CHANGES IN AQUATIC ORGANISM HEALTH AND REPRODUCTION FOLLOWING CHRONIC EXPOSURES TO MULTI-WALLED CARBON NANOTUBES

by

EMILY RAE MCREYNOLDS

(Under the Direction of Marsha C. Black)

ABSTRACT

Engineered carbon nanotubes (CNTs) are revolutionizing the field of biotechnology, with advances in medicine, water purification, construction, and military applications because of their unique properties. With many products on currently on the market containing CNTs, contact with the aquatic environment is inevitable throughout the life cycle of CNT-based products. The U.S. Environmental Protection Agency has listed CNTs and other nanomaterials as "Contaminants of Emerging Concern;" however, the lack of information available, specifically on toxicity to aquatic organisms, hinders the EPA from moving forward with regulations. The purpose of this dissertation was to provide data on chronic exposures of ^{14}C-labeled multi-walled carbon nanotubes (MWCNTs) to model organisms typically used in traditional toxicity tests and determine how the presence of dissolved natural organic matter (NOM) influenced the reproductive toxicity of MWCNTs and overall organism health. We observed that exposure to 2.5 mg/L sonicated MWCNTs decreased the number of offspring released and brood size in *Ceriodaphnia dubia*, but the presence of NOM (2.35 mg C/L) negated this effect, but at a

cost of increased internal accumulation. The presence of NOM also influenced toxicity of MWCNTs to *Americamysis bahia*, where a decrease in the percentage of mature individuals was observed with exposure. We also exposed *Pimpephales promelas* to ^{14}C-MWCNTs-contaminated sediment. *P. promelas* did not accumulate measurable MWCNTs, but we hypothesize that a 24-h depuration period is sufficient for them to clear their gut tract, as measurable MWCNTs were collected in fecal material released during the depuration period. We also observed that bioturbation of sediment by *P. promelas* can influence the ability of MWCNTs to move into the water column but access to the sediment did not change the incidence of morbidity. Finally, we analyzed the literature available on crustacean toxicity after exposure to carbon nanoparticles, integrating my observations from previous studies, to predict mode(s) of action of MWCNTs toxicity to aquatic organisms and give guidance for future research. Overall, these findings provide some of the data needed for a successful risk assessment framework for future regulation of MWCNTs and highlight the importance of NOM in understanding the toxicity of MWCNTs.

INDEX WORDS: nanomaterials, carbon nanotubes, accumulation, *Ceriodaphnia dubia*, natural organic matter, reproductive toxicity, *Americamysis bahia*, ecdysis, *Pimephales promelas*, sediment, liquid scintillation counting, ecotoxicology

CHANGES IN AQUATIC ORGANISM HEALTH AND REPRODUCTION FOLLOWING CHRONIC EXPOSURES TO MULTI-WALLED CARBON NANOTUBES

by

EMILY RAE MCREYNOLDS

BS, University of Tennessee at Knoxville, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

CHANGES IN AQUATIC ORGANISM HEALTH AND REPRODUCTION FOLLOWING CHRONIC EXPOSURES TO MULTI-WALLED CARBON NANOTUBES

by

EMILY RAE MCREYNOLDS

Major Professor: Marsha Black

Committee: Robert Bringolf
Erin Lipp
Matthew Henderson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2013

DEDICATION

To my family, whose encouragement to explore the world around me led me to where I am today.

ACKNOWLEDGEMENTS

First, I thank my advisor, Marsha Black, for giving me the opportunity to work on this project. Even as my mentor, Marsha has treated me as a peer and valued my opinions and suggestions. She has challenged me to think critically, and helped me to use my ecology background to connect my research with the bigger picture. My skills as a toxicologist and a professional have blossomed under her supervision, and I appreciate her unwavering attention in helping me succeed.

My committee has been such a vital resource for me in the development of this dissertation and I appreciate their support and friendship. Drs. Erin Lipp, Robert Bringolf, and Matthew Henderson brought their expertise of their fields to give me advice on experimental designs, helped me problem solve, and explore new routes of research. I could not imagine completing this work without their input and thank them for their dedication in helping me to succeed. I highly value my time working with this interdisciplinary team and give them credit for helping me to become a better scientist and professional.

I am so grateful for being a part of the Georgia Oceans and Health Initiative, which helped me to create meaningful contacts in a truly interdisciplinary field. I appreciate Drs. Marie DeLorenzo and Michael Fulton at the NOAA lab in Charleston for advice and an unforgettable experience that allowed me to collect vital data for Chapter 3. Much credit goes to Erin for being such a strong leader for the GOHI group and encouraging me to attend the Gordon Research Conference. I thank all of the Black lab

members (past and present), colleagues, undergraduate researchers, and staff at the University of Georgia during my graduate career, with special acknowledgement to: Carrie Givens, Jessica Joyner, Deanna Goodwin, Caitlin Smith, Eric Goolsby, and David Brew. A special thank-you goes to Joanne Mauro, who has always gone above and beyond her duties to help me with any problem that needed solving.

So many people have helped shaped my path for me to become the person (and scientist) I am today, even while not physically at UGA with me. I especially cherish my time spent at ENVIRON in Brentwood, TN and truly appreciate the people there who originally sparked my interest in aquatic toxicology. Working at ENVIRON as a lowly high school student and throughout college gave me the fundamental knowledge for the research in this dissertation and allowed me to hit the ground running. Shereé Sheffer and Ryan Towle are two loyal friends who have been supporting me since the day I got my acceptance letter to attend the University of Georgia. Also, my Air Force family has always given me the encouragement and stamina to continue when I have needed it the most, but also provided the best adventures to keep me sane.

Lastly, I thank my amazing family: my dad, Jeff, my mom, Vickie, my sisters, Stephanie and Courtney, and my husband, Josh. They have always been my number one supporters, cheering me on at every stage of graduate school and have never been fair weather fans. Also, I cannot begin to express my gratitude for the emotional, mental, and physical support Josh has provided me over the years. He has heard so many presentations about carbon nanotubes he could present them in his sleep and has also proven to be an excellent editor of this dissertation.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

A REVIEW OF CARBON NANOTUBE BEHAVIOR AND TOXICITY IN THE AQUATIC ENVIRONMENT

INTRODUCTION TO NANOTECHNOLOGY

Scientists of the 21st century are going "small." The newly popular field of nanoscience involves researching materials that are on the nanoscale (0.1-100 nm). Nanotechnology involves manipulating and synthesizing novel products that have unique properties based on this small scale (Moore 2006). The American Society for Testing and Materials (ASTM 2006), British Standards Institution (2007), and the Scientific Committee on Emerging and Newly-Identified Health Risks (2007) define "nanomaterial" as a material with one dimension under 100 nm. ASTM defines a "nanoparticle" as a material that has at least two dimensions between 1 and 100 nm. Nanoparticles are not always engineered materials; nearly 40% of particles produced from diesel- and gasoline-fueled vehicles, as well as combustion sources (like cars) in urban settings, are on the nano-sized scale (Shi et al. 2001). Historically, natural and incidental nanoparticles in air have been referred to as "ultrafine particles," while in soil and water, are termed "colloids" (Klaine et al. 2008). Aquatic colloids contain macromolecular organic materials such as humic and fulvic acids, proteins, as well as inorganic species like hydrous iron and manganese oxide (Hyung et al. 2008).

Fullerenes (C_{60}, also known as buckyballs or Buckminster fullerenes) are one of the eight known allotropes of carbon, the most familiar being diamond and graphite. Carbon nanotubes are a type of fullerene molecule. Carbon nanotubes are a recently discovered allotrope of carbon. Although two Russian scientists first described "graphitic carbon fibers" in 1952, Ijima is credited for the discovery in 1991 after transmission electron microscopy (TEM) technology improved. Manufactured carbon nanotubes (CNTs) have unique properties that make them different from other allotropes of carbon and a more popular choice in biotechnology over fullerenes. Carbon nanotubes have a high surface-to-mass ratio, high electron mobility, conduct electricity, and have extremely high tensile strength (Mwangi et al. 2012). But because CNTs are relatively new to science, researchers still do not have a full understanding of their potential applications.

The United States Government has been a key driver in nanotechnology development, investing $1 billion a year in nanotechnology applications (Brower 2006). While the government is investing large sums of money into the development of nanotechnology, according to a study conducted in 2005 by the Project on Emerging Nanotechnologies, only about 1% of the funding is going towards studying risks. Between 2009 and 2011, the US EPA issued several Significant New Use Rules (SNURs) for nanomaterials under Section 5(a)(2) of the Toxic Substances Control Act (TSCA). These new rules relate to specific applications of CNTs and require companies involved with CNT technology to notify the EPA 90 days prior to production, importation, or CNT processing. After notification, the EPA has to evaluate the intended purpose of the technology and determine whether the company can proceed with the CNT use (USEPA

2011). The SNURs are currently the only regulations imposed on CNT use. Any further regulations will require the development of a risk assessment, needing in-depth knowledge of CNT behavior and toxicity to animals and the environment.

USES OF CARBON NANOTUBES

CNTs can be added to everyday items such as exterior paint, sports equipment, and concrete. They have the potential to be used in the medical field as drug delivery systems targeting individual cells (Liu et al. 2009, Im et al. 2010, Oh et al. 2010). CNTs are currently being used as an additive in marine anti-fouling paint, marketed as an "environmentally-friendly" product. Biofouling, such as barnacle attachment, can increase fuel consumption by 40%, costing the shipping industry more than $30 billion per year (Jackson 2008). Instead of using a paint mixed with a chemical (such as tributyltin) to kill attaching organisms, CNTs mixed in marine paint inhibit organisms from attaching to the ship walls. This paint application allows for less ship maintenance and better fuel economy and has the potential to revolutionize the shipping industry. The US government is also interested in advancement of CNT biotechnology for the defense industry. Military uses of CNTs include strong, lightweight materials for vehicles and buildings, thermal control in aircrafts, physical camouflage, and woven fibers for bulletproof clothing (Kennedy et al. 2008, Wu et al. 2004). The National Science Foundation predicts that the market for products and services involving nanotechnology will reach $1 trillion by 2015.

One of the major advances in CNT technology is water purification. Traditional filtration systems for point of use water systems and drinking water are inadequate in

removing many hazardous chemicals and microbes. Filters containing CNTs are receiving attention for their ability to remove chemical and biological contaminants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, metals, microbes, and cyanobacterial toxins (Yan et al. 2006, Akasaja and Watari 2009, Upadhyayula and Gadhamshetty 2010). Small viruses such as polio and larger pathogens like *E. coli* and *S. aereus* can be caught in the filtration system (Brady-Estévez et al. 2008). Successful case studies using portable nanofiltration units in developing countries such as India and South Africa have increased the awareness of nanofiltration possibilities and the potential to have these systems used on a larger scale (Upadhyayula and Gadhamshetty 2010). Benefits of nanofiltration technology include no need for chlorine addition, creating a CNT filter is inexpensive, and the filtration system is easy to operate and maintain. The addition of a CNT filtration system on a traditional wastewater point of use system also reduces the amount of biofilms on the surface, which requires less maintenance (Upadhyayula et al. 2009).

The same properties that make CNTs useful in water and wastewater treatment are also applicable to bioremediation. The fate, transformation, and transportation of many chemical compounds in the environment are altered in the presence of CNTs (Gao et al. 2008). Carbon nanotubes are able to remove heavy metal ions such as Cu(II), Pb(II), Co(II), and Cd(II) from water (Liang et al. 2004, Stafiej and Pyrzynska 2007). They can also adsorb and remove endocrine disruptors like bisphenol-A and 17 α-ethinyl estradiol from the water column (Pan et al. 2008). Long and Yang (2001) observed that multi-walled carbon nanotubes (MWCNTs) remove dioxin from air more efficiently than activated carbon. The unique characteristics of CNTs (such as large surface area,

functionalization, affinity for aggregation) may play a role in the affinity of CNTs to bind to contaminants (Musee 2010).

PRODUCTION OF CARBON NANOTUBES

Carbon nanotubes are classified as anthropogenic materials even though natural processes, including volcanic events, can produce them (Velasco-Santos et al. 2003). Carbon nanotubes have been found in ice-core samples, deep rock formations, and crude oil (Velasco-Santos et al. 2003, Esquivel and Murr 2004). They can also form incidentally through incineration (Murr et al. 2004); CNTs were produced from high-heat exposure to collapsing building material at the World Trace Center Site and found in dust samples and lungs of workers (Wu et al. 2010). While animals have evolved to have contact with natural nano-sized particles (Klaine et al. 2008), adequate defense mechanisms against particles that are inhaled straight into the lower area of the lungs (smaller than 2.5 μm) have not yet evolved.

Previously, the high cost of production was one of the limiting factors for large-scale application of man-made CNTs. The historical price of CNTs has been $80-100 per gram (Upadhyayula and Gadhamshetty 2010). However, recent advances in technology of continuous production have increased the amount of CNTs a single factory can produce annually, driving down the price. The global production of CNTs was estimated to be about 271 tons multi-walled CNTs per year and about 7 tons single-walled CNTs per year in 2006 (Rakov 2008). However, because there are few regulations globally related to CNT production, these are only crude estimates. Currently, one factory alone has the ability to produce 500 tons a year, with future production expected to be 3000

tons per year (Sherman 2007, Wu et al. 2009). Large-scale production of CNTs can be achieved through multiple routes: electric arc discharge, laser ablation, high-pressure carbon monoxide process, or chemical vapor deposition (Upadhyayula and Gadhamshetty 2010). The majority of commercial-scale producers use chemical vapor deposition (CVD) because it is easily controllable and more cost effective than the other production methods. While CVD provides cheap and easy production, it is not an "environmentally-friendly" technique. Khanna and Bakshi (2009) suggest that the energy required for CNT production using the CVD technique is 20-100 times greater than energy used for steel manufacturing. The CVD process uses non-renewable hydrocarbon feedstocks for CNT synthesis, toxic metals as catalysts, and concentrated acids during CNT purification: all processes that call for environmental concern. However, CNT production does have the potential to be more environmentally conscious. In sight of this problem, the RECYTUBE project, funded by the European Union, was created to develop plastic nanocomposites by reusing CNT scraps. Plastics developed from this project could help offset the carbon footprint of CNT production. If the RECYTUBE project were adopted in other countries that also have high production volumes (such as USA or China), this process would help to decrease the ecological footprint of CNT production.

TYPES OF CARBON NANOTUBES

There are two categories of CNTs: single-walled and multi-walled. Single-walled (SWCNTs) are single layers of graphene rolled into a tube while multi-walled (MWCNTs) are multiple layers of graphene, ranging from a double-walled nanotube to 50 concentric tubes with the interwall space between tubes being 0.34 nm (Yamabe 1995,

Ajayan 1999). Many differences exist in the way MWCNTs and SWCNTs behave in the environment. Multi-walled carbon nanotubes do not have the same conducting properties as SWCNTs, but are more similar to the semi-conductor bulk graphite (Kang et al. 2008b). Lam et al. (2007) reported that SWCNTs have much higher concentrations of metal residues (such as Ni, Co, Fe, Mo) than MWCNTs, which contain minimal amounts of impurities (typically Co or Fe, depending on the manufacturing process). Also, Zhang et al. (2009) observed that MWCNTs disperse better with sonication than SWCNTs. Based on these observations is it apparent that MWCNTs and SWCNTs are two distinct materials and should be treated as such.

ENVIRONMENTALLY RELEVANT CONCENTRATIONS

With the volume of CNT manufacturing increasing annually, the probability of CNTs escaping into the aquatic environment throughout their product life-cycle also increases (Daughton 2004). Detecting engineered carbon nanoparticles in the environment is still a significant challenge. One of the problems for detecting CNTs in environmental media (air, soil, water) is that there is already natural background of nano-sized carbon particles, although the concentration is low (Klaine et al. 2008). Currently, there is no technology available to distinguish between natural background levels of nanomaterials and engineered or anthropogenic ones. Lack of accurate measurements of CNTs in water or sediment inhibits development of accurate exposure predictions. Methods such as thermal oxidation, TEM, or SEM (scanning electron microscopy) are not sufficiently sensitive to determine the difference between engineered nanomaterials and black carbon in the environment (von der Kammer et al. 2012). Raman spectroscopy

and optical absorbance spectroscopies can be used to characterize pure carbon nanomaterials in high concentration, but cannot accurately measure low concentrations or samples in media (von der Kammer et al. 2012). However, recent improvements in Raman spectroscopy technology paired with thermal optical transmittance/ reflectance (TOT/R) have the ability to detect MWCNTs in urban air and cyanobacteria, which suggests that detection in complex environmental media will be possible in the future (Doudrick et al. 2012). Another novel method, microwave induced heating, has proven to be successful in measuring accumulation of MWCNTs in *Eisneia fetida*, the earthworm (Li et al. 2013). MWCNTs have the ability to absorb microwaves and release heat at a much higher temperature than other carbon allotropes, where the change in temperature quantitatively measure the amount of MWCNTs in a sample (Li et al. 2013).

The use of nanotechnology has produced new types of waste streams containing nanomaterial residues or nanowaste. Currently, the quantity of nanowastes can only be estimated from reported production volumes of nanomaterials and predicted increases from commercialization of nano-based products (Musee 2011). The incomplete and sometimes contradictory global statistics on nanomaterial production hinder estimation of country specific and global nanowaste quantities (Musee 2011). Critical exposure routes of CNTs are predicted to be during the production and shipping of bulk CNTs, during production of the final products containing CNTs, during use, and during disposal or recycling (Som et al. 2010).

Further understanding environmental behavior of CNTs is essential for predicting concentrations in the environment. For example, there is controversy in the literature about the ability of CNTs to degrade, which would affect the movement of CNTs in the

environment. It is possible that CNT-containing products will degrade over time, releasing CNTs into the environment. Mixtures of CNTs in composite matrices create a durable product that can withstand physical and chemical processes (Nowack et al. 2011). Alternatively, although matrices are considered stable, some studies suggest that products containing CNTs can degrade. Ngyuen et al. (2009) observed that an epoxy matrix containing MWCNTs undergoes photodegradation resulting in an increase of the MWCNT concentration on the composite surface. Exposure to sunlight can also produce reactive oxygen species from SWCNTs (Chen and Jafvert 2010). Further investigations of the life cycle of CNTs and their degradation ability will provide important factors for regulatory purposes and better predictions of environmental concentrations.

Alternatively, Mueller and Nowack (2008) suggest CNTs are unlikely to be released during product use because they are typically bound within materials (not on the surface) such as polymers or used in closed compartments such as computers or batteries. Compared with predicted concentrations of nano-TiO_2 and nano-Ag modeled in environmental compartments (air, soil, water), predicted CNT concentrations are the lowest of the three nanomaterials (Mueller and Nowack 2008). CNTs are predicted to be partially combusted in the waste incineration process, therefore less will be moved to landfills than other modeled nanoparticles (Mueller and Nowack 2008). In this same study, predicted environmental concentrations of CNTs for Swiss water range from 0.0005 μg/L for a realistic exposure to 0.0008 μg/L for a high emission scenario (Mueller and Nowack 2008). However, Gottschalk et al. (2009) created another model that predicted that the largest amount (54.2%) of CNT waste will be found in landfills, compared to 12.5% being incinerated. CNTs in landfills may leach or run off into aquatic

environments over time. Models by Gottschalk et al. (2009) predicted that the highest concentrations of nanomaterials in the US will be found in sludge-treated soil or the sediment, with expected CNT concentrations ranging from 23.9 to 74.6 ng/kg in sludge treated soil. Models of CNT deposition U.S. sediment is predicted to increase on average 46 ng/kg yearly (Gottschalk et al. 2009). Estimated CNT concentrations in wastewater in the US are 6.6 to 18.4 ng/L (Gottschalk et al. 2009). Mueller and Nowack (2008) suggest that the expected concentrations of CNTs pose little risk based on the data presently available. With the projected increase in CNT use, modeled CNT concentrations will change. However, there are very little data available regarding concentrations in the environment; Gottschalk et al. (2009) suggest that more care should be given to future CNT exposure scenarios, as the modeling by Mueller and Nowack (2009) was only created for a small geographic range (Switzerland) that is not known to produce large quantities of CNTs. Currently, there are no models exploring predicted concentrations in marine environments.

PRECENDENCE FOR CONCERN

Carbon nanotubes have been incorporated in a newly developed anti-corrosion marine coating, which has been on the market since 2009 (Bayer MaterialScience). In recent history tributyltin (TBT), a type of organotin, was mixed in anti-fouling paint for use on ship hulls worldwide. Like current use of CNTs it was heavily used prior to determining toxicity. Low concentrations of TBT in UK marine waters nearly decimated local populations of the dog whelk (*Nucella lapillus*) and periwinkle (*Littorina littorea*) (Bryan et al. 1986, Gibbs et al. 1988, Minchin et al. 1997). In Tokyo Bay and the Strait of

Malacca, elevated TBT concentrations were found in areas with intense shipping and ferry activities (Hashimoto 1998). Because CNTs have a similar use as TBT, areas most likely affected will be shipping lanes and harbors. Also, the majority of large-scale CNT manufacturers are located near marine or estuarine environments (Klaine et al. 2008). This information calls for a more in-depth investigation of CNT toxicity in order to avoid repeating past ecological disasters. In the following paragraphs, we will summarize the majority of experiments exposing a variety of aquatic freshwater and marine organisms to carbon nanoparticles (fullerenes, SWCNTs, and MWCNTs).

ECOTOXICITY IN FRESHWATER

Most published toxicity studies of CNTs have focused on mammalian model systems, with limited research involving other biological systems like invertebrates (which make up 95-97% of faunal species), fungi, plants, reptiles, or amphibians (Musee 2011). The majority of information available about the toxicity of carbon-based nanomaterials has been obtained through short duration, high concentration acute tests. Chronic studies conducted at lower concentrations and for longer durations will yield more environmentally realistic data for long term exposures, while acute studies help predict effects following a spill or large release of nanomaterials (Moore 2006). Not only is there a problem of limited data available, but also tests have yielded no simple conclusions regarding toxicity of CNTs. Furthermore, there is a need to develop standard testing protocols to reduce variance in test results (Mwangi et al. 2012). Inconclusive results could be due in part from species-specific sensitivities, different test protocols,

exposure durations, surface coatings or functionalization (Kennedy et al. 2008). Results of acute studies of carbon nanomaterials in freshwater (FW) or saltwater (SW) are summarized in Table 1.1; results of chronic studies exposing aquatic organisms to carbon nanoparticles are detailed in Table 1.2.

Fullerenes

As one of the first carbon nanoparticles discovered, toxicity data on fullerenes from a variety of organisms is more plentiful than data from other types of carbon nanoparticles. While fullerenes and CNTs are both carbon nanoparticles, research has shown that they have very different behaviors and toxicity in the aquatic environment. *Daphnia magna* exposed to 35 mg/L fullerenes for 48 h did not experience significant mortality (Roberts et al. 2007), while another study exposing *Carassius auratus* (goldfish) to 1.0 mg/L fullerenes for 32 d observed a decrease in weight and length (Zhu et al. 2008). After exposure, Zhu et al. (2008) measured an increase in lipid peroxidase in the *C. auratus* liver while glutathione decreased in all tissues, suggesting oxidative stress in *C. auratus*. *Danio rerio* (zebrafish) embryos exposed to 1.5 mg/L C_{60} experienced delayed development, decreased survival and hatching rates, and pericardial edemas in hatched fish (Zhu et al. 2007). Already, the difficulties in comparison among different species are becoming apparent.

SWCNTs

Exposure of nanomaterials to bacteria and protozoa can provide much information for environmental health and has implications for wastewater treatment. Exposure to 0.3 m/cm^3 SWCNTs caused an increase of *Escherichia coli* gene expression related to stress

factors, such as higher levels of mRNA sigma factors, than exposure to the same concentration of MWCNTs (Kang et al. 2008a). Higher toxicity is predicted to be a result of the smaller diameter of SWCNTs, which can interact more with the outside of *E. coli* cells (Kang et al. 2008a). Another study found that SWCNTs dispersed in natural organic matter have high antimicrobial properties because of individually dispersed SWCNTs piercing the bacterial cell outer membrane; the SWCNTs are hypothesized to be more toxic to Gram-positive bacteria which have softer outer membranes than Gram-negative (Liu et al. 2009). *Tetrahymena thermophile,* a ciliated protozoa typically found in wastewater treatment sites, internalized SWCNTs after an exposure to 11.9 mg/L for 3 d; internalization of SWCNTs caused the protozoa to congregate from loss of mobility, interfering with their ability to ingest and digest prey (Ghafari et al. 2008).

While fullerene exposure was not acutely toxic to *D. magna*, an exposure to 20 mg/L SWCNTs in the same study resulted in 100% mortality in *D. magna* and an LC_{50} of 10 mg/L (Roberts et al. 2007). The scientists hypothesized that the *D. magna* ingested the SWCNTs through its normal feeding behavior; however, precipitates of the SWCNTs adhered to the outside of the daphnids, which is a proposed mechanism of toxicity. The results from this study suggested that nanomaterial shape and size influences toxicity to *D. magna*. Interestingly, *Leptocheirus plumulosus*, another freshwater crustacean, was exposed to 100 g/kg of SWCNT in both sediment and food for 7 d and no measurable accumulation or toxic effects were observed. (Mwangi et al. 2012). Differential species sensitivities may play a role in SWCNT toxicity to freshwater crustaceans.

Exposures to *Oncorhynchus mykiss* (rainbow trout) can provide information about environmental and potential human health implications. Increased activity of glutathione

levels in *O. mykiss* gills and liver were measured after a 10 d exposure to 0.5 mg/L SWCNTs (Smith et al. 2007). Abnormal division of liver cells was observed in exposed *O. mykiss* and SWCNTs were found in the gut lumen (Smith et al. 2007). In another study exposing *O. mykiss* cell cultures to SWCNTs and MWCNTs, exposure to SWCNTs had more of a stimulatory effect on macrophage cells at non-toxic concentrations compared to MWCNTs (Klaper et al. 2010). *O. mykiss* fed SWCNT-contaminated food (500 mg/kg) for 6 wks experienced no significant effects on growth, hematology, tissue ion concentrations, histopathology, osmoregulation, or biochemistry (Fraser et al. 2011). The authors hypothesize that ingestion of 500 mg/kg SWCNTs places fish at a much lower risk than exposure to other dietary contaminants (Fraser et al. 2011). The number of different outcomes for toxicity to the same species (*O. mykiss*) suggests that standard protocols for toxicity testing and CNT development are necessary to make true comparisons.

MWCNTs

Although MWCNTs can be mass-produced more efficiently than SWCNTs, little research has investigated the toxicity of MWCNTs to aquatic organisms. Various freshwater organisms from alga to fish have been studied and have resulted in extremely variable conclusions. When a green alga species (*Chlorella* spp.) was exposed to 100 mg/L of MWCNTs for 96 h, Long et al. (2012) observed inhibited growth, oxidative stress, shading effects interfering with photosynthesis, and agglomeration of MWCNTs on algal cells. *Stylonychia mytilus* (unicellular ciliated protozoan) exposed to 1 mg/L MWCNTs experienced cell growth inhibition and damage to the macronucleus and

external membrane of the cells (Zhu et al. 2006c). Electron microscopy revealed that MWCNTs were localized to cell mitochondria and damage to the mitochondria is a predicted mode of action for overall cell damage in *S. mytilus* after exposure to the MWCNTs. Alternatively, another study observed that cytoxicity to *Tetrahymena pyriformis* (another ciliated protozoa) was related to the type of culture media used; growth of the protozoan could be stimulated in the presence of 100 mg/L MWCNTs and protease peptone yeast extract medium (PPY) (Zhu et al. 2006b). Again, a wide range of observed toxicities results from lack of standard toxicity testing protocols. Also, having only one study for each of the organisms mentioned makes robust comparisons even more limited.

Moving higher in the trophic system, Petersen et al. (2009) observed that *Daphnia magna* accumulated 0.4 mg/L MWCNTs in the gut tract after 24 hours of exposure and could not excrete the ingested MWCNTs. *Ceriodaphnia dubia* exposed to 2 mg/L MWCNTs after 48 h had reduced body length; MWCNTs were also observed to accumulate in the digestive tract and brood chamber (Li and Huang 2011). Interestingly, another study exposed *C. dubia* to 9.5 mg/L MWCNTs with an addition of 15 mg/L NOM and found the only effect on reproduction varied by pH, not by the presence of MWCNTs (Alloy and Roberts 2011).

Amphibians have been used as research subjects in traditional toxicity testing, but only two studies have used them as testing organisms in carbon nanoparticle exposures. Juvenile *Xenopus laevis* (African clawed frog) did not experience genotoxicity after being exposed to 20 mg/L MWCNTs for 12 d, but mortality and growth reduction occurred (Mouchet et al. 2008). The authors hypothesized that toxicity occurred because

of physical blockage of gills and digestive tract. The same results were not seen in another amphibian: 1 g/L acid-cleaned MWCNTs were not acutely toxic or genotoxic to Mexican salamander larvae (*Ambystoma mexicanum*) in a 12 d exposure, although black masses were observed in their gut tracts (Mouchet et al. 2007).

Danio rerio (zebrafish) is a model organism typically used in tradiaitonal toxicity testing; however, only studies with exposures to MWCNTs have used this species. *D. rerio* eggs exposed to 60 mg/L MWCNTs for 72 h had a mucus coating surrounding the chorion as well as exhibited a hatch delay after exposure (Asharani et al. 2008). Alternatively, another study did not report a hatch delay in *D. rerio* until after exposure to 240 mg/L MWCNTs (Cheng et al. 2007). Interestingly, after being injected with 2 ng of functionalized MWCNTs, *D. rerio* embroys developed normally into larvae with no difference in survival rates between treated and control fish (Cheng et al. 2009). However, the second generation of treated fish had lower survival rates than control fish (Cheng et al. 2009). This study in particular calls for further research of MWCNTs, especially for life cycle and multi-generational sub-lethal effects that may not be seen in acute or subchronic studies. Interestingly, *D. rerio* has only been used as a testing organism in MWCNT exposure studies.

TOXICITY IN MARINE ENVIRONMENTS

Comparing sensitivies of marine and freshwater organisms to carbon nanoparticles is extremely important for developing predictions about carbon nanotube toxicity; however, this process is extremely difficult (Klaine et al. 2008). Not only do many marine organisms exist without comparable freshwater species, but the properties

of the water itself raises doubts as to whether data gathered in freshwater exposures may be used to assess risks in marine environments. Major differences likely exist in the behavior and fate of carbon nanotubes in marine water compared to freshwater. Seawater is usually more alkaline, has higher ionic strength, and has a wide concentration of NOM, with estuarine environments typically having high levels of dissolved NOM from both marine and freshwater organic inputs. High ionic strength will cause increased aggregation of nanoparticles (Klaine et al. 2008). Increased aggregation of CNTs occurred with an increase of Ca^{+2} ions; calcium may neutralize negative particles and serve as a binding agent between two CNT particles (Schwyzer et al. 2012). Higher levels of NOM in coastal zones may cause CNTs to behave differently compared to CNTs in pelagic marine environments (Klaine et al. 2008), as the presence of Ca^{+2} ions changes the Zeta potential of NOM (Schwyzer et al. 2012). Because CNTs are extremely hydrophobic, they are predicted to associate with lipids in the surface microlayer of seawater and large aggregates of CNTs may be coated by the microlayer lipid (Moore 2006). This will influence the bioavailability and behavior of CNTs in relation to the ocean ecosystem below the ocean surface. The surface microlayer in industrial harbors is often associated with high concentrations of organochlorines, PAHs, and heavy metals (Wurl and Obbard 2004). Hardy et al. (1990) reported concentrations of PCBs and pesticides 1-100 million times greater in the sea-surface microlayer than measured in bulk-water. Like these other hydrophobic chemicals, CNTs could become trapped and found at much higher concentrations in this surface microlayer. The upper few centimeters of the sea-surface microlayer serve as important habitats for neuston, a unique community of bacteria, protozoa, microalgae, crustaceans, and invertebrate larvae

(Hardy et al. 1990). Kelp bass larvae residing in the sea-surface microlayer experienced an increase in toxicity (mortality, abnormalities, and reduced growth) in polluted areas near shore in Los Angeles, CA compared to larvae living in non-polluted areas (Cross et al. 1987). Also, partitioning of CNTs into the sea-surface microlayer could be aerosolized and possibly expose air breathing marine animals such as sea birds (Klaine et al. 2008). Little data has been gathered on the toxicity of carbon nanoparticle toxicity to freshwater organisms, but even fewer observations have been made on marine organisms.

Fullerenes

Surface sediment- and filter-feeding mollusks are prime targets for uptake of manufactured nanoparticles because they are known to accumulate pollutants associated with suspended particles and sediment (Moore 2006). Following exposures of *Crossostrea virginica* (Eastern oyster) to 100 μg/L fullerenes for 4 d, 40% of the hepatopancreas cells were considered functionally impaired (Ringwood et al. 2009). Exposure to 100 μg/L also caused a decrease in normal embryonic development and this concentration was hypothesized to be a biologically relevant concentration for reproductive failure in the environment (Ringwood et al. 2009).

SWCNTs

The copepod *Amphaiscus tenuiremis* experienced life-cycle mortality, reduced fertilization rates, and reduced molting success after a chronic exposure to 10 mg/L SWCNTs for 28 d (Templeton et al. 2006). Mechanical disruption of the feeding appendages, penetration of the gut wall, and active uptake of CNTs followed by oxidative

stress are all possible modes of action for the observed SWCNT toxicity. Templeton et al. (2006) noted that the smallest fragments of SWCNTs induced the highest levels of toxicity, suggesting that size plays an important role in producing toxic effects. A sediment dwelling marine organism, *Arenicola marina* (lugworm) did not accumulate a significant amount of SWNCTs after being exposed to 30 mg/kg for a 10-d acute exposure (Galloway et al. 2010). SWCNTs also did not cause any measurable DNA or cellular damage in *A. marina*. In a study by Parks et al. (2013), *Americamysis bahia* and *Ampelisca abdita* were exposed to 100 g/kg of SWCNTs in either sediment or food for 7 d. In all cases there was no observed toxicity and the animals were able to excrete the SWCNTs without the material crossing the gut lumen (Parks et al. 2013).

Exposure to 1-10 mg/L SWCNTs for 96 h caused no effect on mortality or hatch success to *Fundulus heteroclitus* (mummichog) embryos (Blickley and McClellan-Green 2008). However, larvae exposed to 1 mg/L SWCNTs for 96 h had significantly higher whole-body glutathione levels (a biomarker used for oxidative stress) compared to control fish. No mortality was observed in adult *F. heterclitus* when exposed to the same SWCNT concentrations, but increased liver glutathione levels were observed to have concentration-dependent response with SWNCT exposure concentrations (Blickley and McClellan-Green 2008).

MWCNTs

At the base of the food chain, *Dunaliella tertiolecta* (marine green alga) exposed to 5 mg/L MWCNTs for 96 h experienced decreased cell growth with an EC_{50} of 0.82 mg/L (Wei et al. 2010). *D. tertiolecta* exposed to 10 mg/L MWCNTs for 96 h

experienced a 22% reduction in photosystem II function with particles also adsorbed onto cell surfaces. However, when the MWCNTs were first filtered through a 0.2 μm filter, no cytotoxicity was observed, suggesting that large MWCNT particles may be more toxic to marine algae than smaller particles. However, having only one study on MWCNT exposures to marine species limits the ability to make true comparisons between species, aquatic environments, and particles types.

DISPERSION OF CARBON NANOTUBES IN SOLUTION

Carbon nanotubes in general are extremely hydrophobic (log K_{ow}=2.69-2.77) and prone to aggregation because of high Van der Waals forces along the length axis (Petersen et al. 2010). Aggregation is a characteristic that differentiates CNTs from other allotropes of carbon, which reduces surface area of CNTs (Hyung et al. 2007, Zhang et al. 2009). Most experiments require some sort of dispersion in aqueous solution to properly observe CNT behavior. Zhang et al. (2009) observed that MWCNTs disperse better with sonication than SWCNTs. Many early studies that observed toxicity of carbon-based nanomaterials used tetrahydrofuran (THF) as a solvent to disperse the nanomaterials (Oberdörster 2004, Zhu et al. 2006a, Porter et al. 2007). Oberdörster et al. (2006) and Lovern and Klaper (2006) hypothesized the smaller size of the THF-nanomaterials particles increased toxicity; however, residual levels of THF may have confounded toxicity results (Andrievsky et al. 2005, Henry et al. 2007). Future CNT toxicity testing needs to investigate possible confounding factors from nanotube preparation and dispersion techniques (Kennedy et al. 2009). The addition of NOM as a

dispersion method is gaining popularity as it is more biologically relevant than other dispersion methods.

PRESENCE OF NATURAL ORGANIC MATTER

CNTs were previously not considered as potential contaminants of the aquatic environment because of their hydrophobicity (Hyung et al. 2007). However, the unexpected dispersal of man-made CNTs may occur in the aquatic environment in the presence of dissolved organic matter. Natural organic matter (NOM) is a complex and heterogeneous mixture of a diverse group of molecules (Kim et al. 2009). It is a naturally occurring substance found in rivers and lakes and serves as an energy source in many lacustrine and riverine food webs (Salonen and Hammar 1986). In freshwater, NOM concentrations can range from 1 to 100 mg/L (Paul et al. 2006). Dissolved organic carbon (a component of NOM) generally ranges from 0.5 to 4.0 mg C/L in lakes and rivers but can reach from 10 to 50 mg C/L in wetlands and marshes (Thurman 1984). NOM is produced from the decomposition of vegetative and animal material in a watershed and it varies in molecular weight and chemical characteristics, having hydrophobic and hydrophilic components (Edgington et al. 2010). Hyung et al. (2007) observed that 500 mg MWCNTs could be suspended for one month in Suwannee River water (59.1 mg C/L). MWCNT adsorption onto NOM was proportional to the aromatic content of the NOM (Hyung and Kim 2008), but MWCNT stability may differ with different NOM sources (Chappell et al. 2009). Chappell et al. 2009 also observed that adsorption increased as ionic strength increased or as pH decreased, which could affect CNT behavior near industrial water discharge locations.

