Environ. Sci. Technol. **2011,** *45,* 1133–1138

Influence of Polyethyleneimine Graftings of Multi-Walled Carbon Nanotubes on their Accumulation and Elimination by and Toxicity to *Daphnia magna*

ELIJAH J. PETERSEN,*,† ROGER A. PINTO,‡ DANIELLE J. MAI,‡ PETER F. LANDRUM,§ AND WALTER J. WEBER, JR.*,‡

Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, United States, Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States, and National Oceanic and Atmospheric Administration, Ann Arbor, Michigan 48108, United States

Received September 2, 2010. Revised manuscript received November 30, 2010. Accepted December 9, 2010.

Modifications of carbon nanotubes (CNTs) for different applications may change their physicochemical properties such as surface charge. Assessments of the extent to which such modifications influence CNT ecotoxicity, accumulation, and elimination behaviors are needed to understand potential environmental risks these variously modified nanoparticles may pose. We have modified carbon-14 labeled multi-walled carbon nanotubes (MWNTs) with polyethyleneimine (PEI) surface coatings to increase their aqueous stability and to give them positive, negative, or neutral surface charges. Uptake and elimination behaviors of *Daphnia magna* exposed to PEI-coated and acid-modified MWNTs at concentrations of approximately 25 and 250 μg/L were quantified. PEI surface coatings did not appear to substantially impact nanotube accumulation or elimination rates. Although the PEI-modified nanotubes exhibited enhanced stability in aqueous solutions, they appeared to aggregate in the guts of *D. magna* in a manner similar to acid-treated nanotubes. The MWNTs were almost entirely eliminated by *Daphnia* fed algae during a 48 h elimination experiment, whereas elimination without feeding was typically minimal. Finally, PEI coatings increased MWNT toxicities, though this trend corresponded to the size of the PEI coatings, not their surface charges.

Introduction

Carbon nanotubes (CNTs) comprise a novel class of nanoparticles manifesting unique structural, electrical, and chemical properties. Two unique aspects of CNTs are that their sidewall ends can be modified and that they can be grafted with copolymers (*1*). Numerous applications are expected to utilize these properties to functionalize or otherwise modify the nanotubes, including polymeric composites with stabilized CNT reinforcement (*2*) and CNTs modified for drug delivery devices (*3*−*5*).

In addition to engineered changing of the surface chemistry of nanotubes for potential applications, environmental processes such as biotic and abiotic degradation, photodegradation, and interactions with natural organic matter (*6*) may impact the surface chemistry of CNTs in natural environments. The impacts of changing nanotube surface chemistries on their environmental fates and ecotoxicities are largely unknown. CNTs with different surface chemistries have been shown to manifest profoundly different toxicities to *Ceriodaphnia dubia* (*7*), with alkyl and amino functional groups dramatically decreasing *Ceriodaphnia* survival and hydrophilic groups having the opposite effect. Various types of surface-modified fullerenes, another carbonaceous nanoparticle, have exhibited substantially different toxicities to *Daphnia pulex* and *Daphnia magna* (*8, 9*). More than 350 tons of CNTs were estimated to have been produced during 2007−2008 (*10*), and increased production is expected in future years. The extent to which the surface chemistries of CNTs affect their ecotoxicity is thus a critical research topic.

A particularly important component of risk assessment is potential for bioaccumulation in organisms. Detection of CNTs in environmentally relevant media previously posed a formidable challenge, but the synthesis of carbon nanotubes radioactively labeled with the carbon-14 isotope has since enabled their quantification in soils, sediments, and organisms (*11*−*15*). While results consistently indicated negligible absorption of CNTs by ecological receptors in terrestrial and sediment ecosystems (*11, 13*−*16*), organism dry mass uptakes of up to 6.8% were recently measured for *D. magna* exposed for 24 h to 400 μg/L of multi-walled carbon nanotubes (MWNTs) (*12*). These *Daphnia* were unable to eliminate MWNTs from their gut tracts in the absence of feeding with algae.

The extent to which the surface chemistries of CNTs influence their accumulation and depuration behaviors, however, remains unknown. Assessment of the impacts of various MWNT surface coatings on their accumulation and elimination behaviors and ecotoxicity in aquatic environments was thus the goal of the study. To accomplish this aim, we tested the accumulation and elimination behaviors and ecotoxicities of MWNTs grafted with polyethyleneimine (PEI), which endows them with positive, negative, or neutral surface charges (*5*), using *Daphnia magna*. *D. magna* have a worldwide distribution, are sensitive to environmental pollution, and filter large volumes of water on a daily basis. They are an established model organism for ecotoxicology testing and have been recently used with various nanomaterials (*7, 9, 12, 17*−*28*). In this study, the toxicity of various PEI-grafted and unmodified MWNTs was assessed using *Daphnia* immobilization tests, and these results were compared to previous in vitro assays that showed substantial cytotoxicity only for positively charged MWNT-PEIs (*5*).

Experimental Methods

Carbon Nanotube Synthesis, Purification, and Characterization. Carbon-14 MWNTs were synthesized and purified as previously described (*12*−*14*). The MWNTs were then treated with HNO_3/H_2SO_4 (v/v = 3:1), filtered, and rinsed with boiling water. These "3:1 MWNTs" were then grafted with PEI as described previously (*5*). Briefly, acyl chloride groups were first introduced to 3:1 MWNTs by extensive reflux

* Corresponding authors phone: (301)-975-8142 (EJP); fax: (301)-975-8505 (EJP), e-mail: Elijah.Petersen@nist.gov (EJP); phone (734) 485-7955) (WJW), e-mail: wjwjr@umich.edu (WJW).
† National Institute of Standards and Technology.
‡ University of Michigan.
§ National Oceanic and Atmospheric Administration.

