Abstract: Graphene layers are potential candidates in a large number of applications. However, little is known about their ecotoxicological risks largely as a result of a lack of quantification techniques in complex environmental matrices. In this study, graphene was synthesized by means of graphitization and exfoliation of sandwich-like FePO₄/dodecylamine hybrid nanosheets, and ¹⁴C was incorporated in the synthesis. ¹⁴C-labeled graphene was spiked to artificial freshwater and the uptake and depuration of graphene by Daphnia magna were assessed. After exposure for 24 h to a 250 μg/L solution of graphene, the graphene concentration in the organism was nearly 1% of the organism dry mass. These organisms excreted the graphene to clean artificial freshwater and achieved roughly constant body burdens after 24 h depuration periods regardless of the initial graphene exposure concentration. Addition of algae and humic acid to water during the depuration period resulted in release of a significant fraction (>90%) of the accumulated graphene, but some still remained in the organism. Accumulated graphene in adult Daphnia was likely transferred to the neonates. The uptake and elimination results provided here support the environmental risk assessment of graphene and the graphene quantification method is a powerful tool for additional studies.

Introduction: Graphene, first isolated in 2004, is a flat monolayer of carbon atoms tightly packed into a two-dimensional lattice.² Two-dimensional graphene consists of a single layer or up to 10 layers,¹⁻⁵ exhibiting exceptionally high crystal and electronic quality that has a number of potential applications.⁶⁻⁷ It is inevitable that graphene will enter the environment in increasing amounts with usage in consumer products, but their ecological risks are largely unknown. Thus far, it has been documented that graphene induces cytotoxic effects such as cell membrane damage and bacterial toxicity.⁸⁻¹⁰ Furthermore, it was also demonstrated recently that graphene can even cause some genotoxic effects on human stem cells like DNA fragmentations and chromosomal aberrations.¹¹,¹²

One critical factor is the extent to which graphene bioaccumulates in organisms and spreads through food chains.¹³ However, this topic has not been investigated yet, largely because methods have not been available to readily quantify graphene in complex environmental or biological systems. Techniques that can be used to measure graphene in relatively pristine samples such as scanning probe microscopy, Raman spectroscopy and X-ray diffraction are severely limited by the presence of other carbonaceous materials.² An imaging technique based on Fourier transform infrared spectroscopy mapping was developed to analyze the graphene spatial distribution inside nematodes,¹⁴ but this approach does not provide quantitative results. Similarly, light microscopy has been used to qualitatively detect graphene in cells.⁸ Thus, a quantitative method that can be used in complex environmental and biological matrices is clearly needed.

To overcome this challenge, we here report the synthesis of carbon-14 labeled graphene for the first time to our knowledge, by means of graphitization and exfoliation of sandwich-like FePO₄/dodecylamine hybrid nanosheets. A similar approach has recently been successfully employed to quantify carbon-14 labeled carbon nanotubes and fullerens in complex biological samples by detecting Beta emissions from the C-14
isotope. Carbon-14 graphene was dispersed in water and its uptake and depuration behaviors measured using aquatic organism Daphnia magna, which is a standard U.S. Environmental Protection Agency test organism. D. magna are filter feeders and have a central position in freshwater food web dynamics. Once accumulation occurs, organisms at higher trophic levels that consume filter feeders might be expected to elevated concentrations.

**EXPERIMENTAL SECTION**

**Materials.** All reagents used are of analytical grade without further purification. The carbon-14 ring-labeled phenol was purchased from (Brea, CA). 14C(U)-phenol had a chemical purity of ≥97% (analyzed with dated HPLC radiochromatogram) and was stored in an ethanol solution. Ferrous chloride tetrahydrate (FeCl2·4H2O), ammonium dihydrogenphosphate (NH4H2PO4) and hydrochloric acid (HCl, 37%) were purchased from Nanjing Chemical Reagent Co., Ltd. Dodecylamine and other chemicals were purchased from J & K Technology Co., Ltd. Suwannee River humic acid (HA) was obtained from the International Humic Substances Society.

