

CARBON NANOMATERIALS IN FRESHWATER ECOSYSTEMS: AN CHRONIC,
MULTI-GENERATIONAL, AND GENOMIC ASSESSMENT OF TOXICITY TO

DAPHNIA MAGNA

by

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A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
in Freshwater Sciences

at

The University of Wisconsin-Milwaukee

May 2014

ABSTRACT
CARBON NANOMATERIALS IN FRESHWATER ECOSYSTEMS: A CHRONIC,
MULTI-GENERATIONAL, AND GENETIC ASSESSMENT OF NANOMATERIAL
TOXICITY TO *DAPHNIA MAGNA*

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The University of Wisconsin Milwaukee, 2014
Under the supervision of professor Dr. Rebecca Klaper

Carbon nanomaterials are synthesized with a variety of core structures and surface chemistries to make them more biocompatible for application in different industries, but variation in core structure and functionalization can change the toxicity of carbon nanomaterials to organisms. In addition, current literature is dominated by data from acute toxicity assays, but meta-data is necessary to improve our understanding of nanomaterial toxicity. This project identifies specific core structures and surface chemistries that make carbon nanomaterials more and less toxic using chronic toxicity assays and multi-generational assays to generate a dataset on the sub-lethal impacts of nanomaterials to *Daphnia magna*. In addition, gene expression was evaluated on organisms from these experiments to assess biochemical pathways that are important in an organism's response to nanomaterial exposure. Results indicate that core structure and surface chemistry influence nanomaterial toxicity to *Daphnia*. Fullerene gamma-cyclodextrin complexes (C60-GCD) induced 100% mortality to daphnids after 17 days of exposure at 5 ppm, while fullerenes that were not bound to gamma-cyclodextrin were the least toxic particle types to daphnids. Carbon nanotubes induced the most consistent

negative impacts to *Daphnia* reproduction and growth, as all types of carbon nanotubes reduced reproduction or adult size at concentrations of 10 ppm or 50 ppm. Multi-generational impacts to mortality and reproduction were observed in *Daphnia* for several particles types, and single-walled carbon nanotubes functionalized with carboxy-amides (SWCNT-CONH₂) significantly reduced reproduction in the F₀, F₁, and F₂ generations. Many of the carbon nanomaterial exposures did not change the expression of glutathione-s-transferase (GST), vitellogenin fused with superoxide dismutase (VTG-SOD), or NADH dehydrogenase, indicating that other biochemical pathways are important in the toxicity of these materials. However, GST expression was reduced in F₀ and F₂ daphnids from SWCNT-CONH₂ exposures, indicating a possible impairment of this particle type to the oxidative response system. Fullerenes functionalized with malonate and non-covalently bound to GCD (C60-malonate-GCD) induced significant changes in the expression of all three investigated genes, and these changes are indicative of oxidative stress in daphnids (increases in GST transcripts), impacts to reproduction (increased VTG-SOD transcripts), and impacts to mitochondrial metabolism (decreased NADH dehydrogenase transcripts). In addition, these data indicate that GST, VTG-SOD, and NADH dehydrogenase have the potential to be used as biomarkers of early detection of nanomaterial exposure for SWCNT-CONH₂ and C60-malonate-GCD particle types. *Daphnia* have a central role in the trophic structure of ecosystems, as they feed on algae and transfer energy from lower trophic structure to higher trophic structures. A decline in daphnid populations would change the composition of phytoplankton communities, with the potential for eutrophication of small lakes and ponds if daphnid populations are completely eliminated from the ecosystem. *Daphnia* are also essential food sources for

juvenile and adult fish, and a loss of daphnid populations would reduce available nutrition for higher trophic organisms. This research provides a thorough and detailed expression of sub-lethal nanomaterial toxicity to *Daphnia* in long-term and multi-generational scenarios, and it can be used to inform the synthesis of nanomaterials such that they cause minimal harm to organisms and the environment.

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Extended Nomenclature</u>
CNM	Carbon nanomaterial
C60	Fullerene
SWCNT	Single-walled carbon nanotube
SWCNT-COOH	Single-walled carbon nanotube functionalized with carboxyl groups
SWCNT-CONH ₂	Single-walled carbon nanotube functionalized with carboxy-amide groups
SWCNT-PEG	Single-walled carbon nanotube functionalized with polyethylene glycol
C60-OH	Fullerene functionalized with hydroxyl groups
C60-amino	Amino substituted methanofullerene derivative
C60-malonate	Disodium malonate derivative of C60
βCD	Beta cyclodextrin
γCD	Gamma cyclodextrin
C60-βCD	Supramolecular complex of C60 with beta cyclodextrin
C60-amino-γCD	Supramolecular complex of C60-amino with γCD
C60-malonate-γCD	Supramolecular complex of C60-malonate with γCD
LC50	Concentration that is lethal to 50% of a population
ppb	Parts per billion
ppm	Parts per million

CHAPTER 1: INTRODUCTION

A nanomaterial is defined as any material with at least one dimension that is measured between 1 and 100 nanometers. Nanomaterials are characterized by very high surface area to volume ratios and they display exaggerated mechanical, thermal, catalytic, electrical, and optical activity when compared to their larger counterparts [1]. The increased chemical and physical reactivity of nanomaterials makes them useful for many applications; however, these unique properties make it difficult to predict nanomaterial toxicity to organisms. As a consequence, literature contains contradictory information about the toxicity of nanomaterials due to slight deviations in particle design and experimental conditions. In addition, there is not enough toxicity data in the literature on chronic, multi-generational, and genetic impacts of nanomaterials to organisms. This is problematic because acute toxicity data does not capture all of the facets of nanomaterial toxicity, leaving many questions unanswered.

Carbon nanomaterials (CNMs) are of particular interest, as they are not biocompatible in their unfunctionalized pristine state. Scientists and engineers have synthesized CNMs with a particularly wide variety of core structures and surface properties to enhance their function in many types of applications [2]. The wide variety of CNM core structures and surface chemistries can change how CNMs interact with biological systems, thereby increasing the complexity of interactions between CNMs and organisms and further muddling our understanding of CNM toxicity. This is problematic, as people and organisms will likely become exposed to CNMs from the manufacture or use of nano-products or from waste generated from these products. Potential exposure routes include direct injection into the blood circulation [3], inhalation from worker

exposure [4] or from commercial product use [5], and absorption into the body through dermal contact [6]. CNM waste from sewage and landfills will be transported to wastewater treatment plants; however, it is not clear if treatment processes will successfully remove these emerging contaminants from wastewater effluent [7]. It is possible that CNMs from wastewater treatment plants and other point and non-point source pollution could infiltrate drinking water sources, and aquatic organisms are at particular risk of exposure as CNM waste accumulates in the environment.

Current literature indicates a wide variety of toxicities across CNM particle types that vary in core structure and surface characteristics. Fullerenes dispersed with gum arabic were not found to be toxic, however SWCNTs dispersed with gum arabic induced 50% mortality to *C. dubia* (LC50) and 50% growth inhibition to *P. subcapita* (IC50) at 0.27 ppm and 2.11 ppm, respectively [8], indicating that core structure has a role in the differential toxicity of CNMs. Surface functionalization has also been shown to impact toxicity. The LC50 for exposure of unfunctionalized fullerenes to zebrafish embryos was found to be 200 ppb, while the LC50 for exposure to hydroxylated fullerenes was over 4,000 ppb, indicating that hydroxylation can decrease the toxicity of fullerenes to zebrafish embryos [9]. In addition, data from our own laboratory indicated significant mortality of daphnids to hydroxylated fullerenes and unfunctionalized fullerenes with concentrations as high as 100 ppm [10], reiterating the wide range of potential toxicities associated with CNMS.

The reasons for these variations in toxicity with differential core structures and surface properties are not entirely clear. Alterations in core structure and surface functionalization can change the characteristics of the particles themselves, and this can

subsequently lead to alterations in toxicity. Core structure and surface functionality of nanomaterials can alter particle characteristics like aggregation state, charge, hydrophobicity, and bioavailability. For example, a previous study indicated that functionalization of SWCNTs altered SWCNT aggregation state, dispersivity, and morphology, and that these changes in aggregation state were responsible for reduced cytotoxicity to bacteria [11]. Literature shows that positively charged particles can disrupt cell membrane potentials with the consequence of inhibited cell proliferation, while negative and neutral charged particles do not have this effect. In addition, charged particles can also disrupt cell membrane bilayers, with positively charged particles inducing fluidity and negatively charged particles causing local gelation, which could lead to differential nanomaterial toxicities. Surface charge can affect the formation of protein coronas in biological media, which can also modify particle toxicity [12]. Functionalization and aggregation state have also been shown to alter the reactivity and bioactivity of fullerenes, as smaller aggregates tend to be more hydroxylated, produce more reactive oxygen species (ROS), and are associated with increased levels of microbial inactivation [13].

Literature is biased toward acute toxicity data that are cheaper and easier to generate, while data on the impacts of chronic nanomaterial exposure are less plentiful. Despite larger data sets that are available for acute nanomaterial toxicity, it is more likely that environmental exposure of organisms to nanomaterials will be long-term with an increased potential for sub-lethal impacts. To further complicate toxicity testing, nanomaterials possess physical-chemical components other than mass and concentration that are more common for traditionally toxic chemicals and contaminants. These physico-

chemical factors that influence nanomaterial interactions are temporally dynamic; they change over time and they change upon interaction with biological systems [14]. This indicates the potential for a change in toxicity of nanomaterials over time and with variations in the exposure setting. Therefore there is a specific need for experimental data on the long-term exposure of multiple types of nanomaterials to freshwater organisms that cover a wide breadth of nanomaterial types. One of the goals of this project is to determine the chronic impacts of twelve types of unfunctionalized and functionalized CNMs to *Daphnia magna*.

In addition to the concerns and necessity for more experiments on chronic nanomaterial toxicity, nanomaterials have the potential to induce multi-generational impacts on whole organisms, and currently there is no literature available on the multi-generational impacts of nanomaterials on organisms after the exposure has been removed. There are already multi-generational studies on other types of contaminants, like endocrine-disrupting compounds and heavy metals [15, 16], and recent evidence demonstrates the potential for nanomaterials to act as endocrine disruptors to vertebrates and invertebrates [17]. Nanomaterials have also been shown to induce changes to the epigenome *in vitro* [18, 19], and if these changes happen in the germ-line they could be propagated to future generations of organisms, further accentuating the need for multi-generational studies to be carried out with nanomaterials.

Multi-generational assays can also offer valuable toxicity data that single generation assays cannot provide. Multi-generational assays allow for toxicity testing of all critical windows of exposure [20], including exposure to nanomaterials during prenatal and perinatal stages of development. Exposure during these sensitive periods of

development can lead to dysregulation of the fetal epigenome, with increased potential for negative consequences to affect future generations of organisms, even after the exposure is removed [21]. This is important, as over the past decade there has been a push for more toxicity testing over longer durations and at more sensitive stages of development. A comprehensive evaluation of chemical toxicity at more life stages can ensure that the needs of more sensitive populations, like children, are being met by safety and regulatory guidelines [22].

Another way to investigate the long-term impacts of nanomaterials is to identify the mechanisms of action of nanomaterials in organisms. Understanding changes in gene expression after nanomaterial exposure can provide additional information about nanomaterial toxicity to organisms that cannot be observed in life cycle and mortality assays, including information about the differences between low-dose and high-dose effects, potential mechanisms of toxicity, and discovery of biomarkers of early exposure [23]. The primary mechanism that is used to describe individual toxicity of CNMs to organisms is oxidative stress. Oxidative stress is associated with an increase in reactive oxygen species (ROS) or a decrease in the effectiveness of anti-oxidant defenses in a living system, and it can result in significant damage to cell membranes and DNA. Some types of CNMs have been shown to increase ROS [24], decrease activity of important anti-oxidant enzymes [25], increase levels of oxidatively damaged DNA [26], and induce lipid peroxidation [27] in biological systems. As organisms react to oxidative stress, certain genetic pathways and biological activity can become sequentially activated, including DNA repair activity and the activation of pro or anti-apoptotic biochemical

pathways [28]; however, it is important to note that this activity could be an organism's response directly to the nanomaterial, as well.

Although oxidative stress is described as the primary mechanism of CNM toxicity, other mechanisms could have a role in CNM toxicity. Cytochrome p450's are important for hormone regulation and xenobiotic metabolism [27], and the expression of these genes could change in response to CNM exposure because nanomaterials might interfere with hormonal activity in organisms or be transformed by p450 enzymatic activity. CNMs could also impact an organism's immune system, as has been shown by impairment to phagocytic activity in human macrophages [29] and increased inflammation observed in trout macrophages [30].

Epigenetics describes changes in gene expression resulting from alterations in the underlying structure of DNA, and changes at this level of biological organization could also describe mechanisms of CNM toxicity. Epigenetic modifications operate through changes in histone and DNA machinery, and these signatures have potential roles in cell growth and differentiation, cell death, and disease and aging. Other chemicals have already been shown to have epigenetic impacts to organisms, including endocrine disruptors like vinclozolin [31] and heavy metals [32].

Daphnia magna constitute an ideal model invertebrate for aquatic toxicity assays. Their life histories are well known, they have a short life span coupled with high rates of reproduction, and they are easy to keep in the laboratory. In addition, *Daphnia* are becoming invaluable model organisms for experiments in environmental genomics. *Daphnia* reproduction is parthenogenetic and *Daphnia* young are exact genetic clones of their mothers. This helps us to isolate changes in gene expression that are a consequence

of specific environmental stressors (*i.e.* nanomaterials). *Daphnia* are also primary consumers in aquatic environments, and they transfer trophic energy from the base of the trophic pyramid to higher trophic levels. Impacts to daphnid populations in the wild would result in a disruption of the balance of aquatic ecosystems in the wild.

Despite the demonstrated advantages of nanomaterials, scientific evidence suggests that exposure could adversely impact ecosystems and human health. The unique size-related properties of CNMs make it difficult to make generalizations about CNM distribution or toxicity in organisms because their behaviors cannot be described singularly by classical physics or quantum physics alone, and literature lacks more detailed comparative toxicity information on multiple types of nanomaterials over a chronic and multi-generational time period. CNMs are shown to distribute to cell and organs in living systems, and particle design and the surrounding environment can alter that distribution and toxicity patterns. CNM exposure can exert significant impacts on important life cycle parameters of aquatic organisms with potential negative implications for populations and ecosystem dynamics. Oxidative stress is described as the primary mechanism of toxicity; however, it is likely that other mechanisms also play a role in nanomaterial toxicity.

This research seeks to provide data that can help to fill the in gaps in the current nano-toxicology literature. The first part of this dissertation seeks to determine how core structure and surface chemistry influence the toxicity of CNMs over a chronic exposure. In addition, we will evaluate how good acute toxicity assays are at predicting chronic nanomaterial toxicity. To answer these questions, *Daphnia magna* will be exposed to various types of CNMs that differ in core structure and surface chemistry for a 21-day

period and mortality, reproduction, and daphnid size at the end of the exposure will be documented. It is hypothesized that carbon nanotubes will induce higher rates of mortality, lower reproduction, and reduce values for growth in daphnids compared to fullerenes. In addition, it is expected that an evaluation of toxicity at 48 hours will not be a good predictor of sub-lethal impacts of nanomaterial toxicity. This research will improve the current knowledge in the field of nano-toxicology because it will provide a more-detailed comparison of nanomaterial toxicity using sub-lethal endpoints over a long-term exposure. The current state of knowledge in the field is dominated by data from acute toxicity assays that are easier to perform and more commonly used by regulators. However, it is more likely that organisms will receive a chronic exposure to nanomaterials in the environment that won't induce acute mortality, but could change essential life cycle parameters such as reproduction and growth over a longer time period.

The second part of this dissertation seeks to determine how core structure and surface chemistry influence the multi-generational toxicity of CNMs to *Daphnia* over two successive generations after the initial nanomaterial exposure is removed. To answer this question, F₁ neonates from the exposed F₀ brood were transferred to control water and raised over a chronic time period, and this was repeated with the F₂ generation. Mortality, reproduction, and adult size were measured during these multi-generational trials. It is hypothesized that carbon nanotubes will negatively affect multi-generational mortality, reproduction, and size compared to fullerenes. Current literature demonstrates multi-generational impacts of other types of toxicants to organisms and cells, and literature also indicates that some types of nanomaterials can induce epigenetic changes to cells *in vitro*. The second part of this dissertation supplies the first data on multi-

generational nanomaterial toxicity to whole organisms after the exposure has been removed, and it provides an even more in-depth comparison of long-term nanomaterial toxicity to organisms.

The third part of this dissertation identified underlying mechanisms of toxicity that were observed in the chronic and multi-generational trials from the first two parts of this work. To identify underlying mechanisms of toxicity, genes from key biochemical pathways will be investigated by Q-PCR. Changes to the expression of glutathione-s-transferase (GST) will evaluate the role of oxidative stress associated with CNM exposure. Expression of vitellogenin fused with superoxide dismutase (VTG-SOD) helps determine the role of CNM exposure on daphnid reproduction, and changes in the expression of NADH dehydrogenase helps to identify the impact of CNMs on glycolysis and mitochondrial metabolism. Although this project investigated changes in the expression of genes in a multi-generational context, it did not directly investigate any epigenetic modifications in daphnids (however, if changes in gene expression appear across multiple generations of daphnids, it is possible that nanomaterials act on the daphnid epigenome, and a direct investigation of epigenetic impacts on daphnids would be included in future work).

It is hypothesized that carbon nanotubes will induce more changes to the expression of these genes than fullerenes. In addition, it is expected that different types of nanomaterials will alter different biochemical pathways, so that not all nanomaterial types will induce the same patterns of expression to GST, VTG-SOD, and NADH dehydrogenase. This data can advance current knowledge in the field of nanomaterial toxicity because a greater understanding of biochemical pathways important in the

nanomaterial stress response of *Daphnia* can provide a uniquely detailed evaluation of nanomaterial toxicity; this in turn would allow for nanomaterial design that would maximize their use in industries, while reducing harmful impacts to biological systems. The molecular processes that are important in the stress response can also be resolved and linked to processes in other species, and relevant genes can be used as potential biomarkers of early exposure of organisms to nanomaterials in the environment.