10.1021/es1030239
Published on Web 12/23/2010

with thionyl chloride. This allowed amide bond formation with PEI in a subsequent reaction in an anhydrous N,N-dimethylformamide (DMF) phase, thus yielding MWNT-PEI. Amine terminals were then reacted with acetic anhydride and succinic anhydride in dimethyl sulfoxide (DMSO) yielding acetylated MWNT-PEI-Ac (Ac denotes acetyl groups) and carboxylated MWNT-PEI-Suc (Suc denotes succinamic acid groups). For each class of PEI-grafted MWNT produced, organic solvent phases, excess reactants, and byproducts were removed by dialysis against deionized water (6 times, 4 L) for 3 days. The purified nanomaterial was either kept in suspension or lyophilized and in both cases stored at 4 °C.

Nanotubes produced by this procedure were thoroughly characterized previously (*5*). ^{1}H NMR spectroscopy qualitatively confirmed the covalent bonding between 3:1 MWNTs and PEI. The zeta potential (mV) for the 3:1 MWNTs, MWNT-PEI, MWNT-PEI-Ac, and MWNT-PEI-Suc were measured as −45.6, 34.6, −0.756, and −20.6, respectively. Transmission electron microscopy (TEM) images revealed that PEI grafting and the surface modifications did not change the morphology of the carbon nanotubes and that aggregation had not occurred in deionized water solutions with PEI-grafted MWNTs. The length distribution of 3:1 MWNTs was previously assessed using scanning electron microscopy, and an average length of 407 nm was determined, although the length distribution was quite broad (*12*). The percentage of the total MWNT composite mass that was attributable to the surface coatings was determined using biological oxidation (OX-500, R. J. Harvey Instruments Company) and comparing the measured radioactivities to those of the 3:1 MWNTs. The percentages were 24 ± 1, 30 ± 2, and 38 ± 4 ($n = 4$; uncertainties always represent standard deviations) for the MWNT-PEI, MWNT-PEI-Ac, and MWNT-PEI-Suc, respectively.

Test Organisms. *Daphnia magna* were cultured in COMBO medium, a freshwater medium that can be used to grow algae and *Daphnia* (21 ± 2 °C, 16:8 h light:dark photoperiod) (*29*). *D. magna* were fed three to five times a week with a culture of green algae *Selenastrum capricornutum* (UTEX 1648) and *Chlamydomonas reinhardtii* (UTEX 90).

Immobilization Test. *Daphnia* neonates (1 to 2 d old) underwent immobilization tests at a range of concentrations (0 to 40 mg/L) for nonradioactive 3:1 MWNTs and for each of the three types of PEI-modified MWNTs (*30*). Immobilization is a common toxicological end point for toxicity to *Daphnia*, tested by mechanically agitating them and observing whether they move; immobilization differs from mortality in that *Daphnia* may still be alive even if they are immobile (*27*). Five replicates of 10 *Daphnia* in 20 mL vials were tested after 24 and 48 h with 3:1 or PEI-coated MWNTs spiked to artificial freshwater ($CaCl_2 \times 2H_2O$ 58.8 mg L^{-1}, $MgSO_4 \times 2H_2O$ 24.7 mg L^{-1}, $NaHCO_3$ 13.0 mg L^{-1}, and KCl 1.2 mg L^{-1}; hardness $[Ca^{2+}]+[Mg^{2+}] = 0.5$ mM). Several additional experiments were performed to verify the validity of these experiments as described in the Supporting Information.

Additionally, solutions of each type of PEI-coated MWNT at the highest concentrations tested were filtered using ashless Whatman cellulose filters (2.5 μm, grade 42), and *Daphnia* were exposed to the filtrate. While the filtrate from the MWNT-PEI-Ac and MWNT-PEI-Suc did not cause immobilization, the filtrate from the MWNT-PEIs caused 18% immobilization. In a secondary study, all three types of MWNT-PEIs were dialyzed, and their toxicity tested as described above. Additional experiments using potassium dichromate were conducted to compare the sensitivity of the *Daphnia* used here to literature results. The PEI polymer by itself was also tested; PEI-Suc and PEI-Ac were not tested as a result of experimental challenges associated with the synthesis, purification, and identification of these polymers in the absence of their covalent bonding to MWNTs prior to dialysis. The percentages of *Daphnia* immobilized after 24 and 48 h of exposure were plotted against test concentrations and the data analyzed by statistical probit method (BioStat 2009, AnalystSoft) to calculate EC_{50} values (i.e., the concentration at which 50% of the *Daphnia* become immobilized) and their 95% confidence limits.

