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Core Structure and Surface Functionalization of Carbon Nanomaterials Alter Impacts to Daphnid Mortality, Reproduction, and Growth: Acute Assays Do Not Predict Chronic Exposure Impacts

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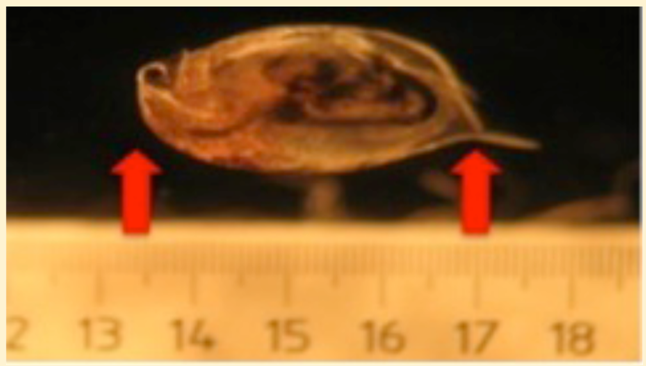
Supporting Information

ABSTRACT: There are currently over ninety products incorporating carbon nanomaterials (CNMs) on the market today for a variety of applications. Modifications in core structure and surface chemistry of manufactured nanomaterials are used to optimize nanomaterials for specific uses. However, there is a notable lack of information on how core structure and surface chemistry may alter toxicity in low-level, chronic exposures. This paper examines the effects of twelve CNMs that differ in their core structure and surface chemistry to *Daphnia magna* over a 21-day chronic exposure. Overall, nanomaterials with a carbon nanotube core were more toxic to daphnids than fullerenes, with the one exception of fullerenes with a gamma-cyclodextrin surface chemistry. Acute mortality was not a good predictor of chronic effects as none of the CNMs induced toxicity at tested concentrations after 48 h, yet chronic assays indicated significant differences in mortality, reproduction, and growth realized after 21 days. Our results indicate that (1) acute exposure assays do not accurately describe the impact of CNMs to biological systems, (2) chronic exposures provide valuable information that indicates the potential for different modes of action for nanomaterials of differing chemistries, and (3) core structure and surface chemistry both influence particle toxicity.



INTRODUCTION

Nanomaterials exhibit unique physical and chemical properties that make them valuable additions to various products including medicines, polymer composites, and electronics.[1−3] However, the benefit of enhanced physical and chemical activity of nanomaterials is also cause for concern as nanomaterials may be released into aquatic environments and also exhibit increased reactivity there. The novel, size-related reactivity of nanomaterials complicates the ability to predict or change nanomaterial toxicity.[4] Carbon nanomaterials (CNMs) are of specific interest, as they are engineered with a wide variety of core structures and surface functionalizations that change their chemical and physical properties to enhance their suitability for several industrial applications.[5,6] Despite the large array of available CNM configurations, research to date has focused on the toxicity of a limited variety of CNM types,[7−9] and much of the data are not comparable across toxicity experiments due to variations in organisms and experimental conditions.[10,11]

Nanomaterial toxicity has been attributed to core structure and surface functionalization, and variations in these factors have been shown to alter the level of toxicity to biological systems.[10] Existing studies find a wide range of toxicities depending on particle type and experimental set up. As an example, single-walled carbon nanotubes (SWCNTs) induce significant cytotoxicity to alveolar macrophages at a dose of 11.3 $\mu g/cm^2$, while fullerenes were cytotoxic at 226 $\mu g/cm^2$ in this experiment.[12] Surface chemistry such as hydroxylation has been shown to alter the mechanism of particle cytotoxicity, as unfunctionalized fullerenes induce reactive oxygen species (ROS) dependent membrane damage and necrosis, while hydroxylated fullerenes are associated with ROS-independent mechanisms of cell death, apoptosis, and DNA fragmentation.[13] Variations in core structure and functionalization have also been shown to impact nanomaterial toxicity in vivo, as 48-h LC_{50} values for SWCNTs indicated much higher toxicity to daphnids compared to MWCNTs and C60,[14] and oxidative stress biomarkers were differentially activated in daphnids after exposure to nanomaterials that varied in core structure and functionalization.[15] Although literature indicates the impor-

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tance of core structure and functionalization for evaluating nanomaterial toxicity, many experiments focus on the investigation of the acute toxicity of a few types of nanomaterials to organisms or cell cultures.

Aquatic organisms have a particular risk of exposure as many chemicals used within consumer products often end up in household wastewater and pass into receiving rivers and lakes. Data already indicate the presence of manufactured nanomaterials in wastewater effluent entering natural environments for C60 fullerenes and *N*-methylfulleropyrrolidine at concentrations as high as 65 ppb,[16] and titanium dioxide nanoparticles at concentrations varying from 5 to 15 ppb.[17] Yet, like other emerging contaminants, these particle types are not acutely toxic at levels found in the environment.