Collectively, the results of these three project elements will help to establish what types of core structures and functionalizations are more toxic to exposed and future generations of *Daphnia*, with the ultimate goal to find ways to synthesize nanomaterials in ways that minimize their potential harms to the environment.

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CHAPTER 2

Core structure and surface functionalization of carbon nanomaterials alter impacts to daphnid mortality, reproduction, and growth: Acute assays do not predict chronic exposure impacts

Published in:

Environmental Science and Technology

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(Received 26 March 2013; Accepted 17 July 2013)

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 the toxicity of 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 hours, 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 toxicity depending on particle type and experimental set up. As an example, single-walled carbon nanotubes (SWCNTs) induce significant cytotoxicity to alveolar macrophages at doses of $11.3 \mu\text{g}/\text{cm}^2$, while fullerenes were cytotoxic at $226 \mu\text{g}/\text{cm}^2$ 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-hr LC₅₀ 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 importance 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 sub-lethal 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 endpoints, including reproduction and adult size, in the aquatic toxicology and ecological model organisms, *Daphnia magna*.

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 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 supplementary material (Figure S2 and S3).

- **Derivative 1 (C60- β CD)**: supramolecular complex of C60 with β -cyclodextrin (β CD): C60 (100 mg, 0.139 mmol) and β CD (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 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- γ CD)**: supramolecular complex of amino-substituted methanofullerene derivative with γ CD: Derivative **2** (50.0 mg, 0.045 mmol) and γ CD (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 bath sonication.
- Derivative **4 (C60-malonic acid)**: malonic acid derivative of C60, synthesized and characterized according to literature [18-21].
- Derivative **5 (C60-malonate)**: disodium malonate derivative of C60. Derivative **4** (50.0 mg, 0.061 mmol) was dissolved first in 0.01 M NaOH (6.1 mL) and then diluted to 1 L of deionized water followed by 1 h bath sonication.
- Derivative **6 (C60-malonate- γ CD)**: supramolecular complex of disodium malonate derivative of C60 with γ CD. Derivative **4** (50.0 mg, 0.061 mmol) and γ CD (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 bath sonication.

Cyclodextrins were non-covalently 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 two weeks at 600 RPM. The remaining particle types were suspended in milli-Q water by sonication for two hours in a water bath. All particle types were sonicated for five minutes before use in exposures. These include fullerenes (C60), hydroxylated fullerenes (C60-OH₂₄), single-walled carbon nanotubes (SWCNTs), carboxylic acid functionalized SWCNTs (SWCNT-COOH), carboxy-amide functionalized SWCNTs (SWCNT-CONH₂), polyethylene glycol functionalized SWCNTs (SWCNT-PEG), and multi-walled 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 nanomaterials. Concentrations below 50 ppm were chosen because they were determined to be sub-lethal 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 two hours 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 β CD and γ CD, 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, β CD or γ CD exposures. Additionally, PEG functional attachments have been previously shown to be non-toxic 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 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 non-parametric 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 in supplementary information.

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 105-175 nm in size (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 micron 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-CONH₂). 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, and 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 hr) 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- γ CD and C60-malonate- γ CD 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, γ CD controls induced no mortality and the equivalent exposures of particle types without γ CD (C60-amino and C60-malonate) induced no mortality, indicating a toxic action specifically associated with fullerenes bound to γ CD. When lowered to 1 ppm, C60-amino- γ CD exposure induced significant mortality to daphnids after 19 days (U=23.5, $p < 0.05$), while C60-malonate- γ CD 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 treatments compared to reproduction in control daphnids (Figure 2a). After nine days of exposure, an average of 7.06 neonates were produced per control daphnid. However, during this time frame 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 nine 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 ($U=6$, $p < 0.05$ and $U=15$, $p < 0.05$). 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 and C60-amino- γ CD 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- γ CD and C60-amino- γ CD at 5 ppm because the exposure induced 100% mortality to daphnids after 17 days. However, daphnids from 1 ppm C60-amino- γ CD 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%, $U=1$, $p < 0.05$ and 58.7%, $U=0$, $p < 0.05$) compared to control daphnids. Daphnids exposed to SWCNT-CONH₂ and SWCNT-PEG significantly reduced reproduction only at 50 ppm compared to controls (reduction of 35.9%, $U=0$, $p < 0.05$ and 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-CONH₂ and SWCNT-PEG produced 55.0% and 68.7% more neonates than daphnids from 50 ppm SWCNT-COOH exposures ($U=1$, $p < 0.05$ and $U=4$, $p < 0.05$). This indicates that

CONH₂ 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, $p < 0.05$ and 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 in size than daphnids exposed to 50 ppm SWCNTs (U=7, $p < 0.05$).

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, $p < 0.05$ and 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-CONH₂, 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, $p < 0.05$ and reduction in size

of 10.0%, $U=0$, $p < 0.05$). When the size of daphnids from functionalized SWCNT exposures (SWCNT-CONH₂, 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-CONH₂ exposures ($U=0$, $p < 0.05$), indicating that SWCNT-COOH is more toxic to daphnid size than SWCNT-CONH₂.

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, sub-lethal concentrations of fullerenes and nano-sized titanium dioxide were found to alter daphnid behavior, another sub-lethal endpoint, 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 MWCNTs³⁰ 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 hours [31]. Nanomaterials may also interfere with feeding mechanics. 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 ($> 1 \mu\text{m}$ 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-CONH₂ or SWCNT-PEG. This indicates that carboxylation of SWCNTs increases their toxicity to daphnids, while functionalization with CONH₂ or PEG decreases SWCNT toxicity to daphnids. Although all three functionalizations improve the dispersability 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-CONH₂ 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 γ CD by non-covalent bonds were the most toxic particle types that were investigated in this experiment. C60-amino- γ CD and C60-malonate- γ CD 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 γ CD bound fullerene derivatives are more bioavailable to daphnids compared to fullerene derivatives that are not bound to γ CD, thereby increasing the toxicity of γ CD bound fullerene derivatives.

Effects of Particle Size, Charge, and Potential Byproducts

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 TiO₂ nanoparticles [42], and previous work in our laboratory also indicated increased mortality of daphnids exposed to smaller size

fractions of TiO₂ 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 like 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 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- γ CD had the largest percentage (70.5%) of particles with diameters under 110 nm, while C60-amino- γ CD 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- γ CD (zeta potential = -9.26 mV) and C60-malonate- γ CD (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 emphasize 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 photo-generation of oxidative species in our suspensions is unlikely. In addition, there is evidence that UV light becomes significantly attenuated at depths of four to six centimeters 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 endpoints 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 to examine nanomaterial impacts would be valuable to elucidating mechanisms of action to better identify ways to create 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.

Acknowledgements

This research was supported by the National Science Foundation (CBET 933720) to R. Klaper and J. Chen, the Charles A. and Anne Morrow Lindbergh Foundation to D. Arndt, and the Fellowship Program and the School of Freshwater Sciences at University of Wisconsin Milwaukee. Special thanks to Adam Nikolaus, Nicholas Neureuther, Joel Weinberger, Tess Leuthner, Bonnie Blalock, and Andrew Kuhn for help conducting chronic toxicity assays.

Supplementary Information

Tables that summarize the nanomaterial characterization and results are in supplementary information. This includes 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).

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CHAPTER 2 FIGURES

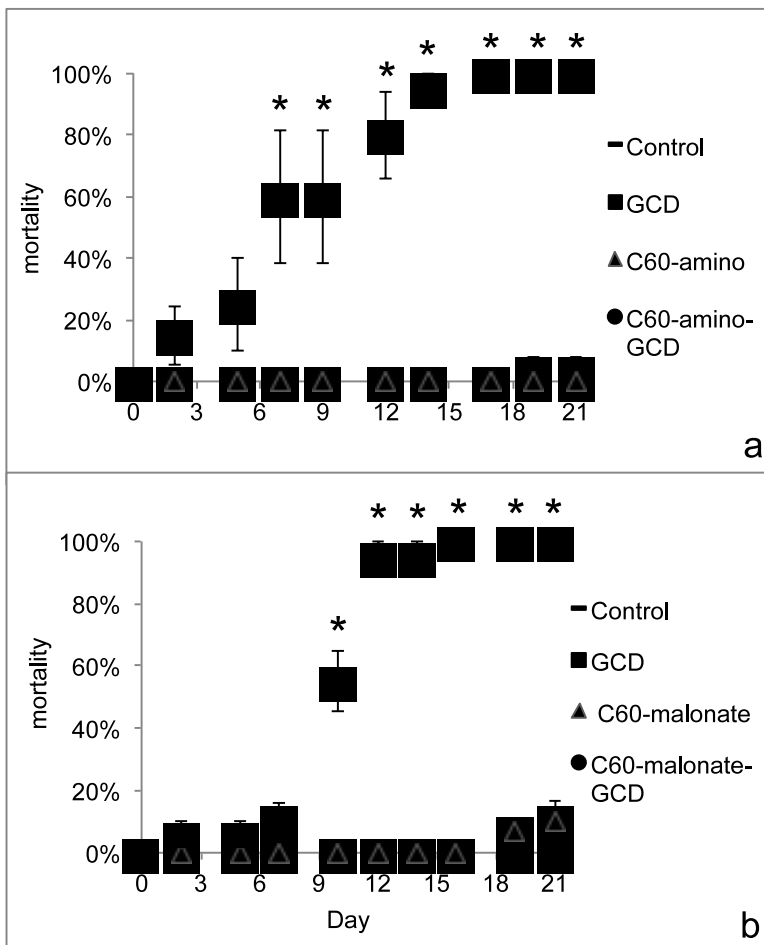


Figure 1 **Impacts of γ CD-bound fullerene derivatives to chronic daphnid mortality.**

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.

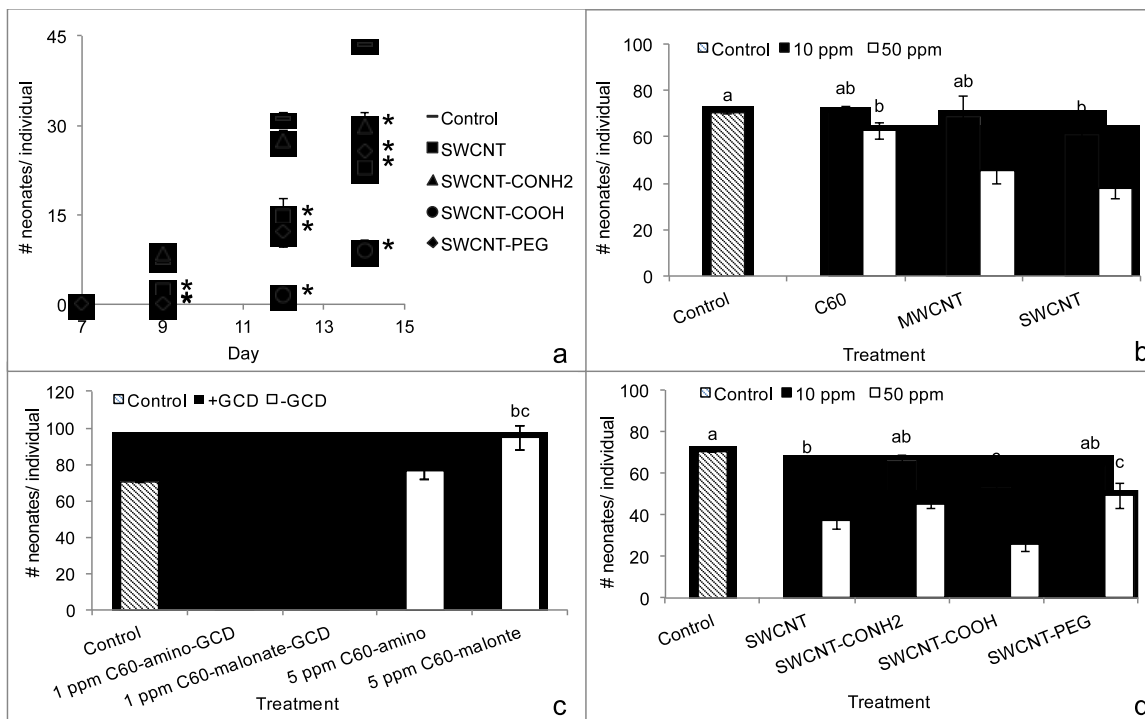


Figure 2 Impacts of CNM exposure on daphnid reproduction. Reproduction evaluated by Mann Whitney U test for two independent samples. Data are significant at $p < 0.05$. Stars indicate significant difference from control. Letters indicate significant difference among treatments and controls. (A) Impact of 50 ppm SWCNT, SWCNT-CONH₂, 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.

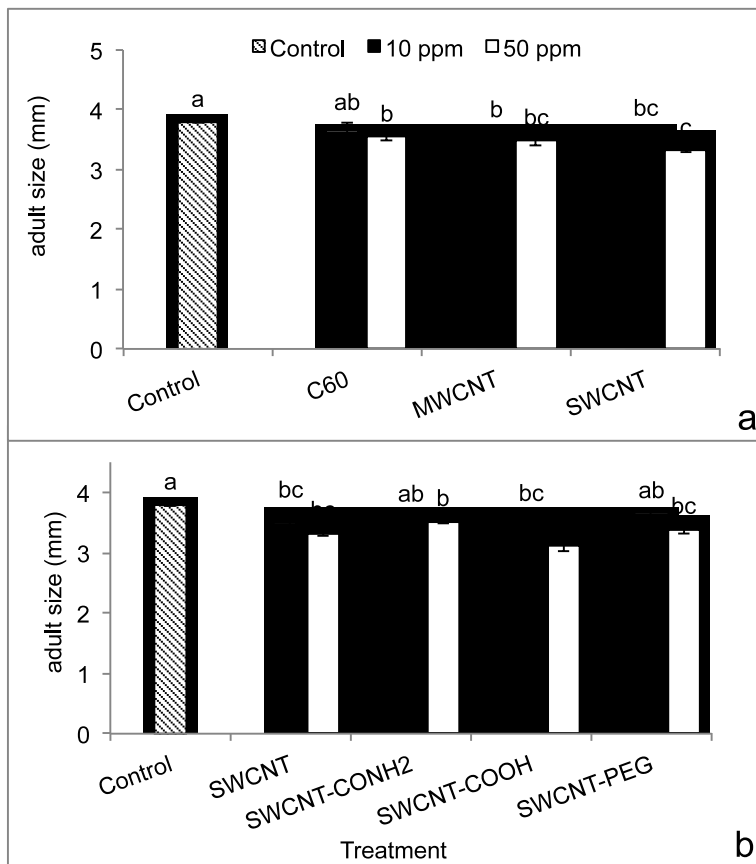


Figure 3 Impacts of CNM exposure on adult daphnid size. Adult size evaluated by Mann Whitney U test for two independent samples. Data are significant at $p < 0.05$. Letters indicate significant difference among treatments and controls. (A) Impact of unfunctionalized CNMs on adult daphnid size at 21 days.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

CHAPTER 2 SUPPLEMENTARY INFORMATION

Core structure and surface functionalization of carbon nanomaterials alter impacts to daphnid mortality, reproduction, and growth: Acute assays do not predict chronic exposure impacts

Number of supplementary information pages: 11

Number of Figures: 4

Number of Tables: 4

Figure S1

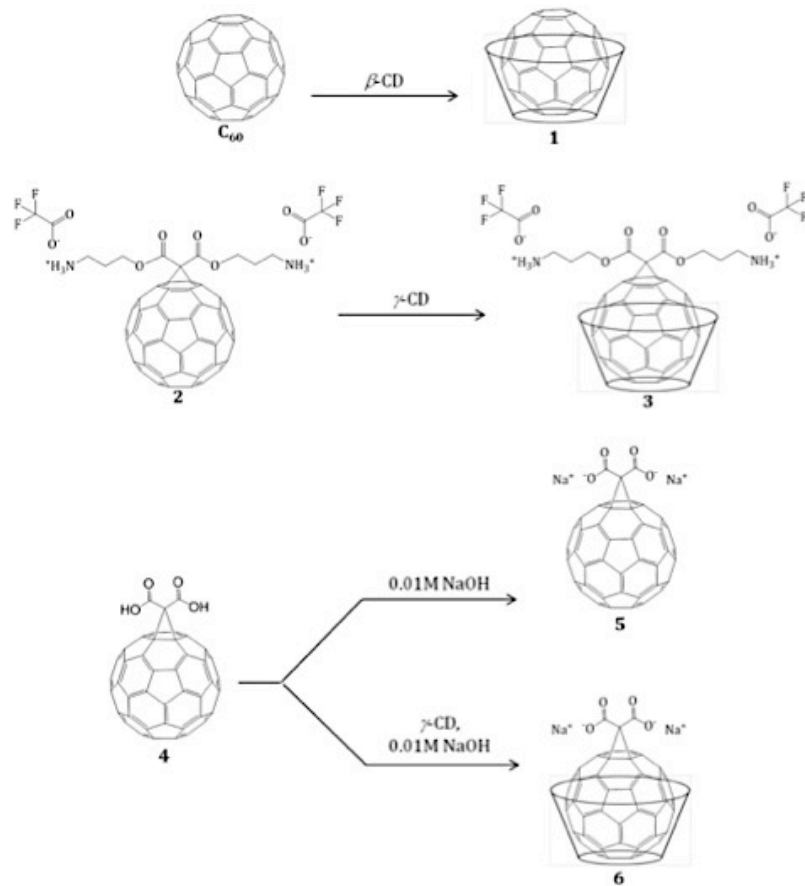


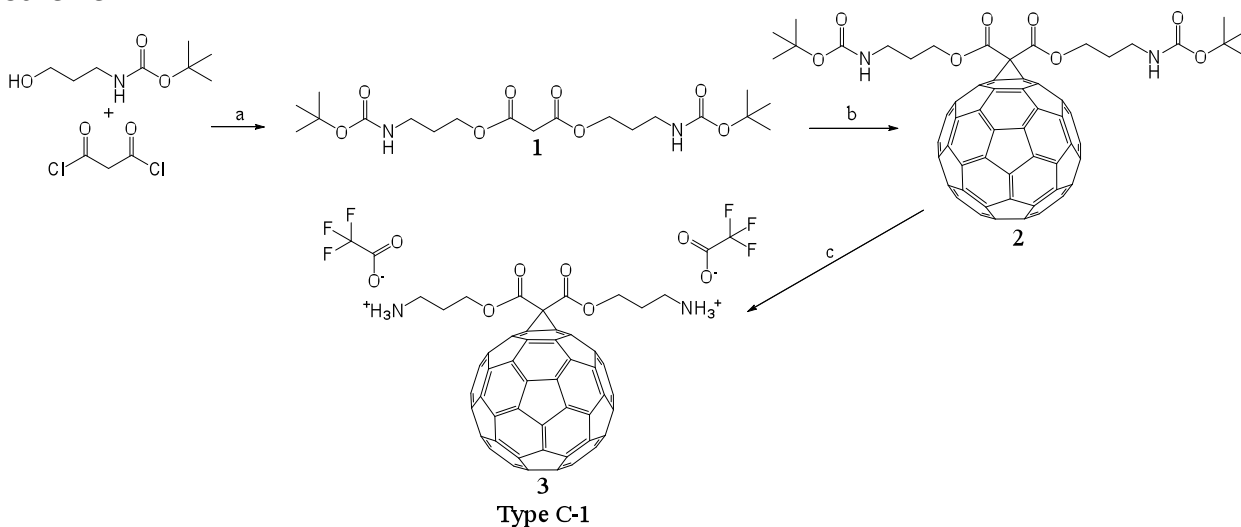
Figure S1 **Synthesis of fullerene derivatives.** Derivative 1 indicates supramolecular complex of C₆₀ with β -cyclodextrin (β CD); Derivative 2 indicates amino-substituted methanofullerene; Derivative 3 indicates supramolecular complex of amino-substituted methanofullerene derivative with γ CD; Derivative 4 indicates malonic acid fullerene derivative (derivative 4 was an intermediate to synthesize 5 and 6. It was not used for exposures); Derivative 5 indicates disodium malonate derivative of C₆₀; Derivative 6 indicates supramolecular complex of disodium malonate derivative of C₆₀ with γ CD.