Uptake Experiments. For experiments using 3:1 MWNTs, a 3.0-mg CNT sample was added to a 1 L beaker containing 900 mL of artificial freshwater. 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 the day before the start of an experiment to allow for MWNT settling overnight. This MWNT solution or stock containers with different PEI-modified nanotubes were diluted to yield concentrations of approximately 250 or 25 μg of MWNT nanotubes/L using artificial freshwater. Uptake experiments were designed such that the *Daphnia* were exposed to similar specific radioactivity values for each sample, even though the mass of the nanotube conjugates would vary among the treatments as a result of the additional masses from the PEI coatings. The 25 μg MWNT/L solution was near the detection limits of the scintillation counting of residual ^{14}C beta emissions for the 3:1 MWNTs and was selected given that environmental modeling studies currently predict very low suspended nanotube concentrations in ecosystems (*10, 31*). Before *D. magna* addition, one 3-mL sample was taken from each 100 mL exposure container and mixed with Insta-Gel Plus cocktail (Packard), and the radioactivity was measured by liquid scintillation counting (LSC). Prior to the experiment, *Daphnia* were removed from the primary culture container and transferred to fresh artificial freshwater without algae for at least 1 h to allow for partial gut purging and help the organisms acclimate. Then, 10 organisms were added to each container with MWNTs. Triplicate containers were sampled after 1, 4, 10, 24, and 48 h, and all 10 *Daphnia* were sacrificed. There was no feeding during these experiments. No toxicity patterns were observed for any of the different types of MWNTs under these experimental conditions, and any immobilized *Daphnia* observed were not included in subsequent measurements. At the end of the exposure period, *D. magna* were placed in beakers containing clean water and pipetted vigorously to remove any nanotubes loosely attached to the carapace or appendages. After this procedure, nanotube aggregates were not visible on the exterior of the organisms. *D. magna* were then added to foil boats, dried, weighed, and combusted using biological oxidation. The radioactivity of each sample was measured using LSC. After *D. magna* removal, the aqueous-phase nanotube concentration of each container was measured as described above.

Elimination Experiments. Elimination experiments were conducted in a manner similar to that employed in the uptake experiments. After allowing the *Daphnia* to partially purge their guts for 1 h in clean artificial freshwater, *D. magna* were exposed for 24 h to artificial freshwater spiked with carbon nanotubes at a ratio of 3 mL solution per organism. Then, *Daphnia* were pipetted to clean water to wash them. Triplicate samples of 10 organisms were sampled and their ^{14}C radioactivities determined as described above. The remaining *Daphnia* were transferred either to clean artificial freshwater or to artificial freshwater amended with 1.0×10^8 algae cells/L (*32*). *D. magna* were removed from these media after 1, 4, 24, or 48 h. For the samples taken after 48 h, an additional 1.0×10^8 algae cells/L was added after 24 h. *Daphnia* immobilization was less than 10% for all experiments.

After the 48 h elimination experiment, *Daphnia* masses decreased on average by 27% in the absence of algae, whereas the masses increased on average by 82% when *Daphnia* were fed. The mass changes followed a linear pattern reasonably well; thus, the masses were accordingly adjusted using a linear fit of the rate of mass change for each individual experiment

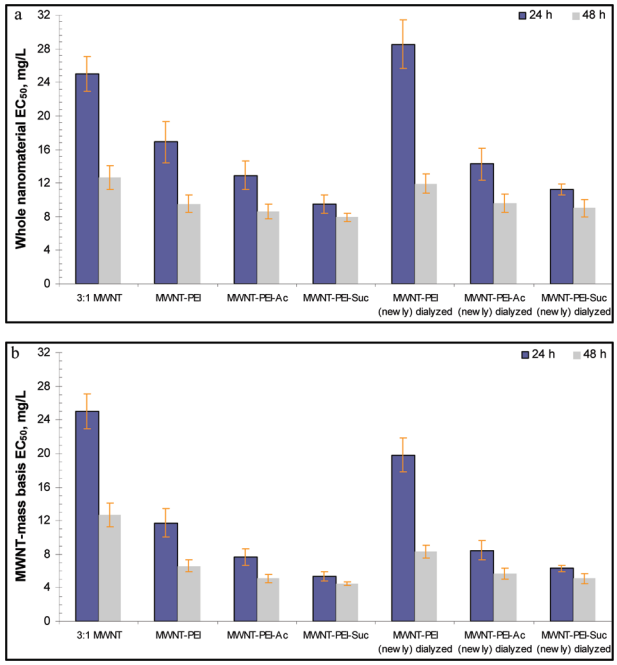

FIGURE 1. EC_{50} values for *Daphnia magna* exposed to regular and PEI-modified MWNTs. Values are given after 24 and 48 h for (a) the whole mass of the MWNT with the PEI coating as indicated by "whole nanomaterial," and (b) on the basis of the MWNT core by itself as indicated by "MWNT-mass basis." Values provided for the MWNTs after they were recently dialyzed are marked "(newly) dialyzed." Error bars represent the 95% confidence intervals.

(*33*). This adjustment is necessary when the rate of mass change is near the rate of change as a result of the elimination processes.

Modeling. Data from uptake experiments were fit to the following first-order accumulation rate model given in eq 1 using nonlinear curve fitting (SAS Institute).

$$C_t = \frac{C_w k_u}{k_e}(1 - e^{-k_e t}) \quad (1)$$

In this equation, C_t is the concentration of the compound in the organism at time t (mg kg^{-1} dry weight), C_w is the initial concentration in the water phase (mg L^{-1}), k_u is the uptake coefficient of the compound from the water phase (L kg^{-1} dry organism mass h^{-1}), k_e is the elimination rate constant of the compound by *Daphnia* (h^{-1}), and t is the time (h). This model assumes relatively constant MWNT concentrations and limited biotransformation so that uptake and elimination mass transfer processes involved can be reasonably described by first-order uptake and elimination rate models and a simple exponential decay between the initial and equilibrium bioaccumulation factors. The aqueous-phase MWNT concentration for all types of MWNTs tested here was found to decrease by 12% on average across all of the conditions over 48 h, thus suggesting that the relatively constant MWNT concentration assumption was fulfilled. Additionally, the average mass decrease during the uptake experiment was only 7.6%; thus, corrections for mass changes were not included.