**Graphene Synthesis and Characterization.** Twenty mL of an ethanol solution containing dodecylamine (5,000 g) was added into 80 mL of a mixed aqueous solution of FeCl2·4H2O (1,590 g) and NH4H2PO4 (900 g) at 50 °C. The solid product was collected by centrifugation and dried under vacuum at 40 °C for 12 h. The mixture was heated at 700 °C for 12 h under argon. After cooling, the black powder and 40 mL of 37% hydrochloric acid were transferred into a Teflon-lined autoclave and sealed before being heated in an oven at 180 °C for 24 h. The black solid product was collected by centrifugation, and then washed by water and anhydrous ethanol over 10 additional times to ensure the purity of the graphene. The powder was then dried under vacuum at 40 °C for 12 h. To disperse the carbon-14 graphene in water, a 500 mL beaker containing graphene and 300 mL of artificial freshwater (AF) (CaCl2·2H2O, 58.8 mg/L; MgSO4·7H2O, 24.7 mg/L; NaHCO3, 130.0 mg/L; KCl, 1.2 mg/L; hardness [Ca2+] + [Mg2+] = 0.5 mmol/L17,22 was seated in ice. The solution was sonicated for 6 h with the probe tip of an ultrasonic processor (100 W, P = 7.52 J/s) approximately 0.4 cm from the bottom of the beaker.

The Fe content in triplicate samples of the graphene powder was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300DV) under the following parameters: 0.55 L/min nebulizer flow; 1.3 mL/min sample pump flow; cyclonic spray chamber; 2 mm alumina injector; and 238 nm wavelength. The minimum detection limit of Fe for ICP analysis was 0.005 mg/L.

To assess potential carbon-14 byproducts from the synthesis procedure, carbon-14 graphene (0.3 mg) was extracted in sequence using dichloromethane (5 mL), n-hexane (5 mL), and dichloromethane (5 mL). These solutions were recombined, and subjected to anhydrous sodium sulfate to remove water. After that, the sample was dried using a gentle nitrogen stream and reconstituted in methanol and dichloromethane (4:1, v/v) for HPLC and GC-MS analysis (see Part I in Supporting Information (SI) for additional details). Filtrate from the sonicated graphene samples were similarly analyzed (see Part I in SI for details). In addition, the reconstituted solution for the graphene powder and a filtrate sample from the sonicated graphene were added to Gold Star scintillation cocktail (Meridian) and their radioactivities were measured in a liquid scintillation counter (LSC) (LS6500; Beckman Coulter, Pasadena, CA).

Transmission electron microscopy (TEM) and High-resolution TEM (HRTEM) measurements were conducted with a JEM-2010 electron microscope, using an accelerating voltage of 200 kV. Raman spectroscopic analysis was performed with a Renishaw InVia system utilizing a 514 nm incident radiation. Scanning electron microscopy (SEM) measurements were conducted with a Hitachi S4800 instrument, using an accelerating voltage of 10 kV. TEM, HRTEM, Raman spectroscopy, and SEM analyses were performed for both the graphene powder and the sonicated graphene. Graphene solution (100 and 250 µg/L) was filtered using a 0.22 μm filter to obtain a mat of the sonicated graphene for additional characterization. X-ray photoelectron spectroscopy (XPS) measurements of graphene powder were performed on a PHI 5000 VersaProbe with a monochromatic Al Ka X-ray source. Nitrogen sorption isotherms of the graphene powder were collected at 77 K using Micromeritics ASAP2020 equipment. Brunauer–Emmett–Teller (BET) and BJH models are respectively used for specific surface area and porosity evaluation.

**Carbon-14 Labeling Quantification.** The purified graphene powder was dispersed in the solution by sonication as described above and the radioactivity of the purified graphene solution was quantitatively measured in a LSC following combustion in a biological oxidizer (BO) or direct addition to scintillation cocktail (Gold Star, Meridian). The BO (OX-500; Zinsser Analytic, Germany) was used to burn the graphene at 900 °C for 4 min under a stream of oxygen gas running at 360 mL/min. The 14CO2 released during the combustion process was captured in alkaline carbon-14 scintillation cocktail (Zinsser Analytic, Germany) and then counted by LSC. The direct addition of the above graphene solution to scintillation cocktail (Gold Star, Meridian) followed by scintillation counting was found to consistently underestimate the radioactivity of the graphene relative to burning the graphene in the BO. The direct addition method yielded only 48% of the radioactivity measured using the BO method (10.9 ± 0.07 mCi/g for direct method and 22.71 ± 1.78 mCi/g for BO method; errors always represent standard deviation values; n = 3). However, since the coefficient of variation of the BO method for triplicate sample (7.86%) was inferior to that of the direct addition method (1.37%), the direct addition method was predominately used. The radioactivity response detected using the direct addition method was linear (R2 = 0.99) with respect to the graphene concentration.

