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Biological Uptake and Depuration of Carbon Nanotubes by Daphnia magna

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It is inevitable that carbon nanotubes (CNTs) will be released to and widely dispersed in environmental ecosystems, given their numerous expected applications. Determination of their potential for bioaccumulation by ecological receptors is thus critical. Previous research involving several different terrestrial and benthic organisms has indicated that CNTs spiked to soils or sediments do not bioaccumulate. Conversely, we report here distinctly different uptake and depuration behaviors for an aquatic organism, Daphnia magna, in a water-only system. After 48 h of exposure of this organism to a 0.4 µg/mL solution of dispersed nanotubes, the CNTs comprised 6.3 ± 1.5% of the residual organism dry mass. Moreover, these organisms were unable to excrete the nanotubes to either clean artificial freshwater or filtered Lake Kontiolampi water after 24 h depuration periods, even though the lake water had a substantial concentration of natural organic matter. Addition of algae to the water during the depuration period did result however in release of a significant fraction (~50–85%) of the accumulated CNTs within the first few hours, but little thereafter. Light microscopy results suggest that the vast majority of the accumulated CNTs remained in the organisms’ guts and were not absorbed into cellular tissues.

Introduction

Carbon nanotubes (CNTs) are currently synthesized and produced on a substantial scale worldwide, the total mass for the 2007/2008 year being recently estimated as 350 tons (1). Given their exciting properties and decreasing production costs, increasing numbers of applications and volumes produced are anticipated. This pattern suggests that CNTs can be expected to enter environment systems with increasing frequency and in increasing amounts, while the risks they may pose are largely unknown.

One of the most significant currently not well understood risks is their potential accumulation by organisms and transfer throughout food chains (2, 3). It has been predicted that carbonaceous nanomaterials may bioaccumulate significantly because of their inherent hydrophobicity (4–6). One novel approach to investigate the uptake potentials of CNTs by ecological receptors is carbon-14 labeling of these materials to enable quantification of their mass accumulation in organisms (7–9). In prior investigations no significant accumulations of radiolabeled CNTs by earthworms (7), the sediment-dwelling oligochaete Lumbriculus variegatus (8), or two estuarine invertebrates (9) were observed. What was unclear in these investigations though has been the extent to which the lack of uptake stemmed from strong sorption of the CNTs to soil/sediment particles, or whether the CNTs were only minimally absorbed across intestinal or dermal tissues, thus limiting systemic circulation. CNT uptake by a few other organisms has been investigated in the absence of soil or sediment. The ciliated protozoan Tetrahymena thermophila was found recently to internalize dispersed single-walled carbon nanotubes (SWNTs), suggesting the possibility of food chain transfer, but internalization by this single-celled organism does not necessarily indicate similar patterns for larger organisms (10). After being fed a yeast paste spiked with SWNTs, only a minute fraction of these nanotubes passed through the gut walls of the fruit fly Drosophila melanogaster and were found in their organs (11). In other experiments conducted with aquatic organisms in the absence of sediment, CNTs have been detected qualitatively in numerous organisms, but their uptake quantities and long-term fates were not determined (12–16). Injected modified nanotubes have generally been shown to be readily excreted from organisms in biomedical studies (17–19), but injected pristine SWNTs have been found to remain in various organs at high concentrations after 28 days of uptake, thus suggesting that CNT modifications may well play significant roles in their biodistribution (20). It is unknown, however, to what extent nanotube biodistribution and excretion behaviors may differ between organisms exposed in environmental systems and those exposed in biomedical studies as a result of different organism physiologies, exposure routes, and nanotube modifications.

To assess quantitatively the bioaccumulation potentials of CNTs in aquatic environments in the absence of soil or sediment particles, we investigated Daphnia magna uptake, depuration, and biodistribution of acid-treated multiwalled carbon nanotubes (MWNTs). D. magna are easy to culture, are sensitive to environmental pollution, and are filter feeders, indicating that they typically filter large volumes of water on a daily basis. They have commonly been used for ecotoxicology testing in which other nanomaterials have been included (21–26).

