AQUATIC TOXICITY OF ONE DIMENSIONAL CARBON NANOMATERIALS

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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ABSTRACT

This research determined the toxicity of one dimensional carbon nanomaterials (CNMs) to benthic invertebrates. The study hypothesized that one-dimensional CNMs in water and sediment were toxic to aquatic organisms and that toxicity was due to metals solubilized from CNMs upon contact with water or sediment, the CNMs with metals or CNMs without metals, and that factors affecting toxicity include sonication, type and sources of CNMs, and sediment characteristics. Tests were conducted with as-produced or modified carbon nanotubes (CNTs) from commercial sources or silicon carbide nanowires (SiCNW). There were three primary studies: (1) toxicity to aquatic invertebrates of SiCNW in water or sediment exposures, (2) toxicity to aquatic invertebrates of CNT in water exposures, and (3) toxicity to aquatic invertebrates of Multi-walled CNT (MWCNT) in sediment exposures. The amphipod *Hyalella azteca*, the midge *Chironomus dilutus*, the oligochaete *Lumbriculus variegatus* and mussels *Lampsilis siliquoidea* or *Villosa iris* were selected as representative test organisms because they are typically used in toxicity testing of contaminants in water and sediment. In the SiCNW study, acute 48-h exposures to sonicated and non-sonicated SiCNW were conducted with amphipods and 96-h exposures to sonicated SiCNW were conducted with midge, oligochaetes and juvenile mussels. In addition, 10-d exposures of amphipods to sonicated SiCNW layered on a sediment surface or mixed with sediment with the daily replacement of the overlying water were performed. In the CNT water study, short-term 14-d water-only tests were conducted by exposing amphipods, midge, oligochaetes, or mussels to a thin layer of CNTs with the periodic replacement of water.

In the MWCNT sediment study, 14- and 28-d whole-sediment toxicity tests were conducted by exposing amphipods to MWCNTs spiked into eight reference sediments (99:1 sediment to MWCNTs on a dry weight basis) also with the periodic replacement of the overlying water. The sediments evaluated in the MWCNTs spiking study had different amounts of total organic carbon (TOC) and acid volatile sulfides (AVS), which could affect the toxicity or distribution of MWCNTs in the sediment and could also affect the potential toxicity associated with dissolved metals associated with the MWCNTs.

In the SiCNW study, sonicated SiCNW were toxic to the amphipods but not to the midge, oligochaetes or mussels. The non-sonicated SiCNW were not toxic to amphipods in acute water exposures. The survival of amphipods exposed to sonicated SiCNW layered on the sediment surface or mixed in with the sediment was not significantly different from amphipod survival in the control. However, the amphipods growth was significantly reduced in both exposures to SiCNW, layered on the sediment surface and mixed in with the sediment, relative to the growth in the control sediment without the addition of SiCNW. In the CNT water-only study, the survival of the invertebrates was significantly reduced in three as-produced CNTs but not in two modified CNT samples (i.e., cleaned with nitric acid and washed with water or mixed with a metal complexing agent) relative to the control. In most cases, the growth of the test organisms was also significantly reduced with exposure to CNTs. During the exposures of the organisms to the CNTs, they were coated with the CNTs and they also ingested and accumulated it in their guts.

In the CNT sediment-spiked study, the survival of the amphipods was typically not reduced. However, the biomass of amphipods was significantly reduced in three out of the eight sediments spiked with CNTs compared to the control sediment. The metal concentrations in the overlying water also were only slightly elevated in the spiked sediments relative to the concentrations in the control sediment. These results show that while metals may be released from the MWCNTs, the binding capacity of the evaluated sediments was likely sufficient to limit the bioavailability of the metals to the amphipods during exposures. Specifically, MWCNTs spiked in sediments with less than approximately 1% TOC or with a high percentage of sand were toxic to amphipods. These results demonstrate that growth was a more sensitive endpoint than survival for the amphipods. The 14-d whole sediment tests also indentified sensitive sediments where 1% MWCNTs spiked in sediment reduced the growth of amphipods but not significantly relative to amphipods exposed to control sediments. The 14-d tests again identified growth as a more sensitive endpoint than survival of the amphipods exposed to CNTs in sediments. The 28-d whole sediment exposures were conducted with selected sensitive sediments. The 28-d tests are relevant in the assessment of the environmental impact of the CNTs because they are hydrophobic and may accumulate in sediments with the potential to adversely affect the growth of amphipods. In the 28-d whole sediment tests with two sensitive sediments, amphipod growth was significantly reduced in exposures to 1% MWCNTs spiked in sediment relative to amphipods in control sediment and demonstrated that growth was a more sensitive endpoint.

Overall, the toxicity of the CNMs (CNTs or SiCNW) appears to be the effect of the coating of respiratory surfaces or the blocking of the digestive tract of the exposed benthic invertebrates. The CNTs appear to smother the organisms and may interfere with their ability to feed. The metals dissolution from the as-produced CNTs could also have contributed to the toxicity. The toxicity test results with the selected CNMs, test organisms and the sediments do not disprove the study hypothesis.

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ACRONYMS

ASTM	American Society for Testing and Materials
AVS	Acid-volatile sulfides
$CaCO_3$	Calcium carbonate
CCC	Criteria continuous concentration
CERC	Columbia Environment Research Center
CMC	Criteria maximum concentration
CNMs	Carbon nanomaterials
CNTs	Carbon nanotubes
Co	Cobalt
CVD	Chemical vapour deposition
DLS	Dynamic light scattering
DWCNTs	Double-walled carbon nanotubes
EC20	Effective concentration at 20%
EDS	Energy-dispersive X-ray microanalysis system
ENMs	Engineered nanomaterials
Fe	Iron
LC50	Median lethal concentration at 50% mortality
Mo	Molybdenum
MWCNTs	Multi-walled carbon nanotubes
Ni	Nickel
NNI	National nanotechnology initiative
NSTS	National science and technology council
OECD	Organisation for Economic Co-operation and Development
Si	Silicon
SI	Supporting information
SiCNW	Silicon carbide nanowires
SiO_2	Silica
SWCNTs	Single walled carbon nanotubes
TOC	Total organic carbon
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
WQC	Water quality criteria
YCT	Yeast-Cerophyll-Trout Chow

CHAPTER 1

INTRODUCTION

Nanomaterials and nanotechnology

Nanomaterials (NM) are natural or engineered matter that is less than 100 nanometers in size in more than one dimension (ASTM 2009b). There are two types of NM; those that occur naturally (e.g., clay, organic matter and iron oxides) (Klaine et al. 2008) and engineered nanomaterials (ENMs) for specific applications. ENMs include: (1) carbon nanomaterials (CNMs), (2) metals and metal oxides, (3) semi-conductor nanocrystals also known as quantum dots, (4) zero-valent metals produced by reduction of solutions of metal salts (e.g., reduction of ferric (Fe (III)) or ferrous (Fe (II)) salts with sodium borohydride), and (5) dendrimers which are multi-functional polymers (Klaine et al. 2008). The ENMs are produced by size reduction of larger materials or are grown from simple molecules (Manocha et al. 2004, Christian et al. 2008).

"Nanotechnology is the science of designing, synthesizing, modifying and manipulating ENMs to create efficient, cheaper and more targeted products or materials (NNI 2008)". Nanotechnology applications have expanded to almost all aspects of daily life (Dekkers et al. 2007) with an estimated global economic impact in industrial, consumer and medical products at $292 billion in 2010 and projected to reach $1 trillion by 2015 (Tinkle 2008). This volume of trade in nanotechnology illustrates the potential for environmental dispersion of the ENMs through intentional and unintentional releases including diffuse releases associated with wear and erosion from the general use of consumer products containing the ENMs (Nowack and Bucheli 2007).

Increased production of ENMs will increase the likelihood of the release of these materials into aquatic systems. CNMs are among the most produced and used in nanotechnology and therefore have attracted great focus in the field of aquatic toxicology (Klaine et al. 2008).

Carbon nanomaterials

CNMs include carbon nanotubes (CNTs) and silicon carbide nanowires (SiCNW) (Hurt et al., 2006). These CNMs have a large aspect ratio (length/diameter) resulting in a nearly one-dimensional structure (Donaldson et al. 2006, Daenen et al. 2003).CNTs are composed of single-walled carbon nanotubes (SWCNTs) or multiple concentric cylinders (multi-walled carbon nanotubes (MWCNTs)) of graphene ideally closed at each end by half a fullerene (Donaldson et al. 2006). The CNTs are commercially produced using heavy metal catalysts and substrates include silica (SiO_2) (Grobert 2007, Donaldson et al. 2006). The CNTs have low mass density, high electrical and magnetic properties, high thermal conductivity, and good mechanical properties (Eklund et al. 2007). The potential applications of CNTs include electronics, optics, materials science, automotive s, textiles, biomedical sciences and biotechnology (Eklund et al. 2007, Dekkers et al. 2007, Daenen et al. 2003, Polizu et al. 2006). SiCNWs are synthesized using SiO_2, silicon (Si), and MWCNTs (Liu and Yao 2005, Tang et al. 2000). Potential uses include electronics composites, polymers, and dental and orthopedic implants (Allen et al. 1995, Xu 2003).

The presence of CNTs and SiCNW in the aquatic environment might adversely affect the health of aquatic organisms. CNTs are distinct from macroscale forms of carbon (USEPA 2008).

Toxicity of chemicals in biological organisms

Toxicology is the science of harmful and deleterious effects of chemicals on living organisms (Hoffman et al. 2002), while toxicants are agents that cause damage to the structure or functions of an organism or actually cause death (Rand and Petrocelli 1985). Toxicants could change the water and or sediment characteristics resulting in unfavorable conditions for aquatic life and there are procedures developed by the American Society for Testing and Materials (ASTM) and the Environmental Protection Agency (EPA) for conducting aquatic toxicity tests in water or sediments (ASTM 2009, EPA 2000). The aquatic toxicity tests evaluate the responses of aquatic species to exposures of suspected contaminants in water and or sediments. The testing principle assumes that the contaminants change the physical-chemical equilibrium of the aquatic system thereby causing stress and physiological effects in the test organisms (Adams and Rowland 2002).

Acute and chronic tests are the two types of aquatic toxicity tests performed. Acute tests evaluate the responses of aquatic test organism to toxicants after short duration exposures (e.g., 24 to 96 hours depending on the species). The objectives of acute tests are to determine the upper limit concentrations of toxicants, to evaluate relative toxicity, and for determining dose responses to chemicals.

Mortality is the endpoint of the acute tests. Chronic tests are conducted for days or longer durations to evaluate sub-lethal effects test to test organisms from chemical exposures which may cause physiological and/or biochemical disruptions of the life cycle leading to behavioral, developmental, or population level effects (Ingersoll et al. 1990). Survival, growth and reproduction are usually the endpoints of the chronic tests.

Cytotoxicity of carbon nanotubes

CNTs in direct contact with cell membranes have been reported to cause cytotoxicity (Kang et al. 2007) and CNTs have the potential to cross cell membranes (Pulskamp et al. 2007). The exposure of mice embryonic stem cells to MWCNT caused cytotoxicity (Zhu et al. 2007) and exposure from SWCNT into the alveolar macrophage produced inflammatory responses and the formation of granuloma (Chou et al. 2008). SWCNT intratracheally instilled into mice for 7-d using carbon black and quartz as reference toxicity standards showed that SWCNT were more toxic than carbon black or quartz on an equal-weight basis (Lam et al. 2004) and MWCNT intratracheally instilled into guinea pigs produced pulmonary toxicity with multiple lesions (Yu et al. 2008). *In vitro* exposures to SWCNT and MWCNT by murine macrophage cell line RAW 264.7 was cytotoxic and caused oxidative damage to DNA (Bergamaschi 2010). An investigation on the effects of CNT length on human acute monocytic leukemia cell line THP-1 showed that longer CNTs induced higher degrees of inflammation than short lengths, probably because the shorter lengths were more readily enveloped by macrophages (Sato et al. 2005).

A study on the effects of purity of CNTs was performed with pristine and purified SWCNT exposure to human macrophage cells and has shown that pristine SWCNT was cytotoxic while purified SWCNT was not (Cherukuri et al. 2004). It has been reported that exposure to purified SWCNT by human keratinocyte cells caused oxidative stress, and a decrease in cell viability compared to the control (Tejral et al. 2009), while exposure to purified MWCNT by human embryonic kidney cells was cytotoxic (Monteiro-Riviere et al. 2005).

The murine lung macrophage cell line exposed to SWCNT and MWCNT with asbestos and carbon black as toxicity reference standards were both toxic relative to the asbestos and carbon black (Murr et al. 2005). Pulskamp et al. (2007) incubated human lung cells with commercial SWCNTs and MWCNTs, carbon black and quartz as reference materials and also with acid-treated SWCNT and reported that none of the CNTs were toxic to cells. However, a dose- and time-dependent increase of intracellular reactive oxygen species and a decrease of the mitochondrial membrane potential with the commercial CNTs occurred, but these effects were not observed with purified CNTs suggesting that metal impurities in the commercial CNTs caused cytotoxicity. Studies compared the cytotoxic effects of human MSTO-211H cells exposed to dispersed CNTs, agglomerated CNTs and asbestos as a reference and show that dispersed CNTs were the most cytotoxic followed by asbestos, while agglomerated CNTs were the least cytotoxic at the same concentrations (Wick et al. 2007). These studies, however, have used different cell lines, culturing conditions, and incubation times, which makes it difficult to compare the results.

Toxicity of carbon nanomaterials to aquatic organisms

With the increasing production and applications of CNMs, the release of these materials into the aquatic environment from production facilities, landfills, industrial and domestic waste water, the atmospheric and the wear of consumer products is inevitable (Organisation for Economic Cooperation and Development (OECD) 2006, Nowack and Bucheli 2007). CNMs generally are more reactive than the bulk forms and potentially could adversely affect the health of aquatic organisms (Dhawan et al. 2009, Chen et al. 2007, Fenoglio et al. 2007, Panessa-Warren et al. 2006, Borm et al. 2006).

In the aquatic environment, CNMs might be accumulated by aquatic biota and eventually by humans through the food chain (Figure 1.1). For example, amphibian larvae *Ambystoma mexicanum* ingested DWCNTs during exposures (Mouchet et al. 2007), copepod *Amphiascus tenuiremis* and polychaete *Streblospia benedicti* ingested SWCNTs spiked in sediments (Ferguson et al. 2008) and oligochaetes (*L. variegatus*) ingested SWCNTs and MWCNTs during exposures (Petersen et al. 2008).

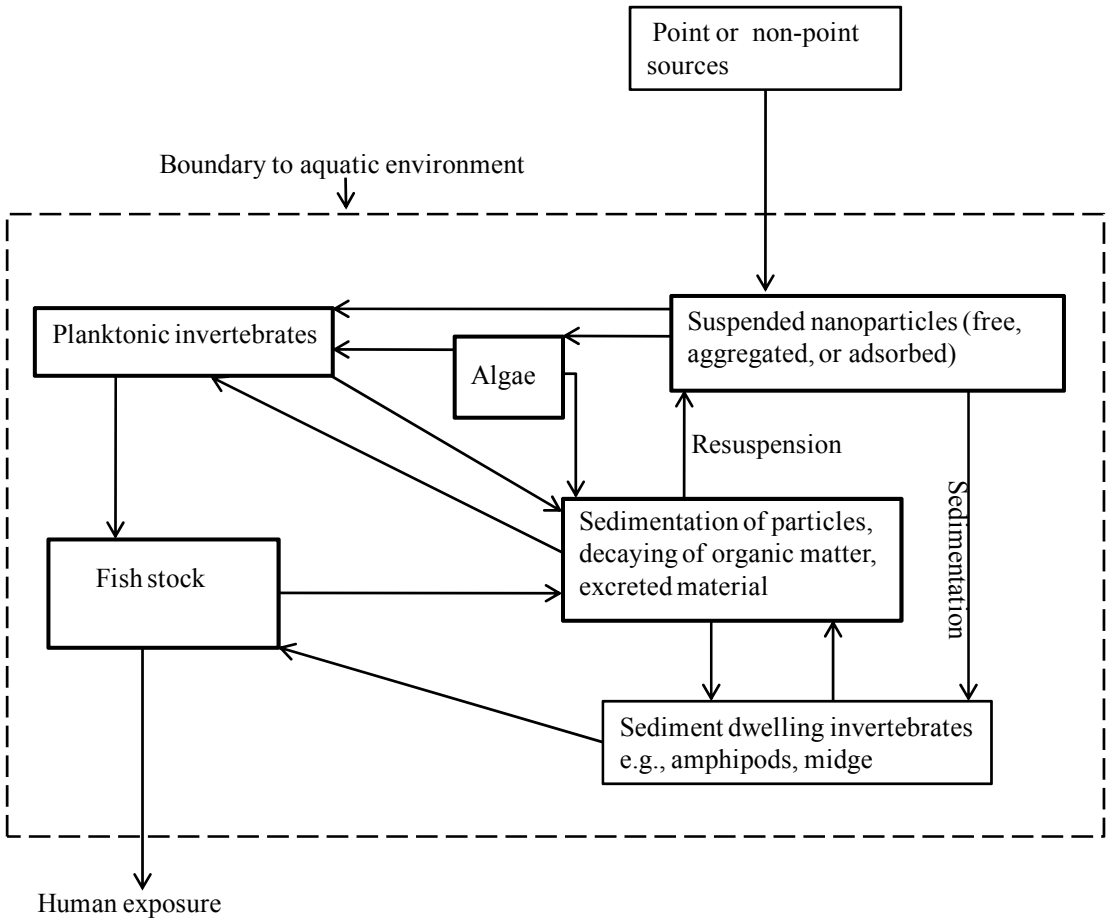

Figure 1.1. Potential exposure routes of engineered nanomaterials to aquatic invertebrates and significance in the aquatic food web (adapted from Baun et al. 2008)

Studies indicate that size, surface area, surface structure/shape, solubility and dissolution, reactivity, coagulation or aggregation, resistance to wear and tear, degradability/stability, chemical composition and purity of ENMs influence their toxicity to organisms (Dekkers et al. 2007, Nowack and Buchelli 2007). The influence of size of CNMs in toxicity to aquatic organisms has been illustrated in several studies.

Templeton et al. (2006) exposed estuarine copepod *Amphibiascus tenuiremis* to different size fractions of SWCNT suspensions and observed size dependent toxicity where the smaller size fractions were more toxic than larger particles. Similarly, the amphipods *L. plumulosus* and *H. azteca* exposed to different sizes of carbon nanoparticles spiked into sediment showed that mortality increased with decreases in particle sizes (Kennedy et al. 2008). A study on exposures of green algae *Pseudorkirchneriella subcapitata* to silica nanoparticles reported 20% effective concentrations for growth rate (EC20) of 20 mg/L for particles with 12.5 nm and 29 mg/L with 29 nm diameter particles while bulk silica was not toxic at exposures up to 1 g/L (Hoecke et al. 2008).

It is expected that nanomaterials will undergo transformations in the environment during their lifetime. The modifications of the nanomaterials could change their effects on biological organisms in the aquatic environment. Kennedy et al. (2008) investigated the influence of engineered surface modifications of CNTs in aquatic systems. In this study, *Ceriodaphnia dubia* were exposed to raw MWCNTs, MWCNT-OH or MWCNT-COOH and it was reported that a significant reduction in survival occurred with raw MWCNTs relative to the control, while the exposures to MWCNT-OH and MWCNT-COOH did not significantly reduce the survival relative to the control (Kennedy et al. 2008). This suggests the introduction of –OH or –COOH to the surface of the raw MWCNT reduced its toxicity.

The above effect is not a general pattern with all CNTs as illustrated with *Daphina magna* exposure to nontoxic lysophosphatidychlorine coated SWCNTs where the daphinds removed the coat on the SWCNTs after ingestion and excreted uncoated SWCNTs which were toxic to the daphnids (Roberts et al. 2007). The mechanisms of toxicity of ENMs to aquatic organisms remain unclear. CNTs toxicity has been partly attributed partly to metal impurities (Zhu et al. 2009; Pulskamp et al. 2007). Studies illustrate that the potential toxic route of the ENMs to aquatic organisms could be via the gut (Baun et al. 2008).

A key issue in aquatic toxicity of ENMs has been the protocol to prepare test exposures because of effects of aggregation and settling from the water column resulting in dynamic exposure concentrations in the bioassay (Klaine et al. 2008). CNMs dissolve poorly in water and tend to form aggregates. Sonication (see Roberts et al. 2007), stirring (see Oberdorster et al. 2006) and surfactants (see Lovern and Klapper 2006, Henry et al. 2007) have been applied individually or in combinations in toxicity testing of NM to reduce their aggregation and enhance dispersion in the testing media (Oberdorster et al. 2005, Christian et al. 2008, Shelimov et al. 1998). For example, Smith et al. (2007) sonicated SWCNTs in sodium dodecyl sulfate (a surfactant) in exposures to rainbow trout (*Oncorhynchus mykiss*). However, it is felt that the use of surfactants in toxicity tests is not an environmentally relevant practice. Sonication is applied in industrial processes in nanotechnology (e.g., to disperse SiCNW into other polymeric materials to make better composites) (Chisholm et al. 2005, Yong et al. 2004).