The real impact of many emerging contaminants may be due to sublethal toxicity over a chronic exposure. Most nanotoxicology studies to date are acute, high-dose exposure studies, and do not provide information as to whether nanomaterials have an impact at a lower dose over a chronic exposure or whether acute toxicity information predicts effects in this type of more realistic scenario. In addition, this type of study can provide a better assessment of mechanisms of CNM toxicity. This project investigates the effects of chronic exposures of *Daphnia magna* to twelve types of CNMs that differ in their core structure and surface functionalization. The goal of this research is to generate detailed and comparative nanotoxicity profiles for a variety of core structures and functionalizations within one study to determine whether nanomaterials that differ in their core structure or functionalization also differ in their chronic toxicological impacts. In addition, this project seeks to determine whether acute assays accurately predict the effects of chronic nanomaterial exposure. In this research, we measure the impacts of chronic nanomaterial exposure on multiple end points, including reproduction and adult size, in the aquatic toxicology and ecological model organism, *Daphnia magna*.

■ MATERIALS AND METHODS

Nanomaterial Preparation and Characterization. Twelve nanomaterials that differed in core structure and surface functionalization were used for these experiments, and were obtained through synthesis or from a manufacturer (see Supporting Information (SI) Table S1 for more information).

Synthesized CNMs. Six fullerenes with various derivatives were synthesized at the University of Wisconsin-Milwaukee (Figure S1). A detailed description of synthesis and purification is included in the SI (Figures S2 and S3).

- Derivative **1** (C60-βCD): supramolecular complex of C60 with β-cyclodextrin (βCD). C60 (100 mg, 0.139 mmol) and βCD (660 mg, 0.582 mmol) were ground in an agate mortar for 1 h to give a uniform brown powder. The resulting powder was then dissolved in 1 L of deionized water followed by 1 h of bath sonication.
- Derivative **2** (C60-amino): amino-substituted methanofullerene derivative. Synthesized and characterized according to literature.[18−21] Derivative **2** (50.0 mg, 0.045 mmol) was dissolved in 1 L of deionized water followed by 1 h bath sonication.
- Derivative **3** (C60-amino-γCD): supramolecular complex of amino-substituted methanofullerene derivative with γCD. Derivative **2** (50.0 mg, 0.045 mmol) and γCD (58.8 mg, 0.060 mmol) were ground in an agate mortar for 1 h to give a uniform brown powder. The resulting powder was then dissolved in 1 L of deionized water followed by 1 h of bath sonication.
- Derivative **4** (C_{60}-malonic acid): malonic acid derivative of C60, synthesized and characterized according to literature.[18−21]
- Derivative **5** (C_{60}-malonate): disodium malonate derivative of C60. Derivative **4** (50.0 mg, 0.061 mmol) was dissolved in 0.01 M NaOH (6.1 mL) and then diluted to 1 L in deionized water followed by 1 h of bath sonication.
- Derivative **6** (C60-malonate-γCD): supramolecular complex of disodium malonate derivative of C60 with γCD. Derivative **4** (50.0 mg, 0.061 mmol) and γCD (78.9 mg, 0.081 mmol) were ground in an agate mortar for 1 h to give a uniform brown powder. The resulting powder was then dissolved in 0.01 M NaOH (6.1 mL) and diluted to 1 L, followed by 1 h of bath sonication.

Cyclodextrins were noncovalently bound to fullerenes by van der Waals forces, and the electronic structures of the cyclodextrin-derivatized fullerenes remain basically unchanged from the equivalent fullerenes without cyclodextrin. All reagents and solvents were used as received unless otherwise noted.

Purchased CNMs. Seven types of nanomaterial powders were obtained from five manufacturers and suspended in milli-Q water. C60 was suspended in milli-Q water by stirring for 2 weeks at 600 rpm. The remaining particle types were suspended in milli-Q water by sonication for 2 h in a water bath. All particle types were sonicated for 5 min before use in exposures. These include fullerenes (C60), hydroxylated fullerenes (C60-OH_{24}), single-walled carbon nanotubes (SWCNTs), carboxylic acid functionalized SWCNTs (SWCNT-COOH), carboxy-amide functionalized SWCNTs (SWCNT-$CONH_2$), polyethylene glycol functionalized SWCNTs (SWCNT-PEG), and multiwalled carbon nanotubes (MWCNTs). Suspension methods did not involve any additional solvents or surfactants as these are known to affect the way the particles interact with biological systems.[11] SWCNTs were prepared by the electronic arc discharge method, and carbonaceous purity information is included in Table S1.

Nanomaterial Characterization. All particle suspensions were characterized using transmission electron microscopy (TEM) to evaluate aggregate size distribution, dynamic light scattering (DLS) with a Zetasizer from Malvern Instruments (Worcestershire, UK) to evaluate suspension stability, and particle tracking with a Nanosight (Wiltshire, UK) to evaluate particle aggregate size distribution in real time within our media. DLS was conducted in milli-Q water and MHRW to measure stability in both types of medium. Inductively coupled plasma mass spectroscopy (ICPMS) was performed by Pace Analytical (St. Rose, LA) to evaluate metal catalyst residue in the stock suspensions. Samples underwent an acid digestion process prior to measurement by ICPMS.[22]

Daphnia Cultures and Toxicity Assays. *Daphnia magna* were obtained from a culture in the Klaper laboratory and maintained with a 16:8 light/dark cycle at 20 °C according to the OECD Guidelines for Testing of Chemicals (1998).[23] They were kept in moderately hard reconstituted water (MHRW)[24] and fed a combination of freshwater algae (*Selenastrum capricornutum*) and alfalfa (*Medicago sativa*). Fluorescent lights that emit in the visible spectrum were used for stock cultures and experiments.