Natural organic matter particles cover the hydrophobic surface of CNTs, increasing stability of individual particles in solution (Wang et al. 2008, Hyung et al. 2007, Hyung and Kim 2008). An increase in CNT stability suggests higher CNT concentrations and longer residence times in the water column, which could lead to increaseed contact with aquatic organisms and toxic effects (Edgington et al. 2010). Edgington et al. (2010) observed that the MWCNT surfaces become saturated with NOM at very low concentrations of dissolved organic carbon, and the addition of more NOM in the solution phase did not change the zeta potential, suggesting no further change in the surface charge. The aromatic content and molecular weight distribution of NOM might be useful parameters to predict NOM adsorption and MWCNTs dispersion in aquatic ecosystems. The adsorption of negatively charged NOM to the surface of activated carbon particles increased as ionic strength increased and pH decreased (McCreary and Snoeyink 1980, Randtke and Jepsen 1982). The strong, absorptive π to π electron-donor interaction (noncovalent force) between the CNT surface (π donors) and aromatic rings of NOM (π acceptors) was hypothesized to be the mechanism for NOM sorption onto MWCNTs (Chen et al. 2007, Hyung et al. 2007, Hyung and Kim 2008). As an alternative view, O'Driscoll et al. (2009) suggested that CNTs might be absorbed onto NOM particles instead of NOM adsorbing to CNTs. Adding NOM as a natural dispersant to toxicity tests with CNTs increases the environmental realism of the studies.

NOM AND OTHER CONTAMINANTS

The presence of NOM is important for the fate and transport of xenobiotic compounds in aquatic environments (Lam et al. 2007). Hydrophobic organic chemicals

(HOCs) typically associate with the organic content of the soil. NOM can serve as a sorbent for organic chemicals in aquatic environments, essentially protecting fish from adsorbing them (McCarthy and Jimenez 1985). Binding of PAHs, polychlorinated biphenyls (PCBs), and DDT to NOM has been shown to reduce the bioavailability of these chemicals for uptake and bioaccumulation by aquatic organisms (Leversee et al. 1983, McCarthy and Jimenez 1985, Black and McCarthy 1988). Black and McCarthy (1988) hypothesized that contaminants bound to NOM did not diffuse across the gill membranes of *O. mykiss*.

Alternatively, NOM has been shown to increase the transport of soluble organics and reduce the bioavailability of Cu and Pb ions (Luider et al. 2004, Sciera et al. 2004, Richards et al. 2001). NOM binds metals by their carboxylic and phenolic groups (Sánchez-Marín et al. 2007). An association of NOM with CNTs may alter the fate and transport of other hydrophobic organic chemicals (Wang et al. 2008). For example, the presence of NOM may decrease the ability of CNTs to bind to hydrophobic organic chemicals by increasing competition of adsorption sites (Wang et al. 2009). Polycyclic aromatic hydrocarbons (PAHs) can be absorbed by CNTs causing an increase in PAH toxicity (Cheng et al. 2004, Yang et al. 2006). Also, MWCNTs dissolved in NOM may alter Cu speciation and bioavailability possibly by deactivating certain functional groups on the NOM, hindering copper ion binding (Kim et al. 2009). The interaction between MWCNTs and other compounds such as hydrophobic organic chemicals and metals should be considered when risks of MWCNTs are explored as there may be competition for binding sites in the presence of NOM.

DATA GAPS

While the amount of available data increases yearly, scientists are still unable to make specific conclusions about CNT toxicity to aquatic organisms. Large data gaps exist that are blocking the progression of government regulations on CNT production, use, and disposal. One of the most obvious data gaps is the lack of toxicity data from MWCNT exposures to aquatic organisms, particularly those that live in marine environments. Many problems exist including lack of standardized CNT production procedures, testing protocols, and known environmentally realistic concentrations of CNTs. The EPA has listed nanomaterials as "Contaminants of Emerging Concern," which will encourage more in-depth studies. Other chemicals listed in this category include endocrine-disrupting chemicals (i.e., 17α-ethynyl estradiol, 17α-estradiol, mestranol), pharmaceuticals and personal care products (i.e., triclosan, methoxyethanol, erythromycin), and perfluorinated compounds (perfluorooctane sulfonate, perfluooctanic acid).

CURRENT RESEARCH

In the following chapters, I report novel findings of toxicity to various aquatic organisms following chronic exposure to ^{14}C-labeled multi-walled carbon nanotubes (MWCNTs). In Chapter Two, I present results on reproductive toxicity (decrease in brood number and size) and accumulation in *Ceriodaphnia dubia,* a freshwater crustacean, following a 7-d exposure to MWCNTs and how the presence of natural organic matter changes toxicity and accumulation of MWCNTs in *C. dubia*. In Chapter Three, I expanded on the information gathered from Chapter Two, and exposed a marine

crustacean, *Americamysis bahia*, to MWCNTs for 7 d measuring reproductive toxicity (percentage mature adults and release of offspring) and accumulation. *A. bahia* were tested in two sensitive life stages 7-14 d old and 14-28 d old to determine which time period is more sensitive to MWCNT exposure. Similar to the experimental design described in Chapter Two, I compared the accumulation potential and toxicity of MWCNTs in the presence and absence of dissolved natural organic matter. Chapter Four describes the subchronic exposure of *Pimephales promelas* (fathead minnow) to MWCNT-contaminated sediment. I measured mortality, morbidity (missing swim bladder, pathogenic growths, and gut impaction), and accumulation of ^{14}C-labeled MWCNTs over the course of 10 d. I also determined if the presence of *P. promelas* affected the movement of MWCNTs into the water column through the use of inclusion nets. Lastly, I measured the concentration of ^{14}C-MWCNTs in the water column before and after 0.45 μm filtration to determine how MWCNTs interact with sediment particles. In Chapter Five, I analyzed the literature available on crustacean toxicity after exposure to carbon nanoparticles (fullerenes, SWCNTs, and MWCNTs), integrating my observations from Chapters Two and Three. For the first time reported in the literature, all the predicted modes of toxicity of MWCNTs to crustaceans were combined and suggestions were made for future toxicity testing to create a usable risk assessment framework.

REFERENCES

Ajayan PM. 1999. Nanotubes from carbon. *Chemical Review*. 99(7): 1787-1800.

Akasaja T and F Watari. 2009. Capture of bacteria by flexible carbon nanotubes. *Acta. Biomater*. 5:602-612.

Alloy MM and AP Roberts. 2011. Effects of suspended multi-walled carbon nanotubes on daphnid growth and reproduction. *Ecotox. Environ. Safety*. 74:1839-1843.

American Society for Testing and Materials. 2006. Standard terminology relating to nanotechnology. E 2456-06. West Conshohocken, PA.

Andrievsky G, Klockkov V, Derevyanchencko L. 2005. Is the C60 fullerene molecule toxic?! *Fullerenes, Nanotubes, and Carbon Nanostructures*. 13:363-376.

Asharani PV, Serina NGB, Nurmawati MH, Wu YL, Gong Z, Valiyaveettil S. 2008. Impact of multi-walled carbon nanotubes on aquatic species. *J. of Nanoscience and Nanotechnol.* 8(7):3603-3609.

Black MC and JF McCarthy. 1988. Dissolved organic macromolecules reduce the uptake of hydrophobic organic contaminants by the gills of rainbow trout (*Salmo gairdneri*). *Environ. Tox. Chem*. 7:593-600.

Blickley TM and P McClellan-Green. 2008. Toxicity of aqueous fullerene in adult and larval *Fundulus herteroclitus. Environ. Tox. Chem.* 27:1964-1971.

Brady-Estévez, AS, Kang S, Elimelec M. A single-walled-carbon-nanotube filter for removal of viral and bacterial pathogens. *Small*. 4(4):481-484.

British Standards Institution. 2007. Terminology for nanomaterials. PAS 136:2007. London, UK.

Brower V. 2006. Is nanotechnology ready for primetime? *J. Natl. Cancer Inst*. 98(1):9-11.

Bryan GW, Gibbs PE, Hummerstone LG, Burt GR. 1986. The decline of the gastropod *Nucella lapillus* around South-West England: evidence for the effect of tributyltin from antifouling paints. *J. Mar. Biol. Assoc. UK*. 66(3):611-640.

Chappell MA, George AJ, Dontsova KM, Porter BE, Price CL, Zhou P, Morikawa E, Kennedy AJ, Steevens JA. 2009. Surfactive stabilization of multi-walled carbon nanotube dispersion with dissolved humic substances. *Environ. Pollut*. 157:1081-1087.

Chen W, Duan L, Zhu D. 2007. Adsorption of polar and nonpolar organic chemicals to carbon nanotubes. *Environ. Sci. Technol.* 41(24):8295-8300.

Chen C-Y, Jafvert CT. 2010. Photoreactivity of carboxylated single-wallEd carbon nanotubes in sunlight: reactive oxygen species production in water. *Environ. Sci. Technol*. 44:6674-6679.

Cheng J, Flahaut E, Chen SH. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environ. Tox. Chem*. 26(4):708-716.

Cheng J, Chan CM, Veca LM, Poon WL, Chan PK, Qu L, Sun Y-P, Chen SH. 2009. Acute and long-term effects after single loading of functionalized multi-walled carbon nanotubes into zebrafish (*Danio rerio*). *Tox. Appl. Pharm*. 235:216-225.

Cheng XK, Kan AT, Tomsom MB. 2004. Naphthalene adsorption and desorption from aqueous C-60 fullerene. *J. Chem. Eng. Data*. 49:675-683.

Cross JN, Hardy JT, Hose JE, Hershelman GH, Antrim LD, Gossett RW, Crecelius EA. 1987. Contaminant concentrations and toxicity of sea-surface microlayer near Los Angeles, California. *Marine Environ. Research*. 23:307-323.

Daughton CG. 2004. Non-regulated water contaminants: emerging research. *Environ. Impact Asses. Rev*. 24:711-732.

Doudrick K, Herckes P, Westerhoff P. 2012. Detection of carbon nanotubes in environmental matrices using programmed thermal analysis. *Environ. Sci. Technol.* 46(22):12246-12253.

Edgington AJ, Roberts AP, Taylor AM, Allow MM, Reppert J, Rao AM, Mao J, Klaine SJ. 2010. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ. Tox. Chem*. 29:2511-2518.

Esquival EV and LE Murr. 2004. A TEM analysis of nanoparticulates in a polar ice core. *Materials Characterization*. 52(1):15-25.

Fraser TWK, Reinardy HC, Shaw BJ, Henry TB, Handy RD. 2011. Dietary toxicity of single-walled carbon nanotubes and fullerenes (C60) in rainbow trout (*Oncorhynchus mykiss*). *Nanotox*. 5(1):98-108.

Gao J, Bonzongo J-CJ, Bitton G, Li Y, Wu C-Y. 2008. Nanowastes and the environment: using mercury as an example pollutant to assess the environmental fate of chemicals adsorbed onto manufactured nanomaterials. *Environ. Toxicol. Chem.* 27(4):808-810.

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

Ghadfari P, St-Denis CH, Power ME, Jin Xu, Tsou V, Mandal HS, Bols NC, Tang Xiaowu. 2008. Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa. *Nature Nanotechnol*. 3(6):347-351.

Gibbs PE, Pascoe PL, Burt BR. 1988. Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J. Mar. Biol. Assoc. UK*. 68(4):715-731.

Gottschalk F, Sonderer T, Scholz RW, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for different regions. *Environ. Sci. Technol*. 43:9216-9222.

Hardy JT, Crecelius EA, Antrim LD, Kiesser SL, Broadhurst VL. 1990. Aquatic surface microlayer contamination in Chesapeake Bay. *Marine Chem*. 28:33-351.

Hashimoto S, Watanabe M, Noda Y, Hayashi T, Kurita Y, Takasu Y, Otsuki A. 1998. Concentration and distribution of butyltin compounds in a heavy tanker route in the Strait of Malacca and Tokyo Bay. *Marine Environ. Res*. 45(2):169-177.

Henry TB, Menn FM, Fleming JT, Wilgus J, Compton RN, Sayler GS. 2007. Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ. Health Perspect*. 115(7):1059-1065.

Hyung H, Fortner JD, Hughes JB, King JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41:179-184.

Hyung H and JH Kim. 2008. Natural organic matter (NOM) adsorption to multi-walled carbon nanotubes: effect of NOM characteristics and water quality parameters. *Environ. Sci. Technol.* 42:4416-4421.

Ijima S. 1991. Helical microtubules of graphitic carbon. *Nature*. 354(6348):56-58.

Im JS, Bai BC, Lee YS. 2010. The effect of carbon nanotubes on drug delivery in an electrosensitive transdermal drug delivery system. *Biomaterials*. 31:141-1419.

Jackson L. 2008. Marine biofouling and invasive species: guidelines for prevention and management. *United Nations Environment Programme*.

Kang S, Herzberg M, Rodrigues Elimelech M. 2008a. Antibacterial effects of carbon nanotubes: size does matter! *Langmuir*. 24:6409-6413.

Kang S, Mauter MS, Elimelech M. 2008b. Physicochemical determinants of multi-walled carbon nanotube bacterial cytotoxicity. *Environ. Sci. Technol.* 42(19). 7528-7534.

Kennedy AJ, Hull MS, Steevens JA, Donstova KM, Chappell MA, Gunter JC, Weiss CA. 2008. Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. *Environ. Tox. Chem*. 27(9):1932-1941.

Kennedy AJ, Gunter JC, Chappell MA, Goss JD, Hull MS, Kirgan RA, Steevens JA. 2009. Influence of nanotube preparation in aquatic bioassays. *Environ. Tox. Chem*. 28(9):1930-1938.

Khanna V and BR Bakshi. 2008. Carbon nanofiber polymer composites: evaluation of life cycle energy use. *Environ. Sci. Technol.* 43:2078-2084.

Kim KT, Edgington AJ, Klaine SJ, Chao JW, Kim SD. 2009. Influence of multiwalled carbon nanotubes dispersed in natural organic matter on speciation and bioavailability of copper. *Environ. Sci. Technol*. 43:8979-8984.

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ. Tox. Chem*. 27(9):1825-1851.

Klaper R, Arndt D, Setyowati K, Chen J, Goetz F. 2010. Functionalization impacts the effects of carbon nanotubes on the immune system of rainbow trout, *Oncorhynchus mykiss*. *Aquat. Tox*. 100:211-217.

Lam B, Baer A, Alaee M, Lefebvre B, Moser A, Williams A, Simpson A. 2007. Major structural components in freshwater dissolved organic matter. *Environ. Sci. Technol*. 41(24):876-883.

Leversee GL, Landrum PF, Geisy JP, Fannin T. 1983. Humic acids reduce bioaccumulation of some polycyclic aromatic hydrocarbons. *Can. J. Fish. Soci*. 40(Suppl. 2):63-69.

Li M and CP Huang 2011. The responses of *Ceriodaphnia dubia* toward multi-walled carbon nanotubes: effect of physical-chemical treatment. *Carbon*. 49(5):1672-1679.

Li S, Irin F, Atore FO, Green MJ, Cañas-Carrell. 2013. Determination of multi-walled carbon nanotube bioaccumulation in earthworms measured by a microwave-based detection technique. *Sci. Total Environ*. 445-446:9-13.

Liang P, Liu Y, Zeng J, Lu H. Multiwalled carbon nanotubes as solid-phase extraction adsorbent for the preconcentration of trace metal ions and their determination by inductively coupled trace metal ions and their determination by inductively coupled plasma atomic emission spectrometry. *J. Anal. At. Spectrom*. 19:1489-1492.

Liu S, Wei Li, Hao L, Fang N, Wook M, Xu R, Yang Y, Chen Y. 2009a. Sharper and Faster "Nano Darts" Kill More Bacteria: A Study of Antibacterial Activity of Individually Dispersed Pristine Single-Walled Carbon Nanotubes. *ACSNano*. 3(12): 3891-3902.

Liu Z, Tabakman S, Welsher K, Dai HJ. 2009b. Carbon nanotubes in biology and medicine: in vitro and in vivo detection, imaging, and drug delivery. *Nano. Res*. 2:85-130.

Long Z, Ji J, Yang K, Lin D, Wu Fengchang. 2012. Systematic and quantitative investigation of the mechanism of carbon nanotubes' toxicity toward algae. *Environ. Sci. Technol.* 46(16):8458-8466.

Long RQ and RT Yang 2001. Carbon nanotubes as superior sorbent for dioxin removal. *J. of Amer. Chem. Soc*. 123(9):2058.

Lovern SB and R Klaper. 2006. *Daphnia magna* mortality when exposed to Titanium dioxide and fullerene (C60) nanoparticles. *Environ. Tox. Chem*. 25(4):1132-1137.

Luider CD, Crusius J, Playle RC, Curtis PJ. 2004. Influence of natural organic matter source on copper speciation as demonstrated by Cu binding to fish gills, by ion selective electrode and by DGT gel sampler. *Environ. Sci. Technol.* 38(10):2865-2872.

McCarthy JF and BD Jimenez. 1985. Interactions between polycyclic aromatic hydrocarbons and dissolved humic material: binding and dissociation. *Environ. Sci. Tech*. 19(11): 1072-1076.

McCreary JJ and VL Snoeyink. 1980. Characterization and activated carbon adsorption of several humic substances. *Water Res*. 14:151-160.

Minchin D, Bauer B, Oehlmann J, Schulte-Oehlmann U, Duggan CB. 1997. Biological indicators used to map organotin contamination from a fishing port, Killybegs, Ireland. Marine Poll. 34(4):235-243.

Moore MN. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ. Internat*. 32(8):967-997.

Mouchet F, Landois P, Flahaut E, Pinelli E, Gauthier L. 2007. Assessment of the potential in vivo ecotoxicity of double-walled carbon nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum. Nanotox*. 1:149-156.

Mouchet F, Landois P, Sarremejean E, Bernad G, Puech P, Pinelli P, Flahaut E, Gauthier L. 2008. Characterization and *in vivo* ecotoxicity evaluation of fouble-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. *Aquatic Tox.* 87:127-137.

Mueller NC and B Nowack. 2008. Exposure modeling of engineered nanoparticles in the environment. *Environ. Sci. Technol*. 42:4447-4453.

Murr LE, Bang JJ, Esquivel EV, Guerrero PA, Lopez DA. 2004. Carbon nanotubes, nanocrystal forms, and complex nanoparticle aggregates in common fuel-gas combustion sources and ambient air. *J. of Nanoparticle Research*. 6(2):241-251.

Musee N. 2011. Nanowastes and the environment: potential new waste management paradigm. *Environ. Intern.* 37:112-128.

Mwangi JN, Wang N, Ingersoll CG, Hardesty DK, Brunson EL, Li H, Deng B. 2012. Toxicity of carbon nanotubes to freshwater aquatic organisms. *Environ. Tox. Chem*. 31(8):1823-1830.

Nguyen T, Pellegrin B, Mermet L, Shapiro A, Gu X, Chin J. 2009. Network aggregation of CNTs at the surface of epoxy/MWCNT composite exposed to UV radiation. *Proceedings*, Nanotech 2009 Conference, May 3-7, Houston TX. 1: 90-93.

Nowack B, Ranville JF, Diamond S, Gallego-Urrea JA, Metcalfe C, Rose J, Horne N, Koelmans AA, Klaine SJ. 2011. Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environ. Tox. Chem*. 31(1):50-59.

Oberdörster E. 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environ. Health Perspect.* 112:1058-1062.

Oberdörster E, Zhu S, Blickley TM, McClellan-Green P, Haasch ML. 2006. Ecotoxicology of carbon-based engineered nanoparticles: effects of fullerenes (C60) on aquatic organisms. *Carbon*. 44:1112-1120.

O'Driscoll NJ, Messier T, Robertson MD, Murimboh J. 2009. Suspension of multi-walled carbon nanotubes (CNTs) in freshwaters: examining the effect of CNT size. *Water Air Soil Poll*. 208(1-3):235-241.

Oh WK, Yoon H, Jang J. 2010. Size control of magnetic carbon nanoparticles for drug delivery. *Biomaterials*. 31:1342-1348.

Pan B, Lin D, Masahayekhi H, Xing B. 2008. Adsorption and hysteresis of bisphenol a and 17α-ethinyl estradiol on carbon nanomaterials. *Environ. Sci. Technol*. 42:5480-5485.

Parks AN, Portis LM, Schierz A, Washburn KM, Perror MM, Burgess RM, Ho KT, Chandler GT, Ferguson PL. 2013. Bioaccumulation and toxicity of single-walled carbon nanotubes to benthic organisms at the base of the marine food chain. *Environ. Tox. Chem*. 32(6):1270-1277.

Paul A, Stösser R, Zehl A, Zwirmann E, Vogt RD, Steinberg CEW. 2006. Nature and abundance of organic radicals in natural organic matter: effect of pH and irradiation. *Environ. Sci. Technol.* 40(19):5897-5903.

Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ Sci. Technol*. 43(8):2969-2975.

Petersen EJ, Huang QG, Weber WJ Jr. 2010. Relevance of octanol-water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes. *Environ. Tox. Chem*. 29(5):1106-1112.

Porter AE, Gass M, Muller K, Skepper JN, Midgley PA, Welland M. 2007. Direct imaging of single-walled carbon nanotubes in cell. *Nature Nano*. 2(11):713.

Rakov EG. 2008. The current status of carbon nanotube and fiber production. *Nanotechnol. Russ*. 3(9-10): 575-580.

Randtke SJ and CP Jepsen. 1982. Effects of salts on activated carbon adsorption of fulvic acids. *J. Am. Water Works Assoc*. 74:84-93.

Roberts AP, Mount AS, Seda B, Souther K, Qiao R, Lin S, Ke PC, Rao AM, Klaine SJ. 2007. In vivo biomodification of lipid-coated carbon nanotubes by Daphnia magna. *Environ. Sci. Technol.* 41:3025-3029.

Richards JG, Curtis PJ, B urnison BK, Playle RC. 2001. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss*) and on metal binding to their gills. *Environ. Tox. Chem*. 20(6):1159-1166.

Ringwood AH, Levi-Polyachenko N, Carroll DL. 2009. Fullerene exposures with oysters: embryonic, adults, and cellular responses. *Environ. Sci. Technol.* 43:7136-7141.

Salonen K and T Hammar. 1986. On the importance of dissolved organic matter in the nutrition of zooplankton in some lake waters. *Oecologia*. 68(2):246-253.

Sánchez-Marín P, Lorenzo JI, Blust R, Beiras R. 2007. Humic acids increase dissolved lead bioavailability for marine invertebrates. *Environ. Sci. Technol.* 41:5679-5684.

Schwyzer I, Kaegi R, Sigg L, Smajda R, Magrez Arnaud, Nowack B. 2012. Long-term colloidal stability of 10 carbon nanotube types in the absence/presence of humic acid and calcium. Environ. Poll. 169:64-73.

Scientific Committee on Emerging and Newly Indentified Health Risks. 2007. The appropriateness of the risk assessment methodology in accordance with the Technical Guidance Documents for new and existing substances for assessing the

risks of nanomaterials, 21-22 June 2007. European Commission. Brussels, Belguim.

Sciera KL, Isely JJ, Tomasso JR, Klaine SJ. 2004. Influence of multiple water quality characteristics on copper toxicity to fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* 23(12): 2900-2905.

Shi JP, Evans DE, Khan AA, Harrison RM. 2001. Sources and concentration of nanoparticles (<10 nm diameter) in the urban atmosphere. *Atmos. Environ.* 35:1193-1202.

Sherman LM. 2007. Carbon nanotubes lots of potential- if the price is right. *Plast. Technol.* 007:1-7.

Som C, Berges M, Chaudhry Q, Dusinska M, Fernandes TF, Olsen SI, Nowack B. 2010. The importance of life cycle concepts for the development of safe nanoproducts. *Toxicology.* 269(2010):160-169.

Smith CJ, Shaw BJ, Handy RD. 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhychus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat. Tox*. 82:94-109.

Stafiej A and K Pyrzynska. 2007. Adsorption of heavy metal ions with carbon nanotubes. *Separation and Purification Technol*. 58(1):49-52.

Templeton RC, Ferguson PL, Washburn KM, Scrivens WA, Chandler GT. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ. Sci. Technol*. 40:7387-7393.

Thurman, E.M. 1984. Organic geochemistry of natural waters. Martinus Nijhoff, The Hague, The Netherlands.

Upadhyayula VKK, Deng S, Mitchell MC, Smith GB. 2009. Application of carbon nanotube technology for removal of contaminants in drinking water: a review. *Sci. Total. Environ*. 408:1-13.

Upadhyayula VKK and V Gadhamshetty. 2010. Appreciating the role of carbon nanotube composites in preventing biofouling and promoting biofilms on material surfaces in environmental engineering: a review. *Biotechnol. Advances*. 28:802-816.U.S.

Environmental Protection Agency. 2011. Enhancing EPA's Chemical Management Program. Washington, DC. http://www.epa.gov/opptintr/exisitingchemicals/pubs/Existing .Chem.Fact.sheet.pdf

von de Kammer F, Ferguson PL, Holden Pa, Masion A, Rogers KR, Klaine SJ, Koelmans AA, Horne N, Unrine JM. 2012. Analysis of engineered nanomaterials in

complex matrices (environment and biota): general considerations and conceptual case studies. *Environ. Tox. Chem*. 31(1):32-49.

Velasco-Santos C., Martinez-Hernández, Consultchi A, Rodriguez R, Castano VM. 2003. Naturally produced carbon nanotubes. *Chem. Phys. Letters*. 373(3-4):272-276.

Wang X, Lu J, Xing B. 2008. Sorption of organic contaminants by carbon nanotubes: influence of adsorbed organic matter. *Environ. Sci. Technol*. 42:3207-3212.

Wang X, Tao S, Xing B. 2009. Sorption and competition of aromatic compounds and humic acid on multiwalled carbon nanotubes. Environ. Sci. Technol. 43:6214-/6219.

Wei L, Thakkar M, Chen Y, Ntim SA, Mitra S, Zhang X. 2010. Cytoxicity effects of water dispersible oxidized multi-walled carbon nanotubes on marine alga, *Dunaliella tertiolecta*. Aquatic Tox. 100:194-201.

Wu M, Gordon RE, Herbert R, Padilla M, Moline J, Mendelson D, Litle V, Travis WD, Gil J. 2010. Case report: lung disease in World Trace Center responders exposed to dust and smoke: carbon nanotubes found in the lungs of World Trace Center patients and dust samples. *Environ. Health. Perspect*. 118(4):499-504.

Wu X. 2009. CNano technology commissions world's largest carbon nanotube manufacturing plant with a capacity of 500 tons per year. Santa Clara, Ca: CNano.

Wu Z, Chen Z, Du X, Logal JM, Sippel J, Nikolou. 2004. Transparent, conductive carbon nanotube films. Science. 305(5688):1273-1276.

Wurl O and JP Obbard. 2004. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. *Marine Pollution Bulletin*. 48:1016-1030.

Yamabe T. 1995. Recent development of carbon nanotubes. *Synthetic Materials*. 1511-1518.

Yan H, Gong A, He H, Zhou J, Wei Y, Lv L. 2006. Adsorption of microcystins by carbon nanotubes. *Chemosphere*. 62:142-148.

Yang K, Zhu L, Zing B. 2006. Adsorption of polycyclic aromatic hydrocarbons by carbon nanomaterials. *Environ. Sci. Technol*. 40(6):1855-1861.

Zhang S, Shao T, Bekaroglu SSK, Karanfil T. 2009. The impacts of aggregation and surface chemistry of carbon nanotubes on the adsorption of synthetic organic compounds. *Environ. Sci. Technol*. 43:5719-5725.

Zhu S, Oberdörster E, Haasch ML. 2006a. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow. *Marine Environ. Research*. 62:55-63.

Zhu Y, Tiecheng R, Li Y, Guo J, Li W. 2006b. Dependence of the cytoxicity of multi-walled carbon nanotubes on the culture medium. *Nanotech*. 17:4668-4674.

Zhu Y, Zhao Q, Li Y, Cai X, Li W. 2006c. The interaction and toxicity of multi-walled carbon nanotubes with *Stylonychia mytilus*. *J Nanosci Nanotechnol*. 6(5):1357-1356.

Zhu X, Zhu L, Lang Y, Chen Y. 2008. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environ. Tox. Chem*. 27(9):1979-1985.

Table 1.1. Summary of published acute toxicity data of carbon nanomaterials.

Form	Media	Exposure Duration	Organism	Concentration	Effects	Reference
Fullerenes	FW[c]	48 h	*Daphnia magna*	35 mg/L	no significant effect on mortality	Roberts et al. 2007
Fullerenes	FW	96 h	*Danio rerio* embryos	1.5 mg/L	delayed embro/larval development, decreased survival/hatching rates, pericardial edema in surviving fish	Zhu et al. 2007
Fullerenes	FW	1 h	*Thamnocephalus platyurus*	3 mg/L	fullerenes agglomerated in gut tract but able to excrete	Patra et al. 2011
Fullerenes	SW	4 d	*Crossostrea virginica*	100 µg/L	40% of hepatopancreas cells functionally impaired; concentration for reproductive failure	Ringwood et al. 2009
SWCNTs[a]	FW	2 h	*Escherichia coli*	0.3 m/cm^2	production of stress-related genes	Kang et al. 2008
SWCNTs	FW	2 h	*Staphyloccocus aerus, Bacillus subtillis*	5 mg/L	cell death, "nano-darts" pierce outer membrane	Liu et al. 2009
SWCNTs	FW	3 d	*Tetrahymena thermophile*	11.9 mg/L	internalization of SWCNTs interefered with prey capture and digestion	Ghafari et al. 2008
SWCNTs	FW	96 h	*Daphnia magna*	10 mg/L	(LC50), 100% mortality at 20 mg/L	Roberts et al. 2007
SWCNTs	FW	24 h	*Oncorhynchus mykiss*	0.5 mg/L	stimulates macrophage cells, functionalized CNTs more toxic	Klaper et al. 2010
SWCNTs	FW	10 d	*Oncorhynchus mykiss*	0.5 mg/L	increased GSH levels in gills/liver, abnormal liver cell division, accumulation in the gut lumen	Smith et al. 2007
SWCNTs	SW[d] sediment	10 d	*Arenicola marina*	30 mg/kg	no significant accumulation in tissues	Galloway et al. 2010
SWCNTs	SW	96 h	*Fundulus heterclitus*	10 mg/L	no effect on mortality of adults/juviles and no effect on hatch success	Blickley and McClellan-Green 2008
SWCNTs	SW	96 h	*Fundulus heterclitus*	1 mg/L	higher levels of glutathione	Blickley and McClellan-Green 2008
SWCNTs	FW	96 h	*Chlorella spp.*	100 mg/L	inhibited growth, oxidative stress, shading effections, agglomeration of MWCNTs on cells	Long et al. 2012

MWCNTs[b]	FW	48 h	*Stylonychia mytilus*	1 mg/L	cell growth inhibition, damage to macronucleus and external membrane	Zhu et al. 2006c
MWCNTs	FW	12 d	*Ambystoma mexicanum*	1 g/L	not acutely toxic or genotoxic, but black masses seen in GI tract	Mouchet et al. 2007
MWCNTs	FW	48 h	*Tetrahymena thermophile*	100 mg/L	growth of protozoan stimulated rather than inhibited (on PPY medium)	Zhu et al. 2006b
MWCNTs	FW	24 h	*Daphnia magna*	0.4 mg/L	accumulation in gut tract, cannot be extreted	Petersen et al. 2009
MWCNTs	FW	48 h	*Ceriodaphnia dubia*	2 mg/L	reduced body length; MWCNT accumulated in gut tract and brood chamber	Li and Huang 2011
MWCNTs	FW	12 d	*Xenopus laevis* larvae	10 mg/L	no genotoxicity, but mortality and growth reduction significant	Mouchet et al. 2008
MWCNTs	FW	72 h	*Danio rerio* embryos	60 mg/L	(LOEC) decreased hatch, slimy mucus coating around embryo	Asharani et al. 2008
MWCNTs	FW	72 h	*Danio rerio* embryos	240 mg/L	hatching delay	Cheng et al. 2007
MWCNTs	SW	96 h	*Dunaliella tertiolecta*	10 mg/L	decreased cell growth (LOEC 5 mg/L) with EC50 of 0.82 mg/L, 22% reduction in Photosystem II function	Wei et al. 2010
MWCNTs	FW	48 h	*Ceriodaphnia dubia*	50.9 mg/L	EC50 (mortality), carapace abnormalities	Kennedy et al. 2008
MWCNTs	FW	96 h	*Ceriodaphnia dubia*	17 mg/L	LC50 (mortality) for stirring MWCNTs, lower than sonicating (LC50=21 mg/L)	Kennedy et al. 2009
MWCNTs	FW	48 h	*Daphnia magna*	4 mg/L	unable to excrete MWCNTs without presence of food	Petersen et al. 2009
MWCNTs	FW	96 h	*Daphnia magna*	2 mg/L	LC50 for reduced growth	Edgington et al. 2010

[a]SWCNTs=single-walled carbon nanotubes

[b]MWCNTs=multiwalled carbon nanotubes

[c]FW=freshwater

[d]SW=saltwater

Table 1.2. Summary of published chronic toxicity data of carbon nanomaterials.

Form	Media	Exposure Duration	Organism	Concentration	Effects	Reference
Fullerenes	FW[c]	21 d	*Daphnia magna*	2.5 mg/L	(LOEC) delayed molting, decreasing in offspring size	Oberdörster et al. 2006
Fullerenes	FW	32 d	*Carassius auratus*	1.0 mg/L	decreased weight and length, increased LPO in liver, GSH decreased in all tissues	Zhu et al. 2008
SWCNTs[a]	SW[d]	28 d	*Amphiascus tenuiremis*	10 mg/L	increased life-cycle mortality, reduced fertilization rates, reduced molting success	Templeton et al. 2006
SWCNTs	SW sediment	7 d	*Americamysis bahia*	100 g/kg	no significant toxicity, SWCNTs excreted without crossing gut lumen	Parks et al. 2013
SWCNTs	SW sediment	7 d	*Ampelisca abdita*	100 g/kg	no significant toxicity, SWCNTs excreted without crossing gut lumen	Parks et al. 2013
SWCNTs	SW sediment	28 d	*Leptocheirus plumulosus*	100 g/kg	animals were able to excrete SWCNTs in fecal pellets	Parks et al. 2013
SWCNTs	FW sediment	14 d	*Hyallela azteca*	1 g/L	reduced mortality and biomass, unable to excrete MWCNTs after depuration	Mwangi et al. 2013
SWCNTs	FW	7 d	*Ceriodaphnia dubia*	9.5 mg/L	reproduction varied by pH, not presence of MWCNTs	Alloy and Roberts 2011
MWCNTs[b]	FW	single-loading	*Danio rerio*	2 ng injection	lower survival rates in second generation	Cheng et al. 2009
MWCNTs	FW	7 d	*Ceriodaphnia dubia*	0.2 mg/L	decrease in reproduction and growth	Edginton et al. 2010
MWCNTs	FW sediment	10 d	*Leptocheirus plumulosus*	99 g/L	reduced mortality	Kennedy et al. 2008
MWCNTs	FW sediment	10 d	*Leptocheirus plumulosus*	30 g/kg	reduced mortality	Kennedy et al. 2009
MWCNTs	FW sediment	14 d	*Hyallela azteca*	1 g/L	reduced mortality and biomass, unable to excrete MWCNTs after depuration	Mwangi et al. 2013
MWCNTs	FW sediment	10 d	*Hyallela azteca*	264 g/L	reduced mortality, but carbon black and activated carbon were more toxic	Kennedy et al. 2008
MWCNTs	FW sediment	10 d	*Hyallela azteca*	300 g/kg	50% reduced mortality only when MWCNTs were sonicated	Kennedy et al. 2009

[a]SWCNTs=single-walled carbon nanotubes

[b]MWCNTs=multiwalled carbon nanotubes

[c]FW=freshwater

[d]SW=saltwater

CHAPTER 2

ASSESSING THE EFFECT OF DISPERSION TECHNIQUES ON REPRODUCTIVE TOXICITY AND ACCUMULATION IN *CERIODAPHNIA DUBIA* AFTER CHRONIC EXPOSURE TO MULTI-WALLED CARBON NANOTUBES [1]

[1] Emily R. McReynolds and Marsha C. Black. In preparation to be submitted to *Chemosphere*.

ABSTRACT

Carbon nanotubes (CNTs) have many uses in the biotechnology field. As they are produced more cheaply in recent years, they can be produced on a large scale with the potential to contact the aquatic environment through product use or accidental and known releases. Many new products on the market incorporate nanomaterials as antifoulants or to increase thermal protection or material strength and provide CNTs a direct exposure route to water systems. *Ceriodaphnia dubia* were exposed to 1.25-5 mg/L MWCNTs solubilized using three different techniques during chronic toxicity tests: water-bath sonication, sonication with an addition of 2.35 mg C/L natural organic matter (NOM), and magnetically stirring for 24 h with an addition of 2.35 mg C/L NOM. Significant mortality was not observed, but exposure to MWCNTs produced negative reproductive effects. An average decrease of 70.03±8.63% in total reproductive output was observed for *C. dubia* exposed to 5 mg/L water-bath sonicated MWCNTs ($p=0.002$). However, when natural organic matter (NOM) was added to the MWCNTs, no reproductive toxicity was observed when bath sonicated ($p=0.33$) or stirring ($p=0.207$). Accumulation was significant in *C. dubia* exposed to the sonicated with NOM (B-CNT+NOM) treatment compared to controls, where the average accumulation was 23.26 ±12.86 ng per organism ($p=0.016$). Neonates released from adults exposed to 3.75 mg/L water-bath sonicated MWCNTs accumulated the highest amount of MWCNTs compared to neonates from other treatments, with an average of 5.12±2.71 ng per organism. We hypothesize that the presence of NOM is protective against reproductive toxicity; the NOM coats the MWCNT particles making them more stable, but more available for consumption.

Continuous exposure of high levels of MWCNTs in aquatic environments with low NOM levels could result in a decrease in microcrustacean reproduction.