Research problem and study approach

The effect of CNMs in aquatic systems is largely unknown and currently unpredictable (Tinkle 2008) and there is need for research data to help in regulation and in ecological risk assessment. As types and quantities of products containing CNMs in the marketplace increase, the probability of intentional and unintentional releases into aquatic environment also increases. CNMs have the characteristics of fibers (Sato et al. 2005, Pacurari et al. 2010), are hydrophobic and tend to aggregate (Eklund et al. 2007, Tasis et al. 2006, Jortner and Rao 2002), are biologically non-degradable (HSE 2004), and generally contain heavy metals (Guo et al. 2007, Pulskamp et al. 2007). Their toxic effects have not been well studied. If released into the environment, the organisms dwelling at the sediment-water interface represents aquatic organisms likely to be exposed to CNMs. Data on the aquatic toxicity of these materials are limited. However, recent studies have reported toxicity of these materials to aquatic organisms in water and sediment (e.g., Kennedy et al. 2008, Zhu et al. 2009).

The objectives of the current study are to investigate the toxicity of one-dimensional CNMs toward aquatic organisms that inhabit sediment-water interfaces and to identify factors controlling the toxicity to these organisms. CNTs and SiCNW were used as representative one dimensional CNMs and their toxicity is determined by adapting methods for conducting toxicity tests with sensitive sediment-dwelling organisms including amphipods (*Hyalella azteca*), freshwater mussels (*Lampsilis siliquoidea*), midge (*Chironomus dilutus*) and oligochaetes (*Lumbriculus variegatus*) (ASTM 2009a, EPA 2000).

These aquatic organisms are sensitive to contaminants in water or sediment and have been used extensively in toxicity studies (e.g., Phipps et al. 1995, Borgmann et al. 2005, Burton et al. 2002, Ingersoll et al. 1994, Ingersoll et al. 2000, Besser et al. 2004, Keithly et al. 2004, Wang et al. 2007).

The hypothesis was that toxicity of the one-dimensional CNMs in water and sediment to aquatic organisms is due to one or more of the following factors: (1) metals solubilised from CNMs upon contact with water, (2) physical contact with CNMs with or without metals, (3) sonication, (4) type and sources of CNMs, and (5) sediment characteristics. The goal of the study is to: (1) determine the toxicity of one-dimensional CNMs in water or sediment to aquatic organisms, (2) determine the sensitive end points (e.g., lethality and growth) in the toxicity tests, (3) determine whether toxicity is from the main structure of the CNMs or from metal impurities, (4) determine whether the physical characteristics of sediments influence the toxicity of the CNMs, and (5) determine the relative sensitivities of the selected test organisms towards the CNMs tested.

Studies were conducted in three tiers. Tier 1 screened the test CNMs for toxicity in water-only tests with four benthic invertebrates.

Tier 2 screened the toxicity of CNMs identified as toxic in Tier 1 using whole-sediment toxicity tests and test organisms identified to be sensitive in Tier 1. Tier 3 consisted of conducting dilutions of CNMs in whole-sediment toxicity tests with test organisms used in Tier 2 and the identified toxic sediment concentrations in Tier 2. These tests were conducted following safety precautions outlined in standard operating procedure developed for conducting toxicity tests with ENMs in the laboratory.

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CHAPTER 2

TOXICITY OF SILICON CARBIDE NANOWIRES TO SEDIMENT-DWELLING INVERTEBRATES IN WATER OR SEDIMENT EXPOSURES

ABSTRACT

Silicon carbide nanowires (SiCNW) are insoluble in water. When released into an aquatic environment, SiCNW would likely accumulate in sediment. The objective of this study was to assess the toxicity of SiCNW to four freshwater sediment-dwelling organisms: amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*), and mussels (*Lampsilis siliquoidea*). Amphipods were exposed to either sonicated or non-sonicated SiCNW in water (1.0 g SiCNW /L) for 48 h. Midge, mussels, and oligochaetes were exposed only to sonicated SiCNW in water for 96 h. In addition, amphipods were exposed to sonicated SiCNW in whole-sediment for 10 d (44% SiCNW on dry weight basis of sediment). Mean 48-h survival of amphipods exposed to non-sonicated SiCNW (83% survival) in water was not significantly different from the control (90% survival), whereas mean survival of amphipods exposed to sonicated SiCNW in two 48-h exposures (0 or 15% survival) was significantly different than the control (90 or 98% survival). In contrast, no effect of sonicated SiCNW was observed on survival of midge, mussels, or oligochaetes. Survival of amphipods was not significantly reduced in 10-d exposures to sonicated SiCNW either mixed in the sediment or layered on the sediment surface.

However, significant reduction in amphipod biomass was observed with the SiCNW either mixed in sediment or layered on the sediment surface, and the reduction was more pronounced for SiCNW layered on the sediment. These results indicated that (1) non-sonicated SiCNW in water were not acutely toxic to amphipods, (2) sonicated SiCNW in water were acutely toxic to the amphipods, but not acutely toxic to midge, oligochaetes or mussels, and (3) sonicated SiCNW in sediment did not affect the survival, but reduced growth or biomass of amphipods in 10-d whole-sediment exposures.

INTRODUCTION

Progress in nanotechnology has resulted in the development of many nanoscale structures and devices with unique chemical, physical and biological properties and functions. One of the most active areas is the synthesis of one-dimensional nanomaterials with wide potential applications, such as carbon-based nanotubes, nanofibers, and silicon carbide nanowires (SiCNW) (Jortner et al. 2002, Monacha et al. 2004, Silva et al. 2004, Liu and Yao 2005, Yang et al. 2005). There is little doubt that some of these manufactured nanomaterials will be released into the environment; however, information on the toxicity of nanomaterials and in particular SiCNW to aquatic organisms inhabiting water or sediment is limited (Savage 2005, Service 2003, Maciangioli and Zhang 2003, United States Congress 2003, Sun Innovations 2006).

The SiCNW are a wide band gap semiconductor with high breakdown field strength, high thermal conductivity, high saturation drift velocity, and strong resistance to harsh environments.

These properties make SiCNW a promising material for use in producing high-temperature, high-power, and high-frequency nano-electronic devices (Neudeck 1994, Li et al. 2002). The SiCNW also have high mechanical strength and therefore are a good candidate for use in metal and ceramic matrix composites, as a polymer ingredient, and as materials for dental and orthopedic implants (Allen et al. 1995, Xu 2003). A study on the effects of SiCNW on three human cell lines (macrophages, fibroblasts and bone cells) showed that SiCNW at a concentration of 1.0 g/L were cytotoxic and caused widespread cell death with evidence of cytoplasmic vacuolation and membrane damage in the cells (Allen et al. 1995). Toxicity data of SiCNW to aquatic organisms is not available in the scientific literature.

The SiCNW are insoluble in water. When released into aquatic environment, SiCNW would likely accumulate in sediment (Sun Innovations 2006), thus representing a potential route of exposure to sediment-dwelling organisms. Commercial SiCNW are expected to have a wide range in length distribution. Sonication is commonly used to disperse SiCNW to other polymeric materials to make better composites (Chisholm et al. 2005, Yong and Hahn 2004). Sonication is one approach to separate and break apart long SiCNW into shorter lengths. By comparing the sonicated and non-sonicated SiCNW in toxicity tests, the effect of sizes and also dispersion of the SiCNW on toxicity to aquatic organisms can be evaluated. In this study we aimed to evaluate the effects of sonicated or non-sonicated SiCNW on the survival or growth of four sediment-dwelling invertebrates using water-only or whole-sediment toxicity tests.

MATERIALS AND METHODS

Preparation of silicon carbide nanowires

The SiCNW were synthesized by a chemical vapor deposition (CVD) method using silica (SiO_2; particle sizes: 0.5 to 10 μm; purity : 99%, Sigma Aldrich, Milwaukee, WI USA), silicon (Si; particle size: 325 mesh or <44 μm; purity: 95%; Sigma Aldrich), and multi-walled carbon nanotubes (MWCNTs) as starting materials (diameters: 60 to 100 nm; lengths: 0.5 to 40 μm; purity: 95%; amorphous carbon: 2%; specific surface area: 40 to 300 m^2/g; Helix Material Solutions, Richardson, TX USA). These ingredients were mixed at a MWCNTs:SiO_2:Si mass ratio of 1:2:4, and the mixture was put in high purity alumina crucibles and transferred to a CVD chamber flushed at 100 standard cm^3 per minute argon (Ar)/hydrogen (H_2; 5% H_2; 25°C and 1 atmosphere). The chamber was then heated to 1500°C at 150°C/hour increment and held for 15 hours, when the following reactions occurred to create SiCNW (Neudeck 1994):

$$Si\ (solid_{(s)}) + SiO_{2\,(s)} \rightarrow 2SiO\ (gas_{(g)})$$

$$SiO_{(g)} + 2C_{(s)} \rightarrow SiCNW_{(s)} + CO_{(g)}$$

$$SiO_{(g)} + CO_{(g)} \rightarrow SiCNW_{(s)} + CO_{2\,(g)}$$

The samples were removed from the crucibles when the CVD chamber cooled to room temperature by blowing through the Ar/H_2 gas. The materials had three distinct layers with different colors, which were manually separated.

The top layer was green and composed of high-purity SiCNW, the middle layer was a gray and green mixture of a relatively lower purity of SiCNW, and the bottom layer was a mixture of Si/SiO_2/MWCNTs (Tang et al. 2000, Pan et al. 2000). The relatively pure SiCNW in the top layer were collected, characterized, and used for toxicity testing in the present study.

The as-prepared SiCNW were characterized for morphology, composition and size distribution by Scanning Electron Microscopy (SEM) on a Hitachi S4700 field emission scanning electron microscope and the associated Thermo-Noran energy-dispersive X-ray microanalysis system (EDS), Transmission Electron Microscopy (TEM) on a JEOL-1400 system, X-Ray Diffraction (XRD), and Dynamic Light Scattering (DLS) (Handy et al. 2008, Murdock et al. 2007). The DLS analysis was conducted on Molecular Sizing Instrument (Proteinsolutions, model DynaPro 99, Charlottesville, VA, USA) attached to a micro sampler (Helma 43μL sample cell, light path 3 mm, zentrum height 15 mm).

Test organisms

About 7-d-old amphipods (*Hyalella azteca)*, 10-d-old midge (*Chironomus dilutus)*, and adult oligochaetes (*Lumbriculus variegatus*) were obtained from laboratory culture at U.S. Geological Survey, Columbia Environmental Research Center (CERC), Columbia, MO, USA.

About one-week old (for Test 1) or two-month-old (for Test 2) juvenile mussels (fatmucket *Lampsilis siliquoidea*) were obtained from Missouri State University, Springfield, MO (see Wang et al. 2007) for a description of methods used to culture the mussels). The test organisms were acclimated to test water and temperature for at least 24 h before the start of the toxicity tests (USEPA 2000, ASTM 2009a,b&c, APHA 2005).

Water-only toxicity tests

Because of the largely unknown human health effect of nanomaterials, procedures were developed for safe handling, storage, and disposal of nanomaterials in the testing laboratory (e.g., use of static renewal rather than flow-through exposures to minimize release of nanomaterials in the testing laboratory). Acute toxicity tests were conducted with amphipods, midge, oligochaetes, and mussels using procedures adapted from test methods developed by the U.S. Environmental Protection Agency (USEPA 2000) and American Society for Testing and Materials (ASTM 2009 a, b, c). A day before the start of an exposure, 30 mg of SiCNW was added to each of four replicate 50-ml glass beakers containing 30 ml test water. The test water was ASTM reconstituted hard water (hardness 160 to 180 mg/L as $CaCO_3$, alkalinity 110-120 mg/L as $CaCO_3$ (ASTM 2009 a &b) in Tests 1 and 2 or well water diluted with deionized water to a hardness of about 110 mg/L as $CaCO_3$ in Test 3 and to a hardness of about 140 mg/L as $CaCO_3$ (Table 2.1). The test concentration of SiCNW was 1.0 g/L. This relatively high concentration was selected to screen for acute toxicity because exposures to 1.0 g SiCNW /L reportedly caused cytotoxicity in human cells (Allen et al. 1995).

A thin layer of clean silica sand (US Silica, Berkeley Springs, WV, USA) was also added into each test beaker and control beaker (without the addition of SiCNW), except for tests with mussels because addition of sand would make recovery of juvenile mussels difficult at the end of the exposures.

Samples of non-sonicated SiCNW (as produced) and sonicated SiCNW were evaluated in the water toxicity tests. The sonicated SiCNW were prepared by placing each replicate beaker containing the mixture of SiCNW into a Bransonic Ultrasonic Cleaner Sonicator (Model 251 OR-MTH, Branson Ultrasonic, Danbury, CT, USA) for sonication at 100 watts for 60 minutes. Amphipods were exposed to sonicated or non-sonicated SiCNW for 48 h, while midge, mussels, and oligochaetes were exposed to sonicated SiCNW for 96 h (Table 2.1).

Ten organisms were impartially transferred into each exposure chambers at the beginning of each test and were not fed during the exposures. The exposures were conducted at 23ºC in a temperature controlled water bath under a photoperiod of 16:8 light: dark with a light intensity of about 200 lux. Survival was determined at the end of the exposures based on lack of movement after stimulation with a blunt probe for amphipods, midge, and oligochaetes, or lack of foot movement (within 5-min observation period) for mussels. The test acceptability criterion was at least 90% survival for the controls (USEPA 2000, ASTM 2009a, b&c).

The pH, dissolved oxygen, conductivity, alkalinity, and hardness of the overlying water were measured at the beginning and end of the exposures in composited samples collected from replicate beakers in each treatment following standard methods (APHA 2005). Considering that MWCNTs used in the SiCNW fabrication contained some metal impurities including cobalt (2.5%), molybdenum (0.6%) and nickel (0.5%), water samples were collected for metal analysis from exposure beakers with 1.0 g SiCNW /L (set parallel to Test 4). The metal concentrations were measured by inductively coupled plasma mass spectroscopy (ELAN DRC-e, Perkin-Elmer Sciex, PerkinElmer, Waltham, MA, USA).

Sediment toxicity tests

Two 10-d whole-sediment toxicity tests were conducted with amphipods using procedures adapted from test methods developed by USEPA (2000) and ASTM (2009b). Amphipods were selected because they showed more sensitivity to the SiCNW in the 48-h water-only exposures than the other invertebrates (see the later section of Results). Because of the limited amount of SiCNW available for testing, the sediment exposures were conducted in 50-ml beakers containing about 300 mg of wet sediment and 30 ml of diluted well water. This volume of sediment formed about a 3 ml layer of sediment at the bottom of the beakers. The sediment used in the exposures was collected from West Bearskin Lake in Minnesota, USA (Ingersoll et al. 1998, Ingersoll et al. 2002).

The sediment had 84% moisture content and on a dry weight basis, 49% sand, 19% silt, 32% clay, 9% total organic carbon, 33 μmol/g acid volatile sulfide, 24 μg nickel/g, 34 μg copper/g, 86 μg zinc/g, 0.13 μg silver/g, 0.69 μg cadmium/g, and 14 μg lead/g (Ingersoll et al. 1998, Ingersoll et al. 2002).

In Sediment Test 1, 270 mg of wet sediment was mixed with 30 mg of sonicated SiCNW (the same amount as in water-only tests) in each of four replicate beakers (44% SiCNW based on dry weight of the sediment) by mechanical stirring the sediment for about 5 minutes. About 30 ml test water was added to all the beakers (control and SiCNW treatments), which were then stored at 4ºC for 7 d to equilibrate. One day before the start of the exposures, the beakers were placed in water bath at 23°C to acclimate to test temperature. In Sediment Test 2, 270 mg of wet sediment was added to each of four replicate beakers, followed by adding 30 mg of sonicated SiCNW on the surface of the sediment without mixing. About 30 ml of test water was added into each control and treatment beakers. The beakers were then kept in water bath at 23°C for one day before start of exposures.

Ten amphipods were impartially transferred into each replicate beaker at the start of exposures. The beakers were covered with watch glasses to reduce evaporation and were held in a water bath at 23ºC. Amphipods were fed once a day with 0.25 ml of 1800 mg/L Yeast-Cerophyll-Trout Chow (YCT) (ASTM 2009b). About 20 ml of overlying water in each beaker was removed daily by siphoning and replaced by the same volume of the test water.

The water was added along the side of the beaker with a 5-ml pipette to minimize the suspension of sediment and SiCNW. Partially renewing the overlying water on a daily basis did not suspend the sediment in the exposure beakers. The overlying water at the beginning and end of the exposures was sampled and analyzed for hardness, alkalinity, dissolved oxygen, conductivity, pH, and ammonia following standard methods (APHA 2005). Conductivity and dissolved oxygen were also measured daily in composite water samples from the four replicate beakers per treatment.

Amphipods were isolated from each beaker at the end of the sediment tests by gently swirling the overlying water with the sediment and pouring the mixture into a glass pan. The beakers were rinsed and the water or remaining sediments or organisms poured into the glass pan and the surviving amphipods were counted. The test acceptability criterion was at least 80% survival for the controls (ASTM 2009a, b, c). The test conditions are summarized in Table S2.1.

The surviving organisms were preserved in 8% sugar formalin solution for subsequent length measurement (Ingersoll et al. 2002). The length of amphipods was measured along the dorsal surface from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface using an EPIX imaging system (PIXCI® SV4 imaging board and XCAP software; EPIX Inc., Buffalo Grove, IL, USA) connected to a computer and a microscope (Ingersoll et al. 1998). The biomass of surviving amphipods from treatment replicates was estimated as the sum of individual amphipod weights calculated from the empirical relationship: Weight (mg) = ((0.177* Length (mm)) – 0.0292)^3 (Ingersoll et al. 2008).

Data analysis

The statistical differences between SiCNW treatment and control for mean survival, length, weight, or biomass were analyzed using *t*-test if the data were normally distributed (Shapiro-Wilk's test) and had homogeneity of variances (Bartlett's test). If assumptions of the *t*-test were not met, the Wilcoxon rank sum test was used. The level of statistical significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Sample characterization

The X-ray diffraction pattern indicated that the prepared material were crystalline SiC. Observations made with SEM indicated that most of the SiCNW were straight (Figure 2.1A), as reported in other studies (Li et al. 2006), and grew along the <111> direction (similar to other reported data (Tang et al. 2000), typically with silica and a thin graphite shell of several nanometers (Li et al. 2006).

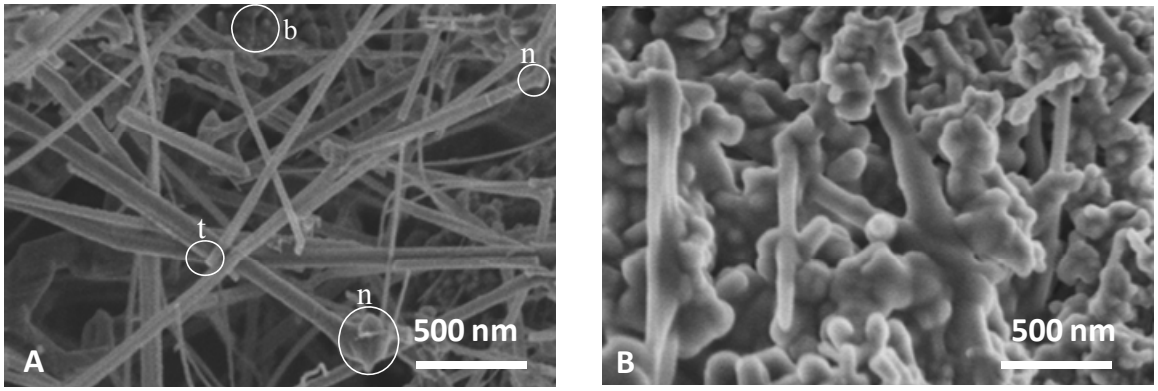

Figure 2.1. Scanning Electron Micrograph images: (A) As-produced silicon carbide nanowires sample with randomly selected locations indicating bulk of sample (b), node (n) and tip (t) of wires targeted in elemental composition analysis, and (B) Aggregated silicon carbide nanowires after one hour of sonication.

The DLS analysis indicated the SiCNW ranged from 40 to 500 nm in diameter (Figure 2.2A) and from 5 to 65 μm in length (Figure 2.2B). About 67% of the SiCNW had diameter of less than 100 nm and more than 78% of the SiCNW were longer than 20 μm. Other studies have reported SiCNW with diameters of 20 to 100 nm and lengths of 1 to 100 μm (Seeger 1999, Wu et al. 2002). The minimum reported length for hazardous fibers that could potentially induce fibrosis and mesothelioma in biological cells is about 20 μm (Oberdorster et al. 2007), indicating that as-prepared SiCNW used in this study were mostly in the length range that had potential for fiber-like toxicity to biological cells.