Results and Discussion

***Daphnia* Immobilization.** The toxicity of the 3:1 and PEI-modified MWNTs to *Daphnia* was investigated by assessing their EC_{50} values as shown in Figure 1 (for the raw data used to calculate the EC_{50} values see Figure S3 of the Supporting Information). The EC_{50} 24 h value for polyethyleneimine itself was 19.3 mg/L (Figure S2 of the Supporting Information), which is similar to that for MWNT-PEI, even though the PEI coating only accounted for 24% of the total nanomaterial mass. The 3:1 MWNTs were the least toxic and yielded a 48 h EC_{50} value of approximately 12.7 mg/L. Previously determined 48 h EC_{50} values for raw MWNTs were 50.9 mg/L and 8.7 mg/L for *Ceriodaphnia dubia* (*34*) and *D. magna* (*27*), respectively. This suggests that *Ceriodaphnia dubia* may be less sensitive to MWNT exposure than *D. magna*. Kennedy and co-workers (*7*) also tested the toxicity of MWNTs with amino functional groups and found 100% mortality after 96 h at a concentration of 2 mg/L. According to these results, MWNT-PEI would be expected to be the most toxic MWNT tested here as a result of their positive surface charge if surface charge is the primary determinant of nanotube toxicity. However, the MWNT-PEIs were found to be less toxic than the other two types of PEI-modified MWNTs on the basis of the mass of the whole composite (Figure 1a) and of the MWNT core by itself (Figure 1b) after 24 h. This implies that the size of the polymer coating the MWNTs plays a larger role in its toxicity than the surface charge, despite previous results, which showed that positively charged dendrimers possessed elevated cytotoxicity as a result of their ability to damage the lipid membrane (*35*).

A second set of experiments with freshly dialyzed MWNTs revealed that the 24 h EC_{50} value of the MWNT-PEI composites increased by 69% after dialysis, but those for the MWNT-PEI-Suc and MWNT-PEI-Ac did not significantly change. This result suggests that there was dissolution of the PEI coatings from the nanotubes to the aqueous phase solution during the 8 month period that the nanotubes remained in solution prior to the finalization of the experiments. While there were large differences for the EC_{50} values after 24 h among the different types of PEI-modified MWNTs, the results were nearly identical after 48 h with the 95% confidence interval for the MWNT-PEI-Ac overlapping with those for MWNT-PEI and MWNT-PEI-Suc. This suggests that varying relative toxicological profiles may be observed after different time points and that the influence of the difference surface coatings and charges was less evident with increased exposure duration. There was still a difference between the 3:1 MWNTs and the PEI-grafted MWNTs after 48 h, which suggests that the presence of a surface coating increased the nanotube toxicity. Given that natural organic matter (NOM) has also been shown to coat the surfaces of CNTs (*6*), NOM coatings may also increase MWNT toxicity. Chronic exposure tests may show an effect from the different charges of the surface coatings if the positively charged coatings interact with the lipid bilayer of the microvilli in the *Daphnia* gut, and such effects may only influence *Daphnia* health after extended time periods.

Uptake Results. Daphnia uptake results indicated that a steady state concentration was usually reached after 10 h for the 250 μg/L exposures and after 24 h for the 25 μg/L exposures (Figure 2). The results are somewhat consistent with data from previous studies on *Daphnia* uptake of MWNTs (0.04, 0.1, and 0.4 mg/L) and fullerenes (0.5 and 2 mg/L), which showed a steady state after 24 h (*12, 23*) and that for TiO_2 which showed steady states after 12 and 24 h for exposure concentrations of 0.08 and 0.52 mg/L, respectively (*24*).

The maximum concentration measured here was 12 $\pm$ 1.1 μg/mg for the MWNTs after exposure to 250 μg/L for 48 h, a value indicating that MWNTs accounted for 1.2% of the total *Daphnia* dry mass. Similarly high nanotube accumulation values have been observed for other nanoparticles such as fullerenes and TiO_2 (*20, 23, 24*). Nevertheless, this value was smaller than a previously measured body burden of 63 $\pm$ 15 μg/mg after exposure to 400 μg/L for 24 h (*12*). The cause of the lower accumulation results measured here is

