5-2012

UPTAKE AND TOXIC EFFECTS OF SURFACE MODIFIED NANOMATERIALS IN FRESHWATER AQUATIC ORGANISMS

Brandon Seda
Clemson University, bcseda@fiu.edu

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A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

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by
Brandon Casey Seda
May 2012

Accepted by:
Stephen J. Klaine, Committee Chair
William S. Baldwin
Cindy M. Lee
Andrew S. Mount
Aaron P. Roberts

ABSTRACT

Nanomaterials are a class of materials with unique properties due to their size, and the association of these properties with the toxicity of nanomaterials is poorly understood. The present study assessed the toxic effects of stable aqueous colloidal suspensions of three distinctly different classes of nanomaterials in aquatic organisms. The fullerene, C_{70}, was stabilized through non-covalent surface modification with gallic acid. Toxicity of C_{70}-gallic acid was confirmed to exhibit similar toxic effects as C_{60}-fullerene, including changes in antioxidative processes in *Daphnia magna*. *Daphnia magna* fecundity was significantly reduced in 21d bioassays at C_{70}-gallic concentrations below quantifiable limits (0.03 mg/L C_{70}). Antioxidant enzyme activities of glutathione peroxidase and superoxide dismutase as well as lipid peroxidation suggested that exposed organisms experienced oxidative stress.

Carbon dots are a class of nanomaterials proposed for use as nontoxic alternatives to semiconductor quantum dots for photoluminescent applications, because of the difference in toxicity of their core components: carbon as opposed to heavy metals. *In vivo* analysis of treated organisms by confocal fluorescence microscopy revealed carbon dots were absorbed and systemically distributed regardless of particle size. The present study did not find any evidence of acute toxicity at concentrations up to 10mg/L carbon dots. These concentrations also failed to produce negative effects in *Ceriodaphnia dubia* bioassays to predict chronic toxicity. Carbon dots also failed to elicit developmental toxic effects in zebrafish.

The toxic effects of semiconductor quantum dots have been partially attributed to the release of heavy metals with their degradation, particularly cadmium. Laser ablation inductively coupled mass spectrometry was used to compare the uptake of cadmium, selenium and zinc in *Daphnia magna* treated to CdSe/ZnS quantum dots or $CdCl_2$. These quantum dots were observed to accumulate primarily in the gut lumen and no evidence of uptake of intact quantum dots was observed. Evidence suggests degradation of the quantum dots release of component ions with accumulation of Cd and Zn in the gut epithelia. Quantum dots elicited acute toxicity at 0.66 mg/L Cd but promoted increased reproduction at 40 µg/L.

DEDICATION

Above all, I dedicate this work to my wife, Jennifer Seda. Thank you for sharing your love and your life with me. I could not have accomplished all that I have without you. I would also like to dedicate this work to my parents, Dawn and Richard Seda, for being a constant source of encouragement and inspiration. I've learned the most important things in life from you. Thank you!

ACKNOWLEDGEMENTS

I would like to acknowledge and thank everyone that has helped with this research project. I would like to especially thank my advisor, Steve Klaine for his guidance during my graduate career. I would like to thank all of my committee members, William Baldwin, Cindy Lee, Andrew Mount, and Aaron Roberts, for all of their time and help with my dissertation research. I also want to thank all of my lab mates for their support, feedback, internet video distractions, and physical labor, especially Aaron Edgington, Brad Glenn and Austin Wray. A special thanks to Jim Smith for his assistance with an intense week filled with LA-ICP-MS. Antonio Nequera provided input in developing methods for *Daphnia* egg extraction. Thanks also to all of my collaborators: Vicki Colvin, Huigang Zhu, Pu-chun Ke, Sijie Lin and the folks at Selah Technologies in Pendleton, SC for providing the materials needed to perform my research. Fluorescence microscopy was performed at the Clemson Imaging Facility with the guidance of Terri Bruce. I'd also like to thank Norm Ellis for providing analyses and Nancy Korn from the Clemson Histopathology lab. Finally, I would like to say a very special 'Thank you!' to all of my family and friends for their constant encouragement and support.

TABLE OF CONTENTS

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

List of Figures (Continued)

Figure Page

PREFACE

The young but rapidly growing field of nanotechnology has given rise for concern over the environmental and human health risks of potentially released engineered nanomaterials. Nanomaterials are any material with at least one dimension measuring less than 100 nm. These materials are actively pursued for a wide variety of applications due to the unique properties nanomaterials possess as a function of their size. It is estimated that more than $1 trillion worth of products will be nano-engineered in some form by the year 2020. Because of the relative novelty of the field of nanotechnology very little is known about the environmental fate, mobility and toxicity of frequently expanding number of engineered nanomaterials. Because nanomaterials possess unique physicochemical properties, they are suspected to behave drastically different from well characterized contaminants. Therefore, existing frameworks for predicting risk may not be sufficient to protect against nanomaterial exposure.

This dissertation is comprised of a literature review and three journal articles. The literature review (Chapter One) discusses nanomaterials with the potential to be used in fluorescence imaging and discusses factors affecting their uptake and toxicity. The first article (Chapter Two) describes the effects of acute and chronic exposure of a water stabilized C_{70}-fullerene complex to *Daphnia magna*, including the measurement of biomarkers of oxidative stress. The second article (Chapter Three) explores the size dependence of nano-sized carbon dots coated with polyethylene glycol on toxicity in three aquatic species and their uptake using confocal fluorescence microscopy. The final article (Chapter Four) describes acute and chronic toxic response of CdSe/ZnS quantum

dots modified with poly(maleic acid) octadecanol further conjugated to polyethylene glycol in two aquatic species. This chapter also evaluates the fate of ions released from quantum dot degradation in *D. magna*.

CHAPTER ONE

LITERATURE REVIEW

NANOMATERIALS

Nanomaterials are generally defined as materials with at least one dimension smaller than 100 nm. At this size range, nanomaterials often exhibit unique properties greatly different from their bulk counterparts as a function of their size. These properties promote the use of nanomaterials for an array of novel applications. Funding for engineered nanomaterials research has grown exponentially in the last 20 years. Although some of these nanomaterials can occur naturally, these engineered materials may be of particular concern because of their novel properties or potential for considerable production.

These engineered nanomaterials can be produced by direct synthesis in nanosize range or by the application of physical grinding or milling of a macroparticulate product. However, the size of nanomaterials produced by mechanical means, such as grinding or milling, is limited by equilibrium, resulting in particles as large as 300nm diameter [Klaine et al. 2008]. However, the milling of highly crystalline particles is able to achieve particles as small as 1-10nm.

Five basic classifications are generally applied to nanomaterials based on their parent material: carbonaceous nanomaterials, nanopolymers, metal oxides, zero-valent metals and semiconducting materials.

Although naturally occurring, cage-like carbon molecules were first proposed in 1970, their existence was only confirmed after 1985 with the production of buckminsterfullerene (C_{60}) by graphite evaporation [Kroto et al. 1985]. Subsequent research produced a variety of fullerene derivatives synthesized by constraining synthesis method and conditions such as environmental parameters and catalysts. Further adjustments of these parameters resulted in a wide variety of fullerene related structures of different sizes (C_{70}, C_{120}, etc) and shapes including carbon nanotubes (CNT), nanohorns, nano wires and much more. Production of carbon-based nanomaterials is typically achieved by isolating the products of graphite arc discharge, laser ablation, or chemical vapor deposition. These materials can also be configured so that multiple concentric spheres or tubes are nested within each other to produce nano-onions and multi-walled carbon nanotubes (MWCNT). Larger particles can also be formed by covalent bonding between fullerenes resulting in fullerene clusters and polymeric fullerene chains.

More traditional polymers can be used to create nanosized dendrimer particles with controlled size topology, flexibility and molecular weight. These materials are constructed by building chains of monomers from a core molecule (e.g. ammonia). Like other nanomaterials, the potential applications of nanodendrimers range greatly. However, research for these nanomaterials remains primarily focused on medical and biological applications because of the facility for the addition of other molecules such as imaging tags, therapeutics and cancer and disease targeting sensors.

Zero-valent metal nanomaterials are commonly synthesized by the reduction of solutions of metal salts. The physicochemical properties of these nanomaterials can be controlled by varying the reductant and reduction conditions. Among the most popular methods is the reduction of ferrous or ferric iron salts with sodium borohydrate. The resultant nano-iron has been applied in the remediation of waters, sediments and soils to remove nitrates as well as the detoxification of organochlorine pesticides [Zhang 2003]. Nanosilver has garnered the greatest interest for commercial nanomaterials application because of its use as an antimicrobial agent in a wide variety of products such as wound dressings, clothes and other textiles, toothbrushes, baby products and washing machines.

Synthesis of zero-valent metal and metal oxide nanomaterials is common and often within the capability of most chemical laboratories [Gu and Soucek 2007]. This feature promotes mass production and possibly greater variability in the properties of similar products between manufacturers. Mechanical grinding is common for production of metal oxide nanomaterials, including ZnO, TiO_2, CeO_2, CrO_2, MoO_3, Bi_2O_3 and binary oxides such as $BaTiO_2$, $LiCoO_2$ and InSnO. Both TiO_2 and ZnO have achieved extensive commercial applications because of their photolytic properties. The estimated use of metal oxides for skin products alone is greater than 1,000 tons/yr [Pitkethly 2004].

Because of their distinct optical properties, semiconductor nanocrystals (quantum dots, QD) are extensively developed for electronics (e.g. photovoltaic cells and optical displays) and biomedical applications (e.g. imaging contrasts and traceable therapeutic vectors). The key component of QD is their reactive core, which controls their optical properties and is typically comprised of metals such as in the case of CdSe, CdTe, or

InAs cores. This core is synthesized from a nucleation reaction, such as high-temperature solution phase synthesis with subsequent crystal growth and size controlled by reaction conditions [Murray et al. 2001] An additional metalsulfide monolayer shell is often incorporated into QD to protect the core against oxidation.

NANOMATERIALS IN THE ENVIRONMENT

With the imminent use of nanomaterials in greater quantities and in consumer products, interest in the implications of this technology has grown considerably [Colvin 2003]. However, the general and scientific communities are each divided as to whether the potential hazards of the growing field outweigh the promised advantages [Maysinger et al. 2007]. The lack of technical data available on the environmental and human health effects nanotechnolgy provides a setting supportive for both proponents and skeptics of nanotechnology to make broad and sometimes contradictory conclusions about the safety of nanomaterials. Most work observing adverse effects of nanomaterials has been centered around human health, especially concerning inhalation of these particles, while very little work has been done in ecological systems in comparison [Klaine et al. 2008].

Because many of these nanomaterials are still being developed and have yet to reach commercial use, predictions about the possible extent of release of nanomaterials into ecosystems is difficult to ascertain. However, nanomaterials release can reasonably be considered to enter the environment by similar mechanisms as other contaminants. Production facilities are a likely source of intentional and unintentional release as

atmospheric emissions and solid or liquid waste streams. Those nanomaterials with environmental applications such as groundwater or soil remediation will be deliberately released. Although widely used in the U.S., nano-iron approved for this usage has been denied in some countries until further research into their possible effects. Commercial products have already been manufactured to include nanomaterials and the continued increase of nanomaterials in commercial products will provide proportional opportunity for their release into the environment via usage, leaching or disposal.

The current level of understanding of how these nanomaterials may behave in the environment is lacking. A disadvantage that further suppresses the study of the fate and behavior of nanomaterials in the environment is the deficiency of analytical methods to adequately characterize and quantify nanomaterials in complex media. However, with rapid advances in analytical equipment and refinement of existing methodologies explicitly for these materials, this obstacle may be considerably reduced in the near future. Until then, existing knowledge and practices can be applied to nanomaterials.

The behavior of nanomaterials in the environment is suggested to exhibit properties relative to those of colloids because of shared size ranges. This shared range includes particles <10 nm, which have been suggested to be the most environmentally relevant size fraction of natural colloids because of the dramatic change in properties. Like colloids, nanomaterial fate and behavior are suspected to be dominated by aggregation [Gustafsson and Gschwend 1997], eventually forming particles (>1 μm) sufficiently large enough that they deposit via sedimentation. This process has been well characterized to understand environmental transport of trace metals, which generally sorb

to high-surface-area colloids that aggregate and settle out of the water column [Honeyman and Santschi 1992]. Similarly, nanomaterials have large ratios of surface area relative to volume and will interact with other colloids, nanomaterials and contaminants, resulting in aggregation. Another property common to nanomaterials that affects their stability in aqueous suspension is their hydrophobicity. Based on known current uses of nanomaterials, a simplified box model suggested environmental concentrations of 1-100 μg/L in freshwater [Boxall 2007]. In contrast, dissolved and colloidal natural organic matter in freshwater can be found at 1-10 mg/L. In estuarine and marine waters, increased ionic strength will likely greatly increase aggregation of nanomaterials as is the case with colloids.

The same properties that prevent nanomaterials from remaining in aquatic environments adversely affect their efficiency for aqueous applications of these particles. Thus, substantial research has been conducted to increase the aqueous stability of these particles, adding another order of variability in the behavior of nanomaterials in the environment. Studying the behavior and toxic effects of these augmented particles is particularly important because they are likely to remain suspended in the water column, increasing the risk of exposure to aquatic organisms.

Aqueous stability of nanomaterials can be enhanced by the addition of hydrophilic moieties from covalent binding of functional groups or physically associated coating. This alteration of the surface chemistry allows for prevention of interactions between nanomaterials that could promote aggregation.

CARBON NANOMATERIALS

Carbon nanomaterials discharged into aquatic systems can become functionalized or derivatized by biomolecules and natural organic matter (NOM) allowing for the dispersion of carbon nanomaterials in freshwater [Hyung et al. 2007, Xie et al. 2008]. Hyung et al. [2007] showed that Suwanee River water, which maintains a naturally high concentration of NOM, maintained greater multi-walled carbon nanotube concentrations than a solution of sodium dodecyl sulfate detergent. Disaggregation of C_{60} by NOM leading to significant changes in particle size and morphology suggests NOM may play a critical role in the transport of fullerenes in aquatic ecosystems [Xie et al. 2008]. Edgington et al. [2010] reported that acute toxicity of multi-walled carbon nanotubes was not significantly influenced by NOM concentrations, although the source of NOM could affect CNT toxicity. This source-dependent difference may be due to differences in NOM composition and thus differences in interaction between NOM and CNT. Gallic acid, a ubiquitously occurring component of NOM, has been shown to interact with the fullerene, C_{70}, to form stable suspensions in water [Salonen et al. 2007].

Much of the existing research on the ecotoxicology of fullerenes has focused on the effects of C_{60} and derivatives of C_{60} [Klaine et al. 2008]. However, the behavior of other fullerenes such as C_{70} are not as well characterized, with almost no published data on biological effects. These fullerenes are assumed to have similar toxicological effects, since they are closely related both physically and chemically to C_{60}. In fact, C_{70} is likely to be prepared coinciding with C_{60} because it is a byproduct of fullerene production. In an

evaluation of toxicity in embryonic zebrafish, C_{60} and C_{70} elicited comparable effects at equivalent concentrations for each mortality and delay in development [Usenko et al. 2007].

Even with the addition of stabilizing coatings or functional groups, exposure is often to clusters of suspended nano fullerenes (i.e. nC_{60}) or colloidal fullerenes. The tendency for fullerenes to aggregate into particles too large for absorption causes tissues that come into direct contact with these particles more susceptible to toxic effects. Fullerene exposure is anticipated to occur through typical pulmonary, dermal or oral pathways. Thus, toxicity at the corresponding sites of exposure—lungs and gills, dermis, and gastrointestinal tract—is of particular concern.

Pulmonary exposure to fullerenes is the primary risk for human health because of the likelihood of workplace exposure. Baker et al. [2008] observed a pulmonary deposition fraction of 14.1% of C_{60} nanoparticles (55nm diameter) compared to 9.3% of C_{60} macroparticles (0.93μm), but both treatments displayed similar elimination with a half-life of 26 d and 29, respectively. Neither treatment displayed gross toxic effects on microscopic analysis although C_{60} was internalized by alveolar macrophage. Further study by Fujita et al. [2009] revealed gene upregulation associated with inflammation, oxidative stress, apoptosis and metalloendopeptidase activity although inflammatory response and tissue injury were deemed not severe by the authors. Multiple studies support that a range of inhaled nanoparticles induce proinflammatory responses [Donaldson and Stone 2008], but Roursgaard et al. [2008] however, demonstrated fullerol reduced the inflammatory response elicited by quartz, a physical irritant. Oberdörster et

al. [2004] suggest that inhaled C_{60} can translocate across olfactory nerves in sinus cavities providing an atypical route of contaminant uptake. A similar neuronal pathway was hypothesized to occur in fish exposed to fullerenes and exhibiting oxidative stress in brain tissue [Oberdörster 2004].

Another potential pathway for human exposure is through dermal contact. Fullerene did not any induce observable irritation by dermal patch exposure in humans [Huczko et al. 1999]. Additional dermal exposure studies of fullerene are exceedingly limited. Although dermally applied fullerenes were not absorbed and distributed systemically, ^{14}C-labled C_{60} were internalized by keratinocytes *in vitro* but did not impact cell proliferation at concentrations up to 2μM [Scrivens et al. 1994]. When derivatized with phenylalanine, C_{60} internalized by keratinocytes caused an inflammatory response with dose-dependent necrosis. Yamawaki and Iwai [2006] observed dose-dependent reduction of cell viability in human umbilical vein endothelial cells with chronic fullerol exposure. Decreased cell attachment and slowed cell growth in these HUVEC cells indicated a possibility for fullerol induced cardiovascular disease. Fullerols also accumulated in ocular cells (human lens epithelial cells) with cytotoxicity enhanced by both UVA and visible light [Roberts et al. 2007]. *In vivo* studies, however, showed no toxicity with installation of fullerene soot in rabbit eye irritation tests [Huczko et al. 1999].

Oral administration of ^{14}C-labeled C_{60} to rats showed that it is not effectively absorbed, though trace amounts were detected in urine suggesting that some absorption occurred [Yamago et al. 1995]. Similar administration of fullerite, a mixture of C_{60} and

C_{70}, resulted in no observed toxic effects (lethality, body weight, or behavior) at doses as high as 2,000 mg/kg unlike the lethality associated with intraperitoneal injection [Mori et al. 2007]. Likewise, pulmonary exposure of C_{60} absorption via inhalation could not be detected in blood samples of treated rats, but possibly absorbed C_{60} was proposed to be below the sensitivity of the detection method [Baker et al. 2008]. Limited absorption of fullerenes coupled with this difficulty in fullerene detection in complex systems prevents adequate determination of dispersion in organisms.