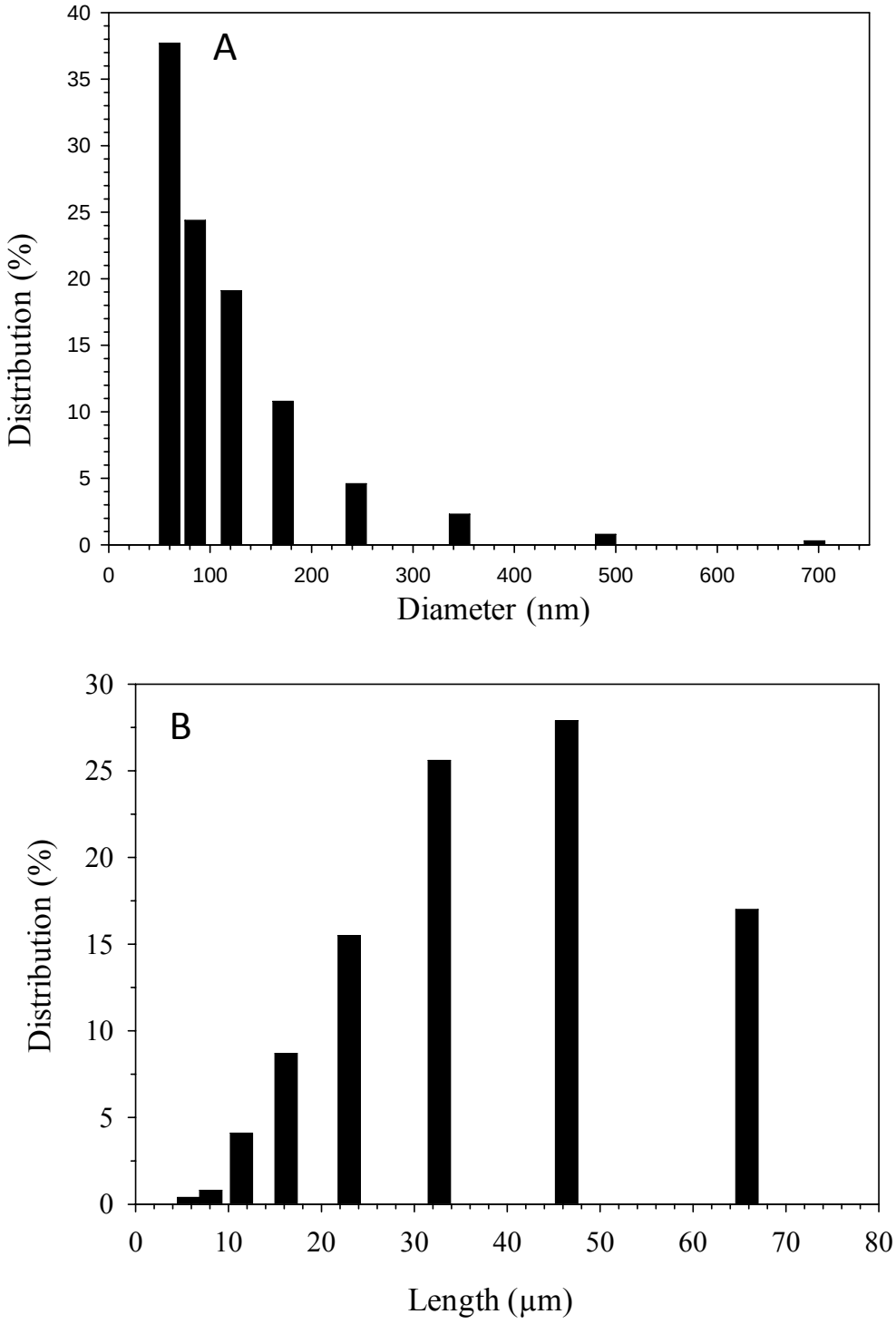

Figure 2.2. Size distribution of as-produced silicon carbide nanowires: (A) Diameter, and (B) Length

The SEM imaging showed that sonication broke down SiCNW into smaller primary particles with lower aspect ratios (Figure 2.1B) and DLS particle size distribution analysis indicated after the SiCNW were broken down they quickly formed aggregates with more than 95% of the aggregates having diameters of over 100 nm (Figure 2.3). It was also observed that the sonicated SiCNW dispersed in water took about 6 hours to settle to the bottom of the beakers after sonication while almost all of the non-sonicated SiCNW settled into clumps to the bottom of beaker in about one hour after addition to the beakers. The SiCNW treatments (sonicated or non-sonicated) were kept for 24 h to allow the suspension of SiCNW to settle to the bottom of the beakers before starting the exposures when the overlying water was clear.

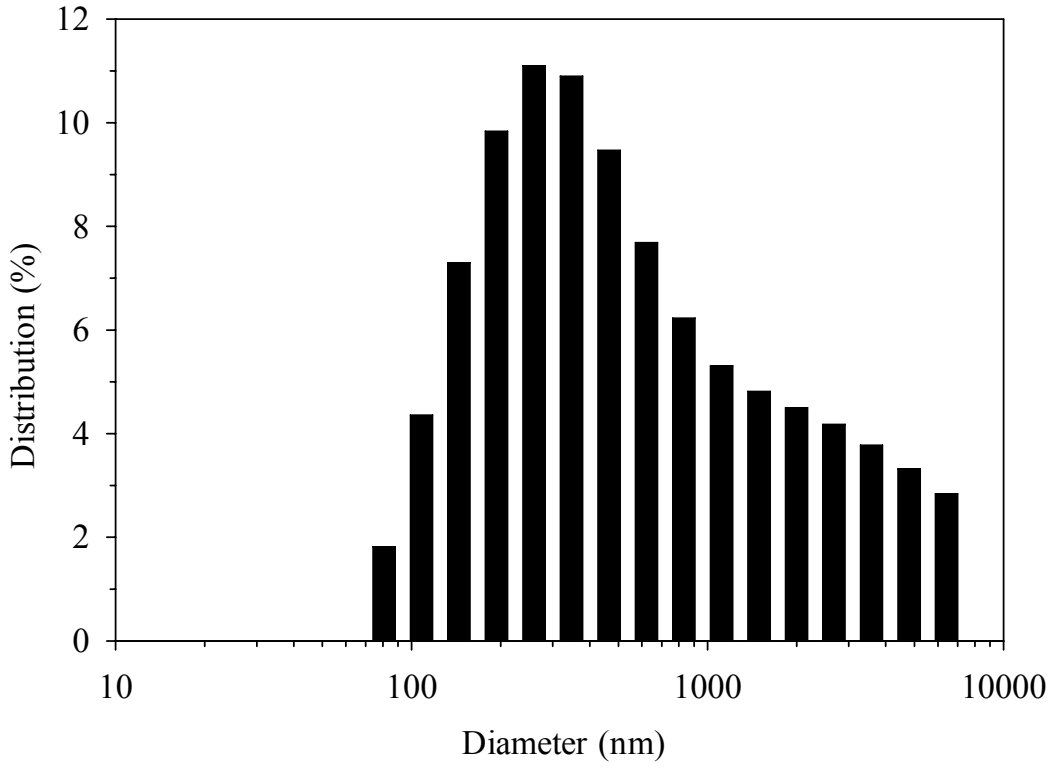

Figure 2.3. Size distribution of sonicated silicon carbide nanowires

The concentrations of metals in overlying water in test treatments were generally low. For example cobalt and molybdenum concentrations in overlying water from treatment in Test 4 were 0.20 µg Co/L and 1.0 µg Mo/L, respectively, which were only slightly higher than the concentrations in the control (0.07 µg Co/L and 0.40 µg Mo/L). These concentrations were not likely toxic to aquatic organisms (e.g., Borgmann et al. 2005, Phipps et al. 1995).

In addition the results of the EDS analysis of the SiCNW sample conducted at the junctions, nodes and tips of the wires (Figure 2.1A) where impurities including metals are usually located and also at random sites in bulk of the sample showed presence of only silicon (57.3%) , oxygen (2.4%), and carbon (40.3%), but not heavy metals indicating the metal concentrations were below detection limits e.g., less than 0.1%.

Water-only toxicity tests

Water quality characteristics were relatively consistent during the exposures (Table 2.1). Mean hardness and alkalinity were within the range of expected concentrations of ASTM reconstituted hard water or diluted well water. The exception was for Test 1 (Table 2.1), where the conductivity, hardness, and alkalinity at the end of the test were higher than expected, probably due to water evaporation from test chambers. Evaporation of water was reduced in subsequent tests by covering the chambers with watch glasses during the exposures. The dissolved oxygen concentration in the exposure water was always >7.0 mg/L.

Table 2.1. Mean water quality characteristics in silicon carbide nanowires (SiCNW) toxicity tests conducted with amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*) and mussels (*Lampsilis siliquoidea)*. Standard deviation in parentheses, n=4

Test	Treatment	Organism	Test duration (d)	Test water	pH	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Water-only tests								
1	Non-sonicated SiCNW	Amphipods	2	ASTM hard[a]	8.5 (0.1)	581(30)	120 (0)	165 (7.1)
	Sonicated SiCNW	Amphipods	2	ASTM hard	8.8 (0.2)	751 (232)	149 (38)	211 (58)
	Sonicated SiCNW	Midge	4	ASTM hard	8.8 (0.1)	833 (348)	173 (72)	245 (106)
	Sonicated SiCNW	Oligochaetes	4	ASTM hard	8.7 (0.2)	849 (371)	71 (69)	251 (115)
	Sonicated SiCNW	Mussels	4	ASTM hard	8.9[b]	587[b]	122[b]	170[b]
2	Sonicated SiCNW	Amphipods	2	ASTM hard	8.5 (0.1)	584 (34)	120 (0)	165 (7.1)
	Sonicated SiCNW	Midge	4	ASTM hard	8.5 (0.1)	602 (59)	125 (7.1)	170 (0)
	Sonicated SiCNW	Oligochaetes	4	ASTM hard	8.4 (0.2)	587 (26)	120 (0)	165 (7.1)
	Sonicated SiCNW	Mussels	4	ASTM hard	8.6 (0.1)	589 (41)	122 (2.8)	168 (2.8)
3	Sonicated SiCNW	Amphipods	2	Diluted well 1	8.3 (0.2)	365 (23)	100 (18)	119 (8.8)
4	Sonicated SiCNW	Amphipods	2	Diluted well 2	8.3 (0.4)	408 (9)	149 (27)	140 (21)
Whole-sediment tests								
1	Sonicated SiCNW mixed in sediment	Amphipods	10	Diluted well 2	8.4 (0.4)	426 (33)	158 (14)	163 (13)
2	Sonicated SiCNW layered on sediment	Amphipods	10	Diluted well 2	8.1 (0)	414 (16)	160 (12)	157 (3.5)

[a]American Society for Testing of Materials (ASTM) reconstituted hard water [23].

[b]Water quality only measured in control treatment at the beginning of the exposures, n=2.

The control survival was ≥90% for all test species in the four tests, except for midge (83% control survival) and mussels (30% control survival) in Test 1 (Table 2.2). Low control survival of midge and mussels may have been due to poor quality of organisms at the start of the exposure or due to the testing of midge in small exposure chambers. Data from these two tests with midge and mussels were excluded from further analysis and discussion. The mean 48-h survival of amphipods exposed to non-sonicated SiCNW in Test 1 was 83%, which was not significantly different from the control (Table 2.2). In contrast, mean survival of amphipods exposed to sonicated SiCNW (Tests 1 and 2), was significantly reduced relative to the survival in control (Table 2.2).

However, the survival of midge, oligochaetes, or mussels exposed to sonicated SiCNW were not significantly reduced relative to the control (Table 2.2), indicating that these organisms were less sensitive than amphipods to the sonicated SiCNW.

Table 2.2. Mean survival of amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*) and mussels (*Lampsilis siliquoidea*) in 48- or 96-h water-only exposure to sonicated silicon carbide nanowires (SiCNW). Standard deviation in parentheses, n=4 (except in Test 2 with mussels, n=3). An asterisk (*) indicates a significant reduction relative to the control ($p<0.05$)

			Test		Survival (%)	
Test	Treatment	Organism	duration (d)	Test water	Control	Treatment
1	Non-sonicated SiCNW	Amphipods	2	ASTM hard[a]	90 (8.2)	83 (5.0)
	Sonicated SiCNW	Amphipods	2	ASTM hard	90 (12)	0 (0)*
	Sonicated SiCNW	Midge	4	ASTM hard	83 (17)[b]	75 (17)
	Sonicated SiCNW	Oligochaetes	4	ASTM hard	100 (0)	100 (0)
	Sonicated SiCNW	Mussels	4	ASTM hard	30 (38)[b]	30 (35)
2	Sonicated SiCNW	Amphipods	4	ASTM hard	98 (5.0)	15 (13)*
	Sonicated SiCNW	Midge	4	ASTM hard	100 (0)	100 (0)
	Sonicated SiCNW	Oligochaetes	4	ASTM hard	100 (0)	100 (0)
	Sonicated SiCNW	Mussels	4	ASTM hard	95 (10)	100 (0)
3	Sonicated SiCNW	Amphipods	2	Diluted well 1	98 (5.0)	73 (9.6)*
4	Sonicated SiCNW	Amphipods	2	Diluted well 2	98 (5.0)	48 (21)*

[a]American Society for Testing of Materials (ASTM) reconstituted hard water [23].

[b]Test did not meet acceptability requirement of 90% control survival. No statistic comparison was made.

This study indicated that sonicated SiCNW, but not non-sonicated SiCNW, were acutely toxic to amphipods. The mechanism for the enhanced toxicity of SiCNW after sonication is not clear, but sonication was observed to break the SiCNW into particles with lower aspect ratios which probably had more contact with the exposed organism compared with exposure to the non-sonicated SiCNW. The effect of sonication on the toxicity of nanomaterials to aquatic organisms has shown conflicting results. For example, sonication of fullerene (C60) reportedly enhanced toxicity to largemouth bass (*Micropterus salmoides*) (Oberdorster 2004) and sonicated lysophophatidylchlorine-coated single walled carbon nanotubes (SWCNTs) was acutely toxic to the cladoceran (*Daphnia magna*) (Roberts et al. 2007).

However, sonication of TiO_2 showed reduced toxicity of the TiO_2 to *D. magna* compared to non-sonicated TiO_2 (Lovern and Klaper 2007).

The sonicated SiCNW were less toxic in exposures in well water (48 to 73% survival, Table 2.2) compared to in ASTM reconstituted water (0 to 15% survival), suggesting that the differences in water chemistry may influence the toxicity of sonicated SiCNW to the *H. azteca*. Additional studies should be conducted over a broad range of water quality conditions to better determine if water quality influences the toxicity of SiCNW or other nanomaterials. For example natural organic matter dissolved in water has been used to keep carbon nanotubes in suspension in column stability and settling tests (Kennedy et al. 2008).

The SiCNW coated the surfaces of the test organisms and were also observed in the digestive tracts of the amphipods, oligochaetes, and midge (it was difficult to observe the distribution of SiCNW inside the shell of the mussels). Studies have shown that ingestion of nanomaterials by test organisms can cause blockage of the digestive tract (Kennedy et al. 2008, Charterjee 2008, Borm et al. 2006, Christian et al. 2008) and coating of nanomaterials may also smother respiratory surfaces, thus contributing to the toxicity of the nanomaterials to aquatic organisms. The higher sensitivity of amphipods in comparison to other test organisms is not completely understood. However, the sensitivity of the amphipods observed in this study is consistent with observation that *H. azteca* are often quite sensitive to a variety of contaminants compared to other organisms (e.g., Ingersoll 1998, Borgmann et al. 2005, Phipps et al. 1995).

Because of the high sensitivity of amphipods in the water-only exposures, the whole sediment toxicity tests with SiCNW were conducted only with the amphipods.

Sediment toxicity tests

In the 10-d sediment toxicity tests with amphipods, water quality characteristics were relatively consistent (Table 2.1). Dissolved oxygen concentration was >7.5 mg/L and total ammonia concentration was <0.4 mg/L. Mean survival of amphipods in the controls was ≥88% (Table 2.3), which met test acceptability requirements (USEPA 2000, ASTM 2009b). Mean survival of amphipods exposed to sonicated SiCNW either mixed in the sediment or layered on the surface of the sediment was 80% and not significantly different from the control (Table 2.3). Hence, the presence of sediment reduced the lethal effects of exposure to the sonicated SiCNW relative to the 48-h water-only toxicity tests (Table 2.2).

When amphipods were exposed to SiCNW mixed in the sediment, mean length or weight of amphipods was not significantly different from the control, but the biomass of amphipods was significantly reduced by 20% relative to the control (Table 2.3). When amphipods were exposed to SiCNW layered on the surface of the sediment, the mean length, weight and biomass of amphipods were all significantly reduced relative to the control, and biomass was reduced by 60% relative to the control (Table 2.3).

Table 2.3. Means of survival, length, weight, and biomass of amphipods (*Hyalella azteca*) in 10-d exposures to sonicated silicon carbide nanowires (SiCNW) mixed in sediment or layered on sediment surface. Standard deviations in parentheses, n=4. An asterisk (*) indicates a significant reduction relative to the control ($p<0.05$)

Test	Treatment	Survival (%)	Length (mm)	Weight (mg)	Biomass (mg)
1	Control	88 (9.6)	1.88 (0.11)	0.030 (0.006)	0.25 (0.03)
	Sonicated SiCNW mixed in sediment	80 (8.2)	1.81 (0.11)	0.026 (0.005)	0.20 (0.02)*
2	Control	93 (5.0)	1.92 (0.06)	0.031 (0.004)	0.28 (0.04)
	Sonicated SiCNW layered on sediment	80 (14)	1.63 (0.06)*	0.019 (0.002)*	0.11 (0.04)*

These results indicated severe inhibition on the growth of amphipods exposed to SiCNW layered on the surface of the sediment compared to SiCNW mixed in sediment. The more severe effect of SiCNW layered on the surface of the sediment compared to SiCNW mixed into sediment was likely due to the increased contact of amphipods with SiCNW layered on sediment surface. The SiCNW mixed in sediment was less available to the amphipods than SiCNW layered on the sediment surface. Both approaches for adding SiCNW to sediment are relevant to environmental processes. The mixing with sediment represents a scenario where SiCNW might be mixed into sediment over an extended period of time. The layering of SiCNW on the surface of the sediment would simulate the initial deposition of SiCNW onto sediment following release or spill of the material into surface water.

Previous studies on toxicity of silicon carbide (SiC) have been inconclusive. For example, the material safety data sheet states that SiC has no known acute or chronic toxicity to either humans or aquatic organisms (Sun Innovations 2006).

While Allen et al. (1995) reported concentrations of up to 0.1 g SiC particles /L in 72-h exposures to three human cell types were not acutely toxic, SiC at 1.0 g/L caused severe cytotoxicity to all of the three cell types. The present study demonstrated that about 0.63 g SiCNW /g sediment on a dry weight basis (30 mg SiCNW spiked into 270 mg wet sediment at about 84% moisture content) were toxic to amphipods in 10-d exposures. The concentrations of SiCNW evaluated in the present study were likely much higher than would be expected in the environment; however, testing of high concentrations has been recommended for initial toxicity screening of nanomaterials (Borm et al. 2006). Toxicity data with high concentrations forms the basis for the design of dilution tests or longer exposures to determine concentration thresholds for sensitive test species. In another study with sediments spiked with MWCNTs, Kennedy et al. (2008) reported a 10-d median lethal concentration of 264 g MWCNTs/kg-sediment to *H. azteca*. That study, however, may not be comparable to the present study because of differences in the type of nanomaterial tested (e.g., morphology, surface area, hydrophobicity, composition, particle sizes (Handy et al. 2008, Murdock et al. 2007, Borm et al. 2006, Christian et al. 2008), difference in sediments tested, and toxicity endpoints (e.g., both survival and growth of amphipods were evaluated in the present study).

Several factors should be considered in the toxicity assessment of nanomaterials to aquatic organisms, including the types of nanomaterials (chemical compositions, physical and surface characteristics with and without sonication), types of organisms and endpoints evaluated, water or sediment characteristics.

The present study was conducted in basic accordance with USEPA (2000) and ASTM (2009b) methods without using artificial procedures such as surfactants to keep the SiCNW suspended in water. The present study demonstrates that the adapted approaches are suitable for testing toxicity of SiCNW in water and sediment. Based on the experience gained in this study, we are conducting water-only or whole-sediment toxicity tests with other nanomaterials (e.g., various carbon nanotubes) and amphipods in 14- to 28-d exposures.

In summary, our results indicated that (1) non-sonicated SiCNW in water were not acutely toxic to amphipods, (2) sonicated SiCNW in water were acutely toxic to amphipods, but not toxic to midge, oligochaetes or mussels, and (3) sonicated SiCNW mixed in sediment or layered on sediment surface were chronically toxic to amphipods. It is possible that the impairment of respiration by smothering the surface of test organisms or adverse effects on other physiological functions including blockage or injury of the digestive tract could have contributed to the toxicity of the SiCNW, however, the specific mode of toxicity needs to be investigated further.

