fig. 3b). Elementary composition of nano-TiO₂ on organism surfaces was confirmed by EDX detector as indicated in Fig. S3. The differences in surface attachment might originate from the differences in particle/organisms surface charge, particle agglomerate size and structure, filtering apparatus, particle/organisms surface areas, and particle-biomolecular interactions (Kumar, 2009; Dabrunz et al., 2011). An extensive discussion of the evidence for these interactions is provided by Nel et al. (2009).

3.3. Photoactivity evaluation – toxic mechanism

The dependence of photocatalytic ROS generation on exposure time, illumination condition, nanoparticle type, and solar radiation

Fig. 2. Agglomerate size (a) of particles and mass of nano-TiO₂ (b, dark; and c, SSR) in water columns during the 48-h bioassay.

Fig. 3. Representative SEM images showing the aggregated particles on organism surfaces at 48 h. (a) represents *O. latipes* after particle exposure, specifically nano-TiO₂ for this image. (b–d) shows graphene, nano-TiO₂ (P25), and GNP on the surface of *D. magna*, respectively.

spectrum is shown in Fig. 4. No ROS generation was observed for graphene, hence, no further investigation was conducted (data not shown). This indicated that graphene was not phototoxic, as

expected, and could not produce significant amounts of ROS under dark conditions. No increase over time was observed in ROS production for all exposure scenarios conducted in the dark. ROS

generation generally increased over time for both nano-TiO₂ and GNP. This confirmed that ROS generation of nano-TiO₂-based materials was dependent on both illumination and exposure time. At the same exposure time, GNP generated more ROS than nano-TiO₂, irrespective of spectrum pattern. For example, under the full spectrum, GNP-induced ROS generation had a 1.3-fold increase compared with that of nano-TiO₂ after a 20-min exposure. This increased photoactivity could be attributed to the fact that: (1) graphene, an excellent acceptor of the generated electrons of nano-TiO₂, could suppress the charge recombination and make more charge carriers to form ROS, and (2) the two-dimensional planar structure of graphene could facilitate the rapid transport of charge carriers, hence leading to an effective charge separation (Zhang et al., 2009; Pan et al., 2012). Interestingly, this difference was most obvious when the 400-nm filter was applied. Specifically, with the 400-nm filter, GNP-induced ROS generation had a 2.3-fold increase compared with that of nano-TiO₂ after a 20-min exposure. This agreed with our previous investigation, in that a sharp absorption edge existed at 400 nm for nano-TiO₂, while increased adsorption in the visible light region was detected for GNP (Pan et al., 2012). The 20-nm red-shift in GNP was caused by the chemical bond formation of Ti–O–C between nano-TiO₂ and graphene (Woan et al., 2009). This result indicated that compared with nano-TiO₂, GNP had visible light photoactivity, one of the features that facilitate its use in environmental remediation. Finally, with the same exposure time (either 10 or 20 min), nano-TiO₂ induced-ROS production followed this order: full spectrum > spectrum with 360-nm filter > spectrum with 400-nm filter = dark

($P < 0.05$); and for GNP, full spectrum > spectrum with 360-nm filter > spectrum with 400-nm filter > dark ($p < 0.05$). These results demonstrated the spectrum-specific ROS generation of nano-TiO₂ and GNP and the extended visible light absorption of GNP for solar light harvesting.

3.4. Toxicity

To our knowledge, this is the first study to investigate the toxicity of graphene to aquatic organisms, with the majority of previous studies focusing on its antibacterial properties (Jastrzebska et al., 2012). In this study, graphene largely agglomerated and exhibited no toxicity to either species even at the highest concentration (100 mg L⁻¹) under either dark or SSR conditions (data not shown). Though acute toxicity of graphene to aquatic organisms was not observed, their impacts, especially chronic effects of graphene-family materials on environmental and human health, should not be ignored, as supported by recently discovered adverse effects such as spontaneous cell membrane penetration of graphene microsheets, antibacterial properties of graphene and graphene oxide nanowalls, and graphene oxide induced necrosis in macrophages (Akhavan and Ghaderi, 2010; Jastrzebska et al., 2012; Qu et al., 2013; Li et al., 2013b).