D. magna were exposed to 0, 10, or 50 ppm concentrations of carbon nanomaterials that were purchased directly from a manufacturer, and to 0, 1, and 5 ppm concentrations of particle types that were synthesized on campus (derivatives **1**−**6**), due to limitations in the quantity of nanomaterials. Concentrations below 50 ppm were chosen because they were determined to be sublethal based on a series of LC50 values that were calculated from acute exposures of *Daphnia* to similar nanomaterials in previous work in our laboratory.[15] Exposures were 21 days with static renewal, where neonates were placed in either 100 mL of control (MHRW only) or nanoparticles (0−50 ppm in MHRW), and medium was changed out three times per week. Mortality and reproductive output were measured during the suspension changes. Daphnid size was measured as the length of the daphnid from the top of the head to the base of the apical spine at day 21.

Additional controls were conducted to determine any impacts from the catalyst used to create the carbon nanotubes. A sample of the catalyst was obtained from Carbon Solutions Inc. and suspended in milli-Q water by sonication for 2 h in a water bath to replicate the conditions of SWCNT suspensions. Concentrations of the catalyst exposures were designed to replicate the highest concentration nickel exposure the daphnids received from nanomaterial experiments (184 ppb Ni from 50 ppm unfunctionalized SWCNT exposures). Free ligand control experiments were also conducted with βCD and γCD, and these ligands were suspended into media in the same manner and at the same concentrations as those found in the nanoparticle suspensions. No chronic toxicity (reproduction, growth, or mortality) was associated with any of the catalyst, βCD, or γCD exposures. Additionally, PEG functional attachments have been previously shown to be nontoxic to organisms.[25,26]

Experiments were optimized to meet the mortality and reproduction requirements outlined by the OECD Guidelines for the Testing of Chemicals.[23] Modifications were made to the exposures to compensate for variation introduced by population density by removal of proportionate volumes of medium and food from the exposures as mortality occurred. Daphnids were kept at a concentration of one daphnid per 20 mL of medium with a food concentration of 400 000 algal cells/mL medium. Total reproductive output was calculated for the number of surviving individuals at the time of measurement and then reported as the average number of neonates produced per surviving individual.

Statistical Analysis. Reproduction and growth measurements were normalized to the total control average to adjust for variation in these parameters over the time period of the experiment. Not all data adhered to assumptions of normality for independent *t* test analysis, so the effects of nanomaterials on daphnid mortality, reproduction, and adult size were compared to controls by nonparametric Mann−Whitney *U* tests for two-independent samples. Values were determined to be significant at $p < 0.05$.

RESULTS

Tables that summarize the nanomaterial characterization and toxicity assay results are provided in the SI.

Nanomaterial Characterization. Using TEM, DLS, and Nanosight analysis, fullerene suspensions were shown to be polydispersed with the presence of many particles less than 100 nm in diameter in the suspensions. Average hydrodynamic diameters for the fullerene suspensions ranged from 105 to 175 nm (Table S2). Reliable aggregate size measurements could not be obtained for nanotube suspensions. However, TEM images showed the presence of individual nanotubes, as well as aggregates of nanotubes larger than one micrometer in the suspensions (Figure S4). SWCNT, MWCNT, and C60-amino were not stable in suspensions with milli-Q water (zeta potential < |30| mV), while the rest of the functionalized fullerenes and nanotubes were stable in milli-Q water (zeta potential > |30| mV). Stability decreased when the nanomaterials were added to MHRW (Table S3); however, the stability of nanomaterials in MHRW-only is not representative of particle stability in the exposure medium, as the presence of algae and alfalfa significantly improved the stability of nanomaterials in suspension.

ICPMS analysis indicated the presence of metals in some of the particle suspensions (Table S1). Fullerenes were synthesized with an iron catalyst, and ICPMS indicated the presence of iron, strontium, and copper in the stock suspensions. Carbon nanotubes were synthesized with a nickel/yttrium catalyst, and ICPMS indicated the presence of nickel in nanotube stock suspensions between 60 and 368 ppb. This was not surprising, as the manufacturer reported the presence of metals in their nanotubes determined by thermogravimetric analysis (4−8% for SWCNTs, 5−8% for SWCNT-COOH, 3−6% for SWCNT-PEG, and 5−8% for SWCNT-CONH$_2$). In addition, supernatant from unfunctionalized SWCNT and MWCNT was examined by ICPMS, and concentrations of nickel were found in supernatant samples, indicating the release of nickel ions into the medium. However, concentrations of nickel in SWCNT and MWCNT supernatant samples were significantly lower (73.3 and 15.0 ppb Ni) than concentrations from the nanomaterial suspensions themselves. A catalyst-only control experiment showed no significant changes to daphnid mortality, reproduction, or size in response to catalyst exposures of 200 ppb nickel. However, it is possible that a synergistic action of the combination of nickel and nanotube exposures could enhance toxicity of nanomaterials to *Daphnia*, but this has yet to be shown.