Experimental Procedures

Carbon Nanotube Synthesis, Purification, and Characterization. Synthesis and purification of carbon-14 MWNTs have been described elsewhere (7, 8). In the work described here, CNTs refer to MWNTs unless otherwise specified. Briefly, nickel nitrate, magnesium nitrate, citric acid, and 20 mL of Milli-Q water were mixed. This solution was then dried at 100 °C and the solid calcined. A 100 mg quantity of this catalyst was added to a quartz boat and hydrogen flown over the boat as the reactor temperature was raised to and held at 600 °C. The flow of hydrogen gas was then stopped, and the reactor was cooled to room temperature in argon. The MWNTs were purified by bath sonication in full-strength hydrochloric acid for 1 h, filtered, and washed with boiling water. They were

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then bath sonicated in an acid mixture composed of a 3:1 ratio of concentrated sulfuric (J. T. Baker, 95–97%) and nitric (J. T. Baker, 65%) acids, a process previously shown to increase the concentration of functional groups on nanotubes (27), then filtered, and washed with boiling water. These CNTs had a specific radioactivity of 0.12 mCi/g as previously determined.

CNTs were thoroughly characterized prior to the bioaccumulation studies. Microscopic analysis of MWNTs was performed using scanning electron microscopy (SEM) with a Philips/FEI XL30 FEG scanning electron microscope operating at an accelerating voltage of 15.0 kV. CNTs were dispersed and then dripped onto cleaned silicon wafers for SEM analysis. Thermal gravimetric analysis (TGA) (Pyris 1 TGA, Perkin-Elmer) was used to determine concentrations of catalyst impurities remaining and to assess the presence of amorphous carbon impurities. Given its less stable chemical configurations, amorphous carbon generally burns at lower temperatures than do the CNTs themselves. As such, carbon impurities can be quantified by analyzing the derivative of mass changes with respect to temperature; a large peak at a higher temperature is generally attributed to the CNTs themselves, while peaks at lower temperatures indicate carbon impurity oxidations. X-ray photoelectron spectroscopy (XPS) spectra taken using a Kratos Analytical Axis Ultra X-ray photoelectron spectrometer were used to assess elemental compositions of the MWNTs. A thick mat of amorphous carbon impurities was removed and sodium kakodylate buffer re-added. For the dehydration steps, Daphnia were refrigerated in the following solutions for 10 min each: 30% acetone, 60% acetone, 90% acetone, removed and sodium kakodylate buffer re-added. This solution was then sonicated for 1 h with the probe tip approximately 0.4 cm from the bottom of the beaker. Sonication was conducted at least two days prior to the start of an uptake experiment because preliminary experiments showed that most MWNT settling occurred during the first two days following sonication. Aqueous-phase radioactivity was determined prior to uptake experiments by mixing 2 mL of this solution with 10 mL of Ultima Gold scintillation cocktail (Packard) in triplicate and measuring radioactivity via liquid scintillation counting (LSC). A variety of exposure conditions were tested, including changing suspended nanotube concentrations (0.04, 0.1, or 0.4 μg/mL) or exposure volumes (30, 100, or 200 mL). Before D. magna addition, duplicate samples were taken from each exposure container of 5 mL for experiments conducted with 30 mL of initial water or 10 mL for all other exposure conditions. These volumes were added to scintillation vials and mixed with equal volumes of Insta-Gel Plus cocktail (Packard) and radioactivities measured using LSC. Ten organisms were then added to each container. Triplicate containers were sampled after 1, 4, 10, 24, and 48 h. D. magna were placed in beakers containing clean water and pipetted vigorously to remove any nanotubes attached to the outsides of their bodies. After this procedure, nanotube aggregates were not visible on the exterior of the organisms, and contributions from the attached nanotubes to the total mass of nanotubes associated with the Daphnia are thus expected to be insignificant. D. magna were then added to foil boats, dried, weighed, added to scintillation vials with 20 mL of Ultima Gold cocktail, ultrasonicated for 10 min, allowed to sit for at least 24 h, and then analyzed using LSC. After D. magna removal, the aqueous-phase nanotube concentration was measured as described above. Statistical analyses were performed using ANOVA and a 95% confidence interval.