**Test Organisms.** Daphnia magna were cultured in AF aerating for more than 3 days (20 ± 1 °C, 16:8 h light:dark photoperiod), as performed using a standard method.22 D. magna were fed three to five times a week with a culture of green algae (mainly Scenedesmus sp.). Daphnia neonates (<1 day old) were used for following uptake and depuration experiments, and adult Daphnia with eggs were used for transfer to offspring experiments.

**Uptake Experiments.** A 0.9 mg graphene sample was weighed on a microbalance (Mettler Toledo, XP56 Microbalance, Readability: 1 µg), added to a 500 mL beaker containing 300 mL of AF, and sonicated as describe above. This suspended graphene solution was diluted within two days using AF to yield exposure concentrations of approximately 250 µg,
100 μg, 50 μg, and 25 μg of graphene L⁻¹ for uptake experiments. Before D. magna addition, one 3 mL sample was taken from each exposure container and mixed with Gold Star scintillation cocktail (Meridian), and the radioactivity was measured by LSC. Another 3 mL of solution was moved into 1 cm-cuvette and scanned by Zeta Potential & Particle Size Analyzer (ZetaPlus, Brookhaven Instrument) under an average count rate of 20.4 kcps to explore the size distribution of
graphene in the solution. Prior to the experiment, Daphnia neonates were removed from the primary culture container and transferred to fresh AF without algae for at least 1 h to allow for partial gut purging and help the organisms acclimate. Thirty of these organisms were then added to each container containing 90 mL of exposure solution. Triplicate control (without Daphnia) containers with 30 mL of exposure solution were prepared to quantify graphene settling during the exposure period. The 30 mL volume was selected to save the radioactivity graphene which is highly expensive and challenging to prepare. Our preliminary result suggested that there was no statistical difference for the graphene settling velocity between 30 and 90 mL of exposure solution (100 μg/L). The measurement of the aqueous-phase radioactivity in each container was determined by mixing 3 mL of this solution with 7 mL of Gold Star scintillation cocktail and measuring radioactivity via LSC. Triplicate containers were sampled after 1, 4, 10, 24, and 48 h. There was no feeding during these experiments. No organisms were immobilized under this experimental condition. After the exposure duration, D. magna were placed in beakers containing clean water and pipetted vigorously to remove graphene particles attached to their carapaces. After this procedure, graphene aggregates could not be observed on the exterior of the organisms using light microscopy (Nikon Eclipse Ti-U), and thus contributions from the attached graphene to the total mass of graphene associated with the Daphnia are expected to be minimal. Then, the 30 Daphnia from each container were added to foil boats, dried, weighed using the Mettler Toledo microbalance, added to scintillation vials with 10 mL of Gold Star cocktail, ultrasonicated for 20 min, allowed to sit for at least 24 h, and then analyzed using LSC; preliminary results suggested that quantification of graphene concentrations using this method was more reproducible in neonates than with adults which motivated the use of neonates. The radioactivity from control samples (i.e., Daphnia without exposure to graphene) was subtracted for all of the uptake, depuration, and transfer to neonates results. After D. magna removal, aqueous-phase radioactivity was also measured as described above to determine the concentration of graphene remaining in solution. Control experiments revealed that there was less than a 26% organism mass decrease between the 0 and 48 h data points for the uptake and depuration experiments.

Additional experiments were also carried out using the same reactor setup and procedure described above to examine how the ratio (per daphnia/mL artificial freshwater) may influence the graphene uptake by daphnia and the tested ratio was 1:3, 1:10 and 1:17 (per daphnia/mL artificial freshwater). An exposure concentration of approximately 100 μg of graphene L⁻¹ was tested for this uptake experiment.

**Depuration Experiments.** Elimination experiments were conducted similarly to the uptake experiments. After acclimating for 1 h in AF without graphene, 39 sets (each with 30 organisms) were prepared and then respectively exposed for 24 h to AF spiked with C-14 graphene at a ratio of 3 mL of solution per organism and then pipetted to clean water to wash them. Three sets of organisms (30 daphnia in each set) were respectively sampled, dried, weighed and their radioactivities determined as described above. The remaining 36 sets of D. magna were randomly separated to be 3 groups and each group (with 12 sets) was respectively added to clean AF, artificial freshwater supplemented with algae at a concentration of 1.0 × 10⁸ cells of algae L⁻¹, and AF with 10 mg (TOC)-L⁻¹. At predetermined intervals (1, 4, 12, and 24 h), 3 sets of D. magna (30 organisms in each set) was sampled from each depuration media and sacrificed to measure graphene concentration in the organisms. Daphnia were observed with light microscopy during this period.