Toxicological profiles of nano-TiO₂ and GNP in this study are provided in Fig. 5. Water quality for all bioassays was well within the acceptable bioassay limits. Specific parameters were as follows: temperature, 21.7–23.0 °C; dissolved oxygen, 8.82–9.57 mg L⁻¹; and conductivity 9.9–12.7 mS m⁻¹. Control survivals of *D. magna* and *O. latipes* were greater than 90% for all bioassays. For *D. magna*, LC50 values were 118 (95% CI, 74–186) mg L⁻¹ for nano-TiO₂ and 138 (95% CI, 93–199) mg L⁻¹ for GNP under dark conditions and 60 (95% CI, 30–100) µg L⁻¹ for nano-TiO₂ and 90 (95% CI, 50–150) µg L⁻¹ for GNP under SSR (Fig. 5a). For *O. latipes*, LC50 values were greater than 500 mg L⁻¹ for both nano-TiO₂ and GNP under dark conditions, 8.5 (95% CI, 7.3–10) mg L⁻¹ for nano-TiO₂, and 11 (95% CI, 9.3–13) mg L⁻¹ for GNP under SSR (Fig. 5b). Several conclusions can be made from this. First, for both species, no nano-TiO₂/GNP toxicity was observed in dark conditions, whereas under SSR, increased toxicity was observed for both nano-TiO₂ and GNP. This agreed well with the increased ROS production under SSR for both nano-TiO₂ and GNP (Fig. 4). Second, no differences in phototoxicity were observed between nano-TiO₂ and GNP under the exposure conditions in this study, though in a well-dispersed state, GNP constantly showed increased ROS generation compared with that of nano-TiO₂ (Fig. 4). The large agglomeration in the current study led to mass transfer and shadowing effects, hence the decreased ability of primary particles to absorb incoming exciting photons which led to less ROS generation (Buxton, 1988; Lin et al., 2006). Also, tightly packed aggregates could facilitate quenching effects that originate either from the recombination of generated holes with electrons from adjacent particles or from ROS recombination with electrons/holes on particle surfaces (Hotze et al., 2010a; Jassby et al., 2012). This indicates that when it comes to phototoxicity, agglomeration is a more important determining factor than material modification at the molecular level. It is possible that phenomenon would apply to photodegradation of contaminants as well. Finally, compared with *O. latipes*, *D. magna* is more sensitive to both nano-TiO₂ and GNP. The SSR LC50 values for nano-TiO₂ and GNP of *O. latipes* were three orders of magnitude higher than those of *D. magna* (Fig. 5). The close proximity of nanoparticles and organism surfaces increases the probability that ROS will reach biological targets rather than being quenched in intervening media. Compared with *D. magna*, minimal surface attachment of nanoparticles existed for *O. latipes*. Other factors are likely to determine responses however, and future studies are needed to evaluate the relative efficiency of biological

Fig. 4. Photocatalytic reactive oxygen species (ROS) production over time by nano-TiO₂ (P25) and GNP in the dark (a) and SSR (b). '360-nm' and '400-nm' represent that 360-, and 400-nm filter were applied, respectively. * $p < 0.05$.

Fig. 5. Toxicity of nanoparticles (nano-TiO₂, P25; and GNP) to *D. magna* (a) and *O. latipes* (b) versus log-concentrations during 48-h exposures in the dark/SSR condition.

defense systems, such as the type and amount of pigmentation or UV-absorbing compounds (i.e., mycosporine-like amino acids, MAAs) in different aquatic organisms (Dunlap and Shick, 1998). Regardless of the mechanisms, nanomaterial-organism direct interaction had a clear effect on toxicity in this study.