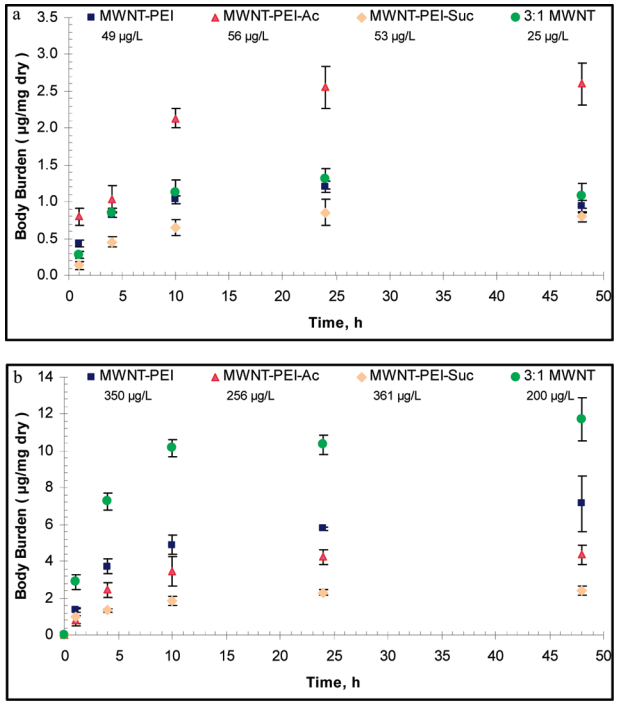

FIGURE 2. MWNT uptake by *Daphnia magna*. The aqueous concentrations ranged from 25 to 53 μg/L in the lower concentration range experiments (a) and from 200 to 361 μg/L in the highest levels tested (b) for 3:1 MWNTs, MWNT-PEI, MWNT-PEI-Ac, and MWNT-PEI-Suc. The initial concentrations vary based on a consistent radioactivity being used among the conditions. Mean and standard deviation values for the body burdens were calculated from triplicate samples.

unclear, but it can be explained in large part as a result of different initial MWNT concentrations and from differences in the *Daphnia* sizes. In the previous study (*12*), the MWNT body burden directly corresponded to the aqueous phase concentration at concentrations of 40, 100, and 400 μg/L. If this pattern was followed here, the *Daphnia* body burden after exposure to 400 μg/L should be twice as large as that for 200 μg/L, but the body burden measured here is comparatively smaller even after this adjustment. While the *Daphnia* used in both studies were 5 to 7 days old, the *Daphnia* in the current experiment were typically fed more frequently, which resulted in body masses four times larger than in the previous study; the *Daphnia* masses after exposure for 1 h to MWNTs in this experiment ranged from 0.0408 (±0.0036) to 0.0612 (±0.0041) mg/per *Daphnia*, while those in the previous experiment ranged from 0.0107 (±0.0006) to 0.0188 (±0.0007) mg/per *Daphnia*. Given that past experiments with carbon nanotubes and fullerenes consistently indicated that the accumulated nanoparticle masses were related to the concentration of carbon nanomaterials in the organism guts and that the relative ratio of the gut volume to the total organism mass is expected to be smaller for larger organisms, the smaller MWNT body burdens in these experiments can be partly explained by the larger *Daphnia* masses. While additional experiments testing the effect of *Daphnia* size on body burdens for nanomaterials are needed to further investigate this hypothesis, comparatively smaller fullerene accumulation was measured for adult *Daphnia* (250 mg/kg wet weight *Daphnia* after exposure to 0.2 mg fullerene/L for 48 h (*22*)) compared to 5 to 7 day old *Daphnia* (4300 ± 700 mg/kg wet weight *Daphnia* after exposure to a 0.5 mg/L solution for 24 h (*23*)). The fullerene suspensions in these two experiments were prepared differently. These differences may have unknown effects on the *Daphnia* body burdens, so this comparison only provides tentative evidence for a *Daphnia* size-related accumulation effect.

No trend was evident among the 3:1 MWNTs and different types of PEI-modified MWNTs with regards to their uptake behaviors on the basis of Figure 2. Exposures to 25 μg/L for 48 h resulted in body burdens for the MWNT-PEI-Ac that were approximately 2.5 times larger than for the other types of MWNTs, which had nearly identical body burdens. Conversely, in 48 h exposures to highest concentrations (≈250 μg/L), the 3:1 MWNTs had the highest body burdens, while the lowest were for the MWNT-PEI-Suc. This shows that the different surface coatings did not cause a systematic trend in body burdens, a result consistent with the hypothesis that MWNT accumulation in *Daphnia* results from gut compaction (*12, 23, 24*). Given that the PEI coatings did not have a systematic effect on the body burdens, CNT wrapping by NOM, a ubiquitous compound in environmental water bodies, would also not be expected to influence MWNT body burdens.

Modeling did reveal, however, some apparent trends in uptake and elimination rates (Table 1). For example, the k_u and k_e values were nearly identical between the two concentrations for the 3:1 MWNTs and the MWNT-PEI-Suc, both of which are negatively charged. However, significant differences in kinetics were observed for the MWNT-PEI and MWNT-PEI-Ac, which have positive and neutral surface charges, respectively. This difference may be a result of different propensities for the nanotubes to aggregate on the basis of their surface charges. Additionally, MWNT-PEI-Suc had much smaller k_u values compared to those of other MWNTs at the lower concentration, but the cause of this difference was unclear.

Elimination Results. The elimination results shown in Figure 3 are largely consistent with those from previous studies with carbon nanoparticles (*12, 23*). As was the case in a previous investigation of MWNT elimination (*12*), elimination data in the presence or absence of algae was not consistently well fit with a first-order exponential decay model (data not shown). These results contrast with elimination behaviors of fullerene which were well fit using a first-order decay model (*23*). A clearer trend may have been observed for MWNTs in this study if there had not been such substantial mass changes. Elimination rate constants were also fit from the uptake data modeling (Table 1), but there were generally no significant differences among the types of nanotubes, and no readily apparent patterns among coefficients between the two concentrations were evident. These elimination coefficients were similar but slightly larger than the values of $0.09 \pm 0.02\ h^{-1}$ and $0.11 \pm 0.02\ h^{-1}$ determined for *Daphnia* exposed to fullerenes at 0.5 and 2 mg/L, respectively (*23*).