The effects of nanomaterials in aquatic organisms are often dependent on the behavior of these materials in water. Multiple studies report that nanomaterials are predisposed to aggregation and deposition in the gut tract of *D. magna* [Roberts et al. 2007, Edgington et al. 2010, Lewinski et al. 2010]. Roberts et al. [2007] demonstrated that the removal of a noncovalently bound surface coating of lysophosphatiyl choline, which was previously conjugated with single-walled carbon nanotubes, occurred in the gut tract. The loss of hydrophilic components promoted precipitation of the CNT, forming precipitates that adhered to the organism and limited organism mobility. Carbon nanomaterial aggregates were observed to remain in the gut tract much longer than other particles such as colloidal clay [Edgington et al. 2010] The extended presence of nanomaterial aggregates in the digestive tract could cause energetic effects due to physical limitations on feeding rates [Roberts et al. 2007]. Transmission electron microscopy suggests MWCNT can disaggregate in the gut lumen but are unable to translocate the gut epithelia [Edgington et al. 2010].

Computer simulations of small fullerene clusters of <10 molecules indicated that

these clusters can localize in lipid bilayer cell membranes without mechanical damage to the membrane, where the clusters disaggregate passively and spontaneously [Wong-Ekkabut et al. 2008]. Interpretation of these results should be taken with care because no other molecular components such as membrane-bound proteins were considered in the model. Molecular dynamic models by Bedrov et al. [2008] indicated C_{60} interacted with lipid head groups and lipid core of the membrane and they were predicted to exhibit high permeability within simulations. This result suggests that fullerenes may act as effective drug delivery carriers into cells.

Although limited uptake has complicated detection for inhalation and oral studies, distribution is more clearly observed by injection of the nanomaterial. Upon intraparitoneal injection in rats, C_{60} was distributed to kidney, liver and spleen. [Chen et al. 1998]. Gharbi et al. [2005] suggested that metabolism of fullerenes occurs following accumulation in the liver, but metabolites have yet been identified. Studies regarding the absorption, distribution, metabolism and excretion of fullerenes are lacking and would benefit from further inquiry.

A common response associated with nanomaterials is the induction of inflammation , an innate immune reaction can occur due to the presence of damaged cells, pathogens or irritants. *In vitro* studies indicate the production of proinflammatory factors like interleukin 8 and tumor necrosis factor α [Rouse et al. 2006]. *In vivo* studies indicating inflammatory responses, however lack such information on mechanistic pathways of toxicity. Roursgaard et al. [2008] indicated the likelihood of concentration dependence in this effect as demonstrated by anti-inflammatory response at low

concentrations in mice and proinflammatory response at higher concentrations. The proinflammatory response of nanoparticles is also partly a factor of their size and subsequent increased oxidative reactivity of their large surface area [Brown et al. 2001]. Oxidative stress and inflammation are closely interconnected. Inflammation can be initiated by the presence of prooxidants, but an inflammatory immune response can cause the production of cellular-derived ROS [Kirkham 2007].

Evidence supporting the probability of oxidative response from fullerene exposure is also common although there is a notable amount of contradictory evidence. Suspended C_{60} has been observed to induce oxidative damage in human cells and in aquatic organisms [Oberdörster et al. 2006, Markovic et al. 2007]. Markovic et al. [2007] demonstrated that the ability of different colloidal C_{60} suspensions to produce reactive oxygen species (ROS) depends on the solvent used for their preparation. Other research has indicated, however, that depending on surface modification and method of preparation, these C_{60} can actually act as highly effective antioxidants by radical scavenging [Dugan et al. 1996, Isakovic et al. 2006, Wang et al. 1999, Witte et al. 2007, Sayes et al. 2004]. Foley et al. [2002] reported that although fullerenes can quench reactive oxygen species, they will produce singlet oxygen when illuminated with ultra-violet radiation.

In cells exposed to C_{60}, cell death can occur because of lipid peroxidation caused by the generation of oxygen radicals; highly derivatized C_{60} systems do not generate these species as readily and thus have lower cellular toxicity [Markovic et al. 2007]. Both lipid-soluble and water-soluble C_{60} derivatives can effectively prevent lipids from radical-

initiated peroxidation and breakdown of membrane integrity [Witte et al. 2007]. The ability of fullerene to disrupt cellular membranes via ROS-mediated lipid peroxidation appears to be dependent on the properties of the fullerene, coating and environmental conditions.

Investigation into effects of fullerene on reproductive success has been limited to toxic response in embryos. Tsuchiya et al. [1996] exposed adult rats to polyvinylpyrrolidone-solubilized C_{60} by vaginal plug resulting in 100% mortality to embryos after 18 days with multiple developmental abnormalities. Fullerol had no observed effect in fullerol-exposed zebrafish embryos, but C_{60} adversely affected survival, hatch rate, heartbeat rate and occurrence of pericardial edema [Zhu et al. 2008]. However, the addition of glutathione attenuated these effects indicating oxidative stress may have been a contributing factor. Reduced light exposure also reduced developmental effects of C_{60} exposure in zebrafish embryos, supporting evidence that ROS production by fullerenes is propagated by UV radiation [Usenko et al. 2008]. Disturbance in energetics due to aggregation of particles in the digestive tract as mentioned previously may also adversely affect reproduction.

Although the vast majority of research of carbon nanomaterials involves fullerene and carbon nanotubes, not all carbon nanomaterials maintain the graphene-like arrangement of carbon atoms as these two types. For instance carbon black, an amorphous material produced by incomplete combustion of heavy petroleum products. Colloidal suspensions of particles substantially larger than atoms or ordinary molecules (1 nm – 10 μm), can include a fraction of nano-sized carbon black particles. Very little

published data exists regarding the toxic effects and environmental behavior of carbon black. Most of this research pertains to pulmonary exposure. Even less data are available for effects specific to the nano-sized fraction of carbon black. Approximately 95% of inhaled nano-sized carbon black remained in the lungs [Mills et al. 2006], but radiolabled nano-sized carbon were detected indicating they can be absorbed and systemically circulated [Nemmar et al. 2002]. Niwa et al. [2007] determined nano-sized carbon black reached alveolar regions of the lungs and was internalized by macrophage. Endotracheal dispersion of carbon black in low-density lipoprotein knockout mice resulted in the development of atherosclerosis [Niwa et al. 2007].

Like carbon black, carbon dots lack the defined shape of fullerenes. These materials possess unique optical properties due to passivated defects on the carbon particle surface acting as excitation energy traps [Sun et al. 2006]. These materials are prepared by the laser ablation of a carbon target in the presence of water vapor. Carbon dots are readily internalized by human breast cancer (MCF-7) cells [Cao et al. 2007]. Carbon dots intravenously injected in mice are primarily excreted via urine and only kidneys and liver exhibited carbon dot associated fluorescence [Yang et al. 2009]. These mice exhibited no toxic response. The appeal of the use of carbon dots lays in their predicted low toxicity due to the lack of inherently toxic components such as similarly fluorescent quantum dots.

QUANTUM DOTS

Photoluminescent nanomaterials are a common focus of nanotechnology research for many reasons, particularly for biological and medical imaging. Semiconductor QD, which typically contain heavy metals such as cadmium, are predominantly studied among this class of nanomaterial. The unique optical properties of QD make them optimal fluorophores for biomedical imaging [Chan et al. 2002]. Bioactive moieties (e.g. antibodies and receptor ligands) can be conjugated to target specific biological events or structures [Gao 2004, Wu et al. 2003], DNA [Dubertret et al. 2002] and cell membrane receptors [Lidke et al. 2004]. This tunable specificity enables QD to be explored for highly specific drug delivery [Scherer et al. 2001, Yu and Chow 2005]. Their optical properties are also ideal for LED displays and semiconductive properties for ultra-high density data storage [Wu et al. 2004]. However, these metal-based QD have frequently been reported to elicit toxic effects and will likely pose a potential environmental hazard (Michalet et al. 2005, Derfus et al. 2004, Kirchnir et al. 2005, Lovric et al. 2005, Juzenas et al. 2008).

Environmental fate and transsport of QD is poorly understood as well as routes of exposure [Hardman 2006]. The potentials for QD to readily become airborne or aerosolized have simply not yet been evaluated is unclear [Pelley et al. 2009]. As with other nanomaterials, QD are inherently hydrophobic and require the addition of coatings or functional groups for aqueous application, thus raising the potential for these materials to remain suspended in an aquatic environment. These coatings also serve to protect the

metalloid core from oxidation and degradation. Comprehension of the general properties and behavior of QD can prove to be a difficult task due to the diversity of QD types synthesized, allowing for core, shell and coating chemistries. Each possesses a unique suite of physicochemical properties to determine its toxicity and behavior in the environment.

Much of the research into the exposure and uptake of quantum dots has been limited to *in vivo* studies because of potential for exposure to humans through a variety of biological applications. QD have been shown to be incorporated into a variety of cell types via endocytotic mechanisms and reside in cells for weeks to months. This potential retention may lead to increased body burdens and a risk of bioaccumulation in organs and tissue [Hardman 2006]. The potential for human exposure has lead *in vivo* studies focus on dosage by oral administration or injection to rodents. Routes of exposure of QD by more environmentally relevant means are poorly understood for [Hardman 2006].

Rats chronically dosed by injection did not exhibit significant toxicological effects at high doses of 15 nmol QD with different coatings [Hauck et al. 2010]. Intravenously injected doses of CdSe/ZnS QD are rapidly (<90 min) distributed to the liver, with small amounts present in the spleen, kidney and bone but are not detected as Cd using inductively coupled plasma mass spectroscopy in the feces or urine for up to ten days after dosing [Fischer et al. 2006]. In a longer-term study with polyethylene glycol-coated CdTe/ZnS QD, similar localization and excretion was observed for 28 days [Yang et al. 2007]. Fitzpatrick et al. [2009] observed decreased fluorescence in the liver after five days, whereas six months were required to observe reduced fluorescence in bone

marrow and more than two years for lymph nodes. However, the emission wavelength fluorescence associated with the lymph nodes was found to be blue shifted over time, which could be evidence of slow degradation of the QD as this property is a function of QD size. The depletion of Cd from the QD was considered to have been low due to the lack of observed toxic effects at the end of two years after dosage.

Xenopus embryos injected with micelle-encapsulated CdSe/ZnS QD could be transferred to daughter cells during cell division [Dubertret et al. 2002]. Incorporation of QD has been shown in multiple studies to occur by endocytosis for a variety of cell types [Derfus et al. 2004, Hoshino et al. 2004, Lovric et al. 2005]. Hoshino et al. [2004] observed CdSe/ZnS coated with sheep serum albumin first adhered to cells surfaces then where endocytosed and to increase cystolic QD concentration in a time-dependent manner. Further uptake into the cell nucleus could be controlled by particle size for CdTe QD [Lovric et al. 2005]. In addition to endocytosis, the conjugation of bioactive moieties on QD surfaces can promote receptor-mediated uptake processes. CdSe/ZnS QD with epidermal growth factor (EGF) proved proved to be highly specific for the EGF receptor (erbB1) with rapid internalization by Chinese hamster ovary cells, with localization into endosomes [Lidke et al. 2004]. Similarly, mice prostate tumors specifically absorbed QD conjugated with prostate-specific antigen [Gao et al. 2004].

Distribution of CdSe/CdS in porcine skin was dependent on coating (COOH or PEG) although these QD could not penetrate beyond the uppermost stratum corneum [Lee et al. 2007]. UV exposure concurrent with QD-COOH treatment to skin resulted in no apparent difference in effects [Zhang et al. 2008]. Application of dermabrasion to

remove the stratum corneum and some epidermis prior to QD exposure allowed for sufficient penetration to distribution to liver and lymph [Gopee et al. 2009].

Lewinski et al. [2010] used a simpler model organism, *Daphnia magna*, in an attempt to observe the differential uptake of CdSe/ZnS QD with different coatings. Dissection of the gut tract and subsequent analysis revealed >90% of the total Cd in the organism was associated with the intestines. Further fluorescence analysis suggests the uptake of intact QD to be dependent on QD coating. Lewinski et al. [2011] further investigated QD uptake in aquatic organisms by feeding CdSe/ZnS exposed *D. magna* to zebrafish (*Danio rerio*) resulting in low Cd assimilation into tissues. After five days of exposure, elevated Cd levels were detected in fish livers and carcass. Livers of fish allowed to depurate after 15 days of exposure remained elevated Cd levels after one day, where as carcass levels returned to baseline after two days. No bioaccumulation of Cd was observed. Biomagnification of CdSe QD was reported by Werlin et al. [2011] to occur in *Pseudomonas aeruginosa* following uptake of contaminated *Tetrahymena thermophila.*

The manner in which QD under go metabolic processes and excretory mechanisms remain inadequately understood regarding their elimination from cells and organisms. Vertebrate systems typical recognize QD as foreign with elimination of the materials through the liver, spleen and lymphatic systems, but discrepancies in the literature exist [Hardman et al. 2004]. Systemic distribution may be variable dependent on individual physicochemical properties of the QD in question. Degradation of QD may be enhanced by the metallothionein-mediated depletion of surface cations from the QD

shell, exposing the core [Aryal et al. 2006].

Determining factors of QD toxicity include particle size, charge, concentration, coating bioactivity, stability against degradation and core composition. Determining how these multiple factors are associated with toxicity is made more difficult by the abundance of varying test methods and particle characterization. Discrepancies regarding QD toxicity are common in published literature, partially attributable to the lack of toxicology-based studies and variability in QD exposure/dosage concentrations reported, and properties of the QD and organisms tested. Many of the published studies from which QD toxicity information is derived were performed by nanotechnology researchers rather than toxicologists, who have a greater knowledge of the intricacies of collecting and interpreting toxicological data [Hardman 2006].

In vivo toxicity bioassays are common because of their potential applications in humans and limited volumes. Each, mercaptopropionic acid-coated, cysteamine-coated and uncoated CdTe QD exhibited cytotoxic effects in rat pheochromocytoma cells [Lovric et al. 2005]. This cytotoxicity was expressed as chromatic condensation and membrane blebbing typically associated with apoptosis and was greatest in uncoated particles. Toxicity of QD treatments was predicted to be due to Cd release, formation of radicals or QD interaction with cellular components. Cadmium release is likely a significant factor because *N*-acetylcysteine, a known Cd toxicity inhibitor, also reduced cell death from QD exposure. Smaller cationic QD (2.2nm) localized in the nucleus whereas larger QD (5.2nm) with equal charge localized in the cytosol. In a similar study, genotoxicity was discovered to be due to a cysteamine coating [Hoshino et al. 2004]. The

toxicity observed by Lovric et al. [2005] is unlikely due exclusively to this coating because toxicity was partially dependent on particle size. Several studies have been cited in the literature as demonstrating a lack of evidence for QD-induced toxicity in both *in vitro* and *in vivo* studies [Hardman 2006].

Cd/CYTOTOX/QUANTUM DOTS DEGRADATION

The importance of Cd ion release in QD toxicity is supported by evidence provided by exposing QD to photolytic and oxidative conditions prior to exposure to organisms. Such conditions degrade the protective coating, exposing capping and core materials for dissolution [Hardman 2006]. Rat hepatocytes exposed to mercaptoacetic acid-tri-*N*-octylphosphine-oxide coated CdSe QD exhibited no significant decrease in cell viability from controls unless the QD were exposed to UV light or air prior to treatment [Derfus et al. 2004]. The adverse effect was dependent on the length of time the QD were exposed to UV light. The addition of at least one monolayer of ZnS capping to the QD greatly reduced these effects. Cadmium ions were also determined to be released as a result of degradation by both hydrogen peroxide and hydrochloric acid produced by phagocytic cells [Mancini et al. 2008]. Degradation was also observed *in vivo* as a decrease in fluorescence assumed to be a result of degradation-mediated deformations in the QD surfaces; these deformations altered the particles' optical properties thus quenching their fluorescence [Gao et al. 2004].

A common theme apparent to nanomaterials is that the formulation of broadly

inclusive hypotheses to predict environmental behavior and toxicity of a single nanomaterial class is difficult due to the vastly different properties that can be incorporated due to differences in preparation, properties of the pristine nanomaterials and the addition of coatings and functional groups to achieve specific applications. The goal of this study was to observe if relationships between distinctly different nanomaterials can be predicted based on essential characteristic properties.

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CHAPTER TWO

TOXICITY OF AQUEOUS C_{70}-GALLIC ACID SUSPENSION IN *DAPHNIA MAGNA*

ABSTRACT

The present study assessed the toxic effects of stable aqueous colloidal suspensions of C_{70}-fullerene to *Daphnia magna*. The suspensions were stabilized through non-covalent surface modification with gallic acid. In addition to whole organism responses, changes in antioxidative processes in *D. magna* were quantified. Acute toxicity was observed with $96LC_{50}$ for C_{70}-gallic acid of 0.4 ± 0.1 mg/L C_{70}. *Daphnia magna* fecundity was significantly reduced in 21d bioassays at C_{70}-gallic concentrations below quantifiable limits (0.03 mg/L C_{70}). Antioxidant enzyme activities of glutathione peroxidase and superoxide dismutase as well as lipid peroxidation suggested that exposed organisms experienced oxidative stress. Microscopy techniques utilized to determine cellular toxicity via apoptosis proved unsuccessful.

INTRODUCTION

The burgeoning development of nanotechnology allows for a wide range of applications with potentially exponential growth in production and use leading to considerable discharge of nanomaterials into the environment. Understanding how these materials interact in the environment is of particular importance in determining bioavailability to organisms. Carbon nanomaterials discharged into aquatic systems can become functionalized or acquire coatings of biomolecules and natural organic matter (NOM) [Hyung et al. 2007]. Gallic acid, a ubiquitously occurring component of NOM, has been shown to self-assemble with the fullerene, C_{70}, to form stable aqueous suspensions [Salonen et al. 2008]. Further, these authors demonstrated translocation of these surface-modified fullerenes into mammalian cells and observed consequential contraction of the cell membranes. These observations laid the foundation for the present research.