Figure S2

Synthesis of C₆₀-amino

Maika Lor and Jian Chen

Scheme 1



- Pyridine, CHCl₂, 4 hrs.
- C₆₀, CBr₄, DBU, toluene, sonicate, 30 min.
- TFA, toluene, 3 hrs.

1: 2.50 g (14.3 mmol) of tert-butyl N-(3-hydroxypropyl)carbamate was added to a pre-dried flask and dissolved with 125 mL of methylene chloride. 0.99 g (7.15 mmol) of malonyl chloride and 1.16 mL (14.3 mmol) of pyridine were added to the flask under Argon and stirred for 4 hours at room temperature. TLC showed the product with an R_f of 0.40 (silica gel, 1:1 hexane/ethyl acetate). The product was purified by column chromatography on silica gel using 1:1 hexane/ethyl acetate solvent. 2.98 g (50.9%) of product was obtained after drying.

¹H-NMR (300 MHz, CDCl₃): δ (ppm)=4.89 (s, 2H), 4.22 (t, 4H), 3.40 (s, 2H), 3.20 (q, 4H), 1.86 (m, 4H), 1.44 (s, 18H).^[1]

2: 0.25g (0.347 mmol) of C₆₀ and 0.17 g (0.521 mmol) of CBr₄ was added to a pre-dried flask and dissolved with 170 mL of toluene, followed by 30 seconds of sonication. The fullerene was fully dissolved after 30 minutes of stirring. 0.22 g (0.521 mmol) of **1** and 0.16 g (1.05 mmol) of 1,8-diazabicycloundec-7-ene were added to the flask under Argon and stirred for 30 minutes at room temperature. TLC showed the product with an R_f of 0.08 (silica gel, 100:5 toluene/ethyl acetate). The product was purified by column chromatography on silica gel using toluene to remove unreacted C₆₀, followed by 10:1 toluene/ethyl acetate. 0.20 g (50.6%) of product was obtained after drying.

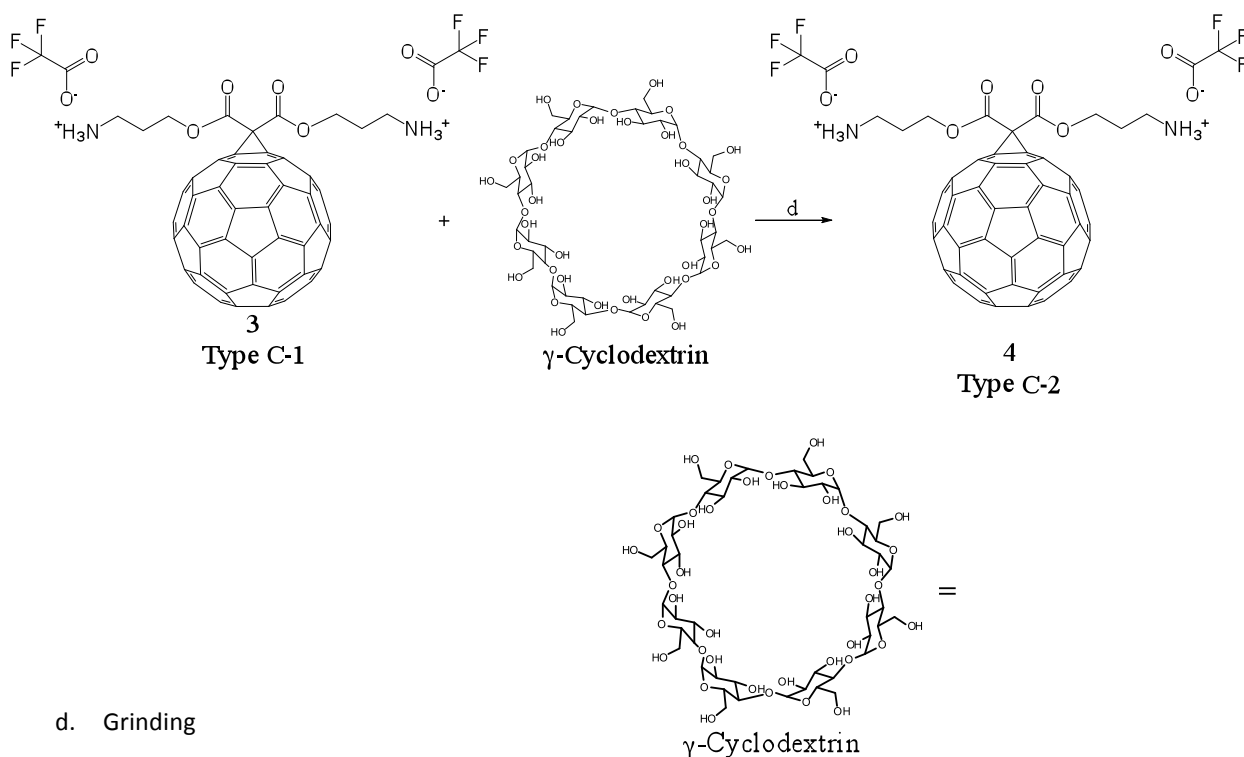
¹H-NMR (300 MHz, CDCl₃): δ (ppm)=4.90 (s, 2H), 4.58 (t, 4H), 3.32 (q, 4H), 2.06 (m, 4H), 1.46 (s, 18H).^[1]

3 (Type C1, C60-amino): 175 mg (0.154 mmol) of **2** was dissolved in 35 mL of toluene, then 35 mL of trifluoroacetic acid was added. The reaction was stirred for 3 hours at room temperature. The solvent was removed. The solid residue was washed with toluene to remove any trace of starting material and dried under vacuum to obtain 155 mg (91.7%) of product.^{[1][2]}

References:

- (1) Richardson, C. F.; Shuster, D. I.; Wilson, S. R. *Org. Lett.* 2000, 2, 1011-1014.
- (2) Kordatos, K.; Ros, T. D.; Bosi, S.; Vazquez, E.; Bergamin, M.; Cusan, C.; Pellarini, F.; Tomberli, V.; Baiti, B.; Pantarotto, D.; Georgakilas, V.; Spalluto, G.; Prato, M. *J. Org. Chem.* 2001, 66, 4915-4920.

Scheme 2 (Our own procedure)



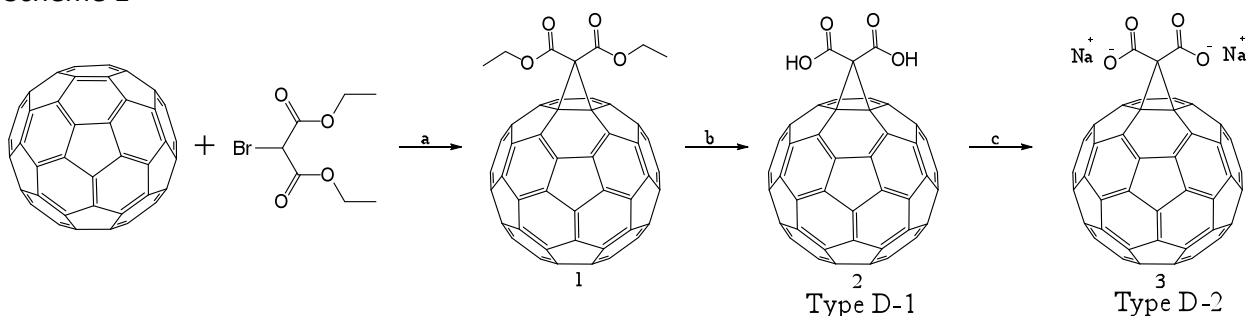
4 (Type C-2, C60-amino-GCD): 20.0 mg of fullerene (type C-1) and 23.5 mg of γ -cyclodextrin were ground for 1h to give a uniform brown powder. The brown powder was dissolved in 400 ml of deionized water followed by 30 min of bath sonication to give a yellow-brown solution of 4(0.05 mg fullerene/ml; 0.06 mg γ -CD/ml).

Figure S3

Synthesis of C₆₀-malonate

Maika Lor and Jian Chen

Scheme 1



- e. NaH, toluene, room temperature, 6.5 hours
- f. NaH, toluene, 60°C, 3 hours
- g. 1 M NaOH, sonication

1: 100 mg (0.139 mmol) of fullerene C₆₀ was added to a pre-dried three-neck flask and dissolved with 46 ml of toluene. 49.8 mg (0.208 mmol) of diethyl bromomalonate and 33.4 mg (1.39 mmol) of NaH were added to the solution under Argon. After 6.5 hours of stirring at room temperature, the reaction was hydrolyzed with 2 drops of 2N H₂SO₄, dried with MgSO₄. After filtering the MgSO₄, the solvent was removed by rotovap at 60°C. TLC showed the product with an *R_f*(silica gel, toluene) of 0.7. The product was purified by column chromatography on silica gel using 1:1 hexane/toluene solvent. After column separation, the material was dissolved in CHCl₃ and precipitated with ethanol to obtain 55.5 mg (46 % yield) of the product after drying.

FT-IR (KBr): ν_{max} (cm⁻¹) = 2979, 1745 (C=O), 1428 (C₆₀), 1295, 1266, 1234, 1206, 1186 (C₆₀), 1095, 1061.

¹H-NMR (300 MHz, CDCl₃): δ (ppm) = 4.57 (q, 4H), 1.50 (t, 6H).^[1]

2 (Type D-1, intermediate to C₆₀-malonate): 50 mg (56.9 μ mol) of **1** was added to a pre-dried flask and dissolved with 25 ml of toluene. 27.4 mg (1.14 mmol) of NaH was added under Argon. The mixture was stirred for 3 hours at 60°C. Once the mixture was cooled to room temperature, the salt was precipitated with 0.5 ml of methanol, causing vigorous gas evolution. The precipitate was collected by centrifugation and washed with toluene, 2M H₂SO₄, and water, respectively. 40.5 mg (86%) of the product was obtained after drying.^[2]

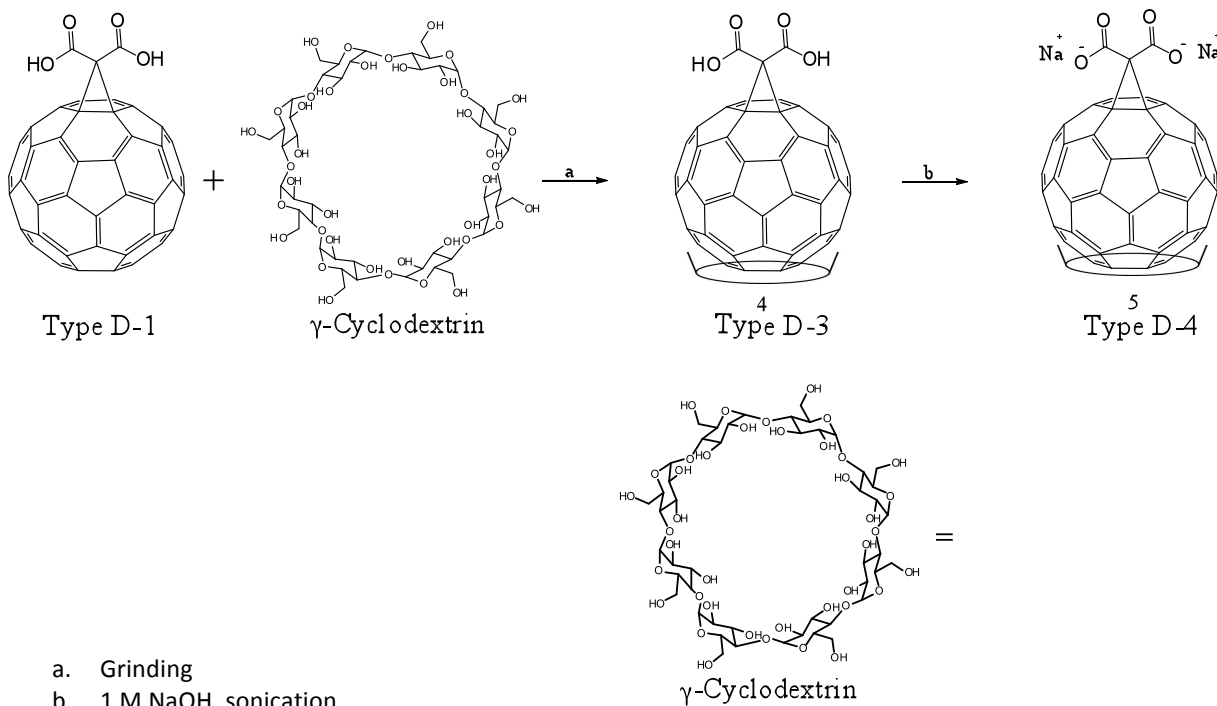
3 (Type D-2, C₆₀-malonate): 20 mg (24.31 μ mol) of **2** was dispersed in 397.7 ml of deionized water and 2.3 ml of 0.01M NaOH solution by 1h bath sonication to give a yellow-brown suspension of **3** (0.05 mg fullerene/ml).

References:

(3) Bingel, C. *Chem. Ber.* 1993, 126, 1957.

(4) Lamparth, I.; Hirsch, A. *J. Chem. Soc., Chem. Commun.* 1994, 1727.

Scheme 2 (Our own procedure)



4 (Type D-3, intermediate to C60-malonate-GCD): 17.3 mg of Fullerene (type D-1) and 27.28 mg of γ -cyclodextrin were ground for 1h to give a uniform brown powder.

5 (Type D-4, C60-malonate-GCD): The above brown powder (**4**, type D-3) was dissolved in 344 ml of deionized water and 2.0 ml of 0.01 M NaOH solution by 30 min of bath sonication to give a yellow-brown solution of **5** (0.05 mg fullerene/ml ; 0.08 mg γ -CD/ml).

Figure S4

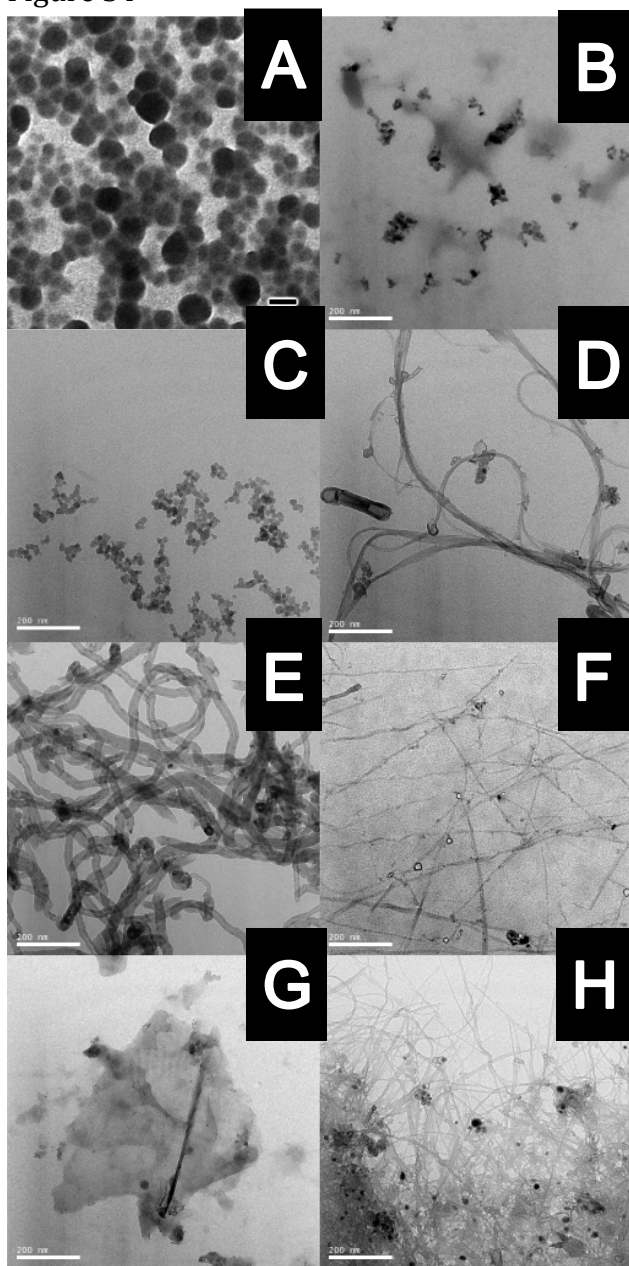


Figure S4

TEM images of (A) C60 (B) C60-OH (C) C60-(β CD) (D) SWCNT (E) SWCNT-CONH₂ (F) SWCNT-COOH (G) SWCNT-PEG (H) MWCNT.