To check results obtained by sonicating D. magna in scintillation cocktail against those by biological oxidation, D. magna were exposed for 24 h to nanotubes at an initial concentration of 0.4 μg/mL in a 200 mL solution. The organisms were removed from the water, cleaned, and weighed. The protocol described above was used to determine the radioactivities of Daphnia in five of the ten containers. Daphnia from the five remaining containers were shipped to the University of Michigan (Ann Arbor, MI), where they were combusted using biological oxidation (OX-500, R. J. Harvey Instruments Co.), and the radioactivity was determined using LSC.

Depuration Experiments. Depuration experiments were conducted in a manner similar to that employed during the uptake experiments. D. magna were exposed for 24 h to spiked artificial freshwater at a ratio of 3 mL of solution per organism and pipetted to clean water to wash them. Triplicate samples of 10 organisms were sampled and their radioactivities determined as described above. The remaining D. magna were added to artificial freshwater, artificial freshwater amended with 1.0 × 10^6 cells of algae/L (29), or filtered water from Lake Kontiolampi collected in Eastern Finland (62°43'46″ N, 29°51'18″ E). The water from Lake Kontiolampi was filtered first through combusted glass fiber filters (Schleicher & Schuell GF52, Dassel, Germany) and then through 0.45 μm filters (sterile membrane filter, Schleicher & Schuell). The organic and inorganic carbon concentrations of the resulting filtrate were 20.9 and 0.64 mg/L, respectively. D. magna were removed from these media after 1, 4, 12, or 24 h. An additional experiment was conducted using artificial freshwater amended with algae to test for longer depuration periods (1 and 48 h). An additional 1.0 × 10^6 algae cells/mL was added after 24 h. A significant number of Daphnia died after 48 h of depuration, and only duplicate samples of the organisms were collected for radioactivity analysis.

Light Microscopy. D. magna exposed to nanotubes also were assessed using light microscopy. They were added after exposure to a 1:1 mixture of 4% glutaraldehyde and 0.2% sodium kakodylate buffer (pH 7.5) and refrigerated overnight. This solution was then removed, sodium kakodylate buffer was re-added, and the organisms were kept in this solution for at least 15 min. This buffer solution was then removed and a 1:1 mixture of 2% OsO4 and 0.2% 0.1 M sodium kakodylate buffer (pH 7.5) added for 4 h. This solution was removed and sodium kakodylate buffer re-added. For the dehydration steps, Daphnia were refrigerated in the following solutions for 10 min each: 30% acetone, 60% acetone, 90% acetone, and 100% acetone twice. The samples were then sequentially placed in the following solutions at room temperature: 100% acetone, 1:1 mixture of 100% acetone and Epon, and a 1:3 mixture of 100% acetone and Epon. Daphnia were then added to 100% Epon in a bath overnight. Slicing was conducted the next day. Daphnia were stained by incubation at 37 °C for 10–20 min in a 1:20 ratio solution of 1% toluidine blue and 2.5% NaHCO3 by incubation.

Results and Discussion Nanotube Characterization. SEM micrographs (Figure 1a,b) suggest an average nanotube length of 407 nm, while transmission electron micrographs have revealed a diameter range mainly from 30 to 70 nm (7, 8). Thermal gravimetric analysis indicated the absence of amorphous carbon in the plot of the rate of mass change per temperature change as shown by the lack of a second peak at a lower temperature. Additionally, the carbon purity was 99.9 ± 0.2 (n = 3), demonstrating almost complete removal of catalyst materials. X-ray photoelectron spectroscopy indicated significant damage to the carbon nanotubes and a high fraction of functional
groups. The elemental percentage of oxygen was 6.8 ± 0.3% ($n = 3$). To what extent, if any, these CNT modifications affect their bioaccumulation by organisms is currently unknown and a subject for additional research.
Nanotube Recovery. Body burdens were 42 ± 7 and 23 ± 2 for samples treated with sonication in scintillation cocktail and biological oxidation, respectively. This indicates that *Daphnia* sonication in scintillation cocktail yielded only 56% of the radioactivity measured using biological oxidation. Preliminary experiments with *Daphnia* exposed to water with 0.04 µg of nanotubes/mL showed a recovery of roughly 61%, suggesting the reproducibility of this result. The lower recovery of organisms sonicated in scintillation cocktail is likely the result of absorption of the carbon-14/β emissions by CNT aggregates (7). All *Daphnia* body burdens were adjusted accordingly.