Impact of Carbon Nanomaterials on Acute and Chronic Mortality. No significant acute mortality (< 48 h) was observed for any of the particle types at the tested concentrations, as doses were chosen to be below LC50 values calculated from acute exposures of a subset of CNMs.[15,27] Differential mortality was observed for some particle types after an extended exposure period. C60-amino-γCD and C60-malonate-γCD induced significant mortality to daphnids (60% mortality, $U = 13.5$, $p < 0.05$; and 55% mortality, $U = 0$, $p < 0.05$) at 5 ppm after 7 and 10 days (Figure 1). In contrast, γCD controls induced no mortality and the equivalent exposures of particle types without γCD (C60-amino and C60-malonate) induced no mortality, indicating a toxic action specifically associated with fullerenes bound to γCD. When lowered to 1 ppm, C60-amino-γCD exposure induced significant mortality to daphnids after 19 days ($U = 23.5$, $p < 0.05$), while C60-malonate-γCD induced no significant mortality after 21 days. Of the carbon nanotubes, only 50 ppm MWCNTs induced significant mortality to daphnids (20% mortality after 19 days, $U = 70.5$, $p < 0.05$). None of the other treatments induced significant mortality to daphnids.

Impact of Carbon Nanomaterials on Reproduction. Reproduction over time was significantly impacted by SWCNT

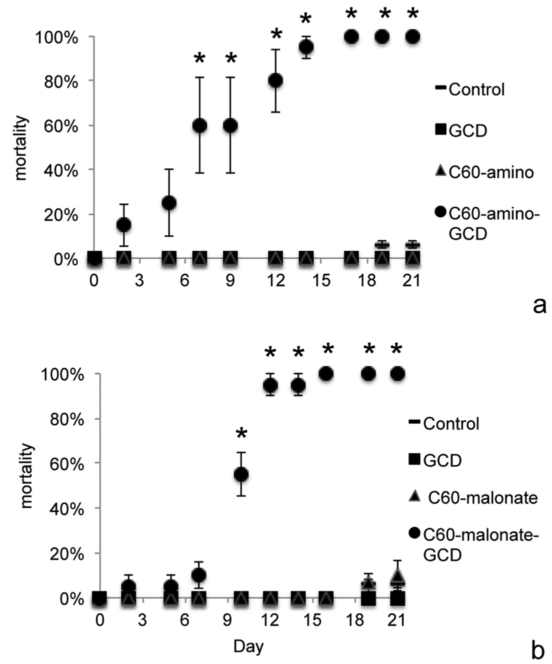

Figure 1. Impacts of γCD-bound fullerene derivatives to chronic daphnid mortality. Mortality evaluated by the Mann−Whitney *U* test for two independent samples. Data are significant at $p < 0.05$ and stars indicate significant difference compared to control. GCD indicates gamma cyclodextrin. (A) Daphnid mortality after chronic exposure to 5 ppm C60-amino and C60-amino-GCD. (B) Daphnid mortality after chronic exposure to 5 ppm C60-malonate and C60-malonate-GCD.

treatments compared to reproduction in control daphnids (Figure 2a). After 9 days of exposure, an average of 7.06 neonates were produced per control daphnid. However, during this timeframe significantly fewer neonates were produced by daphnids exposed to 50 ppm concentrations of SWCNT (2.23 neonates per daphnid, $U = 0$, $p < 0.05$), SWCNT-COOH (zero neonates per daphnid, $U = 0$, $p < 0.05$), and SWCNT-PEG (0.18 neonates per daphnid, $U = 7.5$, $p < 0.05$). Fullerene-based particles and MWCNTs did not show any differences in reproduction compared to controls after 9 days of exposure.

At 21 days, several types of CNMs altered reproductive output in daphnids, and core structure was an important parameter that influenced daphnid reproduction (Figure 2b). All of the unfunctionalized CNMs (C60, SWCNT, MWCNT) significantly lowered reproduction at 50 ppm compared to control daphnids (reduction of 11%, $U = 0$, $p < 0.05$; reduction of 46.5%, $U = 0$, $p < 0.05$; reduction of 35.4%, $U = 25$, $p < 0.05$). In addition, C60 was significantly less toxic to daphnid reproduction compared to carbon nanotubes. Daphnids from 50 ppm C60 produced an average of 62.58 neonates. In contrast, daphnids from 50 ppm SWCNT and MWCNT exposures produced 24.9 and 17.1 neonates ($U = 6$, $p < 0.05$; $U = 15$, $p < 0.05$). Differences in reproduction relative to core structure were not significant at concentrations below 50 ppm ($p > 0.05$).