There was generally only a minimal change in organism body burdens in the absence of feeding with algae, although for some conditions, there was a decrease during the first hour of elimination. These results contrast to some extent with previous results that did not indicate a decrease in MWNT body burdens in the absence of algae feeding (*12*). In the presence of algae feeding in this study, however, there was nearly complete elimination for the 25 μg/L condition (89 to 99% of the initial body burden) and substantial, but not complete, nanotube removal for the 250 μg/L conditions (63 to 96% of the initial body burden). Similarly, Kennedy et al. microscopically observed nearly complete removal of MWNTs from *Ceriodaphnia dubia* after 24 h elimination with algae but minimal elimination without algae (*34*). The results for the 250 μg/L exposures tested here may have been influenced to some extent by the large *Daphnia* mass increase in the presence of feeding and the larger initial body burdens. These results differ from those by Petersen et al. (*12*) in that elimination was only observed during the first few hours of feeding in that study, while elimination during the full 48 h

TABLE 1. Modeled Uptake and Elimination Coefficients

lower C_w level	k_u[a]	k_u 95% C.I.[b]	k_e	k_e 95% C.I.	R^2
MWNT-PEI	13280 (2060)[c]	9150, 17410	0.442 (0.051)	0.332, 0.552	0.920
MWNT-PEI-Ac	10340 (1730)	6320, 14360	0.162 (0.023)	0.118, 0.206	0.951
MWNT-PEI-Suc	2830 (461)	1090, 2950	0.183 (0.017)	0.069, 0.142	0.943
3:1 MWNT	14730 (2130)	9490, 19970	0.298 (0.049)	0.205, 0.392	0.939
higher C_w level	**k_u**	**k_u 95% C.I.**	**k_e**	**k_e 95% C.I.**	**R^2**
MWNT-PEI	3370 (390)	2030, 4710	0.184 (0.024)	0.079, 0.298	0.911
MWNT-PEI-Ac	2040 (340)	860, 3220	0.196 (0.021)	0.133, 0.259	0.950
MWNT-PEI-Suc	1380 (480)	400, 2370	0.264 (0.053)	0.151, 0.376	0.902
3:1 MWNT	12420 (2690)	6860, 18000	0.275 (0.040)	0.207, 0.342	0.953

[a] These k_u values can be converted to a wet mass basis by taking into account that the wet to dry mass ratio for 5 day old daphnia was 16 ± 1. [b] C.I. refers to confidence interval. [c] Data represent the modeled value with the standard error in the parentheses.

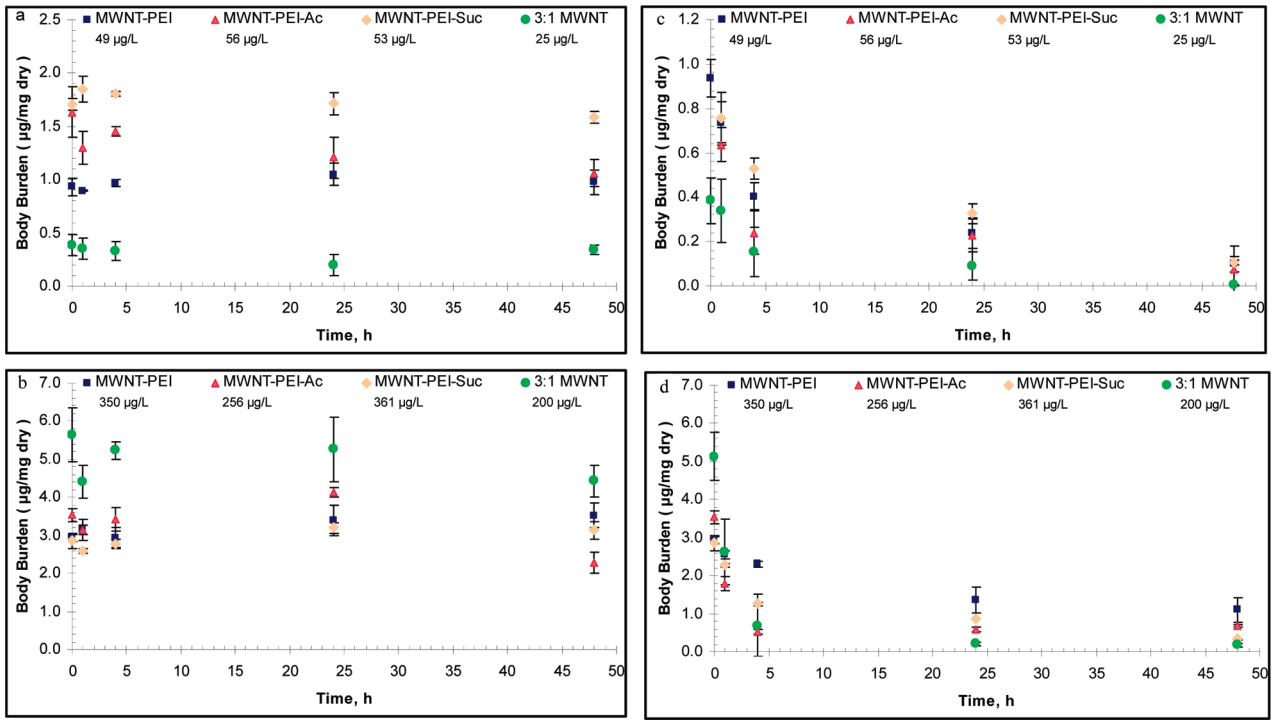

FIGURE 3. MWNT elimination by *Daphnia magna*. The concentration for the 24 h uptake period before elimination was approximately 25 µg/L (a, c) or 250 µg/L (b, d) for 3:1 MWNTs, MWNT-PEI, MWNT-PEI-Ac, and MWNT-PEI-Suc. Elimination occurred either in the absence (a, b) or presence of algae (c, d). Mean and standard deviation values were calculated from triplicate samples.