Much of the existing research on the ecotoxicology of fullerenes has focused on the effects of C_{60} and derivatives of C_{60} [Klaine et al. 2008]. The effects of the fullerene, C_{70} are not as well characterized. Fullerenes, such as C_{70}, may have similar toxicological effects to C_{60} since they share closely related physical and chemical properties.

There are conflicting opinions about the toxicity of C_{60} in aquatic organisms. Suspended C_{60} has been observed to induce oxidative damage in human cells and in aquatic organisms [Oberdörster et al. 2006, Markovic et al. 2007]. However, other research has indicated that, depending on surface modification and method of preparation, these C_{60} can actually act as highly effective antioxidants by radical

scavenging [Dugan et al. 1996, Monti et al. 2000, Gharbi et al. 2005]. Foley et al. [2002] reported that, although fullerenes can quench reactive oxygen species (ROS), they will also produce singlet oxygen when illuminated with ultra-violet radiation. Therefore, oxidative stress may be a significant contributing factor to C_{70} toxicity, but may not be the exclusive cause.

The ability of different colloidal C_{60} suspensions to produce ROS depends on the solvent used for their preparation and environmental conditions (i.e. UV radiation) [Markovic et al. 2007, Sayes et al. 2004]. However, evidence also shows that C_{60}, depending on derivatization and method of suspension, can act as an antioxidant [Isakovic et al. 2006, Wang et al. 1999, Witte et al. 2007]. In cells exposed to C_{60}, cell death occurs because of lipid oxidation caused by the generation of oxygen radicals; highly derivatized C_{60} systems do not generate these species as readily and thus have lower cellular toxicity [Markovic et al. 2007]. Both lipid-soluble and water-soluble C_{60} derivatives can effectively prevent lipids from radical-initiated peroxidation and breakdown of membrane integrity [Witte et al. 2007].

To minimize oxidative damage to cellular components due to ROS production, organisms have developed antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) [Di Giulio et al. 1995, Haliwell and Gutteridge 1999]. Up-regulation of these antioxidant enzymes within organisms can occur in response to elevation of ROS production; therefore differences in the regulation of these enzymes can be used as biomarkers of ROS levels [Livingstone 2003]. Failure to moderate excessive ROS can lead to deleterious effects such as enzyme inactivation, protein degradation,

DNA damage and lipid peroxidation [Halliwell et al. 1999]. Lipid peroxidation is considered to be of particular concern in the case of tissue damage, which can lead to the disruption of essential cellular functions [Rikans and Hornbrook 1997]. Malondialdehyde (MDA), a by-product of lipid peroxidation, can be measured as an oxidative stress biomarker [Liebovitz and Siegal 1980]. *Daphnia magna*, a widely used test organism for aquatic risk assessment, has been shown to express such biomarkers as changes in enzyme regulation and lipid peroxidation due to exposure to ultraviolet light, redox cycling compounds and transition metals [Barata et al. 2005a, Barata et al. 2005b].

These antioxidative measures require energy, which would otherwise be utilized for physiological functions such as reproduction. These functions may also be impaired by carbon nanomaterials via physical stress. For instance, carbon nanomaterials such as carbon nanotubes can accumulate in the gut lumen, possibly reducing efficient absorption of food [Roberts et al. 2007, Edgington et al. 2010]. Therefore, in addition to oxidative damage, C_{70} potentially causes other sublethal effects on organism fitness.

The goal of the present study was to assess the response of *D. magna* to exposures of gallic acid modified C_{70} fullerene. To accomplish this goal we characterized the response of *D. magna* to acute and chronic exposures of C_{70}-gallic acid, and we quantified the oxidative damage following sublethal C_{70}-gallic acid exposure.

MATERIALS AND METHODS

SUSPENSION OF C_{70}-GALLIC ACID

Fullerene C_{70} was purchased from SES Research, and its purity was reported by the distributor to be 95%. To prepare suspensions of C_{70}-gallic acid (C_{70}-GA) 5 mg C_{70} and 25 mg gallic acid were combined in a 100 ml conical glass tube. Increments of synthetic laboratory moderately hard freshwater (MHW) were added to obtain a final volume of 100 mL [US EPA 1993]. The contents of the tube were probe sonicated for one hour. Suspensions were allowed to settle for one hour and the supernatant was transferred to a clean glass tube using a glass pipet and taking care not to transfer settled particles. All exposure suspensions were prepared assuming a nominal stock concentration of C_{70}-GA (50 mg/L C_{70}). Since the suspension procedure was not 100% effective, exposure concentrations were quantified as described below.

CHARACTERIZATION OF PARTICLE SIZE

Test suspensions of C_{70}-GA complex (nominal concentration 10 mg/L C_{70}) were analyzed for particle size by dynamic light scattering (DLS). A Beckman Coulter PCS submicron particle size analyzer was used for DLS analysis. Zeta potentials of C_{70} and C_{70}-GA suspensions were measured by Malvern Zetasizer Z (Malvern). For each

characterization analysis care was taken to avoid samples containing unsuspended materials.

QUANTIFICATION OF C_{70}-GALLIC ACID TEST SUSPENSIONS

Samples of test suspensions were obtained prior to each daily media renewal for determination of C_{70} concentration. Twenty-five ml samples were shaken vigorously with 5 ml hexane to extract the C_{70}. A single aliquot was obtained from each daily-prepared treatment suspension concentration. Extracts were stored in 7 ml glass vials. Concentrations of C_{70} in hexane extracts were quantified spectrophotometrically (λ=550nm) using a Molecular Devices, SpectraMAX Gemini UV spectrophotometer.

A standard curve was prepared using stock C_{70}-GA dissolved in hexane. C_{70}-GA concentrations were calculated as mg C_{70} detected per L aqueous test suspension. The lowest concentration used for the standard curve was 0.015 mg/L C_{70} in hexane, therefore calculated suspension concentrations less than 0.03 mg/L C_{70} were considered beneath the detectable limit of quantification. Concentrations were averaged over the duration of each experiment.

DAPHNID ACUTE BIOASSAYS

Daphnia magna neonates were obtained from an in house laboratory stock maintained at the Institute of Environmental Toxicology, Clemson University (CU-ENTOX; Pendleton,

SC). Routine reference acute toxicity tests have been performed with this culture to ensure consistent culture sensitivity to sodium chloride. Results of these reference toxicity tests are available through CU-ENTOX by contacting the corresponding author. Tests were performed based on United States Environmental Protection Agency standard methods using synthetic freshwater [US EPA 1993]. Test volumes and the number of organisms per replicate were altered to compensate for a limited supply of C_{70}. This synthetic freshwater was used to prepare C_{70}-GA suspensions of the following nominal test concentrations: 10, 8, 4, 2 and 1 mg/L C_{70} by serial dilution for 96 h bioassays for use for acute toxicity bioassays. Test suspensions were prepared daily.

Acute bioassay methods followed standard procedures [US EPA 1993]. *Daphnia magna* neonates aged less than 24 h were exposed in static renewal acute toxicity tests in 30 ml glass beakers containing 25 ml test solutions at 25 ± 1 ºC. Three replicates were tested per treatment. Each treatment contained 5 neonates. Mortality was observed at 24 h intervals and organisms fed a diet of algae (*Selenastrum capricornutum*) and yeast-cereal-trout chow. After allowing organisms to feed for one hour, all living organisms were transferred to test chambers containing fresh test solutions.

DAPHNID CHRONIC BIOASSAYS

Chronic bioassay methods followed standard procedures [US EPA 1993, Klemm et al. 1994] with modifications. Test suspensions of C_{70}-GA of nominal test concentrations 2, 1, 0.5, 0.25, and 0.125 mg/L C_{70} were prepared by serial dilution of stock suspension

with synthetic freshwater for the 21 d bioassays. Neonates aged less than 24 h were exposed in static renewal tests in 500 ml polyethylene beakers containing 400 ml test solutions at 25 ± 1 ºC. Three replicates of five individuals were tested per treatment. Mortality and reproduction were observed at 24 h intervals. At this time all offspring were counted and discarded, and remaining living organisms were transferred to test chambers containing fresh test solutions. After daily transfer, organisms were fed a diet of algae (*Selenastrum capricornutum*) and yeast trout chow.

LIPID PEROXIDATION

For each treatment, twenty 24 h old *D. magna* neonates were placed in each of three 500 ml polyethylene beakers containing 400 ml of appropriate test solutions: C_{70}-GA (0.5 and 2.5mg C_{70}), 10mg/L gallic acid and control. Synthetic MHW and a solution of 50 mg/L gallic acid were each used as controls. Each treatment was carried out in three replicates. After static exposure for 24 hours, organisms from each experiment were subsampled for analysis of lipid peroxidation. Lipids were extracted from whole body daphnids (pooled wet mass 100 – 200 mg, n=3); the extent of lipid peroxidation in these samples was determined by the thiobarbituric acid reactive species (TBARS) assay according to the procedure of Barata et al. [2005a]. Measurements were carried out by fluorescence spectrometer.

ENZYME ACTIVITY AND PROTEIN DETERMINATION

Juvenile *D. magna* (4-5 days old) were exposed for 24 h as in the test described in the lipid peroxidation section above. Treatments included 10 mg/L gallic acid solution, a C_{70}-GA suspension (nominal concentration of 2 mg/L C_{70}), and 20 µg/L copper solution. Copper was used as a positive control as a known oxidative stressor to result in changes in TBARS and antioxidant enzyme expressions [Barata et al. 2005a]. Three replicate tests were performed for each treatment. Gallic acid and copper solutions were prepared using MHW. Juveniles (pooled wet mass 50-100mg) from each sample were homogenized in 1: 4 wet weight: buffer volume ratio in 4 ˚C 100 mM KCl pH 7.4 and 1 mM ethylenediaminetetraacetic acid.

Homogenates were centrifuged at 10,000 x g for 10 min and the supernatants removed for immediate enzyme activity analysis. Measurements were carried out on plate reader spectrophotometer (Molecular Devices SpectraMax 190) at 25 ˚C. Superoxide dismutase (SOD) activity was measured by superoxide dismutase assay kit (Cayman Chemical) as per kit instructions. Glutathione peroxidase (GPX) activity was determined using a glutathione peroxidase assay kit (Cayman Chemical) as per kit instructions. Protein concentrations in the supernatants were measured by modified Lowry Protein Assay kit (Pierce).

APOPTOSIS

Juvenile *D. magna* were exposed to C_{70}-GA (nominal concentration of 2 mg/L C_{70}) for 24 h. For each treatment and control, ten individuals were fixed in 10% buffered paraformaldehyde. Samples were embedded in paraffin. Cross-sections were prepared by ultramicrotome. Samples were prepared and analyzed according to manufacturer's instructions for ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (Chemicon International). Analysis was performed on a Zeiss 510 laser scanning confocal fluorescent microscope.

STATISTICAL ANALYSIS

Survival data from the 96 h test were analyzed by trimmed Spearman-Karber method to derive 96 h median lethal concentration (LC_{50}). Data sets from the 21 d toxicity bioassays, lipid peroxidation and enzyme activity levels were each analyzed for significance by one-way ANOVA with Tukey's post hoc test using SAS Software (SAS Institute). Apoptosis data were analyzed for significance using Student's t-test. Significant differences were established at $p<0.05$.

RESULTS AND DISCUSSION

C_{70}-GALLIC ACID SUSPENSION CHARACTERIZATION

We were able to produce colloidal suspensions of C_{70}-GA in moderately hard water to use in the *D. magna* toxicity bioassays by modifying the method of Salonen et al. [2008] for coating C_{70} with gallic acid. While not 100% effective we were able to quantify the average C_{70} concentrations in treatments by hexane extraction. Measured concentrations were approximately 10% of nominal concentrations indicating that a considerable amount of C_{70} did not remain suspended during sonication of the stock suspensions. The mean particle size of a C_{70}-GA test solution two hours after preparation was calculated as 1432 ± 690nm (Figure 2.1). The size distribution corresponds to the tens of nanometers up to micrometers range of particle sizes reported by Salonen et al. [2008] for unfiltered C_{70}-GA in deionized water.

Zeta potentials for C_{70} and C_{70}-GA suspended in moderately hard water were -29 ± 7.8mV and -32 ± 7.2mV, respectively. These values are not significantly different and indicate that both methods produced suspensions considered to be incipiently to moderately stable. However, a visible portion of C_{70} had precipitated from the suspension without gallic acid prior to zeta potential analysis.

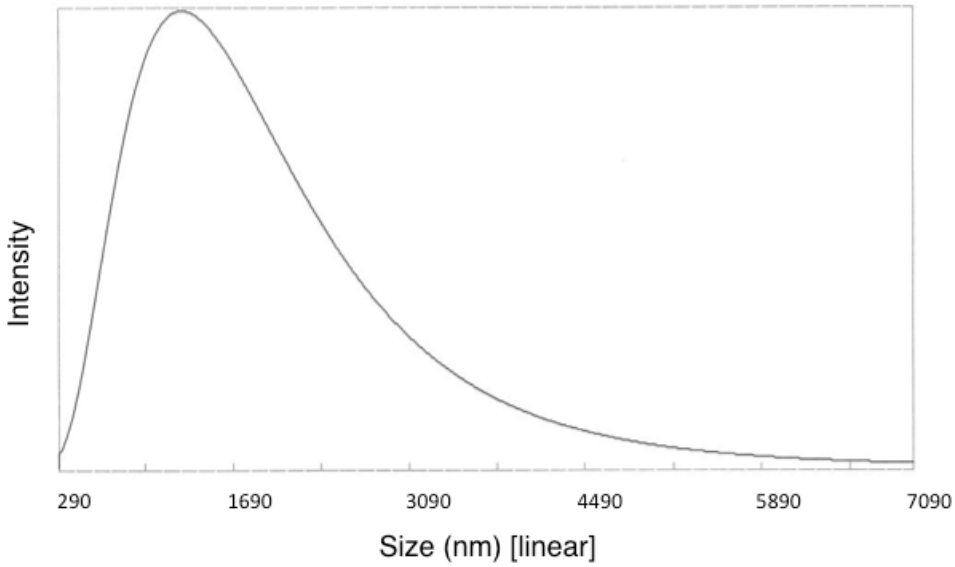

Figure 2.1. Particle size distribution of C_{70}-GA determined by dynamic light scattering within 2 h of preparation. Mean particle size of 1,432 ± 690nm.

Upon visual inspection of suspensions prepared in MHW compared to those prepared as described in Salonen et al. [2008] with deionized water, larger aggregates were observed in suspensions prepared in MHW. A possible explanation for the increase in our suspensions is the greater ionic strength of MHW compared to distilled water. Ionic strength has been shown to increase aggregation of fullerene suspensions in water [Brant et al. 2007].

ACUTE TOXICITY OF C_{70}-GALLIC ACID

A dose dependent decrease in survival was observed for *D. magna* neonates with a 96 h LC_{50} value of 0.4±0.1 mg/L (Figure 2.2). Microscopic examination of the gut tract of exposed organisms indicated substantial amounts of nanoparticles, coinciding with previous data that *D. magna* collected nanomaterial aggregates within their gut tract [Roberts et al. 2007, Edgington et al. 2010]. However, we could not confirm that C_{70}-GA migrated beyond the epithelia of the gut tract.

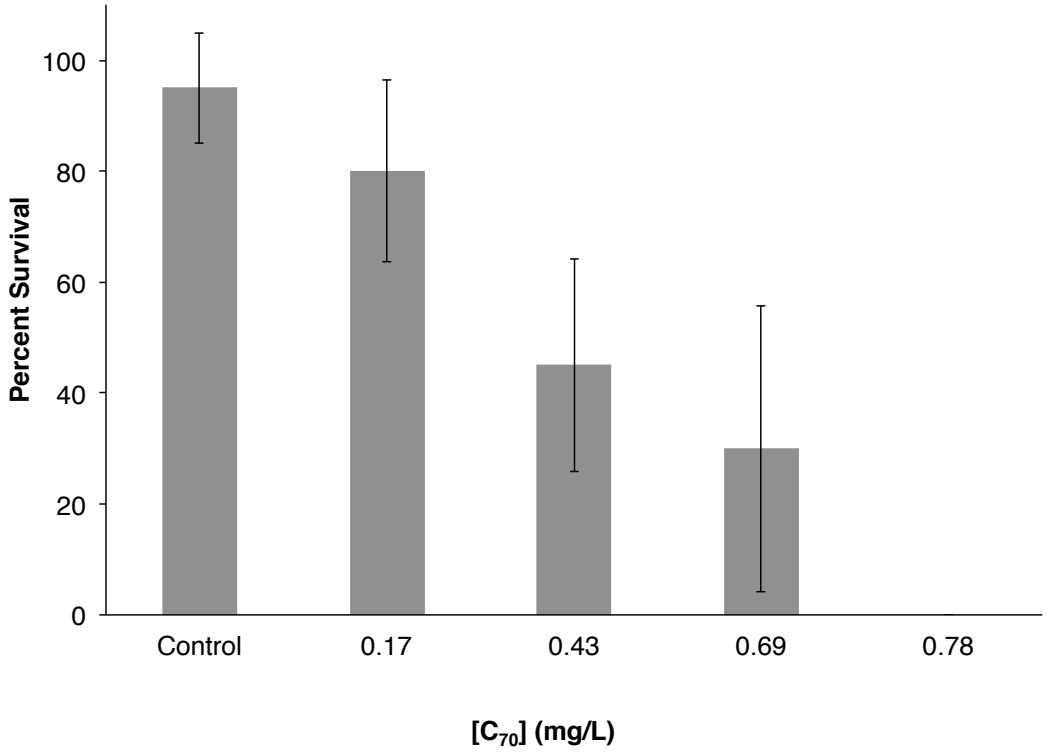

Figure 2.2. Mean percent survival (±SD) of *D. magna* exposed to C_{70}-gallic acid complex (C_{70}-GA) in 96 h static renewal test. X-axis concentrations represent average [C_{70}] of suspensions calculated from mg C_{70} extracted in hexane per L of aqueous suspension.

Time-course micrographs (Appendix) indicated rapid accumulation of fullerenes within the gut tract followed by clearance after the individual was placed in clean MHW. Depuration times of greater than 12 h were required for complete clearance of the material from the gut tract. This time is greater than that reported for clearance of suspended clay by *D. magna* [Robinson et al. 2010] but shorter than the 28 h reported for multi-walled carbon nanotubes (MWNT) suspended in natural organic matter [Edgington

et al. 2010]. However, given that C_{70}-GA in the present study was more toxic than the MWNT reported by Edgington et al. [2010], it is likely that something beyond gut tract clogging and interference with food processing is causing the toxicity.