Fullerene functionalization increased or decreased reproduction in *Daphnia* depending on concentration and the type of functional attachment (Figure 2c). While unfunctionalized fullerenes significantly reduced reproduction by 11%, daphnids from 50 ppm C60-OH exposures did not exhibit any changes in reproduction compared to controls ($p > 0.05$), indicating that functionalization with hydroxyl groups decreases the toxicity of fullerenes to daphnid reproduction. While C60 did not significantly alter daphnid reproduction compared to controls below 50 ppm, exposure of daphnids to 5 ppm C60 malonate

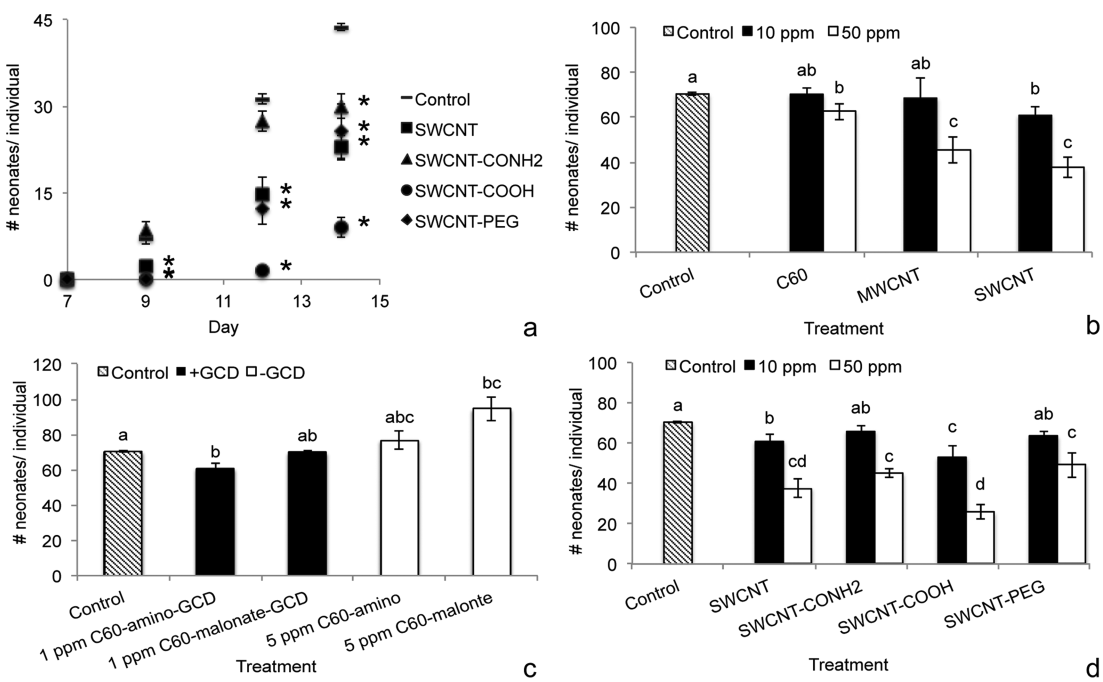

Figure 2. Impacts of CNM exposure on daphnid reproduction. Reproduction evaluated by Mann−Whitney *U* test for two independent samples. Data are significant at $p < 0.05$. Stars indicate significant difference from control. Letters indicate significant difference among treatments and controls. (A) Impact of 50 ppm SWCNT, SWCNT-$CONH_2$, SWCNT-COOH, and SWCNT-PEG over time. (B) Impact of unfunctionalized CNMs on reproduction at 21 days. (C) Impact of derivatized fullerenes on daphnid reproduction at 21 days. (D) Impact of unfunctionalized and functionalized SWCNTs on daphnid reproduction at 21 days.

and C60-amino-γCD significantly changed reproduction compared to controls. Daphnids from 5 ppm C60-malonate exposures increased reproduction by 25.5% compared to control daphnids (U = 9.5, p < 0.05). Reproduction could not be evaluated in equivalent particle types C60-malonate-γCD and C60-amino-γCD at 5 ppm because the exposure induced 100% mortality to daphnids after 17 days. However, daphnids from 1 ppm C60-amino-γCD exposures produced 13.7% fewer neonates compared to controls (U = 6, p < 0.05).

Functionalized SWCNTs also influenced toxicity to daphnid reproduction (Figure 2d). Significant decreases in reproduction were observed in daphnids exposed to 10 and 50 ppm SWCNT-COOH (reduction of 24.6%, U = 1, p < 0.05; 58.7%, U = 0, p < 0.05) compared to control daphnids. Daphnids exposed to SWCNT-$CONH_2$ and SWCNT-PEG significantly reduced reproduction only at 50 ppm compared to controls (reduction of 35.9%, U = 0, p < 0.05; 30.3%, U = 25, p < 0.05). A comparison of reproduction in daphnids from unfunctionalized SWCNTs to functionalized SWCNTs yielded no significant differences in reproduction; however, daphnids from SWCNT-$CONH_2$ and SWCNT-PEG produced 55.0% and 68.7% more neonates than daphnids from 50 ppm SWCNT-COOH exposures (U = 1, p < 0.05; U = 4, p < 0.05). This indicates that $CONH_2$ and PEG functional attachments make SWCNTs less toxic to daphnid reproduction than COOH attachments.

Impact of Carbon Nanomaterials on Adult Daphnid Size. Exposure of daphnids to 50 ppm C60, SWCNT, and MWCNT reduced daphnid size by 6.6%, 12.4%, and 8.2% compared to controls (U = 32, p < 0.05; U = 0, p < 0.05; U = 19, p < 0.05) (Figure 3a). Daphnid size was also significantly decreased in 10 ppm SWCNT and MWCNT exposures (reduction of 7.9%, U = 9, p < 0.05; reduction of 4.6%, U = 36, p < 0.05). C60 did not significantly alter daphnid size compared to controls at 10 ppm. Daphnids exposed to 50 ppm C60 were 6.6% larger than daphnids exposed to 50 ppm SWCNTs (U = 7, p < 0.05).