**Transfer to Offspring.** After 24 h exposure to a 250 μg/L graphene suspension, 10 gravid Daphnia were sampled, dried, weighed, and their radioactivities determined. The remaining gravid Daphnia were added to artificial freshwater supplemented with algae at a concentration of 1.0 × 10⁸ cells of algae L⁻¹ for 2 h. As shown in Figure S1 of SI, gravid Daphnia were able to purge the majority of the graphene from their gut after 1 h when they were fed algae during the depuration period. After removal from the artificial freshwater supplemented with algae container, the Daphnia were vigorously pipetted in clean water to remove any graphene on the organisms’ carapaces. Three groups of 10 of these Daphnia were sampled, weighed using the microbalance, and their radioactivities were measured as described above. Another 10 D. magna with eggs were separately transferred into clean AF for reproduction. All of the offspring produced by each gravid Daphnia during a 42 h period were counted. Because the neonates may ingest graphene excreted by maternal Daphnia, all neonates were transferred into artificial freshwater supplemented with algae at a concentration of 1.0 × 10⁸ cells of algae L⁻¹ for 2 h to minimize possible uptake of the graphene excreted from the gravid adult. After 2 h of algae feeding, the neonates from each maternal Daphnia were collected, weighed, and their radioactivity were measured as described above. The eggs in the remaining 10 Daphnia were carefully removed from the brood pouch with a fine needle, and their radioactivities were also measured after respectively being washed using water and methanol 6 times. The mass of two hundred of eggs was dried and weighed using the Mettler Toledo micro balance. The average mass for each egg was used to quantify the graphene contained in the dried eggs.

**Statistics.** All statistical analyses were performed using SPSS 18.0 (PASW Statistics, IBM Company); differences were considered statistically significant at p < 0.05. Depuration results data were analyzed by one-way analysis of variance (ANOVA). Errors always represent standard deviation values.

**RESULTS AND DISCUSSION**

**Graphene Synthesis and Characterization.** As shown in Figure 1a, high quantities of few-layer graphene sheets were successfully synthesized on the sandwich-like FePO₄/dodecylamine hybrid nanosheets. Graphene layers were obtained by removing the inorganic components of FePO₄ from the hybrid nanosheets using 37% HCl; no FePO₄ was detectable in the graphene by measuring the Fe content of the graphene using ICP-OES (Fe concentration was below limit of detection 0.005 mg/L). The potential for the formation of carbon-14 byproducts during the synthesis and sonication procedure were analyzed using GC-MS, HPLC, and LSC, as described in Experimental Section. Additional chemical peaks were not found for either the graphene powder or the filtrate of the sonicated graphene using either HPLC or GC-MS. The radioactivity readings in disintegrations per minute (DPM) in the extraction solution for the graphene powder and the filtrate of the sonicated graphene was respectively 26.44 ± 9.19 and 41.05 ± 10.11 (n = 3). These values were not statistically different from the background value (37.24 ± 7.92) (n = 3). The specific radioactivity of the purified graphene was 10.9 ±
0.15 mCi/g measured using direct addition method as shown in Materials section. A TEM image of the as-prepared graphene shown in Figure 1b displays the typical crumpled nanosheets of graphene. HRTEM image shown in Figure 1c indicates that the graphene mainly consisted of 4 layers and the interlayer distance is about 0.344 nm, which is the interlayer distance of graphite.\(^{25,26}\) SEM image (Figure 1d) indicates that most of the graphene are crumpled leaf-like nanosheets and agglomerated (also in Figure S2a of SI). The size distribution of graphene in the AF solution is presented in Figure S2b of SI and it suggested that the size distribution of the graphene has two narrow peaks and the average sizes of the peaks are 300 and 2000 nm, respectively. TEM and SEM images confirmed that sonication did not change the morphology of the graphene. The marked strong D, G, and 2D bands in the Raman spectra (Figure 1e) is well indexed to graphene with multilayers structure.\(^{2,25,27,28}\) Raman spectroscopic analysis revealed that there was no change in the Raman spectra bands for the graphene after sonication. A specific surface area of 660 m\(^2\)/g is obtained from the nitrogen sorption/desorption isotherms of the multilayer graphene (Figure 1f) according to the BET model. Considering the specific surface area of the single-layer graphene (2630 m\(^2\)/g),\(^{29,30}\) the obtained graphene are mainly consisted of 4-layer graphene, in accord with the HRTEM result. XPS spectra of the graphene (Figure 1g) indicates that the atomic ratio of C:O is 89:6 (the remaining 5% is 1.4% of H and 3.6% of N).