elimination period was observed here. This outcome may be in part a result of larger and healthier *Daphnia* in this experiment; while *Daphnia* immobilization was observed after 48 h in the previous study, *Daphnia* immobilization was less than 10% in this study. The difference in the immobilization findings between these studies may relate to the larger *Daphnia* size if the lack of feeding during the elimination period during this experiment resulted in mass loss instead of mortality (see Table S1). Similar to the uptake experiments, differences in the elimination rates among the different types of MWNTs was generally not observed. This result suggests that NOM wrapping would not be expected to influence MWNT elimination rates, and that modifications of the surfaces of MWNTs either intentionally for consumer products or through other environmental processes would not be expected to influence their elimination behaviors with *Daphnia magna*.

Environmental Implications. Overall, these results indicate that coating of MWNTs with polymers will increase their toxicity to *Daphnia* as compared to unmodified MWNTs, and that the charge of the coating may not play a significant role in *Daphnia* immobilization. The presence of polymers with different surface coatings did not impact MWNT uptake or elimination rates, and feeding with algae was confirmed to be necessary for elimination. Thus, the availability of food in surface waters may profoundly influence the extent to which MWNTs accumulate in *Daphnia*.

Acknowledgments

This work was supported by awards from the University of Michigan Graham Environmental Sustainability Institute and U.S. EPA grant RD833321. Although research described in this article was funded in part by the U.S. EPA, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the

materials or equipment identified are necessarily the best available for the purpose.

Supporting Information Available

Detailed plots of the immobilization data used to determine the EC_{50} values for the potassium dichromate, polyethyleneimine, and MWNTs, experiments performed to verify the validity of the immobilization results, and *Daphnia* masses during elimination experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Homenick, C. M.; Lawson, G.; Adronov, A. Polymer grafting of carbon nanotubes using living free-radical polymerization. *Polym. Rev.* **2007**, *47* (2), 265–290.

(2) Dalmas, F.; Chazeau, L.; Gauthier, C.; Masenelli-Varlot, K.; Dendievel, R.; Cavaille, J. Y.; Forro, L. Multiwalled carbon nanotube/polymer nanocomposites: Processing and properties. *J. Polym. Sci., Part B: Polym. Phys.* **2005**, *43* (10), 1186–1197.

(3) Bianco, A.; Kostarelos, K.; Partidos, C. D.; Prato, M. Biomedical applications of functionalised carbon nanotubes. *Chem. Commun.* **2005**, (5), 571–577.

(4) Shi, X. Y.; Wang, S. H.; Shen, M. W.; Antwerp, M. E.; Chen, X. S.; Li, C.; Petersen, E. J.; Huang, Q. G.; Weber, W. J., Jr.; Baker, J. R. Multifunctional dendrimer-modified multiwalled carbon nanotubes: Synthesis, characterization, and in vitro cancer cell targeting and imaging. *Biomacromolecules* **2009**, *10* (7), 1744–1750.

(5) Shen, M. W.; Wang, S. H.; Shi, X. Y.; Chen, X. S.; Huang, Q. G.; Petersen, E. J.; Pinto, R. A.; Baker, J. R.; Weber, W. J., Jr. Polyethyleneimine-mediated functionalization of multiwalled carbon nanotubes: Synthesis, characterization, and in vitro toxicity assay. *J. Phys. Chem. C* **2009**, *113* (8), 3150–3156.

(6) Hyung, H.; Fortner, J. D.; Hughes, J. B.; Kim, J. H. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol.* **2007**, *41* (1), 179–184.

(7) Kennedy, A. J.; Gunter, J. C.; Chappell, M. A.; Goss, J. D.; Hull, M. S.; Kirgan, R. A.; Steevens, J. A. Influence of nanotube preparation in aquatic bioassays. *Environ. Toxicol. Chem.* **2009**, *28* (9), 1930–1938.

(8) Klaper, R.; Crago, J.; Barr, J.; Arndt, D.; Setyowati, K.; Chen, J. Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: Changes in toxicity with functionalization. *Environ. Pollut.* **2009**, *157* (4), 1152–1156.

(9) Lovern, S. B.; Strickler, J. R.; Klaper, R. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C_{60}, and $C_{60}HxC_{70}Hx$). *Environ. Sci. Technol.* **2007**, *41* (12), 4465–4470.

(10) Mueller, N. C.; Nowack, B. Exposure modeling of engineered nanoparticles in the environment. *Environ. Sci. Technol.* **2008**, *42* (12), 4447–4453.

(11) Ferguson, P. L.; Chandler, G. T.; Templeton, R. C.; Demarco, A.; Scrivens, W. A.; Englehart, B. A. Influence of sediment-amendment with single-walled carbon nanotubes and diesel soot on bioaccumulation of hydrophobic organic contaminants by benthic invertebrates. *Environ. Sci. Technol.* **2008**, *42* (10), 3879–3885.

(12) Petersen, E. J.; Akkanen, J.; Kukkonen, J. V. K.; Weber, W. J., Jr. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol.* **2009**, *43* (8), 2969–2975.