Keywords- nanomaterial, natural organic matter, sonication, accumulation, reproductive toxicity

INTRODUCTION

Carbon nanotubes (CNTs) have recently emerged as a promising application in the biotechnology field. Advances in the medical field, long-lasting batteries, space exploration, and water purification are all areas where carbon nanomaterials can be applied. Everyday items such as exterior paint, sports equipment, and concrete containing CNTs demonstrate the broad range of use. As an industry, nanotechnology is expected to exceed one trillion dollars per year by 2015 (Nel et al. 2006). The toxicity of these materials is relatively unknown, and there is very little governmental regulation controlling their production or release. The U.S. Environmental Protection Agency (USEPA) has listed CNTs as "Contaminants of Emerging Concern" and suggested that further research into their toxicity is necessary before regulatory legislation is enacted. Currently, the only regulation involved with CNTs involves a pre-manufacturing notice to the EPA and potential uses of the CNTs as part of the Toxic Substances Control Act. There is no requirement for proper disposal or spill scenarios. As a result, the potential for human health implications and possible accumulation in commercially and ecologically important animals are areas that need to be explored. Carbon nanotubes will ultimately be introduced to the aquatic environment through accidental spills or known

releases and will come into contact with aquatic organisms, at an unknown cost. In 2008 a leading producer of MWCNTs reported production of over 70 tons a year of its product, Baytubes (www.baytubes.com). Another company announced that at full capacity its newest plant would produce 500 tons of nanotubes every year (www.cnanotechnology.com). Currently worldwide production quantities of nanomaterials are unknown; however, it is evident that production rates are significant and will only continue to rise in the future (Musee 2011).

Carbon nanotubes are categorized into two main species: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Single-walled nanotubes consist of a single layer of graphene, while MWCNTs contain multiple layers of graphene. The majority of toxicity research on CNTs has been conducted with SWCNTs in acute exposures; however, as commercial processes creating MWCNTs have become more readily available with less cost, acute toxicity of MWCNTs is being characterized, mostly in aquatic invertebrates. Petersen et al. (2009) reported that acute (24 h) exposure of *Daphnia magna* to 0.4 mg/L MWCNTs did not cause significant mortality, but the organism accumulated MWCNTs and then was unable to excrete them. Longer exposures (96 h) and higher concentrations of *D. magna* to 20 mg/L SWCNTs caused significant mortality with an LC_{50} of 10 mg/L and 100% mortality (Roberts et al. 2007). *D. magna* appeared to ingest the SWCNTs and SWCNT precipitates also adhered to the carapace of the daphnids. Ingestion or adherence to lipophilic structures may be potential mechanisms of toxicity. The few models that have predicted realistic environmental exposure concentrations of MWCNTs suggest that concentrations used in laboratory settings are much higher than expected concentrations; Gottschalk et al. (2009)

predicts environmental concentrations of CNTs in United States surface waters to range between 0.0006 and 0.004 ng/L. However, testing high concentrations of MWCNTs still provides valuable information for creating comprehensive testing guidelines and provides the framework for an environmental risk assessment of MWCNTs. Also, techniques for CNT detection currently limit exposure concentrations to large additions of materials in experiments. The literature detailing chronic effects of CNTs is extremely limited; therefore, exposures with longer duration are needed (Edgington et al. 2010, Li and Huang 2011).

Carbon nanotubes are extremely hydrophobic and require some combination of chemical dispersants, stirring, or sonication to keep them in aqueous solution (Ham et al. 2005). Tetrahydrofuran (THF) has previously been used to disperse carbon nanomaterials in solution. However, measures to remove THF may not be sufficient and residual levels have confounded toxicity assessments (Andrievsky et al. 2005, Henry et al. 2007). Recent literature has suggested that magnetic stirring of carbon nanoparticles is more environmentally relevant than adding chemicals to aid in dispersion (Oberdörster et al. 2006); however, MWCNTs are better dispersed using sonication (Kennedy et al. 2009). The literature suggests that there is a difference in toxicity between and stirred and sonicated MWCNTs. Stirred solutions of MWCNTs were significantly more toxic to *C. dubia* (96-h LC50 = 17 mg/L) than sonicated solutions (96-h LC50= 21 mg/L) (Kennedy et al. 2009). The relationship was reversed when *Hyalella azteca* was exposed to the MWCNT solutions, suggesting that there may be differences in species sensitivities. More research involving dispersion by magnetic stirring and sonication needs to be completed to determine toxicity thresholds for at-risk species.

Another dispersion method is use of dissolved natural organic matter (NOM) to aid in MWCNT dispersion. Use of NOM is more environmentally realistic than addition of other chemicals or solvents because it is a naturally occurring substance found in rivers and lakes and serves as an energy source in many lacustrine and riverine food webs (Salonen and Hammar 1986). Natural organic matter is a complex organic substance with both hydrophobic and hydrophilic moieties. Because of the hydrophobic natures of CNTs and NOM, CNTs should associate with NOM upon contact, changing the environmental behaviors of CNTs (Wang et al. 2009). Up to 500 mg/L MWCNTs were dissolved in filtered Suwanee River water (5.91 g C/L) and this solution remained stable for over a month (Hyung et al. 2007). Dispersion of CNTs occurs because NOM coats the extremely hydrophobic surface of CNTs, which provides a more favorable surface for their stability in water (Hyung et al. 2007).

The lack of information on CNT behavior in aquatic environments has resulted in comparisons with other hydrophobic organic contaminants that share similar chemical characteristics, such as polycyclic aromatic hydrocarbons (Petersen et al. 2008). Polycyclic aromatic hydrocarbons (PAHs) are a group of toxic compounds that are carcinogenic, mutagenic, tetratogenic and are fairly liphophilic. While the log K_{OW} (2.69-2.77) calculated for MWCNTs does not suggest a potential to biomagnify, accumulation of this material is still a concern (Petersen et al. 2010). Similarly, PAHs do not biomagnify in food webs; instead the highest concentrations are found in phytoplankton and the lowest levels in animals that ingest the phytoplankton because PAHs are readily metabolized by higher-order animals (Broman et al. 1990). If CNTs can accumulate in organisms in a similar manner as other hydrophobic organic chemicals, this will have a

profound effect on organisms lowest on the food chain. Focusing research on potential target organisms (such as invertebrates in lowest trophic levels of the food chain) will provide crucial information to potential food chain effects. MWCNTs have not been observed to bioaccumulate or bioconcentrate in organisms, but literature documents carbon nanoparticles crossing the gut lumen or aggregating in the digestive tract of organisms (Smith et al. 2007, Petersen et al. 2011).

Investigating the possible interaction between MWCNTs and NOM in the aquatic environment is also important for predictions of toxicity to organisms under natural exposures or conditions. NOM is found at varying levels in the majority of rivers and streams throughout the world and hydrophobic chemicals are known to typically associate with NOM (McCarthy and Jimenez 1985). Binding of PAHs to NOM reduced their bioavailability for uptake and bioaccumulation by aquatic organisms, such as *O. mykiss* (Black and McCarthy 1988) and *D. magna* (Kukkonen et al. 1989). If the environmental behavior of CNTs is similar to PAHs, the presence of NOM may alter bioaccumulation and toxicity of MWCNTs.

Our overall goal was to determine the chronic toxicity of MWCNTs to *C. dubia* and measure accumulation of MWCNTs during a chronic exposure. We tested different dispersion techniques of MWCNTs to help determine what approaches should be used for future biomonitoring and toxicity testing. We also evaluated NOM as a natural solvent and its effects on MWCNT toxicity and accumulation. Better characterization of the accumulation potential of MWCNTs will provide essential information about the possible need for additional regulation of this material.

MATERIALS AND METHODS

Organism

Ceriodaphnia dubia is a freshwater invertebrate found in littoral areas of lakes and ponds throughout the world and is an integral part of many food webs. It is a model organism used in chronic toxicity testing and biomonitoring studies. The *C. dubia* used for this research were obtained from an existing culture at the Ecological Services Laboratory, US EPA Region IV, Athens, Georgia, USA and were maintained and cultured in-house in moderately hard reconstituted water (MHW) (U.S. EPA 2002). Routine reference acute and chronic toxicity tests have been performed with this culture to ensure homogenous culture sensitivity to copper ions over time.

MWCNTs

Multi-walled carbon nanotubes were chosen for this study over SWCNTs because of their availability, lower cost, higher projected rate of production, and greater current use. One of the limitations of researching the behavior of carbon nanotubes is the difficulty in quantifying them at low concentrations in environmental or biological media (Petersen et al. 2008a). Enhanced detection of MWCNTs in water and biological samples can be obtained with radiolabeled MWCNTs. This process is unique in that it allows for quantification of modified or unmodified MWCNTs or aggregates of MWCNTs in digested tissues. Our test materials were created by a vapor deposition of methane on a Ni-MgO catalyst as described by Chen et al. (1997), modified to incorporate the ^{14}carbon isotope into a powdered form of MWCNTs (Petersen et al. 2008a). The purified MWCNTs were sonicated in a strong acid (3:1 ratio of concentration sulfuric and nitric

acid), making them more hydrophilic, with a purity of 99.9±0.2 (Petersen et al. 2009). Beta emissions from ^{14}C can be detected in most samples with a liquid scintillation counter (LSC) following solubilization in Ultima Gold scintillation cocktail (Perkin Elmer). The specific activity of the test materials was 0.12 μCi/mg. Further characterization is described by Petersen et al. (2008a, 2008b, 2009), who obtained test materials came from the same source.

NOM Source

Suwanee River NOM was purchased from the International Humic Substances Society (St. Paul, MN, USA). The NOM was collected by reverse osmosis and dried to a powder form. Elemental composition of the NOM by weight was 52.43% wt carbon, 4.19% wt hydrogen, 42.69% wt oxygen, 1.10% wt nitrogen, 0.65% wt sulfur, 0.02% wt phosphate, and 7% wt ash. For experiments, NOM was dissolved directly into MHW without filtration at a final concentration of 2.35 mg C/L (4.5 mg NOM/L).

MWCNT dispersion

MWCNTs were suspended in MHW (U.S. EPA 2002) through several different dispersion techniques. First, the dry carbon nanotubes were weighed and placed in glass media bottles filled with MHW to create nominal concentrations of 1.5, 2.5, 3.75, and 5 mg/L. Each nanotube solution was sonicated for two hours with a low-powered water-bath sonicator (Branson model #2510) just prior to its use in an experiment and prior to each water change (every 24 h). Carbon nanotubes treated by bath sonication will be referred to as B-CNT. Prior to experimentation, dispersion success was measured in a

solution of 10 mg of multi-walled carbon nanotubes added to one L of synthetically-made moderately hard water (MHW). The nanotube solution was sonicated for 2 h and then two mL samples were collected over 24 h to determine the amount that remained suspended in solution. The 2-h sonication duration was determined to be adequate to keep approximately 75% of the initial MWCNT concentration in solution over a 24 h period, the time between water changes in our exposures (Fig 2.1).

The introduction of Suwanee River NOM (2.35 mg C/L) to CNT solutions served as an additional dispersion technique in MWCNT exposure studies. Additional experiments were completed with addition of Suwannee River NOM at a final exposure concentration of 2.35 mg C/L. Two NOM treatments were tested. NOM was added to the water-bath sonicated MWCNTs after a 2-h sonication (referred to as B-CNT+NOM) or by magnetically stirring the same concentration of NOM and MWCNTs for 24 h (S-CNT+NOM) to assess a possible treatment effect from sonication. Two mL of exposure water were analyzed daily for each dispersion technique by LSC to confirm the nominal exposure concentrations.

Bioassays

Following EPA Method 1002.0, a 7-d chronic exposure of *C. dubia* was used to measure reproductive toxicity and accumulation of MWCNTs. The MHW had a pH range of 7.5 to 7.8, hardness ranging from 80 to 92, and alkalinity ranging from 60 to 64. Following dispersion by the various techniques, a total of 15 mL of each solution was immediately transferred to exposure vessels (1 oz. biodegradable plastic cups, rinsed with Milli-Q water prior to use). Each treatment had 10 replicates, with one randomly chosen

individual *C. dubia* (<24 h old) added to each replicate. As each *C. dubia* was chosen for the experiment in a small time window, all C. dubia were nearly the same size. A randomized complete block design was used to randomize the treatments to control for extraneous variation. A secondary control concentration containing MHW and 2.35 mg C/L NOM was also included to account for any effects of NOM on *C. dubia.* Three replicate tests were completed for each dispersion technique, except for B-CNT, which had n=4. Organisms were placed in an incubator which had a 16:8 light:dark photoperiod and a constant temperature of 25.0±1.0°C. Test solutions were renewed daily with freshly prepared MWCNT exposure solutions and organisms were fed with 100 µL *Selenastrum* spp. and 100 µL YTC (mixture of yeast, cerophyll, and digested trout chow, Aquatic Biosystems, Fort Collins, CO, USA). In a 7-d exposure, a control *C. dubia* will typically release 3 to 4 broods (clutches) of offspring; therefore, daily counts of *C. dubia* reproduction (number of neonates released per day) were made over the course of the chronic exposure. Only the first 3 broods collected from any adult daphnid were used as a part of the data set. When transferring adults, careful pipetting techniques ensured that any CNT aggregates naturally attaching to the carapace of *C. dubia* were not disturbed. Prior to test termination, adult organisms were observed under a stereomicroscope for viewing of possible surface and internal accumulation of MWCNTs.

Each day after the neonates were counted, they were pooled by brood number and concentration and placed in MHW for approximately two minutes to allow for surface desorption of MWCNTs not firmly attached to the carapace. The desorption period was not meant to mimic a depuration period, rather to determine if attachments of CNT aggregates had an effect on mortality or reproductive production. Therefore, only

MWCNTs that had interacted with the carapace were assumed to remain attached to the *C. dubia* prior to analysis. Similar to neonates, adults were collected after 7 days of exposure (or when 80% of the controls had third broods), pooled by treatment and MWCNT concentration and placed in MHW for surface desorption of MWCNTs. Pooled organisms (adults or neonates) were then transferred to a 7-mL plastic scintillation vial containing 200 µL of 40% (w/v) NaOH and digested overnight at room temperature. After digestion, 5 mL of Ultima Gold scintillation cocktail (Perkin Elmer) was added to each vial. The vials were then placed in the liquid scintillation counter (LSC) and counted for 10 min per sample. To ensure that the addition of NaOH (to digest organisms) would not confound LSC readings, preliminary quench tests were performed by adding 100-1000 µL of 40% (w/v) NaOH to vials containing *C. dubia* and MWCNTs and compared to vials containing no NaOH. The results demonstrated a minimal increase in background counts, but no masking of ^{14}C detection when 200 µL 40% (w/v) NaOH was added to digest the organisms (Table 1). To account for this interference, a blank vial with 200 µL of 40% (w/v) NaOH and 1 mL MHW was counted with each LSC analysis and the blank CPM was subtracted from each sample CPM. Neonates were collected and digested in the same method as described above, but pooled by exposure concentration and brood number (1-3).

Data Analysis

Data analyses for reproductive toxicity were conducted using ToxCalc v5.032 (Tidepool Scientific Software, McKinleyville, CA, USA). All data met assumptions for normality; no transformations were needed. Differences in reproductive success

(decreases in brood number and size) between treatments (n=10 individuals for each treatment; n= 3 replicate tests for each suspension method, except for B-CNT which had n=4 replicate tests) were determined using analysis of variance (ANOVA) followed by a Tukey's post-hoc test (α =0.05). Accumulation was determined from the amount of radioactivity measured in pooled samples containing bodies of either adult or neonate *C. dubia*. The average CPM per organism was calculated using the original LSC reading (corrected for the blank CPM) divided by the number of organisms in the vial. Comparisons were made between CPM per organism among treatments rather than individual or average weights because we did not have access to a scale that would accurately weigh sub-mg weights. For reference, adult female (7 d old) *C. dubia* weigh between 10.2±2.0 and 16.2±1.1 µg (±SD) depending on the algal food source (Knight and Waller 1986). Individual accumulation of MWCNTs was converted to ng/organism based on the specific activity of the MWCNTs (0.12 µCi/mg). Differences between treatments in accumulation for both neonates and adults were compared in the same manner as reproductive toxicity by using SAS software v. 9.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Exposure concentrations

Two mL of exposure water from each treatment was sampled daily to determine how accurately sonication methods solubilized the MWCNTs. Bath sonication methods (B-CNT and B-CNT+NOM) provided the most accurate solution concentration, with mean concentration (±SE) of 4.59±1.18 mg/L measured from a target concentration of 5 mg/L (n=24). Stirring the MWCNTs (S-CNT+NOM) proved to be the least effective in

producing a desired concentration, with a target exposure concentration of 5 mg/L and actual mean exposure concentration 3.94±0.97 mg/L (n=18). Exposure characterizations were not completed for this experiment.

Visual Results

Adult organisms at each concentration were observed under a stereomicroscope following 7-d exposure. Control organisms had smooth carapaces without any foreign external attachments and typically had eggs in the brood chamber (Fig 2A). When exposed to the water-bath sonication method (B-CNT), *C. dubia* were observed with MWCNT attached to the carapace of individuals (Fig 2.2B). Across the same exposure concentration range, no agglomerations of MWCNTs were observed to be adhering to the *C. dubia* when exposed to B-CNT+NOM for a 7-d period (Fig 2.2C), but there was black material in their gut tracts, assumed to be MWCNTs (Fig 2.3).

Reproductive Toxicity

Multi-walled carbon nanotubes were not lethal to *C. dubia* at any of the test concentrations (0-5 mg/L) during the 7-d exposure for any treatment. However, significant decreases in brood size and number were noted at higher concentrations tested in the B-CNT treatment group (Fig. 2.4). Some *C. dubia* adults exposed to higher concentrations (3.75, 5 mg/L) of B-CNT ceased producing offspring toward the end of the 7-d exposure, with a mean reduction in total reproductive output (±SE) of 63.80± 10.11% for *C. dubia* exposed to 3.75 mg/L compared to controls. *Ceriodaphnia dubia* exposed to 5 mg/L B-CNT exhibited a decrease of 70.03±8.63% in total offspring

production compared to control *C. dubia* ($F_{4,54}$ =4.86, p=0.002, Table 2). There was no observed reproductive toxicity when *C. dubia* were exposed to CNT treatments with NOM (B-CNT+ NOM, $F_{5,54}$=1.18, p= 0.33; S-CNT+NOM, $F_{5,54}$=1.50, p=0.207). The number of offspring produced by adults in a secondary control of MHW + NOM was not significantly different from the number produced by the control (MHW) adults (p=0.33); therefore, the presence of NOM did not affect offspring production on its own.

MWCNT accumulation

Adults

Although CNTs were visibly attached to *C. dubia* exposed to B-CNTs (Fig 2.2B), no relationship was apparent between exposure concentrations of MWCNTs and CNT accumulation (surface adherence or internal accumulation) by organisms exposed to B-CNT because of a large variability of accumulation among individuals within the same exposure concentration ($F_{4,15}$=1.22, p=0.3419, Fig 2.5). The mean accumulation (±SE) of *C. dubia* after exposure to 5 mg/L B-CNTs was 4.15±1.73 ng per organism. Adults exposed to S-CNT+NOM for 7 days also did not significantly accumulate more MWNTs than the controls, with mean accumulation of 4.21± 3.31 ng per organism in the 5 mg/L exposure ($F_{5,12}$=0.88, p=0.5203). However, *C. dubia* exposed to B-CNT+NOM did accumulate significantly more MWCNTs than controls; those exposed to 5 mg/L accumulated an average 23.26 ±12.86 ng per organism ($F_{5,12}$=4.42, p=0.016).

Neonates

While adults exposed to B-CNTs did not accumulate significantly more MWCNTs than the controls, the neonates produced from adults that were exposed to this

treatment accumulated the highest amount of MWCNTs per individual among treatments and significantly more than control neonates. Significant accumulation occurred when the neonates were exposed to the two highest concentrations tested: 3.75 mg/L B-CNTs (with mean ±SE accumulation of 5.12±2.71 ng per organism) and 5 mg/L with a mean of 5.08±2.25 ng per organism ($F_{4,38}$=3.41, p=0.018, Fig 2.6). Neonates collected from adults exposed to 5 mg/L S-CNT+NOM also accumulated significantly more than the controls, with a mean accumulation of 1.01±0.42 ng per organism ($F_{5,47}$=4.34, p=0.002). When adults were exposed to 5 mg/L B-CNT+NOM, their neonates also accumulated significantly more MWCNTS than control, with mean accumulation of 2.52±1.15 ng per organism ($F_{5,47}$=2.37, p=0.049).

DISCUSSION

Little information is available regarding the comparative toxicity of MWCNTs that have been modified by different dispersion procedures. The results of this study suggest that the type of dispersion technique greatly influences the reproductive toxicity and accumulation potential of MWCNTs by *C. dubia*. We suggest that in the absence of added agents (solvents, NOM), water-bath sonication, although less environmentally relevant, is more effective at suspending MWCNTs in solution than magnetic stirring for 24 h. The sonicator may be more effective in breaking the MWCNTs into smaller pieces, enabling a more uniformly-dispersed sample.

Chronic exposure to MWCNTs caused reproductive toxicity to *C. dubia* when MWCNTs were sonicated in a low-powered bath sonicator (B-CNT) (LOEC 2.5 mg/L). We hypothesize that physical adherence of sonicated MWCNTs to the carapaces of *C.*

dubia most likely prevented molting, thereby restricting the release of neonates and suggest that this is a mode of action for MWCNT reproductive toxicity to *C. dubia*. When a large particle or aggregation of MWCNTs becomes attached to the *C. dubia*, the organism may not have a way to remove it from its carapace (Li and Huang 2011). The MWCNT aggregates may not only inhibit molting, but also may also immobilize the organism, inducing stress and likely preventing it from feeding properly. Other research has suggested exposure to SWCNTs and MWCNTs caused acute toxicity by clogging of the gut tract in *D. magna* and *C. dubia* (Roberts et al. 2007, Edgington et al. 2010). It is also possible that eggs in the brood sac were exposed to the CNTs. The brood pouch is connected directly to the outside media and the adult provides a constant flow of water to the pouch (Rosenkranz et al. 2009). Epiphia (egg cysts produced during stressful periods) were not observed in any concentration for any treatment; instead, we hypothesize that the reproductive toxicity was the result of decreased of egg production because of exposure to the MWCNTs or perhaps egg resorption if the carapace could not be shed because of MWCNT surface agglomeration. These factors combined could explain the reproductive toxicity observed at higher concentrations in the B-CNT (sonication) experiments.

No reproductive toxicity was observed at any concentration tested when 2.35 mg C/L of Suwanee River NOM was added to water-bath sonicated MWCNTs (B-CNT + NOM) or MWCNTs magnetically stirred for 24 h (S-CNT + NOM). The presence of NOM appears to be protective against reproductive toxicity. In microscopic analysis, there were no visible agglomerations adhering to the carapaces of *C. dubia* exposed to MWCNTs. Our work supports that of Hyung et al. (2007), where the aggregations seen in

sonicated MWCNTs can be overcome by NOM addition; NOM solubilized the MWCNTs and prevented aggregation. We suggest that the presence of the NOM allowed the MWCNTs to remain stable in the exposure solution as individual particles for a much longer period of time. In turn, this reduces the number of aggregations that would adhere onto the *C. dubia* or makes the MWCNTs sufficiently soluble, reducing the potential for adherence to carapaces. Microscopic analyses by Hyung et al. (2007) showed that the suspensions of CNTs with NOM contained primarily individually dispersed CNTs. The stabilization of MWCNTs in NOM suggests an increased bioavailability and mobility of smaller particles over that of agglomerates (Hyung et al. 2007).

We observed that NOM was protective against reproductive toxicity; however, other sources suggest otherwise. In another report, *C. dubia* reproduction was reduced after exposure to all MWCNT concentrations greater than 0.125 mg/L with the addition of NOM (ranging from 2-20 mg/L dissolved organic carbon) (Edgington et al. 2010). However this difference in reported toxicity may be attributed to differences in MWCNT behavior caused by the modifications in MWCNT synthesis and purification procedures. Both our MWCNTs and those used by Edgington et al. (2010) were created by a similar chemical vapor deposition technique. However, the use of a different catalyst may help explain a difference in overall toxicity reported. The MWCNTs used in the present study were produced using a Ni-MgO catalyst, while those in the Edgington et al (2010) study used Fe (Andrews et al. 1999). The processes used also created different lengths and purities of the MWNCTs. Edgington et al. (2010) reported an approximate diameter of 25 nm, length of 50 μm, and a purity of approximately greater than 95%. MWCNTs used in the present study had a larger diameter (30-70 μm in diameter), but were shorter in length

(ranging from hundreds of nanometers to a few micrometers), and a purity of 99.9%±0.2 (Petersen et al. 2008). Overall, our MWCNTs were shorter in length, wider in diameter, more heterogeneous in size than those used in the study by Edgington et al. (2010). The literature available suggests that length plays a role in nanotube toxicity and purity may also change CNT behavior (Templeton et al. 2006, Hull et al. 2009, Kennedy et al. 2009).

The preparation of the carbon nanotubes could have also influenced toxicity. Edgington et al. 2010 sonicated MWCNTs with a probe sonicator but used the supernatant collected after 24 h of settling. MWCNTs used for the current study were not allowed to settle before use in the experiments, suggesting that our exposure concentrations had a higher probability for agglomeration and settling. The MWCNTs used in our study also went through an acid purification prior to sonication. Literature has suggested that acids used in functionalization can shorten nanotubes, therefore increasing dispersion potential (Chen et al. 2004) further changing them from those used by Edgington et al. (2010). From our findings, we suggest that functionalization may also reduce reproductive toxicity of MWCNTs in *C. dubia*. Finally, Edgington et al. 2010 did not investigate the toxicity of the MWCNTs alone (without NOM) for comparison.

We suggest that differences in MWCNT preparation and the amount of NOM used influence the toxicity of MWCNTs to adults. The amount of NOM used in our experiments reflects a more environmentally realistic scenario with an average amount of NOM (2.35 mg C/L) present for aquatic ecosystems. High levels of NOM addition may only be applicable to freshwater aquatic areas with large amounts of organic input. We hypothesize that non-natural levels of NOM may increase the dietary availability of MWCNTs, causing an increase of gut impaction within *C. dubia*, inducing stress and

higher levels of toxicity. However, without standardized MWCNT production and toxicity testing methods, it is still extremely difficult to assess MWCNT toxicity between laboratories.

Our MWCNTs were radiolabeled and we were able to measure the amount of MWCNTs associated with exposed organisms, either by agglomeration onto the organism's surface or accumulation within the bodies of *C. dubia*. Microscopic analysis showed large aggregates on the outside of all organisms exposed to B-CNT; but whether the adult *C. dubia* also accumulated the material internally via dietary uptake is unclear. We suggest that the apparent accumulation may more likely be a function of B-CNT agglomerates adhering to the lipophilic carapace of the organism, but no dose-response relationship appeared evident between B-CNT exposure concentrations and *C. dubia* accumulation. We hypothesize that random accumulation of MWCNTs on (or within) individual adult *C. dubia* attributed to the high variation observed among samples (Fig 2.5). This wide variability of MWCNT accumulation among replicates within treatments likely interfered with the statistical significance of treatments; however, the nature of the hydrophobic MWCNTs most likely caused unavoidable random aggregation. *C. dubia* exposed to NOM-treated MWCNTs (B-CNT+ NOM and S-CNT+ NOM) accumulated MWCNTs without large aggregates visible on the carapace. Black material (presumably MWCNTs aggregates) was visible in the gut tracts of organisms exposed to B-CNT+ NOM. Among the dispersion techniques tested, the B-CNT+NOM treated adults had the highest measurable amount of MWCNTs in or on their bodies, with a mean accumulation (±SE) of 23.26±12.86 ng/L when exposed to the 5 mg/L exposure concentration. For NOM treatments, we hypothesize that *C. dubia* accumulated the smaller-sized, NOM-

coated, stable MWCNTs through dietary uptake rather than random agglomeration of large particles on the carapace. Our visual evidence (Figs. 2.1 and 2.3) supports this hypothesis, as does transmission electron microscope (TEM) imagery from Edgington et al. (2010), which confirmed the presence of NOM solubilized MWCNTs in the gut tracts of *D. magna*.

Accumulation of MWCNTs was also measured in neonates produced by parents exposed to the various MWCNT treatments. Developing neonates in the brood pouch are connected directly to the outside media through a constant flow of water through the brood chamber and have the potential to accumulate many sizes of nanoparticles in their storage droplets (Rosenkranz et al. 2009). We were unable to determine if the MWCNTs accumulation by the neonates was due to maternal transfer, exposure while in the brood sac prior to being released, or from being in contact with and filtering exposure water for a <24 h time period between their release and collection. However, as observed for adults in sonicated MWCNT solutions with no NOM, aggregates randomly accumulated on the lipophilic carapace of neonate *C. dubia*. Again, random aggregation of the sonicated MWCNTs could also explain the extreme variability between multiple replicates for each exposure concentration. Interestingly, the highest observed accumulation of MWCNTs in neonates was in those neonates released by adults exposed to the B-CNT treatment. This suggests that while adults may be more at risk for internal accumulation of smaller MWCNT particles (produced by B-CNT+NOM treatment), larger agglomerations produced by water-bath sonication (B-CNT) could influence the health and successful maturation of the second generation (neonates) as well as reducing reproductive output by adults.

Overall, we hypothesize that adult *C. dubia* exposed to MWCNTs stabilized in NOM (B-CNT+NOM and S-CNT+NOM) did not experience reproductive toxicity because of the increase in solubility of MWCNTs and a decrease in MWCNT affinity for adhering to the carapace once they were coated with NOM. Perhaps the smaller pieces of MWCNT coated with NOM mimics a natural food source, and were ingested in a concentration-dependent fashion. Sonication reduces the size of the MWCNTs, which are then coated with NOM, making them more stable in solution and more available for consumption for a longer period of time by filter-feeding organisms such as *C. dubia*. Conflicting observations on CNT size related to gut clearance have been published. Li and Huang 2011 suggest that aggregate size is directly related to the energy required by *C. dubia* for the removal. More energy is consumed removing large particles in *C. dubia* (Li and Huang 2011). However, another study suggested that smaller particles (20 nm) cleared more slowly than large particles (1000 nm) in *D. magna* (Rosenkranz et al. 2009). More research is required to definitively determine what size particles are most resistant to elimination and potentially more toxic to invertebrate gut tract.

While we suggest that exposure to MWCNTs can cause reproductive toxicity in *C. dubia*, the ability for MWCNT accumulation to cause other forms of toxicity is still unclear. There is a possibility that MWCNT accumulation in the gut track could have negative effects on the normal functioning on the *C. dubia* digestive tract. Shortened MWCNTs (created by sonication) and functionalized (using a 3:1 acid solution) MWCNTs are associated with higher potential damage to bacterial cell membranes, whereas longer MWCNTs appeared less toxic (Kang et al. 2008). Accumulated MWCNTs could cause damage on a cellular level in the *C. dubia* gut tract under several

scenarios. If the MWCNTs create reactive oxygen species (Smith et al. 2007, Zhu et al. 2008), it is possible that they could interact with and degrade food sources (algae or NOM), causing the food to lack the essential nutrients necessary for the *C. dubia* (Rosenkranz et al. 2009). When *C. dubia* were exposed to 200 mg/L MWCNTs, they did not grow in size even though food was plentiful (Li and Huang 2011). The authors hypothesized that the gut space was filled with MWCNTs aggregates which hindered further ingestion and digestion of their natural food source or that the food was no longer nutritional because of interaction with MWCNTs. Changing the nutritional status of food may not be the only way in which MWCNTs disrupt *C. dubia* reproductive success. A realistic environmental scenario is that CNTs may bind to hydrophobic organic chemicals (HOCs) or heavy metals (Cheng et al. 2004, Stafiej and Pyrynska 2007, Kim et al. 2009) and upon consumption by *C. dubia*, be transported into the gut where chemical toxicity could occur after absorption. A "Trojan-horse" mechanism could involve CNTs bringing contaminants directly into the cells, causing much higher levels of oxidative stress than presence of metals or HOCs alone (Limbach et al. 2007, Baun et al. 2008). Research providing more realistic scenarios will determine how MWCNT interactions with other contaminants affect the life cycle of *C. dubia* and other non-target organisms.

We did not include a depuration period in our study to see if the MWCNTs accumulated were indeed impacted within the organism or how much material remained after a 7-d exposure study. A study employing TEM showed presence of the MWCNTs in the gut tract, but did not reveal MWCNTs crossing the gut lumen (Edgington et al. 2010). Microvilli of the gut potentially kept the long strands of MWCNTs from penetrating the gut lumen (Edgington et al. 2010). Determining if accumulation can have

negative effects on *C. dubia* fecundity and survival is the next important step in determining the consequences of MWCNT exposure.

ECOLOGICAL SIGNIFICANCE

Concentrations of dissolved organic carbon in rivers range from less than 1 mg/L in alpine streams to more than 20 mg/L in some tropical rivers, rivers draining wetlands, or polluted rivers (Spitzy and Leenheer 1988). Aquatic organisms that live in environments that have naturally high levels of NOM may be less susceptible to reproductive toxicity caused by MWCNTs, whereas *C. dubia* and other crustaceans inhabiting areas that have naturally low levels of NOM (such as alpine water systems) may be more at risk to reproductive effects. From the results presented here, we suggest that MWCNTs with smaller lengths and dispersed as individual particles are most likely to be accumulated by other organisms. Increased accumulation of MWCNTs beyond that observed in the present study may cause additional negative effects. The introduction of MWCNTs into an aquatic environment containing NOM may also change the fate and transport of other chemicals. MWCNTs with a modified surface due to coating by NOM have been shown to decrease the sorption capacity of MWCNTs for selected organic contaminants and metals rendering the chemicals more bioavailable and potentially more toxic (Wang et al. 2008, Kim et al. 2009). Interactions between MWCNTs (±NOM) and other coexisting toxic compounds should be taken into account when considering the aquatic environmental risks of CNTs (Kim et al. 2009). Further exploration of MWCNTs accumulating in particular locations within aquatic invertebrates, such as lipophilic storage compartments, needs to be completed to fully characterize the potential for

toxicity. Rosenkrantz et al. (2009) suggested that if found in storage compartments, even short-term exposure of nanoparticles, could lead to residues persisting in the planktonic community. If nanoparticle exposure and accumulation affects sensitive processes such as growth and molting, as observed in the present study and others, natural populations could be affected, causing ripple effects throughout the aquatic food web. Chronic data gathered on MWCNT toxicity (with and without the presence of NOM) that includes accumulation through invertebrate food webs or multigenerational effects will help provide valuable information for future regulatory purposes and guide future research on organisms at higher trophic levels. Concentrations that were used in this experiment may only be relevant to spill scenarios; therefore, chronic studies involving lower, environmentally realistic concentrations are also needed.

Acknowledgements- The present study was supported by the U.S. Environmental Protection Agency Science to Achieve Results Program (grant R827149) and the University of Georgia Interdisciplinary Toxicology Program. The authors thank Qingguo Huang and Elijah Petersen for providing the labeled carbon nanotubes and Caitlin Smith for assisting with experiments.

REFERENCES

Andrews R, Jacques D, Rao AM, Derbyshire F, Qian D, Fan X, Dickey EC, Chen J. 1999. Continuous production of aligned carbon nanotubes: a step closer to commercial realization. *Chem. Phys. Letters* 303: 467-474.

Andrievsky G, Klockkov V, Derevyanchencko L. 2005. Is the C60 fullerene molecule toxic?! *Fullerenes, Nanotubes, and Carbon Nanostructures.* 13:363-376.

Baun A, Sørensen SN, Rasmussen RF, Hartmann NB, Koch CB. 2008. Toxicity and of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C60. *Aquat. Toxicol*. 86:379–387.

Black MC and JF McCarthy. 1988. Dissolved organic macromolecules reduce the uptake of hydrophobic organic contaminants by gills of rainbow trout (*Salmo gairdneri*). *Environ. Tox. Chem*. 7(7): 593-600.

Broman D, Näuf C, Lundbergh I, Zebühr Y. 1990. An in situ study on the distribution, biotransformation and flux of polycyclic aromatic hydrocarbons (PAHs) in an aquatic food chain (seston-*Mytilus edulis* L.-*Somateria mollissima* L.) from the Baltic: An ecotoxicological perspective. *Environ. Tox. Chem*. 9(4):429-442.

Chen Q, Saltiel C, Manickavasagam S, Schadler LS, Siegel RW, Yang H. 2004. Aggregation behavior of single-walled carbon nanotubes in dilute aqueous suspension. *J. Colloid Interface Sci.* 280:91-97.

Chen P, Zhang HB, Lin GD, Hong Q, Tsai KR. 1997. Growth of carbon nanotubes by catalytic decomposition of CH_4 or CO on a Ni-MgO catalyst. *Carbon*. 35:1495–1501.

Cheng XK, Kan AT, Tomsom MB. 2004. Naphthalene adsorption and desorption from aqueous C-60 fullerene. *J. Chem. Eng. Data*. 49:675-683.

Edgington AJ, Roberts AP, Taylor LM, Alloy MM, Reppert J, Rao AM, Mao J, Klaine SJ. 2010. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ. Tox. Chem*. 29(11): 2511-2518.

Gottschalk F, Sonder T, Scholz Rw, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. *Environ. Sci. Technol*. 43: 9216-9222.

Ham HT, Choi YS, Chung IJ. 2005. An explanation of dispersion states of single-walled carbon nanotubes in solvents and aqueous surfactant solutions using solubility parameters. *J. of Colloid Inferface Sci.* 288(1): 216-223.

Henry TB, Menn FM, Fleming JT, Wilgus J, Compton RN, Sayler GS. 2007. Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ. Health Perspect*. 115:1059-1065.

Hull MS, Kennedy AJ, Steevens JA, Bednar AJ, Weiss CA Jr, Vikesland PJ. 2009. Release of metal impurities from carbon nanomaterials influences aquatic toxicity. *Environ. Sci. Technol.* 43(11): 4169-4174.

Hyung H, Fortner JD, Hughes JB, Kin JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41(1): 179-184.

Kang S, Mauter MS, Elimelech M. 2008. Physicochemical determinants of multiwalled carbon nanotube bacterial cytoxicity. *Environ. Sci. Technol*. 42: 7528-7534.

Kennedy AJ, Gunter JC, Chappell MA, Goss JD, Hull MS, Kirgan RA, Steevens JA. 2009. Influence on nanotube preparation in aquatic bioassays. *Environ. Tox. Chem.* 28(9): 1930-1938.

Kim KT, Edgington AJ, Klaine SJ, Cho JW, Kim SD. 2009. Influence of multiwalled carbon nanotubes dispersed in natural organic matter on speciation and bioavailability of copper. *Environ. Sci. Technol.* 43(23): 8979-8984.