**Uptake Results.** No Daphnia were immobilized after being exposed for 48 h to a graphene concentration of 250 \(\mu\)g/L, the highest graphene concentration in this study, or to clean AF. As shown in Figure 2, uptake results across the range of concentrations tested here (25, 50, 100, and 250 \(\mu\)g/L) showed a general increase during the first 24 h followed by a slight decrease from 24 to 48 h. This reveals that a pseudosteady-state concentration was reached after 24 h; the increase in body burden during this period could not be explained by decreasing organism mass because the mass actually decreased by less than 26% and body burden increased by more than a factor of 3.

Another potential explanation for the pseudosteady state is the change in the aqueous phase graphene concentration. After *D. magna* removal, aqueous-phase radioactivity at each sampling time was also measured to determine the concentration of graphene in solution, and the results were presented in Figure S3 of SI. These results indicate that substantial settling occurred during the first 24 h but then the graphene concentration in the dispersion remained relatively stable from 24 to 48 h. While the graphene concentration in solution remained relatively constant from 24 to 48 h, the body burden values of the tested concentrations (25, 50, 100, and 250 \(\mu\)g/L) at 48 h were, respectively, 38%, 55%, 58%, and 66% of the value at 24 h. Given that the organism mass decrease from 24 to 48 h was only 12%, this decrease in body burden likely stems from the decrease in the aqueous phase concentration during the first 24 h as a result of settling, and the body burdens adjusting to the decreased aqueous phase concentration. In support of this hypothesis, the settling rates during the first 24 h increased with increasing concentration, and there was an increasing rate of decrease in the body burdens with increasing concentration. In addition, the coefficients of variation were larger for higher initial suspended graphene concentrations which would be expected given the higher rates of settling.

During the exposure period, the radioactivity in the exposure solutions without *D. magna* was also measured to assess the aggregation and settling of graphene (see Figure S4 of SI). This figure reveals that roughly 20–25% of the graphene settled from the exposure solution under the tested concentrations at 24 h. The presence of *Daphния* in the exposure solution enhanced the settling rates of graphene; approximately 60% to 85% of the graphene settled from the exposure solution after 24 h (see Figure S5 of SI). The enhancement is likely attributable to the fact that the graphene particles are concentrated in the digestive system as shown the black parts in the body of the Daphnia (see SI Figure S6) and were impacted by passage through the organism gut tract.\(^{31}\)

The high uptake concentrations are similar to those obtained in previous studies with carbon nanotubes and fullerenes. It has been shown previously that *D. magna* intake of the nanotubes was 68 \(\mu\)g/mg of dry tissue after 24 h exposure to a 400 \(\mu\)g/L nanotube suspension.\(^{32}\) When testing a range of multiwall carbon nanotubes (MWCNTs) with various surface charges (concentrations 200 to 361 \(\mu\)g/L), the body burdens after exposure for 24 h ranged from 2.4 to 12 \(\mu\)g/mg,\(^{17}\) a concentration range that covers the maximum graphene body burden measured in this study (7.8 \(\mu\)g/mg). However, these earlier studies on MWCNTs used much larger *Daphния* that were aged 5 to 7 d old, while neonates (<1 d old) were used in this study. Petersen et al.\(^{17}\) suggested that the lower MWCNTs body burdens measured in the later study may have been a result of the larger organisms and the corresponding smaller ratio of the gut tract volume to the whole organism mass. The neonates used to assess graphene uptake were much smaller than those in either of these MWCNTs studies. Thus, an interesting topic for future work would be to investigate how the graphene body burden would compare to those for MWCNTs in similarly sized organisms. A number of studies have also assessed the accumulation of fullerenes by *D. magna*. The body burdens of fullerenes in these organisms typically ranged between 2.3 and 5 \(\mu\)g/mg wet mass,\(^{33–35}\) except for one study by Tao et al.\(^{36}\) who found a maximum body burden of 0.24 mg/mg wet mass for uptake by adult *Daphния*. It is challenging to directly compare results reported on wet and dry
mass bases. A wet to dry mass conversion factor of ~7.5 was determined in this study by weighing neonates before and after drying. Using this factor, the fullerene body burdens were larger than those measured here. A body burden of 3.1 μg/mg wet mass was reported for neonates (<1 d old) after exposure for 24 h to a 200 μg/L concentration, suggesting that higher fullerene concentrations may be accumulated on a dry mass basis compared to graphene. More standardized exposure conditions regarding the age of the Daphnia and reporting results on a wet mass or dry mass basis would facilitate comparisons among studies. While additional studies are needed to assess what impact Daphnia age and size would have on the body burdens for different carbon nanoparticles, measuring the organism mass on a dry mass basis is recommended to remove uncertainty from differences in the water concentration in the organisms.37