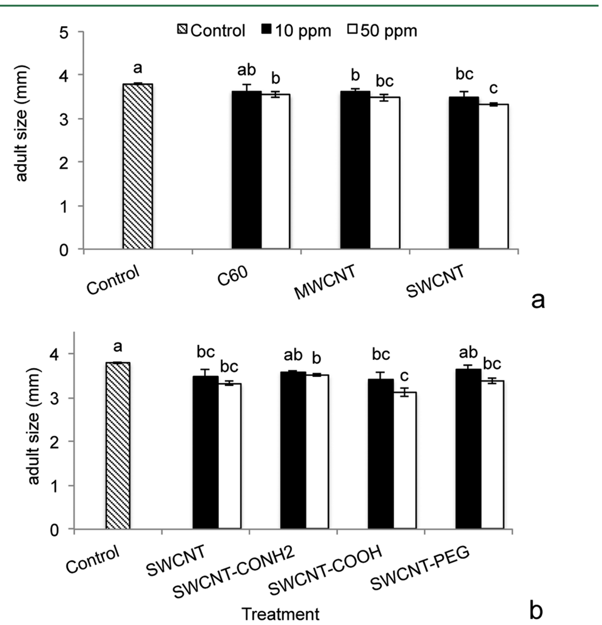

Figure 3. Impacts of CNM exposure on adult daphnid size. Adult size evaluated by Mann−Whitney U test for two independent samples. Data are significant at p < 0.05. Letters indicate significant difference among treatments and controls. (A) Impact of unfunctionalized CNMs on adult daphnid size at 21 days. (B) Impact of unfunctionalized and functionalized SWCNTs on adult daphnid size at 21 days.

Fullerene functionalization influenced daphnid size, depending on the type of functionalization and concentration. Daphnid size in 50 ppm C60-OH treatments was not significantly different from controls (p > 0.05), indicating that functionalization of C60 with hydroxyl groups can mitigate the toxicity of C60 to daphnids. Daphnids exposed to 5 ppm C60-amino and C60-malonate were 3.4% and 5.5% smaller in size than control daphnids (U = 8, p < 0.05; U = 12, p < 0.05). No other fullerene particle types induced changes to adult daphnid size.

Functionalization influenced the toxicity of SWCNTs to adult daphnid size (Figure 3b). Daphnids exposed to 50 ppm SWCNT, SWCNT-$CONH_2$, SWCNT-COOH, and SWCNT-PEG were 12.4%, 7.4%, 17.7%, and 10.2% smaller in size than control daphnids (U = 0, p < 0.05; U = 0, p < 0.05; U = 0, p < 0.05; U = 0, p < 0.05). Daphnids exposed to 10 ppm SWCNT and SWCNT-COOH also exhibited significant decreases in size compared to controls (reduction in size of 7.9%, U = 9, p < 0.05; reduction in size of 10.0%, U = 0, p < 0.05). When the size of daphnids from functionalized SWCNT expsoures (SWCNT-$CONH_2$, SWCNT-COOH, SWCNT-PEG) was compared to the size of daphnids from unfunctionalized SWCNTs, no significant differences were observed. However, daphnids from 50 ppm SWCNT-COOH exposures were 12.3% smaller in size than daphnids from 50 ppm SWCNT-$CONH_2$ exposures (U = 0, p < 0.05), indicating that SWCNT-COOH is more toxic to daphnid size than SWCNT-$CONH_2$.

■ DISCUSSION

Acute Assays Do Not Predict Chronic Impacts of CNM Exposure. Our data indicate that acute assays are not sufficient for predicting chronic toxicity of CNMs. None of the CNMs in this investigation exhibited significant toxicity to daphnids over an acute period, but many impacted chronic mortality, reproduction, and adult size at these concentrations over longer exposure periods. In addition, daphnid responses to nanomaterial treatments varied more over longer exposure periods, indicating that chronic assays may provide a better indication of the differences in the mechanism of toxicity of nanomaterials of differing core structures and surface chemistries. The mechanism of action of nanomaterials may not be accurately captured with acute, high-dose exposures, especially when these materials are modified with coatings, proteins, and functional groups that may act with individual receptors in an organism. Increased sensitivity of chronic toxicity tests with lower exposure concentrations is evident in other studies with different toxicants.[28] In our previous studies, sublethal concentrations of fullerenes and nanosized titanium dioxide were found to alter daphnid behavior, another sublethal end point, making daphnids more visible to predators and with possible increases in metabolic costs.[29] These results may be missed by acute toxicity assays and traditional LC50 values.