Knight JT and WT Waller. 1987 Incorporating *Daphnia magna* into the seven-day *Ceriodaphnia* effluent toxicity test method. *Environ. Tox. And Chem*. 6(8):635-645.

Kukkonen J, Oikaro A, Johnsen S, Gjessing E. 1989. Effects of humus concentrations on benz[a]pyrene accumulation from water to *Daphnia magna*: comparison of natural waters and standard preparations. *Sci. Total Environ.* 72(2):197-207.

Li M and CP Huang. 2011. The responses of *C. dubia* toward multi-walled carbon nanotubes: effect of physical-chemical treatment. *Carbon*. 49(5): 1672-1679.

Limbach LK, Wick P, Manser P, Grass R, Bruinink A, Stark WJ. 2007. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. *Environ. Sci. Technol*. 41:4168-4163.

Musee N. 2011. Nanowastes and the environment: potential new waste management paradigm. *Environ. Int*. 37: 112-128.

McCarthy JF and BD Jimenez. 1985. Interactions between polycyclic aromatic hydrocarbons and dissolved humic material: binding and dissociation. *Environ. Sci. Tech*. 19(11): 1072-1076.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nano level. *Science*. 311(5761): 622-627.

Oberdörster E, Zhu SQ, Blickley TM, McClellan-Green P, Haasch ML. 2006. Ecotoxicology of carbon-based nanomaterials: effects of fullerene (C60) on aquatic organisms. *Carbon*. 44(6): 1112-1120.

Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna. Environ. Sci. Technol*. 43(8):2969-2975.

Petersen EJ, Huang QG, Weber WJ Jr. 2008a. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus. Environ. Health Perspect*. 116(4):496-500.

Petersen EJ, Huang QG, Weber WJ Jr. 2008b. Bioaccumulation of radio-labeled carbon nanotubes by *Eisenia foetida. Environ. Sci. Technol*. 42:3090-3095.

Petersen EJ, Huang QG, Weber WJ Jr. 2010. Relevance of octanol-water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes. *Environ. Tox. Chem*. 29(5): 1106-1112.

Petersen EJ, Pinto RA, Mai DJ, Landrum PF, Weber Jr, WJ. 2011. Influence of polyethyleneimine graftings of multi-walled carbon nanotubes on their accumulation and elimination by and toxicity to *Daphnia magna*. *Environ. Sci. Technol.* 45:1133-1138.

Roberts AP, Mount AS, Seda B, Souther J, Qiao R, Lin Sijie, Ke PC, Rao AM, Klaine SJ. 2007. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol*. 41(8):3025-3029.

Rosenkranz P, Chaundhry Q, Stone V, Fernandes T. 2009. A comparison of nanoparticle and fine particle uptake by *Daphnia magna*. *Environ. Tox. Chem*. 28(10): 2142-2149.

Salonen K and T Hammar. 1986. On the importance of dissolved organic matter in the nutrition of zooplankton in some lake waters. *Oecologia*. 68(2): 246-253.

Spitzy A and Leenheer J. 1988. Dissolved organic carbon in rivers. Biochemistry of major world rivers. *M S Swaminathan Research Foundation*: Chennai, India.

Smith CJ, Shaw BJ, Handy RD. 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Tox*. 82: 94-109.

Stafiej A. and K. Pyrznska. 2007. Adsorption of heavy metal ions with carbon nanotube. *Separat. and Purif. Technol*. 58: (2007) 49–52

Templeton RC, Ferguson PL, Washburn KM, Scrivens WA, Chandler GT. 2006. Life-cycle effects of single-walled carbon nanotubes (SWCNTs) on an estuarine meiobenthic copepod. *Environ. Sci. Technol.* 40:7387-7393.

U.S. Environmental Protection Agency. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA 600-/4-91/002. Cincinnati, OH.

Wang X, Lu J, Xing B. 2008. Sorption of organic contaminants by carbon nanotubes: influence of adsorbed organic matter. *Environ. Sci. Technol*. 42:3207-3212.

Wang X, Tao S, X Baoshan X. 2009. Sorption and competition of aromatic compounds and humic acid on multiwalled carbon nanotubes. *Environ. Sci. Technol*. 43(16):6214-6219.

Zhu X, Zhu L, Lang Y, Chen Y. 2008. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene agreegates. *Environ. Tox. Chem*. 27(9):1979-1985.

Table 2.1. Quench curve demonstrating no interference of 200 μL NaOH added to vials to digest *Ceriodaphnia dubia,* measured by counts per minute (cpm).

μL NaOH added	Average cpm	Standard Error
0	39.67	2.27
100	39.67	2.30
200	41.33	3.17
500	44.83	2.70
1000	47.17	2.30

Table 2.2. Concentrations of multi-walled carbon nanotubes (MWCNTs, mg/L) that related to reproductive toxicity to *Ceriodaphnia dubia* after chronic exposures with three different dispersion techniques.

	Reproductive Toxicity	
Treatment	LOEC (mg/L)	NOEC (mg/L)
B-CNT[a]	2.5	1.25
B-CNT+NOM[b]	>5	>5
S-CNT+NOM[c]	>5	>5

[a] B-CNT = bath sonicated MWCNT
[b] B-CNT+NOM = bath sonicated MWCNTs + 2.35 mg C/L natural organic matter
[c] S-CNT+NOM = stirred MWCNTs + 2.35 C/L natural organic matter

FIGURE LEGENDS

Figure 2.1. Dispersion of multi-walled carbon nanotubes (10 mg/L) in synthetically-made moderately hard water (MHW) for 24 h following 2 h sonication. By 24 h, approximately 75% of the initial solution remained dispersed.

Figure 2.2. Representative control adult *Ceriodaphnia dubia* (A) and adults exposed to 2.5 mg/L bath-sonicated carbon nanotubes (B-CNTs, LOEC for reproduction) with the absence (B) and presence (C) of Suwanee River natural organic matter (NOM, 2.35 mg C/L) at 40x.

Figure 2.3. Visible multi-walled carbon nanotubes (MWCNTs) in the gut tract of adult *Ceriodaphnia dubia* after 7-d exposure to [2.5 mg/L] of B-CNT+NOM (bath-sonicated carbon nanotubes and natural organic matter) treatment denoted by a black arrow (40x). No agglomerations were noted on any of the exposed adults but there was significant accumulation of MWCNTs at the [5 mg/L] concentration (23.26±12.86 ng per organism), suggesting internal accumulation of MWCNTs by adults.

Figure 2.4. Average offspring production by *Ceriodaphnia dubia* adults (neonates per adult) over the course of three broods during exposure to 2.5 mg/L multi-walled carbon nanotubes (MWCNTs) in three dispersion treatments (B-CNT= bath sonicated MWCNTs; B-CNT+NOM= bath sonicated MWCNTs with the addition of 2.35 mg C/L natural organic matter; S-CNT+NOM= stirred MWCNTs with the addition of 2.35 C/L natural organic matter). Data from MWCNT-exposures are represented by dashed lines

Controls (moderately hard water and moderately hard water with the addition of 2.35 C/L natural organic matter) are represented by solid lines. (*) denotes statistical significance compared to control (Tukey's test, $p<0.05$).

Figure 2.5. Average accumulation (±SE) of multi-walled carbon nanotube (MWCNTs) by adult *Ceriodaphnia dubia* after a 7-d exposure. Accumulation includes ingested MWCNTs and MWCNTs adhering to the carapace of exposed organismTen *C. dubia* were exposed per test concentration, with n=3 replicate tests per treatment except for bath sonicated exposures, which had n=4 replicate tests. Only the *C. dubia* adults exposed to the B-CNT+NOM (bath sonicated MWCNT with 2.35 mg C/L) had significant accumulation when exposed to 5 mg/L MWCNTs, with an average of 23.26±12.86 ng MWCNTs per organism. (*) denotes statistical significance versus control (Tukey's test, $p<0.05$).

Figure 2.6. Average accumulation (±SE) of multi-walled carbon nanotubes (MWCNT) in *Ceriodaphnia dubia* neonates released from chronically exposed adults. Accumulation includes ingested MWCNTs and MWCNTs adhering to the carapace of exposed organisms. Neonates exposed to 5 mg/L MWCNTs (all dispersion treatments) accumulated significantly more MWCNTs than controls. The neonates whose maternal generation was exposed to 3.75 mg/L of the bath sonicated treatment (B-CNT) of MWCNTs accumulated the highest amount of MWCNTs of all treatments, with a mean concentration of 5.12±2.71 ng per organism (range of n=1 to n=104, 31 vials total for 4 experiments). Reproductive toxicity resulted in the decrease in production of neonates

over time, resulting in an n=0 for some replicates. (*) denotes statistical significance from control (Tukey's test, $p<0.05$).

Figure 2.1.

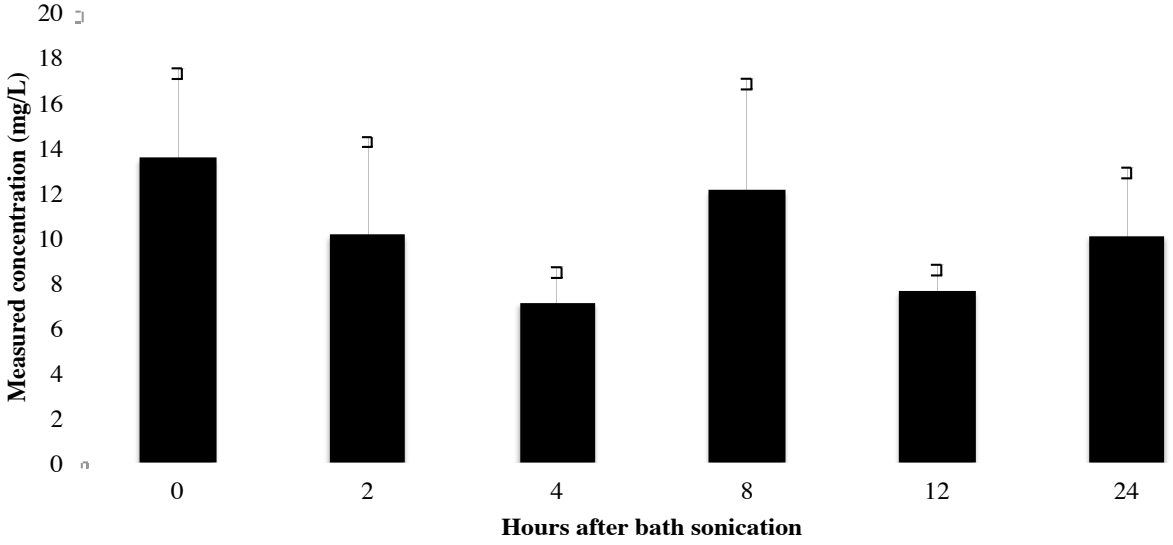

Measured concentration (mg/L)
20
18
16
14
12
10
8
6
4
2
0
0
2
4
8
12
24
Hours after bath sonication

Figure 2.2.

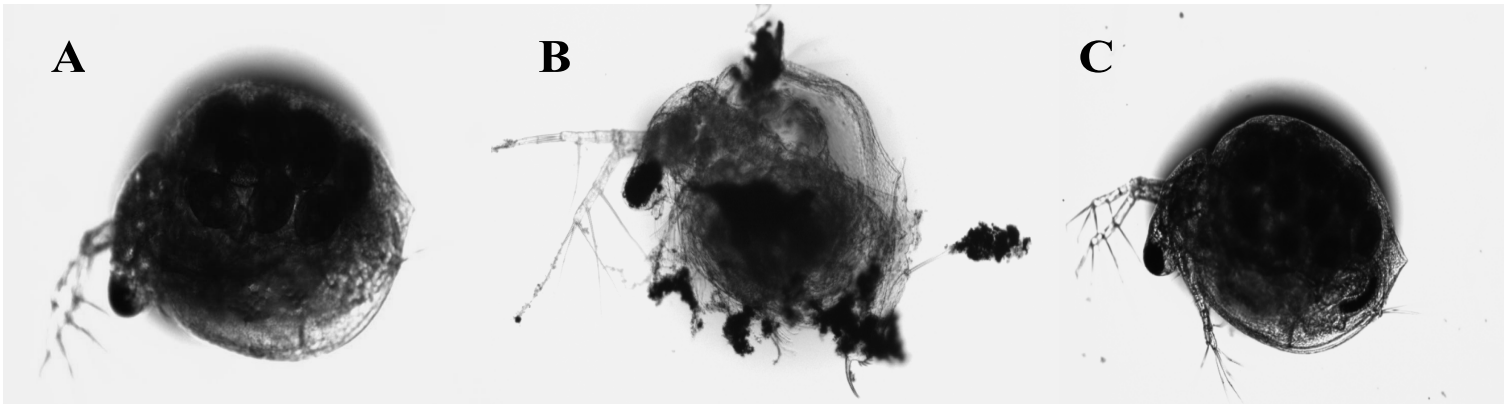


Figure 2.3.

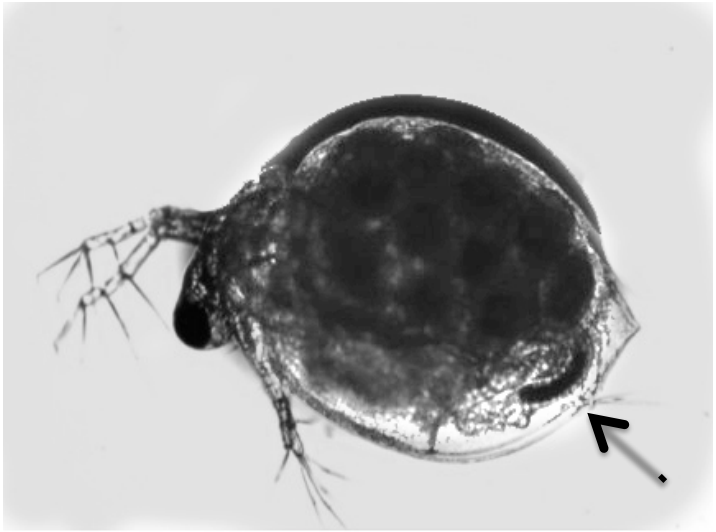


Figure 2.4.

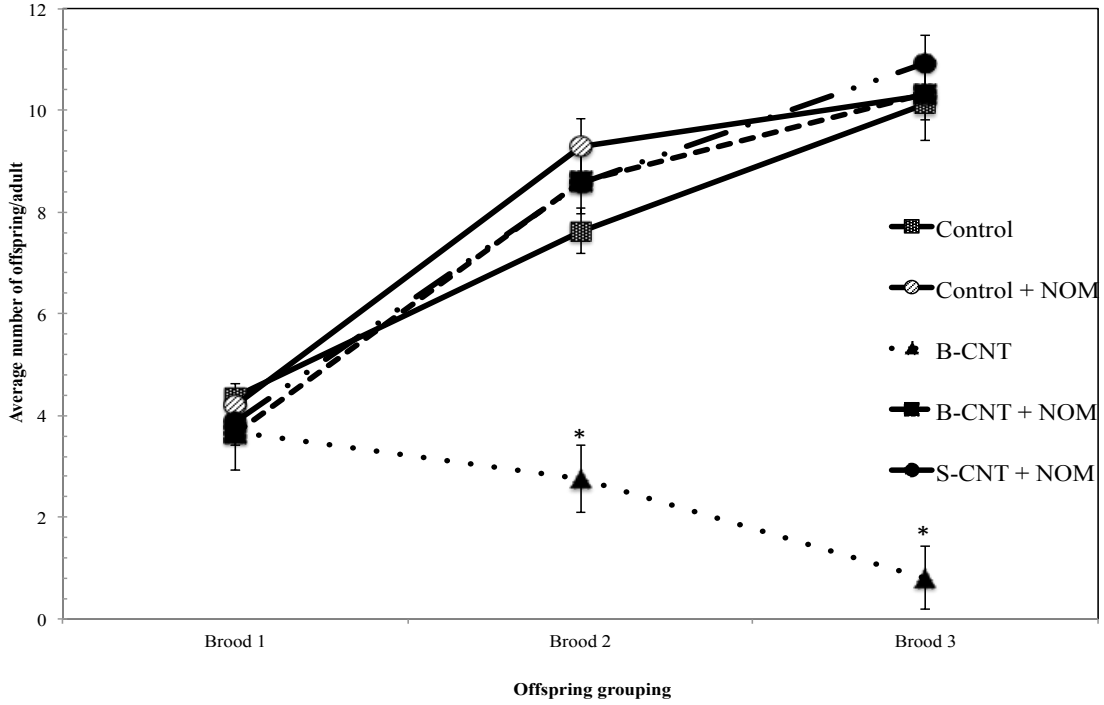

12
10
8
6
4
2
0
Average number of offspring/adult
Brood 1
Brood 2
Brood 3
Offspring grouping
Control
Control + NOM
B-CNT
B-CNT + NOM
S-CNT + NOM
*
*

Figure 2.5.

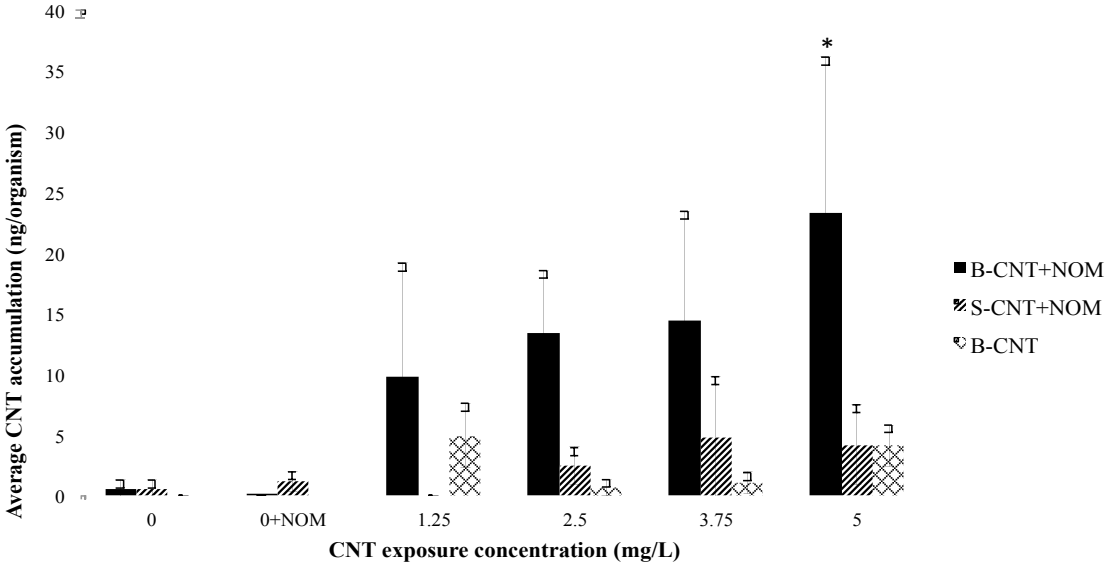
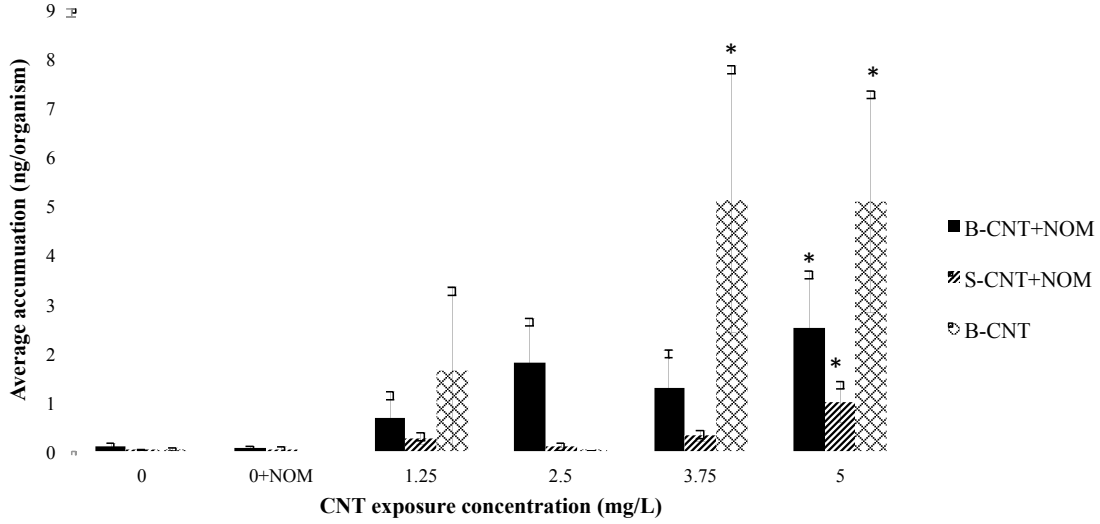

Average CNT accumulation (ng/organism)
40
35
30
25
20
15
10
5
0
*
0
0+NOM
1.25
2.5
3.75
5
CNT exposure concentration (mg/L)
B-CNT+NOM
S-CNT+NOM
B-CNT

Figure 2.6.


Average accumulation (ng/organism)
9
8
7
6
5
4
3
2
1
0
0
0+NOM
1.25
2.5
3.75
5
CNT exposure concentration (mg/L)
B-CNT+NOM
S-CNT+NOM
B-CNT

CHAPTER 3

REPRODUCTIVE TOXICITY AND ACCUMULATION IN AN ESTUARINE CRUSTANCE AFTER CHRONIC EXPOSURE TO MULTI-WALLED CARBON NANOTUBES: THE ROLE OF DISSOLVED NATURAL ORGANIC MATTER [1]

[1] Emily R. McReynolds, Marie E. DeLorenzo, and Marsha C. Black. In preparation to be submitted to *Carbon*.

ABSTRACT

Carbon nanotubes (CNTs) have many uses in the biotechnology field. As production efficiency improves with new technology, they are produced on a large scale with impending contact with the aquatic environment. Many products on the market, such as marine-antifouling paint, provide CNTs a direct exposure route to water systems. Little information has been provided on the toxicity of nanomaterials following exposures of aquatic organisms, particularly those that live in marine and estuarine environments. We report the adverse chronic effects of multi-walled carbon nanotubes (MWCNTs) on *Americamysis bahia*, an estuarine crustacean, in a water-only system in the presence of natural organic matter from a natural seawater source and in the absence of natural organic matter from a synthetic seawater source. Exposures were conducted in two developmental windows (7-14 and 14-21 days old) to identify sensitive reproductive stages. For each exposure, four concentrations of MWCNTs were used, ranging from 0.1 mg/L to 10 mg/L. Test endpoints were mortality, maturation, and MWCNT accumulation. Significant mortality was not observed, but exposure to MWCNTs produced negative reproductive effects. In the 7-14 d developmental window, a 59.5% decrease in the number of mature individuals exposed to 5 mg/L MWCNTs in natural seawater was observed compared to the controls ($p=0.040$). In contrast, individuals exposed to 10 mg/L MWCNTs dispersed in synthetic seawater showed 23.8% increase in the number of mature individuals ($p=0.003$). Accumulation of MWCNTs increased at higher exposure concentrations for each development stages tested; the largest MWCNT accumulation measured when dispersed in natural seawater (31.5±12.8 ng per organism), but the lowest NOEC for accumulation (0.1 mg) was measured when MWCTNs were

dispersed in synthetic saltwater (p=0.0276). Continuous exposure of MWCNTs could result in a decrease in wild populations or a disruption in estuarine food webs. This experiment documents previously unknown delays of maturation in marine organisms caused by MWCNTs exposure and the importance of natural organic matter increasing environmental realism of laboratory observations.

Keywords: *Americamysis bahia*, ecdysis, molting, nanomaterial, accumulation, reproduction, natural organic matter

INTRODUCTION

Revolutionary advances in medical science, military technology, and commercial applications has increased worldwide interest in nanomaterials like carbon nanotubes (CNTs) over the past decade. As an industry, nanotechnology is expected to exceed $1 trillion per year by 2015 (Nel et al. 2006). One of the uses of CNTs relevant to the marine environment is as an ingredient in anti-fouling paint marketed as “environmentally friendly”. Used on ships of all sizes, upon leaching or weathering this paint provides a direct exposure route for CNTs to organisms in marine and estuarine environments. The hurdles to widespread commercial production, cost, purification, and assembly methods are rapidly being overcome. A single factory was able to produce 40 tons of CNTs in 2007 but production methods have improved to enable a factory to produce 500 tons per year (Global Industry Analysts 2007). As production increases, CNT contact with the aquatic environment is inevitable, which is a concern because the toxicity of these

materials is relatively unknown and there is very little governmental regulation controlling the production or release. The only regulation controlling CNTs in the United States is a Significant New Use Rule (SNUR) under the Toxic Substances Control Act in effect as of June 2011(https://www.federalregister.gov/articles/2013/06/26/2013-15032/significant-new-use-rules-on-certain-chemical-substances). This SNUR requires manufacturers, importers, or processors of MWCNTs must give the United States Environmental Protection Agency (USEPA) 90 d notice before beginning the activity and the EPA has the ability to limit or prohibit the action. Manufacturers can voluntarily give information about predicted production volumes or environmental and human health data, but are not required. The EPA has listed CNTs as "Contaminants of Emerging Concern" and suggested that further research into their toxicity is necessary before more strict regulatory legislation is enacted. As a result, the potential for human health implications and possible accumulation in commercially and ecologically important animals are areas of high research priority.

Few studies have investigated the effects of carbon nanotubes on marine and estuarine organisms likely because CNTs are extremely hydrophobic and even more insoluble in seawater than freshwater, and were originally not thought to be bioavailable in the water column. However, CNTs are now being engineered to be more hydrophilic through the addition of functional groups, which may increase their bioavailability in the aquatic environment. To mimic what may occur in aquatic environments, the addition of natural organic matter (NOM) is a dispersion method to increase hydrophilicity for laboratory testing of MWCNTS. Use of NOM is more environmentally realistic than addition of other chemicals or solvents because it is a naturally occurring substance in

aquatic environments and serves as an energy source in many aquatic food webs (Salonen and Hammar 1986). Carbon nanotubes become dispersed because NOM coats the extremely hydrophobic surfaces of CNTS, increasing their stability in water (Hyung et al. 2007, Wang et al. 2009).

To date, no data on acute or chronic toxicity of multi-walled carbon nanotubes (MWCNTs) to marine or estuarine organisms are available in the literature. This data gap likely exists in large part from the idea that MWCNTs are not bioavailable in marine or estuarine environment (Klaine et al. 2008, Lin et al. 2010). With this present data gap, other types of carbon-based nanomaterials can be used for comparison. Single-walled carbon nanotubes (SWCNTs) are similar to MWCNTs as tube-like carbon-based nanomaterials, but consist only of one sheet of graphene, whereas MWCNTs have many. Neither sublethal effects (cytotoxicity, burrowing behavior) nor significant bioaccumulation post-depuration were measured in a sediment-dwelling lugworm (*Arenicola marina*) exposed to 30 mg/kg SWCNTs in sediment over a 10-d period (Galloway et al. 2010). The researchers suggest that the SWCNTs were ingested and not absorbed, but instead moved straight through the gut tract and were excreted. In an acute study with the estuarine fish, *Fundulus heteroclitus*, no effects were evident on hatch success or juvenile survival after a 96-h exposure to 10 mg/L fullerenes (spherical C_{60}) (Blickley and McClellan-Green 2008). However, higher levels of glutathione were measured in adults exposed to 1 mg/L fullerenes in a 96-h acute exposure, which suggests the presence of oxidative stress.

Although acute toxicity studies may be relevant to spills, determining what happens over is important. There has been one life-cycle study that can be used as a basis

for understanding the chronic toxicity of CNTs. An estuarine meiobenthic copepod (*Amphiascus tenuiremi*s), was exposed to 10 mg/L SWCNTs for 28 d (Templeton et al. 2006). Increased mortality, reduced fertilization rates, and reduced molting success in this crustacean were all noted as a result of the SWCNT exposure. The authors hypothesized that mechanical disruption of the feeding appendages, molting inhibition, and active uptake of SWCNTs followed by oxidative stress were the most likely modes of action for CNT toxicity to *A. tenuiremis*. These same modes of action could be present for other marine invertebrates exposed to other CNTs. However, without data available on MWCNT exposures, assessing the differences in MWCNT toxicity versus SWCNTs is difficult. Furthermore, the unique modes of action of SWCNTs in *A. tenuiremis* require further investigation to determine if toxicity is similar among crustacean species.

The largest invertebrate phylum is Arthropoda, in which insects and crustaceans are the two largest groups. Because few insects have aquatic larvae, crustaceans are considered the most numerous and ecologically important group of invertebrates in the marine environment (Baun *et al.* 2008). Our model organism is the mysid shrimp, *Americamysis bahia* (formally *Mysidopsis bahia*). It is a small, shrimp-like crustacean that is endemic to inshore waters throughout the Gulf of Mexico and has been reported along the Atlantic coast of the United States from Florida to Rhode Island (Price et al. 1994). *Americamysis bahia* is an omnivore and feeds on a wide range of food items including detritus, phytoplankton, and zooplankton (Mauchline 1980). It is found in high densities and serves as prey for many fish and bird species. Mysid shrimp are important to many estuarine food webs and serve as an important link in energy transfers to higher trophic levels (Mees et al. 1994, Roast et al. 1998). Because it is easy to culture and

maintain in the laboratory, has a short reproductive cycle, is tolerant to a range of salinities, and is relatively sensitive to toxicants, *A. bahia* is an EPA-recommended organism for whole-effluent toxicity (WET) tests and regulatory biomonitoring in marine and estuarine environments (USEPA 1993). Some of the key knowledge gaps we help bridge in these experiments are providing MWCNT toxicity data on an estuarine organism, measuring chronic toxicity of MWCNTs to pinpoint possible mode of actions, determining the role of NOM in MWCNT toxicity, and finally, measuring accumulation by using ^{14}C-labeled MWCNTs.

MATERIALS AND METHODS

Natural culture water and organisms

Americamysis bahia used in the natural seawater exposures (subsequently referred to as NSW) were cultured in-house at the Coastal Center for Environmental Health and Biomolecular Research mysid culturing facility (Charleston, SC). The original *A. bahia* stock culture was obtained from Aquatic Indicators, Inc., St. Augustine, FL. Test organisms were cultured in a flow-through system with seawater from Charleston Harbor, SC under aerated conditions. Natural seawater was obtained from Charleston Harbor estuary, SC, (N 32° 45’ 11.52’’; W 79° 53’ 58.31’’). The seawater was filtered through a 5 μm particle filter and activated carbon, UV-sterilized and mixed with deionized water to obtain salinity of 20 ppt. Total organic carbon was measured using a Shimadzu 5050, which involves a combustion and non-dispersive infrared gas analysis method. The TOC of the filtered seawater was 13.52 mg/L, suggesting a high level of dissolved NOM (< 5 μm in size).

Synthetic culture water and organisms

Americamysis bahia used in the synthetic seawater exposures (SSW) were obtained from Aquatic Biosystems, Inc., Ft. Collins, CO, where they were cultured following traditional methods as suggested by the EPA (USEPA 1993). Upon receipt, organisms were acclimated for 48 h in 20 L aquaria with aeration and static renewal at 24 h. Synthetic seawater was created by mixing Instant Ocean Aquarium Sea Salt (Spectrum Brands, Inc.) with MilliQ deionized water to yield a salinity of 20 ppt. No TOC was detected in the SSW (Shimadzu 5050).

Routine-reference acute and chronic toxicity tests were performed with both of these cultures to ensure homogenous culture sensitivity to the surfactant sodium dodecyl sulfate (SDS, Dirilgen and Ince 1995, Mariani et al. 2006).

MWCNTs

Multi-walled carbon nanotubes were chosen for use in this study over SWCNTs because of their availability, lower cost, higher projected rate of production, and greater current use (Volder et al. 2013). To quantify the amount of MWCNTs in environmental media and organisms, experiments were conducted with radiolabeled MWCNTs (^{14}C). The synthesis of ^{14}C-MWCNTs was previously described (Chapter 2; Petersen et al. 2008). Radiolabeled MWCNTs had a specific activity of 0.12 μCi/mg and a purity of 99±0.2% (Petersen et al. 2009).

MWCNT dispersion in seawater

The dry, powdered form of the MWCNTs was weighed and added to control

seawater (either SSW or NSW) to make a stock solution of 10 mg/L MWCNTs, verified through liquid scintillation couting (LSC). Concentrations of the stock were confirmed by daily analysis of 2 mL aliquots prior to dilution. The hydrophobic MWCNTs were suspended immediately prior to use in an experiment in control seawater (SSW or NSW) through the use of a low-powered water bath sonicator (Branson model 2510). The 2-h sonication period was sufficient to keep approximately 85±8% of the initial MWCNT concentration suspended in solution over a 24 h period, the time between water changes in the exposures (data not shown).

Bioassays

Following a modified version of USEPA Method 1007.0, a 7-d chronic exposure of *A. bahia* was conducted to measure reproductive toxicity and accumulation of MWCNTs (USEPA 1993). This method is based on a 7-14 d toxicity test. The method was modified to assess toxicity in two age ranges of *A. bahia*: 7-14 d, and 14-21 d old. These two age ranges were chosen to represent critical time periods in the mysid shrimp reproductive cycle. During these juvenile stages, *A. bahia* undergo several moltings through the process of ecdysis to become sexually mature and mate successfully. Four ^{14}C-MWCNT concentrations (0.1, 1, 5, 10 mg/L) were added to control SSW or NSW and were sonicated just prior to exposures, which included seawater-only controls. Exposures were conducted in 200-mL beakers with six replicates for each concentration and 150 mL total volume of exposure water for each vessel. Ten randomly selected, age-appropriate *A. bahia* were placed in each replicate. Beakers were randomly placed in an incubator that had a 16:8 light:dark photoperiod and a constant temperature of 26.0±1.0

°C. Daily renewal of exposure solution included a transfer of all organisms to fresh solution as well as feeding with brine shrimp nauplii (*Artemia* spp.) *ad libitum*.

Mortality and reproductive toxicity

Survival of *A. bahia* was monitored in daily observations of each beaker for each 7-d experiment. Animals were considered dead when they were viewed lying at the bottom of the beaker, grey in appearance, and without appendage movement. Reproductive toxicity was assessed (Omano OM36 compound microscope) in the 7-14 d exposure window as the percentage of mature adults on day 14 compared to controls. Mature adults included females containing a developed brood sac with or without eggs in the oviduct and males with gonads (USEPA 1993). Immature individuals did not have any of these characteristics. The toxicity endpoint for the 14-21 d exposure window was offspring production. In the 14-21 d age group, daily counts were recorded of *A. bahia* reproduction (number of juveniles released per day in each replicate).

Accumulation

All organisms were viewed microscopically to observe accumulation of MWCNTs at the termination of the test and prior to liquid scintillation counting (LSC) of ^{14}C-MWCNT accumulation. An iPhone 4 (Apple, Inc., Cupertino, CA) with a ScopeMonkey attachment (Microfacturing, Inc., Atlanta, GA) fitted to an Olympus SZX9 research stereomicroscope was used to take photographs of mysids in the SSW-MWCNT exposures. Photographs were not taken during the NSW exposures because there was no access to a microscope camera in the radioactive-restricted areas of the facility. Adult

organisms were then pooled by replicate and placed in control seawater for 2 min to allow for surface desorption of MWCNTs to remove potentially confounding MWCNTs not physically attached to the outside of the organisms. Pooled organisms were transferred to 7-mL plastic scintillation vials and 400 µL of 2 N NaOH was added to each vial to digest the organisms. Quality control tests have shown that the addition of NaOH does not mask the ^{14}C readings, based on a quench curve analysis of a range of NaOH values (data not shown). After digestion, 5 mL of ScintiVerse BD scintillation cocktail fluid (Fisher Scientific) was added to each vial. The vials were the placed in the liquid scintillation counter (LKB Wallac 1211 RackBeta) and counted for 10 min per sample. A blank sample containing only 400 µL of 2 N NaOH and control seawater (5 mL) was counted for 10 min and subtracted from all subsequent samples (blank cpm±SE = 21±2).

Data analyses

Assumptions of normality for all statistical analyses were tested using SAS software (Shapiro-Wilkes, SAS Institute Inc., v. 9.3); subsequent analysis used the same software unless otherwise stated. All data met assumptions for normality therefore no transformations were needed. Data analyses for mortality at all age groups were conducted with an analysis of variance (ANOVA; $\alpha = 0.05$). Difference in the number of mature individuals in each replicate (for 7-14 d window for both SSW and NSW) among treatments was determined by ANOVA followed by a Dunnet's test for each data set compared to the controls ($\alpha = 0.05$). This procedure was also used to determine differences in offspring production in the 14-21 d time window for SSW and NSW exposures.

Accumulation was calculated from the amount of radioactivity measured in pooled samples containing bodies of *A. bahia*. The average counts per minute (cpm) per organism was calculated from the original LSC reading (corrected for the blank CPM) divided by the number of organisms in the vial. Values were converted to ng per organism using the specific activity of the MWCNTs (0.12 μCi/mg). Differences in accumulation of MWCNTs by *A. bahia* by treatment at each time window were compared in the same manner as assessing reproductive toxicity. Linear regressions and coefficients of determination (r^2 value) of MWCNT accumulation versus exposure concentrations for all data sets were conducted in Microsoft Excel (Microsoft Office 2011) and p-values for regression analysis were calculated in SAS (SAS Institute Inc., v. 9.3).

RESULTS

Exposure concentrations

Mean exposure concentrations of stock MWCNT solutions made daily throughout both NSW experiments was 9.8±1.1 mg/L with a target stock concentration of 10 mg/L. The mean stock concentration for the 7-14 d experiment was 11.7±2.7 mg/L, and for the 14-21 d experiment it was 9.9±1.0 mg/L. The mean stock exposure concentration for both SSW experiments was 9.6±4.9 mg/L. The stock concentration for the 7-14 d experiment was 8.7±4.9 mg/L and for the 14-21 d experiments was 10.5±4.9 mg/L. Because measured values (±SE) overlapped the target concentration, nominal values are reported.