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SUPPORTING INFORMATION

Table S2.1. Conditions for water or sediment toxicity tests with silicon carbide nanowires (SiCNW) using amphipods (*Hyalella azteca)*, midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*), and juvenile mussels (*Lampsilis siliquoidea*) under static renewal conditions based on method adapted from ASTM (2009a) and USEPA (2000).

1. Test species:	*H. azteca, C. dilutus, L. variegatus, L. siliquoidea*
2. Test chemicals:	SiCNW
3. Test type:	1. Static non water renewal for water toxicity tests 2. Static water renewal for sediment toxicity tests
4. Test Duration:	1. Water exposures: 48 h for *H. azteca* and 96 h for others 2. Sediment exposure: 10 days with *H. azteca*
5. Temperature:	23±1°C
6. Light quality:	Ambient laboratory light
7. Light intensity:	200 lux
8. Photoperiod:	16L:8D
9. Test chamber:	50-ml glass beaker (water exposures: containing a thin layer of sand for *H. azteca, C. dilutus, L. variegatus*)
10.Test solution volume:	30 ml
11. Renewal of solution:	None
12. Age of test organism:	7-d amphipods, midge, adult oligochaetes , and 5-d to 3-month-mussels
13. Organisms/chamber	10
14. Replicates/treatment	4
15. Feeding:	None
16. Aeration:	None
17. Dilution water:	Reconstituted ASTM hard water (160-180 mg/L as $CaCO_3$) or diluted CERC well water (hardness 110 to 140 mg/L as $CaCO_3$)
18. Dilution factor:	None
19. Test concentration:	1. 30 mg SiCNW in 30 ml test water 2. 30 mg SiCNW in 270 mg sediment with 30 ml overlying water
20. Chemical residues:	None
21. Water quality:	Dissolved oxygen, pH, conductivity, hardness, and alkalinity
22. Endpoint:	Survival
23. Test acceptability:	≥90% survival in controls for all organisms in acute water-only tests ≥ 80% survival for amphipods in 10-d sediment tests

CHAPTER 3

TOXICITY OF CARBON NANOTUBES TO FRESHWATER AQUATIC INVERTEBRATES

ABSTRACT

Carbon nanotubes (CNTs) are hydrophobic in nature and tend to accumulate in sediments when released into aquatic environments. As part of our overall effort to examine the toxicity of carbon-based nanomaterials to sediment-dwelling invertebrates, we evaluated the toxicity of different types of CNTs to amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*), and mussels (*Villosa iris*) in 14-d water-only tests. The results showed that 1.00g/L (dry weight) of commercial sources of CNTs significantly reduced the survival or growth of the invertebrates. Toxicity was influenced by the type and source of the CNTs, whether the materials were pre-cleaned, whether sonication was used to disperse the materials, and species of the test organisms. Light and electron microscope imaging of the surviving test organisms showed the presence of CNTs in the gut as well as on the outer surface of the test organisms, although no evidence was observed to show penetration of CNTs through cell membranes. The study demonstrated that both the metals such as nickel solubilized from CNTs and 'metal-free" CNTs contributed to the toxicity.

INTRODUCTION

Carbon nanotubes (CNTs) are hollow graphene cylinders that are microns to millimeters in length (Eklund et al. 2007), and can be single-walled (SWCNTs) with a diameter of 0.7 to 3 nm or multiple-walled (MWCNTs) with a diameter of 10 to 25 nm (Baughman et al. 2002). CNTs are produced by chemical vapor deposition, carbon arc discharge, laser ablation, and electrolysis methods, using carbon compounds as feedstock and metals such as Ni, Co, Mo, Fe, Cr, Cu, and Al as catalysts (Donaldson et al. 2006). Metal catalyst residuals and amorphous carbon are the main impurities in the as-produced CNTs (Donaldson et al. 2006). The cleaning or modification of as-produced CNTs can be achieved by oxidation, acid treatment, annealing, sonication, filtration, and functionalization processes (Donaldson et al. 2006). CNTs have low mass density, high mechanical strength, high electron/hole mobility; and high thermal conductivity. They are expected to be widely used in areas such as medical sectors, electronics, composites and materials science (Eklund et al. 2007). The world commercial production capacity of CNTs in 2007 was about 300 tons/yr MWCNTs and 7 tons/yr SWCNTs, with total commercial sales of over $200 million (Thayer 2007). Consumer products in the market that contain CNTs include sporting goods, textiles and shoes, vehicle fenders, electronics, x-ray tubes and batteries (Dekkers et al. 2007).

CNTs are expected to enter aquatic environments through sources such as general weathering, disposal of CNT-containing consumer products, accidental spillages, and waste discharges (OECD 2006, Nowack and Bucheli 2007).

Because CNTs are hydrophobic and non-biodegradable (Donaldson et al. 2006), these materials can accumulate in aquatic biota when released into aquatic environments. CNTs effects on aquatic organisms have however not been fully evaluated, and the results of previous toxicity studies are not conclusive. For example, a 48-h acute test with SWCNTs, which had been mechanically stirred for 4 months, was not toxic to the cladoceran (*Chydorus sphaericus*) at exposure concentrations up to 100 mg/L (Velzeboer et al. 2008), and acid-cleaned double walled CNTs were not toxic to the salamander (*Ambystoma mexicanum*) in 12-d exposures to concentrations up to 1.0 g/L (Mouchet et al. 2007). No significant effects on mortality, development, and reproduction was observed in estuarine copepods (*Amphiascus tenuiremis*) exposed to purified SWCNTs, but mortality increased, fertilization rates were reduced, and molting success decreased when the copepods were exposed to the as-produced SWCNTs (Templeton et al. 2006). In another study, the hatching of zebra fish (*Danio rerio*) embryos was delayed by the presence of CNTs but not by carbon black under comparable conditions, and Ni or Co impurities in the CNTs potentially contributed to delayed hatching (Cheng et al. 2007). These inconclusive results could be due to different test protocols, organisms, and exposure durations (Kennedy et al. 2008, Zhu et al. 2009). Furthermore, toxicity could be complicated by surface coatings/functionalizations that are often introduced for various applications. To illustrate, the cladoceran (*Daphnia magna*) was able to modify the solubility of the nanotubes after ingesting a water-soluble, lysophophatidylcholine coated SWCNTs during normal feeding (Roberts et al. 2007).

Other studies have shown that as-produced CNTs are more toxic than functionalized CNTs (with hydroxyl- or carboxyl-groups) to cladoceran (*Ceriodaphnia dubia*) (Kennedy et al. 2008) , and CNTs were more toxic than carbon black to *D. magna* (Zhu et al. 2009). Apparently, no simple conclusion exists regarding the toxicity of CNTs, and there is a need to develop Standard Test Protocols with a standard suite of test organisms to eliminate some of the variance in test results. The role of metal impurities introduced during the manufacturing of CNTs to toxicity of aquatic organisms needs to be elucidated.

The objectives of this study were to assess the toxicity of CNTs to the amphipod (*Hyalella azteca*), the midge (*Chironomus dilutus*), the oligochaete (*Lumbriculus variegatus*), and the mussel (*Villosa iris*) in 14-d water-only tests and to determine the potential contribution of metals to any observed toxicity. These sediment-dwelling aquatic invertebrates were selected due to their sensitivity to contaminants in water or sediment and importance in aquatic systems (e.g., Phipps et al. 1995, Borgmann et al.2005, Ingersoll et al. 2005 and 2008, Keithly et al. 2004). Results of the water-only tests could then be used to design sediment toxicity tests (Mwangi 2010). Toxicity tests were conducted in which all four benthic invertebrates were exposed to as-produced or purified CNTs, and additional tests were conducted with the amphipod exposed to spiked nickel and/or EDTA to illustrate the effects of dissolved metals. The test methods used were adapted from those described by ASTM (2009a, b).

MATERIALS AND METHODS

Materials

The commercially available CNTs evaluated included: (1) SWCNTs from Shenzhen Nanotech Port, China (SWCNT-S) with >90wt% (weight percent) purity, 2 nm average diameters, 5-15 μm average lengths, >400 m^2/g specific surface area, <2wt% ash, <5wt% amorphous carbon; (2) MWCNTs from Helix Material Solutions Inc., TX, USA (MWCNT-H) with >95wt% purity, and <0.2wt% total impurities; and (3) MWCNTs from Shenzhen Nanotech Port, China (MWCNT-S) with >95%wt purity, <0.2% ash, < 3% amorphous carbon, 10-20 nm diameters, 5-15 μm lengths, and 40- 400 m^2/g specific surface area. Other chemicals used included nitric acid (15.9 N, ACS grade), ethylenediaminetetraacetic acid (EDTA, 99.9% purity), and nickel (II) chloride ($NiCl_2.6H_2O$, 99% purity).

Toxicity Tests

Stock suspensions were prepared by weighing 800-mg CNTs in a fume hood into each of the two 80-ml beakers, each containing 60 ml of test water. One stock was sonicated for 2 min at 65 W (Sonifier 450, Branson Ultrasonic Corporation) to disperse the CNTs and the other was not sonicated. The stock solutions were mixed using a magnetic stirrer, and four aliquots of 15-ml suspension each containing 200 mg CNTs were transferred into four 300-ml glass beakers with a pipette. Five-ml of fine sand was placed into each exposure beaker to provide a substrate for the test organisms (ASTM 2009a), except for the tests with mussels (ASTM 2009b).

The exposure water (hardness 100 mg/L as $CaCO_3$, alkalinity 80 mg/L as $CaCO_3$, and pH 8.0) was prepared by blending appropriate amounts of well water with deionized water. The exposure water was added to each test beaker to maintain volumes of 200 ml.

About 7-d old amphipods, 7-d old midge, adult oligochaetes, and 6 to 8-month old mussels were obtained from laboratory cultures at the U.S. Geological Survey, Columbia Environmental Research Center (CERC), Columbia, Missouri. Test organisms were acclimated to test temperature and water for at least one day.

To initiate each test, 10 amphipods, midge, oligochaetes, or 5 (for MWCNT-S) or 10 (for MWCNT-H) mussels were impartially transferred into each of 4 replicate test beakers. The beakers were placed in a water bath maintained at 23±1ºC and a photoperiod of 16h light: 8h dark. To record the initial body sizes of the test organisms, 20 amphipods were preserved in 8% sugar formalin solution and 20 mussels in 80% ethanol (Ingersoll et al. 2005). Four replicates each of ten midge or oligochaetes were oven dried at 60°C for 24 h, weighed, and then burned at 500°C for 4 h to determine the ash-free biomass. Additionally, to obtain images of the test organisms, separate tests were conducted parallel to the toxicity tests and organisms were recovered from these tests at 6 and 14d, photographed and analyzed by light or transmission electron microscopy.

During the tests, exposure beakers were constantly aerated with about 3 air bubbles /sec from the bottom of the beakers to maintain suitable oxygen content in overlying water. On test days 2, 5, 7, 9 and 12, aeration was stopped for about 1h to allow any potentially suspended CNTs to settle, and about100 ml of water (50% volume) was replaced with an equal volume of fresh test water.

Following every water renewal, amphipods in each beaker were fed 0.5 ml of Yeast-Cerophyl-Trout Chow (1800 mg/L) once daily , and midge or oligochaetes were fed 1.0 ml Tetrafin® flake fish food (4 g/L stock suspension, ASTM 2009a). Mussels were fed 2 ml of algal mixture twice daily (ASTM 2009b). Preliminary tests with different types of feed and feeding rates were conducted to select the feeding conditions that provided the best survival and growth for the test organisms and maintained acceptable water quality in the 14-d water only tests.

Water quality parameters, including dissolved oxygen, conductivity, pH, total alkalinity, total hardness and total ammonia, were determined on composite water samples collected on test days 0, 7, and 14 from each treatment following standard methods (APHA 2005). Test conditions are summarized in Table S3.1A.

Range finder toxicity tests were conducted to determine the highest non-toxic EDTA concentration to amphipods. The EDTA solution was prepared on test day -1 and stored in a cooler until needed for water renewal on Monday, Wednesday and Friday during the test. The test treatments in two replicates each in 300-ml beakers with 200 ml solution were (1) 200 ml well water (control), (2) 0.16 g EDTA/L, (3) 0.08 g EDTA/L, and (4) 0.04 g EDTA/L, and 0.02 g EDTA/L. The reported 4-d LC50 for *H. azteca* as 0.16 g EDTA/L in moderately hard water (USEPA 2007) and therefore was the highest EDTA concentration in the range finder tests conducted as summarized in Table S3.1B.

The effects of nickel on the toxicity of CNTs to amphipods were evaluated in a set of toxicity tests with (1) 200-ml well water as control, (2) 200 mg MWCNT-S in 200-ml water, (3) 200 mg MWCNT-S + 0.04 g/L (0.14mM) EDTA in 200-ml water; and (4) 1.50 mg/L (0.026 mM) of Ni; and (5) 1.50 mg/L of Ni + 0.04 g/L of EDTA. In the Ni tests, EDTA was added as a chelating agent to reduce the toxicity of metals (Burkhard and Ankley 1989). Preliminary tests showed that good control survival or growth was possible with exposures to 0.04 g EDTA/L. Test methods and conditions were otherwise the same as for CNTs tests as described previously.

The acceptability criteria for the tests was at least 80% control survival for amphipods and mussels, 70% control survival for midge, and a positive biomass gain for oligochaetes (ASTM 2009a, b). Both survival and growth were measured in the various test treatments and compared to the responses in the control treatments. The mortality of amphipods, midge, and oligochaetes were determined based on the lack of movement following stimulation with a blunt probe (ASTM 2009a). Mussels that exhibited foot movement within a 5-min observation period were classified as alive under a dissecting microscope (ASTM 2009b).

Surviving amphipods were preserved in vials with 8% sugar-formalin solution and mussels in 80% ethanol for subsequent length measurement. Amphipod length was measured along the dorsal surface from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface. Mussel shell length was measured as the maximum shell length. Length measurements were made using a computer digitizing system equipped with video micrometer software obtained from Image Caliper (Resolution Technology, Dublin, OH, USA) (Ingersoll et al. 2005).

The biomass of amphipods was calculated as the sum of individual amphipod weights within a replicate which was estimated from the measured lengths using the following empirical relationship by Ingersoll et al. (2008): Weight (mg) = ((0.177* Length (mm)) – 0.0292)^3. Surviving oligochaetes or midge isolated from each replicate at the end of the test were placed in an aluminum weigh pan and dried at 60ºC for 24h and weighed to determine biomass. The dried organisms were then burned at 500ºC for about 4 h to obtain the ash-free dry biomass (ASTM 2009a).

Dissolved concentrations of the trace metals (Co, Ni, Cr, Fe, and Mo) in water from each treatment were analyzed with inductively coupled plasma optical emission spectroscopy (VISTA-MPX CCD Simultaneous ICP-OCS, Varian). Samples were collected following filtration through 0.45-µm membrane and preserved in 1.0% of high purity nitric acid at 4^{o}C prior to analysis. The instrument detection limits were $\leq$ 0.9 µg/L for these metals.

Sample Characterization and Analysis

The tested CNTs samples and organisms exposed to these nanomaterials were characterized by the transmission electron microscopy (TEM; JEOL 1400, Tokyo, Japan) and scanning electron microscopy (SEM, Hitachi S4700 FESEM, Tokyo, Japan). CNTs were also analyzed by a Thermo-Noran energy-dispersive X-ray microanalysis system (EDS) equipped with the SEM. The effects of sonication on aggregation and dispersion of the CNTs was evaluated by measuring diameters of randomly selected nanotubes from SEM images using Java-based image processing software ImageJ.

The test organisms were prepared for TEM imaging according to established procedures (Panessa-Warren et al. 2006). Live organisms were photographed using a DXM 1200C Nikon camera attached to an SMZ 1500 Nikon microscope. Prior to the photographing, 1-3 drops of 0.20g/L of tricaine methanesulfonate (MS-222) in well water was used as an anesthetic to immobilize the organisms.

Data Analysis

Survival and growth were arcsine (log) transformed and tested for normality and homogeneity of variance. If the data were normally distributed (Shapiro-Wilk's test) and had homogeneity of variance (Bartlett's test). Statistical differences in mean survival or growth among treatments were determined by analysis of variance (ANOVA), with mean comparison made by Fisher's Least Significant Difference (LSD) test, or by *t*-test if only two treatments were compared in a test. If the assumptions of the ANOVA or *t*-test were not met, Wilcoxon Rank Sum test was used (USEPA 2000). The level of statistical significance was set at $p \leq 0.05$. The data analysis was generated using SAS/STAT® software, Version 9.1.3, SAS Institute Inc., Cary, NC, USA.

RESULTS

Characteristics of CNTs

The primary element found in both the SWCNTs and MWCNTs by EDS was carbon, as expected, but there were also significant amounts of oxygen in the MWCNTs (Table 3.1).

Metal impurities included Co (4.9%) and Mo (1.5%) in SWCNTs sample; Ni (3.0%) and Mo (1.2%) in MWCNT-S sample; and Fe (10.1%), Co (2.5%), Mo (0.6%), and Ni (0.5%) in MWCNT-H sample. When the material was modified by 3.0 M nitric acid (NAM MWCNT-S), metal impurities were largely removed from the sample (i.e., <0.01%).

Table 3.1. Elemental compositions (percent weight) of single-wall carbon nanotubes from Shenzhen (SWCNT-S), multi-wall carbon nanotube from Shenzhen (MWCNT-S) or Helix USA (MWCNT-H), and nitric acid modified (NAM) MWCNT-S samples analyzed with Energy Dispersive Spectrometer

Sample	Carbon	Oxygen	Silicon	Iron	Cobalt	Molybdenum	Nickel
SWCNT-S	93.4	<0.1	<0.1	0.3	4.9	1.5	<0.1
MWCNT-S	89.7	11.8	0.9	<0.1	0.2	1.2	3.0
NAM MWCNT-S	86.1	13.5	<0.1	0.4	<0.1	<0.1	<0.1
MWCNT-H	70.9	15.3	0.1	10.1	2.5	0.6	0.5

The as-produced MWCNT-H and MWCNT-S are in entangled rope-like bundles with scattered dark spots, indicating the presence of particulate metal impurities **(**Figure 3.1A, B**)**. Following 3.0 M nitric acid treatment for 12 hours, the population of dark spots representing metal impurities was reduced **(**Figure 3.1D**)** when compared with the un-treated sample (Figure 1B). This is consistent with the EDS results showing that MWCNT-S modified with nitric acid had lower concentrations of Co, Mo, and Ni **(**Table 3.1**))**. SWCNT-S tends to form bundles as observed and also had dark spots indicating the presence of metal impurities **(**Figure 3.1C, Table 3.1**)**.

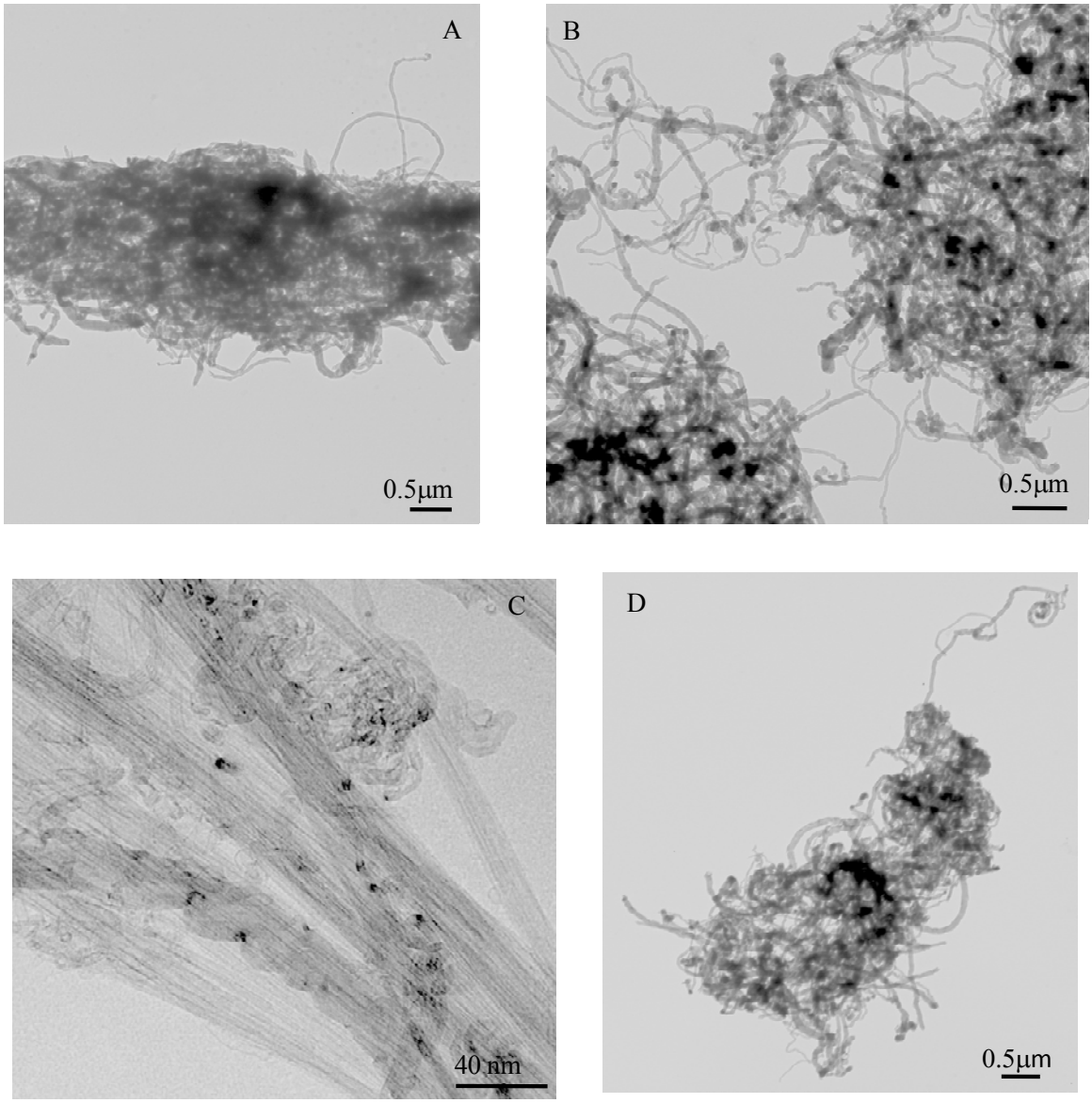

Figure 3.1. Transmission electron microscope images of: (A) Multi-wall carbon nanotube from Helix (MWCNT-H); (B) MWCNT from Shenzhen (MWCNT-S); (C) Single-wall carbon nanotube from Shenzhen (SWCNT-S); and (D) nitric acid modified MWCNT-S

Similarly, the SEM images of MWCNT-H show the existence of bright spots on some carbon nanotubes, which generally are indicative of impurities such as amorphous carbon or encapsulated metal catalyst particles.