Table S1: Manufacturer Information and Purity Characterization

	CNM Core	Surface Chemistry	Purity (%)	ICPMS (ug/L)
(¹)	C60	none	99.5%	9.49 Fe 0.1 Sr
(²)	C60	hydroxyl	99%	34.6 Fe 6.88 Cu
(³)	C60	β CD	98%	20.1 Fe 29.7 Cu
(³)	C60	amino	98%	20.1 Fe 29.7 Cu
(³)	C60	amino- γ CD	98%	20.1 Fe 29.7 Cu
(³)	C60	malonate	98%	20.1 Fe 29.7 Cu
(³)	C60	malonate- γ CD	98%	20.1 Fe 29.7 Cu
(⁴)	SWCNT	none	>90%	368.0 Ni 73.3 Ni (supernatant)
(⁴)	SWCNT	COOH	>90%	212.0 Ni
(⁴)	SWCNT	CONH ₂	>90%	158.0 Ni
(⁴)	SWCNT	PEG	>90%	60.9 Ni
(⁵)	MWCNT	none	>95%	151.0 Ni 15 Ni (supernatant)

Column 1 denotes manufacturer (¹Alfa Aesar, ²MER Corporation, ³UWM Chemistry, ⁴Carbon Solutions Inc., and ⁵NanoAmor Inc.); Columns 2 and 3 denote core structure and surface chemistry; Column 4 denotes carbonaceous purity of the starting material as reported by the manufacturer; Column 5 metal content by ICPMS results. Detection limit (DL) for ICPMS is 5 ppb for Cu, 100 ppb for Fe, 1 ppb for Ni, 1 ppb for Sr. Method Detection Limit (MDL) for ICPMS is .024 ppb Cu, 5.27 ppb Fe, .024 ppb Ni, and .0548 ppb Sr. Supernatants from unfunctionalized SWCNT and MWNCT were also examined by ICPMS and are noted in column 4 of the SWCNT and MWCNT rows.

Table S2: Nanomaterial Size Characterization

CNM Core	Surface Chemistry	Diameter Peaks (nm)	Ave. Diameter (nm)	% CNMs <110 nm
C60	none	117, 141	141 ± 59	40%
C60	hydroxyl	64, 137	144 ± 68	46.5%
C60	βCD	79, 114	107 ± 51	67.2%
C60	amino	65, 84	142 ± 73	46%
C60	amino-γCD	109, 150	152 ± 67	36.6%
C60	malonate	99, 164	175 ± 116	30%
C60	malonate-γCD	85, 102	105 ± 40	70.5%

Columns 1 and 2 denote core structure and surface chemistry. Fullerene size characteristics were generated by Nanosight. Column 3 denotes hydrodynamic diameter size peaks; Column 4 denotes average hydrodynamic diameter; Column 5 denotes the percentage of particles with diameters under 110 nm. CNM indicates carbon nanomaterial.

Table S3: Nanomaterial Stability Characterization

CNM Core	Surface Chemistry	ζ-potential (MQ water) (mV)	ζ-potential (MHRW water) (mV)
C60	none	-39.6	-32.8
C60	hydroxyl	-54.0	-8.6
C60	βCD	-42.2	-19.6
C60	amino	-17.1	-24.6
C60	amino-γCD	-9.3	-5.85
C60	malonate	-63.8	-16.4
C60	malonate-γCD	-47.9	-20.9
SWCNT	none	23.1	-19.0
SWCNT	COOH	-61.1	-23.8
SWCNT	CONH ₂	-52.0	-23.9
SWCNT	PEG	-58.1	-23.4
MWCNT	none	23.0	-19.3

Columns 1 and 2 denote core structure and surface chemistry; Column 3 denotes zeta (ζ) potential of nanomaterials in purified milli-Q (MQ) water; Column 4 denotes ζ -potential of nanomaterials in MHRW. Note that the stability of suspensions in MHRW-only is not representative of nanomaterials in the exposure medium, as the presence of algae and alfalfa in the medium significantly improved suspension stability.

Table S4: Results summary for chronic exposures

CNM Core	Surface Chemistry	Mortality	Reproduction	Adult Size
C60	none	-	50 ppm	50 ppm
C60	hydroxyl	-	-	-
C60	β CD	-	-	-
C60	amino	-	-	5 ppm
C60	amino- γ CD	1 ppm	1 ppm	-
C60	malonate	-	5 ppm (i)	5 ppm
C60	malonate- γ CD	5 ppm	-	-
SWCNT	none	-	10 ppm	10 ppm
SWCNT	COOH	-	10 ppm	10 ppm
SWCNT	CONH ₂	-	50 ppm	50 ppm
SWCNT	PEG	-	50 ppm	50 ppm
MWCNT	none	50 ppm	50 ppm	10 ppm

Summary of chronic exposure results. Only treatments that were statistically significant ($p < 0.05$) are noted. The lowest concentrations that induced significant differences from controls are reported. Values indicate decreases in mortality, reproduction, and growth unless followed with (i), denoting an increase in the observed endpoint. Dashes indicate no significant difference from controls.

CHAPTER 3

Multi-generation impacts on *Daphnia magna* of carbon nanomaterials with differing core structures and functionalizations

Published in:

Environmental Toxicology and Chemistry, DOI 10.1002/etc.2439

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(Received 24 June 2013; Accepted 23 October 2013)

ABSTRACT

Several classes of contaminants have been shown to have multi-generational impacts once a parental generation has been exposed. Acute and chronic toxicity are described for several types of nanomaterials in the literature, however, no information is available on the impact of nanomaterials on future generations of organisms after the exposure is removed. In the present study, we examined the impacts of carbon nanomaterials (CNMs), including fullerenes (C_{60}) and single and multi-walled carbon nanotubes (SWCNTs and MWCNTs) with neutral, positive, and negative functional groups to first (F_1) and second (F_2) generation daphnids after a parental exposure (F_0). Data from the present study indicate that multi-generational toxicity is present with certain nanomaterial exposures and is highly dependent upon the surface chemistry of the nanomaterial. Many CNMs that showed toxicity to exposed F_0 daphnids in previous experiments did not induce multi-generational toxicity. However, certain nanomaterials such as C_{60} -malonate, SWCNT, SWCNT- $CONH_2$, and MWCNT cause a significant decrease in either survival or reproduction in F_1 daphnids, and SWCNT- $CONH_2$ decreased reproduction out to the F_2 generation. Impacts of nanomaterials on F_1 and F_2 size were small and lacked clear patterns, indicating that carbon nanomaterials have minimal multi-generational impacts to size. Industries should take into account how surface chemistry influences nanomaterial toxicity to future generations of organisms to create sustainable nanomaterials that do not harm freshwater ecosystems.

Keywords: Aquatic toxicology, Nanoecotoxicology, Nanomaterials, Reproductive toxicology, Multi-generational toxicology, Epigenetics

INTRODUCTION

Engineered nanomaterials are emerging contaminants that have novel physical and chemical properties. They have already been widely commercialized in today's marketplace despite the uncertainties regarding how they will interact with biological systems [1]. Most notably, carbon nanomaterials (CNMs) have been synthesized with a particularly wide array of shapes and functionalities for applications in medicine, clothing, cosmetics, electronics, and polymer composites. As the production and application of CNMs increases, the likelihood that CNMs will end up in the environment and in aquatic systems also increases [2].

Multi-generational impacts of chemicals have been demonstrated for several other classes of compounds, including endocrine disrupting chemicals, PFOs and PFOAs, and heavy metals [3-5]. These impacts include elevated mortality and decreased size and reproduction in second and third generation offspring of various organisms including rats, *Daphnia magna*, and Japanese medaka. A continuous exposure of multiple generations of organisms to a chemical may cause physiological changes to support adaptation or acclimatization [6-8]. In addition, a single exposure of parent organisms to a chemical can result in exposure of offspring to the chemical during sensitive prenatal stages of development, which can lead to significant adverse outcomes later in life, and these effects can show up a generation or more after the exposure is removed [9].

There are growing data for acute and chronic toxicity for a variety of nanomaterials [10]; however, no data is available on the multi-generational impacts of nanomaterials to whole organisms after the exposure has been removed. In vitro assays have demonstrated that nanomaterials can induce changes to the epigenome (DNA

methylation, histone modifications, and gene silencing by non-coding RNAs) [11, 12], suggesting the possibility of impacts to future generations. If the effects of nanomaterial exposure are transferred to future generations of organisms, there could be long-term ecological consequences. Therefore an understanding of how nanomaterial exposure will affect populations of organisms after the nanomaterial exposure has been removed is essential to increase our knowledge about the long-term ecological impacts of nanomaterials, and it is relevant for any scenarios where remediation is necessary [13].

The current study investigates the multi-generational response of future generations of *Daphnia magna* from an F₀ exposure to multiple types of CNMs that differ in core structure and surface functionalization. *Daphnia* are a model aquatic invertebrate for toxicity assays because of their Holarctic distribution in freshwater systems, parthenogenetic reproductive strategy, and the quantity of data regarding their life history and responses to environmental stressors. They are ideal for multi-generational studies as they are genetic clones, allowing the potential for epigenomic impacts to be measured. In our previous study, we demonstrated that nanoparticle structure and functionalization influence particle toxicity to first-generation exposed *Daphnia* (F₀) [14]. In the present study, the consequences of F₀ exposures to future generations of daphnids (F₁ and F₂) are evaluated, using impacts on survival, reproduction, and adult size as adverse outcome endpoints.

MATERIALS AND METHODS

Nanomaterial preparation and characterization

Six carbon nanomaterials were synthesized by J. Chen at UWM. These particles include C₆₀-βCD (derivative 1), C₆₀-amino (derivative 2), C₆₀-amino-γCD (derivative 3), C₆₀-malonic acid (derivative 4), C₆₀-malonate (derivative 5), and C₆₀-malonate-γCD (derivative 6) (Figure 1). Beta and gamma cyclodextrins (βCD and γCD) were ground with fullerenes in an agate mortar to yield derivatives 1, 3, and 6. Particles were suspended in de-ionized water by 1 h bath sonication in the absence of solvents and surfactants, as this has been shown to change how the particles interact with organisms [15]. The smallest average diameter was observed with C₆₀-malonate-γCD particles (105 nm), followed by C₆₀-βCD (107 nm), C₆₀-amino (142 nm), C₆₀-amino-γCD (152 nm), C₆₀-amino-γCD (175 nm). The most stable of these particle types were C₆₀-malonate and C₆₀-malonate-γCD, with more negative zeta (ζ) potential values (-63.8 mV and -47.7 mV), followed by C₆₀-βCD (-42.2 mV), C₆₀-amino (-17.07 mV), and C₆₀-amino-γCD (-9.26 mV). Analysis by ICPMS indicated low levels of iron and copper in the suspensions (Table S1).

An additional 7 particle types were obtained as powders from manufacturers and suspended in milli-Q water. No additional solvents or surfactants were used to suspend the particles. These include fullerenes (C₆₀; Alfa Aesar), hydroxylated fullerenes (C₆₀-OH; MER Corp.), single-walled carbon nanotubes (SWCNTs; Carbon Solutions), carboxylic acid functionalized SWCNTs (SWCNT-CO₂H; Carbon Solutions), carboxylamide functionalized SWCNTs (SWCNT-CONH₂; Carbon Solutions), polyethylene glycol functionalized SWCNTs (SWCNT-PEG; Carbon Solutions), and multi-walled carbon nanotubes (MWCNTs; NanoAmor). The average diameters for C₆₀ and C₆₀-OH were 141 nm and 144 nm, respectively. Average diameters for nanotubes ranged from

800 nm to over 2 microns; however, due to the high aspect ratio of the nanotubes, the size of the aggregates is not uniform, and some aggregate sizes are smaller and larger than these reported average diameters. Functionalized nanotubes (SWCNT-COOH, SWCNT-PEG, and SWCNT-CONH₂) were the most stable in suspension with milli-Q water (ζ potential of -61 mV, -58.07 mV, and -52.04 mV), followed by fullerenes (C₆₀-OH and C₆₀ ζ potentials of -54.02 mV and -39.6 mV), and unfunctionalized nanotubes (SWCNT and MWCNT ζ potentials of 23.07 mV and 22.98 mV). Analysis by ICPMS indicated the presence of 9.49 ppb and 34.6 ppb iron in C₆₀ and C₆₀-OH suspensions, and also the presence of 0.1 ppb strontium in C₆₀ suspensions and 6.88 ppb copper in C₆₀-OH suspensions (Table S1). Nickel was present in all carbon nanotube suspensions. The highest nickel concentration was found in SWCNT (368 ppb), followed by SWCNT-COOH (212 ppb), MWNCT (151 ppb), SWCNT-CONH₂ (60 ppb), and SWCNT-PEG (60 ppb) (Table S2). A sample of the catalyst that was used to synthesize the carbon nanotubes was obtained directly from the manufacturer (Carbon Solutions, Inc. Riverside, CA), and acute and chronic toxicity experiments with this catalyst indicated that it does not influence daphnid mortality, reproduction, and adult size at the concentrations in treatments.

Daphnia cultures

Daphnia magna were obtained from cultures in the Klaper laboratory at the UWM School of Freshwater Sciences and maintained in a 16:8 light/dark cycle at a temperature of 20 °C in moderately hard reconstituted water (MHRW) [16]. Cultures were fed a combination of freshwater algae (*Selenastrum capricornutum*) and alfalfa

(*Medicago sativa*). Adult females were chosen from stock cultures for breeding purposes and maintained in 500 mL beakers at constant population of 1 *Daphnia*/80 mL MHRW.

Multi-generation assays

The F₀ generation daphnids were exposed to 0, 10 and 50 ppm concentrations of carbon nanomaterials obtained from commercial sources and 0, 1, and 5 ppm for those nanomaterials that were synthesized by J. Chen due to limitations in quantity as well as higher toxicity found with a few of these nanomaterials [14]. The maximum concentrations chosen reflected exposure levels that were determined to be sub-lethal, based on a series of LC50 values calculated from acute exposures of *Daphnia* to nanomaterials in previous work in our laboratory [14, 17]. Additional controls with βCD and γCD were conducted to evaluate the potential toxicity of these surface attachments. Five F₁ generation female daphnids were chosen from second or third broods of F₀ daphnids. F₁ daphnids were born in the exposure medium, but were placed in control MHRW within 24 h. Five F₂ generation female daphnids were then chosen from second or third broods of F₁ daphnids. F₁ and F₂ generations of daphnids were raised in control medium (MHRW only) for 21 d with static renewal where medium was replenished 3 times per week.

Mortality and reproductive output were measured during medium 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. Experiments met the mortality and reproduction requirements of controls outlined by the OECD Guidelines for the Testing of Chemicals [18]. Changes in population density and food availability were eliminated with removal

of proportionate volumes of medium and food from the exposures as mortality occurred. Daphnids were kept at a concentration of 1 daphnid per 20 mL medium with a food concentration of 400,000 algal cells/mL of 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

Effects of nanomaterials to daphnid mortality, reproduction, and adult size were compared to controls by *t*-test or by non-parametric Mann-Whitney U tests. The effect of nanomaterials to daphnid mortality, reproduction, and size was compared across treatments within each generation. Values were determined to be significant at $p < 0.05$.

RESULTS

Multi-gen impact of CNMs on mortality

Multi-generational effects on survival were observed for some carbon nanomaterial treatments in F₁ daphnid generations (Figure 2). Of the unfunctionalized nanoparticle types, MWCNTs decreased the survival rate of F₁ daphnids compared to controls (77.2% survival, U=27, $p < 0.05$) (Figure 2A). Although unfunctionalized C₆₀ did not significantly impact survival, some types of surface chemistries were found to increase the toxicity of C₆₀ to *Daphnia*. This includes 10 ppm C₆₀-βCD (84% survival, U=18, $p < 0.05$) and 5 ppm C₆₀-malonate (64% survival, U=18, $p < 0.05$) (Figure 2B). None of these treatments decreased survival of F₀ daphnids in our previous experiment,

however; MWCNTs at the higher 50 ppm concentration were found to decrease F_0 survival [14].

Multi-gen impacts of CNMs on reproduction

Select carbon nanomaterials did have an impact on reproduction up to two generations after parental exposure. Carbon nanomaterial core structure was an important parameter that influenced multi-generational reproduction in daphnids. Of the unfunctionalized carbon nanomaterials, only 50 ppm SWCNTs significantly decreased F_1 daphnid reproduction compared to controls (decrease of 23%, $t = 2.767$, $p < 0.05$) (Figure 3A), but this effect was not observed in the F_2 generation. At a concentration of 50 ppm, C_{60} reduced reproduction by 17% in F_2 daphnids, ($t = -4.137$, $p < 0.05$) (Figure 3B). Finally, 10 and 50 ppm MWCNTs reduced reproduction in the F_1 and F_2 generation, but this was only significant for 10 ppm exposures in the F_2 (decrease of 18%, $t = -2.192$, $p < 0.05$) (Figure 3A and 3B). The addition of COOH or PEG surface chemistry to SWCNT did not change reproduction compared to controls in any generation; however, SWCNT- $CONH_2$ significantly reduced reproduction in both F_1 (decrease of 17%, $t = -6.351$, $p < 0.05$) and F_2 daphnids (decrease of 17%, $t = -3.956$, $p < 0.05$) (Figure 4A and 4B), indicating that this surface chemistry increases the toxicity of SWCNTs to future generations of daphnids.