The impact of time and initial concentration on phototoxicity of nano-TiO₂ and GNP is shown in Fig. 6a (*D. magna*) and 6b (*O. latipes*). First, phototoxicity was dependent on the initial concentration of nanoparticle, as also indicated in Fig. 5. For example, 0.5 mg L⁻¹ GNP led to 97% 48-h mortality of *D. magna*, while only a 17% mortality was seen at a concentration of 0.25 mg L⁻¹. Since no concentration-dependent formation of large agglomeration was observed in the current study, increased initial concentrations of nanoparticles could lead to more particles available for photoactivation (Fig. 2). Second, phototoxicity was also dependent on time (both 4-h exposure time and following 20-h dark period). Specifically, 4-h UV exposure generally led to increased toxicity compared with 8-h UV exposure and no evidence existed for effective damage repair/recovery during dark periods since delayed deaths frequently happened in the 20-h period between the two 4-h illuminated periods. This occurred because increased illumination time could deliver more photon energy to activate the nanoparticles. Third, species-specific dependence of nanoparticle toxicity on time was observed. Generally, for the 48-h exposure period, the majority of *D. magna* mortality occurred during the second 24-h period, while most *O. latipes* died during the first 24-h period. For example, for 0.25-mg L⁻¹ GNP, no mortality of *D. magna* was observed at 24 h, yet 87% were dead at 48 h. While for 8 mg L⁻¹ GNP, 71% of *O. latipes* were dead at 24 h and this mortality rate stayed constant through the next 24-h exposure. Although both are considered water-column species, *D. magna* tended to move around on the bottom of the test beakers compared with *O. latipes*.

Fig. 6. Phototoxicity of *D. magna* (a) and *O. latipes* (b) versus concentration and time when exposed to nano-TiO₂ (P25)/GNP.

Hence, they were exposed to more nanoparticles which exhibited increased settling over time. This again demonstrates the necessity of monitoring particle behavior for nanotoxicity studies.

4. Conclusions

While the advances in sciences are producing an array of increasingly sophisticated materials, the majority of current nanotoxicology studies remain focused on simple forms of nanomaterials. By comparing the phototoxicity of nano-TiO₂, one of the most studied nanomaterials, and GNP, a new nano-TiO₂-based nanocomposite with increased photoactivity, the current study highlighted the need to investigate the toxicity of complicated nanomaterials. A simple assumption that increased reactivity will cause increased toxicity of nanomaterials could lead to inaccurate and even incorrect predictions of their environmental risk, as supported by the similar phototoxicity of nano-TiO₂ and GNP. Physicochemical properties of particles, their behavior in environmental media, and various environmental factors, such as illumination, temperature and natural organic matters, can all contribute to their ultimate toxicity. Also, from an engineering perspective, the application of photocatalytic nanocomposites should not only consider increased photoactivity at the molecular level, but also the reactivity in suspension or on the substrate where large agglomeration and increased quenching by various environmental factors could occur.

Acknowledgements

The present study was performed while one of the authors, S. Li, held a National Research Council Research Associateship Award at the U.S. Environmental Protection Agency, Mid-Continent Ecology Division. This document has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemosphere.2014.03.058>.