CNTs diameters are 91 ± 31nm for the non sonicated CNTs **(**Figure 3.2A**)** and 48 ± 11 nm for the sonicated CNTs **(**Figure 3.2B**)**. These diameters are statistically different (*t*-test, $p<0.05$), indicating the occurrence of MWCNTs breakdown upon sonication.

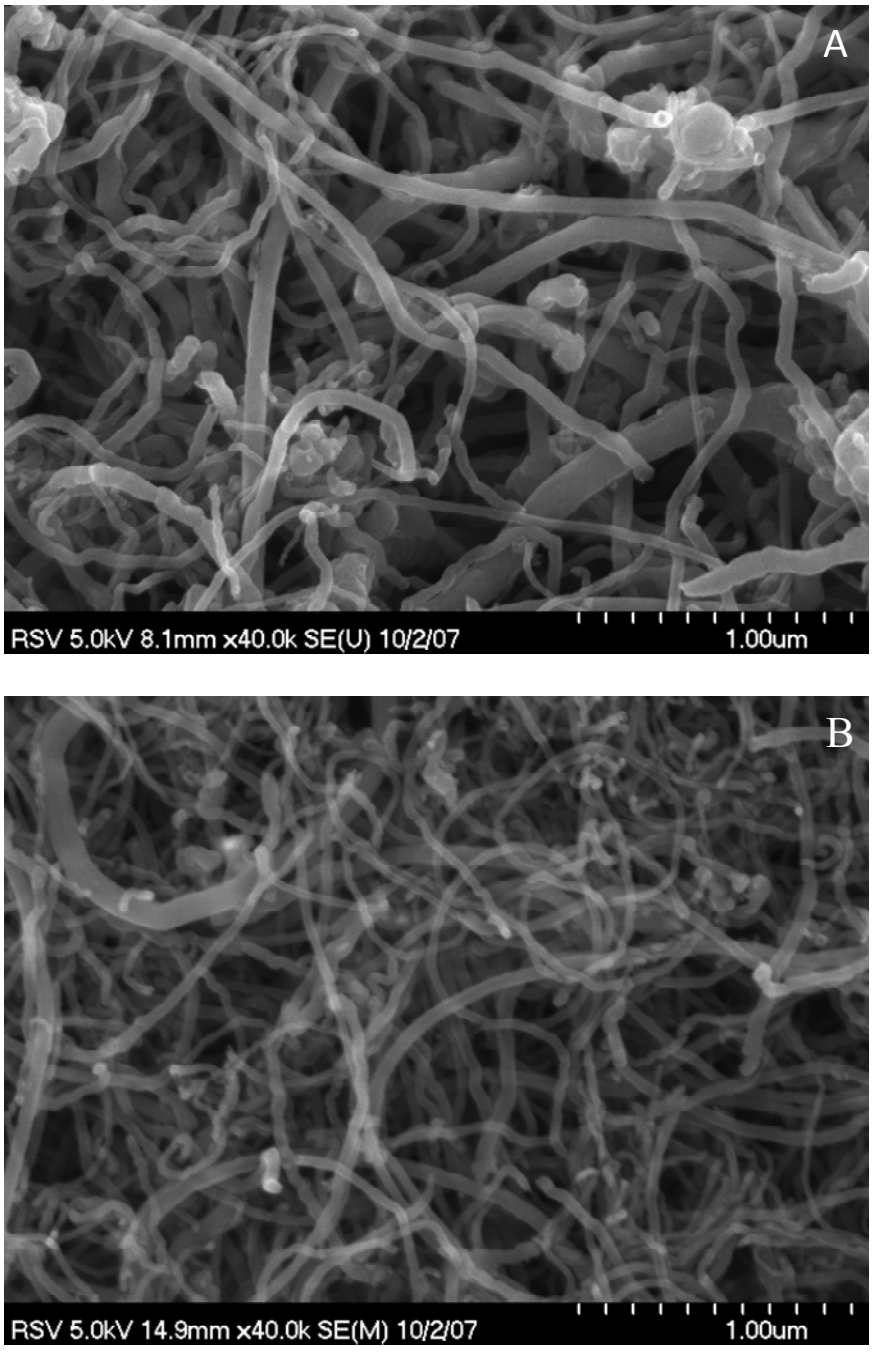

Figure 3.2. Scanning electron microscopy (SEM) images showing morphology of multi-wall carbon nanotubes from Helix (MWCNT-H):(A) non sonicated MWCNT-H, and (B) sonicated MWCNT-H in water

Toxicity of CNTs

The water quality parameters for the toxicity tests were all within the acceptable ranges: dissolved oxygen 7.5 to 9.0 mg/L, conductivity 232 to 343 μS/cm, pH 8.1 to 8.7, alkalinity 71 to 123 mg/L as $CaCO_3$, hardness 103 to 135 mg/L as $CaCO_3$, and total ammonia 0.10 to 1.37 mg/L **(**Table S3.2**)**.The results of the toxicity tests (Table 3.2**)** show **c**ontrol survival of amphipods, midge, and mussels was ≥80%, meeting the test acceptability criterion, except control survival of midge in Test 3. The result for the midge in Test 3 is thus not included for discussion below.

Mean survival or biomass of the test organisms exposed to MWCNTs or SWCNTs with and without sonication were significantly lower than survival or biomass of the controls in 22 of 24 tests (Table 1). For example, the survival of amphipods exposed to non- sonicated MWCNT-H was 5.0% or 2.5% with sonicated MWCNT-H relative to the control survival of 88%. There was no consistent difference in survival or biomass of organisms exposed to CNTs with or without sonication. The mean survival of amphipods, midge and mussels with nitric acid-modified MWCNT-S was not significantly different from the controls but biomass of amphipods, midge and oligochaetes were significantly reduced (Table 3.2).

Table 3.2. Mean response of amphipods (*Hyalella azteca*), midge (*Chironomus dilutus)*, mussels (*Villosa iris*), and oligochaetes (*Lumbriculus variegatus*) exposed to sonicated or non sonicated single- or multi-wall carbon nanotubes from Shenzhen (SWCNT-S or MWCNT-S) or from Helix (MWCNT-H) or nitric acid modified (NAM) MWCNT-S in 14-d toxicity tests. Standard deviations in parenthesis, n=4. Different letters for survival, length, or biomass in a column for a test indicate a significance difference among treatments ($p<0.05$)

		Amphipod		Midge		Mussel		Oligochaete
Test	Treatment	Survival (%)	Biomass (mg)	Survival (%)	Biomass[a] (mg)	Survival (%)	Length (mm)	Biomass[b] (mg)
1	Control	88 (5.0)x	1.2 (1.0)	80 (8.2)x	9.3 (2.6)x	98 (5.0)x	2.2 (0.1)	2.8 (0.2)x
	Non sonicated MWCNT-H	5.0 (10)y	NR[c]	43 (9.6)y	3.7 (0.6)y	23 (17)y	NR[c]	1.2 (1.0)z
	Sonicated MWCNT-H	2.5 (5.0)y	NR	60 (8.2)z	1.4 (0.2)z	43 (19)y	NR	2.3 (0.4)y
2	Control	100 (0)x	NR[d]	83 (5.0)x	NR[d]	NT[e]	NT	3.7 (0.8)x
	Non sonicated SWCNT-S	20 (12)y	NR	10 (8.2)y	NR	NT	NT	1.4 (0.4)z
	Sonicated SWCNT-S	0 (0)z	NR	0 (0)z	NR	NT	NT	2.8 (0.2)y
3	Control	100 (0)x	NR[d]	NR[f]	NR	80 (28)x	NR[d]	5.2 (4.0)x
	Non sonicated MWCNT-S	8.0 (10)y	NR	NR	NR	35 (26)y	NR	0.8 (0.2)z
	Sonicated MWCNT-S	5.0 (10)y	NR	NR	NR	3.0 (10)z	NR	2.2 (0.6)y
4	Control	100 (0)x	0.8 (0.2)x	75 (19)x	5.5 (2.6)x	100 (0)x	1.3 (0.1)x	3.7 (1.2)x
	Non sonicated NAM MWCNT-S	95 (5.8)x	0.3 (0.1)y	60 (14)x	2.6 (0.9)y	98 (5.0)x	1.1 (0.04)y	1.3 (0.4)y

[a]Dry weight.

[b]Ash free dry weight.

[c]Not reported due to <50% survival in CNT treatments.

[d]Not reported because recovered organisms were used for photographs or Transmission Electrom Microscopy imaging.

[e]Not tested.

[f]Not reported because control survival was less than test acceptability criteria (70%, ASTM 2009a).

CNTs coated the outside surfaces of all the four test organisms. The amphipods, midge and oligochaetes also ingested the CNTs as indicated by the blackish material in the guts (Figure 3.3) which was confirmed to be CNTs with the TEM images **(**Figure 3.4). It was not possible to examine the gut of the mussels in these tests.

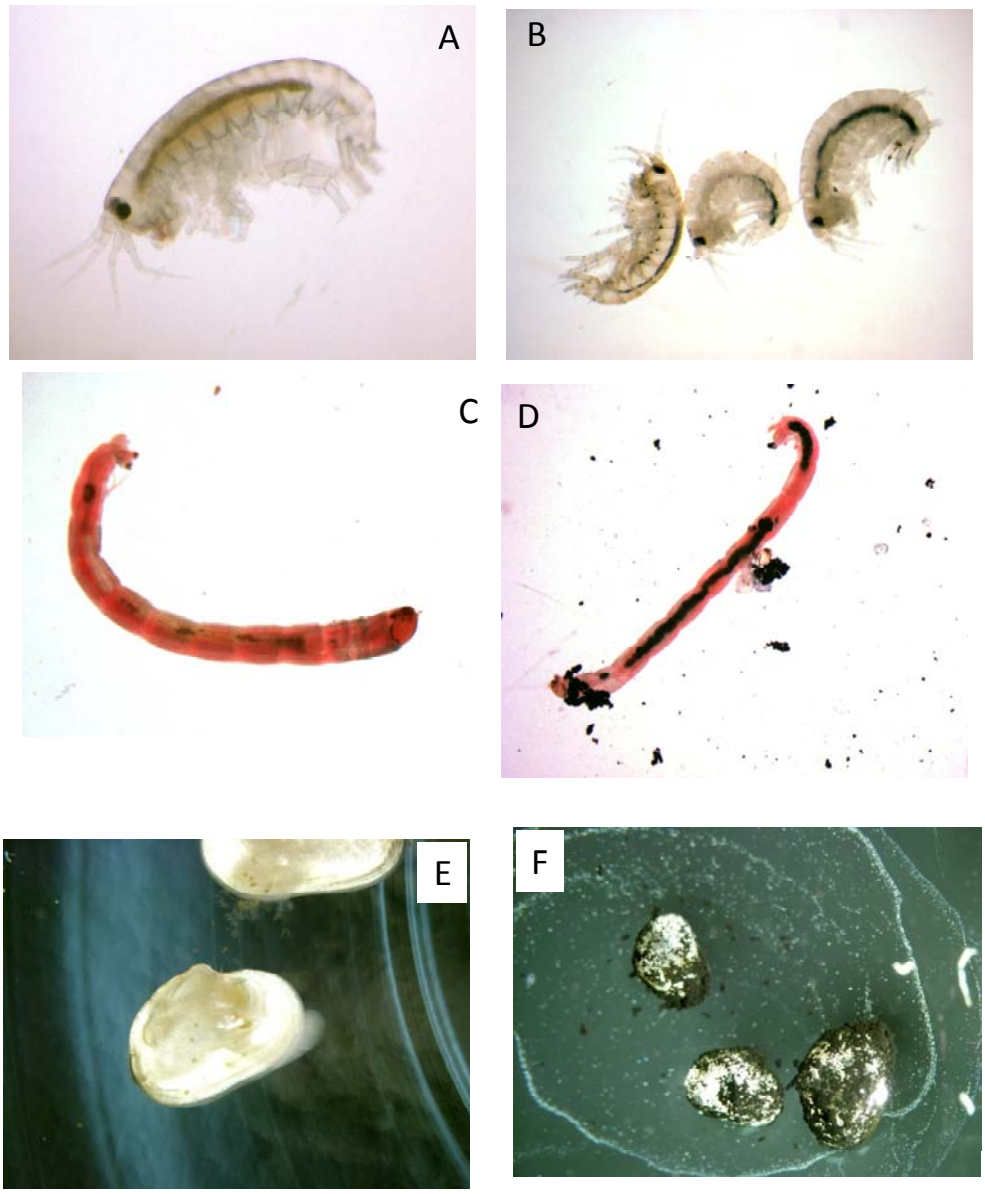

Figure 3.3. Test organisms from the water-only treatments: 1. Amphipods (*Hyalella azteca)* on day 6 in: (A) control (water), and (B) non sonicated multi-wall carbon nanotube from Shenzhen (MWCNT-S); 2. midge (*Chironomus dilutus*) on day 6 in: (C) control (water), and (D) non sonicated MWCNT-S; 3. rainbow mussels (*Villosa iris*) on day 14 in: (E) control (water), and (F) non sonicated MWCNT-S

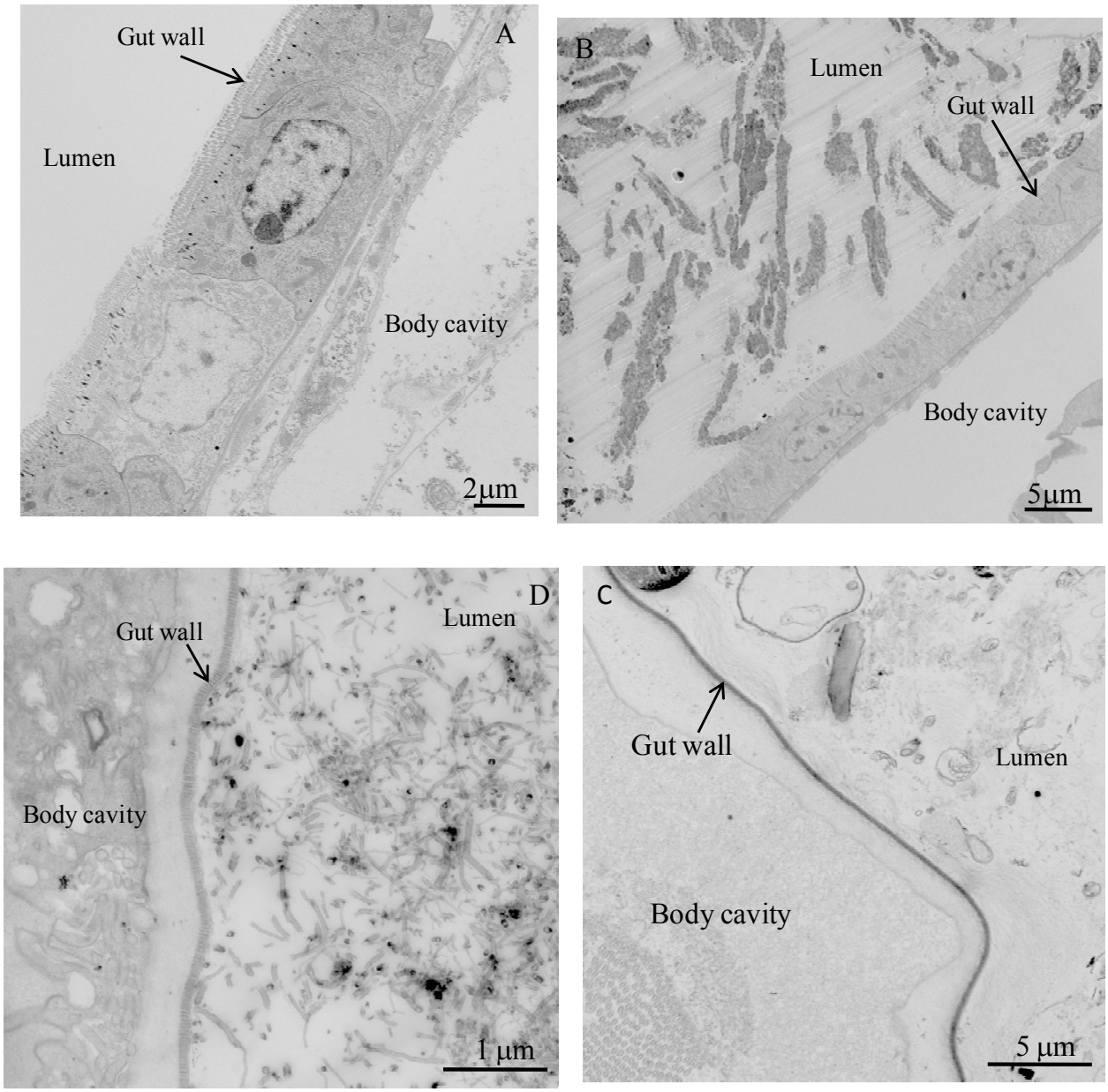

Figure 3.4. Transmission electron microscope (TEM) images of gut of test organisms : amphipod (*Hyalella azteca*) in: (A) control (water), and exposed to (B) non sonicated single-wall carbon nanotube from Shenzhen (SWCNT-S); midge (*Chironomus dilutus*) in: (C) control (water), and (D) non sonicated multi-wall carbon nanotube from Shenzhen (MWCNT-S)

EDTA range finder tests

The water quality parameters did not vary much and were within the expected range (Table S3.3). The results show 0.04 g EDTA/L exposed to amphipods was the highest non-toxic concentration which did not significantly reduce the survival or growth relative to the control (Table 3.3).

Table 3.3. Mean responses of amphipods (*Hyalella azteca*) exposed to ethylenediaminetetraacetic acid (EDTA) in 14-d range finder toxicity tests. Standard deviations in parenthesis, n=2. Different letters for survival, length, weight and biomass in a column indicates a significance difference among treatments ($p<0.05$)

Treatment	Survival (%)	Length (mm/individual)	Weight (mg/individual)	Biomass (mg)
Water (control)	85 (7.1)	3.2 (0.44)	0.17 (0.06)	1.5 (0.09)x
0.02 g EDTA	100 (0)	2.8 (0.56)	0.12 (0.08)	1.2 (0.46)x
0.04 g EDTA	90 (14)	2.9 (0.53)	0.13 (0.08)	1.1 (0.64)x
0.08 g EDTA	90 (14)	2.5 (0.34)	0.08 (0.03)	0.69 (0.14)x
0.16 g EDTA	20 (14)	NR[a]	NR	0.083 (0.065)y

[a]Not reported due to <50% survival in treatment.

Soluble metals during toxicity tests

The metals (Co, Cr, Fe, Mo, and Ni) that could be solubilized from CNTs were measured in the overlying water during the exposure **(**Table S3.3, S3.4**)** decreased in concentrations with time of exposure. In the tests controls, highest metal concentrations measured in 14-d were 65 µg/L for Co, 47 µg/L for Cr, 168 µg/L for Fe, 160 µg/L for Mo, and 20 µg/L for Ni.

In tests examining the potential impact of soluble Ni and EDTA on amphipods, the survival of amphipods was 15% with MWCNT-S and amphipods survival was 95% in the control. Addition of 0.04 g EDTA/L (0.137 mM) to the MWCNT-S treatment resulted in slightly higher survival of amphipods (33%).