Uptake Results. There was not a difference in acute toxicity between *Daphnia* exposed for 48 h to a nanotube concentration of 0.4 µg/mL, the highest nanotube concentration in this study, and those exposed to clean artificial freshwater. As shown in Figure 2, uptake results across the range of concentrations and volumes tested here showed a general increase during the first 24 h followed by a leveling off from 24 to 48 h. This suggests that a pseudo-steady-state concentration was reached after 24 h. There was a slight decrease in measured body burdens from 24 to 48 h, likely as a result of some nanotube settling. This suggests that settled nanotubes do not become readily associated with *Daphnia*. Nanotube aggregates attached to *Daphnia* carapace, appendages, and antennae, as had been shown in previous investigations of nanotubes or fullerenes with *Daphnia* (3, 16, 22), were removed by vigorous pipetting and should not be included in body burden values. Still, they would likely affect the health of *Daphnia* in the environment as a result of such effects as facilitated predation or hindered swimming.

Changing the volume of water used had a significant effect on the *Daphnia* body burdens after exposure for 48 h (P < 0.05). Our initial hypothesis was that the presence of the nanotubes would substantially increase the settling of the nanotubes from solution because of enhanced aggregation during passage through organism guts. However, a pattern was not found between settling rates and solution volume (data not shown). This difference in nanotube uptake for
Exposures to a 30 mL solution is instead attributed to the substantial mass of nanotubes accumulated in the organisms. After 48 h, 29% and 4.6% of the total mass of suspended nanotubes added to the containers were associated with *D. magna* for volumes of 30 and 200 mL, respectively.

*D. magna* were also exposed to a range of suspended CNT concentrations (Figure 2b). Normalizing the body burdens after 48 h of exposure by the suspended nanotube concentration at the conclusion of the experiments yielded values of 360000 ± 40000, 440000 ± 190000, and 350000 ± 80000, for *Daphnia* exposed respectively to 0.04, 0.1, or 0.4 µg/mL. These values are not significantly different (*P* > 0.05), indicating that CNT concentrations in the organisms are directly proportional to the suspended nanotube concentrations over the concentration range tested. These normalized body burden values are equivalent to bioconcentration factors (BCFs) that are typically utilized for hydrophobic organic chemicals (HOCs), but that term is not used because CNTs do not appear to be absorbed into organism tissues.

It has been shown previously that *D. magna* intake of fullerene was approximately 2.3 µg/mg of wet tissue after 48 h of exposure to a 30 ppm fullerene suspension (25). If these results are adjusted to a dry weight basis by assuming that the *Daphnia* dry weight is roughly 8% of the *Daphnia* wet weight as indicated by preliminary results not shown, this fullerene concentration in the *Daphnia* is approximately 29 µg/mg of dry tissue. The higher comparative nanotube accumulation by *D. magna*, 63 ± 15 µg of nanotubes/mg of dry tissue after 48 h of exposure to a solution with an initial nanotube concentration of 0.4 µg/mL, may result from feeding *Daphnia* with algae during the fullerene uptake experiments. Alternatively, this may partly stem from differences in the physicochemical properties (i.e., agglomeration status, surface functional groups, particle morphologies) of CNTs and fullerene aggregates.