Core Structure Impacts Nanomaterial Toxicity. Nanomaterial toxicity significantly differed depending on the core structure of the nanomaterial. The unique structuring of carbon in SWCNTs, MWCNTs, and nC60 leads to different aggregate size distributions, surface areas, and physical and chemical properties, which may have an impact on toxicity. Unfunctionalized carbon nanotubes (SWCNT and MWCNT) were more toxic to daphnids than unfunctionalized fullerenes (C60), with more significant impacts to mortality, reproduction, and size. Other studies indicate different levels of toxicities of MWCNTs, but this is likely due to variations in surface chemistry from our MWCNTs[30] and the presence of natural organic matter in one of the studies.[31] Variation in the stability of the suspensions could account for some of the differential toxicity observed for these unfunctionalized CNMs. Zeta potential for the nC60 suspension was −40 mV, indicating that it is more stable in milli-Q water compared to SWCNTs and MWCNTs (+ 23 mV for both). Less stable nanotube suspensions contain larger aggregates, and it is possible that SWCNT and MWCNT suspensions aggregate, and are therefore more difficult for daphnids to eliminate,[30] as opposed to smaller particulates or aggregates, which have been shown to be eliminated within 24 h.[31] Nanomaterials may also interfere with feeding mechanics. A study on the impacts of suspended clay particles less than 2 μm in diameter on *Daphnia magna* indicated that ingested clay particles reduce the beating rate of the feeding appendage and interfere with the uptake of algae by *Daphnia*, thereby reducing fitness of the daphnids by decreasing ingestion of algae.[32] Although unfunctionalized carbon nanotubes used in these suspensions had lengths over 5 μm and large aggregates (>1 μm diameter) in the suspensions that are readily seen within the gut of the *Daphnia*. It is possible that unfunctionalized carbon nanotubes physically interfere with uptake and feeding of algae by *Daphnia*.

Effects of Variations in Surface Chemistry. Surface chemistry impacted both fullerene and carbon nanotube toxicity, and either increased or decreased toxicity of these particle types depending on the functional attachment. The type of functionalization had a significant impact on the toxicity of SWCNTs. Daphnids exposed to SWCNT-COOH produced significantly fewer neonates at lower concentrations than daphnids exposed to SWCNT-CONH$_2$ or SWCNT-PEG. This indicates that carboxylation of SWCNTs increases their toxicity to daphnids, while functionalization with CONH$_2$ or PEG decreases SWCNT toxicity to daphnids. Although all three functionalizations improve the dispersibility and stability of SWCNTs in biological media, carboxylated SWCNTs are shown to have more amorphous carbon fragments as a result of increased oxidation of carbon, and these amorphous fragments can induce higher levels of toxicity to biological systems.[33] SWCNT-COOH have been shown to inactivate bacteria[34] (a potential food source for daphnids)[35] and produce reactive oxygen species.[36] In contrast, SWCNT-CONH$_2$ and SWCNT-PEG have previously been shown to be less toxic in general.[26,37]

Fullerene toxicity also varied with surface functionalization and bonding chemistry. Unfunctionalized fullerenes decreased reproduction and growth at a concentration of 50 ppm. Hydroxylation of fullerenes improved this outcome, as no significant effects to reproduction or growth were observed compared to controls at the same concentration. Fullerene hydroxylation has been previously shown to decrease fullerene toxicity in vitro[13] and in vivo,[38] and these results are in agreement with the decreased toxicity observed with daphnids exposed to C60-OH in this study. In contrast, fullerenes with disodium malonate (C60-malonate) significantly increased daphnid reproduction at 5 ppm compared to control daphnids, indicating a positive effect of this nanoparticle type on daphnid reproduction. Literature indicates the ability of C60 malonate derivatives to improve antioxidant enzymatic activity in microglia after insult with LPS,[39] and these malonate-derived fullerenes have potential applications in medicine.[40]

Although carbon nanotubes as a whole induced more consistent decreases in reproduction and size in daphnids, fullerene derivatives that were attached to γCD by noncovalent bonds were the most toxic particle types that were investigated in this experiment. C60-amino-γCD and C60-malonate-γCD induced 100% mortality to daphnids at a concentration as low as 5 ppm. Previous research on cyclodextrins highlights their ability to increase the bioavailability of insoluble substrates, and they are used as drug solubilizers and carrier systems.[41] It is possible that γCD bound fullerene derivatives are more bioavailable to daphnids compared to fullerene derivatives that are not bound to γCD, thereby increasing the toxicity of γCD bound fullerene derivatives.

Effects of Particle Size, Charge, and Potential By-products. Much of the toxicity that was observed in this experiment did not reveal any patterns regarding aggregate size and suspension stability. Nanomaterial size has been shown to be important for toxicity of TiO$_2$ nanoparticles,[42] and previous work in our laboratory also indicated increased mortality of daphnids exposed to smaller size fractions of TiO$_2$ nanoparticles and fullerenes.[27] Surface charge has been shown to significantly influence the mechanism of cytotoxicity of gold nanoparticles[43] and of in vivo toxicity of quantum dots.[44] In addition, characteristics of nanomaterials, such as size, surface charge, and functionalization, can influence suspension stability and aggregation state, with further implications for nanomaterial transport and fate.[45,46] Our experimental design specifically avoided the use of surfactants and dispersants because these chemicals can change how the nanomaterials interact with biological systems. As a consequence, our suspensions were polydisperse with a wide range of aggregate sizes and particle stabilities. Despite the settling of some particles out of suspension, daphnids are continuously exposed to the particles for the duration of the experiment. Daphnids are known to swim to the bottom of the beaker to pick up algae, and nanoparticles could be visually observed in the gut during the exposure.