CHRONIC TOXICITY OF C_{70}-GALLIC ACID

Daphnia magna exposed to C_{70}-GA for 21 d exhibited significant mortality at fullerene concentrations equal to or greater than 1 mg/L (Figure 2.3). Sublethal effects of a 21-d C_{70}-GA exposure on *D. magna* indicate a significant decrease in fecundity at concentrations <0.1 mg/L (Figure 2.4). Unlike the use of organic solvents such as THF in preparing aqueous fullerene suspensions has been controversial, which has been shown to exhibit neurotoxicity [Henry et al. 2007], gallic acid is a widely used antioxidant and did not exhibit significant toxicity when tested alone in this study. The surface modified fullerenes tested in the present study exhibited similar toxicity to the *Daphnia magna* tested by Oberdörster et al. [2006] to the C_{60} prepared with tetrahydrofuran (5ppm nC_{60}).

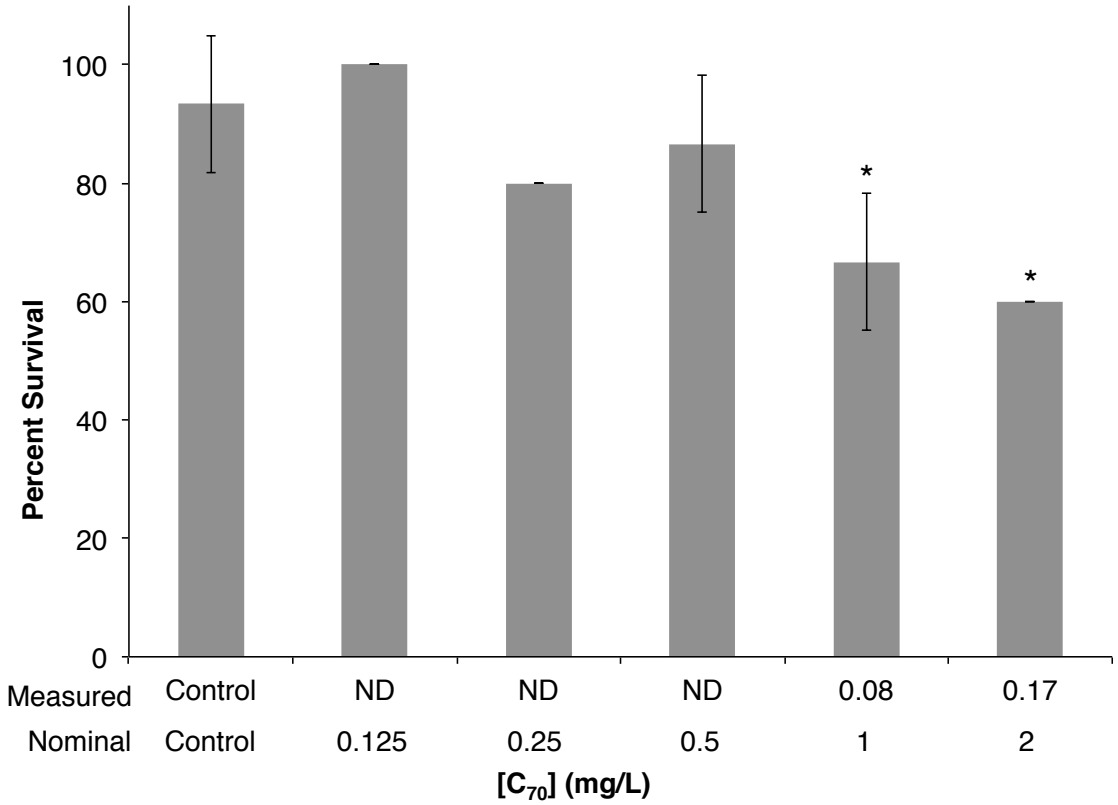

Figure 2.3. Mean percent survival (±SD) of *D. magna* exposed to C_{70}-gallic acid complex (C_{70}-GA) in 21d static renewal bioassay. X-axis concentrations represent average [C_{70}] of suspensions calculated from mg C_{70} extracted in hexane per L of aqueous suspension. Concentrations below the limits of the standard curve (0.03 mg/L) are labeled as ND. *Significantly greater than control. ($P<0.05$).

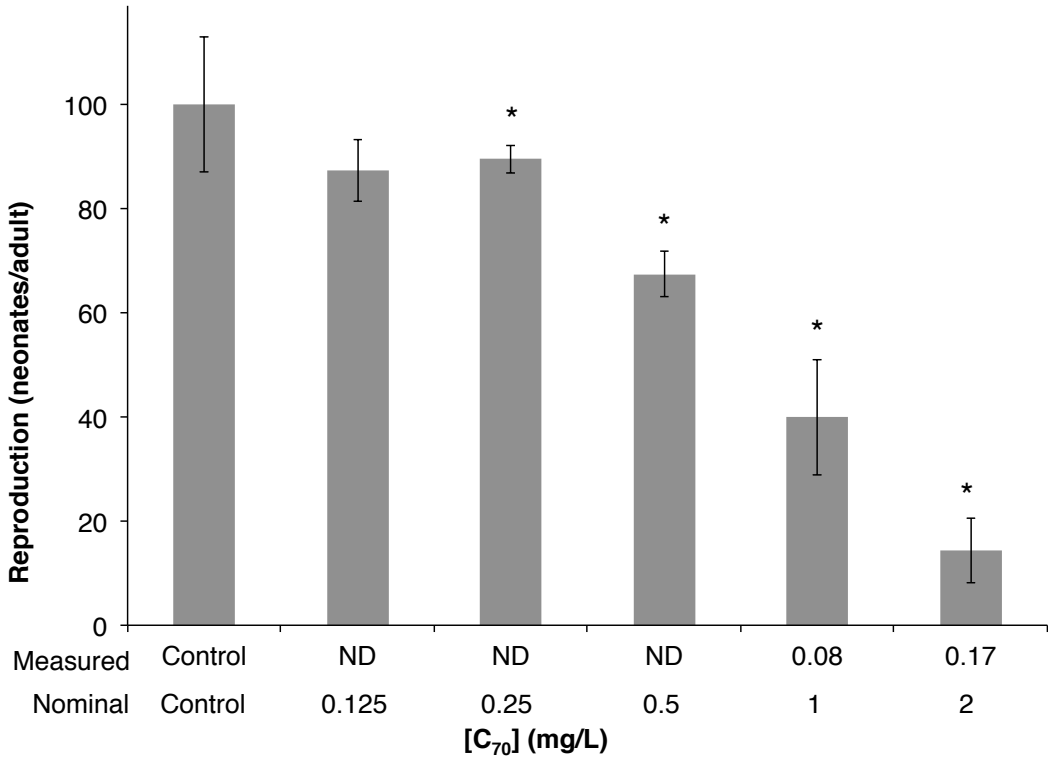

Figure 2.4. Mean number of neonates per adult (±SD) of *D. magna* exposed to C_{70}-gallic acid complex (C_{70}-GA) in 21d static renewal test. X-axis concentrations represent average [C_{70}] of suspensions calculated from mg C_{70} extracted in hexane per L of aqueous suspension. Concentrations below the limits of the standard curve (0.03 mg/L) are labeled as ND. *Significantly greater than control. ($P<0.05$).

There are few other reports in the literature on the effects of chronic exposure of fullerenes to aquatic organisms. Juvenile carp exposed for 32d to 0.2 mg/L C_{60} exhibited reduced body length and weight [Zhu et al. 2008].

To the best of our knowledge this is the first study to report a significant reproductive effect on a eukaryotic organism due to fullerene exposure. Ringwood et al.

[2009] postulated fullerene exposures greater than 10 µg/L could reduce the reproductive success of adult oysters. The observed decrease in fecundity of *D. magna* exposed to C_{70}-GA could be a result of energetics effects caused by interference with food processing as previously proposed for single-walled nanotube aggregates [Roberts et al. 2007]. Additionally, should the observed elevated rates of enzyme activity continue over the course of the daphnia life-cycle, the energy required for reproduction could be reallocated to maintain these enzymatic pathways.

LIPID PEROXIDATION

The presence of malondialdehyde (MDA), a product of lipid peroxidation that reacts with thiobarbituric acid, was determined for each treatment by the TBARS assay. No significant difference in lipid peroxidation was observed in the gallic acid treatment. C_{70}-GA significantly increased lipid peroxidation but the effect was not dose-dependent (Figure 2.5).

From the data, no dependence on concentration could be determined, suggesting that at least one limiting factor exists for the induction of lipid peroxidation in *D. magna* by C_{70}-GA. A contributing factor that may constrain lipid peroxidation could be that much of the C_{70}-GA is retained unabsorbed within the gut tract. If C_{70}-GA was absorbed by *D. magna*, the amount could be limited by the gut epithelium.

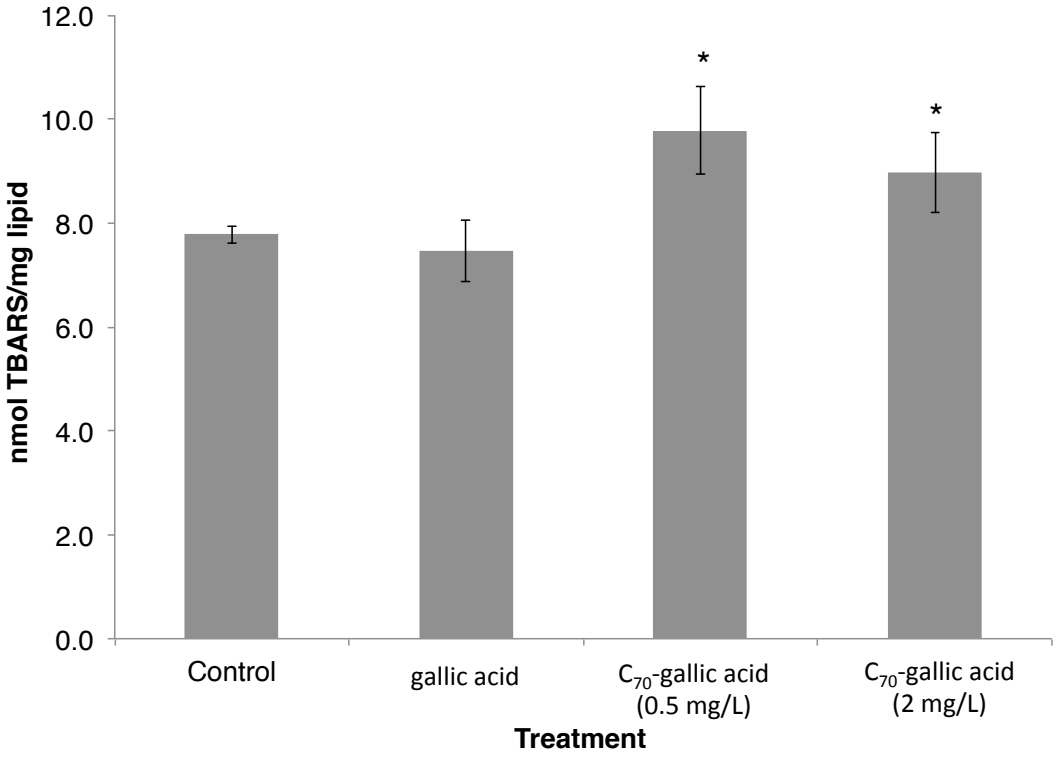

Figure 2.5. Mean TBARS detected as relative to mass of lipid extracts (±SD) of *D. magna* exposed to C_{70}-gallic acid (C_{70}-GA) in 24 h static test (*n*=3). Treatments include MHW control, gallic acid (GA), and treatments to nominal concentrations of C_{70}-GA: 0.5 and 2 mg/L C_{70}. *Significantly greater than control. ($P<0.05$).

ANTIOXIDANT ENZYME ACTIVITY

Daphnia magna exposed to C_{70}-GA exhibited increased SOD and GPX activity (Figures 2.6 and 2.7, respectively). Juveniles exposed to gallic acid alone were observed to have a decreased SOD activity response. This decreased activity could be due to the fact that gallic acid can act as an antioxidant. However, no significant change in GPX activity was observed in *D. magna* juveniles exposed to gallic acid alone. Because gallic acid was observed to have an opposite action on GPX activity, the gallic acid may have an ameliorative effect counter to the apparent oxidative stress caused by the C_{70}-GA.

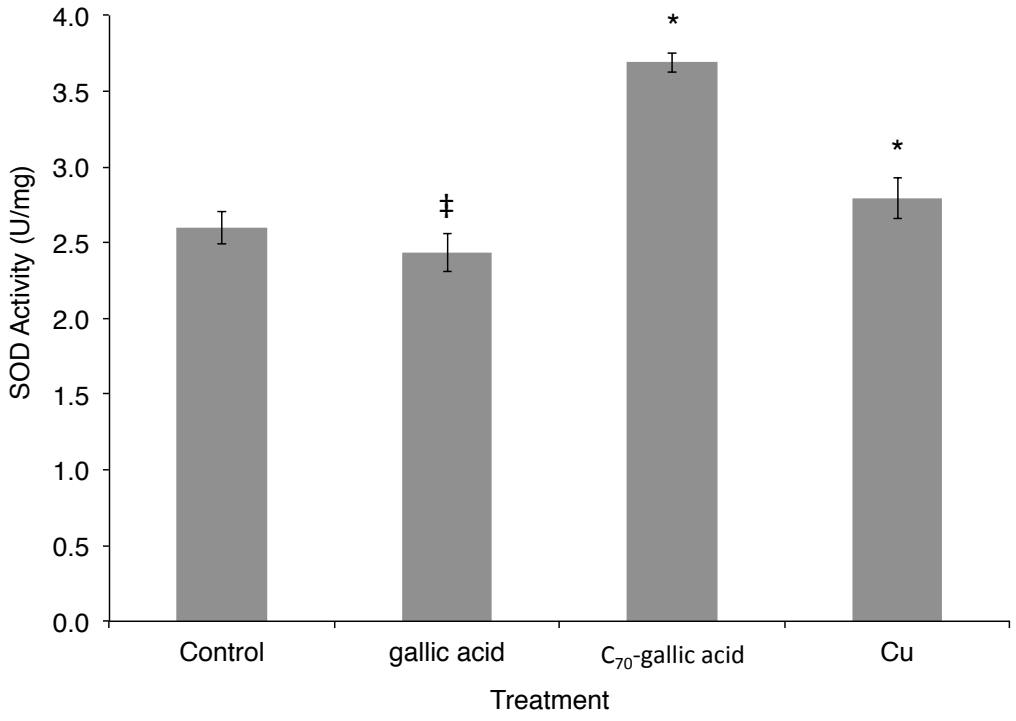

Figure 2.6. Activity of superoxide dismutaste (SOD) in *D. magna* juveniles exposed to gallic acid, C_{70}-gallic acid, and Cu (n=3). Values are expressed as mean±S.D. Normalized to protein concentration. ‡ Significantly less than controls. *Significantly greater than control. ($P<0.05$).

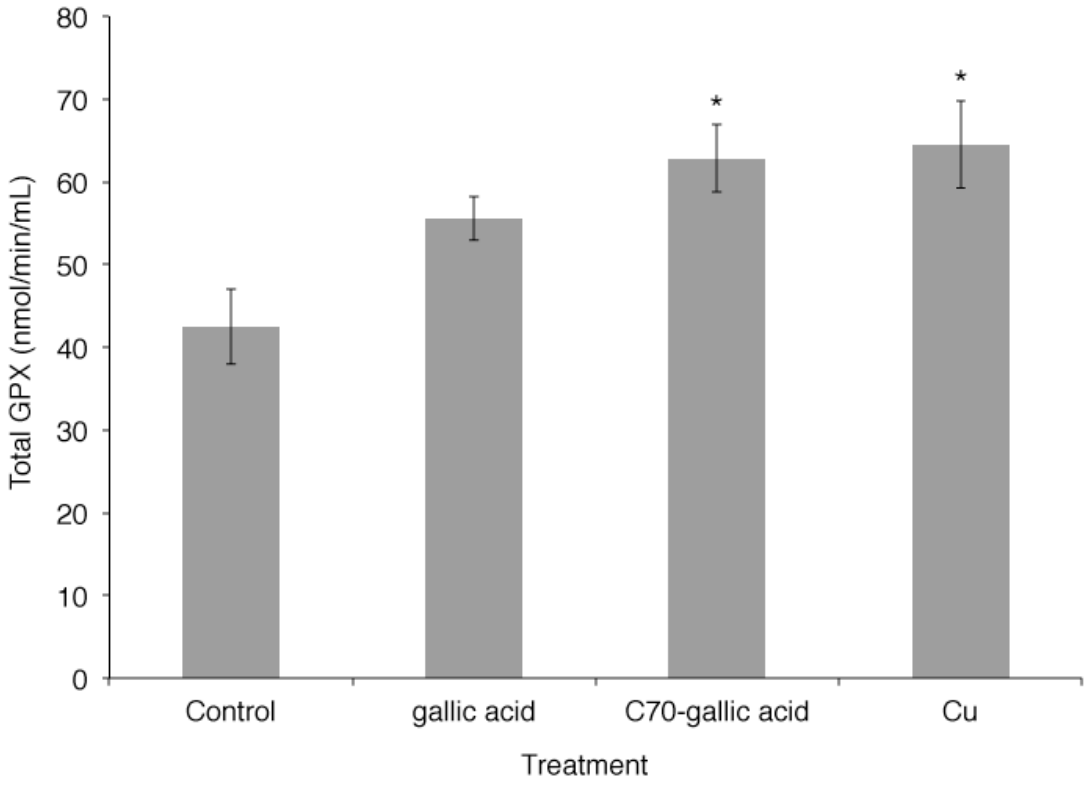

Figure 2.7. Activity of glutathione peroxidase (GPX) in *D. magna* juveniles exposed to gallic acid, C_{70}-gallic acid, and Cu (n=3). Values are expressed as mean±S.D. normalized to protein concentration. ‡ Significantly less than controls. *Significantly greater than control. ($P<0.05$).

APOPTOSIS

The ISOL assay is designed to detect DNA fragmentation resulting from two DNase classes. However, autofluorescence of *D. magna* tissue samples was detected in the same fluorescence wavelength range as the DNase Type II label probe, carboxyfluoroscein (FAM). Therefore, only Cal Fluor Red 590 labeled probes bound to fragmented DNA resulting from Type I nucleases were detected.