In comparison, survival of amphipods exposed to 1.50 mg Ni/L was 0% and was 95% when 0.04 g/L of EDTA was also present. The growth of amphipods was similarly affected by MWCNT-S, Ni, and/or EDTA **(**Table 3.4**)**. Total soluble metals in varying systems were also measured directly (Table SI-4). The measured Ni concentrations in treatments at the start of exposures were 860 µg/L (Ni-only test), 1243 µg/L (Ni + EDTA), 880 µg/L (MWCNT-S), and 1436 µg/L (MWCNT-S + EDTA), which were quite comparable.

Table 3.4. Mean responses of amphipods (*Hyalella azteca*) exposed to non sonicated multi-wall carbon nanotubes from Shenzhen (MWCNT-S) with or without the addition of 40 mg/L ethylenediaminetetraacetic acid (EDTA) or nickel (Ni) solution (1.50 mg Ni/L) with or without the addition of 40 mg EDTA/L in 14-d toxicity test. Standard deviations in parenthesis, n=4. Different letters for survival, length, weight and biomass for a test indicate a significance difference

Treatment	Survival (%)	Length (mm/individual)	Weight (mg/individual)	Biomass (mg)
Control	95 (5.8)x	2.9 (0.34)x	0.12 (0.04)x	1.10 (0.16)x
MWCNT-S	15 (10)y	NR[a]	NR	0.11 (0.10)y
MWCNT-S+EDTA	33 (20)y	NR	NR	0.31 (0.18)y
Ni	0 (0)z	NR	NR	0 (0)z
Ni+EDTA	98 (5.0)x	3.0 (0.44)x	0.13 (0.02)x	1.30 (0.22)x

[a]Not reported due to survival <40% in the CNT treatments.

DISCUSSION

CNTs tested in water are toxic to amphipods, midge, mussels, or oligochaetes. The degree of the toxicity depended on the type of the material, whether the material was pre-treated (i.e., sonication, or acid washed), and the test organism. The CNTs samples consisted of bundles and aggregates with metal impurities mostly at nodes and tips, typical for CNTs (Grobert 2007).

Metal impurities in CNTs were reported to have contributed to the hatching delay of zebra fish (Cheng et al. 2007) and enhance production of intracellular reactive oxygen species in mammalian cells (Pulskamp et al. 2007).

CNTs formed a coating on the surfaces of the test organisms , which may result in adverse effects on the respiratory systems similar to those observed with juvenile trout (*O. mykiss*) exposed to SWCNTs (Smith et al. 2007). The ingestion of the CNTs by the amphipods, midge and oligochaetes **(**Figure 3.2**)** likely caused adverse effects to these organisms. In another study, dietary CNTs blocked the digestive tract and caused mortality in fruit flies (*Drosophila melanogaster)* (Leeuw et al. 2007). For the amphipods and midge, it is observed that CNTs in their guts was not depurated when the exposed organisms (14-d) were transferred into clean test water for 24-h. CNTs in the guts may have caused some digestive tract function disorders such as loss of appetite or blockage of food passage, leading to starvation or death. The TEM images of sections of gut of amphipods and midge showed CNTs deposits between and around the microvilli (Figure 3.3), but there was no evidence of the CNTs penetrating cell membranes and tissues. This is consistent with a recent report that CNTs were ingested by oligochaetes (*L. variegatus*) and accumulated in the guts but was not absorbed into the cellular tissues (Petersen et al. 2008). In our study, CNTs were likely in aggregates larger than pores of cell membranes and therefore failed to enter the tissues. Cheng et al. (2007) reported that SWCNTs aggregates could not enter the chorion of zebrafish (*Danio rerio*) embryos because the sizes were larger than those of the pores on the embryo chorion.

The toxicity of CNTs, therefore, would be different from other acutely toxic substances (e.g., acids), where the toxicants produce immediate destruction of epithelial surfaces and are rapidly absorbed through the contact surface and distributed to neighboring tissues (Sweet and Strohm 2006).

Sonication is commonly used in industry to disperse CNTs for composite reinforcements (Kim et al. 2009), and it is known to result in shorter nanotubes (Saleh et al. 2008). Sonication in aquatic toxicity studies with carbon nanomaterials such as SWCNTs (Smith et al. 2007) and fullerene (C60) (Oberdorster et al. 2006) might have increased their observed toxicity. In the current study, sonication of SWCNT-S increased toxicity of CNTs to amphipods and midge, but not to oligochaetes. Sonication of MWCNTs did not increase toxicity of these materials to amphipods or midge, but either increased or decreased their toxicity to mussels or oligochaetes. These results differ from those observed with silicon carbide (SiC) nanowires, where increased toxicity of SiC to amphipods *H. azteca* was always observed with sonication (Mwangi et al. 2010).

Pre-cleaning of MWCNTs with nitric acid significantly decreased their lethal effects to amphipods, midge, and mussels. Nevertheless, the biomass of amphipods, midge, and oligochaetes was reduced with exposure to pre-cleaned MWCNT-S. Hence, the toxicity of the MWCNT-S was not only due to metals. The acid treatment process adapted in this study removes 49% of Ni and 34% of Fe while the remaining metals are considered to reside inside MWCNTs and unlikely to dissolve in water during the toxicity test (Hua et al. 2008). Analysis of the acid treated MWCNTs by EDS, which probes surface compositions, show the metal impurities were preferentially removed.

Another study similarly show that treatment of CNTs with acid removes impurities including most of the metals in CNTs except for those encased inside the nanotubes (Pulskamp et al. 2007).

In amphipod tests with SWCNT-S, concentrations of Cr, Fe, Mo, and Ni at day 2 and 5 were all below the estimated effect concentrations (Table SI-3), except for Co with 227 μg Co/L for non-sonicated treatment and 317 μg Co/L for the sonicated treatment that were higher than the 7-d LC50 of 89 μg Co/L for *H. azteca* (Borgmann et al. 2005). In tests with amphipods exposed to MWCNT-S, the highest Co concentration was 121 μg Co/L and concentration was much lower in the tests with mussels, midge, and oligochaetes. The difference could be caused by the different foods used and/or trace metal absorption characteristics of the different organisms. The measured concentrations of Cr, Fe, and Mo in water were low compared with their reported 7-d LC50 of >3150 μg/L for *H. azteca* (Borgmann et al. 2005). However, the concentration of Ni in non-sonicated or sonicated MWCNT-S (test 3, Table SI-3) exceeded the 14-d effect concentration at 20% (EC20) of 61 μg Ni/L for *H. azteca* (Keithly et al. 2004) and the U.S. Environmental Protection Agency (USEPA) water quality criteria maximum concentration (470 μg Ni/L) for the protection of aquatic community in freshwater at similar hardness (USEPA 2009).

EDTA range finder test showed 0.04-g EDTA/L (1.369×10^{-4} mol/L) was non-toxic to amphipods in 14-d. This 1.369×10^{-4} mol/L EDTA could potentially bind 8.036 mg-Ni/L (0.0001369 mol/L x 58.7 g Ni/mol) assuming no competition for binding sites with other metals e.g., Ca, Mg or Co (Nowack and Sigg 1996).

The 0.04 g EDTA/L was adequate to bind the 1420 μg/L Ni measured from non-sonicated MWCNT-S treatment and thus eliminate the Ni potential adverse effects on the amphipods.

Based on the observations that pre-cleaning of CNTs with nitric acid removed a large fraction of the metal impurities and the pre-cleaned CNTs demonstrated no significant effects on the survival of amphipods, midge, or mussels, we hypothesize that dissolved metals from the CNTs in the water contribute to the toxicity. It has been reported that transition metals are effective catalysts of oxidative stress in cells, tissues, and biofluids and combined with the presence of carbon nanotubes could induce great oxidative damage to macromolecules (Pulskamp et al. 2007). Nickel is considered one of the main metals contributing to the toxicity of the CNTs because measured concentrations in the overlying water with the CNTs treatments were often above estimated Ni effect concentrations in the first several days of exposures. This is further confirmed in the tests with amphipods with and without EDTA as well as spiked Ni. The EDTA is known to form a strong complex with Ni, which could decrease Ni toxicity (Burkhard and Ankley 1989). The results indicate that while 1.50 mg/L (0.00255 mM) soluble Ni is highly toxic to amphipods (0% survival), the presence of 40 mg/L(0.137mM) EDTA at the same time eliminated the toxicity of the Ni to amphipods. The presence of EDTA also increased the survival of amphipods from 15% to 30% with MWCNTs, which indicates a decrease in toxicity, although the effect was not completely eliminated. Hence, toxicity of the CNTs was not solely due to the release of metals to the water.

In summary, two factors in the as-prepared CNTs are responsible for the observed toxicity to aquatic invertebrates: (1) *Metals solubilized from CNTs*. The dissolved amounts of toxic metals in the water were higher than the reported toxic effect concentrations for amphipods, at least for Ni in the first 2-3 days of exposures (Borgmann et al. 2005, Schubauer-Berigan et al. 1993). Thus the dissolved metals are likely responsible, in part, for the toxicity. Pre-cleaning of CNTs with acid reduces the soluble metal concentrations and thus reduces the observed toxicity of the CNTs to amphipods, midge and mussels. Similarly, the presence of a strong complexing agent (e.g., EDTA) that sequesters toxic metals reduced toxicity. (2) *Intrinsic toxicity of CNTs*. Nitric acid cleaning removes soluble metals from CNTs. Nevertheless, while the acid cleaned CNTs did not affect the survival of amphipods, midge and oligochaetes, they reduced their growth (biomass) significantly. Also, while EDTA can completely eliminate the toxicity of soluble Ni, it does not eliminate the toxicity MWCNT-S. Presumably, any solubilized metals from the CNTs are sequestered by EDTA. So toxicity observed for the acid-cleaned CNTs and in the presence of EDTA must be caused by CNTs.

Environmental implication of this study will become significant when large scale manufacturing, use, and disposal of CNTs occur. The results suggest that releases of as-produced CNTs will cause toxic effect to the benthic invertebrates. This will not only reduce the population of the invertebrates but also likely interfere with the ecological balances with potential to disrupt the food chain in the impacted area.

The study re-enforces the recommendation that all known information on elements in the nanomaterials in the market should be included in the materials safety data sheets (Bernard 2006). It also suggests that acid cleaning of CNTs, which removes significant amounts of easily soluble toxic metals, should be practiced prior to potential applications.

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Table S3.1A. Conditions for water-only toxicity tests with (1) As-produced carbon nanotubes (CNTs) with or without addition of ethylenediaminetetraacetic acid (EDTA), (2) Nitric acid modified CNTs, or (3) nickel (Ni) solution with or without EDTA addition using amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegatus*), and mussels (*Villosa iris*) under static renewal conditions using procedures adapted from ASTM (2009) and USEPA (2000).

1. Test type:	Static renewal
2. Test material	1. As-produced CNTs 2. Nitric acid modified as-produced CNTs 3. CNTs + EDTA
3. Test Duration:	14 d
4. Temperature:	23±1ºC
5. Light quality:	Ambient laboratory illumination
6. Light intensity:	Wide-spectrum fluorescent lights at 200 lux
7. Photoperiod:	16L: 8D
8. Test chamber:	300-ml glass beaker with 5-ml fine sand except for mussel test with no sand addition
9. Water volume:	200 ml
10. Water renewal:	100 ml overlying water on Mondays (M), Wednesdays (W), and Fridays (F)
11. Organisms/age:	About 7-d old amphipods or midge, 5- or 8- month old mussels, or adult oligochaetes
12. Organisms/chamber:	10
13. Replicates/treatment:	4 (+ 1 for photographing organisms)
14. Feeding:	1. Amphipods: 0.5 ml Yeast-Cerophyl-Trout Chow (YCT) after water replacement on MWF 2. Midge and oligochaetes:1.0 ml of Tetrafin®flake fish food after water replacement on MWF 3. Mussels: 2 ml of non-viable algal mixture twice daily
15. Aeration:	About 3 air bubbles/second from the bottom of the test beakers
16. Overlying water:	Diluted well water (Hardness of 100 mg/L as $CaCO_3$, pH 8.2)
17.Test concentrations/beaker:	Negative controls, and 1. 200 mg CNTs in 200 ml test water 2. 200 mg CNTs + 0.04 g EDTA/L in test water 3. 1.5 mg Ni/L 4. 1.5 mg Ni/L + 0.04 g EDTA/L in water
18. Mixing conditions:	Sonication or non-sonication of CNTs
19. Dilution factor:	None
20 Chemical residues:	Ni, Co, Mo, Fe, Cr analyzed in overlying water weekly
21. Water quality:	Dissolved oxygen, pH, conductivity, hardness, alkalinity, ammonia in overlying water measured weekly
22. Endpoints:	1. Images of test organisms exposed to CNTs in water 2. Survival or growth or biomass for amphipods, mussels, midge, and biomass for oligochaetes
23. Test acceptability:	≥80% survival in controls for amphipods and mussels ≥70% survival in control for midge 14-d biomass > 0-d biomass in control for oligochaetes

Table S3.1B. Test conditions for range finder 14-d toxicity test with EDTA using amphipod (*Hyalella azteca)* under static renewal in basic accordance with ASTM (2009) and USEPA (2000)

Parameter	**Details**
1. Test type:	Static renewal
2. Test material	EDTA
3. Test Duration:	14 d
4. Temperature:	23±1ºC
5. Light quality:	Ambient laboratory illumination
6. Light intensity:	Wide-spectrum fluorescent lights at 200 lux
7. Photoperiod:	16L: 8D
8. Test chamber:	300-ml glass test beaker with 5-ml fine sand substrate
9. Water volume:	200 ml
10. Water renewal:	100-ml EDTA solution or test water (control) on Mondays (M), Wednesdays (W), and Fridays (F)
11. Organisms/age:	7-d old amphipod
12. Organisms/chamber:	10
13. Replicates/treatment:	2
14. Feeding:	0.5 ml YCT on M, W, F after water renewal.
15. Aeration:	About 3 air bubbles /second from bottom of exposure beakers
16. Overlying water:	100 mg/L EDTA solution
17. Treatments:	i.Test water (control) ii. 0.16 g EDTA /L iii. 0.08 g EDTA /L iv. 0.04 g EDTA //L v. 0.02 g EDTA /L
18. Preparation EDTA stock solution:	Dissolve 3.2-g EDTA compound in 100-ml de-ionized water
19. Dilution factor:	0.5
20. Chemical residues:	None
21. Water quality:	Dissolved oxygen, pH, conductivity, hardness, alkalinity and ammonia on test Days 0, 7, and 14.
22. Endpoints:	i. Survival. ii. Average length, weight, and biomass.
23. Test acceptability:	≥ 80% control survival

Table S3.2. Mean water quality characteristics measured on Day 0, 7, and 14 in toxicity tests with single- or multi-wall carbon nanotubes from Shenzhen (SWCNT-S or MWCNT-S) or from Helix (MWCNT-H) or nitric acid modified (NAM) MWCNT-S or nickel (Ni) solution (1500 µg Ni/L) with or without the addition of 40 mg/L ethylenediaminetetraacetic acid (EDTA) using amphipods (*Hyalella azteca*), midge (*Chironomus dilutus*), mussels (*Villosa iris*), and oligochaetes (*Lumbriculus variegatus*) as test organisms.
Standard errors in parenthesis, n=4

Test	Species	Treatment	Dissolved Oxygen (mg/L)	Conductivity (µs/cm)	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Total ammonia (mg/L)
1	Amphipods	Control[a]	7.7 (0.1)	306 (12)	8.4 (0.10)	105 (4.0)	114 (8.0)	0.45 (0.14)
		Non-sonicated MWCNT-H	7.9 (0.1)	312 (14)	8.4 (0.10)	106 (2.5)	117 (7.5)	0.54 (0.10)
		Sonicated MWCNT-H	7.8 (0.1)	306 (15)	8.4 (0.05)	111 (8.0)	119 (9.0)	0.46 (0.06)
	Midge	Control	8.2 (0.2)	316 (29)	8.3 (0.05)	108 (10)	125 (12)	0.27 (0.04)
		Non sonicated MWCNT-H	8.0 (0.1)	320 (31)	8.3 (0.05)	112 (12)	123 (12)	0.71 (0.23)
		Sonicated MWCNT-H	8.2 (0.1)	322 (33)	8.3 (0.10)	112 (12)	123 (12)	0.64 (0.45)
	Mussels	Control	8.2 (0.2)	339 (39)	8.3 (0.10)	114 (13)	129 (13)	0.52 (0.14)
		Non sonicated MWCNT-H	8.0 (0.1)	337 (31)	8.3 (0.05)	116 (12)	131 (11)	0.43 (0.11)
		Sonicated MWCNT-H	8.3 (0.1)	331 (37)	8.3 (0.10)	116 (15)	122 (16)	0.40 (0.15)
	Oligochates	Control	8.3 (0.20)	324 (33)	8.3 (0.10)	118 (15)	125 (13)	0.73 (0.25)
		Non sonicated MWCNT-H	8.1 (0.10)	324 (32)	8.3 (0.10)	116 (14)	137 (16)	0.61 (0.23)
		Sonicated MWCNT-H	8.2 (0.10)	316 (31)	8.3 (0.10)	116 (15)	131 (14)	0.55 (0.21)
2	Amphipods	Control	8.5 (0.10)	275 (14)	8.4 (0.10)	106 (7.0)	118 (4.0)	0.10 (0.01)
		Non-sonicated SWCNT-S	8.5 (0.05)	283 (17)	8.4 (0.10)	103 (6.0)	121 (5.0)	0.40 (0.25)
		Sonicated SWCNT-S	8.5 (0.05)	266 (19)	8.4 (0.10)	103 (6.0)	127 (5.0)	0.10 (0.01)
	Midge	Control	8.0 (0.2)	295 (26)	8.5 (0.01)	105 (2.5)	136 (17)	0.52 (0.41)
		Non sonicated SWCNT-S	8.0 (0.2)	289 (24)	8.5 (0.02)	111 (1.0)	130 (13)	0.94 (0.40)
		Sonicated SWCNT-S	8.1 (0.2)	292 (25)	8.5 (0.02)	113 (1.5)	125 (11)	0.69 (0.32)
	Oligochates	Control	8.0 (0.20)	295 (26)	8.5 (0.02)	114 (7.0)	126 (5.5)	1.37 (0.61)
		Non sonicated SWCNT-S	8.0 (0.30)	294 (26)	8.5 (0.01)	115 (4.0)	126 (11)	1.10 (0.50)
		Sonicated SWCNT-S	8.0 (0.30)	291 (25)	8.5 (0.02)	115 (5.0)	128 (12)	1.02 (0.45)
3	Amphipods	Control	9.0 (0.05)	339 (35)	8.4 (0.10)	121 (10)	129 (15)	0.77 (0.42)
		Non-sonicated MWCNT-S	9.0 (0.05)	337 (34)	8.4 (0.20)	120 (9.0)	124 (11)	0.44 (0.17)
		Sonicated MWCNT-S	8.8 (0.15)	335 (33)	8.4 (0.10)	121 (9.5)	133 (14)	0.27 (0.11)
	Midge	Control	8.9 (0.1)	343 (32)	8.1 (0.10)	120 (10)	133 (12)	0.23 (0.12)
		Non sonicated MWCNT-S	8.9 (0.1)	343 (39)	8.1 (0.10)	120 (9.0)	133 (19)	0.39 (0.14)
		Sonicated MWCNT-S	8.6 (0.3)	342 (38)	8.4 (0.20)	124 (12)	133 (16)	0.28 (0.11)
	Mussels	Control	8.7 (0.1)	333 (35)	8.5 (0.15)	123 (10)	121 (9.0)	0.37 (0.14)
		Non sonicated MWCNT-S	8.8 (0.1)	340 (36)	8.4 (0.15)	123 (10)	124 (11)	0.30 (0.12)
		Sonicated MWCNT-S	8.8 (0.3)	343 (38)	8.3 (0.05)	117 (7.0)	127 (10)	0.21 (0.13)
	Oligochates	Control	8.8 (0.1)	331 (32)	8.4 (0.10)	123 (10)	122 (13)	0.42 (0.08)
		Non sonicated MWCNT-S	8.9 (0.1)	335 (36)	8.4 (0.10)	121 (10)	130 (19)	0.37 (0.08)
		Sonicated MWCNT-S	9.0 (0.1)	333 (34)	8.7 (0.20)	123 (10)	125 (15)	0.33 (0.07)
4	Amphipods	Control	7.7 (0.2)	257 (10)	8.3 (0.10)	92 (2.0)	107 (3.0)	0.36 (0.15)
		Non-sonicated NAM MWCNT-S	7.6 (0.3)	252 (17)	8.3 (0.10)	92 (3.0)	106 (5.0)	0.50 (0.04)
	Midge	Control	7.8 (0.1)	267 (15)	8.4 (0.20)	97 (3.5)	107 (3.0)	0.50 (0.21)
		Non sonicated NAM MWCNT-S	7.7 (0.1)	256 (10)	8.3 (0.10)	93 (3.0)	106 (2.5)	0.67 (0.29)
	Mussels	Control	7.8 (0.1)	273 (18)	8.3 (0.10)	99 (4.0)	105 (2.5)	0.34 (0.13)
		Non sonicated NAM MWCNT-S	7.7 (0.2)	271 (17)	8.3 (0.05)	94 (3.5)	103 (3.0)	0.46 (0.19)
	Oligochates	Control	7.7 (0.2)	294 (30)	8.4 (0.05)	108 (10)	108 (5.5)	0.30 (0.20)
		Non sonicated NAM MWCNT-S	7.5 (0.3)	264 (13)	8.3 (0.05)	98 (4.0)	103 (3.0)	0.35 (0.30)
Ni	Amphipods	Control	7.8 (0.15)	252 (22)	8.3 (0.10)	95 (3.0)	121 (11)	0.10 (0.05)
		Non-sonicated MWCNT-S	7.8 (0.15)	259 (20)	8.3 (0.10)	95 (6.0)	123 (10)	0.20 (0.10)
		Non-sonicated MWCNT-S +EDTA	7.9 (0.10)	244 (11)	8.2 (0.10)	72 (8.0)	125 (10)	0.20 (0.10)
		Ni	7.8 (0.15)	269 (37)	8.2 (0.20)	89 (5.0)	116 (14)	0.20 (0.05)
		Ni+EDTA	7.9 (0.10)	232 (16)	8.1 (0.10)	71 (4.0)	120 (10)	0.30 (0.10)

[a]Water-only.