Mortality

The mortality of *A. bahia* exposed to MWCNTs was not significantly different from control mortality in either experiment ($F_{9,50}$=0.594, p=0.795). In exposures conducted in NSW mean mortality (±SE; n=60 individuals) in the 7-14 day exposure window was 1.9±0.5 and for 14-21 d exposure window was 2.3±0.2. For exposures conducted in SSW mean mortality (±SE) for 7-14 d exposure window was 2.0±0.5 and 2.5±0.5 in the 14-21 d exposure window. Maximum level of mortality for controls in a 7-d experiment is <10% (USEPA 1993). In these experiments, control mortality did not exceed 3.8±1.0% in NSW or SSW experiments in both windows.

Reproductive toxicity: sexual maturation

Fewer mature individuals were observed in *A. bahia* exposed to NSW-MWCNTs in the 7-14 d exposure (range of 40.0±13.2 to 58.0±7.3% mature) compared to controls with 78.0±4.7% mature ($F_{4,25}$=2.886, p=0.043; Fig. 3.1). The largest significant decrease in maturity was seen in *A. bahia* exposed to 5 mg/L, where only 40±13.1% adults became mature ($F_{4,25}$=2.886, p=0.043). However, linear regression analysis suggests that there is no concentrations relationship with a decrease in mature individuals when MWNCTs are dispersed in NSW (r^2=0.51, p=0.149, y=-6.8x+75). In contrast, *A. bahia* exposed to MWCNTs dispersed in SSW during the 7-14 d window were more successful in becoming sexually mature than controls, with 59.6±4.7% mature in the highest MWCNT concentration, 10 mg/L, versus 35.6±6.4 % mature in controls ($F_{4,25}$=3.014, p= 0.03). The increase in percentage of mature individuals was positively related to increasing MWCNT exposure concentrations dispersed in NSW(p=0.029, r^2=0.78, y=6.3x+28.11).

Reproductive toxicity: offspring production

Throughout the 14-21 d exposure, offspring production remained constant regardless of exposure concentration and did not differ from controls ($F_{9,50}$=1.856, p=0.811). Average production for adults in any replicate in any treatment dispersed in NSW was 4.5±2.0 offspring. Number of offspring produced by adults exposed to MWCNTs dispersed in SSW during the 14-21 d time window also did not show a difference in the number of offspring produced between exposure concentrations, with an overall average production of 4.7±1.7 ($F_{9,50}$=1.856, p=0.811).

Accumulation: 7-14 d window

In exposures conducted in synthetic seawater MWCNTs were visibly accumulated on the exoskeletons, appendages, and in the gut tracts of *A. bahia* in both tested age groups, 7-14 d old and 14-21 d old (Fig. 3.2). Accumulation was confirmed by quantifying the amount of ^{14}C-labeled MWCNTs through the use of LSC; however, it was not possible to separate external and internal accumulation with this method. In NSW exposures 7-14 d old *A. bahia* exposed to 5 mg/L and 10 mg/L MWCNTs accumulated significantly more MWNCTs than controls (25.10±6.3 ng/ organism and 31.5±12.8 ng/organism, respectively; $F_{4,25}$=5.340, p=0.003; Fig. 3.3A). A concentration response relationship was observed with MWCNT concentration and ng accumulated in 7-14 d *A. bahia* when MWCNTs were dispersed in NSW (p=0.012, r^2=0.905, y=8.8146x-13.227) as well as in SSW (p=0.004, r^2=0.954, y=4.6053x-6.3272). But, unlike exposure to MWCNTs dispersed in NSW, measurable accumulation was statistically significant at the lowest concentration tested in SSW (2.42±0.99 ng MWCNTs per organism,

$F_{4,25}$=3.2667, p=0.0276). Interestingly, there was no linear correlation between an increase in MWCNT accumulation in 7-14 d *A. bahia* and decrease in mature individuals when MWCNTs were dispersed in NSW (p=0.285, r^2=0.358, y=1.234x+37.769) nor SSW (p=0.52, r^2=0.68, y=-0.6158x+62.739).

Accumulation: 14-21 d window

Only *A. bahia* exposed to 5 mg/L MWCNTs dispersed in NSW and SSW accumulated statistically significant amounts of MWCNTs (NSW, $F_{4,25}$=3.445, p=0.0225; SSW, $F_{4,25}$=2.918, p=0.0414, Fig. 3.3B). Interestingly, accumulation measured in *A. bahia* exposed to 5 mg/L MWCNTS dispersed in NSW was nearly double that measured in SSW exposed (20.90±6.65 compared to 10.55±4.93 ng per organism). Although 14-21 d old *A. bahia* accumulated an average 26.42±11.39 ng per organism when exposed to 10 mg/L MWCNTs dispersed in NSW, this was not statistically different from the controls (0.0±0.0 ng per organism) due to a wide variability among samples ($F_{4,25}$=1.817, p=0.157). Similarly, *A. bahia* in the 14-21 d exposed to 10 mg/L MWCNTs dispersed in SSW did not accumulate significantly more MWCNTs ($F_{4,25}$=2.347, p=0.082). Although not all exposures to MWCNT concentrations resulted in accumulation was statistically significant from control, there was a significant linear relationship between MWCNT concentration and accumulation in 14-21 d *A. bahia* when dispersed in NSW (p=0.008, r^2=0.820, y=7.3749x-12.391) but not when dispersed in SSW (p=0.09, r^2=0.65, y=2.7967x-3.652).

Interestingly, we observed *A. bahia* exposed to MWCNTS at all concentrations in SSW and NSW able to excrete ingested MWCNTs without a depuration period following

the exposure. Many *A. bahia* were observed to completely clear their gut tracts of visible CNT accumulation (Fig. 3.4), but we were unable to quantify this excretion.

DISCUSSION

When two age groups of *A. bahia* were exposed to a 0.1-10.0 mg/L range of MWCNTs dispersed in synthetic and natural seawater, mortality was not observed at any concentration. However, other sublethal effects (reproductive toxicity and accumulation) were observed and call for concern. We hypothesize that the decrease in mature individuals observed in NSW-dispersed exposures is related to the inability of *A. bahia* to successfully molt as well as the decrease in energy reserves following an increased accumulation of NSW-dispersed MWCNTs in the gut tract. Understanding the behavior of the *A. bahia* is important to understanding our hypothesized mode of action (inability to molt) of MWCNT toxicity to *A. bahia*. We hypothesize that the presence of MWCNTs in seawater overwhelms normal grooming processes, interrupts molting timing, as well as depletes the allotment of energy reserves for maturation, and are discussed below.

With an increased amount of MWCNTs in and on the *A. bahia* bodies, the animals must remove this foreign material by cleaning their bodies. Grooming behavior in *A. bahia* and related crustacean species has evolved to counteract the effects of environmental fouling, which consists of particulates or organisms such as bacteria or cyanobacteria attaching to the body of a mysid (Clutter 1969). The intermediate age group of 7-14 d in both SSW and NSW appears to be the most sensitive to carbon nanotube exposures when measuring accumulation and reproductive toxicity. *Americamysis bahia* invests much energy in grooming activities; up to 80% of all its

movements relate to grooming the flagella of the antennules and antennae (Acosta and Poirrier 1992). Although extensive grooming occurs elsewhere on the body, *A. bahia* does not clean its eyes or carapace, which suggests that these could be susceptible locations for MWCNT accumulation. If females successfully mate, egg incubation in females may restrict foraging and cleaning behavior to decrease risk of losing eggs (O'Brien and van Wyk 1985), thereby allowing more MWCNTs to remain on the exoskeleton. The cleaning activities of *A. bahia* possibly were overwhelmed in the presence of MWCNTs, causing the observed increase in surface accumulation in immature individuals, and leaving them with less available energy to become sexually mature.

Other behaviors unique to *A. bahia* could contribute to the observed reproductive toxicity in NSW. Mysids must complete several molts before becoming sexually mature through a process referred to as ecdysis. If molting of an individual is disrupted or inhibited for any length of time during ecdysis, it may not become sexually mature at the same time as the rest of the population. Theories of molting timing include adaptation to avoid predation and cannibalism as well as limiting competition (Conan 1985). The inability to molt can create a domino effect that leads to unsuccessful reproduction. Copulation occurs a few hours after successful female molting and only occurs at night. Both the emission of a specific chemical attractant by molting adult females and congregation of *A. bahia* are essential for successful mating (Clutter 1969). The physical adherence of MWCNTs could have interrupted any of these crucial steps and interfered with the mating process. *Americamysis bahia* may not be able to go to the surface to copulate if weighed down by foreign particles such as MWCNTs and if molting is

interrupted by only a few hours, eggs will not mature, but will resorb within 24 h (Cuzin-Roudy and Tchernigovtzeff 1985). Reproductive energy demands can interrupt molting cycles as females typically do not molt while carrying eggs or young in their marsupia and males typically do not molt during courtship and copulation (Mauchline 1973, Hartnoll 1985). Also, stored spermatozoa are lost with a female molt after breeding (Hartnoll 1985), so synchronization of copulation, mating, and molting is an extremely vital process. Grooming is especially important during times when molting cannot occur because molting helps remove foreign particles (Mauchline 1973). The female cleans the inside of the marsupial pouch up to five times an hour (Acosta and Poirrier 1992), which suggests that the young inside may not come into continuous contact with MWCNTs when cleaning is adequate to remove all foreign materials. However, we observed MWCNTs in several female marsupia where eggs were not present (Fig 3.2D) and it is possible that the cleaning of the marsupia was inadequate to remove MWCNTs on eggs or embryos and therefore, embryos never developed and were resorbed. Immature embryos complete three moltings within female marsupia as they mature (Mauchline 1985), and are possibly more sensitive to MWCNT exposure during the molting process.

Successful development and metamorphosis of marine crustacean larvae is dependent on balanced, efficiently-used energy reserves (Sasaki et al. 1986, Lovrick and Ouellet 1994). If crustaceans are unable to obtain energy from exogenous sources, they must use endogenous reserves, such as lipids (normally used for production of eggs in adult females) in order to maintain basal metabolism (Barclay et al. 1983, Capuzzo et al. 1984, Ouellet et al. 1992, Whyte et al. 1986). Crustaceans require additional feeding activities to become mature, which is extremely stressful and requires large amounts of

energy (Hartnoll 1985). With an insufficient diet, *A. bahia* is unable support growth and maturity, thereby increasing the length of the molt cycle in order to conserve energy (O'Brien and van Wyk 1985) which may be the case in *A. bahia* exposed to MWCNTs dispersed in NSW.

It is unclear whether the observed decrease in mature individuals during exposures in the younger age group (7-14 d) was caused by inhibited maturation or if it was only delayed. A threshold effect may exist for those exposed to MWCNTs in NSW; more adults reached maturation when exposed to 10 mg/L than when exposed to 5 mg/L and 1 mg/L exposures. However, more MWCNTs accumulated in and on the organisms exposed to the highest concentration of MWCNTs dispersed in NSW (10 mg/L). The presence of a low percentage of control *A. bahia* becoming mature in SSW (compared to NSW) may provide explanation for the observed increase of sexually mature individuals at the highest concentration MWCNTs (10 mg/L) when dispersed in SSW. *A. bahia* perform better in toxicity tests when using natural seawater, compared to synthetic seawater made from Instant Ocean. The presence of MWCNTs in SSW at higher concentrations may have provided the extra carbon energy source necessary to become sexually mature.

Number of offspring released by *A. bahia* did not decrease at any concentration for the 14-21 d exposures in either treatment (NSW or SSW), which suggests that at this later stage of development, organisms may not be as sensitive to MWCNT exposure. Based on differential toxicity in these two developmental windows, we hypothesize that the interruption of ecdysis, not mating, is the main mode of action for reproductive toxicity caused by exposure to MWCNTs. Proper ecdysis may have been delayed or

inhibited by *A. bahia*'s inability to successfully remove the MWCNTs adhering to its body through their normal grooming procedure and any of the molts could have been affected. Inhibition of ecdysis and increased length of time for reproductive development have been observed in crustaceans exposed to pesticides (Mirex, Methoxychlor, Kepone), industrial chemicals (PCBs, PCPs), and crude oil (Fingerman 1985). As mentioned previously, interruption of molting caused by SWCNT exposure was hypothesized by Templeton et al. (2006) to cause reproductive toxicity in *A. tenuiremus* and is also a proposed mode of action for the freshwater crustacean *Daphnia magna* exposed to SWCNTs (Roberts et al. 2007) and MWCNTs (Edgington et al. 2010; Chapter 2). The tendency of both SWCNTs and MWCNTs to adhere to crustacean bodies and negatively affect molting suggests that they have similar modes of action. However, species sensitivities to CNTs concentrations may be different.

Although there were no significant differences in offspring release, we were unable to successfully determine if there was reproductive toxicity associated with *A. bahia* exposure to MWCNTs during the 14-21 d period because confounding factors may have affected responses. Although our experimental design followed the EPA 1007.0 protocol (USEPA 1993), we hypothesize that the more mature *A. bahia* were stressed due to the lack of sufficient horizontal space in the experimental vessels used for the 14-21 d NSW exposure. The control *A. bahia* did not release a typical number of offspring for this time window, which is indicative of a stressed environment. When females sense a stressful environment, they have the ability to induce longer intermolt periods to cause offspring reduction. Mis-synchronization of molting and breeding cycles has been observed under natural conditions (Conan 1985). Some females may have postponed

molting so that copulation could not occur, thereby not releasing offspring in the given time window. However, although we increased the vessel size for the SSW exposures to accommodate the growth of the 14-21 d old *A. bahia*, control females still did not produce a large number of offspring as expected. Different results are possible with a longer adult exposure to incorporate the shift in intermolt periods. Also, by increasing the exposure time to 14 days to include the more sensitive 7-14 d period (7-21 d exposure period), these experiments may provide results more indicative of a natural exposure.

We also hypothesize that internal and external accumulation of MWCNTs ultimately had a negative effect on *A. bahia* fecundity. Multi-walled carbon nanotube accumulation in *A. bahia* varied drastically based on the presence (NSW) or absence (SSW) of dissolved NOM. Linear concentration responses of MWCNT exposures to average ng accumulated per organism were observed for both age groups exposed to MWCNTs dispersed in NSW and 7-14 *A. bahia* exposed to MWCNTs dispersed in SSW; however, the rate of accumulation is much higher for NSW than SSW during the 7-14 d exposures, based on the slope of the regression lines for NSW (y=8.8146x-13.227) and SSW (y=4.60x-6.33). Visually, MWCNTs were observed adhering to the exoskeleton of the organisms and in the gut tract of exposed *A. bahia*. However, using LSC analysis, these two modes of accumulation could not be differentiated. We hypothesize that random accumulation of MWCNTs on or within individual *A. bahia* attributed to high variation among individuals, leading to wide variability of MWCNT accumulation among replicates within treatments. This variability likely interfered with the statistical significance of the results, as the trends are evident (Fig. 3.3). However, the nature of the hydrophobic MWCNTs most likely caused unavoidable random aggregation leading to

statistically insignificant results, but the results are still biologically relevant. We also observed MWCNTs being excreted from exposed *A. bahia*, which suggests that external accumulation may contribute more to the apparent body burden than retention of MWCNTs in the gut tract. Even though the 14-d old *A. bahia* were smaller than the 21-d old at the end of the exposure periods, on average they accumulated more MWCNTs in and on their bodies.

Interestingly, it appears that 7-14 d *A. bahia* are more susceptible to MWCNT accumulation at lower exposure concentrations when dispersed in SSW than NSW, with significant accumulation at the lowest concentration tested (0.1 mg/L, Fig 3.3A). However, MWCNTs in NSW were more likely to remain suspended in the water column for longer durations in the presence of dissolved NOM compared with exposures in SSW, increasing the likelihood of interaction with *A. bahia*. *Americamysis bahia* exposed to NOM-stabilized MWCNTs accumulated higher concentrations compared to those exposed to MWCNTs in synthetic seawater with no dissolved NOM. We also observed an increase in accumulation in *Ceriodaphnia dubia* exposed to MWCNTs in the presence of NOM (Chapter 2) and suggest that in the presence of dissolved NOM, MWCNTs are viewed as an acceptable food source and consumed more readily than MWCNTs without a coating of NOM. We suggest that a competition among food sources may exist between NSW-dispersed MWCNTs and *Artemia* spp., and as *A. bahia* selects more MWCNTs over *Artemia* spp., more energy is expended to obtain the same nutrition needed to complete maturation and energy reserves used for maturation are most likely depleted. The choice in water source (synthetic or natural) had a profound effect on the interpretation of the data gathered; therefore, we suggest future studies further investigate

the role of dissolved NOM in toxicity to marine crustaceans. Finally, pinpointing the mode(s) of toxicity of MWCNTs in *A. bahia* and other crustaceans will help provide invaluable information to researchers and the government for future regulatory purposes.

ECOLOGICAL SIGNIFICANCE

Given the sensitive nature of successful maturation and mating of *A. bahia*, a small change inhibiting or delaying ecdysis can have great effect on the organism's reproductive output. Changes in energy allocation to life-history activities such as sexual maturation and reproduction will have important consequences on population growth (Vernberg et al. 1978, Calow and Sibly 1990). However, the concentrations used in the current study may only be relevant for spill scenarios. There have not been direct measurements of CNTs in water systems, but models have estimated CNT concentrations may range between 0.5-0.8 ng/L in surface freshwaters and 8.6-18.4 ng/L in wastewater effluent (Mueller and Nowack 2008, Gottschalk et al. 2009). Freshwater NOM flow sources are an important food sources for crustaceans living close to river deltas (Riera et al. 2000). While the dissolved NOM in the NSW was <5 μm in size, we have evidence with the freshwater crustacean, *C. dubia,* that more NOM-sorbed MWCNTs are ingested than MWCNTs alone (Figure 3.3, Chapter 2) suggesting that these particles may be seen as more palatable food sources to *A. bahia* than MWCNTs alone. And as NOM particles and MWCNTs interact, forming larger particles, crustaceans that utilize larger NOM particles as food sources are more likely to be exposed to higher MWCNT concentrations. Because of the decrease in mature individuals observed after exposure, if

a high enough concentration of MWCNTs is present in the marine or estuarine environment, there may be a possible decrease in the wild populations of *A. bahia*. This, in turn, may cause disruption in the estuarine food web, particularly those that rely heavily on *A. bahia* as a food source.

Trophodynamic implications of MWCNT are more complex than a decrease in the population of *A. bahia*. Ingestion of MWCNT-contaminated feces from exposed *A. bahia* by marine organisms is likely to occur in environments with a high exposure concentration of MWCNTs in the water column. *A. bahia* and *Amplelisca abdita* exposed to 100 µg/kg SWCNTs in sediment and algae for 7 d did not accumulate SWCNTs or experience decreased mortality but were able to excrete SWCNTs in fecal pellets during a depuration period (Parks et al. 2013). The excretion of CNTs in fecal pellets was also observed in *A. tenuiremus* (Templeton et al. 2006) and *D. magna* (Petersen et al. 2009). Predators or other organisms may consume CNTs repackaged into fecal pellets, providing another viable exposure route. In areas with low food productivity, many crustaceans result to eating their own feces or fecal pellets of other organisms to obtain energy (Johannes and Satomi 1966). *Fundulus heteroclitus* (mummichog), *Nassa obsolete* (sea snail), *Uca pugnax* (fiddler crab), and *Pagurus longicarpus* (long-clawed hermit crabs) have been observed to obtain energy this way (Johannes and Satomi 1966). Because we observed *A. bahia* excreting MWCNTs in their fecal pellets, this method of exposure should be considered in future exploration of CNT toxicity in different food webs. Animals living in estuaries with high NOM content are expected to encounter higher bioavailable concentrations of MWCNTs through stabilized particles in the water column or fecal pellets within surficial sediments.

Our findings suggest that the 7-14 d developmental window is the most sensitive time of the *A. bahia* life cycle to MWCNT exposure, given that it had the highest MWCNT accumulation and reproductive toxicity occurred after exposure. We suggest that the EPA should continue to use this time window when assessing MWCNTs for future regulatory purposes. However, a full-life cycle study with *A. bahia* would be a better indicator of true reproductive toxicity of MWCNTs, compared to a sensitive time-window approach. Although *A. bahia* exposed to MWCNTs dispersed in SSW in the 7-14 d window had measurable accumulation in the lowest exposure concentration (0.1 mg/L), we realize that testing conditions which contain 0 mg C/L do not provide realistic predictions for MWCNT behavior in a natural estuary, which would typically have high levels of natural organic matter. Heightened environmental realism is extremely important in understanding the potential toxicity of MWCNTs to marine crustaceans; without the addition of NOM in our research, the environmental implications of our findings would have been very different. We suggest that future research of MWCNT behavior in estuarine environments should continue to include test conditions that mimic the natural environment.

Acknowledgements- The present study was supported by the Georgia Oceans and Human Health Initiative, U.S. Environmental Protection Agency Science to Achieve Results Program (grant# R827149), and the University of Georgia Interdisciplinary Toxicology Program. The authors thank Mike Fulton for insightful discussions, Marie DeLorenzo, Katy Chung, Eric Goolsby and David Brew for assisting, and Qingguo Huang for providing the labeled carbon nanotubes.

REFERENCES

Acosta CA and MA Poirrier. 1992. Grooming behavior and associated structures of the mysid *Mysidopsis bahia*. *J. Crustacean Bio.* 12(3):383-391.

Barclay MC, Dall W, Smith DM. 1983. Changes in lipid protein during starvation and moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. *J. Exp. Marine Bio. Ecol.* 68(3)-229-224.

Baun A, Hartmann NM, Grieger K, Kusk KO. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotox.* 17(9):387-395.

Blickley TM and P McClellan-Green. 2008. Toxicity of aqueous fullerene in adult and larval *Fundulus heteroclitus*. *Environ. Tox. Chem.* 27:1964-1971.

Calow P and RM Sibly. 1990. A physiological basis of population processes: ecotoxicological implications. *Funct. Ecol*. 4:283-288.

Capuzzo JM, Lancaster BA, Sasaki GC. 1984. The effects of petroleum hydrocarbons on lipid metabolism and energetics of larval development and metamorphosis in the American lobster (*Homarus americanus* Milne Edwards). 14(1-4):201-228.

Clutter RI. 1969. The microdistribution and social behavior of some pelagic mysid shrimp. *J. Experi. Mar. Biol. Ecol*. 3:125-155.

Conan GY. 1985. Periodicity and phasing in molting. Crustacean issues 3: factors in adult growth. AM Wenner, ed. Rotterdam, Netherlands: AA Balkema. 73-99.

Cuzin-Roudy J and C Tchernigovtzeff. 1985. Chronology of the female molt cycle in *Siriella armata* M. Edw. (Crustacea: Mysidacea) based on marsupial development. *J. Crustacean Biol.* 5(1):1-14.

Dirilgen N and N Ince. 1995. Inhibition effect of the anionic surfactant SDS on duckweed, *Lemna minor* with considerations of growth and accumulation. *Chemosphere*. 31:4185-4196.

Edgington AJ, Roberts AP, Taylor AM, Alloy MM, Reppert J, Rao AM, Mao J, Klaine SJ. 2010. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ. Tox. Chem.* 29:2511-2518.

Fingerman SW. 1985. Non-metal environmental pollutants and growth. *Crustacean issues 3: factors in adult growth.* AM Wenner, ed. Rotterdam, Netherlands: AA Balkema. 219-250.

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

Global Industry Analysts. 2007. Carbon nanotubes-a global strategic business report. Research report #GIA-MCP1507. 360 pp.

Gottschalk F, Sonder T, Scholz Rw, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. *Environ. Sci. Technol*. 43: 9216-9222.

Hartnoll RG. 1985. Growth, sexual maturity, reproductive output. *Crustacean issues 3: factors in adult growth.* AM Wenner, ed. Rotterdam, Netherlands: AA Balkema. 101-128.

Hyung H, Fortner JD, Hughes JB, Kin JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41(1): 179-184.

Johannes RE and M Satomi. 1966. Composition and nutritive value of fecal pellets of a marine crustacean. *Limn. Ocean*. 11(2): 191-197.

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ. Tox. Chem.* 27(9);1932-1941.

Lin D, Liu N, Yang K, Xing B, Wu F. 2010. Different stabilities of multiwalled carbon nanotubes in fresh surface water samples. *Environ. Pollution*. 158:1270-1274.

Lovrich GA and P Ouellet. 1994. Patterns of growth and triacylglycerol content in snow crab *Chionoecetes opilio* (Brachura: Majidae) zoeal stages reared in the laboratory. *Mar Biol*. 120:585-591.

Mariani L, De Pascale D, Faraponova O, Tornambe A, Sarni A, Giuliani S, Ruggiero G, Onorati F, Magaletti E. 2006. The use of a test battery in marine ecotoxicology: the acute toxicity of sodium dodecyl sulfate. *Environ. Tox*. 21(4):373-379.

Mauchline J. 1975. The broods of British Mysidacea (Crustacea). *J. Mar.Biol. Assoc. UK.* 53:801–817.

Mauchline J. 1980. The biology of mysids. *Advances in Marine Biology* 18:1-369.

Mauchline J. 1985. Growth in mysids and euphausids. *Crustacean issues 3: factors in adult growth.* AM Wenner, ed. Rotterdam, Netherlands: AA Balkema. 337-354.

Mees J, Abdulkerim Z, Hamerlynck O. 1994. Life history, growth, and production of *Neomysis integer* in Westerschelde estuary (S.W. Netherlands). *Mar. Ecol. Progress Series* 109:43-57.

Mueller NC and B Nowack. 2008. Exposure modeling of engineered nanoparticles in the environment. *Environ. Sci. Technol.* 42: 4447-4453.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nano level. *Science.* 311 (5761): 622-627.

O'Brien J and van Wyk P. 1985. Effects of crustacean parasitic castrators (Epicaridean isopods and Rhizocephalan barnacle) on growth of crustacean hosts. *Crustacean issues 3: factors in adult growth*. AM Wenner, ed. Rotterdam, Netherlands: AA Balkema. 191-218.

Ouellet P, Taggart CT, Frank KT. 1992. Lipid condition and survival in shrimp (*Pandalus borealis*) larvae. *Can. J. Fish. Aquat. Sci*. 49:368-378.

Parks AN, Portis LM, Schierz PA, Washburn KM, Perron MM, Burgess RM, Ho KT, Chandler GT, Ferguson PL. 2013. Bioaccumulation and toxicity of single-walled carbon nanotubes to benthic organisms at the base of the marine food chain. *Environ. Tox. Chem.* 32(6):1270-1277.

Petersen EJ, Huang QJ, Weber WJ Jr. 2008. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus. Environ. Health Perspect.* 116(4):496-500.

Petersen EJ, Akkanen, Kukkonen JVK, Weber WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol.* 43(8):2969-2975.

Price WW, Heard RW, Struck L. 1994. Observations on the genus *Mysidopsis* Sars, 1864 with the designation of a new genus *Americamysis*, and the description of *Americamysis alleni* and *A. stucki* (Percarida: Mysidacea Mysidae), from the Gulf of Mexico. *Proceedings of the Biological Society of Washington* 107:680-698.

Riera P, Montagna PA, Kalke RD, Richard P. 2000. Utilization of estuarine organic matter during growth and migration by juvenile brown shrimp *Penaeus aztecus* in a South Texas estuary. *Mar. Ecol. Prog. Ser*. 199:205-216.

Roast SD, Thompson RS, Widdows JJ, Jones MB. 1998. Mysids and environmental monitoring: a case for their use in estuaries. *Marine and Freshwater Research* 49:827-832.

Roberts AP, Mount AS, Seda B, Souther J, Qiao R, Lin S, Ke PC, Rao AM, Klaine SJ. 2007. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol*. 41(8):3025-3029.

Salonen K and T Hammar. 1986. On the importance of dissolved organic matter in the nutrition of zooplankton in some lake waters. *Oecologia*. 68(2): 246-253.

Sasaki GC, Capuzzo JM, Biesior P. 1986. Nutritional and bioenergetic considerations in the development of the American lobster Homarus americanus. *Can. J. Fish Aquat. Sci*. 43:2311-2319.

Templeton RC, Ferguson PL, Washburn KM, Scrivens WA, Chandler GT. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ. Sci. Technol*. 40:7387-7393.

USEPA. 1993. Short-term methods for estimating the chronic toxicity of effluents and surface waters to marine and estuarine organisms. 2nd edition. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. EPA-600/4-91/003.

Vernberg WB, McKellar H Jr, Vernberg FJ. 1978. Toxicity studies and environmental impact assessment. *Environ. Manag*. 2:239-243.

Volder MFL, Tawfick SH, Baughman RH, Hart AJ. 2013. Carbon nanotubes: present and future commercial applications. *Science*. 339:535-539.

Wang X, Tao S, X Baoshan X. 2009. Sorption and competition of aromatic compounds and humic acid on multiwalled carbon nanotubes. *Environ. Sci. Technol*. 43(16):6214-6219.

Whyte JNC, Englar Jr, Carswell BL, Medic KE. 1986. Influence of starvation and subsequent feeding on body composition and energy reserves in the prawn *Pandalus platyceros. Can. J. Fish Aquat. Sci*. 43:1142-1148.

FIGURE LEGENDS

Figure 3.1. Average percentage of mature adults post-exposure (7-14 d window) ±SE for both natural (NSW) and synthetic seawater treatments (SSW). *Americamysis bahia* exposed to 5 mg/L MWCNTs in NSW had a maximum decrease of 59.6% in the percentage of mature adults compared to percentage of mature controls ($F_{4,25}$=2.886, p=0.043). *A. bahia* exposed to MWCNTs in SSW had a maximum increase of 23.8% in the percentage of mature adults at 10 mg/L MWCNTs compared to the controls ($F_{4,25}$=3.014, p= 0.03). (*)denotes statistical significance from control, $p<0.05$.

Figure 3.2. Visual comparison of multi-walled carbon nanotube (MWCNT) accumulation in *Americamysis bahia* exposed to 10 mg/L MWCNTs dispersed in synthetic seawater. Female controls at 14 d (A) and 21 d (B) did not have any black accumulation on their bodies. Exposed 14 d *A. bahia* (C) and 21 d *A. bahia* (D) accumulated MWCNTs in the gut tract and on the exoskeleton (black arrows). The 21 d control female (B) has eggs in the marsupial pouch (white arrow), while the exposed 21 d *A. bahia* (D) appears to have MWCNTs in the marsupial pouch (white arrow) (40x magnification). Similar observations were made when *A. bahia* were exposed to MWCNTs in natural seawater, but photographs were not taken.

Figure 3.3. Comparison of average multi-walled carbon nanotube (MWCNT) accumulation (±SE) per *Americamysis bahia* during developmental window of 7-14 d (A) and 14-21 d (B) in natural seawater and synthetic seawater. In exposures a linear concentration response with relationship for MWCNTs accumulation was observed in

both natural seawater ($p=0.012$, $r^2=0.905$, $y=8.8146x-13.227$) and synthetic ($p=0.004$, $r^2=0.954$, $y=4.6053x-6.3272$). However, *A. bahia* exposed to MWCNTs in synthetic seawater had significant accumulation at a lower exposure concentration (0.1 mg/L, $p=0.0276$). In 14-21 d exposures, a linear concentration response with relationship for MWCNTs accumulation was observed in natural seawater ($p=0.008$, $r^2=0.820$, $y=7.3749x-12.391$). (*) denotes statistical significance from control, $p<0.05$.

Figure 3.4 *Americamysis bahia* exposed to 10 mg/L in synthetic saltwater shown excreting multi-walled carbon nanotubes in fecal pellets (black arrow). Similar observations were made with *A. bahia* exposed to multi-walled carbon nanotubes in natural saltwater, but photographs were not taken.

Figure 3.1.

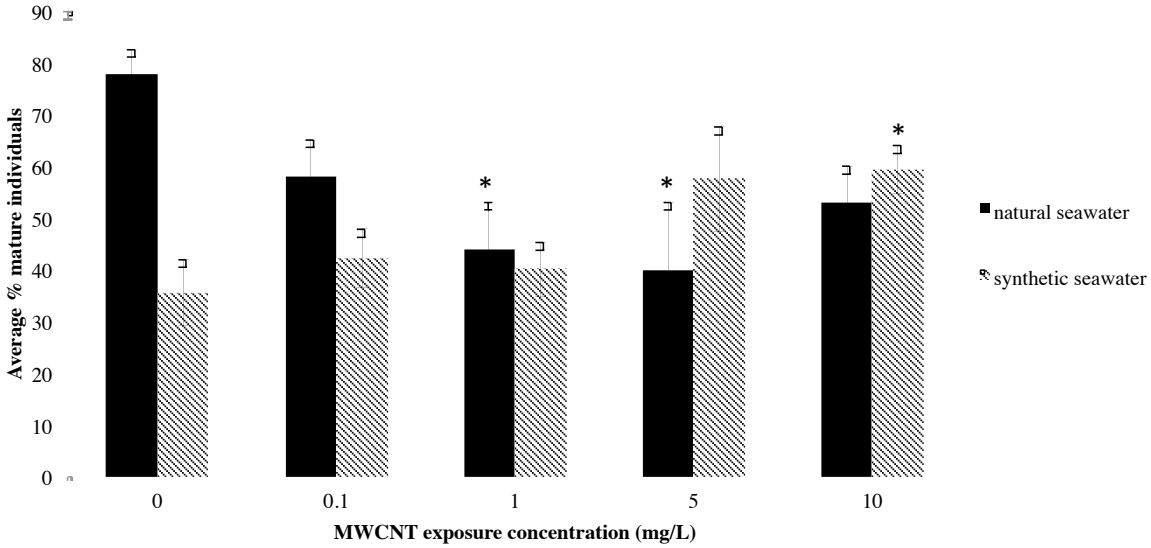

Average % mature individuals
90
80
70
60
50
40
30
20
10
0
0
0.1
1
5
10
MWCNT exposure concentration (mg/L)
natural seawater
synthetic seawater
*
*
*

Figure 3.2.

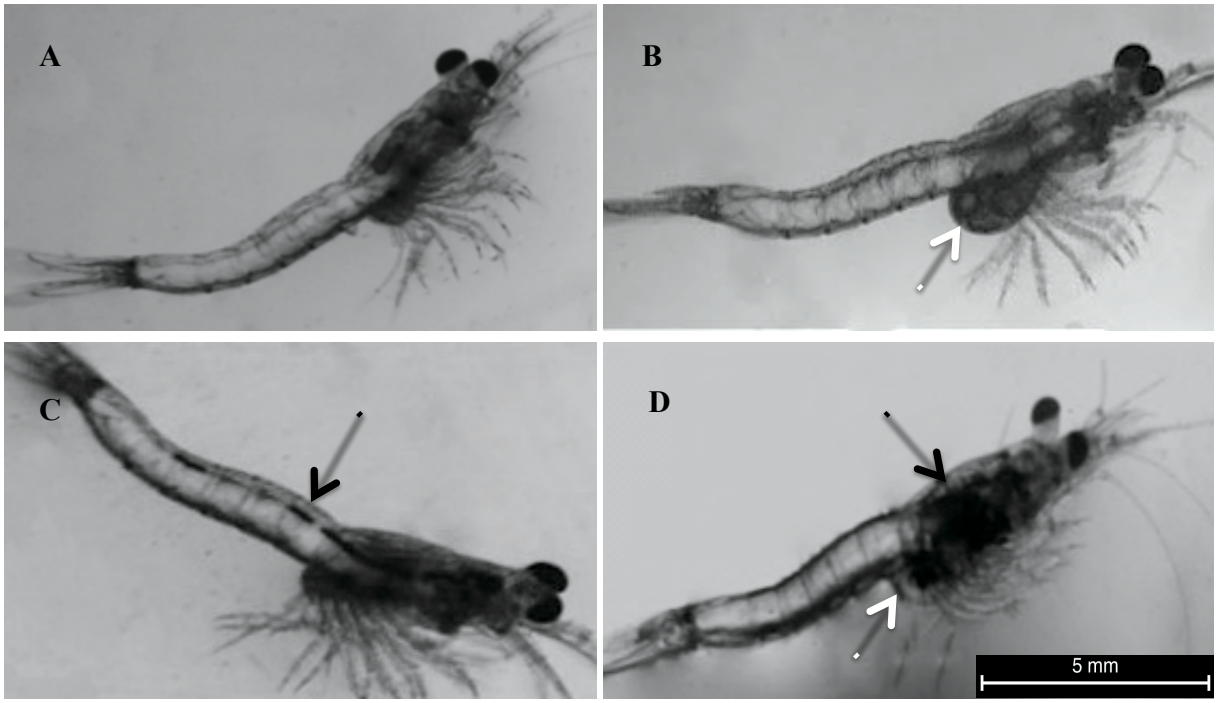


Figure 3.3.