Fluorescence micrographs of cross sections of both control and C_{70}-GA exposed *D. magna* juveniles were observed for fluorescence associated with label probes. Only cells of the gut tract were considered for analysis for ease of tissue identification and proximity to C_{70}-GA aggregates.

There was no significant difference between mean apoptotic cell counts between control and C_{70}-GA exposed *D. magna* juveniles (Appendix). From the data, there was no evidence of a significant induction of apoptosis in *D. magna* gut tissue by the C_{70}-GA complex.

CONCLUSIONS

Results of this work demonstrate that chronic exposure to C_{70}-GA can cause deleterious effects on fecundity in *D. magna* at concentrations below 0.03 mg/L C_{70}, the detection limit of our quantification assay. These chronic effects may result from physical stress of fullerene aggregates in the gut impeding the ability of individuals to feed efficiently.

However, it is likely that this physical effect may not be the only adverse outcome since C_{70}-GA exhibited toxicity at lower concentrations despite having a shorter gut tract clearance time [Edgington et al. 2010]. This mechanism could be oxidative stress as suggested by the results of the biochemical assays in the present study. Previous studies examining fullerene-induced oxidative stress may have been confounded due to the use of solvents such as THF and dimethylsulfoxide to produce the aqueous suspensions [Kim et al. 2010]. While these solvents were inherently toxic, the gallic acid used in the present study to stabilize C_{70} was not toxic. Results of this research underscore the need for additional chronic studies with nanomaterials on aquatic organisms.

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CHAPTER THREE

CARBON DOT UPTAKE AND TOXICITY IN *DAPHNIA MAGNA* AND ZEBRAFISH (*DANIO RERIO*)

ABSTRACT

Carbon dots are a class of nanomaterials proposed for use as nontoxic alternatives to semiconductor quantum dots for photoluminescent applications, because of the difference in toxicity of their core components: carbon and heavy metals. *In vivo* analysis of treated organisms by confocal fluorescence microscopy revealed carbon dots were absorbed and systemically distributed regardless of particle size. The present study did not find any evidence of acute toxicity at concentrations up to 10mg/L carbon dots. These concentrations also failed to produce negative effects in *Ceriodaphnia dubia* bioassays to predict chronic toxicity. Carbon dots also failed to elicit developmental toxic effects in zebrafish.

INTRODUCTION

Carbon dots are a relatively new class of carbon nanomaterials possessing many beneficial properties including high stability over time, decreased photo bleaching and chemical degradation, high quantum yields, and other beneficial optical properties , which promote their use as fluorescence imaging agents [Gonçalves and Silva 2010]. Unlike many other photoluminsecent nanomaterials, carbon dots can be synthesized in water and are highly stable in aqueous suspension [Gonçalves and Silva 2010]. While this is beneficial for many applications it also increases the potential for introduction into aquatic environments. Presently, few studies have examined the consequences of the interactions of carbon dots with biological systems.

In addition to being used for material applications such as electronics, photoluminescent nanomaterials can be used for biomedical imaging. Quantum dots (QD) have been exploited for this purpose because of their exceptional optical properties. However, quantum dots are typically composed of a core of heavy metals that degrades to release metal ions such Cd^{2+}, Se^{2+} and Te^{2+}. Due to the inherent cytotoxicity of these ions, concern over their release has hindered final adoption of QD for in vivo applications [Coto-García et al. 2011]. These quantum dots have been shown to have deleterious effects beyond that of the ion alone [Cao et al. 2007]. One factor that could explain this effect is that quantum dots alter the uptake pathways of these metals, essentially bypassing typical sequestration and elimination pathways of the ionic species making the ions more bioavailable. Multiple approaches to decrease possible toxicity of these

materials are being studied, including encapsulation with inert matrices or modification of the surfaces to reduce degradation [Coto-García et al. 2011].

Another approach is the development of alternative nanomaterials with comparably lower toxicity. Carbon dots have been shown to exhibit performance competitive to traditional CdSe/ZnS dots without observed toxicity in mice or in vitro mouse cells [Yang et al. 2004].

This reduced toxicity in comparison to CD is postulated to be a result of the innocuous properties of the carbon. However, other research has demonstrated that nanomaterials can exhibit physicochemical properties and deleterious effects markedly different from their bulk counterpart, carbon black. Carbon based nanomaterials such as carbon nanotubes and fullerenes have been shown to cause adverse effects in a variety of terrestrial and aquatic species including physical interference [Roberts et al. 2007], lung damage [Lam et al. 2004] oxidative stress [Oberdörster etl al. 2006, Markovic et al. 2007] and developmental abnormalities [Zhu et al. 2008].

No data on the long-term stability of these materials in environmental systems have been published, but because they are stable in aqueous suspensions, carbon dots may relocate to aquatic ecosystems from medical applications via wastewater runoff.

In the only published *in vivo* study [Yang et al. 2009], rats were exposed via injection. The likelihood of uptake by unintentional exposure is unknown. This study provides initial uptake and toxicity data for aquatic organisms exposed to carbon dots. An objective of this study was to make use of the fluorescent properties of these materials, to observe uptake and translocation via fluorescence microscopy.

MATERIALS AND METHODS

MATERIALS

Polyethylene glycol-coated carbon dot suspensions were obtained from Selah Technologies, Inc. (Pendleton, SC) in three sizes 8, 40 and 113 nm. Suspension concentrations were determined gravimetrically from evaporated aliquots. Particle size was confirmed with transmission electron microscopy. Zeta potentials of carbon dot suspensions were measured by Malvern Zetasizer Z (Malvern).

DAPHNID ACUTE BIOASSAYS

Daphnia magna neonates were obtained from an in house laboratory stock maintained at the Institute of Environmental Toxicology, Clemson University (CU-ENTOX; Pendleton, SC). Routine reference acute toxicity tests have been performed with this culture to ensure consistent culture sensitivity to sodium chloride. Results are available through CU-ENTOX by means of the corresponding author. Tests were performed for each size of carbon dots based on United States Environmental Protection Agency standard methods using synthetic freshwater [US EPA 1993]. This synthetic freshwater was used to prepare carbon dot suspensions of the following nominal test concentrations: 10, 5, 2.5 and 1.25 mg/L by serial dilution for 96 h acute toxicity bioassays. Test suspensions were prepared daily.

Acute bioassay methods followed standard procedures [Klemm et al. 1994]. *Daphnia magna* neonates aged less than 24 h were exposed in static renewal acute toxicity tests in 30 ml glass beakers containing 25 ml test solutions at 25 ± 1 ºC. Five replicates were tested per treatment. Each treatment contained five neonates. Mortality was observed at 24 h intervals and organisms fed a diet of algae (*Selenastrum capricornutum*) and yeast-cereal-trout chow (YCT).

SHORT-TERM METHOD FOR ESTIMATION OF CHRONIC EXPOSURE

Short-term bioassay methods using *C. dubia* to estimate chronic exposure followed standard procedures [US EPA 1993, Klemm et al. 1994] with modifications because limited carbon dot supply prohibited chronic *D. magna* bioassays. Test suspensions of carbon dots of nominal test concentrations 10, 5, 2.5, and 1.25 mg/L C_{70} were prepared by serial dilution of stock suspension with synthetic freshwater for 7 d bioassays. Neonates aged less than 24 h were exposed in static renewal tests in 50 ml polyethylene beakers containing 20 ml test solutions at 25 ± 1 ºC. Ten replicates of one individual were tested per treatment. Mortality and reproduction were observed at 24 h intervals. At each interval offspring were counted and discarded, and remaining living organisms were transferred to test chambers containing fresh test solutions. After daily transfer, organisms were fed a diet of *S. capricornutum* YCT.

DANIO RERIO FISH EMBRYO TOXICITY TEST

Fertilized eggs were obtained from the natural mating of adult zebrafish from an in house laboratory stock. Zebrafish culturing and egg retrieval were carried out following methods outlined by Westerfield [2000]. Fertilized embryos were collected within two hours of spawning and placed in a clean petri dish, for each carbon dot size. These eggs were exposed to carbon dot suspensions for 96 h in synthetic freshwater described above at nominal concentrations of 10, 5, 2.5 and 1.25 mg /L. For each treatment one embryo was placed in each of ten 2 mL wells of a 24-well well plate. Organisms were observed at 24 h intervals for mortality and developmental abnormalities, such as spinal curvature, deformations and formation of edemata.

MICROSCOPIC ANALYSIS OF UPTAKE

The uptake of carbon dots in *D. magna*, *C. dubia* and zebrafish was observed via fluorescence. For *D. magna* and *C. dubia*, twenty neonates (<24 h) were exposed for 24 h to 25 mg/L suspensions of carbon dots in MHW for each size. Organisms were not fed during exposure to prevent fluorescence from algae. Experiments were repeated so that organisms were rinsed and placed in clean MHW for 24 h, allowing clearance of the gut tract and molting of the carapace. All samples were embedded in agarose gel for microscopic analysis.

Fertilized zebrafish eggs were retrieved as above and exposed for 24 and 96 h. Eggs exposed for 24 hours were immediately embedded in agarose gel for analysis. Zebrafish larvae that hatched after 96 h were embedded for analysis.

Whole organisms were analyzed within 12 h for fluorescence using a Nikon Ti-E confocal laser scanning microscope (LSM). Samples were observed with a 404 nm excitation laser and a 430-460 band-pass filter. For each round of analyses, microscope settings were adjusted to minimize autofluorescence in controls, and these settings were used for all samples for comparability. For each sample, micrographs of multiple focal planes along its z-axis were captured for the creation of three-dimensional projections.

MATERNAL TRANSFER OF CARBON DOTS

For each carbon dot size, *D. magna* adults (14 d) and neonates (< 24 h) were treated for 48 h and 21 d, respectively. For each treatment, ten adults were exposed to 25 mg/L carbon dot suspensions. Similarly, ten neonates were exposed for 21 d. Eggs were extruded from each of five gravid adults by forcing clean water into the body cavity via a modified Pasteur pipette. Collected eggs were embedded in agarose gel for analysis by confocal fluorescence LSM as described above.

STATISTICAL ANALYSIS

Survival data from the 96 h test were analyzed by trimmed Spearman-Karber method to derive 96 h median lethal concentration (LC_{50}). Data sets from the 7 d toxicity bioassays and fish embryo tests were each analyzed for significance by one-way ANOVA with Tukey's post hoc test using SAS Software (SAS Institute). Significant differences were established at $p<0.05$.

RESULTS AND DISCUSSION

TOXICITY BIOASSAYS

For each size class, carbon dots failed to elicit mortality significantly different from controls (Figure 3.1) for any species tested. Thus, no 96 h LC50 values could be obtained from the acute toxicity data for any of the tested carbon dot sizes. Another carbon nanomaterial, C_{60}, has been estimated to have lethal concentrations (48 h LC_{50}) to *Daphnia* between 460 µg/L and 7.9 mg/L depending on method of nanomaterial preparation [Lovern and Klaper 2006]. *Daphnia* sp. also exhibited erratic swimming behavior in lower concentrations of C_{60} exposure [Lovern and Klaper 2006]. Toxicity of QD is highly dependent on core chemistry and surface coating with reported 48 h LC_{50} values as low as 3.1 nM QD (0.244 ppm Cd) for *D. magna* exposed to CdSe/ZnS QD with differing coating types. [Lewinski et al. 2010]

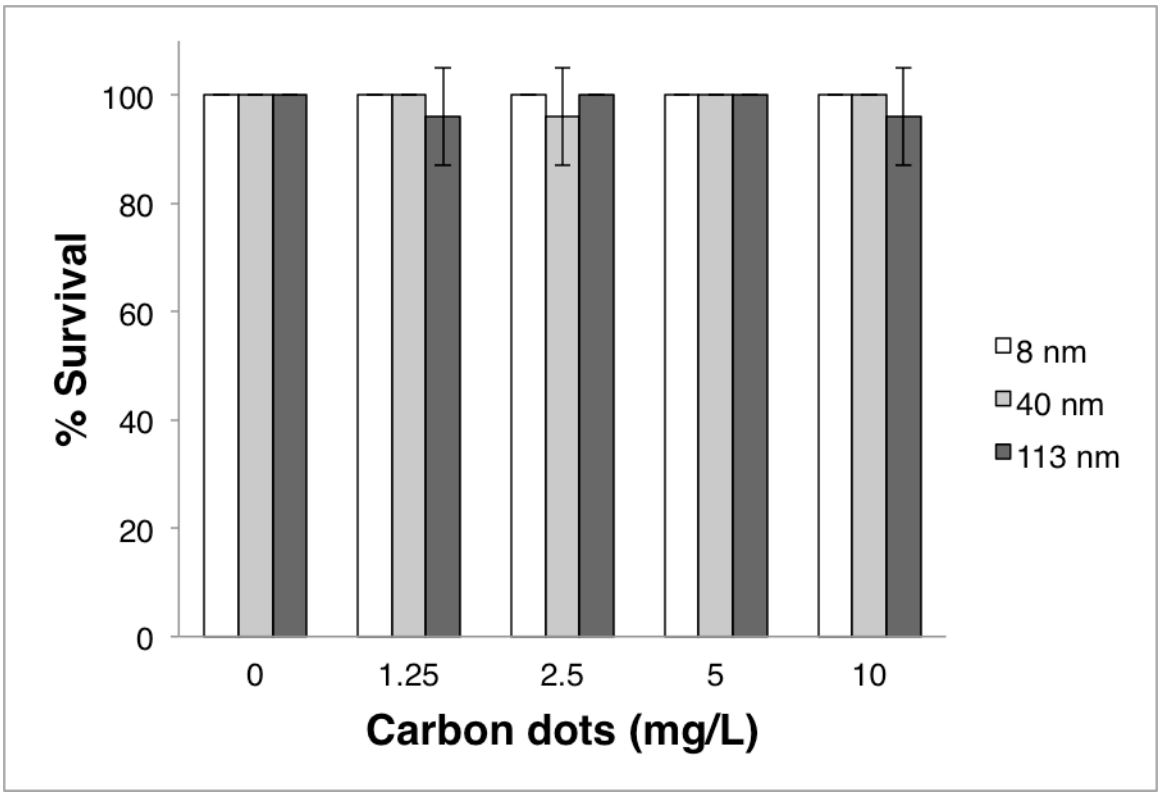

Figure 3.1. Mean percent survival (±SD) of *D. magna* exposed to carbon dots of three diameters (8, 40 and 113nm) in 96 h static renewal test.

For each size class, carbon dots failed to elicit significantly different changes in reproduction of *C.dubia* when compared to controls (Figure 3.2). Fullerene C_{70} exposure significantly reduced fecundity of *D. magna* at concentrations less than 1 mg/L [Seda et al. 2012]. To the best of our knowledge no data has been published on the chronic effects of quantum dots on cladocerans or on their effects on fecundity to any organism.

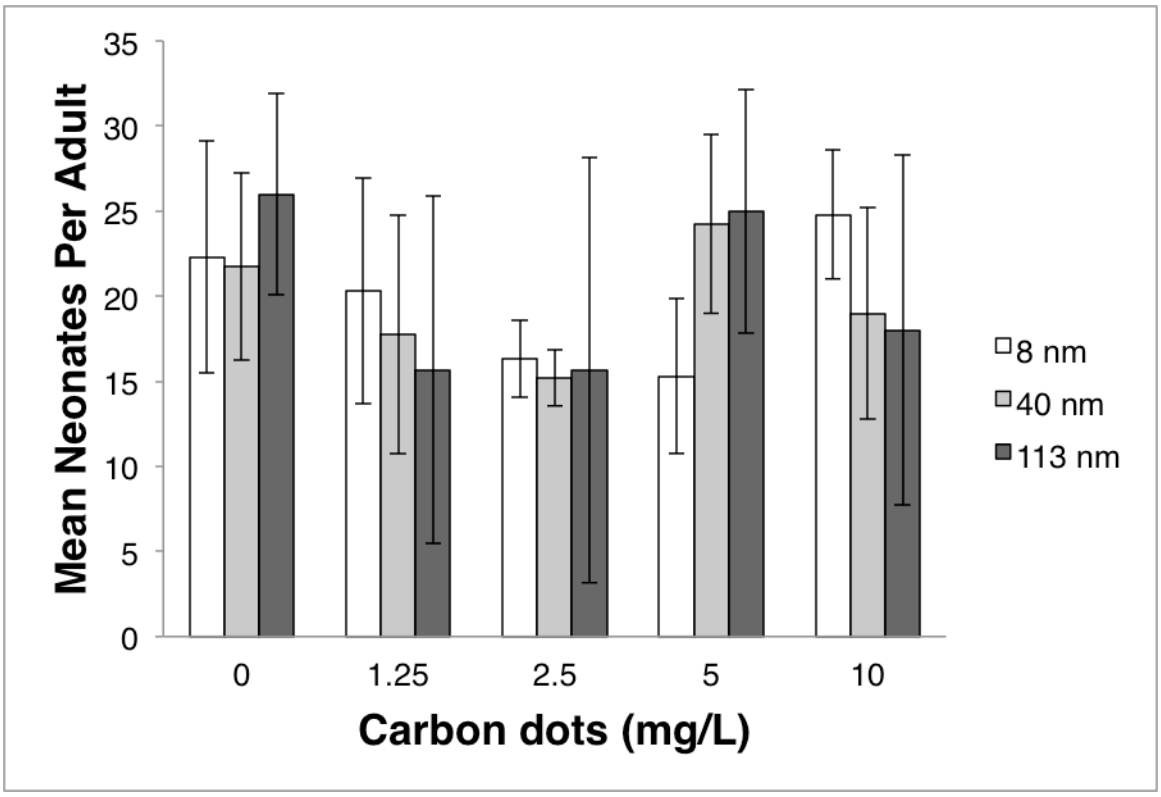

Figure 3.2. Mean number of neonates per adult (±SD) *C. dubia* exposed to carbon dots of three diameters (8, 40 and 113nm) in 7 day static renewal test.