Table S3.3. Mean water quality characterisitics measured on day 0, 7 and 14 in ethylenediaminetetraacetic acid (EDTA) range finder 14-d toxicity tests with amphipods (*Hyalella azteca*). Standard deviation in parenthesis, n=3

Treatment	Dissolved oxygen (mg/L)	Conductivity (µS/cm)	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Total ammonia (mg/L)
Water (control)	6.8 (0.3)	233 (10)	8.3 (0.1)	101 (17)	105 (5.0)	0.1 (0.1)
0.02 g EDTA	6.9 (0.1)	223 (2.1)	8.1 (0.1)	80 (23)	102 (2.8)	0.2 (0.2)
0.04 g EDTA	7.1 (0.4)	213 (5.0)	8.1 (0.09)	79 (13)	96 (5.7)	0.3 (0.1)
0.08 g EDTA	7.1 (0.2)	190 (2.8)	7.9 (0.08)	61 (27)	85 (7.1)	0.7 (0.3)
0.16 g EDTA	7.1 (0.1)	159 (4.2)	7.9 (0.05)	56 (65)	62 (11)	0.9 (0.01)

Table S3.4. Measured concentrations (µg/L) of metals in overlying water in 14-d toxicity tests with single-wall carbon nanotubes from Shenzhen (SWCNT-S) or multi-wall carbon nanotubes from Shenzhen (MWCNT-S) with amphipods (*Hyalella azteca*), mussels (*Villosa viris*), midge (*Chironomus dilutus*), and oligochaetes (*Lumbriculus variegatus*). Numbers in bold exceed U.S. Environmental Protection Agency (US EPA) water quality criteria (USEPA 2009)

				Test day						WQC[a]		Borgmann et al. 2005[b]	Keithly et al. 2004[c]
Test	Species	Treatment	Element	2	5	7	9	12	14	CMC	CCC	7-d LC50	14-d EC20
2	Amphipods	Control	Co	<1.2[d]	<1.2	NM[e]	NM	NM	NM	NA[f]	NA	89	NA
			Cr	<1.0	<1.0	NM	NM	NM	NM	570	74	3150	NA
			Fe	29	<0.9	NM	NM	NM	NM	NA	NA	3150	NA
			Mo	<2	<2	NM	NM	NM	NM	NA	NA	3,150	NA
			Ni	<2.1	<2.1	NM	NM	NM	NM	470	52	147	61
		Non-sonicated SWCNT-S	Co	227	124	NM	NM	NM	NM	NA	NA	89	NA
			Cr	<1.0	<1.0	NM	NM	NM	NM	570	74	3150	NA
			Fe	<0.9	73	NM	NM	NM	NM	NA	NA	3150	NA
			Mo	1387	782	NM	NM	NM	NM	NA	NA	3,150	NA
			Ni	<2.1	<2.1	NM	NM	NM	NM	470	52	147	61
		Sonicated SWCNT-S	Co	317	116	NM	NM	NM	NM	NA	NA	89	NA
			Cr	<1.0	<1.0	NM	NM	NM	NM	570	74	3150	NA
			Fe	<0.9	78	NM	NM	NM	NM	NA	NA	3150	NA
			Mo	1586	1037	NM	NM	NM	NM	NA	NA	3,150	NA
			Ni	<2.1	<2.1	NM	NM	NM	NM	470	52	147	61
3	Amphipods	Control	Co	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	NA	NA	89	NA
			Cr	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	570	74	3150	NA
			Fe	<0.9	168	47	6.4	<0.9	<0.9	NA	NA	3150	NA
			Mo	<2	<2	<2	<2	<2	<2	NA	NA	3,150	NA
			Ni	<2.1	<2.1	<2.1	<2.1	8.2	<2.1	470	52	147	61
		Non-sonicated MWCNT-S	Co	89	<1.2	44	<1.2	<1.2	22	NA	NA	89	NA
			Cr	<1.0	4.8	<1.0	3.4	<1.0	<1.0	570	74	3150	NA
			Fe	7	20	110	30	13	<0.9	NA	NA	3150	NA
			Mo	1331	1027	559	513	537	281	NA	NA	3,150	NA
			Ni	**1422**	**848**	383	217	97	45	470	52	147	61
		Sonicated MWCNT-S	Co	121	25	51	18	6	<1.2	NA	NA	89	NA
			Cr	<1.0	<1.0	<1.0	<1.0	<1.0	2.0	570	74	3150	NA
			Fe	14	2	5	<0.9	<0.9	<0.9	NA	NA	3150	NA
			Mo	1816	1250	736	454	434	346	NA	NA	3,150	NA
			Ni	**2516**	**797**	**740**	395	227	162	470	52	147	61
3	Mussels	Control	Co	8	<1.2	<1.2	<1.2	<1.2	<1.2	NA	NA	NA	NA
			Cr	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	570	74	NA	NA
			Fe	<0.9	<0.9	<0.9	25.8	13.1	15	NA	NA	NA	NA
			Mo	47	97	<2	<2	6.8	4	NA	NA	NA	NA
			Ni	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	470	52	NA	NA
		Non-sonicated MWCNT-S	Co	50	22	26	<1.2	<1.2	<1.2	NA	NA	NA	NA
			Cr	38	<1.0	60	<1.0	1.3	96	570	74	NA	NA
			Fe	72	16	11	<0.9	<0.9	25	NA	NA	NA	NA
			Mo	1464	1326	415	313	484	240	NA	NA	NA	NA
			Ni	**1704**	**900**	**479**	184	92	199	470	52	NA	NA
		Sonicated MWCNT-S	Co	74	36	54	<1.2	<1.2	<1.2	NA	NA	NA	NA
			Cr	78	54	44	<1.0	<1.0	128	570	74	NA	NA
			Fe	17	49	29	1.1	<0.9	53	NA	NA	NA	NA
			Mo	1862	1095	713	483	373	373	NA	NA	NA	NA
			Ni	**2094**	**1020**	**608**	248	197	304	470	52	NA	NA

Table S3.4 continued

3	Midge	Control	Co	22	16	39	<1.2	2	<1.2	NA	NA	NA	NA
			Cr	5	<1.0	13	<1.0	14	<1.0	570	74	NA	NA
			Fe	<0.9	22	84	50	53	36	NA	NA	NA	NA
			Mo	<2	10	<2	<2	142	99	NA	NA	NA	NA
			Ni	<2.1	<2.1	10	13	<2.1	9	470	52	NA	NA
		Non-sonicated MWCNT-S	Co	52	5	<1.2	<1.2	10	40	NA	NA	NA	NA
			Cr	<1.0	17	50	1	<1.0	71	570	74	NA	NA
			Fe	<0.9	<0.9	<0.9	<0.9	17.1	28	NA	NA	NA	NA
			Mo	1535	1021	252	672	252	240	NA	NA	NA	NA
			Ni	**2023**	**912**	**430**	**491**	285	323	470	52	NA	NA
		Sonicated MWCNT-S	Co	112	14	69	<1.2	<1.2	<1.2	NA	NA	NA	NA
			Cr	19	65	70	45	49	144	570	74	NA	NA
			Fe	14	35	<0.9	<0.9	<0.9	<0.9	NA	NA	NA	NA
			Mo	1670	1195	645	479	189	410	NA	NA	NA	NA
			Ni	**2433**	**1110**	**782**	**582**	236	344	470	52	NA	NA
	3 Oligochaetes	Control	Co	25	38	65	<1.2	31	9	NA	NA	NA	NA
			Cr	9.6	<1.0	47	26	<1.0	153	570	74	NA	NA
			Fe	<0.9	41	<0.9	<0.9	61	18	NA	NA	NA	NA
			Mo	33	87	<2	160	35	<2	NA	NA	NA	NA
			Ni	25	<2.1	9	20	<2.1	22	470	52	NA	NA
		Non-sonicated MWCNT-S	Co	79	58	45	41	60	<1.2	NA	NA	NA	NA
			Cr	<1.0	<1.0	44	66	<1.0	135	570	74	NA	NA
			Fe	<0.9	53	45	46	49	29	NA	NA	NA	NA
			Mo	1628	967	587	307	379	313	NA	NA	NA	NA
			Ni	**1910**	**966**	**777**	**612**	**579**	185	470	52	NA	NA
		Sonicated MWCNT-S	Co	29	22	26	<1.2	<1.2	<1.2	NA	NA	NA	NA
			Cr	<1.0	21	59	99	5.2	<1.0	570	74	NA	NA
			Fe	<0.9	16	34	39	<0.9	37	NA	NA	NA	NA
			Mo	1456	1326	313	456	208	329	NA	NA	NA	NA
			Ni	**2412**	**1020**	**810**	**573**	253	372	470	52	NA	NA

[a]Water quality criteria (USEPA 2009):

CMC- Criteria maximum concentration (hardness 100 mg/L as $CaCO_3$).

CCC- Criteria continuous concentration (hardness 100 mg/L as $CaCO_3$).

[b]7-d lethal concentration at 50% mortality (LC50) to *H. azteca* in water (hardness 124 mg/L and alkalinity 84 mg/L as $CaCO_3$ (Borgmann

[c]14-d 20% effective concentrations (EC20) to *H. azteca* in water at a hardness of 96 mg/L as $CaCO_3$ (Keithly et al 2004).

[d]Less than the inductively coupled plasma optical emission spectrometer (ICP -OES) detection limit.

[e]Not measured.

[f]Not available.

Table S3.5. Measured concentration (μg/L) of metals in 14-d toxicity test with multi-wall carbon nanotubes from Shenzhen (MWCNT-S) with or without addition of 40 mg/L ethylenediaminetetraacetic acid (EDTA), Ni solution (1.50 mg Ni/L) with or without addition of 40 mg/L EDTA (Ni+EDTA) with amphipod (*Hyalella azteca*). Numbers in bold exceed the acute water quality criteria (WQC, USEPA 2009)

	Ni			Mo			Co			Fe			Cr		
Treatment	0 d	8 d	14 d	0 d	8 d	14 d	0 d	8 d	14 d	0 d	8 d	14 d	0 d	8 d	14 d
Control	<2.1[a]	11	22	<2	56	70	5	<1.2	13	<0.9	10	<0.9	62	<1	36
Ni	860	1065	982	<2	<2	39	7	20	<1.2	2	<0.9	<0.9	<1	14	53
Ni + EDTA	1243	1133	1293	146	88	137	<1.2	22	<1.2	<0.9	<0.9	<0.9	92	<1	91
MWCNT-S	**880**	178	189	1397	321	121	4	<1.2	<1.2	5	5	<0.9	11	57	49
MWCNT-S + EDTA	1436	511	260	1539	220	142	30	<1.2	<1.2	6	23	35	28	41	28
CMC[b]	470			NA[f]			NA			NA			570		
CCC[c]	52			NA			NA			NA			74		
Borgmann et al. 2005[d]	147			3150			89			3150			3150		
Keithly et al. 2004[e]	61			NA			NA			NA			NA		

[a]Less than detection limit.

[b]Water quality criteria maximum concentration (CMC) of nickel (hardness 100 mg/L as $CaCO_3$), (USEPA 2009).

[c] Water quality criteria continuous concentration (CCC) of nickel (hardness 100 mg/L as $CaCO_3$), (USEPA 2009).

[d]7-d lethal concentration at 50% mortality (LC50) to amphpipod *H. azteca* in water hardness 124 mg/L as $CaCO_3$, alkalinity 84 mg/L as $CaCO_3$ (Borgmann et al 2005).

[e]14-d effective concentrations (EC20) to *H. azteca* in water at a hardness of 96 mg/L as $CaCO_3$ (Keithly et al 2004).

[f] Not available.

CHAPTER 4

TOXICITY OF MULTI-WALLED CARBON NANOTUBES IN WHOLE SEDIMENTS TO AMPHIPOD (*HYALELLA AZTECA*)

ABSTRACT

Whole-sediment toxicity tests were conducted with amphipods (*Hyalella azteca*) exposed to multi-walled carbon nanotubes (CNT) at up to 1% (dry weight) of sediment. The eight sediments tested (including a silica sand) had a broad range of total organic carbon (TOC; 0 to 11%), acid volatile sulfides (AVS; 0 to 42 μmole/g), sand percentage (11 to 100%), and clay content (0 to 61%). The 14-d tests with 1% CNT showed that survival of amphipods was significantly reduced only in the silica sand and Florissant soil. With other six sediments, the survivals were essentially the same with the respective controls. The biomass recovered at the end of the tests, however, tended to be reduced in six out of eight sediments relative to the respective controls but the differences were not statistically significant. Additional 14-d tests with Florissant soil showed that the impact of CNT on the amphipods biomass was statistically significant at 0.1%. Even with 0.01% CNT, there was 30% reduction in the amphipod biomass but it was not significantly different relative to control. Because biomass of amphipods in the 14-d exposures tended to be reduced in several sediments spiked with CNT, two of the sediments (Dow Creek and Raisin River) were therefore selected for subsequent 28-d testing at 1% CNT.

The results showed that the mean biomass was significantly reduced for both sediments relative to their respective controls. For the spiked Dow Creek sediment, survival was also statistically significant reduced relative to control. In summary, the results suggest that biomass was a more sensitive endpoint compared to survival, and the 14-d exposure was not enough to evaluate sub-lethal effects of CNTs spiked in sediment.

A number of factors associated with the properties of CNT and sediments might affect the observed toxicity of CNTs spiked into sediments. First, the metal impurities in CNT could be solubilized. The dissolved metal concentrations measured in overlying water in 14- and 28-d sediment tests, however, were mostly comparable between treatments and respective controls, except for silica sand where Ni and Mo were significantly elevated relative to the control. The results suggested that while metals might be released from the CNT, the binding capacity of the sediments evaluated were likely sufficient to sequester the metals, limiting their exposure to the amphipods. In the silica sand where significant releases of metals into the overlying water occurred, the metals including Ni contributed to the observed toxicity. Second. the CNT could coat the respiratory surfaces of the amphipods and/or block the digestive tract and contribute to the observed toxicity. Additionally, it was observed that there was an apparent separation and layering of CNT in sediments even if they were initially well homogenized in the sediment. Since CNT had low density, its concentration at the sediment/water interface was likely caused by physical and biological disturbance in the sediments.

Consequently, the amphipods dwelling at the interface of water and sediment would experience a level of exposure that could be much higher than what was indicated by the average sediment concentration or average aqueous concentration.

INTRODUCTION

Carbon nanotubes and other carbon-based nanomaterials are fundamentally important in the advent of nanotechnologies because of their exceptional chemical, physical, and biological properties, which could lead to wide applications in material science, medical field, electronics, textiles, automotives and in aviation industry (Seo et al. 2007). Carbon nanotubes may consist of a single graphene layer with 0.4 to 3nm in diameter (single wall carbon nanotubes) or multiple concentric layers of 1.4 to 100 nm in diameter (multi-walled carbon nanotubes) (Eklund et al. 2008). Most carbon nanotubes manufactured commercially are multi-walled (Thayer 2007), and by 2007, its production capacity had reached about 300 tons/year (Eklund et al. 2007). Since the carbon nanomaterials industry is posed for further rapid growth, there is little doubt that some of the materials will be released into the environment, potentially causing harm to human health and ecosystems. The multi-walled carbon nanotubes (CNT) mostly contain metal impurities (Guo et al. 2007, Donaldson et al. 2006), have fiber characteristics (Poland et al. 2008), and are barely biodegradable (Health and Safety Executive, HSE 2004). They are relatively insoluble in water (Hyung et al. 2007) and would likely deposit onto sediments in aquatic environment (Nowack and Bucheli 2007).

In a column study on the stability and settling of carbon materials in aqueous solutions, CNT settled more rapidly than carbon black and activated carbon, suggesting that sediments may be a repository for CNT (Kennedy et al. 2008).

In water-only exposures of CNT to amphipods, midge (*Chironomus dilutus*), oligochaetes (*Lumbriculus variegates*) and mussel (*Virosa iris*), mortality in CNT treatments ranged from 80 to 100%, with the amphipod consistently showing the highest sensitivity of the four invertebrates (see Chapter 3). The toxicity has been attributed to the presence of metal impurities and/or the fiber characteristics (Smart et al. 2005, Poland et al. 2008). The toxicity of CNTs mixed into sediments has barely been investigated. In one study, 10-d whole sediment tests were conducted and showed CNT in sediment had mild toxicity to amphipods (*Leptocheirus plumulosus* and *Hyalella azteca)* (Kennedy et al. 2008). The exposure of CNTs in sediment to oligochaetes (*Lumbriculus variegatus*) was not toxic although the organism ingested and accumulated the CNT in the gut (Peterson et al. 2008).

The objectives of this study were to (1) assess toxicity of CNT spiked in whole sediments to the amphipod, (2) investigate the distribution of CNT spiked into sediment, and (3) identify the factors that may be controlling the toxicity of CNT spiked into the sediment.

MATERIALS AND METHODS

Testing materials

The CNT sample was obtained from Shenzhen Nanotech Port Inc., China and was tested as received. As reported by the manufacturer's Materials Safety Data Sheet (MSDS), the CNT had diameter of 10-20 nm, length of 5-15 μm, and specific surface area of 40-300 m^2/g. It had a purity > 95 %, ash < 0.2 %, and amorphous carbon < 3 %, all by weight. Energy dispersive X-ray spectroscopy (EDS) study showed the sample elemental compositions (% wt) were carbon 89.7, oxygen 11.8, silicon 0.9, cobalt 0.2, molybdenum 1.2, and nickel 3.0 (see Chapter 3), suggesting partial oxidation of the sample. Eight sediments tested included silica sand (quartz), Florissant soil, and sediments from West Bearskin, Spring River, Dow Creek, St. Joseph River, Raisin River and Mill Creek, which had broad ranges in concentrations of total organic carbon (0 - 11 %), acid volatile sulfide (0 - 42 μmol l/g dry weight), and clay content (0 – 61%) (Table 4.1). These sediments had low concentrations of contaminants and good control performance for a variety of sediment testing organisms (e.g., amphipods, mayflies, oligochaetes) (John Besser, USGS, Columbia, MO, unpublished data). Due to their significant differences in properties such as TOC, AVS and clay content, they were selected with a goal to illustrate how these properties might affect the toxicity of CNT.

Table 4.1. Physical chemical properties of the sediments tested with multi-wall carbon nanotubes (MWCNT)

Sediment	Source	Acid volatile sulphides (µmol/g dw)	Total organic carbon (%)	Clay (%)	Silt (%)	Sand (%)	H2O (%)	Reference
Silica sand	Commercial	-	-	-	-	100	-	U.S. Silica Company 2006.
Florissant soil	Missouri	0.1	1	61	26	13	32	Besser et al. 2007.
West Bearskin	Minnesota	42	11	32	19	49	84	Ingersoll et al. 1998.
Raisin River	Michigan	0.6	0.6	2.8	4.6	93	30	Brumbaugh 2009.
Spring River	Missouri	0.8	0.5	7.9	16	76	30	Brumbaugh 2009.
Dow Creek	Michigan	1	1.6	6.1	6.5	87	47	Brumbaugh 2009.
St. Joseph River	Michigan	1.4	2.7	13	24	63	36	Brumbaugh 2009.
Mill Creek	Michigan	13	11	20	69	11	59	Brumbaugh 2009.

Spiking sediment with CNT

Effort was made to mix CNT into various sediments thoroughly to get a relatively homogeneous distribution of CNT in sediments. The quartz sand was rinsed with de-ionized water and dried prior to use. Florissant soil was ground on a glass plate using a wooden pin and sieved through #20 U.S. Standard stainless steel sieve (850 µm-opening) (Ingersoll et al. 2005). Other sediments were used as received. Duplicate samples of about100 ml for each sediment were placed in 500-ml glass jars with a polytetrafluoroethylene (PTFE) lined cap. A pre-determined amount of CNT was added to each jar to produce 1% CNT in each sediment (on a dry weight basis). In addition, proportionally smaller amounts of CNT were also prepared to produce 0.01% and 0.1% CNT in Florissant soil.