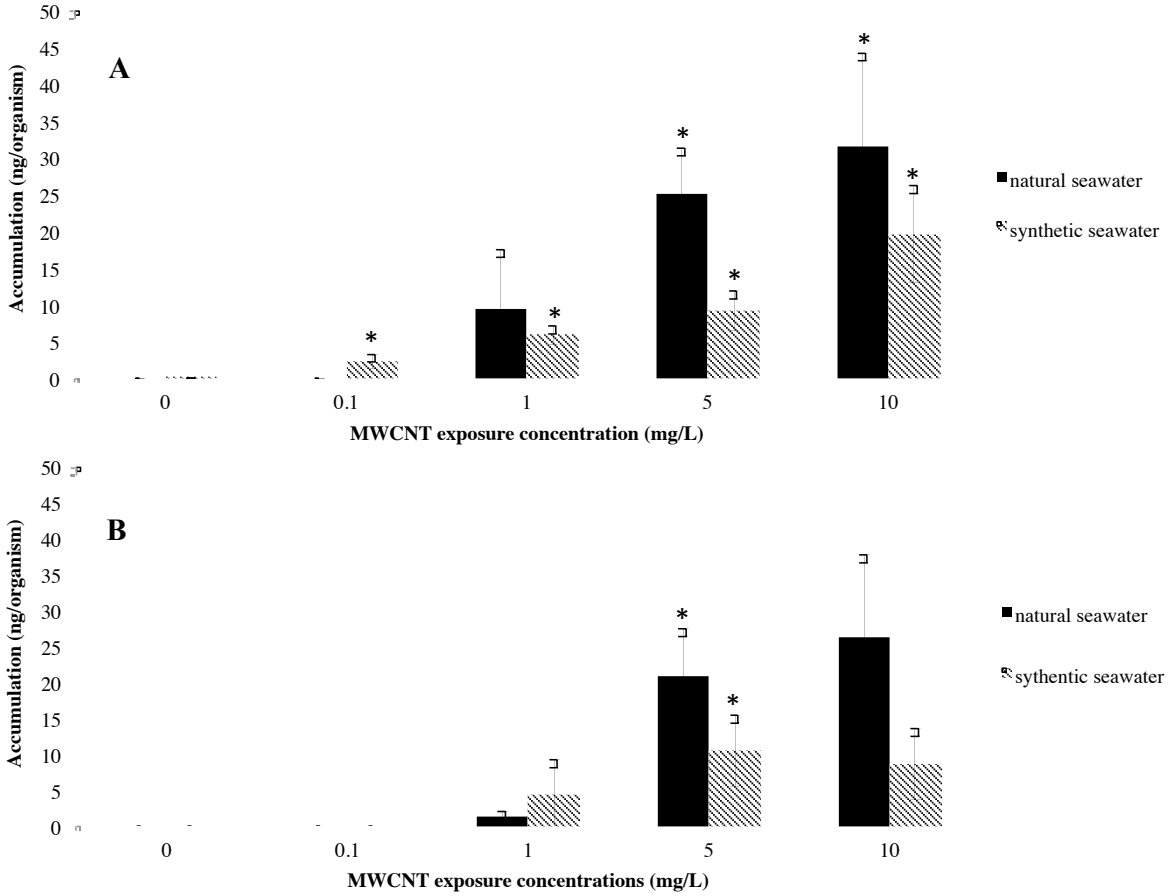
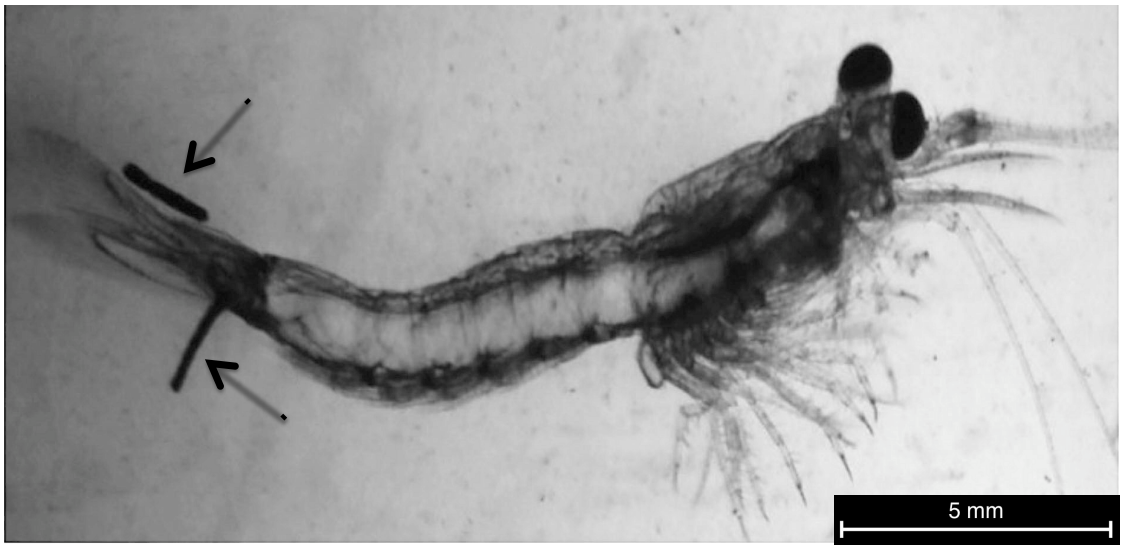

A
Accumulation (ng/organism)
50
45
40
35
30
25
20
15
10
5
0
0
0.1
1
5
10
MWCNT exposure concentration (mg/L)
natural seawater
synthetic seawater
B
Accumulation (ng/organism)
MWCNT exposure concentrations (mg/L)
natural seawater
sythentic seawater

Figure 3.4.



CHAPTER 4

EXPOSURE TO CARBON NANOTUBE CONTAMINATED-SEDIMENT INCREASES MORBIDITY IN JUVENILE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) [2]

[2] Emily R. McReynolds and Marsha C. Black. In preparation to be submitted to *Environmental Toxicology and Chemistry*.

ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) are produced on a large scale with the potential to contact the aquatic environment through product use or accidental releases. Sediment is predicted to be the ultimate sink for MWCNTs in aquatic environments, as MWCNTs are extremely hydrophobic. The objectives of this study were to determine the fate and toxic effects of MWCNTs in a realistic aquatic exposure. Juvenile fathead minnows (*Pimephales promelas*, 14-d old) were exposed to ^{14}C-labeled MWCNTs (0, 0.5, 5, and 50 mg/kg) added to aged sediment collected from a United States Department of Agriculture reference site. Exposure vessels contained 500 g of sediment and 2 L of moderately hard water. Fish in half of the replicates for each concentration were excluded from the sediment to determine the bioavailability of MWCNTs in the water column without sediment disturbance by fish. Toxicity endpoints were growth, morbidity (swim bladder deflation, gill infections, and GI tract abnormalities), and MWCNT accumulation. Water samples collected at 0, 1, 5, and 10 days confirmed the presence of MWCNTs in the water, with highest MWCNT concentrations detected when fish had access to sediments (5, 50 mg/kg; $p<0.0001$), suggesting that fathead minnow behavior increased MWCNT transport from sediments to the water column. Morbidity was observed in fish at all exposure concentrations, but was most frequently detected at the 50 mg/kg exposures, including anal protrusion of the GI tract (6.5% incidence) and filamentous, pathogenic growths on gills (3% incidence). Following a 24 h depuration, fish had no statistically significant accumulation of MWCNTs at any exposure concentration ($p=0.515$). We suggest that *P. promelas* are able to excrete the MWCNTs once placed in clean water, but with the physiological cost of gut protrusions from

exposure. Future research should measure biological responses during longer chronic sediment exposures to evaluate the effect of MWCNTs on the health status of fish. Traditional toxicity testing protocols should be adjusted to include endpoints relevant to the unique behavior of MWCNTs.

Keywords: *nanomaterials, bioturbation, accumulation, depuration, natural organic matter*

INTRODUCTION

Carbon nanotubes (CNTs) have a high surface-to-mass ratio and high electron mobility, can conduct electricity, and have extremely high tensile strength (Mwangi et al. 2012). These unique properties make CNTs extremely popular for potential use in the biotechnology field. There are two categories of CNTs: single-walled and multi-walled. Single-walled (SWCNTs) are single layers of graphene rolled into a tube while multi-walled (MWCNTs) are multiple layers of graphene. In 2007, commercial sales of CNTs and CNT-containing products were over $200 million with world production capacity of MWCNTs estimated to be 300 tons/year and 7 tons/year for SWCNTs (Thayer 2007), with the amount of MWCNTs predicted to rise in the future (Musee 2011). In 2009, CNano Technology announced it had scaled its manufacturing technology to boost production to 500 tons/year for MWCNTs (www.cnanotechnology.com). Because CNTs are relatively new to science, researchers still do not have a full understanding of their potential applications or toxicity. The U.S. Environmental Protection Agency has listed

nanoparticles as “Contaminants of Emerging Concern,” requiring further research prior to future federal regulations.

Carbon nanoparticles can enter the aquatic environment through degradation of products, waste streams from factories, or accidental spills during transportation (Nowack and Bucheli 2007, Musee 2011). Models of carbon nanotube deposition in U.S. sediment predict an average increase of 46 ng/kg each year (Gottschalk et al. 2009). Sediment is considered to be the ultimate sink for CNTs as they are extremely hydrophobic and are known to interact with organic content (Hyung et al. 2007, Klaine et al. 2008, Hyung and Kim 2008). Consequently, sediment dwelling organisms or organisms that interact with sediment are targets for CNT exposure and toxicity. Literature has conflicting reports on the extent of toxicity to benthic organisms exposed to SWCNTs and MWCNTs in sediment. A sediment dwelling marine organism, *Arenicola marina* (lugworm) exposed to 0.03 g/kg in sediment for 10 days did not accumulate a significant amount of SWNCTs and no measurable effects on DNA or cellular damage were observed (Galloway et al. 2010. In a study by Parks et al. (2013), *Americamysis bahia, Ampelisca abdita*, and *Leptocheirus plumulosis* were exposed to 100 g/kg of SWCNT in marine sediment for 28 d. There was no observed toxicity to any organisms and all animals were able to excrete the SWCNTs without the material crossing the gut lumen (Parks et al. 2013). In contrast, *Hyallella azteca* exposed for 14 d to 1 g/L of SWCNTs and MWCNTs in separate exposures had decreased survival and biomass in response to both types of CNTs (Mwangi et al. 2012). Interestingly, unlike the marine crustaceans in the previously mentioned study, *H. azteca* were unable to excrete either SWCNTs or MWCNTs after depuration for 24 h (Mwangi et al. 2012). With little information available, it is unclear if

these observed toxic effects are environmentally realistic. As scientists predict that MWCNTs will be present in higher concentrations in the environment than other carbon nanoparticles, future studies should focus on MWCNT toxicity on animals that have constant contact with the sediment.

Literature on water exposures of carbon nanoparticles to higher-order animals such as aquatic vertebrates is sparse and a lack of standardized testing makes comparisons extremely difficult among studies of fullerenes, SWNCTs, and MWCNTs. Data collected on different fish species reveal that not only the type of carbon nanoparticle but also species sensitivities affect the degree of toxicity observed. For example, decreases in weight and length were observed in *Carassius auratus* (goldfish) after exposure to 1.0 mg/L fullerenes for 32 d (Zhu et al. 2008). Levels of lipid peroxidase were elevated in liver of exposed *C. auratus*, while glutathione was decreased in all tissues, suggesting toxicity was mediated by oxidative stress. Increased activities of glutathione in gills and liver of *Oncorhynchus mykiss* (rainbow trout) were measured after a 10-d exposure to 0.5 mg/L SWCNTs (Smith et al. 2007). Abnormal division of liver cells was observed in exposed *O. mykiss* and SWCNTs were found in the gut lumen. *Danio rerio* embryos exposed to 60 mg/L MWCNTs for 72 h developed a slimy mucus coating on the outer membrane and exhibited decreased hatch success (Asharani et al. 2008). Another study observed that exposure to MWCNTs only caused a hatching delay in *D. rerio* embryos after exposure to 240 mg/L for 72 h and the chorion appeared to be an effective protective barrier (Cheng et al 2007).

Incorporation of CNTs into food or sediment increases the environmental realism of toxicity studies with carbon nanoparticles and these types of studies are becoming

more available in the literature. *Chironomus riparius* exposed to 0.36 to 0.55 mg/cm^2 fullerenes in aquatic sediment had significantly decreased growth (Waissi-Leinonen et al. 2012). However, juvenile *O. mykiss* fed SWCNT-contaminated food (500 mg/kg) for 6 weeks experienced no significant effects on growth, hematology, tissue ion concentrations, histopathology, osmoregulation, or biochemistry, suggesting that food-contamination is not a concern (Fraser et al. 2011).

Because production of MWCNTs is expected to be higher than other carbon nanoparticles, focusing on the toxicity of MWCNTs to address data gaps is vital. Increasing the environmental realism of studies and moving beyond traditional toxicity testing approaches is extremely important as well. The overall goal of this study was to investigate the subchronic toxicity of MWCNTs to an important small freshwater fish model, *Pimephales promelas,* by measuring changes in growth, while monitoring the incidence of morbidity and bioaccumulation of ^{14}C-labeled MWCNTs. We also wanted to determine if access of *P. promelas* to sediment has an effect on MWCNT toxicity or accumulation through bioturbation. The natural aquatic sediment is extremely important for *P. promelas* communities; detritus helps sustain high densities of minnows and contributes to a large flux of nutrients and energy from wetland sediments into the water column (Herwig and Zimmer 2007). By designing an experiment involving sediment contamination of MWCNTs as well as using a test species (*Pimephales promelas*) whose behavior inherently links it with the sediment as a food source, this experiment mimics a sensitive exposure route.

MATERIALS AND METHODS

Organism

Juvenile (13 d old) *Pimephales promelas* were obtained from Aquatic Biosystems (Fort Collins, CO) and acclimated for 24 h in synthetic moderately hard water (US EPA 2002). Less than 10% mortality was observed during this holding time. Fathead minnows were chosen for this experiment because they are standard United States Environmental Protection Agency (EPA) toxicity test organisms and are important in many freshwater food webs across North America. *P. promelas* has also become one of the most valuable baitfish in North America (Davis 1993, Etnier and Starnes 1993). Although fathead minnows are not sediment dwelling organisms, they are known to interact with and consume superficial sediments (McCarthy et al. 2003, Wall et al. 2009)

Sediment

Sediment was collected from a freshwater pond at the United States Department of Agriculture (USDA) research station near Watkinsville, GA. Jones et al. (2007) characterized the sediment from this site as actively methanogenic with a pH of 6.7, 0.3% (w/w) total nitrogen, and 4.0 % (w/w) total organic carbon. Sediment was stored in a sealed 5-gallon plastic bucket at room temperature. Total organic carbon (TOC) release from sediments to overlying water was measured prior to experimentation in treatments (n=3) mimicking experimental conditions (see below) that contained fathead minnows exposed to 1 part sediment: 4 parts moderately hard water, without added ^{14}C-MWCNTs. After 10 days of exposure, water samples (n=9) were collected and TOC content was analyzed on a Shimadzu 5050 TOC Analyzer, which is based on a combustion and non-dispersive infrared gas analysis method. The TOC content of the overlying water was

21.6±1.6 mg C/L, indicating significant release from sediments to the water column. Having measured dissolved organic carbon in the water column increases the of MWCNTs in the water column (Hyung and Kim 2007, Hyung et al. 2008).

MWCNTs

Multi-walled carbon nanotubes were chosen for use in this study over SWCNTs because of their availability, lower cost, higher projected rate of production, and greater current and projected use. One of the limitations of researching the behavior of CNTs is the difficulty in quantifying them at low concentrations in environmental media or organism tissues (Petersen et al. 2008). Enhanced detection of MWCNTs in water and biological samples can be obtained using radiolabeled MWCNTs. This unique process allows for quantification of modified or unmodified MWCNTs or aggregates of MWCNTs in digested tissues and experimental media. Test materials were created by a vapor deposition of methane on a Ni-MgO catalyst as described by Chen et al. (1997), modified to incorporate a ^{14}C isotope into a dry form of MWCNTs (Petersen et al. 2008). The purified ^{14}C-MWCNTs were sonicated in a strong acid (3:1 ratio of concentrated sulfuric and nitric acid), which made them more hydrophilic (Petersen et al. 2008). The purity of MWCNTS used in this experiment was 99%±0.2 (Petersen et al. 2009). Further characterizations of the MWCNTs used in this experiment are described in Petersen et al. (2008, 2009). Water samples containing ^{14}C-MWCNTs were mixed with scintillation cocktail (ScintiVerse BD, Fisher Scientific) and beta emissions were detected and quantified by a liquid scintillation counter (LSC, Beckman LS 6500). Concentrations of

MWCNTs in water and digested tissue samples were calculated from the specific radioactivity of the radiolabeled test material (0.12 μCi/mg).

MWCNT Solution Preparation

The dry, powdered form of the MWCNTs was weighed and added to synthetic moderately hard water (MHW) to make a stock solution of 50 mg/L. MHW used in the experiments had a pH of 8.3±0.03, alkalinity of 66.3±1.19, and hardness of 98.3±1.83. The hydrophobic MWCNTs were suspended in water through sonication for 2 h in a low-powered, water bath sonicator (Branson model 2510) immediately prior to use in an experiment. The 2-h sonication period was determined to maintain approximately 75% of the initial MWCNTs concentration suspended in solution over a 24 h period (Chapter 1). Dissolved organic carbon released from the sediments through interaction with fathead minnows was assumed to stabilize the MWCNTs for a longer period of time (Hyung et al. 2007, Hyung and Kim 2008).

Sediment Bioassays

Toxicity and accumulation of MWCNTs by *P. promelas* was measured in a 10-d sub-chronic exposure. Exposures were conducted in 3.79 L containers, each containing 1 part sediment and 4 parts overlying water. Following sonication, three concentrations of ^{14}C-MWCNTs prepared from the 50 mg/L stock solution were added to 500 g sediment to provide final MWCNT concentrations of 0.5, 5, 10, and 50 mg/kg. Overlying water (2 L MHW) was carefully added to glass containers with MWCNT-amended sediment. There were eight replicates per concentration, with 25 14-d old *P. promelas* added to

each replicate. Within each concentration treatment, 4 replicates were fitted with exclusion netting (1/8" Ace nylon netting, Aquatic Eco-Systems) placed just above the sediments to prevent contact of *P. promelas* with sediments and 4 replicates remained without netting, providing fish with full access to the sediments. For the duration of the toxicity test, containers were gently aerated to maintain sufficient oxygen concentrations (>7.5 mg/L) in the water column. Fish were fed *Artemia* spp. nauplii *ad libitum* daily. Tests were conducted in an incubator with a16:8 h light:dark photoperiod and a constant temperature of 25.0±1.0°C. Ammonia and pH were monitored daily in overlying water. Ammonia was never measured above 0.25 mg/L for any concentration on any day throughout the experiment and pH was measured at a range of 7.7 to 8.3 for all concentrations; therefore, daily water changes were not necessary. On days 0, 1, 2, 5 and 10, one mL of water was collected from each exposure container and samples were composited by treatment and exposure concentration to measure MWCNT concentrations in the water column. On days 1, 2, 5 and 10 post-exposure five fish from each replicate were removed and placed in 500 mL of clean MHW for a 24 h depuration period; no food or sediment was available to fish at this time. After the depuration period for each time point, fish were euthanized with a lethal dose of buffered (pH 7) MS-222 and were immediately analyzed microscopically for total length and morbidity prior to digestion and measuring accumulation of ^{14}C-MWCNTs.

Morbidity

Euthanized *P. promelas* were observed with a compound microscope and a blind examination of treatments without knowing exposure concentrations was conducted.

Specific endpoints examined were posterior or anterior swim bladder deflation, pathogenic growth on gills, and gut protrusions. An iPhone 4 (Apple, Inc., Cupertino, CA) fitted with a ScopeMonkey attachment (Microfacturing, Inc., Atlanta, GA) was used to take photographs through an Olympus SZX9 research stereomicroscope. Fish were then pooled by replicate, weighed, and dried for 24 h at 100°C.

Accumulation

Dried organisms were pooled by concentration and treatment (with or without exclusion netting) then transferred to 7-mL plastic scintillation vials and 400 µL of 2 N NaOH was added to each vial to digest the organisms. Quench tests have shown that the volume of NaOH used to digest the organisms does not mask the ^{14}C readings (data not shown). After digestion, 5 mL of ScintiVerse BD scintillation cocktail fluid (Fisher Scientific) was added to each vial. The vials were placed in the liquid scintillation counter and counted for 5 min per sample. A blank sample containing only 400 µL of 2 N NaOH added to 5 mL control MHW was counted for 10 min and subtracted from all sample vials.

Data analyses

All data met assumptions for normality (Shapiro-Wilks test); no transformations were needed. Data analyses for growth were conducted using an analysis of variance (ANOVA; $\alpha = 0.05$). Differences in MWCNT concentrations in the water column among treatments were determined using an ANOVA followed by a Tukey's post-hoc test ($\alpha = 0.05$). All statistical tests were performed with SAS v. 9.3 (SAS Institute Inc., Cary, NC).

Bioaccumulation of MWCNTs was determined from the amount of radioactivity measured in digested *P. promelas,* pooled by replicate. The average counts per minute (CPM) per organism was corrected for the blank CPM and divided by the number of *P. promelas* in the vial (n=5). Bioaccumulation of MWCNTs (ng per organism) was calculated from the specific activity of the MWCNTs (0.12 μCi/mg).

RESULTS

MWCNTs in the water column

Statistically significant MWCNT concentrations were only measured in the water column of exposure vessels in which *P. promelas* had access to the sediment (e.g., no net exclusion) (Fig. 4.1). Water sampled from exposure vessels containing P. *promelas* exposed to 5 mg/kg (without net exclusion) had a significant amount of MWCNTs by 10 d (0.021±0.008 mg/L, $F_{39,80}$=28.66, p<0.0001). Water samples collected from exposure vessels containing fish exposed to 50 mg/kg (without net exclusion) had a significant concentration of MWCNTs measured on day 5 (0.056±0.010 mg/L) and day 10 (0.062±0.001 mg/L, $F_{39,80}$=28.66, p<0.0001).

Growth and Morbidity

After 10 days of exposure, growth in MWCNT-exposed fish was not significantly different compared to control fish (length, $F_{7,24}$=1.39, p=0.225; wet weight, $F_{7,24}$=2.03, p=0.092). Morbidity (endpoints characterized as deflated or missing swim bladder, pathogenic gill growth, or gut protrusion) was observed at all MWCNT exposure concentrations and ranged from 1 to 11% incidence (LOEC = 0.5 mg/kg, Fig. 4.4).

Interestingly, sediment exclusion was not a factor in the incidence of morbidities ($F_{7,24}$=1.74p=0.146). *P. promelas* exposed to 50 mg/kg MWCNTs (net inclusion and no net combined) had a 6.5% incidence of protruded gut tract over the 10-day exposure period, 3% incidence of fungal growth on gills, and 1% incidence of deflated anterior swim bladder (Figs. 4.4 and 4.5). No morbidity was observed in control fish.

Accumulation

After a 24-h depuration period in clear water and no food addition, fish were able to depurate MWCNTs to control levels, with no statistical differences in MWCNT concentration measured between controls and exposed groups at any time period ($F_{31,96}$=1.19, p=0.26). Even at the highest exposure concentration (50 mg/kg) depurated *P. promelas* had reduced body burdens of MWCNTs ranging from 0±0 to 0.17 ng/organism (Fig 4.2 A). While *P. promelas* without access to the sediment appeared to have a lower body burden when exposed to 50 mg/kg (a range of 0±0 to 0.03 ng/organism), there was no difference in depurated body burdens regardless of treatment (exclusion netting vs. sediment access) ($F_{31,96}$=31.96, p=0.5159). Fecal matter and water collected after 24 h depuration contained measurable amounts of ^{14}C –MWCNTs (Fig 4.3 B); however, there were no significant differences between treatments or concentrations compared to controls because of wide variability among collected fecal samples ($F_{31,96}$=1.19, p=0.26). To give an idea of the variability of the concentration MWCNTs within the same treatment, there was a range of 0.44±0.51 to 97.46±56.78 ng/L measured in depuration water from fish exposed to 50 mg/kg without nets sampled at day 10.

DISCUSSION

Access of *P. promelas* to sediment influenced the amount of MWCNTs in the water column. Concentrations of MWCNTs in the water column increased from control levels (0±0) to 0.062±0.001 ng/L when *P. promelas* had access to sediment with 50 mg MWCNT/kg (day 10), indicating that bioturbation of MWCNT-contaminated sediment by *P. promelas* had a profound effect on movement of MWCNTs into the water column. Fathead minnows are known to interact with aquatic sediments and juvenile fish are less selective about food sources, consuming higher amounts of detritus than adults (Herwig and Zimmer 2007). Because of these behaviors juvenile fish may be more at risk for toxicity from exposure to MWCNT-contaminated sediments if they consume more sediment than adults. Importantly, larger sediment particles that interact with MWCNTs are more likely to be seen as a food source to the *P. promelas* than smaller particles. Through natural foraging behavior, *P. promelas* may resuspend MWCNT particles from the sediment to the water column, where they remain stable, possibly interacting with other organisms in a natural setting. Filter feeders like bivalves may also consume these large MWCNT-sediment particles as food sources. Literature has suggested that bivalves are targets for CNT toxicity through sediment contamination (Klaine et al. 2008, Ringwood et al. 2009).

We suggest current EPA testing protocols for *P. promelas* may not be suitable for regulatory testing of carbon nanomaterials in sediment exposures. For example, growth (measured as a change in fish length and wet weight), is a typical indicator of toxicity, but was not affected by MWCNT exposure in our experiment. However, longer exposure durations may reveal different results. Other literature has reported adverse effects caused

by CNT exposure not typically monitored by traditional toxicity testing. When a saltwater fish, *Fundulus heteroclitus*, was exposed to 10 mg/L SWCNTs for 96 h, there was no effect on traditional endpoints such as mortality of adults or juveniles and also no effect on hatch success (Blickley and McClellan-Green 2008). However, exposure of 1 mg/L SWCNTs to *F. heteroclitus* resulted in higher whole body glutathione levels (a non-traditional endpoint), which suggests oxidative stress (Blickley and McClellan-Green 2008). Interestingly, another study observed that after being injected with 2 ng of functionalized MWCNTs, *D. rerio* embryos developed normally into larvae with no difference in survival rates between treated and control fish. However, the second generation of treated fish had lower survival than control fish, which would not be observed in traditional toxicity methods conducted for short durations (Cheng et al. 2009). Focusing on non-traditional methods for toxicity testing may provide more information than traditional endpoints (such as growth or mortality) for understanding the toxicity of MWCNTs.

While MWCNT accumulation in fish bodies was not measured prior to depuration, following a 24 h depuration period very low concentrations of MWCNTs remained in fish bodies (less than x ng/5 fish) exposed to MWCNTS in sediments (Figure 4.2A), while depuration water representing the total amount of MWCNTs excreted by the five depurated fish contained between x and y ng/500 ml (Figure 4.2B). Depurated amounts of MWCNTs were highly variable and were not related to exposure concentrations or conditions (net exclusion or access to sediments). These results suggest that fish accumulated MWCNTs not only from ingestion of contaminated sediment particles, but also from exposure to overlying water, containing small amounts of

MWCNTs likely dispersed in the water column by colloidal organic carbon. Although a 24 h depuration period appeared sufficient for *P. promelas* to excrete most accumulated MWCNTs, excretion may have come at a cost of extended energy and gut damage. Many researchers have focused on the potential accumulation of MWCNTs; however, research suggests that some animals are able to excrete carbon nanoparticles, while others are unable to excrete CNTs unless they are fed during depuration. Cheng et al. (2009) observed that 2 ng MWCNTs injected into the circulatory system of *D. rerio* were cleared by the body within 96 h. Kennedy et al. (2008) hypothesized that the presence of food is critical in decreasing CNT toxicity in *D. magna* by increasing gut clearance rates. Waissi-Leinonen et al. (2012) observed fullerene aggregates in the gut of *C. riparius* larvae, but did not detect gut epithelial cell absorption. Interestingly, nanoparticle size is directly related to energy required by an organism for excretion; larger MWCNT particles require more energy to clear the gut tract (Li and Huang 2011). Depletion of energy reserves required by *P. promelas* to excrete large aggregates of MWCNTs associated with sediment particles could possibly explain the gut impaction leading to protrusion and possible damage to gut epithelium observed in exposed *P. promelas*.

While there were very low concentrations of MWCNTs remaining in fish bodies following depuration, sublethal effects are still a concern. We observed an increase in morbidity after exposure to 0.5-50 mg/kg MWCNTs in sediments. Although morbidity was observed at a low incidence (≤6.5% occurrence in organisms in any exposure concentration) and was not dependent on exposure concentration or condition, no incidence of morbidity was observed in any control fish. Thus we infer that sediment was not the cause of these adverse health effects observed in MWCNT-exposed *P.*

promelas. The most prevalent morbidity observed was the gastrointestinal tract abnormality (anal protrusion measured in 6.5% of fish exposed to 50 mg/kg MWCNTs) (Fig. 4.4d). We hypothesize that the presence of MWCNTs in the gut tract inhibited proper excretion of fecal material. The observed extension of the intestinal tract in exposed *P. promelas* was possibly a result of MWCNT exposure and compaction of ingested material. However, from our observations, it is unclear whether the intestinal obstruction occurred before or during the depuration period and was a direct consequence of MWCNT exposure or damage caused by movement of MWCNTs through the gut tract without food during the depuration period.

Other studies have implicated the gut as a site where CNTs accumulated and in some cases caused cellular damage. Ingested material appeared to be more densely compacted in the gut of *C. riparus* exposed to fullerenes. Shortened microvilli and areas without microvilli layer were observed in the gut epithelium of fullerene-exposed organisms, suggesting gut epithelial damage (Waissi-Leionen et al. 2012). Petersen et al. (2009) also observed that *D. magna* were unable to excrete MWCNTs during a depuration study without food and hypothesized that presence of MWCNTs may have limited nutrient uptake. MWCNTs and SWCNTs were observed to aggregate between and around microvilli in *Hyallela azteca, Lumbriculus variegatus*, and *Chironomus dilutus* (Mwangi et al. 2012). Aggregation of MWCNTs and damage to microvilli may also have occurred in the *P. promelas* intestinal system in the current study, but these were not endpoints measured in this study. Fish may be more prone to MWCNT or SWCNT toxicity because of their ability to uptake large particles through the gut by endocytosis. But to date no studies have shown evidence of CNTs crossing gut epithelial

cells (Petersen et al. 2008, Petersen et al. 2009, Mwangi et al. 2012), although these observations were made with animals exposed to CNTs for a short periods of time (acute or subchronic). With severe inflammation or damage to the gut or gill, many substances could diffuse directly into the blood through damaged tissue (Handy et al. 2008). Longer exposures such as life-cycle tests may reveal the true behavior of CNTs in fish gut tracts and may provide more information about their ability to cause intestinal damage or cross gut epithelial cells.

The second most prevalent morbidity observed in exposed *P. promelas* was a pathogenic gill infection (3% of fish exposed to 50 mg/kg MWCNTs). Gills are considered a target organ for nanoparticle toxicity (Handy et al. 2008), as only a thin layer of mucus and epithelial cells separate a fish's circulatory system from the aquatic environment (Bols et al. 2001). With 0% incidence of morbidity in the controls but morbidity observed in MWCNT-exposed fish, we suspect that a natural filamentous pathogen commonly found in aquatic sediment was able to colonize the gills in the presence of MWCNTs. Although not confirmed, we hypothesize *Flexibacter columnaris, Saprolegnia* spp., or a similar species is responsible for the gill infections seen in exposed *P. promelas* (personal communication with Dr. Al Camus, University of Georgia). Depending on the temperature, mortality occurs in fish after 1-7 days of exposure to *F. columnaris* (Wakabayashi 1991). The optimum temperature for a *F. columnaris* outbreak ranges between 20^{o} and 30^{o} C (Wakabayashi 1991) and our exposures were conducted at 25^{o} C. Therefore, a longer exposure duration may have revealed lethal effects over time. Another common pathogenic species, *Saprolegnia* spp., requires a surface abrasion on an organism in order to colonize (Singhal et al. 1987, Hatai

et al. 1990). The MWCNTs in the water column could have abraded the *P. promelas* gills through normal respiration, providing a medium for filamentous pathogen growth (like *F. columnaris* or *Saprolegnia* spp.) and perhaps a pathway for MWCNTs to enter the body more effectively. Literature has reported that the number of ectoparasites (attaching to the outside of fish gills) increases in natural fish populations after exposure to pollutants in effluents such as heavy metals and polychlorinated biphenyls (Poulin 1992, Mackenzie 1999, Bols et al. 2001). However, limitations of using ^{14}C-labeled materials did not allow confirmation of a specific pathogen or further histopathological investigations. Interestingly, although pathogenic growth on the gills was observed, no MWCNT aggregates were visible on the gills through microscopic analysis.

Although we did not measure immunological responses, other studies agree with our hypothesis that exposure to MWCNTs affects innate immune responses in fish. Aggregated SWCNTs were observed within sloughed mucus of rainbow trout after exposure to 0.5 mg/L SWCNTs (Smith et al. 2007). Excessive mucus production is the first line of fish immune defense against many environmental stressors (Bols et al. 2001, Chivers et al. 2007). We hypothesize that although mucus sloughing appears rapidly in fish exposed to SWCNTS (Smith et al. 2007), eventually the supply of mucus could be depleted (Handy et al. 2008). Other studies exposing fish and fish cell lines to nanoparticles have documented innate immune responses. For example, in *O. mykiss* cell cultures exposed to CNTs, SWCNTs were observed to have a stimulatory effect on macrophage cells at non-toxic concentrations (Klaper et al. 2010). Jovanović and Palić (2012) hypothesized that nanoparticles can cause physical damage to alarm cells in the mucus layer of fish gills. Other studies have observed that fish immunosuppression leads

to an increase in disease susceptibility (Poulin 1992). If exposure to carbon nanoparticles impairs protective actions of innate immune systems in fish, more severe consequences could be observed in longer studies. Experiments that also measure biomarkers of immune response in fish following chronic exposures to MWCNTs under natural conditions (such as sediment exposures) are needed to elucidate the potential role of immunity in fish morbidity.

We also observed a very small incidence (1%) of *P. promelas* exposed to 50 mg/kg MWCNTs with a deflated anterior swim bladder. Proper inflation of the swim bladder enables a fish to move horizontally and vertically within the water column. Fish without fully inflated swim bladders must continuously move in order to stay suspended and use additional energy to do so (Marty et al. 2005). For example, larval Japanese medaka *Orvzias latipes* had higher oxygen consumption rates when deflated swim bladders were present compared to fish that had normal swim bladder inflation (Marty et al. 2005). No incidence of deflated swim bladders was observed in control fish, suggesting a role of MWCNTs in this response. Affected *P. promelas* could have been unable to swim to the surface to properly fill swim bladders following hatching. Alternatively, MWCNTs could have aggregated in the pneumatic duct connecting the swim bladders to the gut tract and blocked proper air flow into the anterior swim bladder. No published reports of deflated swim bladders following exposure to carbon nanoparticles are available. However, Laban et al. (2010) found a concentration dependent effect of silver nanoparticles (AgNP) on swim bladder inflation in fathead minnow embryos exposed to 2.5-20 mg/L AgNPs. A complete absence of visible swim bladders was noted in fish exposed to concentrations greater than 15 mg/L AgNPs.

Therefore, we suggest that future nanoparticle research should investigate this endpoint in chronic exposures.

Interestingly, all increases in morbidity were observed in exposed *P. promelas* regardless of net exclusion. We suspect that low levels of MWCNTs are able to diffuse into the water column in the presence of dissolved organic matter released from sediment and sediment pore water but we were unable to separate morbidity attributed to contact with contaminated sediment versus solubilized MWCNTs. Concomitant exposures designed to separate these routes of exposure are needed to elucidate the form of MWCNTs causing adverse effects. Future MWCNT toxicity investigations should also include measurement of sensitive biomarkers to investigate modes of action of MWCNT toxicity, rather than the potential to accumulate the materials. Finally, chronic or partial lifecycle exposures may allow even more sublethal effects to emerge than were observed in the present study.

While *P. promelas* can excrete MWCNTs, a 6.5% incidence of gut protrusions suggests that the intestinal tract is damaged by exposure to or through excretion of MWCNTs. These observed incidences of morbidity are a concern because small forage fish like *P. promelas* are the base of many freshwater food webs and are extremely important in aquaculture. Monitoring fish populations provides insight into the overall health of the aquatic environments and may serve as a warning to potential human and ecological health effects. Our data suggest that exposure to MWCNTs has negative effects on the health of *P. promelas*. This is a concern because changes in health status can influence the health status of natural fish populations by increasing the risk of disease susceptibility in individuals (Bols et al. 2001). Reducing the number of healthy fish in a

population could cause disruption in many aquatic food webs. With many critically imperiled watersheds today, government entities should proactively investigate potentially toxic materials like MWCNTs and focus on low-level exposures over chronic durations with ecologically relevant endpoints.

Acknowledgements- The present study was supported by the U.S. Environmental Protection Agency Science to Achieve Results Program (grant# R827149) and the University of Georgia Interdisciplinary Toxicology Program. The authors thank Chi-yen Tseng and Jessica Joyner for assisting, Suzy Baird and Al Camus for insightful discussions, and Qingguo Huang for providing the ^{14}C-labeled carbon nanotubes.

REFERENCES

Asharani PV, Serina NGB, Nurmawati MH, Wu YL, Gong Z, Valiyaveettil S. 2008. Impact of multi-walled carbon nanotubes on aquatic species. *J. Nanosci. Nanotechnol*. 8(7):3603-3609.

Blickley TM and McClellan-Green. 2008. Toxicity of aqueous fullerene in adult and larval *Fundulus heteroclitus*. *Environ. Tox. Chem*. 27(9):1964-1971.

Bols NC, Brucbacher JL, Ganassin RC, Lee LEJ. 2001. Ecotoxicology and innate immunity in fish. *Devel. Compar. Immun.* 25: 853-873.

Chen P, Zhang HB, Lin GD, Hong Q, Tsai KR. 1997. Growth of carbon nanotubes by catalytic decomposition of CH_4 or CO on a Ni-MgO catalyst. *Carbon*. 35:149501501.

Cheng J Chan CM, Veca LM, Poon WL, Chan PK, Q L, Sun YP, Cheng SH. 2009. Acute and long-term effects after single loading of funtionalized multi-walled carbon nanotubes into zebrafish (*Danio rerio*). *Tox. Applied Pharm*. 235:216-225.

Cheng J, Flauhaut E, Cheng SH. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environ. Tox. Chem*. 26(4):708-716.

Chivers DP, Wisenden BD, Hindman CJ, Michalak TA, Kusch RC, Kaminskyj SGW, Jacj KL, Ferrari MCO, Pollock RJ, Halbgewachs CF, Pollock MS, Alemadi S, James CT, Savaloja RK, Goater CP, Corwin A, Mirza RS, Kiesecker JM, Brown GE, Adrian JC, Krone PH, Blaustein AR, Mathis A. 2007. Epidermal alarm substance cells of fishes maintained by non-alarm functions: possible defence against pathogens, parasites and UVB radiation. *Proc. R. Soc. B.: Biol. Sci.* 274:2611-2619.

Davis JT. 1993. Culture of nonsalmonid freshwater fish. *Baitfish*, R.R. Stickley, editor. CRC Press, Boca Raton, Florida. p. 307-321.

Divino JN and WM Tonn. 2008. Importance of nest and paternal characteristics for hatching success in fathead minnow. *Copeia*. 920-930.

Etnier DA and WC Starnes. 1994. The Fishes of Tennessee. 1st ed. *Univ. Tenn. Press*., Knoxville, TN.