FISH EMBRYO TOXICITY TEST

No developmental abnormalities of zebrafish embryos were observed in any treatments. Positive controls of 3.7 mg/L 3,4-dichloroanaline induced 100% embryo mortality and typical developmental abnormalities. Carbon dots failed to elicit dose dependent mortality for any of the carbon dots sizes (Figure 3.3). Estimated 96 h LC_{50} values for C_{60}, C_{70} and $C_{60}(OH)_{24}$ ranged between approximately 200 and 4000 ppb with significant [Usenko et al. 2007]. Concentrations $\geq$ 200 ppb C_{60} also elicited a significant increase in pericardial edema as compared to controls. Exposures of 100 μM QD (poly(acrylic acid)-octylamine copolymer coated CdSe/ZnS QD) elicit significant mortality, yolk sac malformation and pericardial edema [Lewinski et al. 2011].

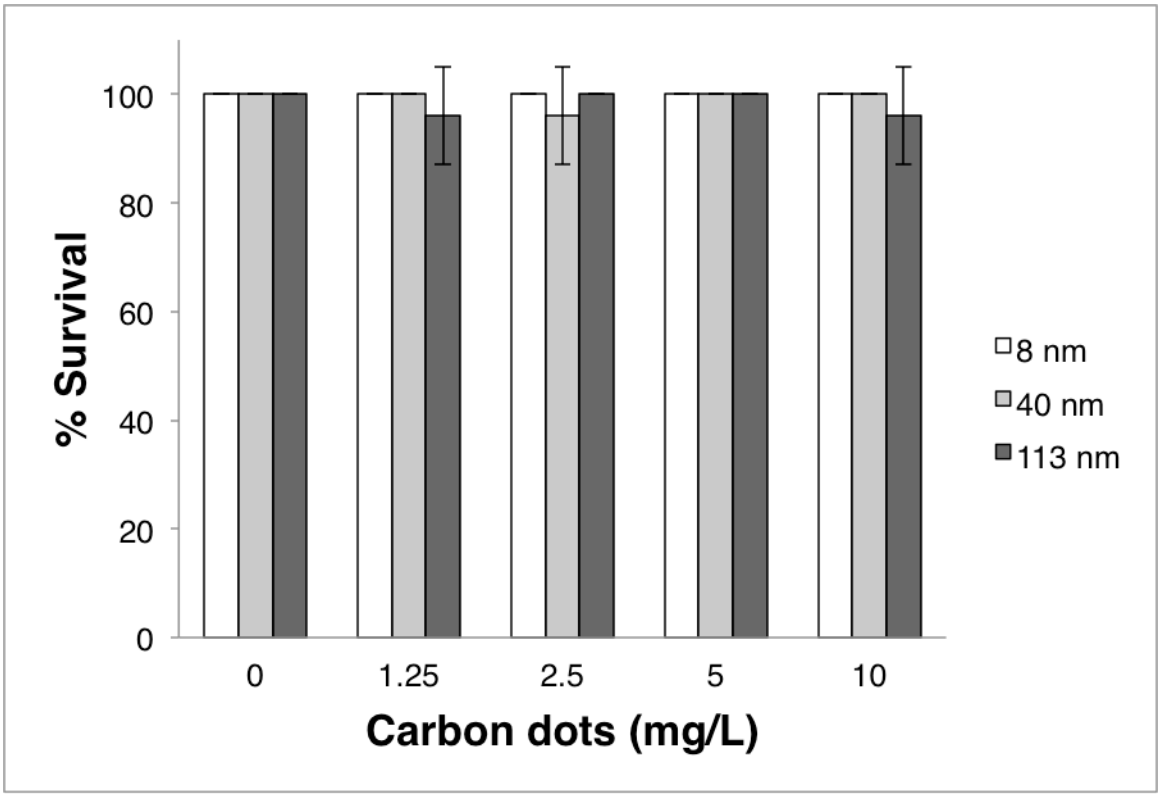

Figure 3.3. Mean percent survival of *D. rerio* exposed to carbon dots of three diameters (8, 40 and 113nm) in 96 hour fish embryo test.

MICROSCOPIC ANALYSIS OF UPTAKE

Analysis of control organisms resulted in the detection of autofluorescence of the carapace of *D. magna* and *C. dubia*. For each experiment, display contrast was adjusted in the micrograph analysis software NIS Elements (Nikon) to visually eliminate this autofluorescence from captured micrographs of control samples (Figure 3.4A). These

visual settings were applied to all similarly exposed samples for comparability. However, fluorescence intensity data were conserved for analysis (Figure 3.4D-F). Figure 3.4 displays *D. magna* treated to 8 nm carbon dots. Results for all three carbon dot sizes were comparable for both *D. magna* and *C. dubia*.

In organisms exposed for 24 h, carbon dots were observed to be predominantly accumulated in the gut tract or adsorbed to carapace (Figure 3.4B & 3.4E). In these samples absorption of carbon dots beyond the barriers of the gut epithelia or carapace could not be determined. Transferring organisms to untreated water for 24 h allowed for clearance of the gut tract and molting of contaminated carapaces. Clearance was unassisted by the presence of food in this study. The reduction of fluorescence signal associated with carbon dots in the gut tract or sorbed to the carapace allows for the detection of lower intensities.

After 24 hours in clean water, fluorescence associated with the carapace reduced in intensity to a range comparable to controls (Figure 3.4F). Fluorescence detected throughout treated organisms indicates *D. magna* carbon dots were absorbed and distributed throughout the organism (Figure 3.4C). In similar microscopy analysis of QD with four different polymer coatings in *D. magna*, fluorescence associated with QD was observed in the gut tract but was not completely removed within 48 h of purging time and absorption was not reported [Lewinski et al. 2010].

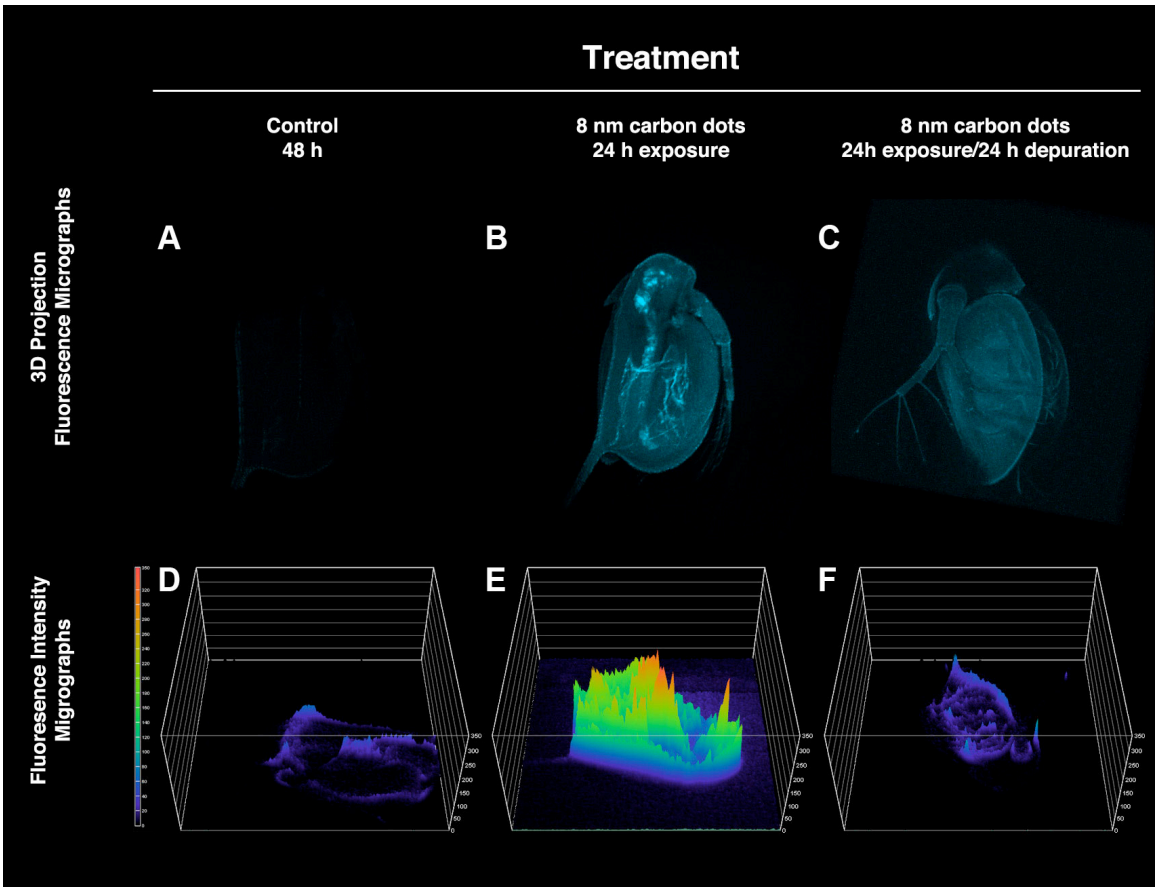

Figure 3.4. Typical confocal fluorescence micrographs of *D. magna* exposed to 8 nm carbon dots. Three dimensional projections of multiple fluorescence micrographs from A) 48 h old control B) 24 h carbon dot exposure and C) 24 h carbon dot exposure after additional 24 h purge time in untreated water. D-F) Fluorescence intensity plots of single focal plane micrographs from respective samples shown in A-C.

In *D. magna* exposed to 8 and 40 nm carbon dots fluorescence was no longer associated with the gut tract after the depuration period (Figure 3.5B and C, respectively) However, a portion of the posterior end of the gut tract continued to exhibit fluorescence attributed to 113 nm carbon dots. Thus, 113 nm carbon dots may have a longer residence time in *Daphnia* gut tracts than other size carbon dots (Figure 3.5D). The presence of approximately 50 nm particles were observed by transmission electron microscopy analysis of these 113nm suspensions. Thus, systemically distributed carbon dots from this treatment may only be from smaller sized portion. With the exception of accumulation within the gut tract and carapace, no preferential target of accumulation could be determined for the carbon dots once absorbed. Similarly, no size-dependent differences in distribution could be determined.

Similar results were observed in *C. dubia* exposed to these carbon dots. However, because of their rate of maturation, many of the *C. dubia* at the end of 24 h in clean water exhibited internal fluorescence associated with eggs or gestating neonates.

Treated zebrafish eggs exhibited no fluorescence after 24 h exposures. The chorion of the egg may be sufficient to prevent absorption of carbon dots. Larvae hatched from exposed eggs varied greatly in intensity and location of fluorescence. This variability is likely due to differences in time between hatching and sampling.

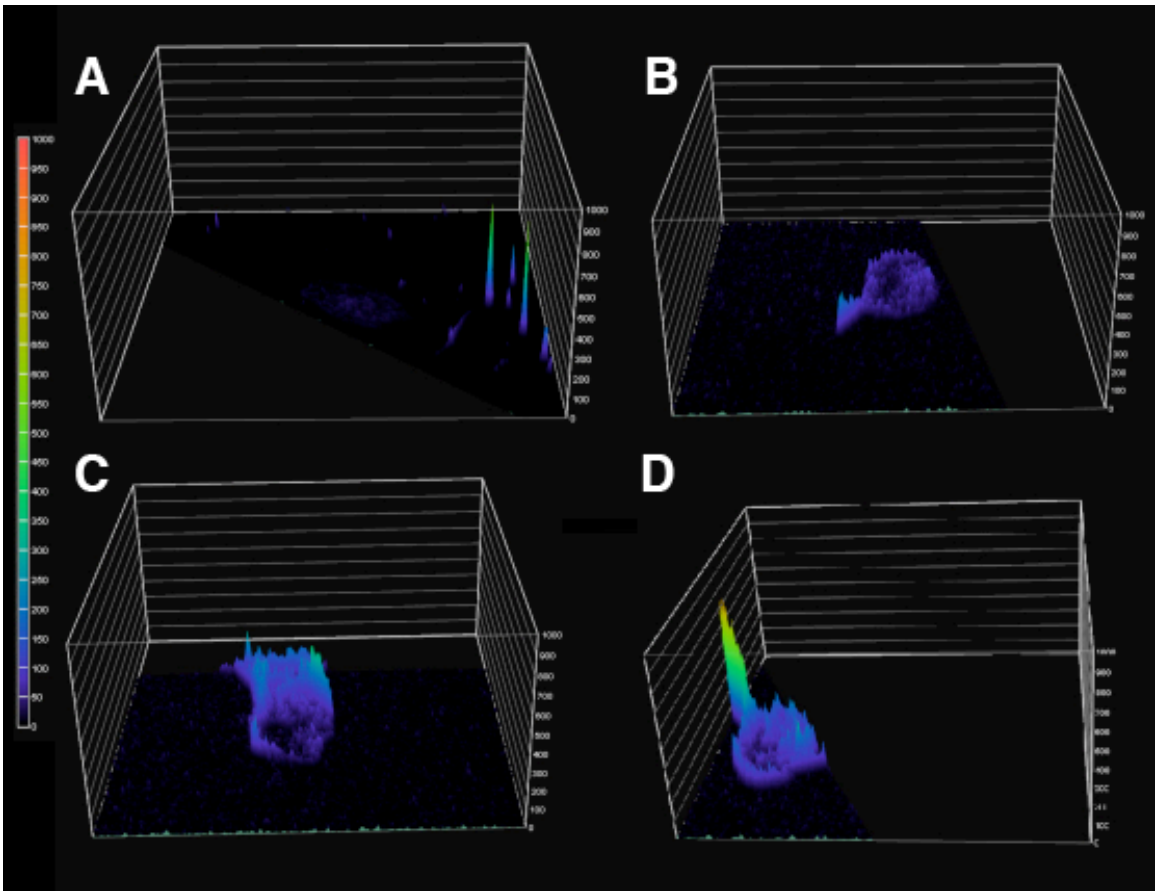

Figure 3.5. Typical confocal fluorescence intensity plots of micrographs of *D. magna* after 24 h exposure and 24 depuration in clean media A) Control B) 8nm carbon dots C) 40nm carbon dots and D) 113nm carbon dots

MATERNAL TRANSFER

No differences from controls were detected in fluorescence of any of the treated eggs of *D. magna* for both acute and chronic exposures. Although carbon dots are readily absorbed by *D. magna*, carbon dots are not transferred to offspring of exposed *D. magna* under the conditions tested.

CONCLUSIONS

The present study suggests that *D. magna* can readily absorb carbon dots, which subsequently undergo systemic distribution. These carbon dots, however do not exhibit deleterious effects at concentrations as great as 10 mg/L, regardless of carbon dot size. These data are important because they demonstrate that carbon dots may serve as less toxic alternatives to the use of semiconductor quantum dots for *in vivo* biomedical applications.

Our data give no evidence of the transfer of carbon dots from exposed *D. magna* to their offspring. Because carbon dots only retain their fluorescence when coated, no conclusions can be drawn about the transfer of possible degradation products. Degradation products of semiconductor quantum dots such as cadmium, zinc and selenium can be transferred to offspring of exposed organisms.

Zebrafish embryos exhibited no malformations or other adverse effects from carbon dot treatment. These data suggest carbon dots have little to no effect on offspring

viability in fish. Although these carbon dots were observed to accumulate in the gut tract, the lack of observed reproductive effects in *C. dubia* treated to carbon dots suggests chronic exposures are unlikely to produce adverse effects in *Daphnia* from diminished ability for to assimilate food. Although carbon dot size was observed to have an effect on residence time in the gut tract, our data show no evidence that the uptake or distribution of carbon dots is dependent on their size.

Nanoparticle size can still have a drastic effect on the bioavailability and toxicity of other nanomaterials. Further research into the toxic effects of carbon dots should consider different surface coatings.

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CHAPTER FOUR

DIFFERENTIAL UPTAKE OF CADMIUM FROM QUANTUM DOTS IN *DAPHNIA MAGNA*

ABSTRACT

The toxic effects of semiconductor quantum dots have been partially attributed to the release of heavy metals with their degradation, particularly cadmium. Laser ablation inductively coupled mass spectrometry was used to compare the uptake of cadmium, selenium and zinc in *Daphnia magna* treated to CdSe/ZnS quantum dots or $CdCl^{2+}$. These quantum dots were observed to accumulate primarily in the gut lumen and no evidence of uptake of intact quantum dots was observed. Evidence suggests degradation of the quantum dots release of component ions with accumulation of Cd and Zn in the gut epithelia. Quantum dots elicited acute toxicity at 0.66 mg/L Cd but promoted increased reproduction at 40 µg/L.

INTRODUCTION

Semiconductor quantum dots (QD) are of particular interest for a variety of applications, particularly because their unique optical properties make them well suited for their use in electronic displays and biomedical imaging. [Brown et al. 2008, Michalet et al. 2005] Other applications include photovoltaic cells and traceable therapeutic vectors [Michalet et al. 2005, Qing et al. 2008]. These optical properties are a product of their nanoscale size; a size dependent band gap in emission energy allows for tunable emission wavelengths and resistance to photobleaching to the point they are regarded as more efficient than many other flourophores [Yu et al. 2003]. However, these materials have been implicated in causing toxicity due to the degradation and subsequent release of heavy metal components such as Cd. Their commercial use has been limited to *in vitro* and some small animal models [Lewinski et al. 2010].

Although many of these studies using *in vivo* models have tracked the distribution and elimination of these materials, doses have generally been applied by injection [Hauck et al. 2010, Fischer et al. 2006]. For environmental applications these studies are not suitable for understanding distribution and toxicity of QD and their associated release of Cd. Of particular concern is that QD in general are often coated in hydrophilic ligands or covalently bound functional groups to make these particles more bioactive. The increase in the bioactivity of these compounds in this manner may lead to increased risk of exposure via extended residence in the water quality and increase in bioavailability.

Daphnia magna, a freshwater zooplankton, is a simple model well suited to study the uptake and distribution of fluorescent materials because their relative transparency and small size allows for whole body microscopic analysis [Lewinski et al. 2010]. They are also model organisms with standard procedures for toxicity testing with multiple regulatory agencies. For this reason, a substantial amount of knowledge already exists for the toxicity of Cd in *D. magna.* Typical pathways of exposure for nanomaterials include sorption through external surfaces and ingestion of particles or contaminated food particles.

QD aggregate and are immobilized in the digestive tract of the *Daphnia* [Lewinski et al. 2010]. Their residence time in the gut tract and their propensity for assimilation have been found to be dependent on the coating of the QD. The effect of coating on uptake and distribution had also been observed in larger organisms models [Hauck et al. 2010].

Because fluorescence microscopy can only detect intact QD that maintain their photoluminescent properties, this technique does not adequately track the uptake of components of QD that may be released by degradation. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been successfully used for microscopic elemental analysis in biological samples [Barst 2011]. This study included the analysis of differential Cd, Se and Zn dispersion detected by LA-ICP-MS to determine differences in uptake of these elements from CdSe/ZnS QD as compared to ionic Cd exposure. The results of this study can be used to determine if a pathway of uptake exists for QD and their component elements.