References

- Akhavan, O., Ghaderi, E., 2010. Toxicity of graphene and graphene oxide nanowalls against bacteria. *ACS Nano* 4, 5731–5736.
- Buxton, G.V., 1988. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (OH/O) in aqueous solution. *J. Phys. Chem. Ref. Data* 17, 513.
- Clemente, Z., Castro, V.L., Feitosa, L.O., Lima, R., Jonsson, C.M., Maia, A.H.N., Fraceto, L.F., 2013. Fish exposure to nano-TiO₂ under different experimental conditions: methodological aspects for nanoecotoxicology investigations. *Sci. Total Environ.* 463–464, 647–656.
- Dabrunz, A., Duester, L., Prasse, C., Seitz, F., Rosenfeldt, R., Schilde, C., Schaumann, G.E., Schulz, R., 2011. Biological surface coating and molting inhibition as mechanisms of TiO₂ nanoparticle toxicity in *Daphnia magna*. *PLoS ONE* 6, 5.
- Dunlap, W.C., Shick, J.M., 1998. Ultraviolet radiation-absorbing mycosporine-like amino acids in coral reef organisms: a biochemical and environmental perspective. *J. Phycol.* 34, 418–430.
- Elimelech, M., 1998. Particle Deposition and Aggregation Measurement, Modelling, and Simulation. Butterworth-Heinemann, Boston.
- EPA, 1996. Methods for the Determination of Metals in Environmental Samples. Elsevier, Norwich.
- Hotze, E.M., Bottero, J.Y., Wiesner, R., 2010a. Theoretical framework for nanoparticle reactivity as a function of aggregation state. *Langmuir* 26, 11170–11175.
- Hotze, E.M., Phenrat, T., Lowry, G.V., 2010b. Nanoparticle aggregation: challenges to understanding transport and reactivity in the environment. *J. Environ. Qual.* 39, 1.
- Ishigami, M., Chen, J.H., Cullen, W.G., Fuhrer, M.S., Williams, E.D., 2007. Atomic structure of graphene on SiO₂. *Nano Lett.* 7, 1643–1648.
- Jassby, D., Budarz, J.F., Wiesner, M., 2012. Impact of aggregate size and structure on the photocatalytic properties of TiO₂ and ZnO nanoparticles. *Environ. Sci. Technol.* 46, 6934–6941.
- Jastrzebska, A.M., Kurtycz, P., Olszyna, A.R., 2012. Recent advances in graphene family materials toxicity investigations. *J. Nanopart. Res.* 14, 1–21.
- Kumar, C.S.S.R., 2009. Metallic Nanomaterials: Nanomaterials for Life Sciences. Wiley, Weinheim.
- Leary, R., Westwood, A., 2011. Carbonaceous nanomaterials for the enhancement of TiO₂ photocatalysis. *Carbon* 49, 741–772.
- Li, S., Wallis, L.K., Ma, H., Diamond, S.A., 2013a. Phototoxicity of TiO₂ nanoparticles to a freshwater benthic amphipod: Are benthic systems at risk? *Sci. Total Environ.* 466–467, 800–808.
- Li, S., Turaga, U., Shrestha, B., Anderson, T.A., Ramkumar, S.S., Green, M.J., Das, S., Canas-Carrell, J.E., 2013b. Mobility of polyaromatic hydrocarbons (PAHs) in soil in the presence of carbon nanotubes. *Ecotox. Environ. Safe.* 2013 (96), 168–174.
- Li, S., Anderson, T.A., Green, M.J., Maul, J.D., Canas-Carrell, J.E., 2013c. Polyaromatic hydrocarbons (PAHs) sorption behavior unaffected by the presence of multi-walled carbon nanotubes (MWNTs) in a natural soil system. *Environ. Sci. Process Impacts* 15, 1130–1136.
- Li, S., Canas-Carrell, J.E., Irin, F., Atore, F.O., Green, M.J., 2013d. Determination of multi-walled carbon nanotube bioaccumulation in earthworms measured by a microwave-based detection technique. *Sci. Total Environ.* 445–446, 9–13.
- Lin, H., Huang, C.P., Li, W., Ni, C., Shah, S.I., Tseng, Y.H., 2006. Size dependency of nanocrystalline TiO₂ on its optical property and photocatalytic reactivity exemplified by 2-chlorophenol. *Appl. Catal. B* 68, 1–11.
- Linsebigler, A., Lu, G., Yates, J., 1995. Photocatalysis on TiO_n surfaces: principles, mechanisms, and selected results. *Chem. Rev.* 95, 735–758.
- Ma, H., Diamond, S.A., 2013. Phototoxicity of TiO₂ nanoparticles to zebrafish (*Danio rerio*) is dependent on life stage. *Environ. Toxicol. Chem.* 32, 2139–2143.
- Ma, H., Brennan, A., Diamond, S.A., 2012a. Photocatalytic reactive oxygen species production and phototoxicity of titanium dioxide nanoparticles are dependent on the solar ultraviolet radiation spectrum. *Environ. Toxicol. Chem.* 31, 2099–2107.
- Ma, H., Brennan, A., Diamond, S.A., 2012b. Phototoxicity of TiO₂ nanoparticles under solar radiation to two aquatic species: *Daphnia magna* and Japanese medaka. *Environ. Toxicol. Chem.* 31, 1621–1629.
- Ma, H., Williams, P.L., Diamond, S.A., 2012c. Ecotoxicity of manufactured ZnO nanoparticles – a review. *Environ. Pollut.* 172, 76–85.
- Miller, R.J., Bennett, S., Keller, A.A., Pease, S., Lenihan, H.S., 2012. TiO₂ nanoparticles are phototoxic to marine phytoplankton. *PLoS ONE* 7.
- Mohamed, A.R., Minoo, T., Piao, C.S., 2007. Advances in nanocatalysis research for carbon nanotubes formation and photocatalytic degradation of phenol. *Bull. Chem. React. Eng. Catal.* 2, 11–17.
- Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., et al., 2009. Understanding biophysicochemical interactions at the nanobio interface. *Nat. Mater.* 8, 543–557.
- Pan, X., Zhao, Y., Liu, S., Korzeniewski, C.L., Wang, S., Fan, Z., 2012. Comparing graphene–TiO₂ nanowire and graphene–TiO₂ nanoparticle composite photocatalysts. *ACS Appl. Mater. Inter.* 4, 3944–3950.
- Qu, G., Liu, S., Zhang, S., Wang, L., Wang, X., Sun, B., Yin, N., Gao, X., Xia, T., Chen, J.-J., Jiang, G.-B., 2013. Graphene oxide induces toll-like receptor 4 (TLR4)-dependent necrosis in macrophages. *ACS Nano* 7, 5732–5745.
- Sant, P.A., Kamat, P.V., 2002. Interparticle electron transfer between size-quantized CdS and TiO₂ semiconductor nanoclusters. *Phys. Chem. Chem. Phys.* 4, 198–203.
- Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H.J., Nagano, T., 2003. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J. Biol. Chem.* 278, 3170–3175.
- Sharma, V.K., 2009. Aggregation and toxicity of titanium dioxide nanoparticles in aquatic environment – a review. *J. Environ. Sci. Health A* 44, 1485–1495.
- Weber, C., 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Environmental Monitoring Systems Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio.
- Woan, K., Pyrgiotakis, G., Sigmund, W., 2009. Photocatalytic carbon-nanotube–TiO₂ composites. *Adv. Mater.* 21, 2233–2239.
- Yang, K., Lin, D.H., Xing, B.S., 2009. Interactions of humic acid with nanosized inorganic oxides. *Langmuir* 25, 3571–3576.
- Zhang, H., Lv, X., Li, Y., Wang, Y., Li, J., 2009. P25-graphene composite as a high performance photocatalyst. *ACS Nano* 4, 380–386.