Farré M, Gajda-Schrantz K, Kantiani L, Barceló D. 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. *Anal Bioanal Chem.* 393:81–95.

Fraser TWK, Reinardy HC, Shaw BJ, Henry TB, Handy RD. 2011. Dietary toxicity of single- walled carbon nanotubes and fullerenes (C_{60}) in rainbow trout (*Oncorhynchus mykiss*). *Nanotox.* 5(1):98-108.

Galloway T, Lewis C, Dolciotti I, Johnston BD, Moger J, Regoli F. 2010. Sub-lethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. *Environ. Pollution.* 158:1748-1755.

Gottschalk F, Sonderer T, Scholz RW, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. *Environ. Sci. Technol*. 43:9216-9222.

Hatai K, Wiloughby LG, Beakes GW. 1990. Some characteristics of *Saprolegnia* obtained from fish hatcheries in Japan. *Mycological Research.* 94(2):182-190.

Herwig BR and KD Zimmer. 2007. Population ecology and prey consumption by fathead minnows in prairie wetlands: importance of detritus and larval fish. *Ecol. Fresh. Fish.* 16(3):282-294.

Handy RD, Henry TB, Scown TM, Johnston BD, Tyler CR. 2008. Manufactured nanoparticles; their uptake and effects on fish- a mechanistic analysis. *Ecotox*. 17:396-409.

Hyung H, Fortner JD, Hughes JB, Kim JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41(1): 179-184.

Hyung H and JH Kim. 2008. Natural organic matter (NOM) adsorption to multi-walled carbon nanotubes: effect of NOM characteristics and water quality parameters. *Environ. Sci. Technol*. 42:4416-4421.

Jones WJ, Mazur CS, Kenneke JF, Garrison AW. 2007. Enantioselective microbial transformation of the phenylpyrazole insecticide fipronil in anoxic sediments. *Environ. Sci. Technol*. 41(24):8301-8307.

Jovanović B and D Palić. 2012. Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms with special emphasis on fish—Review of current knowledge, gap identification, and call for further research. *Aquat. Tox*. 118:141-151.

Kennedy AJ, Hull MS, Steevens JA, Donstova KM, Chappell MA, Gunter JC, Weiss CA. 2008. Factors influencing the partitioning and toxicity of nanotubes in the aquatic vironment. *Environ. Tox. Chem*. 27(9):1932-1941.

Klaper R, Arndt D, Setyowati K, Chen J, Goetz F. 2010. Functionalization impacts the effects of carbon nanotubes on the immune system of rainbow trout, *Oncorhynchus mykiss. Aquat. Tox.* 100:211-217.

Laban G, Nies LF, Turco RF, Bickham JW, Sepúlveda MS. 2010. The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. *Ecotoxi.* 19:185-195.

Li M and CP Huang. 2011. The responses of *Ceriodaphnia dubia* toward multi-walled carbon nanotubes: effect of physical-chemical treatment. *Carbon*. 49(5):1672-1679.

Klaine SJ, Alvarez PJ, Bately GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ. Tox. Chem*. 27:1825-1851.

Mackenzie K. 1999. Parasites as pollution indicators in marine ecosystems: a proposed early warning system. *Marine Poll. Bull*. 38(11):955-959.

Marty GD, Hinton DE, Cech JJ Jr. 1995. Notes: oxygen consumption by larval Japanese medaka with inflated or uninflated swim bladders. *Trans. Amer. Fish. Soc.* 124(4):623- 627.

McCarthy JF, Burns LW, Tolbert VR. 2003. Bioaccumulation of benzo(*a*)pyrene from sediment by fathead minnows: effects of organic content, resuspension and metabolism. *Arch. Environ. Contam. Toxicol.* 45:364-370.

Musee N. 2011. Nanowastes and the environment: potential new waste management paradigm. *Environ. Int*. 37:112-128.

Mwangi JN, Wang N, Ingersoll CG, Handesty DK, Brunson EL, Li H, Deng B. 2012. Toxicity of carbon nanotubes to freshwater aquatic organisms. *Environ. Tox. Chem*. 31(8):1823-1830.

Nowack B and TD Bucheli. 2007. Occurrence, behavior and effects of nanoparticles in t he environment. *Environ Pollut*. 150:5-22.

Parks AN, Portis LM, Schierz A, Washburn KM, Perror MM, Burgess RM, Ho KT, Chandler GT, Ferguson PL. 2013. Bioaccumulation and toxicity of single-walled carbon nanotubes to benthic organisms at the base of the marine food chain. *Environ. Tox. and Chem*. DOI:10.1002/etc.2172.

Petersen EJ, Huang QG, Weber WJ Jr. 2008. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus. Environ. Health Perspect.* 116(4):496-500.

Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna. Environ. Sci. Technol*. 43(8):2969-2975.

Poulin R. 1992. Toxic pollution and parasitism in freshwater fish. *Parasitol. Today*. 8(2):58-61.

Ringwood AH, Levi-Polachenko N, Carroll DL. 2009. Fullerene exposures with oysters: embryonic, adult, and cellular responses. *Environ. Sci. Technol*. 43:7136-7141.

Singhai RN, Swarm J, Davies RW. 1987. Experimental transmission of *Saprolegnia* and *Achlya* to fish. Aquaculture. 64(1):1-7.

Smith CJ, Shaw BJ, Handy RD. 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat. Tox*. 82: 94-109.

Thayer AM. 2007. Carbon nanotubes by metrician: Anticipating new commercial applications, producers increase capacity. *Chem. Eng. News Business*. 85:29-38.

U.S. Environmental Protection Agency. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 4th ed. EPA-821-R-02-013. Washington, DC.

Waissi-Leinonen GC, Petersen EJ, Pakarinen K, Akkanen J, Leppänen MT, Kukkonen JVK. 2012. Toxicity of fullerene (C_{60}) to sediment-dwelling invertebrate *Chironomus riparius* larvae. *Environ. Tox. Chem.* 31(9):2108-2116.

Wakabayashi H. 1991. Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish. *J. Fish Diseases.* 14:279-290.

Wall SB, La Point TW, Isley JJ. 2009. Fish bioturbation of cadmium-contaminated sediments: factors affecting Cd availability to Daphnia magna. Environ. Tox. Chem. 15(3):294-298.

Zimmer KD, Hanson MA, Butler MG. Effects of fathead minnow colonization and removal on a prairie wetland ecosystem. 2001. *Ecosystems*. 4(4):346-357.

Zhu X, Zhu L, Lang Y, Chen Y. 2008. Oxidative stress and growth inhibition in the f reshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environ. Tox. Chem*. 27(9):1979-1985.

FIGURE LEGENDS

Figure 4.1. Multi-walled carbon nanotubes (MWCNTs) measured in the water column for three exposure concentrations (0, 0.5, 50 mg/kg sediment). Exposure vessels containing *Pimephales promelas* having sediment access are indicated by the solid bars; those without access (net exclusion, N) are indicated with dotted bars. MWCNTs were most often detected in the column of exposure vessels when *P. promelas* had access to the sediment (no net) ($p<0.0001$). Controls measured 0.0 mg/L.
(*)denotes statistical significance from control, $p<0.05$.

Figure 4.2. Average amount of MWCNTs measured in *Pimephales promelas* bodies (n=5 for each replicate) after 24 h depuration period (A) and depuration water (500 mL) with suspended fecal material (B). Exposure vessels containing *P. promelas* having sediment access are indicated by the solid bars; those without access (net exclusion, N) are indicated with dotted bars.

Figure 4.3. Total incidence of morbidity in juvenile *Pimephales promelas* by treatment. Solid bars indicate morbidity in fish with sediment access; dotted bars indicate morbidity incidence in fish without sediment access (net exclusion, N). Fish exposed to multi-walled carbon nanotubes (MWCNTs) at all concentrations exhibited morbidity. No significant differences were measured with or without net exclusion. The most common observations were anal protrusion of the gut tract (n=13 for 50 mg/kg) and fungal growths on gills (n=6 for 50 mg/kg). Deflated or missing swim bladders were also

observed (n=3 for all exposures). Controls had no incidence of morbidity.

Figure 4.4. Representative control *Pimephales promelas* (15x) at 2 d (A) compared with those exposed to multi-walled carbon nanotubes (B-D). Nanotube- exposed *P. promelas* exhibited multiple forms of morbidity: deflated swim bladders (B), pathogenic gill infections (C), and anal protrusion of the gut tract (D), which were present at all exposure concentrations.

Figure 4.5. A detailed view of the pathogenic gill infection in a representative *Pimephales promelas* exposed to 50 mg/kg multi-walled carbon nanotubes at 40x.

Figure 4.1.

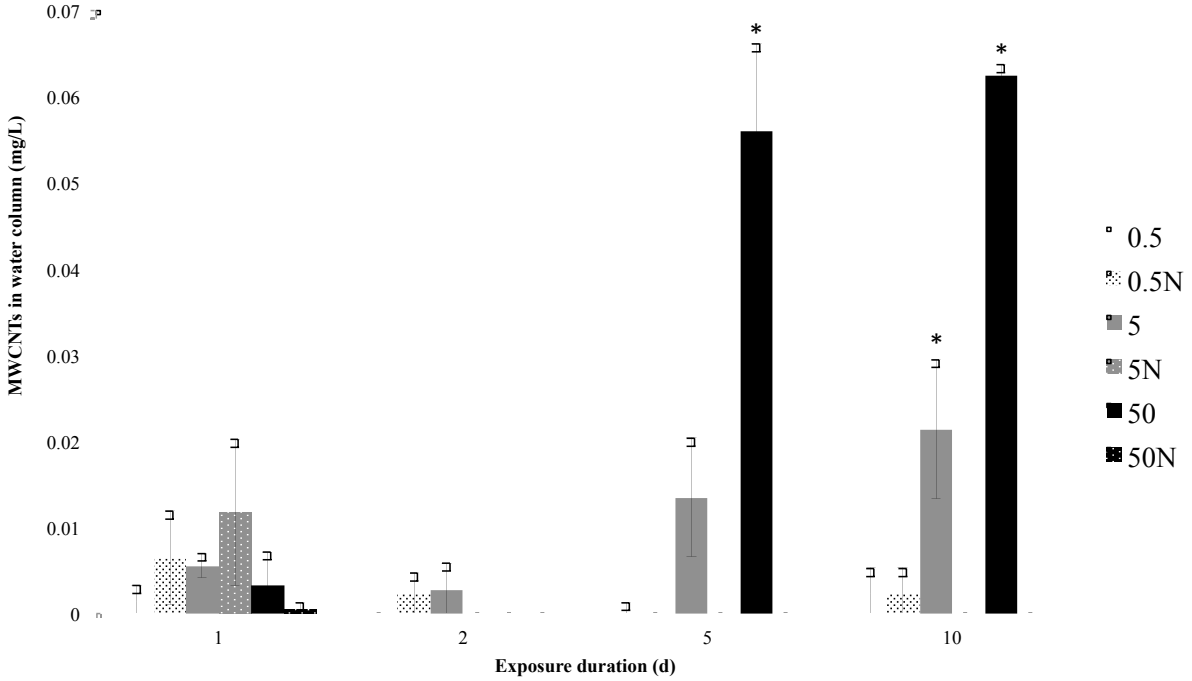
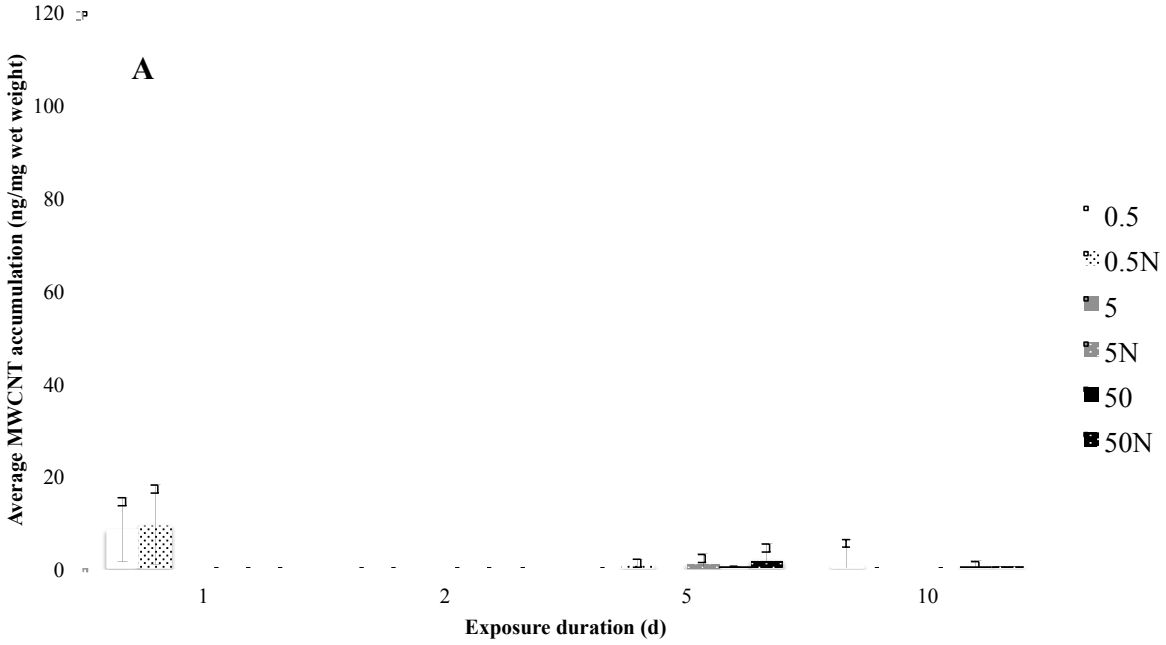

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
MWCNTs in water column (mg/L)
1
2
5
10
Exposure duration (d)
*
*
*
0.5
0.5N
5
5N
50
50N

Figure 4.2


A
Average MWCNT accumulation (ng/mg wet weight)
120
100
80
60
40
20
0
1
2
5
10
Exposure duration (d)
0.5
0.5N
5
5N
50
50N

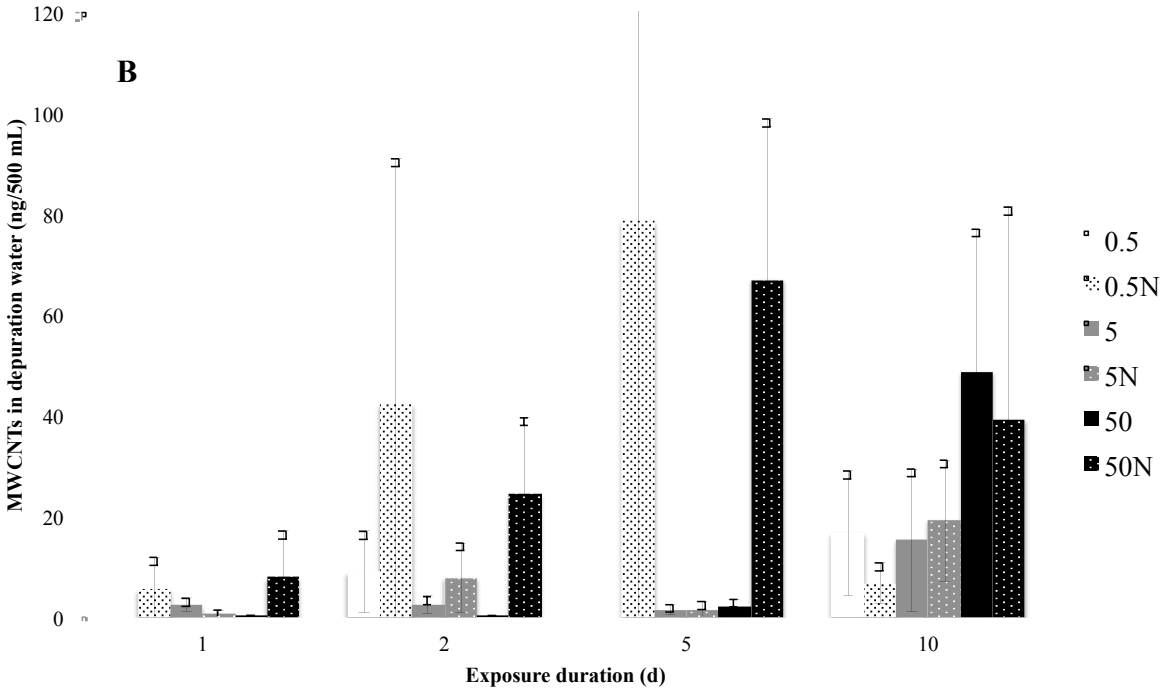

B
MWCNTs in depuration water (ng/500 mL)
120
100
80
60
40
20
0
1
2
5
10
Exposure duration (d)
0.5
0.5N
5
5N
50
50N

Figure 4.3.

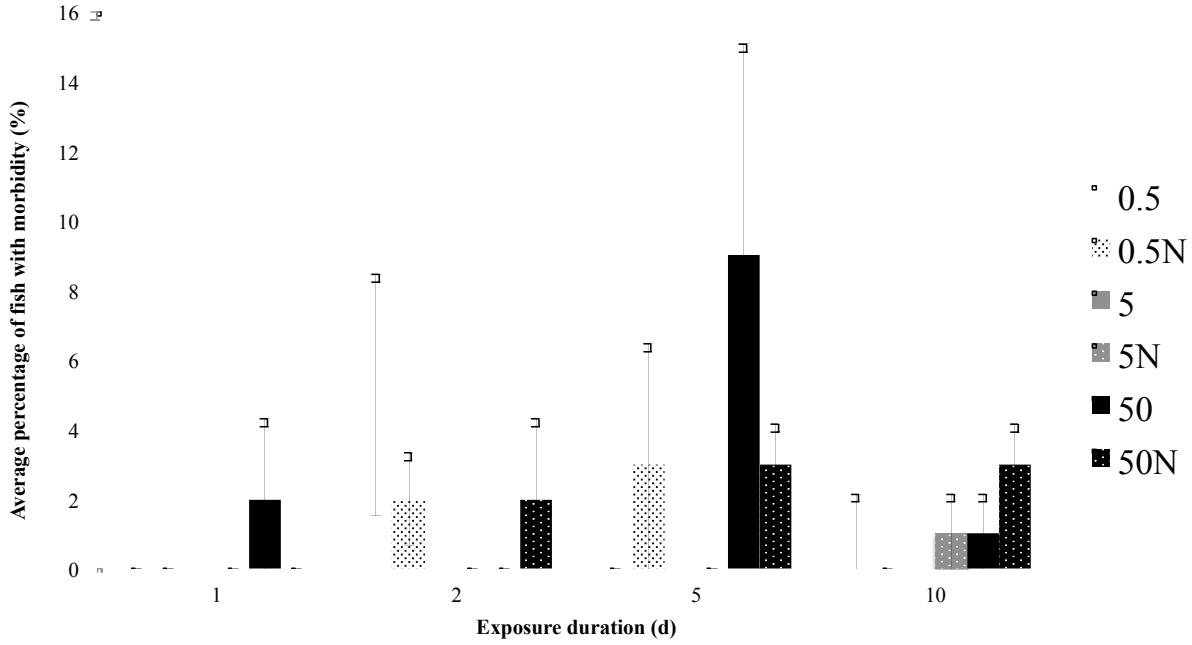

Average percentage of fish with morbidity (%)
16
14
12
10
8
6
4
2
0
1
2
5
10
Exposure duration (d)
0.5
0.5N
5
5N
50
50N

Figure 4.4.

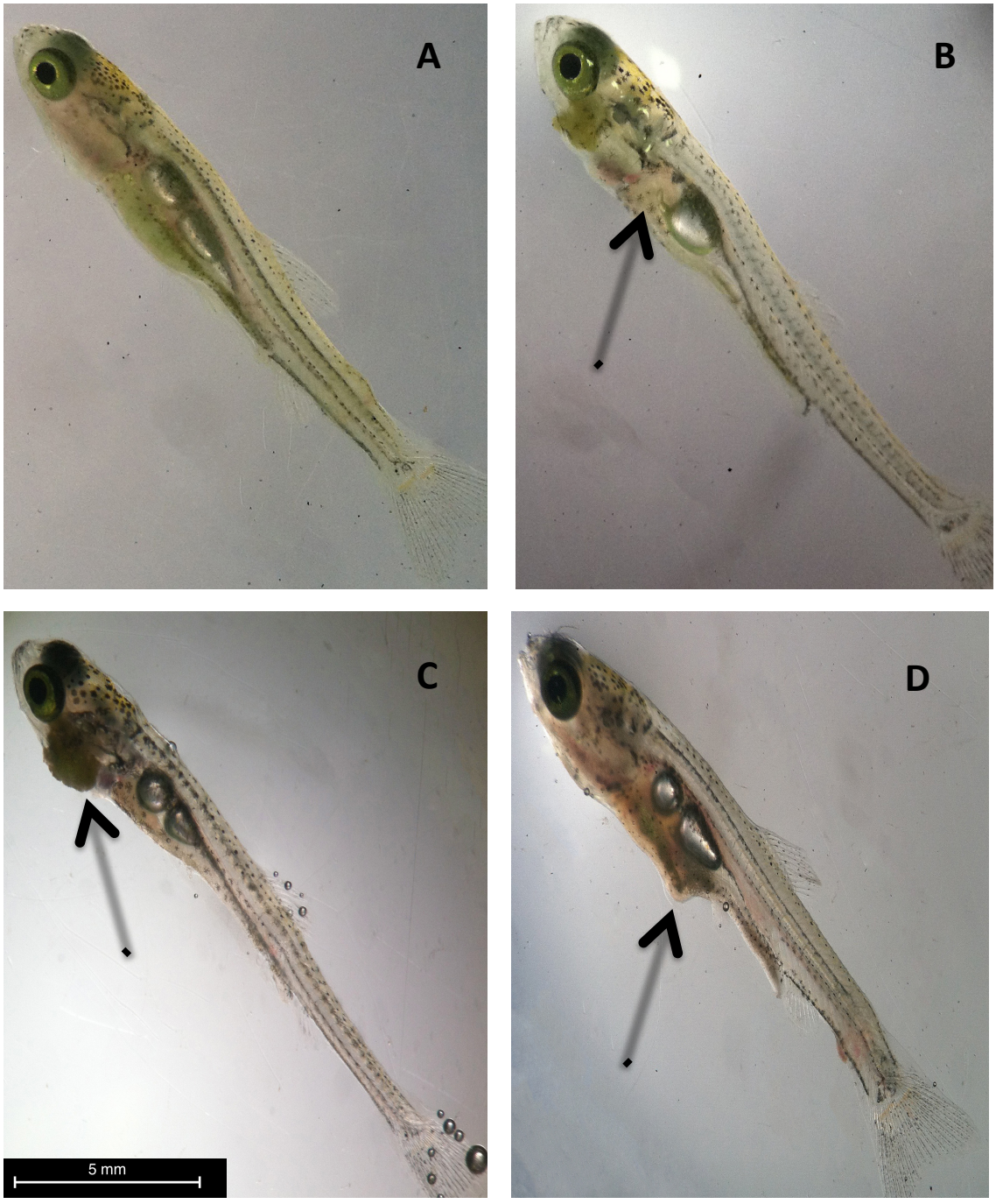


Figure 4.5.

CHAPTER 5

A CRITICAL REVIEW OF CARBON NANOPARTICLE TOXICITY TO AQUATIC CRUSTACEANS AND GUIDANCE FOR FUTURE TESTING[3]

[3] Emily R. McReynolds and Marsha C. Black. In preparation to be submitted to *Integrated Environmental Assessment and Management*.

ABSTRACT

Recent advances in the nanotechnology field and subsequent release of carbon nanoparticles in the environment have raised concerns about environmental safety. This paper gives a critical overview of the toxic effects in aquatic crustaceans after exposure to carbon nanoparticles. *Ceriodaphnia dubia* is the most frequently used test species in carbon nanoparticle testing and is one of the many crustaceans at risk for carbon nanoparticle exposure. Results of this review reveal an extreme deficit of available data in the nanoecotoxicology field. We describe four predicted modes of toxicity for exposure to carbon nanoparticles, with one (interference with ecdysis) being unique to crustaceans. Based on a literature review, this paper provides recommendations for future toxicity testing specific to crustaceans. Scientists are encouraged to use this research as a reference for future toxicity testing.

Key words: fullerenes, carbon nanotubes, crustacean, nanoecotoxicology, natural organic matter

INTRODUCTION

The nanotechnology industry has developed rapidly over the past decade and questions surrounding the environmental safety of carbon nanoparticles (CNPs) continue to go unanswered. The unique properties of CNPs present unknown toxicities compared to stable, elemental carbon. Currently, there are three types of engineered carbon nanoparticles (all allotropes of carbon) available for use in a wide variety of applications:

fullerenes (C_{60}), single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs). Fullerenes (also known as Buckminster fullerenes) are hollow, spherical allotropes of carbon consisting of 60 carbon atoms. Single-walled (SWCNTs) are single layers of graphene rolled into a tube, while multi-walled (MWCNTs) resemble SWCNTS, but are composed of multiple layers of graphene. As production rates of CNPs increase over time, so does the probability of introduction of these engineered materials to the aquatic environment. Throughout the lifecycle of CNPs from production through product use and degredation, there are many avenues for release into aquatic environments and subsequent contact with aquatic organisms is imminent (Nowack and Bucheli 2007, Musee 2011).

The majority of the research completed on organism exposure to CNPs is through acute exposures. While incorporating high exposure concentrations in a short period of time does not represent realistic environmental exposures, this method can provide some basic information in the field of nanoecotoxicology where it is lacking. Baun et al. (2008) recommends that invertebrates such as *Daphnia magna* be used as test organisms to advance nanoecotoxicology research through short-term mortality testing. However, many researchers have already observed that mortality testing requires large amounts of carbon nanoparticles that are not environmentally realistic for predicted exposure concentrations (Handy et al. 2008, Klaine et al. 2008, Gottschalk et al. 2009). A critical need exists for an environmental risk assessment framework that the public, industry, and the government can accept (Kapustka et al. 2009). The U.S. Environmental Protection Agency has already listed nanoparticles as "Contaminants of Emerging Concern," highlighting the need for an environmental risk assessment, but also requiring further

research prior to enacting future federal regulations. However, differences in CNP preparations, experimental designs, and species sensitivities have made comparisons extremely difficult across literature sources (Handy et al. 2008, Kapustka et al. 2009, Klaine et al. 2012). The inability to create a robust data set for a risk assessment has developed from the inability to detect CNPs in the environment, estimate environmental exposure concentrations, predict behavior, and assess risks (Klaine et al. 2012). National toxicity databases such as Toxline (U.S. National Library of Medicine, Bethesda MD) and AQUIRE (U.S. Environmental Protection Agency, Duluth, MN) are ill-equipped to handle the current data available on nanoparticles in general, and therefore, very few studies are available for comparison. Databases such as these require robust data sets while researchers can only provide limited information that results in high levels of uncertainty (Klaine et al. 2012). This lack of cohesion among studies makes data for computer modeling unavailable to complete environmental risk assessments for CNPs and leaves little help to guide decision makers towards action.

The goal of this paper is to give an overview of available literature as well as the inclusion our own experiments to critically evaluate the toxicity of CNPs to crustacean species as sensitive and reliable test organisms. We compare the sensitivities of both freshwater and marine crustaceans to varying concentrations of CNPs, highlighting similarities in predicted modes of toxicity. The purpose of this literature synthesis is to give scientists a better understanding of what data are available to begin creating a useable environmental risk assessment and also to make the data gaps in toxicity testing more visible to policy makers in charge of research funding. Finally, we suggest

directions for future toxicity tests and regulations with specific attention to the molting of crustaceans as a unique component of freshwater and marine environments.

SIGNIFICANCE OF CRUSTACEANS IN TOXICITY TESTING

The subphylum Crustacea has 66,000 identified crustacean species. The majority of this subphylum is aquatic, although some species are terrestrial (i.e., some species of Isopoda), while others use both habitats (Decapoda) (LeBlanc 2007). Crustaceans are considered the most numerous and ecologically important group of invertebrates in both freshwater and marine environments (Baun et al. 2008). Their ecological importance makes crustaceans a particularly significant subphylum to the ecotoxicology field. Smaller crustacean species are well-suited for chronic studies because their life-cycles are much shorter than larger crustaceans (Baun et al. 2008). Whole life-cycle tests with daphnids such as *Daphnia magna* or marine copepods such as *Amphiascus tenuiremus* can be completed in less than three weeks, which decreases the cost and labor intensity to obtain biologically relevant information (Baun et al. 2008). Crustaceans as test organisms have also been observed to be sensitive to many xenobiotic organic chemicals (Baun et al. 2008). While crustaceans are not necessarily good predictors of human toxicity, as many of the modes of action for toxicity relate to contaminants that interfere with the molting process, they do serve as good indicators of overall environmental health (Breitholz et al. 2006). Crustaceans are the only known invertebrates (besides some arthropods) that have true endocrine glands that function similar to vertebrate glands (Verslycke et al. 2007). Therefore, crustacean hormone signaling for physiological

processes like growth and reproduction may be more important to understanding human health than previously thought (Verslycke et al. 2007).

The diversity in crustacean morphology shows their success in occupying many types of niches. Different types of habitats suggest that some crustaceans may be more at risk than others for CNP exposure because of location or species sensitivity. We discuss CNP toxicity to various aquatic crustacean species categorized by niche location: freshwater or marine, water column or sediment-associated.

TOXICITY TO WATER COLUMN CRUSTACEANS

Freshwater exposures

Daphnia magna, Ceriodaphnia dubia, and other daphnid species are the most common invertebrate species used in regulatory chemical testing; therefore, daphnids are an appropriate choice as test organisms for performing ecotoxicological testing on CNPs (Baun et al. 2008). Other planktonic crustaceans similar to daphnids are equally as important in the environment as they are considered the food and energy link between primary producers (algae) and secondary consumers (fish and fish larvae) (Baun et al. 2008). As *D. magna* and *C. dubia* are traditional toxicity testing organisms, the majority of information collected on CNPs is through use of these organisms. Table 5.1 summarizes the literature available on CNP exposures to crustaceans that live in the water column of freshwater environments.

Ceriodaphnia dubia is a freshwater crustacean found in littoral lakes and streams and is the most frequently used test species in CNP exposure studies. *C. dubia* exposed to 2 mg/L MWCNT for 48 h had reduced body length with MWCNTS accumulated in the

gut tract and brood chamber (Li and Huang 2011). MWCNT aggregates were attached to the thoracic appendages and the abdominal claws and this type of attachment was one of the predicted modes of toxicity for reduction in growth (Li and Huang 2011). However, placing *C. dubia* in clean exposure water for 15 min was sufficient to the clear gut tract, suggesting that the *C. dubia* can easily excrete the MWCNTs during a short depuration period (Li and Huang 2011). Interestingly, MWCNT aggregate size was directly related to the energy required to remove the MWCNTs in the gut tract: the smaller the particles, the less time was needed to remove them (Li and Huang 2011). Another study observed that a 7-d exposure of 1 mg/L MWCNTs to *C. dubia* was not lethal, but a significant decrease in reproduction and growth was observed after exposure to 0.25 mg/L (Edgington et al. 2010). In the same study, an addition of 1.79 to 18.5 mg/L dissolved organic carbon in the form of natural organic matter (NOM) to stabilize the MWCNTs in solution may have changed the environmental behavior of the MWCNTs (Edgington et al. 2010). However, there were no experiments that exposed organisms to the same MWCNT source without the presence of NOM in the control for comparison. Kennedy et al. (2008) also stabilized MWCNTs in 100 mg/L NOM, exposing *C. dubia* for 48 h with an EC_{50} (mortality) of 50.9 mg/L. Exposed *C. dubia* developed carapace abnormalities and MWCNTs were visible in the gut tracts (Kennedy et al. 2008). Individuals appeared to have difficulty clearing the aggregates, possibly resulting in mortality or immobilization (Kennedy et al. 2008). This study also observed that during a 24 h depuration period in clean water, *C. dubia* could excrete MWCNTs, but the availability of algae as a food source was necessary for clearance of the gut. Mechanical dispersion techniques also appeared to affect the toxicity of MWNCTs in the presence of 100 mg/L

NOM to *C. dubia*. Kennedy et al. (2009) reported that a 96 h exposure to magnetically stirred MWCNTs induced slightly more mortality (LC_{50}=17 mg/L) than observed in exposures where MWCNT were sonicated (LC_{50}=21 mg/L). Our research suggests that 2.5 mg/L sonicated MWCNTs aggregate on exoskeletons of *C. dubia* which inhibits proper feeding, successful ecdysis, and release of eggs (Chapter 2). We measured significant accumulation of MWCNTs in exposed adult *C. dubia* (without a depuration period) as well as a significant decrease in brood number and size after exposure to 2.5 mg/L (Chapter 2).

Daphnia magna is another daphnid species used in standard toxicity tests as well as nanotoxicity tests. *Daphnia magna* growth was reduced when exposed to 5 mg/L MWCNTs for 7 d (Alloy and Roberts 2011). *Daphnia magna* showed a 50% reduction in reproduction after exposure to 0.5 mg/L MWCNTs, while the same exposure concentration did not significantly decrease *C. dubia* reproduction (Alloy and Roberts 2011). From this study, the authors hypothesize that *D. magna* may be more sensitive to reproductive effects caused by MWCNT exposure than *C. dubia*. The predicted mode of action of observed toxicity was feeding inhibition of *D. magna*, leading to nutrient deficiency (Alloy and Roberts 2011). Interestingly, the authors also suggest that particle size is not biologically relevant to CNP toxicity given the wide range of food particles that *D. magna* ingests (Alloy and Roberts 2011). Petersen et al. (2009) also observed that *D. magna* was unable to excrete MWCNTs after a 48 hr depuration period without food following a 48 h exposure of 4 mg/L MWCNTs (Petersen et al. 2009). However, with the presence of algae as a food source, *D. magna* were able to purge MWCNTs. The authors observed that some of the accumulated MWCNTs remained in the organism's gut, but

did not absorb into cellular tissue (Petersen et al. 2009). An exposure to a significant quantity of MWCNTs is hypothesized to ultimately limit food digestion by *D. magna* (Petersen et al. 2009). Edgington et al. (2010) observed that the presence of food increased the elimination of MWCNTs in *D. magna* after an exposure to 2 mg/L dissolved in a range of NOM concentrations (1.79-18.5 mg/L dissolved organic carbon). Interestingly, this exposure level of MWCNTs was acutely toxic (mortality LC_{50}=2 mg/L) to *D. magna* growth after a 96 h exposure (Edgington et al. 2010).

Thamnocephalus platyurus is a freshwater crustacean found in ephemeral water bodies in the southwestern United States. While not routinely used in toxicity testing, *T. platyurus* has been used in toxicity testing of surfactants (Brausch and Smith 2007) and is an important species in the food chain in water-sensitive areas. When *Thamnocephalus platyurus* was exposed to 3 mg/L of fullerenes for 1 h, fullerenes agglomerated in the gut tract measured on an order magnitude larger than suspended fullerenes in solution (Patra et al. 2011). However, *T. platyurus* was able to excrete these aggregated fullerenes during the exposure duration with and without the presence of food. The authors hypothesize that the ability of organisms to excrete fullerenes may increase agglomerate size of fullerene particles that enters into the aquatic environment, creating stable pellets that may provide an exposure route to benthic organisms. The movement of carbon nanoparticles to the sediment when incorporated into fecal pellets is a possible route of exposure that needs to be further examined (Patra et al. 2011).

Marine exposures

Currently, there is no literature available documenting exposure of carbon

nanoparticles (fullerenes, SWCNTs, or MWCNTs) to pelagic marine crustaceans. With exposure, these animals that live in the marine water column may be the first to come into contact with CNPs as they settle to the bottom or associate with the sea surface microlayer. We suggest that more research focus on this specific data gap, as many marine crustaceans that associate with the water column are at the base of the food chain and are ecologically as well as economically important.

TOXICITY TO SEDIMENT-ASSOCIATED CRUSTACEANS

Freshwater exposures

Because the sediment is the ultimate sink for many contaminants and is considered the ultimate sink for carbon nanoparticles, animals that live in or on the sediment are target species and should be used for nanoecotoxicity testing (Baun et al. 2008, Klaine et al. 2008). Table 5.2 details data available on CNP exposures to crustaceans that associate with freshwater sediment. The only CNP exposure studies incorporating this niche are crustaceans that already have been extensively studied in traditional toxicity tests. These organisms are *Leptocheirus plumulosus* and *Hyallela azteca.*

Leptocheirus plumulosus, a benthic freshwater crustacean, was exposed to 100 μg/g ^{14}C-labeled SWCNTs in sediment and algae for 28 d (Parks et al. 2013). Prior to depuration, sediment-exposed *L. plumulosus* had an elevated CNT body burden nearly 50 times higher than controls and even after the depuration period the body burden for sediment-exposed *L. plumulosus* was significant (Parks et al. 2013). The authors observed that SWCNTs were bioavailable and the most probable route for accumulation

was ingested sediment (Parks et al. 2013). However, survival of *L. plumulosus* was not affected by SWCNT exposure (Parks et al. 2013). Alternatively, Kennedy et al. (2008) observed that mortality in *L. plumulosus* was reduced in 10 d exposures compared to control mortality when exposed to 99 g/L MWCNTs. But both carbon black and activated carbon were observed to be more toxic than MWCNTs in sediment exposures. Mechanical dispersion methods appear to affect *L. plumulosus* toxicity; Kennedy et al. (2009) observed that survival was significantly reduced when *L. plumulosus* was exposed to 30 g/kg MWCNTs and interestingly, the sonicated MWCNT treatment was more toxic than stirred MWCNTs. Kennedy et al. (2009) hypothesized that benthic organisms may be more sensitive to the fragmented MWCNTs produced by the sonication treatment.