(13) Petersen, E. J.; Huang, Q. G.; Weber, W. J., Jr. Bioaccumulation of radio-labeled carbon nanotubes by *Eisenia foetida*. *Environ. Sci. Technol.* **2008**, *42* (8), 3090–3095.

(14) Petersen, E. J.; Huang, Q. G.; Weber, W. J., Jr. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus*. *Environ. Health Perspect.* **2008**, *116* (4), 496–500.

(15) Petersen, E. J.; Huang, Q. G.; Weber, W. J., Jr. Relevance of octanol-water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes. *Environ. Toxicol. Chem.* **2010**, *29* (5), 1106–1112.

(16) Galloway, T.; Lewis, C.; Dolciotti, I.; Johnston, B. D.; Moger, J.; Regoli, F. Sublethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. *Environ. Pollut.* **2010**, *158* (5), 1748–1755.

(17) Heinlaan, M.; Ivask, A.; Blinova, I.; Dubourguier, H. C.; Kahru, A. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere* **2008**, *71* (7), 1308–1316.

(18) Baun, A.; Sorensen, S. N.; Rasmussen, R. F.; Hartmann, N. B.; Koch, C. B. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C_{60}. *Aquat. Toxicol.* **2008**, *86* (3), 379–387.

(19) Lovern, S. B.; Klaper, R. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C_{60}) nanoparticles. *Environ. Toxicol. Chem.* **2006**, *25* (4), 1132–1137.

(20) Oberdörster, E.; Zhu, S. Q.; Blickley, T. M.; McClellan-Green, P.; Haasch, M. L. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C_{60}) on aquatic organisms. *Carbon* **2006**, *44* (6), 1112–1120.

(21) Lovern, S. B.; Owen, H. A.; Klaper, R. Electron microscopy of gold nanoparticle intake in the gut of *Daphnia magna*. *Nanotoxicology* **2008**, *2* (1), 43–48.

(22) Tao, X. J.; Fortner, J. D.; Zhang, B.; He, Y. H.; Chen, Y. S.; Hughes, J. B. Effects of aqueous stable fullerene nanocrystals (nC_{60}) on *Daphnia magna*: Evaluation of sub-lethal reproductive responses and accumulation. *Chemosphere* **2009**, *77* (11), 1482–1487.

(23) Tervonen, K.; Waissi, G.; Petersen, E. J.; Akkanen, J.; Kukkonen, J. V. K. Analysis of fullerene-C_{60} and kinetic measurements for its accumulation and depuration in *Daphnia magna*. *Environ. Toxicol. Chem.* **2010**, *29* (5), 1072–1078.

(24) Zhu, X. S.; Chang, Y.; Chen, Y. S. Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*. *Chemosphere* **2010**, *78* (3), V–215.

(25) Kim, K. T.; Edgington, A. J.; Klaine, S. J.; Cho, J. W.; Kim, S. D. Influence of multiwalled carbon nanotubes dispersed in natural organic matter on speciation and bioavailability of copper. *Environ. Sci. Technol.* **2009**, *43* (23), 8979–8984.

(26) Roberts, A. P.; Mount, A. S.; Seda, B.; Souther, J.; Qiao, R.; Lin, S.; Ke, P.; Rao, A. M.; Klaine, S. J. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol.* **2007**, *41* (8), 3025–3029.

(27) Zhu, X. S.; Zhu, L.; Chen, Y. S.; Tian, S. Y. Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. *J. Nanopart. Res.* **2009**, *11* (1), 67–75.

(28) Lewinski, N. A.; Zhu, H. G.; Jo, H. J.; Pham, D.; Kamath, R. R.; Ouyang, C. R.; Vulpe, C. D.; Colvin, V. L.; Drezek, R. A. Quantification of water solubilized CdSe/ZnS quantum dots in *Daphnia magna*. *Environ. Sci. Technol.* **2010**, *44* (5), 1841–1846.

(29) Kilham, S. S.; Kreeger, D. A.; Lynn, S. G.; Goulden, C. E.; Herrera, L. COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* **1998**, *377*, 147–159.

(30) *Daphnia* sp. Acute Immobilisation Test; OECD Guideline 202; Organization for Economic Cooperation and Development (OECD): Paris, 2004.

(31) Gottschalk, F.; Sonderer, T.; Scholz, R. W.; Nowack, B. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environ. Sci. Technol.* **2009**, *43* (24), 9216–9222.

(32) Standard Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests; E1193-97; American Society for Testing and Materials (ASTM): West Conshohocken, PA, 2004.

(33) Lydy, M. J.; Lasater, J. L.; Landrum, P. F. Toxicokinetics of DDE and 2-Chlorobiphenyl in *Chironomus tentans*. *Arch. Environ. Contam. Toxicol.* **2000**, *38*, 163–168.

(34) Kennedy, A. J. H., M. S.; Steevens, J. A.; Dontsova, K. M.; Chappell, M. A.; Gunter, J. C.; Weiss, C. A., Jr. Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. *Environ. Toxicol. Chem.* **2008**, *27* (9), 1932–1941.

(35) Hong, S. P.; Leroueil, P. R.; Janus, E. K.; Peters, J. L.; Kober, M. M.; Islam, M. T.; Orr, B. G.; Baker, J. R.; Holl, M. M. B. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. *Bioconjugate Chem.* **2006**, *17* (3), 728–734.

ES1030239