Depuration Results. As shown in Figure 3, *Daphnia* were not able to fully purge CNTs from their guts. There was no decrease in concentrations of accumulated nanotubes after

![FIGURE 4. Light microscope pictures of *D. magna* exposed to nanotubes at a concentration of roughly 0.4 µg/mL for 1 h (a) (100×) and 24 h (b (100×), c (250×)).](image-url)
a depuration period of one day in clean artificial freshwater or filtered lake water. While natural organic matter (NOM) has previously been shown to separate nanotube aggregates and suspend nanotubes (33), it does not appear here to facilitate nanotube excretion. These results are interesting in light of the uptake results indicating that nanotube uptake is independent of concentration and that roughly constant body burdens were reached after exposure for 24 h. These factors suggest that *D. magna* are able to ingest and excrete CNTs at roughly the same rate when in solutions with dispersed nanotubes, yet elimination does not occur in the absence of dispersed CNTs.

When *Daphnia* were fed algae during the depuration period, the body burdens decreased 50–85% during the first few hours (see Figure 3b). Even under these conditions, however, *Daphnia* were unable to purge CNTs completely from their systems after 48 h. A smaller mass of nanotubes was excreted for experiments conducted for 48 h with algae (~50%) as compared to those for 24 h (~85%). This may stem from differences in *Daphnia* health. The results largely accord with those previously found qualitatively for *Ceriodaphnia dubia*, which showed that feeding with algae was necessary for gut clearance of nanotubes (14), but differ from those for gold nanoparticles (diameters 17–23 nm) which were excreted without algae feeding (26). This is speculated to be a result of the smaller size of the gold nanoparticles compared to the nanotubes. The depuration behavior found for nanotubes was also similar to those previously found for sediment particles (32). Gillis and co-workers found no apparent depuration of sediment ingested by *D. magna* after 48 h in clean water, but substantial removal after 24 h when the *Daphnia* were fed algae (32).

While nanotube exposure did not result in acute *Daphnia* toxicity, the lack of MWNT excretion observed might still provide insight to toxicokinetics in environmental systems. It is possible that the prolonged presence of significant quantities of CNTs in *D. magna* could limit digestion of food such as algae as a result of interactions between algae and nanotubes remaining in the guts. The lack of CNT depuration also raises a question about potential CNT food chain transfer to organisms at higher trophic levels, an effect that might be exacerbated by decreased *Daphnia* health and increased susceptibility to predation. Moreover, CNTs are known to be strong sorbents for a wide range of organic and inorganic materials (33, 34). The potential long-term presence of nanotubes in *Daphnia* guts and sorption interactions between HOCs and CNTs may well substantially affect HOC toxicity and accumulation, a possibility recently investigated for *Daphnia* exposed to fullerenes and HOCs (22). As noted above, however, the feeding of *Daphnia* on algae or bacteria could retard CNT accumulation.

**Light Microscopy.** It appears from light microscopy images and observations of live *Daphnia* that the vast majority of the ingested nanotubes reside in the organism guts, a finding that accords with SWNT exposure results observed with *D. melanogaster* (11). Comparison of part a with parts b and c of Figure 4 reveals that a substantially smaller fraction of the *Daphnia* gut fills with CNTs during the first hour of exposure than after 24 h. It was not possible, however, to differentiate visually between the gut contents of *D. magna* after exposures of 4 or 24 h or after 24 h depuration periods in clean artificial freshwater or Lake Kontiolampi water.

In closure, the results presented indicate significant nanotube accumulation and limited depuration behaviors for *D. magna* across a relatively limited range of experimental conditions. Further research over a broader range of experimental conditions will yield more comprehensive understandings of CNT fate in aquatic ecosystems. In particular, unmodified nanotubes or those mixed with NOM or algae prior to *D. magna* exposure could potentially influence accumulation and excretion behaviors. Additionally, the presence of algae and consequently continuous feeding by *D. magna* may decrease the extent to which CNTs aggregate in the guts of these organisms, or alternatively, CNTs associated with algae may serve as a new pathway to carry nanotubes into the organisms.

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**Literature Cited**


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