MATERIALS AND METHODS

MATERIALS

Cadmium selenide quantum dots with a zinc sulfide shell and coating of poly(maleic anhydride-alt-1-octadecene conjugated to polyethyelene (CdSe/ZnS-PMAO-PEG) in a borate buffer were obtained from Professor Vicki Colvin, Rice University. The diameter of the particles was 30-40nm (core diameter of 5.5 nm). The emission wavelength of the QD was 590 nm.

DAPHNID ACUTE BIOASSAYS

Daphnia magna neonates were obtained from an in house laboratory stock maintained at the Institute of Environmental Toxicology, Clemson University (CU-ENTOX). Routine reference acute toxicity tests have been performed previously with this culture to ensure consistent culture sensitivity to sodium chloride. Results of these reference toxicity tests are available through CU-ENTOX by contacting the corresponding author. Tests were performed based on U.S. Environmental Protection Agency standard methods using synthetic freshwater [EPA 1993]. Test volumes and the number of organisms per replicate were altered to compensate for the limited supply of C_{70}. This synthetic freshwater was used to prepare C_{70}-GA suspensions of the following nominal test concentrations: 0.125, 0.5, 1 and 2 mg/L C_{70} by serial dilution for acute toxicity

bioassays. Test suspensions were prepared daily.

Acute bioassay methods followed standard procedures [EPA 1993]. *Daphnia magna* neonates less than 24 h old were exposed in static renewal acute toxicity tests in 30 ml glass beakers containing 25 ml test solutions at 25 ± 1 °C. Three replicates per treatment were tested, and each treatment contained five neonates. Mortality was observed at 24 h intervals with organisms fed a diet of algae (*Selenastrum capricornutum*) and yeast-cereal-trout chow. After allowing organisms to feed for 1 h, all living organisms were transferred to test chambers containing fresh test solutions.

DAPHNID CHRONIC BIOASSAYS

Chronic bioassay methods followed standard procedures [EPA 1993, Klemm 1994] with modifications. Test suspensions of C_{70}-GA of nominal test concentrations 1, 2, 4, 8 and 16 µg/L C_{70} were prepared by serial dilution of stock suspension with synthetic freshwater for the 21 d bioassays. Neonates less than 24 h old were exposed in static renewal tests in 500 ml polyethylene beakers containing 400 ml test solutions at 25 ± 1 °C. Three replicates of five individuals were tested per treatment. Mortality and reproduction were observed at 24 h intervals. At this time all offspring were counted and discarded, and remaining living organisms were transferred to test chambers containing fresh test solutions. After the daily transfer, organisms were fed a diet of algae (*S. capricornutum*) and yeast-cereal-trout chow.

LA-ICP-MS

Glass mounted *D. magna* sections were analyzed by LA-ICP-MS for the differences in trace element uptake and distributions between organisms exposed to either QD or ionic Cd^{2+}. Twenty *D. magna* neonates (<24 h) were exposed to QD (20 mg/L Cd) and CdCl (10 mg/L Cd) for 24 h in MHW without food. After treatments, all surviving organisms were sacrificed and fixed in 10% buffered formalin for 48 h. Fixed samples were dehydrated by a series of ethanol washes an embedded in paraffin wax. Embedded samples were sectioned by ultramicrotome and sections mounted on glass slides. Samples were stained with hematoxylin.

Microscope slides were placed into the chamber of a 213nm Nd: YAG laser ablation source (New Wave Research, Fremont CA). Areas of interest were chosen at random for each location type (gut lumen, gut epithelia and internal tissue) for each of five organisms per treatment using a charge-coupled detector camera. Areas of interest were ablated with a 30 μm beam diameter. Ablated samples were analyzed for ^{111}Cd, ^{78}Se and ^{66}Zn isotopes by a Varian 820 ICP-MS coupled to the laser source. Mean isotope counts for the first 30 s of the mass detector signal were used to estimate background noise for each run because this time period occurred prior to sample ablation. These values were subtracted from the remainder of each run to calculate a signal.

STATISTICAL ANALYSIS

Survival data from the 96 h test were analyzed by trimmed Spearman-Karber method to derive 96 h median lethal concentration (LC_{50}). Data sets from the 21 d toxicity bioassays, FET, and LA-ICP-MS analyses were each analyzed for significance by one-way ANOVA with Tukey's post hoc test using SAS Software (SAS Institute). Significant differences were established at $p<0.05$ except where otherwise indicated.

RESULTS AND DISCUSSION

TOXICITY BIOASSAYS

Quantum dots exposure resulted in a dose dependent reduction in survival of *D. magna* (Figure 4.1). A 96 h LC_{50} of 0.66 ± 0.19 40 µg/L total Cd was calculated for these QD. Although there appeared to be a downward trend in survival in the chronic bioassay, the QD did not exhibit any statistically significant change in survival up to 160 µg/L total Cd, whereas $CdCl_2$ exposures resulted in 100% mortality at 40 µg/L Cd (Figure 4.2). Cadmium chloride resulted in a dose dependent reduction in the average number of neonates per adult, with a significant reduction compared to controls at 10 µg/L Cd (Figure 4.3). In contrast, the QD exhibited no significant reduction in reproduction at concentrations up to 160 µg/L Cd. A hermetic response was apparent, as significantly increased fecundity was observed at the QD exposure equivalent to 40 µg/L.

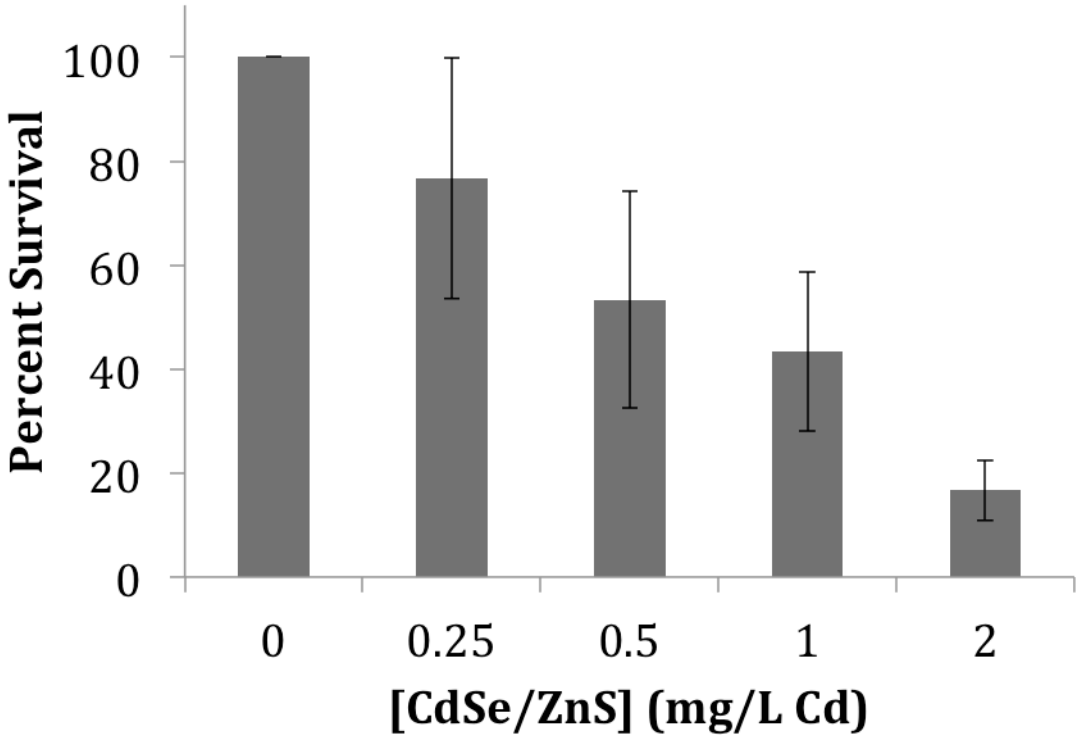

Figure 4.1. Average percent survival (±SD) of *Daphnia magna* exposed to CdSe/ZnS-PMAO-PEG quantum dots in 96 h static renewal bioassay. X-axis concentrations measured total Cd concentrations.

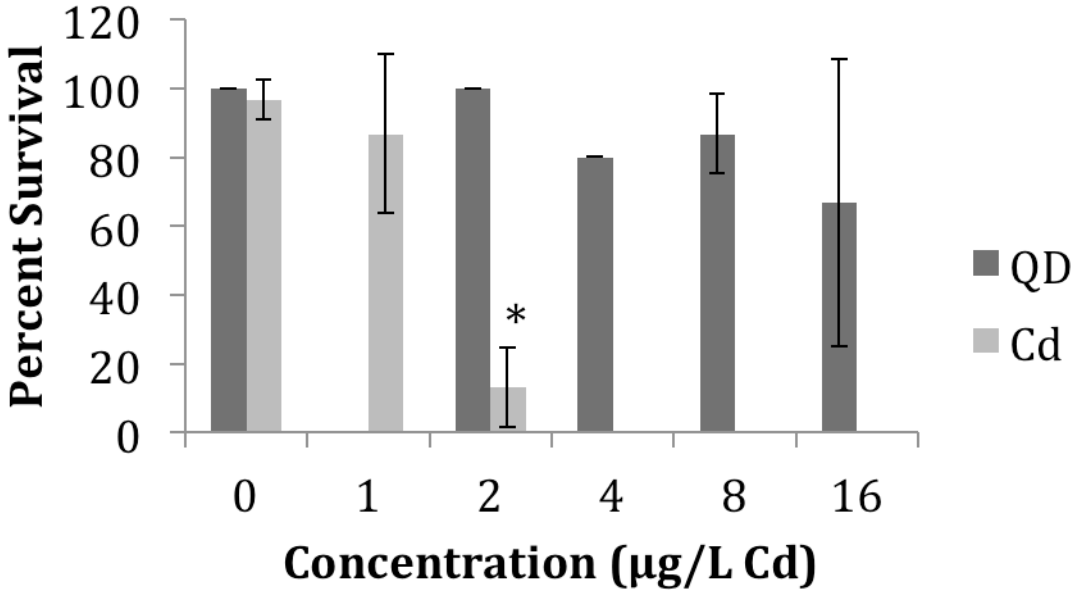

Figure 4.2. Average percent survival (±SD) of *Daphnia magna* exposed to Cd^{+} or CdSe/ZnS-PMAO-PEG quantum dots in 21d static renewal bioassay. X-axis concentrations measured total Cd concentrations. * Significantly less than control ($p<0.05$).

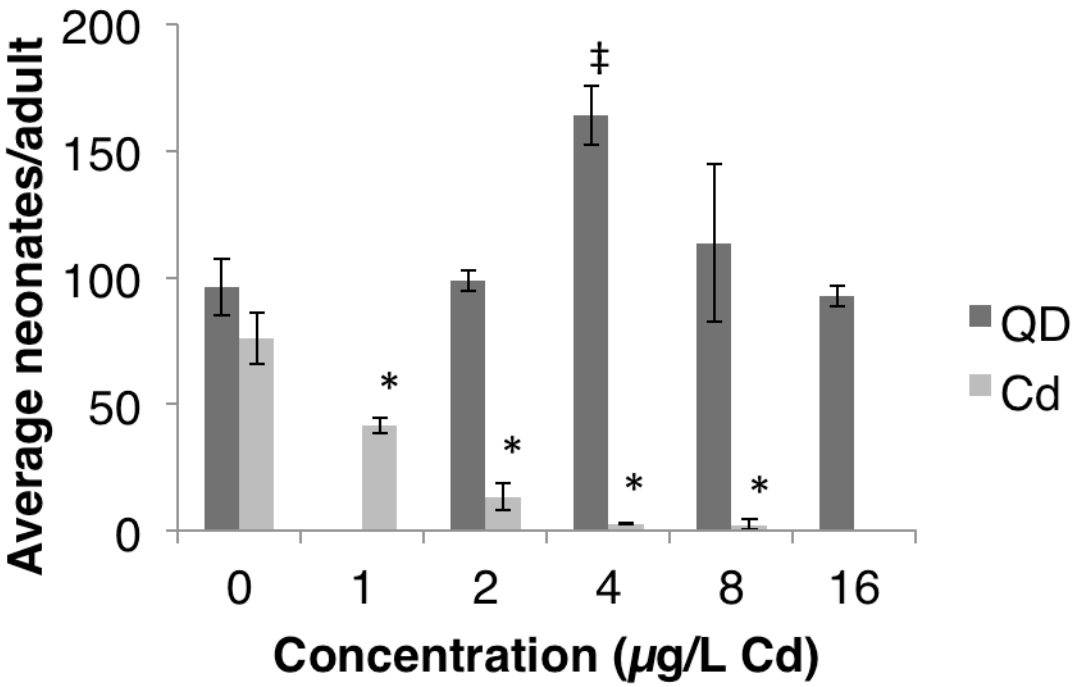

Figure 4.3. Average total neonates/adult (±SD) of *Daphnia magna* exposed to Cd or CdSe/ZnS-PMAO-PEG quantum dots in 21d static renewal bioassay. X-axis concentrations measured total Cd concentrations. * Significantly less than control ($p<0.05$). ‡ Significantly greater than control ($p<0.05$).

LA-ICP-MS

Selenium, Zn, and Cd were present in greater quantities in the gut lumen of QD-exposed daphnids compared to controls and $CdCl_2$ exposures (Figure 4.4). Similarly, QD-exposed organisms had more Cd and Zn associated with the gut epithelia than did controls and $CdCl_2$ exposures (Figure 4.5). Selenium, however was lower in the gut epithelia of QD exposed organisms than in controls and $CdCl_2$ treatments. No significant differences were observed for elemental analyses of internal tissue samples (Figure 4.6).

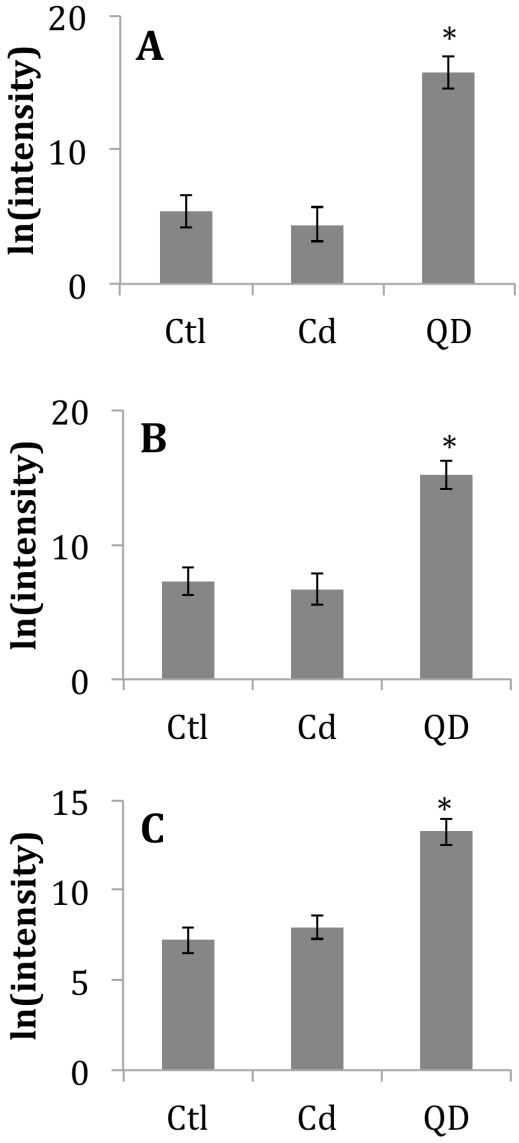

Figure 4.4. Natural log intensity of *Daphnia magna* gut lumen obtained by LA-ICP-MS for A) Cd, B) Zn and C) Se. * Significantly greater than control ($p<0.05$).

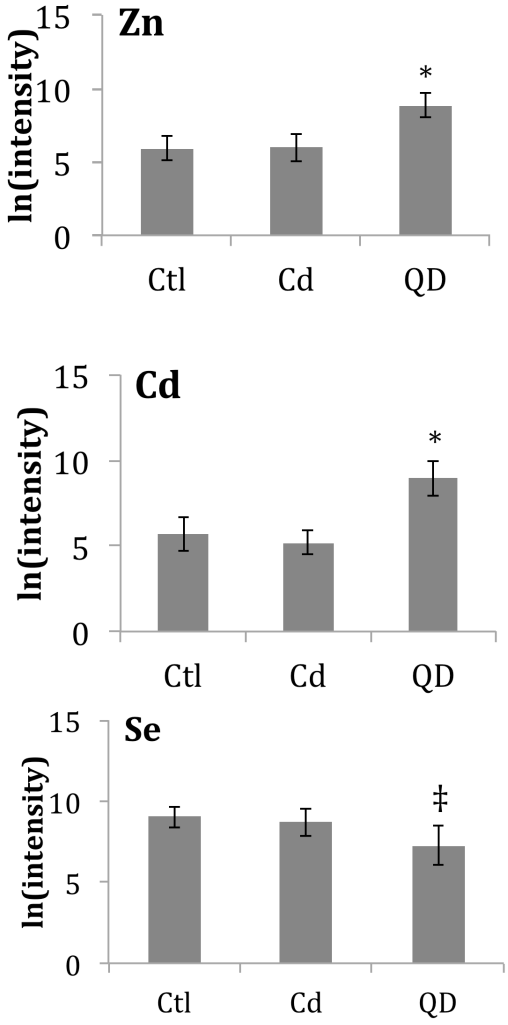

Figure 4.5. Natural log intensity of *Daphnia magna* gut epithelia obtained by LA-ICP-MS for A) Cd, B) Zn and C) Se.* Significantly greater than control ($p<0.05$). ‡ Significantly less than control

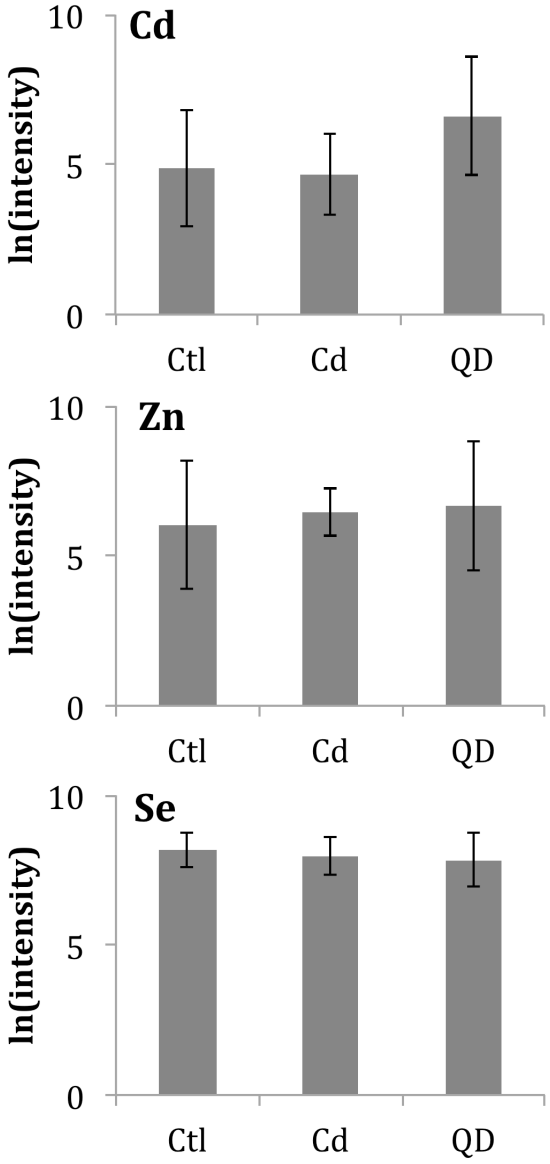

Figure 4.6. Natural log intensity of *Daphnia magna* tissue obtained by LA-ICP-MS for A) Cd, B) Zn and C) Se. * Significantly greater than control ($p<0.05$).