Some functionalized fullerenes had significant impacts to F_1 and F_2 reproduction. While 10 ppm C_{60} had no significant impacts to multi-generational reproduction, reproduction in the F_1 was significantly decreased by 5 ppm C_{60} -malonate (decrease of 9%, $t = 2.361$, $p < 0.05$) and increased by 10 ppm C_{60} - β CD (increase of 22%, $t = 4.863$, p

< 0.05) (Figure 5A). However, these effects were not found in the F₂ generation (Figure 5B). In addition, impacts of 10 ppm C₆₀-βCD on increased reproduction was not significantly different from 10 ppm C₆₀, indicating that the attachment of βCD to C₆₀ doesn't change the impacts of unfunctionalized fullerenes to F₁ reproduction. Finally, 50 ppm C₆₀-OH decreased F₁ reproduction by 12% ($t = -3.608, p < 0.05$) and increased F₂ reproduction by 10% ($t = 2.336, p < 0.05$).

Multi-gen impact of CNMs on adult size

Carbon nanomaterials also had a marginal multi-generational impact on daphnid size, and this was dependent on core structure and functionalization. At 10 and 50 ppm, C₆₀ significantly decreased F₁ adult size by 5.5% and 4% ($t = -4.083, p < 0.05$ and $t = 3.351, p < 0.05$). For functionalized fullerenes, significant decreases in F₁ size were observed for 10 ppm C₆₀-OH, 5 ppm C₆₀-amino, and 5 ppm C₆₀-malonate (decrease of 4%, 5.8%, and 7%) ($t = 3.036, p < 0.05$; $t = -4.863, p < 0.05$; $t = -6.687, p < 0.05$) (Figure 6A); however, none of these treatments were significantly different from controls in the F₂ generation (Figure 6B). In addition, none of these results are significantly different from F₁ daphnids from C₆₀ exposures, indicating that functionalization with these surface chemistries does not change the toxicity of unfunctionalized fullerenes to F₁ daphnid size. In addition, SWCNT-CONH₂ also significantly decreased F₁ adult size at a concentration of 50 ppm compared to controls (decrease of 5%, $t = -6.439, p < 0.05$). Increases in F₁ size were observed with 10 ppm MWCNTs (increase of 2.8%, $t = 2.374, p < 0.05$) and 10 ppm SWCNT-CONH₂ (increase of 6%, $U=3, p < 0.05$). In the F₂ generation, a decrease in adult size was observed for 10 ppm SWCNT-COOH (decrease of 4.9%, $t = -2.876, p <$

0.05) and increases in size were observed for 50 ppm C₆₀ (increase of 1.7%, $t = 2.003$, $p < 0.05$), 50 ppm MWCNT (increase of 5%, $t = 3.711$, $p < 0.05$), and 10 ppm SWCNT-PEG (increase of 6.5%, $t = 4.401$, $p < 0.05$).

DISCUSSION

Carbon nanomaterials exert a multi-generational effect on *Daphnia* survival, reproduction, and growth, as exposure of an original population of daphnids (F₀) to specific nanomaterials had a consequence for the embryo (F₁), or germ-line (F₂) to nanomaterials. The nature of the effect is dependent upon the core nanomaterial structure and surface functionalization; however, the reason for the observed change in toxicity with specific surface chemistries is unclear. Others have proposed surface charge as playing a large role in toxicity [19, 20], but our data show toxicity associated with positive, negative, and neutral particle types. Similarly, nanomaterials represented here encompass a wide range of stabilities (zeta potentials ranging from -60 mV to +23 mV) and sizes (approximate diameters of 150 nm for fullerene particle types and diameters of several microns for carbon nanotubes) with no clear correlation between aggregation state and multigenerational impacts. It is possible that the interaction of the specific surface chemistry of the nanomaterials that reduced multi-generational reproduction in *Daphnia* led to specific interactions within the daphnid that need to be explored further, such as chemical specific interactions with receptors in the organism [21], environmental or protein coronas that dictate interactions with the organism [22], or differences in genomic impacts of nanomaterials across generations. Results in the present study emphasize the importance of testing for multi-generational impacts of nanomaterials on

sub-lethal endpoints, as the results seen here would not have been evident from single generation assays.

Impacts of CNMs on F₁ and F₂ Reproduction

Carbon nanomaterials impacted reproduction across generations, but this impact was specific to the type of nanomaterial to which the original organism was exposed. F₁ reproduction was decreased by several treatments including 50 ppm SWCNT, SWCNT-CONH₂, and C₆₀-OH, and 5 ppm C₆₀-malonate; and F₂ reproduction was decreased by 50 ppm C₆₀ and SWCNT-CONH₂ treatments. F₁ daphnids are born in the nanomaterial medium and receive an initial exposure to the nanomaterials as neonates, and *Daphnia* neonates are already pregnant when they are born so it is possible that F₂ daphnids also receive an initial exposure to nanomaterials during sensitive developmental stages.

Multi-generational nanomaterial toxicity could be mechanical in nature. The daphnid feeding current has been shown to be diverted to the brood chamber to oxygenate the neonates as they develop [23], and if nanomaterials are present in this feeding current they could disrupt the flow of oxygen and other nutrients to the embryos. The transfer of nanomaterials across the epithelial lining of the parent daphnid digestive tract to lipid storage compartments could also impact F₁ daphnids. Lipid storage compartments are used for sustenance during periods of low food resources and for the synthesis of vitellogenin [24], which is an essential protein required for embryogenesis in *Daphnia*. Many nanomaterials have lipophilic properties, and it is possible for nanomaterials to accumulate in lipid storage compartments of *Daphnia* as they are ingested from feeding with subsequent impacts to vitellogenin synthesis and activity.

Some carbon nanomaterials have already been shown to inhibit protein activity [25] and disrupt membrane transport activities in the cell [26], and these actions could impede normal daphnid reproduction and growth.

Multi-generational reproductive toxicity could also result from changes in gene expression [27]. Nanomaterials have been shown to generate oxidative stress in organisms [28, 29], increase DNA damage [30, 31], and induce immune system activity [32]. Molecular models also indicate the potential for carbon nanomaterials to bind to DNA and alter DNA conformations [33]. Previous work in our laboratory shows differential expression of oxidative stress biomarkers glutathione-s-transferase and catalase in daphnids exposed to carbon nanomaterials [17]. Other types of nanomaterials have also been shown to induce genomic changes in *Daphnia*, as zinc oxide nanoparticles induced differential expression of multicystatin, ferritin, and C1q genes [34]. Changes in gene expression have also been found in *C. elegans* after exposure to silver nanoparticles regarding the expression of SOD and Daf12 genes [35].

Interestingly, reproduction was increased in F₁ daphnids from 10 ppm C₆₀-βCD treatments. It is possible for cyclodextrins to be utilized for additional nutritional value with a consequence of increased reproduction in F₁ daphnids, as daphnids have been shown to utilize lipids that are non-covalently bound to SWCNTs for nutritional value in conditions of starvation [36]. However, reproduction in F₁ *Daphnia* was not increased by βCD treatments alone. In addition, reproduction was not increased in C₆₀ treatments that were non-covalently bound to γCD, as C₆₀-amino-γCD and C₆₀-malonate-γCD were too toxic to F₀ daphnids to conduct multi-generational trials at concentrations higher than 1 ppm. F₁ and F₂ daphnids from SWCNT-PEG treatments also exhibited trends for

increased size and reproduction, and it is possible for daphnids to use PEG attachments for nutritional value in ways similar to the cyclodextrins discussed above.

Impacts of CNMs on F₁ and F₂ Size

Some carbon nanomaterials induced changes to the size of adult *Daphnia*. However, while these changes were statistically significant, they were relatively small in nature, with potentially negligible biological implications to the overall fitness of the individual or population. This is in contrast to the same nanomaterial exposures causing a decrease in adult size in F₀ daphnids of more than 10% for many of these nanomaterials in our previous study [14]. There was a slight decrease in size of F₁ daphnids from 10 and 50 ppm C₆₀ exposures, however; F₂ daphnids exhibited sizes that were comparable to controls, indicating that daphnid populations can recover from this effect. Increased size was observed in F₁ and F₂ daphnids for some nanomaterial types in this experiment, and this could support the idea of a life-strategy shift to produce fewer neonates (reduced number of offspring) of higher quality (larger neonate size) in times of environmental stress [37]. Overall, no nanomaterial treatments impacted size by more than 6.5%, and these results suggest that carbon nanomaterials do not have strong multi-generational impacts to daphnid size.

Potential for Trans-generational Toxicity

The multi-generational toxicity of carbon nanomaterials investigated in this experiment could also be explained by toxic impacts to the daphnid epigenome, suggesting the potential for trans-generational toxicity of nanomaterials. Trans-

generational toxicity is defined as an exposure of a previous generation of organisms to a chemical that induces a change to the germ-line that is propagated to future generations of organisms that never received a direct exposure to the chemical. Epigenetic impacts from DNA methylation and histone modifications have already been shown with other types of toxicants [38]. Changes in the epigenome are heritable and can appear in future generations of organisms even after the exposure is removed [39]. Many of the effects to *Daphnia* that were observed in the nanomaterial treatments across generations disappeared by the F₂ generation, indicating that most of the treatments do not likely have an epigenomic effect on daphnids. However, exposure of daphnids to 50 ppm SWCNT-CONH₂ resulted in changes to reproduction that were consistently decreased across F₀, F₁, and F₂ generations, and it is possible that this exposure could have trans-generational impacts to *Daphnia*. If exposure of *Daphnia* to SWCNT-CONH₂ resulted in epigenetic changes to germ line cells, the effect would be observed in future generations, even after the exposure was removed. Future work will include an evaluation of genetic and epigenetic marks (DNA methylation) of F₃ and F₄ generations to observe whether any patterns arise regarding genetic or epigenetic expression and reduced reproduction for this particle type.

Impacts of CNMs on ecological viability of Daphnia

Daphnids play an essential role in aquatic food webs [40], and a sudden decrease in daphnid population viability over several generations could be detrimental to the balance of an aquatic ecosystem. The results seen in this experiment describe survival and reproductive impacts of some nanomaterial types up to 20% that persisted past the

initial F_0 exposure, and this could have important ecological consequences for population dynamics in natural environments. The present study calls for more detailed information on the types of surface chemistries that may be appropriate for creating nanomaterials that have lower toxicity across generations and are therefore more sustainable. Acquiring toxicity information about how a nanomaterial can influence sensitive early developmental stages of an organism and future generations of organisms is an essential component to understanding the potential ecological impacts of nanomaterials on ecosystems.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation (CBET 1134013) to R. Klaper. Special thanks to individuals who helped with experiments; A. Nikolaus, J. Bozich, N. Neureuther, and B. Blalock. Special thanks also to my committee members who offered expert advice on this research; R. Hutz, A. Udvardia, J. Kaster, and M. Carvan, and my two anonymous reviewers.

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FIGURES

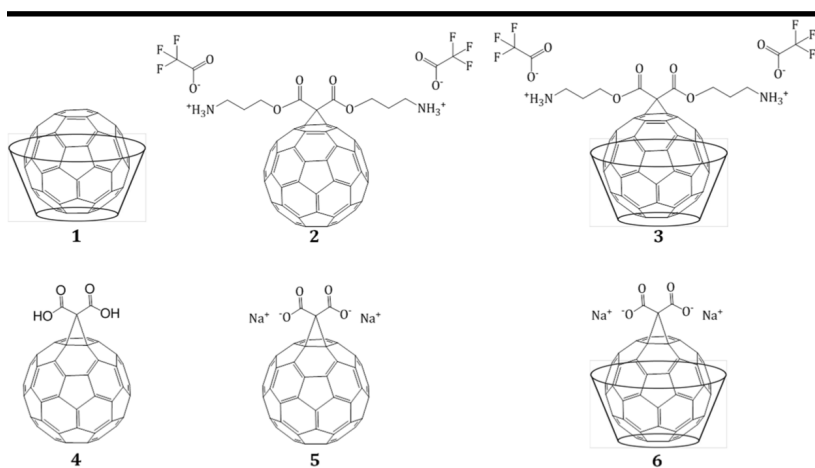


Figure 1 **Fullerene structures synthesized at UWM.** Derivative 1: C₆₀-βCD.

Derivative 2: C₆₀-amino. Derivative 3: C₆₀-amino-γCD. Derivative 4: C₆₀-malonic acid, which was a precursor used to synthesize Derivatives 5 and 6. Derivative 4 was not used for toxicity investigations. Derivative 5: C₆₀-malonate. Derivative 6: C₆₀-malonate-γCD. βCD indicates beta cyclodextrin and γCD indicates gamma cyclodextrin.

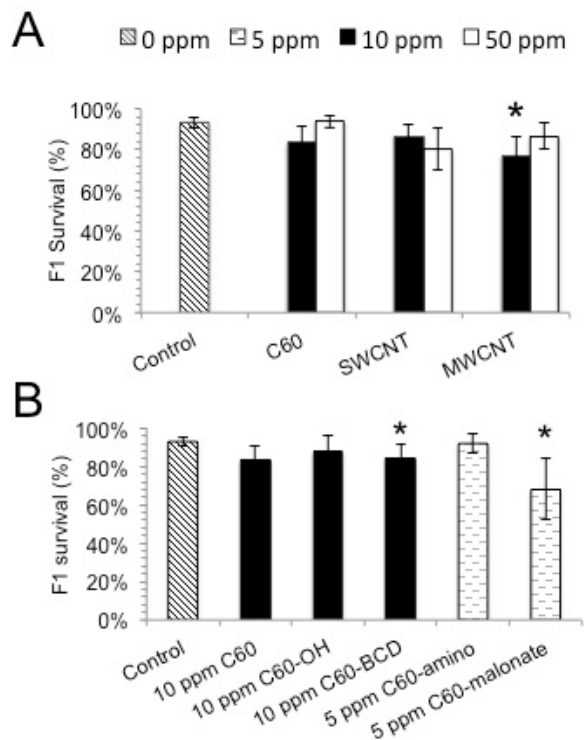


Figure 2 **Multi-generational impacts of carbon nanomaterials on daphnid survival.**

Survival of daphnids after exposure to A) unfunctionalized carbon nanomaterials and B) functionalized fullerenes. Error bars indicate standard error. Values determined to be significant by Mann Whitney U test with $p < 0.05$.

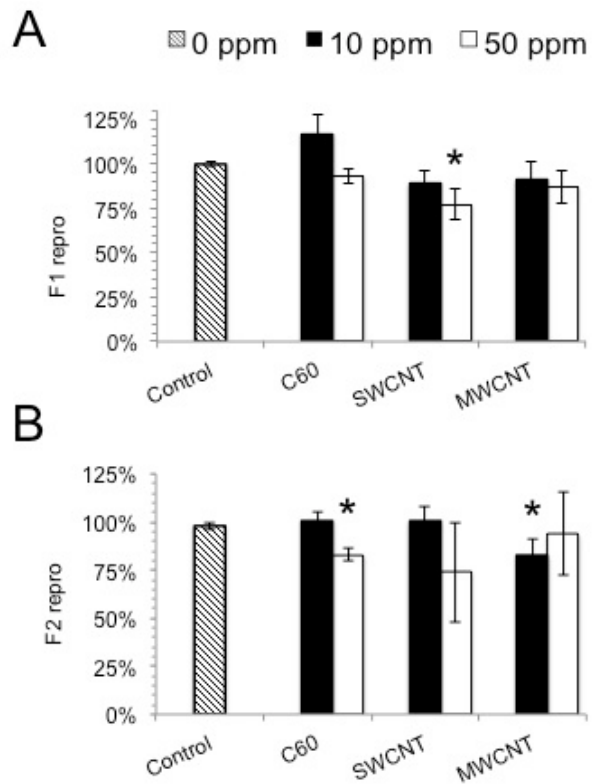


Figure 3 **Reproduction impacts of unfunctionalized carbon nanomaterials** to a) F₁ and b) F₂ generation daphnids. Error bars indicate standard error. Values determined to be significant by *t* test with $p < 0.05$. SWCNT indicates single-walled carbon nanotube and MWCNT indicates multi-walled carbon nanotube.

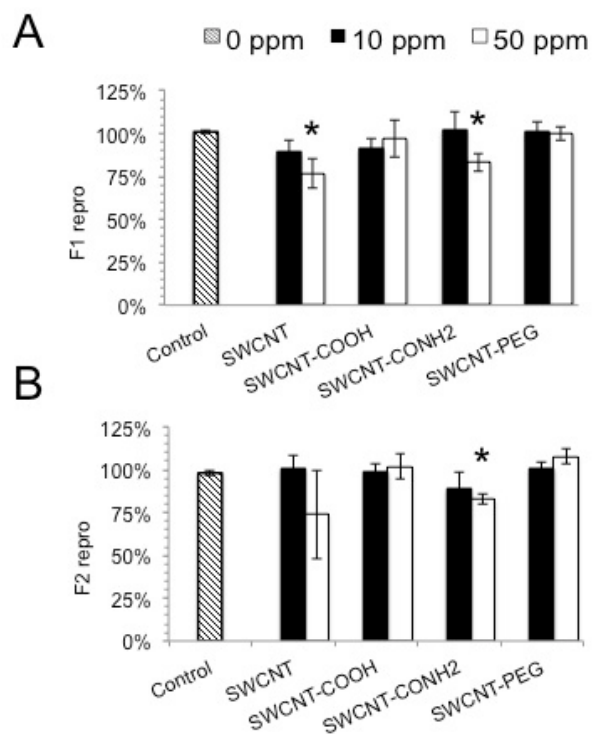


Figure 4 **Reproduction impacts of functionalized single-walled carbon nanotubes (SWCNT) to A) F₁ and B) F₂ generation daphnids.** Error bars indicate standard error. Values determined to be significant by *t* test with $p < 0.05$.