Another freshwater crustacean, *Hyallela azteca*, when exposed to 1 g/L (MWCNTs and SWCNTs in separate experiments) in a water-only exposure for 14 d, was observed to have decreased survival and biomass after exposure to both types of CNTs (Mwangi et al. 2012). Exposed *H. azteca* were unable to excrete either the SWCNTs or MWCNTs after being depurated for 24 h. In this study, sonication increased the toxicity of SWCNTs, but not MWCNTs; pre-cleaning of the MWCNTs with nitric acid also decreased lethal effects (Mwangi et al. 2012). Blockage of the digestive tract by carbon nanotube aggregates may have decreased efficient nutrient uptake by *H. azteca*, but the aggregates did not appear to penetrate the gut wall (Mwangi et al. 2012). Carbon nanotubes may also promote the production of radical oxygen species in organisms and the coating of CNTs on an organism's outer surface may interfere with respiration processes (Mwangi et al. 2012). In a 10-d whole sediment bioassay, mortality was reduced compared to control mortality when *H. azteca* was exposed to 264 g/L

MWCNTs (Kennedy et al. 2008). Similar to observations with exposures to *L. plumulosus*, carbon black and activated carbon were both more toxic to *H. azteca* in sediment exposures than MWCNTs stabilized with 100 mg/L NOM (Kennedy et al. 2008). Interestingly, another study observed that a significant difference in mortality compared to control mortality only occurred in the *H. azteca* exposed to the sonicated treatment of 300 g/kg MWCNTs relative to the control (Kennedy et al. 2009). Exposed to the same source of MWCNTs used by Edgington et al. (2008), *L. plumulosus* appears to be much more sensitive to exposure (LC50=30 g/kg) than *H. azteca* (LC50=300 g/kg). Even within the same species, a wide variety of observed results makes the margin of error for toxicity predictions extremely large.

Marine toxicity

Marine crustaceans that associate with the sediment such as *Amphiascus tenuiremis, Americamysis bahia, and Ampelisca abdita* are also important food sources for fish larvae (Baun et al. 2008). Benthic crustaceans are important to the processes of organic materials degradation and nutrient cycling. A summary of studies exposing marine crustaceans that associate with the sediment is found in Table 5.3. Although some studies did not include sediment as the media for CNP exposure, these studies are still included as the organisms naturally interact with sediment and sediment porewaters.

When *A. tenuiremus* was exposed to 10 mg/L of the smallest SWCNT by-products in a 28 d lifecycle experiment, Templeton et al. (2006) observed reproductive toxicity. Nanotube ingestion was clearly visible, with purified SWCNTs aggregating in the gut followed by repackaging of SWCNTS into fecal pellets, which likely resulted in

morphological alterations of the SNTs (Templeton et al. 2006). Incorporation into fecal pellets may be an ultimate sink of SWCNTs as meobenthic copepods are the second most numerous benthic metazoans (Templeton et al. 2006). Smaller sized SWCNTs may be more bioavailable for digestive and dermal uptake (post-molting) by *A. tenuiremus*, most likely causing oxidative stress. The predicted modes of action for toxicity of SWCNTs to *A. tenuiremus* were mechanical disruption of feeding appendages, penetration of gut wall, and oxidative stress (Templeton et al. 2006). Because no other studies with *A. tenuiremis* have been conducted, exposures with MWCNTs should be high on the priority list given the results of this particular study.

Another crustacean associated with the marine sediment is *Americamysis bahia, which* has been used extensively for traditional toxicity testing and biomonitoring of estuarine environments. Exposure of <48 h old *A. bahia* to 10 µg SWCNT/g for 7 d had no effect on survival (Parks et al. 2013). Results of a 7 d exposure to 100 µg/g (sediment) and food-borne exposure of 100 µg SWCNTs/g (algae) suggested that SWCNTs are bioavailable to *A. bahia* for uptake, but do not appear to accumulate or cause toxicity (Parks et al. 2013). SWCNTs were excreted without crossing the gut lumen and SWCNTs were not detected in either non-depurated or depurated organisms (Parks et al. 2013). Alternatively, we exposed *A. bahia* to 5 mg/L MWCNTs suspended in natural seawater for 7 d and found that although there was not significant mortality, we observed a decrease in the number of mature individuals at 14 d old as well as significant accumulation of ^{14}C-labeled MWCNTs by exposed organisms (Chapter 3). *A. bahia* in our studies were not placed in clean water for a depuration period, so it is unclear

whether the amount of MWCNTs visibly excreted was significant compared to the amount remaining within the gut.

Ampelisca abdita is a tube-dwelling benthic amphipod. *A. abdita* is not a traditional choice for toxicity testing, but guidelines have been established for use this crustacean in future CNP research (Redmond et al. 1994, Schlekat et al. 1995). Similar to the SWCNT exposure to *A. bahia* by Parks et al. (2013), a 7 d exposure to 10 µg/g SWCNTs in had no effect on *A. abdita* survival. After a 7 d exposure to 100 µg SWCNT/g in sediment and food, a significant amount of SWCNTs were measured in *A. abdita* bodies prior to depuration, but no SWCNTs were measured in *A. abdita* after depuration, suggesting that *A. abdita* was able to easily excrete the SWCNTs in fecal pellets. Interestingly, the accumulation route of SWCNT in benthic crustacean appeared to be ingested algae, compared to *L. plumulosus*, which was sediment exposure (Parks et al. 2013).

THE ROLE OF NATURAL ORGANIC MATTER IN TOXICITY

Many literature sources suggest that natural organic matter (NOM) stabilizes CNTs in solution (Hyung et al. 2007, Hyung and Kim 2008). From our experiments with NOM, we hypothesize that as NOM stabilizes the MWCNTs in solution it is less likely to aggregate on the chitinous exoskeleton of *C. dubia*, *A. bahia*, and perhaps other crustaceans. We also observed that the addition of 4.5 mg/L NOM was protective against reproductive toxicity in *C. dubia* when exposed to 5 mg/L MWCNTs for 7 d (Chapter 2). Yet, *C. dubia* exposed to MWCNTs in the presence of NOM appeared to accumulate more MWCNTs in the gut tract (Fig 2.3) compared to *C. dubia* exposed to MWCNTs

without the addition of NOM and an increase in accumulation was confirmed through liquid scintillation counting (Chapter 2). We also observed that *A. bahia* exposed to 5 mg/L in the presence of NOM (in natural seawater) accumulated nearly twice the measurable amount of MWCNTs than *A. bahia* exposed to 5 mg/L in synthetic seawater, which contained no measurable NOM (Chapter 3). Based on these results we hypothesize the NOM surrounds the CNTs, and is then treated as a food particle to filter feeding organisms, increasing the concentration aggregated in the gut. These results in addition to data available in the literature suggest that the interaction with NOM alters the environmental behavior MWCNTs and other CNPs. As many studies have used NOM to disperse CNPs, this may have ultimately influenced the interpretation of results. For example, many studies have used NOM addition as a way to disperse CNTs (Kennedy et al. 2008, Kennedy et al. 2009, Edgington et al. 2010, Alloy and Roberts 2011), but did not include a secondary control without the presence of NOM. Differences in results observed between exposures with and without the presence of NOM make comparison between literature sources even more difficult. As wide ranges of NOM are found naturally in marine and freshwater environments, laboratory exposures of CNPs that include NOM are more environmentally realistic. However, we suggest incorporating secondary controls or separate exposure concentrations without the presence of NOM to help determine the true environmental fate and behavior of CNPs in a variety of aquatic environments.

PREDICTED MODE(S) OF TOXICITY

Literature sources have predicted a myriad of different modes of action for CNP toxicity. We discuss modes of toxicity that have been mentioned by multiple sources and are predicted to occur in crustaceans. Finally, we emphasize the mode of action that is unique to crustaceans.

1. *Nutritional deficiency*

Many studies have suggested that the exposure of crustaceans causes reduced nutritional efficiency of food intake (Petersen et al. 2009, Alloy and Roberts 2011, Patra et al. 2011, Mwangi et al. 2012, Chapters 2 and 3). CNPs may aggregate with algae particles or other food particles, reducing the nutrients of the food source as well as impeding the ingestion or digestion processes. This specific hypothesis is not unique to crustaceans, as studies exposing other organisms have suggested the same response (Mouchet et al. 2007, Ghadfari et al. 2008).

2. *Gut impaction*

After ingestion of CNPs, studies suggest that gut impaction may cause toxicity as many organisms cannot easily excrete CNPs in the absence of food (Templeton et al. 2006, Kennedy et al. 2008, Kennedy et al. 2009, Petersen et al. 2009, Mwangi et al. 2012, Chapters 2 and 3). As mentioned by Patra et al. (2011), fullerene aggregate concentrations were an order of magnitude higher within the gut of *T. platyurus* compared to the exposure concentration in the water column. Gut impaction has also been observed in studies with other organisms and does not appear to be unique to

crustaceans (Mouchet et al. 2007, Ghadfari et al. 2008, Chapter 4). While depuration studies are becoming more widely used, it is still unclear what negative effects result from the presence of CNPs in the gut tract as long as they can be excreted. Histopathology could lead to a greater understanding of this potential mode of action.

3. Reactive oxygen species

The creation of reactive oxygen species (ROS) has also been predicted as a consequence of CNP exposure to crustaceans (Templeton et al. 2006, Mwangi et al. 2012) as well as in other species (Smith et al. 2007). However, it is unclear if the presence of ROS species is a result of natural ROS production, an increase in production from endogenous (produced by the crustacean as a response) or exogenous (produced by CNPs interacting with crustacean) sources, or development of reactive oxygen compounds as a defense mechanism. When exposed to certain chemical stressors, many species produce an "oxidative burst" used to destroy exogenous material. For example, superoxide production *in vitro* has been observed in hyaline cells of *Carcinus maenus*, the shore crab following stimulatory exposure to phorbol 12-myristate 13-acetate (PMA), a known activator of the burst response in fish, and concanavalin A (con A), which has been observed to stimulate H_20_2 production in scallop amoebocytes (Bell and Smith 1993). Crustaceans exposed to CNTs may use this defense mechanism, but no literature is available documenting the measurement of oxidative stress in crustaceans.

4. Molting disruption

The inability to molt (Chapters 2 and 3) and sensitivity in post-molt stages

(Templeton et al. 2006, Kennedy et al. 2008) are predicted toxic effects that are unique to crustaceans. Crustaceans have a lipophilic exoskeleton consisting of chitin, requiring many species to go through several growth instars to remove old exoskeletons and replace them with new ones. The reproductive success and ultimate survival of crustaceans revolves around the timing of these moltings, as both ecdysis and reproduction require extreme amounts of energy. If a crustacean is not in a suitable environment, it will be unable to complete its molt and will die before leaving its old carapace (Conan 1985). Also, mortality by predation is much higher during the molting period, increasing the time sensitive nature of completing ecdysis (Conan 1985). As crustaceans require multiple rounds of molting, the obstruction of CNPs on the carapace could have produce negative effects on reproduction, growth, and other metabolic processes. Through combating foreign particle attachment on the carapace organisms could waste energy typically reserved for growth or reproduction. Interference with ecdysis could also be confounded by aggregation on outside of crustacean bodies, which has been observed to occur in *C. dubia* and *A. bahia* (Li and Huang 2011, Chapters 2 and 3). Delay of ecdysis has been observed in crustaceans exposed to a wide variety of pollutants including heavy metals and aromatic hydrocarbons (Weis et al. 1992). Interestingly, carbon nanotubes are replacing tributyltin (TBT) in anti-fouling paint on ships to discourage barnacle attachment. Exposure to TBT also decreased molting success in crustaceans such as *Uca pugilator* (fiddler crab), but at a much lower exposure concentration of TBT (0.5 μg/L) compared to the concentration of CNPs needed to produce an effect (Weis et al. 1987). Also, the mode of action for TBT is quite different that those predicted for CNPs, as the hypothesized mode of action for TBT toxicity is

from chemical interference with growth and reproduction causing development of imposex organisms, while the mode of toxicity for CNPs is most likely mechanical disruption of reproduction from the physical presence of the CNPs.

RECOMMENDATIONS FOR FUTURE RESEARCH

Based on this review of the most recent literature available on the toxicity of CNPs to crustacean species, we have several recommendations for future toxicity testing. These guidelines for future research can create a pathway for bridging important data gaps with robust data and increasing the feasibility of development of a risk assessment framework in the near future.

1. *The most significant suggestion for the progression in nanotoxicology research is the standardization of exposure studies*. The standardization should include pre-treatment of nanoparticles, size, duration, exposure concentration and controls to make comparisons of results with more robust data points that are biologically significant. Greiger et al. (2009) noted that the lack of standard protocols produces the greatest uncertainty in CNT toxicity testing. Based on the available literature, we suggest that future research be directed toward standardized long-term, low-level exposures with chronic endpoints specific to crustacean biology. Chronic exposures represent more realistic exposure scenarios, and scientists are more likely to observe sublethal effects with longer durations. We suggest that investigations should continue using crustaceans as toxicity testing organisms because there seems to be a varying range of sensitivity depending on the type of CNP, exposure duration, concentration, and species. Baun et al. (2008) also suggests that more research should emphasize accumulation of nanoparticles

during realistic exposures and our investigations agree. Standardization of testing is essential to closing many of the data gaps surrounding CNP exposures.

2. *Test a range of species in the presence and absence of food*. Many authors have observed that the presence of food increases the speed of excretion of CNPs (Kennedy et al. 2008, Kennedy et al. 2009, Petersen et al. 2009). However, most aquatic environments are nutrient limited (Baun et al. 2008) so the availability of food may determine if CNPs can be easily excreted. Crustaceans may also selectively choose CNPs as a food source if other options are limited. Food limitation could change the ability of crustacean species to successfully remove CNPs from their gut tracts. Therefore, we suggest that future studies include a depuration period and treat food availability as a potential confounding factor for gut impaction.

3. *Researchers should focus on the bioavailability of CNTs in fecal pellets after organisms are able to excrete CNPs, helping to predict the aquatic fate and behavior of CNPs.* Research should also include potential exposures of other organisms via consumption of contaminated fecal pellets. (Patra et al. 2011). Experiments determining the role of fecal pellets in the movement of CNPs with in the environment would be a detour from the traditional toxicity test. However, from this literature review, it appears to be the most logical next step in future toxicity testing as many crustaceans have been observed to repackage and excrete CNPs in feces. Studying the interaction of fecal pellets and CNPs in the aquatic environment will provide more biologically relevant information to help bridge the data gap of target species of CNP exposures compared to a short-

duration, high-exposure concentration acute toxicity test. Nevertheless, future fate and bioaccumulation studies will require improved analytical methods.

4. *Future research should include toxic effects of CNPs to estuarine and marine crustacean species.* Few published studies are available for these types of environments, particularly for exposures to crustacean species. Our literature search suggested that crustaceans that associate with the water column have been studied the least, making this niche hard to compare with its freshwater counterparts such as *D. magna* or *C. dubia*. Having few toxicity tests makes comparison between species and environments extremely difficult. Unique features of the marine environment, including tidal action, fluxes in pH and natural organic matter and other abiotic factors, will influence the behaviors of CNPs in marine and estuarine environments, limiting the usefulness of data from freshwater environments for predicting CNT effects in marine environments (Klaine et al. 2008).

5. *Conduct "-omic" studies to pinpoint mode of action.* Future research should determine biochemical effects of CNP exposures to crustaceans by taking advantage of new technology, such as toxicogenomic and metabolomic studies. These methods could determine if ROS production is increased beyond normal levels after exposure to CNPs and if it is an exogenous or endogenous ROS source. Toxicogenomic and metabolomics studies also have the potential to determine any biochemical causes of the molting inhibition observed with exposure to CNPs. Hormones involved with the molting process could also be negatively affected by the presence of CNPs but there are currently no

studies examining this phenomenon. The molting inhibiting hormone (MIH) produced in the eye-stalk of crustaceans is responsible for regulating the synthesis of ecdysteriods, which control molting (LeBlanc 2007). Because crustaceans are known to not expend energy cleaning their eyes (Acosta and Poirrier 1992), this may be a sensitive location for CNP interaction with hormones that are extremely important to crustacean molting and growth.

6. *Finally, exposure studies incorporating larger crustacean species should also be completed to determine if CNP toxicity affects growth instars of larger organisms with the same modes of action as smaller crustaceans.* Given that small crustaceans have a wide variety of sensitivities, research is needed examining CNP exposure and accumulation in larval stages of larger crustacean species, specifically those that have significant economic importance for coastal communities in the United States such as *Farfantepenaeus aztecus* (brown shrimp) or *Callinectes sapidus* (blue crab). Crustacean species may not be suitable test organisms for all contaminants, but as a subphylum they seem to be sensitive to CNP exposure because of the unique behaviors of hydrophilic CNPs in water and the unique life history strategies as crustaceans.

REFERENCES

Acosta CA and MA Poirrier. 1992. Grooming behavior and associated structures of the mysid *Mysidopsis bahia. J. Crustacean Bio.* 12(3):383-391.

Alloy MM and Roberts AP. 2011. Effects of suspended multi-walled carbon nanotubes of daphnid growth and reproduction. *Ecotox. Environ. Safety*. 74:1839-1843.

Baun A, Hartmann NB, Grieger K, Kusk K. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotox*. 17(9):387-395.

Bell KL and VJ Smith. 1993. In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). *Dev. Comp. Immunol*. 17:211-219.

Brausch JM and PN Smith. 2007. Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. *Arch. Environ. Contam. Toxicol*. 52:217-221.

Breitholz M, Rudén C, Hansson SO, Bengtsson BE. 2006. Ten challenges for improved ecotoxiocological testing in environmental risk assessments. *Ecotox.Environ. Safety*. 63:324-335.

Conan GY. 1985. Periodicity and phasing in molting. *Crustacean issues 3: factors in adult growth*. AM Wenner, ed. Rotterdam, Netherlands: A.A. Balkema. 73-99.

Edgington AJ, Roberts AP, Taylor AM, Alloy MM, Reppert J, Rao AM, Mao J, Klaine SJ. 2010. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ. Tox. Chem*. 29:2511-2518.

Ghadfari P, St-Denis CH, Power ME, Jin Xu, Tsou V, Mandal Hs, Bols NC, Xiaowu T. 2008. Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa. *Nature Nanotechnol*. 3(6):347-351.

Gottschalk F, Sonder T, Scholz Rw, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. *Environ. Sci. Technol*. 43: 9216-9222.

Greiger KF, Hansen RF, Baun A. 2009. The known unknowns of nanomaterials: describing and characterizing uncertainty within environmental, health and safety risks. *Nanotox*. 31(1):1-17.

Handy RD, Owen R, Valsami-Jones E. 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotox*. 17(5):315-325.

Hyung H, Fortner JD, Hughes JB, King JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41:179-184.

Hyung H and JH Kim. 2008. Natural organic matter (NOM) adsorption to multi-walled carbon nanotubes: effect of NOM characteristics and water quality parameters. *Environ. Sci. Technol.* 42:4416-4421.

Kaputska L, Chan-Remillard S, Goudey S. 2009. Developing an ecological risk framework to assess environmental safety of nanoscale products. I Linkov and J Stevens (eds.). *Nanomaterials: Risks and Benefits*. Dodrecht, Netherlands: Springer Science. 149-159.

Kennedy AJ, Gunter JC, Chappell MA, Goss JD, Hull MS, Kirgan RA, Steevens JA. 2009. Influence of nanotube preparation in aquatic bioassays. *Environ. Tox. Chem*. 28(9):1930-1938.

Kennedy AJ, Hull MS, Steevens JA, Donstova KM, Chappell MA, Gunter JC, Weiss CA. 2008. Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. *Environ. Tox. Chem*. 27(9):1932-1941.

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ. Tox. Chem*. 27(9):1825-1851.

Klaine SJ, Koelmans AA, Horne N, Carley S, Handy RD, Kapustka L, Nowack B, von de Kammer F. 2012. Paradigms to assess the environmental impact of manufactured nanomaterials. *Environ. Tox. Chem*. 31(1):3-14.

Li M and CP Huang. 2011. The responses of *Ceriodaphnia dubia* toward multi-walled carbon nanotubes: effect of physical-chemical treatment. *Carbon*. 49(5):1672-1679.

LeBlanc GA. 2007. Crustacean endocrine toxiciology: a review. *Ecotox*. 16:61-81.

Mouchet F, Landois P, Flahaut E, Pinelle E, Gauthier L. 2007. Assessment of the potential in vivo ecotoxicity of double-walled carbon nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum. Nanotox*. 1:149-156.

Musee N. 2011. Nanowastes and the environment: potential new waste management paradigm. *Environ. Int*. 37:112-128.

Mwangi JN, Wang N, Ingersoll CG, Hardesty DK, Brunson EL, Li H, Deng B. 2012. Toxicity of carbon nanotubes to freshwater aquatic organisms. *Environ. Tox. Chem*. 31(8):1823-1830.

Nowack B and TD Bucheli. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environ Pollut*. 150:5-22.

Parks AN, Portis LM, Schierz PA, Washburn KM, Perron MM, Burgess RM, Ho KT, Chandler GT, Ferguson PL. 2013. Bioaccumulation and toxicity of single- walled carbon nanotubes to benthic organisms at the base of the marine food chain. *Environ. Tox. Chem*. 32(6):1270-1277.

Patra M, Ma X, Isaacson C, Bouchard D, Poynton H, Lazorchak JM, Rogers KR. 2011. Changes in agglomeration of fullerenes during ingestion and excretion in *Thamnocephalus platyurus*. *Environ Tox. and Chem*. 30(4):828-835.

Petersen EJ, Akkanen J, Kukkonen JVK, Weber, WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ. Sci. and Technol*. 43(8):2969-2975.

Redmond MS, Jones JKP, Scott KJ, Swartz RC. 1994. Preliminary culture and life-cyle experiments with the benthic amphipod *Ampelisca abdita*. *Environ. Tox. Chem*. 13(8):1355-1365.

Schlekat CE, Scott, JK, Swartz RC,Albrect B, Antrim L, Doe K, Douglas S, Ferretti JA, Hansen DJ, Moore DW, Mueller C, Tang A. 1995. Interlaboratory comparison of a 10-day sediment toxicity test method using *Ampelisca Abdita, Eohaustorius Estuarius* and *Leptocheirus Plumulosus*. *Environ. Tox. Chem*. 14(32):2163-2174.

Smith CJ, Shaw BJ, Handy RD. 2007. Toxicity of single walled carbon nanotubes to rain trout (*Orchorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat. Tox.* 82:94-109.

Templeton RC, Ferguson PL, Washburn KM, Scrivens WA, Chandler GT. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ. Sci. Technol*. 40:7387-7393.

Verslycke T, Ghekiere A, Raimondo S, Colin J. 2007. Mysid crustaceans as standard models for the screening and testing of endochrine-disrupting chemicals. *Ecotox*. 16:205-219.

Weis JS, Cristini A, Rao KR. 1992. Effects of pollutants on molting and regeneration in Crustacea. *Amer. Zoo*. 32(3):495-500.

Weis JS, Gottlieb J, Kwiatkowski J. 1987. Tributyltin retards regeneration and produces deformities of limbs in the fiddler crab *Uca pugilator. Arch. Environ. Contam. Toxicol.* 16:321-326

Table 5.1. Summary of carbon nanoparticle exposures to crustaceans that live in the water column of freshwater environments.

Form	Species	Exposure duration	Suspension preparation	Concentration (mg/L)	Presence of NOM[b]	Effects	Reference
MWCNTs[a]	*Ceriodaphnia dubia*	48 h	ozone and ultrasound, ultrasound, or sonication	2	-	reduced body length; MWCNTs accumulated in gut tract and brood chamber	Li and Huang 2011
MWCNTs	*Ceriodaphnia dubia*	7 d	sonication and supernatant settling	0.25	+	decrease in reproduction and growth	Edginton et al. 2010
MWCNTs	*Ceriodaphnia dubia*	48 h	functionalized then stirred	50.9	+	EC50 (mortality), carapace abnormalities	Kennedy et al. 2008
MWCNTs	*Ceriodaphnia dubia*	96 h	functionalized then stirred	17	+	LC50 (mortality) for stirring MWCNTs, lower than sonicating (LC50=21 mg/L)	Kennedy et al. 2009
MWCNTs	*Ceriodaphnia dubia*	7 d	acid treatment then sonication	2.5	+/-	decrease in reproduction only in study without NOM, higher accumulation in presence of NOM	Chapter 2
MWCNTs	*Daphnia magna*	7 d	sonication	0.5	+/-	reduced reproduction, reduced growth (5 mg/L)	Alloy and Roberts et al. 2011
MWCNTs	*Daphnia magna*	48 h	acid treatment then sonication using supernatant	4	-	unable to excrete MWCNTs without presence of food	Petersen et al. 2009
MWCNTs	*Daphnia magna*	96 h	sonication/ supernatant settling	2	+	LC50 for reduced growth	Edgington et al. 2010
Fullerene	*Thamnocephalus platyurus*	1 h	stirring	3	-	fullerenes agglomerated in gut tract but able to excrete	Patra et al. 2011

[a]MWCNTs= multi-walled carbon nanotubes

[b]NOM=natural organic matter

Table 5.2. Summary of published reports on carbon nanoparticle exposures to freshwater crustaceans that live in or on the sediment.

Form	Species	Exposure duration	Suspension preparation	Concentration	Presence of NOM[c]	Effects	Reference
MWCNTs[a]	*Leptocheirus plumulosus*	10 d	functionalized then stirred	99 g/L	-	reduced mortality	Kennedy et al. 2008
MWCNTs	*Leptocheirus plumulosus*	10 d	functionalized then stirred	30 g/kg	-	reduced mortality	Kennedy et al. 2009
MWCNTs	*Hyallela azteca*	14 d	acid treatment then sonication	1 g/L	-	reduced mortality and biomass, unable to excrete MWCNTs after depuration	Mwangi et al. 2013
MWCNTs	*Hyallela azteca*	10 d	functionalized then stirred	264 g/L	+	reduced mortality, but carbon black and activated carbon were more toxic	Kennedy et al. 2008
MWCNTs	*Hyallela azteca*	10 d	functionalized then stirred	300 g/kg	+	50% reduced mortality only when MWCNTs were sonicated	Kennedy et al. 2009
SWCNTs[b]	*Hyallela azteca*	14 d	acid treatment then sonication	1 g/L	-	reduced mortality and biomass, unable to excrete MWCNTs after depuration	Mwangi et al. 2013
SWCNTs	*Leptocheirus plumulosus*	28 d	dispersed 2% w/v SDC using sonication	100 µg/g	-	measurable body burdens in sediment exposed before and after depuration	Parks et al. 2013

[a]MWCNTs=multi-walled carbon nanotubes
[b]SWCNTs=single-walled carbon nanotubes
[c]NOM=natural organic matter

Table 5.3. Published data on carbon nanoparticle exposures to marine crustaceans that live in or on the sediment.

Form	Species	Exposure duration	Suspension preparation	Concentration	Presence of NOM[c]	Effects	Reference
MWCNTs[a]	*Americamysis bahia*	7 d	acid treatment then sonication	5 mg/L	+/-	reduced sexual maturity, measured accumulation in higher concentrations	Chapter 3
SWCNTs[b]	*Americamysis bahia*	7 d	dispersed 2% w/v SDC using sonication	10 µg/g	-	no effect on survival	Parks et al. 2013
SWCNTs	*Americamysis bahia*	7 d	dispersed 2% w/v SDC using sonication	100 µg/g	-	did not accumulate or cause toxicty, were easily excreted, exposed in sediment or food	Parks et al. 2013
SWCNTs	*Amplelisca abdita*	7 d	dispersed 2% w/v SDC using sonication	10 µg/g	-	no effect on survival	Parks et al. 2013
SWCNTs	*Amplelisca abdita*	7 d	dispersed 2% w/v SDC using sonication	100 µg/g	-	detectable body burdens prior to depuration in food exposures, readily excreted	Parks et al. 2013

[a]MWCNTs=multi-walled carbon nanotubes
[b]SWCNTs=single-walled carbon nanotubes
[c]NOM=natural organic matter

CHAPTER 6

CONCLUDING REMARKS

SUMMARY

In the preceding chapters, I attempted to bridge some of the important data gaps associated with lack of information available to create a useable risk assessment framework for future regulation of multi-walled carbon nanotubes (MWCNTs). We determined the chronic toxicity and accumulation potential of MWCNTs to three aquatic species: *Ceriodaphnia dubia*, *Americamysis bahia*, *Pimephales promelas* in the presence and absence of natural organic matter (NOM). These three species were chosen as test species because of their traditional use in standardized toxicity testing and their ecological relevance as important food sources in the aquatic food chain. We also analyzed the literature and provided the first evaluation for predicted modes of MWCNT toxicity to crustaceans.

In a rapidly advancing field such as nanoecotoxicology, the amount of information gathered in a year is enough to change the traditional school of thought. Determining that NOM alters the behavior of MWCNTs in aquatic environments (Hyung et al. 2007, Hyung and Kim 2008, Chappell et al. 2009) as well as toxicity to aquatic organisms (Chapters 2 and 3) can be considered a "paradigm shift" in the way future experiments will be conducted in this research realm. When *C. dubia* were exposed to 2.5 mg/L MWCNTs, we observed a decrease in brood number and clutch size. However, in the presence of 4.5 mg/L natural organic matter, there was no reproductive toxicity at any

concentration tested, up to 5 mg/L. Although the presence of natural organic matter appeared to be protective against reproductive toxicity, we did measure an increase in accumulation within the *C. dubia* compared to no addition of natural organic matter (Fig 2.5).

The observations made in Chapter 2 led us to question if the results are comparable in an estuarine crustacean. Interestingly, unlike the protective role of NOM against reproductive toxicity for *C. dubia*, exposure to 5 mg/L MWCNTs in natural seawater containing 13.5 mg C/L caused a 59.5% decrease in the percentage of mature *A. bahia*. However, similar to exposures with *C. dubia*, we observed that the presence of NOM caused an increase in MWCNT accumulation compared to *A. bahia* exposed to synthetic seawater, which contained no NOM (Fig 3.3). Therefore, we conclude that the behaviors of MWCNTs appear to be similar in freshwater and saltwater in the presence of NOM, but sensivities are species-specific and not directly comparable.

When we started this research project, we assumed that a modified traditional United States Environmental Protection Agency (EPA) toxicity test would provide the best information for the data gaps mentioned in Chapter 1. However, as our research progressed from exposing small crustaceans (Chapters 2 and 3) to vertebrates (Chapter 4), we realized that a non-traditional approach might provide more environmentally realistic data on the behavior and toxicity of MWCNTs. Consequently, we adjusted our experimental design to reflect this idea. *P. promelas* were exposed to sediment contaminated with a range (0-50 mg/kg) of MWCNTs. We used inclusion netting on half of the replicates to determine if *P. promelas* rooting behavior affected the movement of MWCNTs into the water column or if MWCNTs move into the water column on their

own. We discovered that when *P. promelas* had access to the sediment the concentration of MWCNTs that moved into the water column increased; however, inclusion netting had no effect on the morbidity seen in exposed fish, suggesting that solubilized MWCNTs may have more of an effect on fish overall fish health. Particle-bound MWCNTs may have played a role in the gut impaction we observed in exposed fish. Although we did not observe MWCNTs aggregating in *P. promelas* bodies, *C. dubia* exposed to NOM-dispersed MWCNTs had black aggregates in the gut tract (Fig. 2.3). We hypothesize that particle-bound MWCNTs may be viewed by organisms as a food source, increasing the concentration in the gut tract.

The last chapter (Chapter 5) was originally planned to be a risk assessment framework as an approach for future regulation of MWCNTs. However, a thorough inquiring of the literature revealed that scientists are still years away from creating a usable risk assessment framework. The point of this critical review was to analyze all published data related to carbon nanoparticles, add our own findings, and determine what data gaps were still left and why. One of the biggest data gaps is exposure information on marine crustaceans that live in the water column. We determined that marine exposure data are still lagging behind freshwater data, although compared to toxicity data for other chemicals, the information that is available on both is miniscule. One of the most important findings we reported was a complete list of the predicted modes of toxicity to crustaceans after exposure to MWCNTs from our observations and those in the published literature. One of the predicted modes of toxicity, inhibition of molting, is unique to crustaceans, and therefore they should be considered sensitive, target organisms for MWCNT toxicity. Further research is needed to increase our understanding of the

underlying mechanisms of toxicity, but using Chapter 5 as a database of information is certainly a start.

LIMITATIONS AND FUTURE APPROACHES

Problems encountered with ^{14}C-labeled materials

The ability to use radiolabeled MWCNTs was both a blessing and a curse: while we were able to accurately track the movement of ^{14}C-labeled MWCNTs within exposure media and organisms in our studies, we had many debilitating factors associated with use of radioactive materials. For example, we received some information on the characterization of the MWCNTs (Petersen et al. 2008), but we were unable to obtain more information ourselves through transmission electron microscopy (TEM) or any other analysis typically used in characterization of materials because of ^{14}C-contamination issues. Contamination issues also created a problem at the Charleston NOAA lab, as certain laboratory equipment such as scales and a microscope with a camera attachment were unavailable for use with radioactive materials (Chapter 3). Not having access to this equipment meant that we were not able to collect some important data that would have been useful for comparison to *A. bahia* exposed to synthetic seawater. One of the most disappointing limitations with the use of radioactive materials in our research project was that we could not properly identify the pathogenic cause of the growth seen on gills from *P. promelas* exposed to MWCNT-contaminated sediment (Fig 4.5). Identifying a specific pathogen associated with exposure would have been advantageous to our research, as this information would have permitted us to continue more in-depth investigations in the literature.

Having a finite amount of ^{14}C-labeled materials meant that our scope for studies was extremely limited, and many times we could not afford (material-wise) to repeat experiments or investigate further when we found compelling data. Both the amount of test materials needed to explore and limitations based on the radioactivity of the materials hindered research. For example, several other *C. dubia* exposures were completed using different dispersion techniques, but were not included in Chapter 2 because we did not feel comfortable reporting only one study from each unique dispersion method.

Standardized toxicity testing

As mentioned previously, we first attempted to modifiy existing EPA protocols to create experimental designs for MWCNTs exposures. EPA protocols are clearly defined and have been in use for decades. At first glance, the largest benefit to MWCNT exposures incorporating EPA protocols is that there would already be a framework established for future regulations. However, as we collected more data and our experiments progressed into more complex work, we began to see that this "benefit" might actually be a hindrance in discovering the true behavior of MWCNTs in the aquatic environment. For example, one of our failed attempts made us reassess our thought processes towards utilizing traditional toxicity tests. A good idea in essence, we attempted to use a Microtox assay, which calculates an EC50 in a matter of hours by employing bioluminescent marine bacteria (*Vibrio fisheri*) and measuring a change in light output. This is a comparatively cheap toxicity test, extremely fast, and is accepted as a standardized test method by the EPA for discharge, effluent, and other wastes. Unfortunately, repeated tests were not sensitive enough to detect toxicity. Increasing the

concentration of the extremely dark MWCNTs was a confounding factor in our ability to measure the bioluminescence and confer toxicity to *V. fisheri*. Many other researchers have observed sublethal effects that are non-traditional endpoints (Blickley and McClellan-Green 2008, Zhu et al. 2008, Cheng et al. 2009). The change in strategy, moving from traditional toxicity test to more complex designs and endpoints is evident in the experimental design in Chapters 2 and 3 (*C. dubia* and *A. bahia*) compared to Chapter 4 (*P. promelas*). Although we believe that standardized MWCNT exposure protocol is extremely important to be able to make comparisons between exposures, this does not necessarily mean that standard EPA protocols are the best choice. In tests with *P. promelas* (Chapter 4), we observed that innate immune system and gills of exposed fish may have been affected by exposure to MWCNTs, and to continue research on that hypothesis will require a departure from traditional approaches.

Hindsight

While we remained current on literature published on the topic, one of the downfalls was that "current" technically described research was actually completed several years prior, with the amount of time taken to write and publish. One of the strategies that did not become popular until several years into our research was to include a depuration period. Authors had completed this extra step in the past (Petersen et al. 2008, Petersen et al. 2009), but it was not until many started reporting that organisms had the ability to excrete the MWCNTs that a depuration period was deemed vital. This new-found knowledge was incorporated into our research in Chapter 4, where we included a 24 h depuration period and, ultimately, this process changed the interpretation

of our data. While we collected timely and relevant data when exposing *C. dubia* and *A. bahia* to MWCNTs, perhaps we would have been able to distinguish between accumulation and bioaccumulation if we had also included a depuration period in each of these experiments.

CONCLUSIONS

Although there are still many hurdles to go through (e.g. standardized protocol, detection in complex media), we believe that the information reported here adds to the existing knowledge of MWCNT behavior and toxicity in the aquatic environment. We hope that other scientists in the field can use this information to build on the observations we noted and continue to fill in data gaps on exposures to aquatic organisms. We also believe it is important to increase environmental realism in experiments. With every experiment, the knowledge of ecology and behavior of the test organisms became more important. We have concluded that if the concentration of MWCNTs were high enough in the aquatic environments where *C. dubia*, *A. bahia*, or *P. promelas* reside, there could be negative effects on the stability of natural populations. As MWCNTs are a futuristic type of material, toxicity testing should reflect that by incorporating advanced testing approaches (such as the fields of genomics and metabolomics) combined with the knowledge gained in our exposures. Future testing should include more sensitive endpoints (such as molecular, cellular, or behavioral) that would better predict MWCNT toxicity.

REFERENCES

Blickley TM and McClellan-Green. 2008. Toxicity of aqueous fullerene in adult and larval *Fundulus heteroclitus. Environ. Tox. Chem.* 27(9):1964-1971.

Chappell MA, Georgia AJ, Donstova KM, Porter Be, Price CL, Zhou P, Morikawa E, Kennedy AJ, Steevens JA. 2009. Surfactive stabilization of multi-walled carbon nanotube dispersion with dissolved humic substances. *Environ. Pollut.* 157:1081-1087.

Cheng J, Chan CM, Veca LM, Poon WL, Chan PL, Qu L, Sun Y-P, Chen SH. 2009. Acute and long-term effects after single loading of functionalized multi-walled carbon nanotubes into zebrafish (*Danio rerio). Tox. Appl. Pharm*. 235:216-225.

Hyung H, Fortner JD, Hughes JB, King JH. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ. Sci. Technol*. 41:179-184.

Hyung H and JH Kim. 2008. Natural organic matter (NOM) adsorption to multi-walled carbon nanotubes: effect of NOM characteristics and water quality parameters. *Environ. Sci. Technol*. 42:4416-4421.

Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ Jr. 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ. Sci. Technol*. 43(8):2969-2975.

Petersen EJ, Huang QG, Weber Jr. 2008. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus*. *Environ. Health Perspect*. 116(4):496-500.

Zu X, Zhu L, Lang Y, Chen Y. 2008. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environ. Tox. Chem*. 27(9):1979-1985.