Changing the volume of water used impacted the Daphnia body burdens during the 48 h exposure period (see Figure S7 of SI). Higher volumes of test solution resulted in substantially increased body burdens during the first 10 h, but the difference in the body burdens decreased from 10 to 48 h. The body burdens among the different exposure volumes were not significantly different after 48 h (ANOVA, p < 0.05).

Depuration Results. As shown in Figure 3, Daphnia exposed to a graphene concentration of 50 μg/L, apparently in clean AF for Daphnia exposed to different concentrations during the uptake experiments.

When Daphnia were fed algae during the depuration period, the body burdens decreased more than 90% during the first 4 h (see Figure 3). Light microscopy of the Daphnia during the first 40 min of elimination with algae indicated that there was rapid removal of the graphene (see SI Figure S6); while the gut tract initially was full with graphene, there was a clear decrease in the gut fullness with time as the dark color (indicative of graphene in the gut tract) was replaced by a light green color indicative of algae. Figure 3 provides quantitative results from the elimination study and often shows that the Daphnia were able to completely eliminate the graphene. The graphene concentration in Daphnia that had been depurred in AF amended with 1.0 × 10^8 cells of algae L^{-1} for 24 h after being exposed for 24 h to a 50 or 250 μg/L graphene suspension, was not statistically greater than 0. As shown in Figure 3, the presence of 10 mg/L HA in AF also increased the graphene excretion rate compared to the clean AF condition. The enhancement may be attributed to the interactions between HA and graphene or the effect of HA molecule size that is similar to that of algae. It was previously reported that maximum adsorption of HA on graphene was 637 μg/m².38 Additional studies are needed to understand the mechanism of the enhanced graphene excretion in the presence of HA.

Graphene elimination measured in this study was similar in some regards and differed in others in comparison to previous data on MWCNTs and fullerenes. Neither MWCNTs study showed MWCNTs elimination in AF or freshwater with natural organic matter (NOM), but both showed elimination with algae feeding. For the algae feeding condition, Petersen et al.17 showed nearly complete (89–99% of initial body burden remained) or substantial but not complete (63–96% of the initial body burden remained) MWCNTs elimination after 24 h at the lower and higher MWCNTs concentrations, respectively. In contrast, Petersen et al.32 showed significant (50–85%) MWCNTs elimination during the first 3 h with algae feeding but then no subsequent elimination. The graphene depuration results from this study differ from previous studies with MWCNTs in that elimination is observed in clean freshwater, and that the addition of NOM lead to nearly complete elimination (82% and 92% elimination of graphene after 24 h at the 50 μg/L and 250 μg/L concentrations, respectively). However, graphene elimination results are similar in that algae feeding led to nearly complete elimination (90% and 98% elimination of graphene after 24 h at the 50 μg/L and 250 μg/L concentrations, respectively). In the elimination study with fullerenes, only 54% and 26% of the initial concentration remained after the Daphnia were in clean AF for 24 and 48 h, respectively. Unlike this study which showed a similar graphene body burden being reached after depuration for 24 h in clean AF regardless of the initial graphene concentration, an earlier fullerene elimination study showed a first order elimination rate.35 Fullerene elimination by D. magna in the presence of algae has not been quantified to our knowledge. Overall, it seems that the presence of algae facilitates elimination of carbon nanoparticles, but that NOM only facilitated graphene elimination. While fullerene elimination proceeded at a steady rate in clean freshwater, graphene elimination in clean freshwater only occurred for higher body burdens. Thus, elimination behaviors of carbon nanoparticles seem to vary based on their morphology. However, additional studies which test multiple carbon nanoparticles simultaneously.