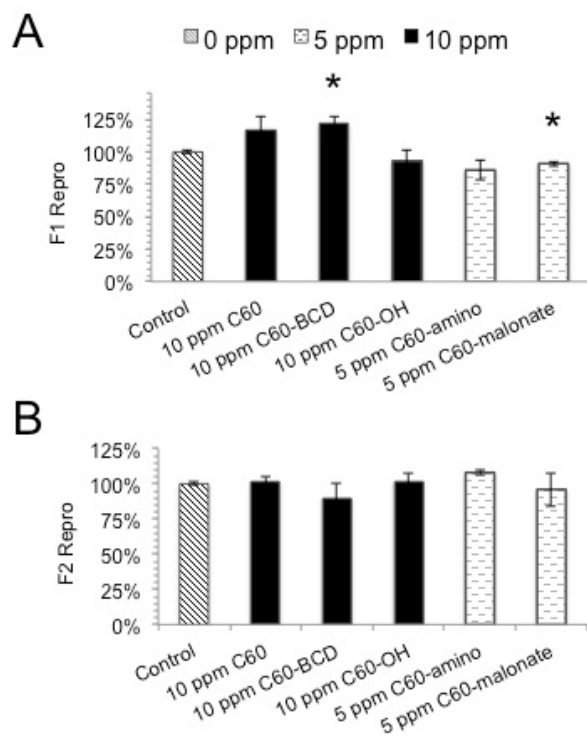


Figure 5 **Reproduction impacts of functionalized fullerenes** to A) F₁ and B) F₂ generation daphnids. Error bars indicate standard error. Values determined to be significant by *t* test with $p < 0.05$.

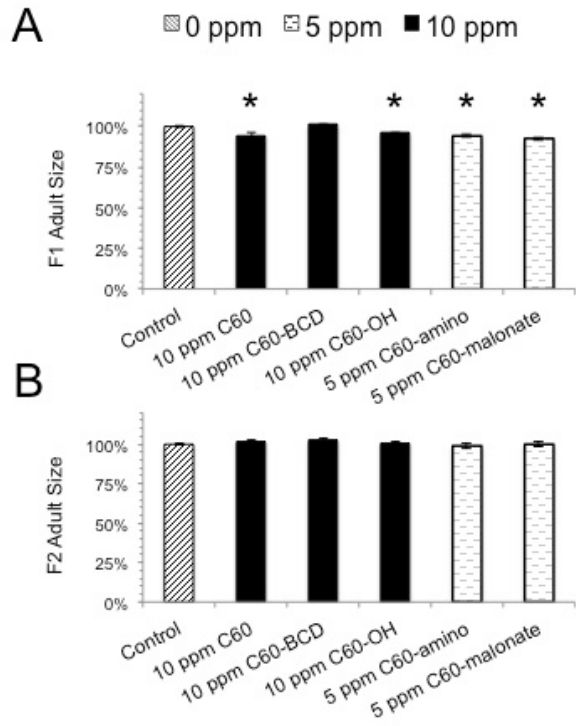


Figure 6 **Size impacts of functionalized fullerenes** to A) F₁ and B) F₂ generation daphnids. Error bars indicate standard error. Values determined to be significant by *t* test with $p < 0.05$.

CHAPTER 3 SUPPLEMENTARY MATERIAL

Multi-generation impacts on *Daphnia magna* of carbon nanomaterials with differing core structures and functionalizations

Supplementary Table 1. Characterization summary for fullerene particle types

CNM type	Diameter (nm)	% CNMs < 110 nm	ICPMS (ppb)	!-potential (mV) (in milli-Q water)	!-potential (mV) (in MHRW)
C ₆₀	141 ± 59	40%	9.49 Fe, 0.1 Sr	-39.6	-32.8
C ₆₀ -OH	144 ± 68	46.5%	34.6 Fe, 6.88 Cu	-54.0	-8.6
C ₆₀ - ^β CD	107 ± 51	67.2%	20.1 Fe, 29.7 Cu	-42.2	-19.6
C ₆₀ -amino	142 ± 73	46%	20.1 Fe, 29.7 Cu	-17.1	-24.6
C ₆₀ -amino- ^γ CD	152 ± 67	36.6%	20.1 Fe, 29.7 Cu	-9.3	-5.85
C ₆₀ -malonate	175 ± 116	30%	20.1 Fe, 29.7 Cu	-63.8	-16.4
C ₆₀ -malonate- ^γ CD	105 ± 40	70.5%	20.1 Fe, 29.7 Cu	-47.9	-20.9

CNM = carbon nanomaterial; C₆₀ = Fullerene; ^βCD = beta cyclodextrin; ^γCD = gamma cyclodextrin; ICPMS = inductively coupled plasma mass spectroscopy; !-potential = zeta potential; MHRW = moderately hard reconstituted water

Supplementary Table 2. Characterization summary for nanotube particle types

CNM type	ICPMS (ppb)	!-potential (mV) (in milli-Q water)	!-potential (mV) (in MHRW)
SWCNT	368.0 Ni	23.1	-19.0
SWCNT-COOH	212.0 Ni	-61.1	-23.8
SWCNT-CONH ₂	158.0 Ni	-52.0	-23.9
SWCNT-PEG	60.9 Ni	-58.1	-23.4
MWCNT	151.0 Ni	23.0	-19.3

CNM = carbon nanomaterials; ICPMS = inductively coupled plasma mass spectroscopy; !-potential = zeta potential; MHRW = moderately hard reconstituted water; SWCNT = single-walled carbon nanotube; COOH = carboxyl; CONH₂ = carboxamide; PEG = polyethylene glycol; MWCNT = multi-walled carbon nanotube

CHAPTER 4

Core structure and surface functionalization of carbon nanomaterials impact gene expression in the freshwater invertebrate *Daphnia magna* in chronic exposures and in a multi-generational study

ABSTRACT

Although the toxicity of carbon nanomaterials on daphnids has been demonstrated, the mechanisms of toxicity are still unclear, and the impact of core structure and surface chemistry on the mechanism of toxicity is also unclear. Some studies indicate that nanomaterials are toxic because of oxidative stress, but it is likely that nanomaterials induce toxicity by other mechanisms, as well. In this project, we evaluated how adverse outcomes from carbon nanomaterials are related to the expression of key genes associated with oxidative stress (glutathione s-transferase; GST), reproduction (vitellogenin fused with superoxide dismutase; VTG-SOD), and energy metabolism (NADH dehydrogenase). We investigated multi-generational changes in the expression of these genes to F₁ and F₂ daphnids. Results show decreased expression of GST in daphnids after exposure to SWCNT and SWCNT-CONH₂. Results also demonstrate increases in GST and VTG-SOD transcripts and decreases in NADH dehydrogenase transcripts associated with daphnid exposure to fullerene-gamma cyclodextrin complexes. Only carboxy-amide functionalized single-walled carbon nanotubes exhibited changes in GST in future generations of daphnids. *Daphnia* are emerging as a promising ecotoxicogenomic model organism, and knowledge of adverse outcome pathways in exposed and future generation daphnids will improve the predictive power of ecotoxicology.

INTRODUCTION

Carbon nanomaterials (CNMs) are an important component in several industries including electronics, medicine, biosensors, and synthetic materials. The successful application of CNMs to various industries requires that they are dispersible and biocompatible, and as a result carbon nanomaterials exist in a variety of shapes with many different surface chemistries. The increasing production rate of carbon nanomaterials elevates exposure potential of these materials to people and organisms. In addition, the toxicity of these materials has been questioned, and the wide array of shapes and surface functionalities of carbon nanomaterials complicates testing for their potential toxicity to biological systems. Several CNMs have been shown to induce toxicity to aquatic and terrestrial organisms [1-4]. In addition, much of the current literature focuses on acute, high-dose studies that fail to capture the more subtle impacts of nanomaterial exposure that are likely to occur in chronic exposure scenarios and multi-generational scenarios where the initial exposure is removed, and where the mechanism of toxicity of nanomaterials is likely to change over longer exposure periods.

Much of the literature indicates that a major cause of CNM toxicity is oxidative stress. CNMs have been shown to generate oxygen radicals in the presence of UV, which can then induce toxicity to organisms and cells [5]. The organism's immune system can also generate reactive oxygen species (ROS) as a natural biological response to get rid of invading pathogens, and studies show that the activation of inflammatory biomarkers is paralleled by activation of biomarkers of oxidative stress and that there are triggered in response to exposure to nanomaterials in aquatic species as well as in other vertebrates [6-8]. However, a majority of these studies investigate the acute, high-dose exposure of

nanomaterials in vitro, while it is more likely that organisms in the environment will be chronically exposed to nanomaterials at lower concentrations that are not acutely toxic. This is problematic, as the induction of oxidative stress can be part of a more global stress response in acute and high-dose scenarios, and oxidative stress can often be an early response of an organism to environmental stress [9]. It is necessary to investigate nanomaterial exposure in chronic scenarios and also in multi-generational scenarios so that more subtle impacts of nanomaterials can be identified.

In the present study, we used *Daphnia magna* to investigate changes in gene expression related to oxidative stress, reproduction, and energy metabolism after a chronic and multi-generational exposure to CNMs. In our previous work, we demonstrated differential toxicity associated with nanomaterials relative to their core structure and functionalization. We found that carbon nanotubes were more consistently toxic to daphnids than were fullerenes, and we found that fullerenes that are noncovalently bound to gamma cyclodextrins were more toxic to daphnids than any other investigated particle type. We also found differential toxicity associated with some nanomaterial types in a multi-generational context. Most notably, single-walled carbon nanotubes functionalized with carboxy-amides reduced daphnid reproduction in both the F₁ and F₂ generations. In the current study, we investigated gene expression of the daphnids used in our previous work. This will help us better understand nanomaterial toxicity in a more detailed, chronic, and multi-generational framework.

Using a targeted Q-PCR approach, we investigated changes in the expression of glutathione-s-transferase (GST), vitellogenin fused with superoxide dismutase (SOD), and NADH dehydrogenase (NADH) in daphnids chronically exposed to various types of

carbon nanomaterials, and also in future generations of daphnids after the initial nanomaterial exposure was removed. GST is involved in the detoxification process, and it has a role in the elimination of hydrogen peroxide radicals [4]. Vitellogenin is an essential yolk protein with an important role in embryo development, and VTG-SOD has been shown to possess a crucial function in the transition of reproduction to diapause in crustaceans [10]. NADH dehydrogenase is important for glycolysis and energy metabolism via the electron transport chain. Hyperactivation of the electron transport chain can also result in the formation of radical species, and changes in the expression of NADH could have implications for oxidative stress as well [11].

The goal of this research was to examine gene expression patterns in low-level chronic exposures to nanomaterials to clarify the role of oxidative stress in chronic exposures, and also to elucidate other mechanisms that are essential for a more comprehensive understanding of nanomaterial toxicity. We investigated how exposures to the F₀ generation impacted gene expression in the F₁ and F₂ generations, and we found that certain nanomaterials with select surface chemistries caused an impact on reproduction and growth in subsequent generations [3]. The objective of this research was to identify the underlying mechanisms of carbon nanomaterial toxicity by investigating the impacts of various types of CNMs on *Daphnia magna* gene expression over a chronic and multi-generational exposure using Q-PCR.

METHODS

***Daphnia magna* cultures and toxicity assays**

Daphnia magna were obtained from Aquatic Ecosystems (Apopka, FL) and were cultured in the Klaper laboratory in moderately hard, reconstituted water [12] at 20 °C in a 16:8 hour light/dark cycle. Neonates less than 24 hours old were used for 21-day chronic toxicity assays. Organisms were placed in 100 mL of control MHRW or nanoparticle exposure medium, and medium was changed three times per week. Reproduction and mortality were evaluated during medium changes. At the end of the 21-day exposure, growth was measured and organisms were flash frozen in liquid nitrogen for RNA extraction. For multi-generational trials, female neonates were sampled from second or third broods of exposed parents (F₀) and transferred to control MHRW. Control medium was replenished three times per week and mortality and reproduction were evaluated during medium changes. Growth of the organisms in each generation was evaluated at the end of the 21-day trial. This was repeated for the F₁ and F₂ generations.

Nanomaterials and characterization

The toxicity of a total of twelve types of carbon nanomaterials with differing core and surface chemistries were investigated in this experiment. Five fullerene derivatives were synthesized at the Chen Laboratory in the Department of Chemistry and Biochemistry at UWM. An amino substituted fullerene derivative (C60-amino) was synthesized according to the literature [13-16] and suspended in one liter of deionized water with one hour of sonication. A disodium malonate derivative of fullerene (C60-malonate) was synthesized by dissolving a malonic acid-derived fullerene in 0.01 M NaOH and then diluting the resulting mixture to one liter with deionized water using one hour of sonication. Grinding fullerene nanopowders with beta cyclodextrins (β CD) or gamma cyclodextrins (γ CD) in

an agate mortar for one hour created three additional types of fullerene derivatives that were investigated for this experiment. This includes a supramolecular complex of C60 with BCD (C60- β CD), C60-amino with γ CD (C60-amino- γ CD), and C60-malonate with γ CD (C60-malonate- γ CD). For more information about the synthesis of these nanomaterials please refer to our previous work [3].

An additional seven types of purified carbon nanomaterials were purchased from a manufacturer and suspended in water upon arrival. This includes unfunctionalized fullerenes (C60) (Alfa Aesar), hydroxylated fullerenes (C60-OH) (MER Corporation), unfunctionalized single-walled carbon nanotubes (SWCNT) (Carbon Solutions Inc.), carboxylated SWCNTs (SWCNT-COOH) (Carbon Solutions Inc.), carboxy-amide functionalized SWCNTs (SWCNT-CONH₂), SWCNTs functionalized with polyethylene glycol (SWCNT-PEG) (Carbon Solutions Inc.), and unfunctionalized multi-walled carbon nanotubes (MWCNTs) (NanoAmor, Inc.).

All of these carbon nanomaterials were characterized by transmission electron microscopy (TEM) for size and surface structure, dynamic light scattering with a Malvern Zetasizer (Worcestershire, UK) for aggregate size distribution and zeta potential, particle tracking with a Nanosight (Wiltshire, UK) to determine aggregate size distributions, and ICPMS with an acid digestion preparation by Pace Analytical (St. Rose, LA) to determine catalyst residue in the suspensions.

RNA extraction, cDNA synthesis, primer design, and Q-PCR

After chronic exposures, daphnids were flash frozen in liquid nitrogen and stored at -80 °C for RNA extraction with a Direct-zol RNA isolation kit (Zymo Research, Irvine, CA).

RNA concentration was measured by Nanodrop and quality analyzed using a Bioanalyzer for degradation. To analyze for differences in gene expression, 500 ng RNA per sample was DNase treated and reverse transcribed to cDNA with Oligo(dT) 15 primers and Superscript III reverse transcriptase (Life Technologies, Grand Island, NY). Primers for Q-PCR (GST, VTG-SOD, and NADH dehydrogenase) were derived from a genetic backbone that was produced by the Klaper laboratory using from 454 sequencing of *Daphnia magna* RNA from a previous experiment (Table 1). Actin was also derived from the genetic backbone, and the actin contig that was used for primer design did not show any changes in expression in response to carbon nanomaterial treatment. Contigs that were used for primer design were blasted against the non-redundant protein database by BLASTX to ensure high identity and query cover with the genes from the database. Q-PCR was conducted using an iTaq SYBR green master mix (Bio-Rad, Hercules, CA) with a primer melting temperature of 62 °C on STEP-ONE Q-PCR real time PCR system and software (Life Technologies, Grand Island, NY). Only daphnids from 50 ppm C60 and 50 ppm SWCNT-CONH₂ treatments were evaluated for multi-generational changes in gene expression, as these treatments resulted in consistent decreases to F₀, F₁, and F₂ reproduction in our previous work [17]. An investigation of multi-generational impacts of other treatments will be included in future work.

Statistical Analysis

Cycle threshold (C_T) values for GST, VTG-SOD, and NADH dehydrogenase were normalized to Actin (delta C_T). The delta C_T value was used for statistical analysis by paired samples t-tests where significance was determined at $p < 0.05$. Data are reported

in the form of fold change, and fold change was calculated by the Livak ($\Delta\Delta C_T$) method [18].

RESULTS

Nanomaterial Characterization

The smallest average diameter was observed with C₆₀-malonate- γ CD particles (105 nm), followed by C₆₀- β CD (107 nm), C₆₀-amino (142 nm), C₆₀-amino- γ CD (152 nm), and C₆₀-amino- γ CD (175 nm). Analysis by ICPMS indicated low levels of iron and copper in the suspensions. The average diameters for C₆₀ and C₆₀-OH were 141 nm and 144 nm, respectively. Average diameters for nanotubes ranged from 800 nm to over 2 microns; however, due to the high aspect ratio of the nanotubes, the size of the aggregates was not uniform, and some aggregate sizes were smaller or larger than these reported average diameters.

The most negative zeta (ζ) potentials were found in C₆₀-malonate and SWCNT-COOH suspensions (-63.8 mV and -61.0 mV). These were followed by SWCNT-PEG (-58.07 mV), C₆₀-OH (-54.02), SWCNT-CONH₂ (-52.04), C₆₀-malonate- γ CD (-47.7 mV), unfunctionalized C₆₀ (-39.6 mV), C₆₀-amino (-17.07 mV), C₆₀-amino- γ CD (-9.26 mV), MWCNT (22.98 mV), and SWCNT (23.07 mV). Suspensions of MWCNT and SWCNT are highly neutral in nature due to the lack of surface functionalization, and it is likely that the positive zeta potentials here reflect positive nickel catalyst residue in the suspensions.

Analysis by ICPMS indicated the presence of 9.49 ppb and 34.6 ppb iron in C₆₀ and C₆₀-OH suspensions, and also the presence of 0.1 ppb strontium in C₆₀ suspensions

and 6.88 ppb copper in C₆₀-OH suspensions. Nickel was present in all carbon nanotube suspensions. The highest nickel concentration was found in SWCNT (368 ppb), followed by SWCNT-COOH (212 ppb), MWNCT (151 ppb), SWCNT-CONH₂ (60 ppb), and SWCNT-PEG (60 ppb). A sample of the catalyst that was used to synthesize the carbon nanotubes was obtained directly from the manufacturer (Carbon Solutions, Inc. Riverside, CA), and acute and chronic toxicity experiments with this catalyst indicated that it does not influence daphnid mortality, reproduction, and adult size at the concentrations in treatments. For a more complete description of nanomaterial characteristics, please refer to our previous research [3, 17].