Some patterns were found between size and toxicity for the nanomaterials used in this study. Carbon nanotube aggregates were larger than fullerene aggregates, and carbon nanotubes were more consistently toxic to daphnid reproduction and final size than particles with fullerene cores. As discussed above, this could be a result of interference with food consumption or nutrient uptake, but could also be a result of differences in uptake of the nanomaterials themselves. *Daphnia* are known to selectively graze phytoplankton,[47] and the presence of nanomaterials could interfere with their ability to select phytoplankton of optimal size or nutrient content.

Many particle types exhibited no patterns between particle size or particle charge and toxicity. C60-malonate-γCD had the largest percentage (70.5%) of particles with diameters under 110 nm, while C60-amino-γCD had one of the smallest percentages (36.6%) of particles with diameters under 110 nm, and both of these particle types were found to be highly toxic to daphnids at concentrations of 5 ppm and higher. SWCNT (zeta potential = 23.07 mV) and SWCNT-COOH treatments (zeta potential = −61.07 mV) had highly dissimilar zeta potential values, however they both significantly decreased daphnid reproduction and body length. C60-amino-γCD (zeta potential = −9.26 mV) and C60-malonate-γCD (zeta potential = −63.8 mV) also had dissimilar zeta potentials, however both particle types significantly increased daphnid mortality. The lack of toxicity patterns for size and stability in this diverse array of

CNMs emphasizes the importance of functionalization for evaluating carbon nanomaterial toxicity. The results of this study indicate that the actual chemistry of a carbon nanomaterial is an important factor for determining nanomaterial toxicity.

It is possible that the observed toxicity associated with these materials could be a result of other mechanisms. Carbon nanotubes have been shown to generate oxidative byproducts under high-energy ultraviolet lights that emit in the solar spectrum.[48] However, the lights used in these experiments were fluorescent lights that emit in the visible spectrum, and therefore the photogeneration of oxidative species in our suspensions is unlikely. In addition, there is evidence that UV light becomes significantly attenuated at depths of 4−6 cm in freshwater samples that are not purified and contain phytoplankton and organic matter.[49] An investigation of the formation of superoxides in our carbon nanotube suspensions was conducted by measuring the oxidatively reduced XTT product at 470 nm. Results indicated that superoxides are not present in our suspensions. Nanomaterials could also be toxic to algae, which could consequently affect the daphnids. A recent study indicates that while there is some affinity of algal particles (*P. subcapita*) to the surface of carbon nanotubes, the algae remains viable, as no changes in cell physiology were observed and photosynthetic activity was still present.[50] Although this study indicated that there was growth inhibition of algae associated with carbon nanotubes, this would not impact our current study, as fresh algae was provided every other day when media was refreshed.

Population Level Effects of CNMs. Changes in daphnid fitness upon nanomaterial exposure are important because zooplankton and aquatic invertebrates are important players in lower trophic structures, and they have a fundamental role in the success of higher organisms, such as fish. Therefore, if CNMs lead to damage of invertebrate populations in a freshwater environment, the effect could be felt at multiple trophic levels of an ecosystem. Physiological effects of a toxicant on reproductive output and growth can have a significant effect on population end points such as population size and population growth,[51] and therefore the fact that the investigated CNMs impact reproduction and adult size is an important result. Nanomaterials may impact energy allocation or alter metabolic costs of processing toxins. Nanomaterials could also influence specific biochemical pathways (such as vitellogenin production, oxidative stress, or chitin production) that are more difficult to measure, but may be very relevant to nanomaterial toxicity as they have been shown to be important with other toxicants.[52] More systems biology approaches that examine nanomaterial impacts would be valuable to elucidate mechanisms of action, and to better identify ways to cause nanomaterials that do not create negative environmental impacts.

The use of CNMs in industry has the potential to generate substantial technological advances.[53] However, the uncertainties surrounding CNM toxicity must be better resolved before these technological innovations can be fully realized. Scientists and engineers have synthesized CNMs with remarkable structural diversity for industrial applications, but these design variations have been shown to change particle toxicity to aquatic organisms.[54] Our results indicate that variations in core structure and surface chemistries of nanomaterials may result in different physiological and ecological impacts to freshwater ecosystems. More research is required to better understand the mechanism of differential nanomaterial toxicity described by these investigations to best protect freshwater invertebrate populations and the overall integrity of freshwater ecosystems. Identifying the characteristics of nanomaterials that make them more or less toxic is valuable for creating a sustainable technology. Here we suggest that alterations in surface chemistry play a large role in doing this.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables that summarize the nanomaterial characterization and results, including images of fullerene derivatives **1−6** (Figure S1) and detailed methods for synthesis of fullerene derivatives **2−6** (Figure S2 and S3), TEM images (Figure S4), nanomaterial purity characterization (Table S1), size characterization (Table S2), stability characterization (Table S3), and results summaries for chronic daphnid mortality, reproduction, and adult size (Table S4). This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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