Supporting Information Cover Sheet

Comparison of TiO₂ Nanoparticle and Graphene-TiO₂ Nanoparticle Composite Phototoxicity to *Daphnia magna* and *Oryzias latipes*

Shibin Li^{1*}, Xuan Pan², Lindsay K. Wallis¹, Zhaoyang Fan², ZuLiang Chen³, and Stephen A. Diamond⁴

¹ *U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, MN, USA*

² *Department of Electrical and Computer Engineering and Nano Tech Center, Texas Tech University, Lubbock, TX, USA*

³ *School of Chemistry and Materials Science, Fujian Normal University, Fuzhou 350007, Fujian Province, China*

⁴ *Nanosafe Inc., Blacksburg, VA, USA*

* Corresponding author: li.shibin@epa.gov Tel: 218-529-5214 Fax: 218-529-5003

Number of pages: 4

Number of figures: 3

Figure S1. Fourier transform infrared (FTIR) spectra of nano-TiO₂ (NP) and graphene-TiO₂ nanoparticles (GNP). Background bands of carbon dioxide (near 2300 cm^{-1}) were removed.

Figure S2. X-ray diffraction (XRD) patterns of nano-TiO₂ (NP) and graphene-TiO₂ nanoparticle (GNP). A represents anatase phase, and R refers to rutile phase.

Figure S3. Elementary confirmation of nano-TiO₂ on the surface of *Daphnia. magna* based on a secondary electron scanning electron image by QUANTAX Energy disperse detector. Titanium element was mapped by red while carbon was depicted by green.