GST expression

Levels of GST were increased or decreased in response to treatment with various types of CNMs. At a concentration of 50 ppm, SWCNTs decreased GST expression by 7.91 fold (df = 5, p < 0.05) and SWCNT-CONH₂ decreased GST expression by 3.52 fold (df = 15, p < 0.05) (Figure 1a). Although unfunctionalized fullerenes did not change the expression levels of GST at any of the tested concentrations, fullerenes that were functionalized with γ CD increased GST expression in daphnids. At a concentration of 1 ppm, C60-malonate- γ CD increased GST expression by 3.23 fold (df = 3, p < 0.05) (Figure 1b). GST expression was also increased 1.74 fold by 1 ppm C60-amino- γ CD, but this was not found to be significant.

Only 50 ppm SWCNT-CONH₂ was found to have a multi-generational impact on GST expression. This particle type decreased GST expression in the F₂ generation by

3.34 fold ($df = 3$, $p < 0.05$). None of the other treatments exerted a multi-generational change in expression of GST in daphnids.

VTG-SOD expression

VTG-SOD expression was significantly increased by two treatments in F_0 generation daphnids. At a concentration of 1 ppm, C60-amino- γ CD increased VTG-SOD expression by 39 fold ($df = 5$, $p < 0.05$) and C60-malonate- γ CD increased VTG-SOD expression by 30 fold ($df = 3$, $p < 0.05$) (Figure 2). None of the other treatments altered VTG-SOD expression, and expression of VTG-SOD was not changed in F_1 or F_2 generation daphnids.

NADH dehydrogenase expression

Expression of NADH dehydrogenase did not change appreciably in daphnids exposed to any of the investigated particle types. Only one treatment, 1 ppm C60-malonate- γ CD, significantly decreased NADH dehydrogenase expression 2 fold ($df = 4$, $p < 0.05$) (Figure 1a). Expression of NADH dehydrogenase was also decreased by 5.2 fold in daphnids exposed to 50 ppm SWCNT (Figure 3b), but this was not found to be significant due to large amounts of variation.

DISCUSSION

In order to better understand overall nanomaterial toxicity, it is essential to understand the underlying mechanisms of nanomaterial toxicity to organisms at lower doses and over longer exposure periods. Current literature demonstrates that

nanomaterials can be toxic at high concentrations over shorter durations of exposure. However, it is likely that organisms will receive an extended exposure to lower concentrations of nanomaterials in the environment, and while there are some data on the chronic and sub-lethal impacts of nanomaterials to organisms, there are few data available on the mechanisms of toxicity for these more environmentally realistic exposure scenarios. In addition, it is important to understand the potential lasting impacts of nanomaterial exposure to organisms after the exposure is removed, and there are no data on the multi-generational impacts of nanomaterials to whole organisms after the exposure has been removed.

In our previous work, we demonstrated that core structure and functionalization can influence nanomaterial toxicity in chronic exposure scenarios [3] and multi-generational scenarios where the initial exposure has been removed. In the present study, we investigate potential underlying mechanisms of chronic and multi-generational nanomaterial toxicity to *Daphnia*. Overall, nanomaterials that elicited a stronger response to daphnid mortality, reproduction, and growth in previous work also induced changes in the expression of at least one of the investigated genes (GST, VTG-SOD, and NADH dehydrogenase), indicating the potential for using these genes to identify environmental stress in organisms from nanomaterial exposure.

GST expression

Several nanomaterials induced changes to the expression of GST, indicating that GST expression might be a sensitive biomarker even across generations and may indicate broader changes in physiology of daphnids in response to chronic exposures to

nanomaterials. GST is a cyto-protective enzyme that becomes elevated to free a cell or an organism from oxygen radicals or other harmful materials. In the F₀ generation, reproduction and adult size were significantly reduced by functionalized and unfunctionalized SWCNTs. Here we show that expression of GST was decreased by SWCNT and SWCNT-CONH₂ treatments, indicating that the toxicity of these particle types could be partially attributed to oxidative mechanisms. It is possible that SWCNT and SWCNT-CONH₂ particle types decrease the daphnid's capacity for cyto-protection by anti-oxidant pathways, resulting in a decreased expression of GST mRNA levels. Decreases in GST expression have been observed in female bullheads after exposure to persistent organochlorines, and this was found to be related to altered sex steroid homeostasis [19]. It is also possible that SWCNT and SWCNT-CONH₂ interfere with algae or with the *Daphnia* gut in ways that decrease algal uptake or algae nutrient availability for *Daphnia*, resulting in decreased levels of GST expression and an impairment to the *Daphnia*'s antioxidant defense system. There is evidence that GST and catalase enzymatic activity in *Daphnia commutata* are reduced when *Daphnia* are fed with lower quality food [20].

Expression of GST was significantly increased by 1 ppm C60-malonate- γ CD, indicating that this particle type can also induce toxicity to an organism by an oxidative stress mechanism. It has been previously shown that GST activity can be induced by cadmium exposure in *Daphnia* [21], and GST enzymatic activity has been increased in *Daphnia* after exposure to other types of nanomaterials, like TiO₂ [22]. However, not all nanoparticle types induce GST expression and activity, as the other particle types in this experiment did not induce GST expression. Literature also illustrates that some

nanomaterials do not induce GST expression, as silver nanoparticles were not found to induce GST expression in *Daphnia* [23]. This indicates that the induction of GST expression by C60-malonate- γ CD is specific to the particle type and functionalization.

Decreases in the expression of GST were also observed in F₁ and F₂ daphnids from 50 ppm SWCNT-CONH₂ exposures, and this was found to be significant in F₂ daphnids. In previous work, we demonstrated that reproduction was significantly decreased by exposure of daphnids to 50 ppm SWCNT-CONH₂, and this effect was observed in F₁ and F₂ daphnids after the exposure was removed [3, 17]. It is possible that GST expression is decreased to the F₂ generation by an epigenetic mechanism that could be transferred to future generations of organisms. It has been demonstrated that CpG hypermethylation of the GST-M2 promoter leads to decreased Sp1 binding with reduced transcription of GST in lung cancer cells [24].

VTG-SOD expression

Expression of VTG-SOD was significantly increased by 1 ppm C60-amino- γ CD and 1 ppm C60-malonate- γ CD. The response was strong, with an increased fold change of more than 30 for both treatments. This is particularly noteworthy, as these particle types were the most toxic to F₀ daphnids in our previous work [3]. After a chronic exposure, C60-amino- γ CD and C60-malonate- γ CD induced 100% mortality to daphnids at 5 ppm. When the exposure concentration was dropped to 1 ppm, daphnids survived the chronic exposure with minimal impacts on reproduction and size (only 1 ppm C60-amino- γ CD resulted in a significant decrease in reproduction, and neither particle type impacted size). Vitellogenin is a major yolk protein found in most females that lay eggs, including vertebrate and invertebrate species. It supplies the oocyte with essential

nutrients that are required for normal embryo development and maturation [25]. In recent literature, a superoxide dismutase domain was discovered at the N-terminus of the vitellogenin gene for some crustaceans, including brine shrimp and *Daphnia*. In brine shrimp, this domain has been implicated in the reproductive switch to embryonic diapause and the formation of diapause embryos [10]. In *Daphnia*, this domain is hypothesized to be important for the detoxification of superoxides that are generated specifically from vitellogenin metabolism [26]. Therefore the large increase in VTG-SOD transcripts that are observed in C60-amino- γ CD and C60-malonate- γ CD exposed *Daphnia* from this experiment could be indicative of the *Daphnia* transitioning to diapause embryo formation, and increases in VTG-SOD transcripts could also be indicative of oxidative stress.

NADH dehydrogenase expression

Expression of NADH dehydrogenase was decreased in *Daphnia* after exposure to 1 ppm fullerene- γ CD complexes, but this was only found to be significant for C60-malonate- γ CD and not for C60-amino- γ CD. A primary function of NADH dehydrogenase is energy transduction in mitochondria by the oxidation of NADH to NAD⁺. It is known that a decrease in mitochondrial oxidative phosphorylation can lead to the production of superoxide anion radicals, and of the five complexes involved in oxidative phosphorylation, NADH dehydrogenase (complex I) is one of the complexes that can generate superoxide anion radical species [27]. Therefore, decreased expression of NADH dehydrogenase transcripts in C60-malonate- γ CD exposed *Daphnia* could indicate a disruption in the electron transport chain and energy metabolism pathways, with potential consequences for increased production of reactive oxygen species and

oxidative stress. In addition, NADH dehydrogenase has been found to be down-regulated by other chemicals, including pesticides, neurotoxins, and heavy metals like cadmium [21, 28].

The differential regulation of GST, VTG-SOD, and NADH dehydrogenase by different types of carbon nanomaterials indicates that core structure and functionalization play an important role in the toxic mechanism associated with these various particle types. Although many particle types reduced daphnid reproduction and growth in our previous experiments, only a fraction of the *Daphnia* exposed to these particle types exhibited changes in the expression of these investigated genes, indicating the probability of other mechanisms of toxicity to be important for many of these particle types. In addition, exposure of daphnids to fullerene- γ CD complexes resulted in increases in GST and VTG-SOD expression and a decrease in NADH-dehydrogenase expression, and this reinforces the higher toxicity of these particle types that was observed in previous work [3]. It is possible that these genes could be used to make up a suite of biomarkers to detect early exposure of organisms to these carbon nanomaterial types. However, it appears that there are unique gene expression signatures with distinct nanomaterial types, and the use of these genes as biomarkers cannot be universally applied to all particle types, as the investigated carbon nanomaterials did not elicit a universal response in GST, VTG-SOD, and NADH dehydrogenase expression in *Daphnia*. This is also the case for other types of nanomaterials and biochemical pathways [9].

This research offers some of the first data on the impacts of nanomaterials to gene expression in long-term exposure and multi-generational scenarios, and it provides a more comprehensive understanding of carbon nanomaterial toxicity and potential

mechanisms associated with nanomaterial toxicity. These data can be used to influence nanomaterial design so as to maximize the application of nanomaterials in industry, while also minimizing potential harms to the environment.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation (CBET 1134013) to R. Klaper. Special thanks to individuals who helped with experiments; Jared Bozich and Nicholas Niemuth. Special thanks also to my committee members who offered expert advice on this research; R. Hutz, A. Udvardia, J. Kaster, and M. Carvan, and my two anonymous reviewers.

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Table 1: Q-PCR Primers designed from contigs from genetic the backbone generated from 454 sequencing of *Daphnia magna* samples.

Gene	Forward	Reverse
GST	GTGACAACATGAAACCCGAATAC	CCATACGCGTTGACCAGATAA
VTG-SOD	TCGCAATGCCACCATCAA	TCTCGACAGTGATCTGGTTCT
NADH dehydrogenase	GCAGGAAACAATAAGGCAAACC	GGTGGCACAGACCATTCTTA

CHAPTER 4 FIGURES

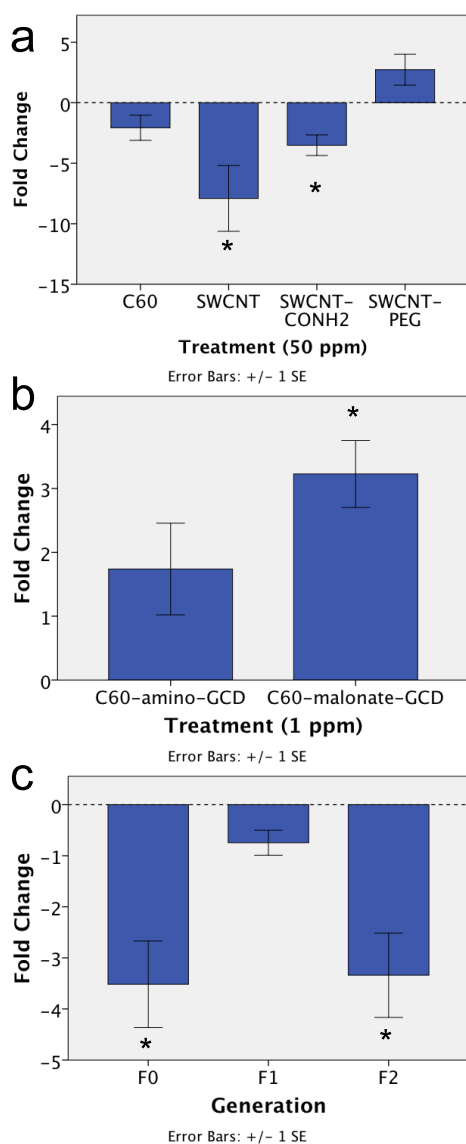


Figure 1 **Change in expression of GST** in a) F₀ *Daphnia* exposed to 50 ppm C60, SWCNT, SWCNT-CONH₂, and SWCNT-PEG, b) F₀ *Daphnia* exposed to 1 ppm C60-amino-γCD and 1 ppm C60-malonate-γCD, and c) F₀, F₁, and F₂ *Daphnia* from 50 ppm SWCNT-CONH₂ exposures. Data expressed as fold change by the delta delta C_T method and paired samples t tests. Values determined to be significant at p < 0.05.

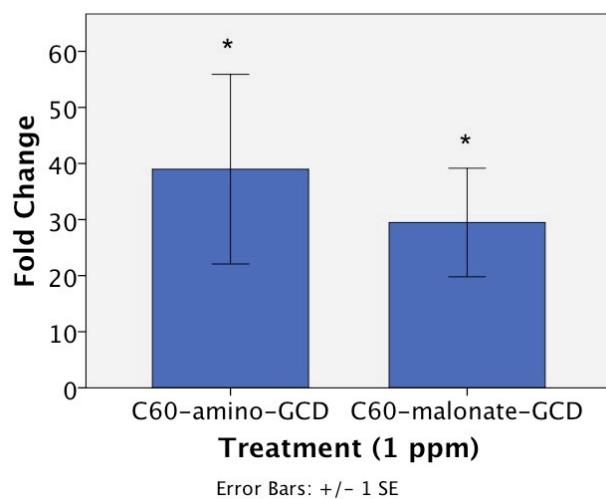


Figure 2 **Change in expression of VTG** in *F₀ Daphnia* after exposure to 1 ppm C60-amino-γCD and C60-malonate-γCD. Data expressed as fold change by the delta delta C_T method and paired samples t tests. Values determined to be significant at $p < 0.05$.

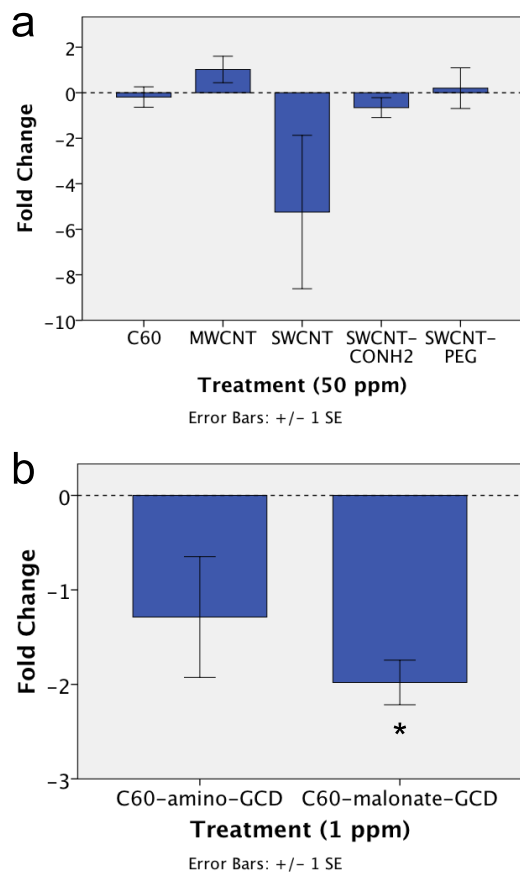


Figure 3 **Change in expression of NADH dehydrogenase in *F₀ Daphnia* after exposure to a) 1 ppm C60-amino- γ CD and C60-malonate- γ CD and b) 50 ppm fullerenes and carbon nanotubes. Data expressed as fold change by the delta delta C_T method and paired samples t tests. Values determined to be significant at $p < 0.05$.**

Chapter 5 DISCUSSION AND CONCLUSIONS

The experiments carried out in this investigation provide data that are essential for obtaining a comprehensive understanding of nanomaterial toxicity to aquatic organisms. Current literature in nano-toxicology is heavily biased towards the impacts of the acute toxicity of nanomaterials to biological systems at high concentrations, using mortality or cell viability as the key endpoint to measure toxicity [1, 2]. However, exposure of organisms to nanomaterials in the environment is more likely to happen over longer exposure periods and at lower doses that do not induce acute mortality.

This research investigated the chronic and multi-generational toxicity of nanomaterials to organisms, and it included reproduction, adult size, and gene expression changes as additional endpoints of toxicity in chronic and multi-generational trials. Negative impacts to these endpoints can damage organism fitness and population dynamics, with negative implications for overall ecosystem health. A reduction in daphnid reproduction can result in an overall decline of the daphnid population, while a change in daphnid size can alter the population structure of *Daphnia* in freshwater ecosystems, with subsequent impacts to the composition of phytoplankton communities and negative impacts to predators in higher trophic structures that rely on daphnids for food. In addition, changes in gene expression can precede physiological changes that would be observed in whole organism toxicity assays. Genes that are identified to be important in the *Daphnia* response to nanomaterials could be used as biomarkers of early exposure of organisms to nanomaterials, and knowledge of relevant changes in gene expression

can help scientists and engineers better understand the underlying mechanisms of nanomaterial toxicity [3].

Ecological implications of CNMs on Daphnia populations

Population growth rate is one of the most robust endpoints that can be used to measure toxicity as the population level; and mortality, reproduction, and growth parameters are basic measurements that can be used to describe population growth. Reproduction and growth are inextricably linked, as reproduction can only begin after puberty is reached [4], and smaller sized daphnids have lower reproduction rates compared to larger daphnids due to energetic constraints [5]. Results from this work indicate that carbon nanotubes could have a greater toxic effect on daphnid reproduction and growth compared with the fullerenes (with the exception of C60- γ CD complexes), resulting in populations that are made up of fewer, smaller individuals.