![Graphene depuration by D. magna. Daphnia were exposed to graphene in artificial freshwater for 24 h with an initial suspended graphene concentration of 50, 100, or 250 μg/L. Depuration occurred in a range of clean media: artificial freshwater (AF) or artificial freshwater with 10 mg (TOC)-L^{-1} humic acid (HA) or the artificial freshwater supplemented with algae at a concentration of 1.0 × 10^8 cells of algae L^{-1} (AL). Mean and standard deviation values were calculated from triplicate samples. The symbols (red dot) and (green star) indicate not significantly or significantly different from zero, respectively.](image-url)
are needed to better understand the exact role of particle morphology. This type of test would help minimize the variability among the *Daphnia* populations used in different studies enabling a focus on morphology.

While *Daphnia* immobilization was not observed under the conditions tested, these substantial graphene concentrations measured in the organisms may cause toxic effects in longer studies, potential risks from cocontaminants present in ecosystems such as metals and organic pollutants which may adsorb onto the graphene nanoparticles, and the potential for trophic transfer.

**Transfer to Offspring Results.** To explore the transfer of graphene to the next generation of neonates, gravid *Daphnia* were exposed to graphene, and the radioactivity of the gravid *Daphnia*, their eggs, and offspring were measured separately. The detailed results are presented in Tables S1 and S2 of the SI. Our data in SI Table S1 suggested that the body burden of the gravid *Daphnia* after a depuration period of 2 h in artificial freshwater supplemented with algae at a concentration of 1.0 × 10^8 cells of algae L^-1 was (0.096 ± 0.018) μg of graphene/mg of dry tissue, a decrease of 75%. Eggs in the gravid *Daphnia* were carefully separated, washed and their radioactivities were measured. Our results suggested that the graphene contained in the eggs was (0.056 ± 0.013) μg of graphene/mg of dry tissue (n = 3). Ten gravid *D. magna* were separately transferred into clean AF for reproduction and the neonates produced by each gravid *Daphnia* in 42 h were counted and their radioactivities were measured (see SI Table S2). Before the radioactivity measurement, the neonates were transferred into artificial freshwater supplemented with algae at a concentration of 1.0 × 10^8 cells of algae L^-1 for 2 h to minimize possible uptake of graphene excreted by gravid *Daphnia*. As shown in Figure 3, the neonates, exposed to the graphene concentration of 50 and 250 μg/L, were able to excrete >94% of the accumulated graphene from their guts after 1 h when they were fed algae during the depuration period. It is evident in the table that the radioactivity detected in each group of neonates and the offspring was (0.068 ± 0.036) μg of graphene/mg of dry tissue; this value was significantly greater than 0 (p = 0.001, f test). Based on the radioactivity comparison of control organisms to exposed organisms (eggs and neonates), we postulate that the retained graphene was partly stored in the brood pouch of the gravid *Daphnia* and can be transferred to the neonates. Gravid *Daphnia* are known to flow water through the brood pouch to provide oxygen to the developing embryos, and this likely lead to exposure to suspended graphene particles. Fullener transfer to eggs of gravid *Daphnia* has also been observed. However, only a very small graphene mass was transferred to the neonates as compared to the large detected fullerene mass in the eggs in the earlier study.

**ACKNOWLEDGMENTS**

Financial support for this project was from the National Natural Science Foundation of China (21377049, 21107046, and 21237002), the Natural Science Foundation of Jiangsu Province (BK2011576), and Research Fund for the Doctoral Program of Higher Education, Ministry of Education of China (20110091120036), and the Fundamental Research Funds for the Central Universities. We thank Prof. Rong Ji for his experimental assistance. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

**REFERENCES**


(22) Water Quality-Determination of the Inhibition of the Mobility of *Daphnia magna* *Straus* (Cladocera, Crustacea)-Acute Toxicity Test, ISO 6341, 1996


(42) Henry, T. B.; Petersen, E. J.; Compton, R. N. Aquoeous fullerene aggregates (nC60) generate minimal reactive oxygen species and are of low toxicity in fish: A revision of previous reports. *Curr. Opin. Biotechnol.* 2011, 22, 533–537.