Daphnia magna exposed to QD had higher concentrations of Cd, Se and Zn in the gut lumen compared to the $CdCl_2$ treatment and the control. This was expected because of the accumulation of QD, containing all three elements, in the gut tract as observed by fluorescence microscopy. However, there was no significant difference of these elements in the gut lumen of $CdCl_2$ exposed organisms as compared to controls. The lack of Cd in this location despite an exogenous source in Cd and QD treated samples may be attributed to dissolution of Cd from the samples during the multiple solvent treatment stages of sample preparation. Organisms fed during the exposure would likely exhibit greater intraluminal metals concentrations due to sorption to food particles preventing such dissolution.

Although there is no significant difference from controls in regards to any measured elements in the gut epithelia of $CdCl_2$ exposed *D. magna*, QD exposed organisms maintained a much greater concentration of Cd and Zn than other treatments. Because Se and Cd are present in the QD core, a similar increase in Se would be suggestive of intact QD. The disparity between Cd and Se in the gut epithelia indicates degradation of the QD and subsequent ion release.

Despite the presence of excess Se from QD the gut epithelia concentrations of Se were decreased compared to controls (α=0.05) and $CdCl_2$ treatments (α=0.1). Selenium released from the QD may be translocated across the gut epithelia, but there was no significant indication of elevated Se in tissue other than the gut epithelia. Elimination of Se could not be determined from the present study because Se concentrations in the stock media were not measured. A considerable difficulty in analyzing trace quantities of Se by

ICP-MS involves signal noise contributions of Ar_2 dimers produced from the Ar carrier gas during ICP-MS analysis, which can be falsely detected as Se isotopes.

Although Kungolos and Aoyama [1993] found aqueous exposure to be a more important pathway of Cd uptake than dietary exposure, Barata et al. [2002] observed *D. magna* can retain more Cd from food than from water. This disparity is partially explained by the hypothesis that differences in lethal and sublethal responses of *D. magna* to Cd by different exposure pathways are controlled physiological mechanisms [Taylor et al. 1998, Barata et al. 2000]. Feeding inhibition in *D. magna* was almost entirely due to Cd bound to algae, rather than dissolved Cd [Taylor et al. 1998]. Barata et al. [2002], however postulated that survival was solely governed by dissolved Cd in both exposure pathways and Cd toxicity is not necessarily related to uptake [Barata et al. 2002]. Total body burden is similarly not a good indicator of dietary zinc toxicity [De Schamphelaere and Janssen 2004].

Evidence suggests that gut physiology and Ca metabolism are impaired by ionic Cd^{2+} exposure with the mid-gut diverticulla being the main target for the uptake of Cd [Griffiths 1980, Taylor et al. 1998]. Differences in metals assimilation efficiencies between aqueous and dietary exposures are likely due to differences in bioavailability.

As with Se, there were no significant differences between treatments for Cd or Zn associated with internal tissues of *D. magna*. Because the variation of size and shape of tissue selected for analysis, the areas selected for analysis were typically not completely occupied by sample, allowing for variation in total surface area analyzed and underestimation of element concentrations. This variation would explain the lack of

significance differences in average intensity values despite considerable differences in maximum intensity values, such as the detection of Cd in QD exposed organisms up to an order of magnitude greater than that of controls.

Both Se and Zn are nutritionally relevant elements for *Daphnia* and are readily absorbed. Although Zn is preferentially absorbed compared to Cd [Barata et al. 2002], Zn remains at highly elevated levels with the gut epithelia. Because the amount of exogenous Cd from the QD core is potentially much greater than Zn released from the monolayer shell, the ionic Cd concentrations present in the gut epithelia may be sufficient to inhibit Zn absorption.

The increase in fecundity observed in *D. magna* exposed to QD at low concentrations (4 µg/LCd) is suggestive of absorption of Se and Zn released via QD and used as nutrients to promote neonate production. Neonates production has been shown to be a significant elimination pathway for both Se and Zn [Barata et al. 2002]. Lack of Se in culture media has been shown to cause abortion of eggs (Winner and Whitford 1987). Exposure to $CdCl_2$ at the equivalent (4 mg/L) total Cd caused significant mortality in *D. magna*, indicating that QD do not degrade completely. The toxic effect in the presence of QD may also have a positive effect on the reproductive rate of *D. magna*; Jenson and Marshal observed the presence of a toxicant such as Cd can induce a reproduction to compensate for the death rate in a population of *D. magna* [1983]. Although at extremely low concentrations QD are less toxic than the equivalent of free Cd^{2+} ions, the QD in the present study is acutely toxic to *D. magna* at concentrations less than 1 mg/L Cd (48 h LC_{50} 0.66 ± 0.19 mg/L total Cd).

CONCLUSIONS

The present study provides evidence that metals common to QD can be differentially absorbed by *D. magna* when the organism is exposed to QD as compared to ionic Cd^{2+} from $CdCl_2$. The apparent lack of absorbed, intact QD suggests that their effects are primarily attributable to their interaction with the cells of the gut wall or the release of their constituents. Accumulation of QD in the gut tract permits the release of these metals and provides a route of exposure analogous to dietary metals exposure. Dietary exposure allows for decreased acute toxicity, development of Cd tolerance and greater assimilation of Cd by *D. magna* compared to aqueous exposure. These factors can positively influence trophic transfer which has been shown to biomagnify in freshwater food webs [Croteau et al. 2003].

Cadmium exposure typically decreases *D. magna* feeding rates which may be partially responsible for the lengthy residence time of QD in the gut tract compared to carbon dots. Alternatively, QD may be absorbed and retained by gut epithelial cells. Further microscopic and elemental analyses are necessary to determine the state of *in vivo* QD degradation. Further understanding of the importance of ion release on the toxic effects of QD could be obtained from the exposure of tolerant strains of *D magna* for each of the QD constituents.

Although this evidence further supports the hypothesis that bioburdens of metals may not be suitable for predicting toxicity, it suggests that QD released Cd toxicity may involve a physiological pathway different from aqueous Cd exposure. Further study

would be necessary to determine if Cd bioburden would be predictive for QD toxicity. Because of current limitations of LA-ICP-MS for absolute quantification future studies of this relationship would benefit from the use of other analytical methods. However, LA-ICP-MS remains a powerful tool to determine relative quantitative relationships at the microscopic scale. Further research with this technique would benefit from the use of larger sample organisms, including adult *D. magna* or alternative species.

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CHAPTER FIVE

CONCLUSIONS AND IMPLICATIONS

One of the major uses of engineered nanomaterials is *in vivo* imaging for biomedical and diagnostic purposes. Because there is such diverse selection of nanomaterials and derivatives of these materials, it is difficult to ascertain which properties best serve in predicting toxicity of novel nanomaterials. In this dissertation, the hazards associated with three aqueously suspended nanomaterials (fullerene C_{70}, quantum dots and carbon dots) with potential biomedical applications was characterized for freshwater aquatic organisms. These nanomaterials were chosen because they are distinctly different yet share some similar properties with each other and other nanomaterials. One aspect of particular interest was that each of these materials maintained a potential for detection by fluorescence, which could be used for *in vivo* analysis of uptake. The goal of this study was to determine if toxic effects could be predicted based on the properties of the nanomaterials studied. Conclusions of this research include the following:

1. **Carbon dots are the least toxic of the three nanomaterials tested.** Carbon dots were predicted to be less toxic than quantum dots (QD) because QD are composed of toxic metals rather than carbon. A summary of the acute toxicity tests for each nanomaterial studied is provided in Table 5.1.
2. **C_{70} toxicity to *Daphnia magna* is a result of oxidative stress**. Although C_{70} is also comprised of carbon like carbon dots, these two nanomaterials have distinct structural

differences providing for different chemical and physical properties. This specific toxic response of oxidative stress was predicted to occur due to the similarity in properties to C_{60}, another fullerene. The 96hLC_{50} value of 0.44 mg/L C_{70} is comparable to the lowest values reported for C_{60} (48hLC_{50} = 0.460mg/L C_{60}) [Lovern and Klaper 2009].

3. **Carbon dots are distributed within *Daphnia magna* while fullerenes and quantum dots stay within the intestinal tract**. Although these materials each maintained similar terminal surface chemistries and were observed at comparable sizes, the differences in core composition affected the ability for uptake of intact nanomaterials.
4. **Quantum dot toxicity to *Daphnia magna* is a result of ionic Cd release**. Retention of fluorescence in the gut tract associated with intact QD and reduced toxicity compared to equivalent Cd^{2+} exposure indicate incomplete degradation of the QD.
5. **Innovative imaging techniques can be useful to assess nanomaterial fate in organisms and their effects**. Carbon dots and quantum dots were each observed *in vivo* using fluorescence techniques. Inductively coupled plasma mass spectrometry coupled with microscopic laser ablation may be highly beneficial in the determination of the biological fate of metals.
6. **No single particle characteristic can be used to predict nanomaterial toxicity**. This research provided evidence that core composition had a notable effect on nanomaterial toxicity. Carbon dots are much less toxic compared to C_{70} although both

are comprised of carbon. Carbon dots and QD exhibited very different behaviors despite sharing similar physical properties and surface chemistries.

Table 5.1. Summary of acute toxicity values and nanomaterial properties and the proposed mechanism of toxicity.

Nanomaterial	96 h LC_{50}	Core Chemistry	Surface Chemistry	Toxicity Mechanism
C_{70}-Gallic acid	0.4 mg/L C_{70}	Carbon – fullerene	Gallic acid	Oxidative stress
Carbon dots	>10 mg/L carbon dots	Carbon - amorphous	PEG	NA
Quantum dots	0.66 mg/L total Cd	CdSe/ZnS	PMAO-PEG	Cd^{2+} release

This study supports that general predictions can be made about nanomaterials based on shared physical and chemical properties with other nanomaterials. A systematic approach to comparing the toxicity of nanomaterials of varying physical and chemical properties such as size, shape, composition, charge, etc. would be beneficial in determining the interrelationship of these properties and toxic response. Such studies, however are cost prohibitive. Literature-based studies of such relationships are not feasible due to a lack of standardization in testing methods and lack of published characterization of the test substance.

The cases of fullerene aggregation and QD degradation in this study illuminate the importance of understanding the influence of nanomaterials behavior on their toxicity. Nanomaterials behave drastically different from other contaminants, which follow predictable behaviors in regards to fate and transport that can be confirmed by analytical methods. Although techniques for the analysis of nanomaterials are rapidly advancing, nanomaterial behavior is poorly understood. Further study into the assessment of nanomaterial hazards would benefit from the use of such techniques to determine relation of nanomaterial behavior in the environment to toxic effects.

Characteristic behaviors of nanomaterials should be considered in determining their modes of toxicity. For instance, the toxic effect of C_{70} in this study may be in part due to the aggregation of carbon nanomaterials into larger particles that can obstruct the gut tract resulting in diminished food absorption [Edgington et al. 2010]. Morales et al. determined that prolonged starvation promotes oxidative stress [2004]. Comparative studies using materials that similarly cause physical blockage such as suspended clay particles would be beneficial in determining if physical stress can initiate a similar response to that of fullerene exposure. Such information would provide a distinctive argument in conflicting reports of fullerenes acting as either prooxidants or antioxidants.

As manufactured forms of nanomaterials are less likely to occur in the environment than their environmentally altered counterparts, Nowack et al. [2012] proposed that risk assessment frameworks for nanomaterials should include additional categories to reflect the diversity of altered end products of nanomaterials and further

pose that studies using pristine nanomaterials may not be relevant for assessing the behavior of the nanomaterials actually used.

Although the conjugation of particular compounds, such as proteins, have been shown to affect the ability of cells to take up nanomaterials, it is important to note that the bioavailability of these altered nanomaterials to aquatic organisms may be influenced more by the effect the conjugate has on the aqueous stability of these nanomaterials.

Future understanding of the role of metal ions in QD would benefit from the study of exposures to degraded QD. The degradation of these QD prior to ingestion would affect the exposure route of released ions, which could greatly affect the degree of toxic response since uptake of ions through the gut may be protective in the case of Cd^{2+} compared to aqueous Cd^{2+} exposure. The role of metal ions in QD toxicity could be further elucidated by studying QD exposure to organisms conditioned to be tolerant for each of the ions that comprise the QD. A disadvantage of this method is that the mechanisms by which such organisms compensate for elevated levels of the metals could also be protective of any effects directly caused by intact QD.

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APPENDICES

Appendix A

C_{70} Gallic Acid Results

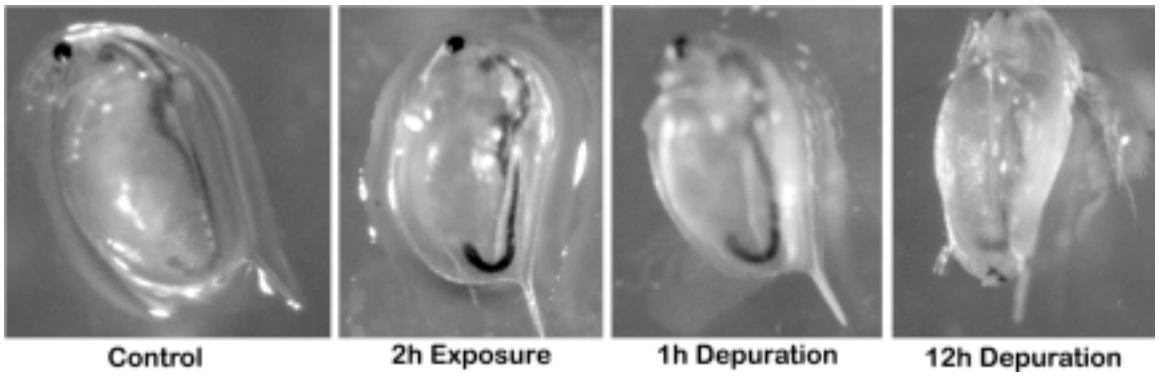

Figure A-1. Time-course micrographs of *Daphnia magna* exposed for 2 hours to C_{70} gallic acid and then placed in clean media without food.

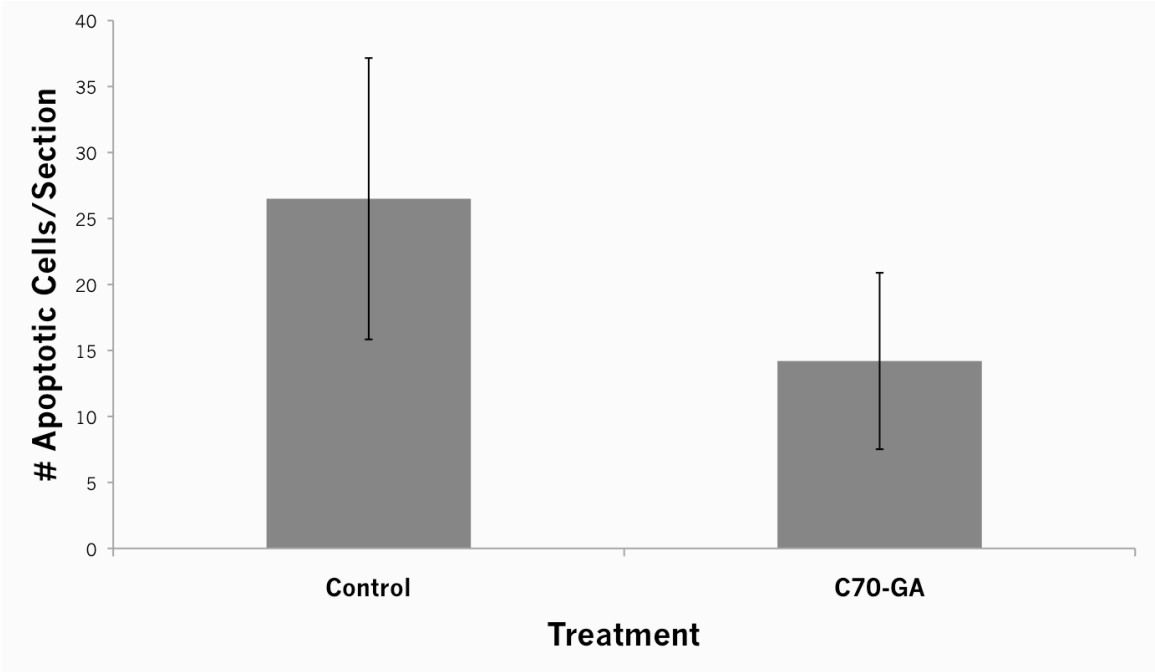

Figure A-2. Mean number of apoptotic cells in *Daphnia magna* juveniles (n=10) exposed to C_{70} gallic acid (C_{70}GA) as determined as CR590 fluorescently labeled cells. Values are expressed as mean ± S.D.