A reduction in the body size of individuals in a population can lead to cascading effects in other trophic levels of the aquatic ecosystem. *Daphnia* are selective grazers of phytoplankton communities, and they can select their food based on algal size and quality [6]. Smaller daphnids might consume algae with different selection tendencies than larger daphnids [7], thereby altering the phytoplankton communities of an ecosystem compared to a population of larger or mixed size daphnids. In addition, *Daphnia* are a preferred food source for juvenile and adult fish, as well as a primary food source for many invertebrates. Larger daphnids are more susceptible to predation by fish, whereas smaller daphnids are

more susceptible to predation by invertebrates [5]. Therefore a daphnid population composed of mostly smaller individuals due to carbon nanotube exposure would provide a more abundant food source for aquatic invertebrates, while also decreasing available nutrition for fish with possible implications for an increase in aquatic invertebrate populations and a decrease in planktivorous fish populations in an ecosystem.

A decrease in the population growth rate to the point where daphnid populations are eliminated from an aquatic ecosystem would also have harmful effects on an aquatic ecosystem. Potential adverse outcomes include the eutrophication of small lakes and ponds, and the loss of a food source for higher trophic structures of the ecosystem. Exposure of daphnids to 5 ppm C60- γ CD complexes resulted in 100% mortality to daphnids after 17 days, indicating that it is possible for exposure of daphnid populations to C60- γ CD complexes to result in the above described adverse outcomes.

Although population growth rate is frequently derived from mortality, reproduction, and growth endpoints after a chronic exposure of daphnids to a pollutant, some scientists argue that these parameters have little ecological relevance. Organisms are particularly vulnerable to toxic exposure during prenatal and perinatal life stages [8], and chemicals that do not appear toxic to juvenile and adult organisms could be toxic to developing embryos. It has been proposed that measuring the intrinsic growth rate has more ecological relevance, as this experimental method can better illustrate impacts of a pollutant on age dependent mortality, reproduction, and development. One of the ways to measure the intrinsic

growth rate is to measure neonate fitness (measure reproduction and growth in next generations of daphnids in the absence of pollutant exposure) [9]. The experiments described in chapter three of this work investigate neonate fitness in F₁ and F₂ generations in the absence of nanomaterial exposure, and results from this chapter indicate that most of the investigated carbon nanomaterials do not have a significant multi-generational impact on daphnids. However, reproduction was reduced in F₁ and F₂ daphnids from C60 and SWCNT-CONH₂ treatments, and this was significant for both F₁ and F₂ generations from SWCNT-CONH₂ treatments. This indicates that these particles types might have a more significant negative impact on the intrinsic population growth rate than originally explained based on chronic toxicity data alone.

Chapter four investigated changes in gene expression that could demonstrate how an organism responds to nanomaterial exposure. Fullerene- γ CD complexes were highly toxic to daphnids at 5 ppm; however, when the concentration was dropped to 1 ppm, few toxic impacts were observed to *Daphnia* mortality, reproduction, and growth. Despite the absence of changes to reproduction and growth parameters, expression of GST and VTG-SOD were increased in C60- γ CD exposed daphnids and expression of NADH dehydrogenase were decreased in C60- γ CD exposed daphnids, indicating that these genes are good predictors of C60- γ CD induced toxicity to *Daphnia*. In addition, GST transcripts are expected to be elevated in organisms undergoing a response to oxidative stress; however, GST expression was decreased in SWCNT and SWCNT-CONH₂ exposed *Daphnia*. This indicates that SWCNT and SWCNT-CONH₂ could impair the oxidative stress mechanism in

Daphnia. The absence of a change in expression of GST, VTG-SOD, and NADH dehydrogenase in daphnids exposed to other carbon nanomaterials indicates that other mechanisms of toxicity, including mechanisms other than oxidative stress, are important in the daphnids response to chronic and multi-generational nanomaterial exposure. This is an important finding, as many studies indicate oxidative stress to be a primary mechanism of toxicity of nanomaterials [10-12].

Recommendations to minimize toxic effects of CNMs on aquatic ecosystems

The experiments described in chapter two, three and four illustrate some important findings for carbon nanomaterial toxicity. First, acute toxicity assays are not good predictors of impacts of chronic and multi-generational nanomaterial exposure. Second, core structure and surface functionalization have an important role in the toxicity of carbon nanomaterials to organisms, and this is true for chronic exposures and for multi-generational exposure scenarios where the initial exposure is removed. Third, carbon nanomaterials induced different changes to the expression of GST, VTG-SOD, and NADH dehydrogenase, indicating nanomaterials can influence its toxicity to organisms by different mechanisms depending on the particle type. Based on these findings, the toxicity of carbon nanomaterials can be reduced by manipulating the particles to have core structures and surface chemistries that minimize toxicity to organisms.

Gamma cyclodextrins are commonly used as drug solubilizers and drug carriers [13]; however, C60- γ CD complexes induced significant toxicity to F₀ daphnids. The use of this particle type in the medicine industry should be avoided

until its toxicity to aquatic organisms is better understood. Scientists are designing other types of fullerene derivatives for drug directed delivery and anti-cancer therapy applications [14, 15], so it is possible to continue to explore the application of other, less toxic fullerene derivatives in medicine.

Carbon nanotubes induced more consistent decreases to reproduction and growth in F_0 daphnids compared with fullerenes. Carbon nanotubes have properties that are unique from fullerenes due to their core structures and high aspect ratios, and replacing nanotube applications with fullerenes would reduce the potential usefulness of their application. However, carbon nanotube toxicity was found to vary in daphnids with respect to specific surface chemistry, indicating that carbon nanotubes can be functionalized to reduce their toxicity to living systems. Unfunctionalized SWCNT and SWCNT-COOH particle types induced more toxicity to F_0 reproduction and growth compared to other functionalized single-walled carbon nanotubes. It is possible that unfunctionalized SWCNT induced higher toxicity to *Daphnia* because this particle type is not compatible in biological media. In addition, SWCNT-COOH particle types are more likely to have amorphous carbon due to oxidation, and amorphous carbon is more toxic to organisms. Functionalization of carbon nanotubes to make them more biocompatible might decrease the general toxicity associated with unfunctionalized particles, but caution needs to be used regarding surface chemistry to reduce potential adverse impacts from oxidation of the nanotubes.

Exposure of F_0 daphnids to SWCNT-CONH₂ decreased multi-generational reproduction out to the F_2 generation. This function group (-CONH₂) is similar to

urea, which is commonly found in fertilizer and contributes to the eutrophication of coastal wetlands and other aquatic habitats. In addition, there is evidence that urea can induce colony formation in *Scenedesmus*, resulting in grazing protection of the algae from *Daphnia* [16]. Perhaps CONH₂ functionality results in change in the algal environment or nutrient quality of the algae that results in higher toxicity to *Daphnia* that can be propagated to future generations of organisms. The expression of GST was significantly down-regulated in F₀ and F₂ daphnids, indicating that exposure of daphnids to this particle type can impair the anti-oxidant defense system.

Based on results from this work, functionalization of carbon nanotubes with COOH and CONH₂ should be avoided. However, functionalization of SWCNTs with PEG induced the least negative impacts to parental and multi-generational reproduction and growth. Functionalization with PEG makes SWCNTs more biocompatible for applications in medicine, and this particle type is also being studied for cancer therapy applications [17]. SWCNTs should be functionalized to improve their biocompatibility in biological systems, but the type of functionality should be carefully considered before use in industrial applications, as carbon nanotube toxicity varies greatly with differing surface chemistries.

Environmental fate of CNMs and implications for toxicity

This research indicates that carbon nanomaterials are toxic to aquatic organisms at concentrations in the ppm range. This is significantly higher than estimated concentrations of engineered carbon nanomaterials in the environment

[18]. However, carbon nanomaterials are expected to be more persistent in the environment, and it is possible that the accumulation of carbon nanomaterials in the environment could reach higher concentrations that could induce toxicity to aquatic organisms that are described in this work. In addition, the carbon nanomaterials can be transformed in the environment upon exposure and interaction with environmental components like natural organic matter, proteins, suspended solids, UV light. This can lead to changes in particle characteristics like aggregation state and hydrophobicity, which can alter toxicity and make carbon nanomaterials more or less toxic to organisms [19]. This work offers preliminary assessment of carbon nanomaterial toxicity to *Daphnia*, but more work needs to be done to elucidate how the toxicity of carbon nanomaterials will change after transformation and degradation in the environment.

Summary and conclusions

This research provides an in depth and comprehensive description of chronic and multi-generational nanomaterial toxicity to *Daphnia*. A comprehensive understanding of nanomaterial toxicity is essential for the establishment of a more effective regulatory framework for nanomaterials, so nanomaterials can be synthesized in ways that minimize the potential harms they pose to organisms and the environment. Despite the demonstrated advantages of the use of nanomaterials for many applications, the data reported in this research indicates that carbon nanomaterials can induce chronic and multi-generational toxicity to aquatic

organisms and that nanomaterial toxicity is highly specific to particle type, making it difficult to draw generalizations about carbon nanomaterial toxicity.

Chapter two and three are published in peer-reviewed scientific journals, and chapter two has already been cited in two additional peer-reviewed journal articles [20, 21]. In addition, the results from this research have been presented at multiple local, national, and international conferences, including the National Society of Environmental Toxicity and Chemistry (SETAC), the International Conference on the Environmental Effects of Nanoparticles and Nanomaterials (ICEEN), and the National Conference for the Society of Toxicology (SOT). This research emphasizes the importance of acquiring sub-lethal toxicity data for nanomaterial toxicity to better inform the industry and regulators.

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DEVRAH ARNDT

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Research Interests

My research examines the impacts of nanomaterial exposure on freshwater ecosystems through evaluation of life cycle and genomic changes in *Daphnia magna*. This information will identify early signs of nanoparticle-induced stress and discover genetic bio-markers which can provide a sensitive assay for determining environmental stress.

Education

University of Wisconsin – Milwaukee, School of Freshwater Sciences; Ph.D. Freshwater Sciences degree expected December 2013. GPA 3.9/4.0
University of Wisconsin – Madison; B.S. Biology and Anthropology; May 2006. GPA 3.3/4.0

Positions and Employment

Ph.D. Graduate Research Assistant (2009 to present); UW – Milwaukee School of Freshwater Sciences.
 Dissertation Topic: “Carbon nanomaterials in freshwater ecosystems: A chronic, multi-generational, and genomic assessment of nanomaterial toxicity to *Daphnia magna*.”
Research Specialist (Sept. 2006 to Aug. 2009); UW-Milwaukee WATER Institute, Milwaukee, WI.
 Investigated the impact of oxidative stress on heart development and function in fathead minnow embryos. Tested impacts of nanoparticle exposure to rainbow trout primary macrophage cell cultures.
Research Technician (May 2006 to Sept. 2006); Invitrogen; Brown Deer, WI.
 Conducted Q-PCR and ELISA’s for new assay development.
Undergrad Research Assistant (May 2005 to Sept. 2006); UW-Madison Agronomy Dept., Madison, WI.
 Supported research on impacts of manure application to soil compaction and weed pressure in cash grain farms. Gained experience with soil and weed surveys, and identification of mid-western invasive species plants.
International Student Conservation Intern (June 2004 to July 2004); University of California-Davis, Davis, CA and Action Stations, Papamoa, New Zealand.
 Contributed to a project to optimize watershed management through assessment of cockle-shell populations. Planted endemic vegetation in a local wetland to support a local wetland restoration project. Worked with eco-tourism companies to learn about endangered species preservation, habitat restoration, and sustainable management of natural resources.

Publications

- (1) Klaper R, Arndt D, Bozich J, Dominguez G. 2013. Molecular Interactions of Nanomaterials and Organisms: Defining Biomarkers for Toxicity and High-Throughput Screening Using Traditional and Next-generation Sequencing Approaches. *The Analyst*.
- (2) Arndt D.; Lor, M.; Chen, J., Klaper, R., Multi-generation impacts of carbon nanomaterials with differing core structures and functionalizations on *Daphnia magna*. *In review* **2013**.
- (3) Arndt D.; Lor, M.; Chen, J.; Klaper, R., Variations in core structure and surface functionalization of carbon nanomaterials alter impacts to daphnid mortality, reproduction, and adult size: Acute assays do not predict chronic exposure impacts. *Environmental Science & Technology* **2013**. Doi 10.1021/es4030595
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Honors and Awards

2013	Midwestern SETAC 2013 Travel Award	(\$250.00)
2012	Water Tech of America Scholarship	(\$700.00)
2012	UWM Graduate Student Travel Award for National SETAC conference	(\$450.00)
2012	ICEENN Travel Award (Banff, CA)	(\$500.00 CAD)
2012	UWM Graduate Student Travel Award for ICEENN conference	(\$450.00)
2011	UWM School of Freshwater Sciences Fellowship	(\$22,700.00)
2011	Charles A. and Anne Morrow Lindbergh Grant	(\$10,580.00)
2010	National 2010 SETAC Conference Travel Award	(\$335.00)

Skills and Techniques

RNA extraction and purification, cDNA synthesis, Real-time PCR, primer design, transmission electron microscopy, cell viability assays, scanning electron microscopy, primary cell cultures, OECD Testing the Toxicity of Chemicals Guidelines (1998), daphnid, algae, and fish culture maintenance, fish dissection, Microsoft Office (Word, Powerpoint, Excel), Sigma Plot, SPSS Statistics Version 20, Endnote X4, Photoshop, BLAST, Public Speaking, Grant Writing

Grants

Arndt, D., Evaluating the short and long-term effects of manufactured nanomaterials on freshwater ecosystems. In Charles A. and Anne Morrow Lindbergh Association: Milwaukee, WI, 2011; Vol. \$10,580.00.

Presentations

- Devrah Arndt**, Maika Moua, Jian Chen, Rebecca Klaper. Impacts of acute and chronic nanomaterial exposure on the genomic response of *Daphnia magna*. Nashville, TN. November 17-21, 2013.
- Devrah Arndt** and Rebecca Klaper. Impacts of acute and chronic nanomaterial exposure on the genomic response of *Daphnia magna*. La Crosse, WI. March 19-20, 2013.
- Devrah Arndt** and Rebecca Klaper. Part I: Toxicity and the Great Lakes. Part II: Nanomaterials and Toxicity. Milwaukee Sailing Club, Milwaukee, WI. *December 11, 2012*.
- Devrah Arndt**, Maika Lor, Jian Chen, Rebecca Klaper. Impacts of structure and functionalization of carbon nanotubes on the toxicological and genomic response of *Daphnia magna*. North American Society of Toxicology and Chemistry (SETAC) Conference, Long Beach, CA. *November 12-15, 2012*. (poster)
- Devrah Arndt**, Maika Lor, Jian Chen, Rebecca Klaper. The chemistry of sustainable nanotechnology and impacts of nanomaterial chemistry on *Daphnia magna*: From population to molecular responses. Sustainable Nanotechnology Organization (SNO) Conference. Arlington, Virginia. *November 4-6, 2012*.
- Devrah Arndt**, Maika Lor, Jian Chen, Rebecca Klaper. Acute and chronic toxicity of carbon nanomaterials to the model organism *Daphnia magna*. International Conference of Environmental Effects of Nanomaterials and Nanoparticles (ICEENN). Banff, Alberta, Canada. *September 10-12, 2012*. (poster)
- Devrah Arndt**, Maika Lor, Jian Chen, Rebecca Klaper. Differences in effects of fullerenes, SWNTs, and MWNTs on survival and reproduction of aquatic organisms. National American Chemical Society (ACS) Meeting. San Diego, California. *March 25-29, 2012*.
- Devrah Arndt** and Rebecca Klaper. SOT 2012. Multi-generational impacts of carbon nanomaterial exposure on the model organism *Daphnia magna*. National Society of Toxicology (SOT) Meeting. San Francisco, California. *March 11-15, 2012*. (poster)
- Devrah Arndt** and Rebecca Klaper. Multi-generational impacts of carbon nanomaterial exposure on the model organism *Daphnia magna*. North American Society of Toxicology and Chemistry (SETAC) Conference. Boston, Massachusetts. *November 14-17, 2011*.
- Devrah Arndt**, Jian Chen, Rebecca Klaper. Impacts of nanoparticle structure and functionalization on the toxicological response of the model organism *Daphnia magna*. Midwest Society of Toxicology and Chemistry (SETAC) Conference. Lake Geneva, Wisconsin. *March 23-24, 2011*.

- Devrah Arndt** and Rebecca Klaper. Impacts of nanoparticle structure and functionalization on the toxicological response of *Daphnia magna*. National Society of Toxicology (SOT) Meeting. Washington, D.C. *March 6-10, 2011*.
- Devrah Arndt**, Jian Chen, Frederick W. Goetz, and Rebecca Klaper. The cellular and gene expression effects of manufactured nanoparticles on primary cell cultures of rainbow trout macrophages. EPA Nano Grantees Meeting. Portland, Oregon. *November 8-9, 2010*.
- Devrah Arndt** and Rebecca Klaper. Effects of Nanoparticle Structure and Functionalization on Acute and Chronic Toxicity to *Daphnia*. North American Society of Toxicology and Chemistry (SETAC). Portland, Oregon. *November 6-12, 2010*.
- Devrah Arndt** and Rebecca Klaper. Impacts of nanoparticle structure and functionalization on the toxicological response of the model organism *Daphnia magna*. International Conference of Environmental Effects of Nanomaterials and Nanoparticles (ICEENN). Clemson, South Carolina. *August 21, 2010*.
- Devrah Arndt**, Jian Chen, Frederick W. Goetz, and Rebecca Klaper. The Cellular and Gene Expression Effects of Manufactured Nanoparticles on Primary Cell Cultures of Rainbow Trout Macrophages. EPA Nano Grantees Meeting. Las Vegas, Nevada. *November 2009*.
- Devrah Arndt** and Rebecca Klaper. Impacts of Nanoparticles on the Environment. Nanotechnology Hearing. Madison, Wisconsin. *October, 2009*.
- Devrah Arndt** and Rebecca Klaper. Evaluating the environmental impacts of nanomaterials. Special Committee of the Wisconsin State Legislature. Madison, Wisconsin. *September 29, 2009*.