Mixing of CNT with silica sand or Florissant soil was accomplished by tumbling on a laboratory jar mill (model 75RMV, US Stoneware, East Palestine, OH) for 1 hr at 40 revolutions/minute (RPM). CNT was mixed with other sediment samples using the same jar mill but rotated at 20 RPM. Also at 20 min intervals, the samples were removed from the jar mill, shaken by hand for one minute and returned onto the jar mill.

After 1hr mixing, test water was added to silica sand and Florissant soil to maintain about 1 cm depth of overlying water. All water saturated sediment samples with CNT as well as original sediments as controls were placed in a water bath at 23ºC for 7 days prior to toxicity testing.

Toxicity tests

Tests were conducted under static renewal conditions using procedures adapted from U.S. Environmental Protection Agency (USEPA 2000) and American Society for Testing and Materials (ASTM 2009). Static water renewal instead of constant renewal was necessary to prevent release of nanomaterials with unknown health impact in the work place and the environment (see Chapter 3).

The tests were conducted for 14 to 28 days measuring effects on survival, length, weight, or biomass of amphipods. A 20-ml aliquot of each sediment sample was placed in 300-ml exposure beakers in four replicates using a 5-ml plastic teaspoon and rinsed with test water into the exposure beakers. 200 ml of test water (dissolved oxygen 7.5 mg/L, conductivity 286 µS/cm, pH 8.2, alkalinity 80 mg/L as $CaCO_3$, hardness 100 mg/L as $CaCO_3$ and total ammonia < 1.0 mg/L) was slowly added to minimize suspension of the sediment. The beakers were kept in the water bath at 23ºC for 24 h for any suspended sediment to settle before the start of the exposures (Day -1). About 20-ml of each sediment, including the controls, was stored in a freezer at -20°C until it was used to determine the distribution of spiked CNT in the sediments.

Ten 7-d-old amphipods were added into each exposure beaker on Day 0 and about 20 of these amphipods were preserved in 8% sugar formalin solution to record their initial body sizes at the start of the exposures (Ingersoll et al. 2005). The exposure beakers with amphipods were kept at 23°C on a 16:8 h light: dark cycle and a light intensity of about 200 lux. Aeration at 3-5 air bubbles/second was provided to each exposure beaker just above the sediment–water interface to maintain optimal dissolved oxygen levels in overlying water. During tests 50% of the overlying water in exposure beakers was replaced with fresh test water three times/week (e.g., Monday, Wednesday, and Friday) using a 50-ml syringe and a needle. One hour before replacing the water, aeration was shut off to let any suspended materials in overlying water to settle.

Amphipods in each beaker were fed 0.5 ml Yeast-Cerophyl-Trout Chow (YCT;1800 mg/L of YCT stock solution; ASTM 2009) after each water renewal. Preliminary tests had established that this feed rate was appropriate to maintain overlying water quality for satisfactory survival and growth of amphipods in control sediments.

Dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia in composited overlying water samples from each treatment were measured at least weekly following standard methods (APHA 2005). Water samples were also collected weekly for trace metal analysis. The samples were filtered through a 0.45-µm polypropylene membranes and preserved by adding 1% high purity nitric acid at 4°C. Dissolved metals were analyzed using inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian).

At the end of the tests on Day 14 or 28, sediment samples from exposure beakers were sieved through a U.S. Standard stainless steel sieve #40 (425 μm-opening) and the contents held in the sieve were washed into an examination glass pan. The surviving amphipods were counted and then preserved in glass vials by adding 8% sugar formalin solution and later their lengths were measured under a microscope (Ingersoll et al. 2005). The collected growth endpoint data included average length, average weight, and biomass. Weight of amphipods was estimated from measured length using the empirical relationship: Weight (mg) = $((0.177* \text{ Length (mm)}) - 0.0292)^3$ (Ingersoll et al. 2008). Biomass in each exposure beaker was then calculated as the sum of weight of the amphipods.

Distribution of CNT in sediment

Distribution of CNT in the selected sediments (Dow Creek, Raisin River, Spring River, and West Bearskin) was evaluated at the beginning and end of the 14-d toxicity tests. The assessment used parallel set-ups to the toxicity tests with aeration and water renewal, but no amphipods and food were introduced. On day 0 and 14, sediment samples were collected near the top and bottom of the sediment column using a Pasteur capillary pipette and preserved in 2 ml cryo tube vials at 4°C. The sediment was placed on 300 mesh copper grids with carbon film support and oven dried at 60°C for about 10 minutes prior to TEM analysis (JEOL 1400 TEM, Japan). The sediment samples were also examined on a FEI Quanta 600F environmental scanning electron microscope (SEM) (FEI, Hillsboro, Oregon, USA).

In addition, digital photographs were taken of the test sediments at the end of tests and later after overlying water had evaporated from the beakers, leaving only the dry sediments. The test conditions are summarized in Table S4.1.

Statistical analysis

The data is presented as mean values with variations about mean represented by standard deviations. The data was log transformed and tested for normality. The statistical differences in survival or growth data between treatments were determined by *t*-test or ANOVA followed by Fisher's Least Significant Difference (LSD) test if the data were normally distributed (Shapiro Wilk's test) or by Wilcoxon Rank Sum test when data failed normality test (USEPA 2000). Differences between treatments were statistically significant when $p<0.05$. Statistical analysis was performed with Statistical Analysis Systems (SAS Institute Inc., Cary, NC, USA) software, Version 9.1.3 for windows.

RESULTS

Water quality

The overlying water quality parameters in the 14- and 28-d whole sediment tests were within the expected ranges across treatments in all exposures (Table S4.2 and S4.3). Dissolved oxygen was 6.8 - 8.0 mg/L, conductivity 221 - 340 µS/cm, pH 7.9 - 8.4, alkalinity 42 - 119 mg/L as $CaCO_3$, hardness 78 - 156 mg/L as $CaCO_3$, and total ammonia 0.2 - 0.7 mg/L.

Concentrations of dissolved metals (Ni, Mo, Co, Fe) in sediment tests with 1% CNT were slightly elevated compared to that in the controls in most of the tests (Table S4.4). In test with silica sand spiked with 1% CNT, however, trace metal concentrations were 172 - 2,495 µg Ni/L, 241-1,588 µg Mo/L, < 26 µg Co/L, and < 39 µg Fe/L. These levels were much higher than those in the controls with < 29 µg Ni/L, < 85 µg Mo/L, < 1.2 µg Co/L, and <16 µg Fe/L (Table S4.4). These concentrations were also higher at the beginning of the exposures but then decreased with time. This trend was expected because of dilution effects with about 50% of overlying water replaced at three times /week during the exposure. The 14-d mean concentration of Ni was 1012 µg/L, much higher than the reported Ni effect concentrations for the amphipods (Keithly et al. 2004, Borgmann et al. 2005, USEPA 2009).

Toxicity of CNT-Spiked Sediments

14-d tests

The mean survival of amphipods in the controls in all 14-d tests was ≥ 93% (Table 4.2 and 4.3), indicating the tests met the acceptability criteria of 80% survival (ASTM 2009, USEPA 2000). Mean survival of amphipods in silica sand and Florissant soil spiked with 1% CNT was significantly reduced relative to the controls (test 1 and 2; Table 4.2). In six other treatments, the sediments with 1% CNT had survival rates comparable to the respective controls (West Bearskin and St. Joseph) or somewhat lower (Spring River, DOW Creek, Raisin River, and Mill Creek), but the differences were not statistically significant.

The growth of amphipods in 1% CNT treatment, as indicated by length, weight and biomass, showed significant reduction for Florissant soil (test 2, Table 4.2). No growth data was collected for the silica sand because no organisms survived in the treatment. The growth reduction, to a lesser degree, was also observed in four of the other six sediments. Additional 14-d tests with Florissant soil at lower CNT levels showed that the reduction in biomass was statistically significant at 0.1% level, and even with 0.01% CNT, there was a 30% reduction in biomass of the amphipods.

Table 4.2. Mean response of amphipods (*Hyalella azteca*) in 14-d whole sediment toxicity tests with multi-wall carbon nanotubes (MWCNT) spiked into nine sediments. Standard deviation in parenthesis, n=4. Asterisk (*) indicates a significant reduction relative to the control ($p<0.05$)

Test Number	Treatment	Survival (%)	Length (mm)	Weight (mg)	Biomass (mg)
1	Silica sand (control)	95 (5.8)	2.96 (0.16)	0.13 (0.22)	1.23 (0.24)
	Silica sand+1% MWCNT	0 (0)*	ND[a]	ND	0 (0)*
2	Florissant soil (control)	95 (5.8)	3.21 (0.18)	0.16 (0.04)	1.55 (0.26)
	Florissant soil +1% MWCNT	78 (13)*	2.65 (0.22)*	0.09 (0.02)*	0.69 (0.14)*
3	Florissant soil (control)	93 (5.0)	3.51 (0.29)	0.22 (0.06)	2.04 (0.56)
	Florissant soil + 0.01% MWCNT	83 (5.0)	3.26 (0.34)	0.17 (0.05)	1.42 (0.47)
	Florissant soil + 0.1% MWCNT	90 (8.2)	2.94 (0.07)	0.12 (0.01)	1.09 (0.18)*
	Florissant soil + 1% MWCNT	85 (5.8)	2.92 (0.07)	0.12 (0.01)	1.02 (0.15)*
4	Spring River (control)	100 (0)	3.16 (0.35)	0.16 (0.05)	1.55 (0.51)
	Spring River +1% MWCNT	95 (5.8)	2.85 (0.22)	0.13 (0.06)	1.24 (0.62)
5	West Bearskin (control)	98 (5.0)	3.04 (0.20)	0.14 (0.03)	1.33 (0.29)
	West Bearskin + 1% MWCNT	98 (5.0)	3.04 (0.10)	0.14 (0.01)	1.32 (0.11)
6	DOW Creek (control)	95 (5.8)	3.60 (0.07)	0.23 (0.02)	2.17 (0.21)
	DOW Creek +1% MWCNT	88 (9.6)	3.34 (0.33)	0.18 (0.06)	1.60 (0.52)
7	St. Joseph River (control)	98 (5.0)	3.06 (0.10)	0.14 (0.01)	1.34 (0.19)
	St. Joseph River +1% MWCNT	98 (5.8)	3.21 (0.20)	0.16 (0.03)	1.56 (0.33)
8	Raisin River (control)	100 (0)	3.81 (0.25)	0.28 (0.06)	2.78 (0.55)
	Raisin River +1% MWCNT	90 (12)	3.50 (0.21)	0.21 (0.04)	1.93 (0.48)
9	Mill Creek (control)	100 (0)	3.62 (0.20)	0.24 (0.04)	2.35 (0.41)
	Mill Creek +1% MWCNT	98 (5.0)	3.49 (0.42)	0.22 (0.09)	2.15 (0.91)

[a]Not reported due to survival <50% in the CNT treatments.

28-d tests

Two sediments (Dow Creek and Raisin River) were selected for 28-d testing at 1% CNT because biomass of amphipods in the 14-d exposures tended to be reduced in sediments spiked with 1% CNT, but these differences were not statistically significant. Mean survival, length, weight and biomass of amphipods with Dow Creek sediments were all significantly reduced relative to the control (Table 4.3). For Raisin River sediment containing 1% CNT, mean survival of amphipods was reduced but not significantly while mean length, weight and biomass of amphipods were significantly reduced relative to control. The growth was therefore a more sensitive endpoint than survival. With the increase in exposure duration from 14- to 28-d, the toxic effects of the 1% CNT in the sediments became more evident, suggesting that longer durations would be needed in sediment toxicity tests with CNT.

Table 4.3. Mean response of amphipods (*Hyalella azteca*) in 14-d and 28-d whole sediment tests with multi-wall carbon nanotubes (MWCNT) spiked into two sediments. Standard deviation in parenthesis, n=4. Asterisk (*) indicates a significant reduction relative to the control ($p<0.05$)

	14 d				28 d			
Treatment	Survival (%)	Length (mm)	Weight (mg)	Biomass (mg)	Survival (%)	Length (mm)	Weight (mg)	Biomass (mg)
Dow Creek (control)	100 (0)	3.77 (0.27)	0.27 (0.06)	2.73 (0.61)	100 (0)	4.61 (0.16)	0.50 (0.05)	4.96 (0.50)
Dow Creek +1% MWCNT	93 (4.3)*	3.17 (0.27)*	0.16 (0.04)*	1.45 (0.32)*	90 (8.2)*	4.15 (0.08)*	0.36 (0.02)*	3.22 (0.32)*
Raisin River (control)	95 (5.0)	3.64 (0.14)	0.24 (0.03)	2.30 (0.13)	100 (0)	4.48 (0.32)	0.45 (0.10)	4.55 (1.04)
Raisin River +1% MWCNT	95 (5.0)	3.35 (0.31)	0.19 (0.06)	1.78 (0.50)	95 (10)	3.99 (0.16)*	0.32 (0.04)*	2.99 (0.05)*

To assess whether the observed toxicity of CNT in sediments could be affected by sediment characteristics, the mean normalized biomasses of the amphipods in 14-d tests were plotted against AVS, TOC, or clay content (Figure 4.1).

The results indicated that the biomass was positively correlated with AVS and TOC, but negatively correlated with clay content.

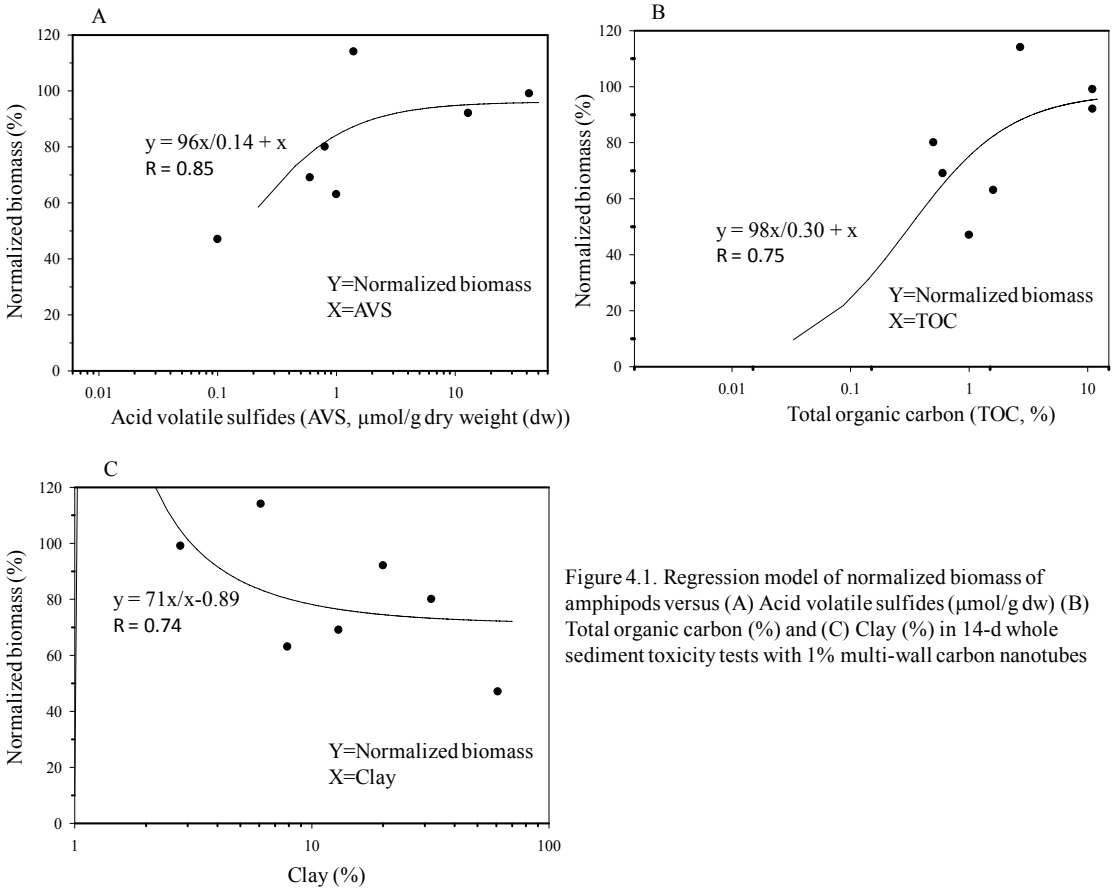

Figure 4.1. Regression model of normalized biomass of amphipods versus (A) Acid volatile sulfides (µmol/g dw) (B) Total organic carbon (%) and (C) Clay (%) in 14-d whole sediment toxicity tests with 1% multi-wall carbon nanotubes

Distribution of CNTs in sediment

The TEM and SEM images of whole sediment treatments showed 1% CNT mixed well with most of the sediments (Figure 4.2 and 4.3) except with silica sand where CNT remained in clumps. The metal catalysts in CNT remained intact 7 days after mixing with the sediments.

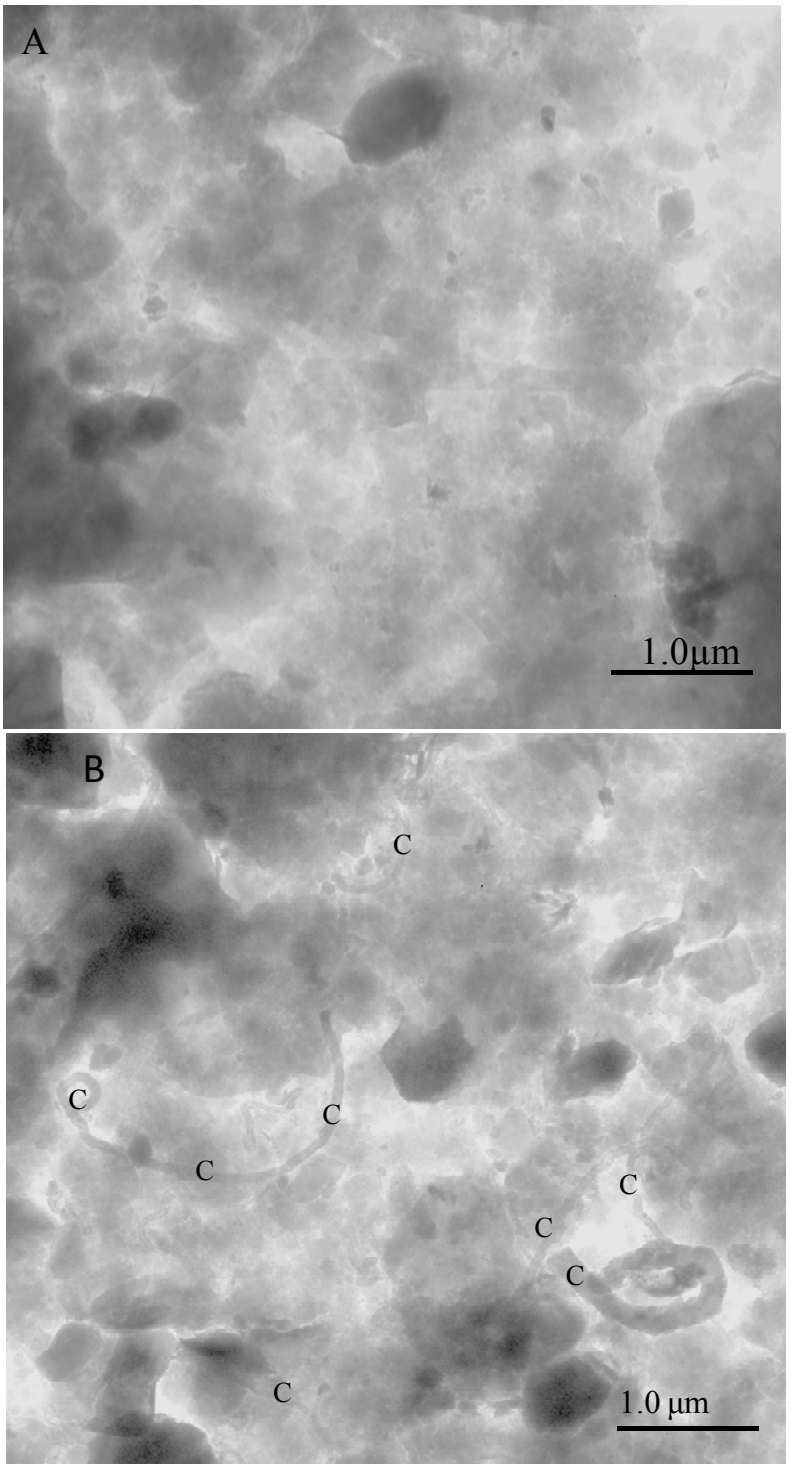

Figure 4.2. Transmission electron microscopy of whole sediment treatment samples on day 0 (A) Control sediment and (B) Sediment with 1% multi-wall carbon nanotubes marked C.

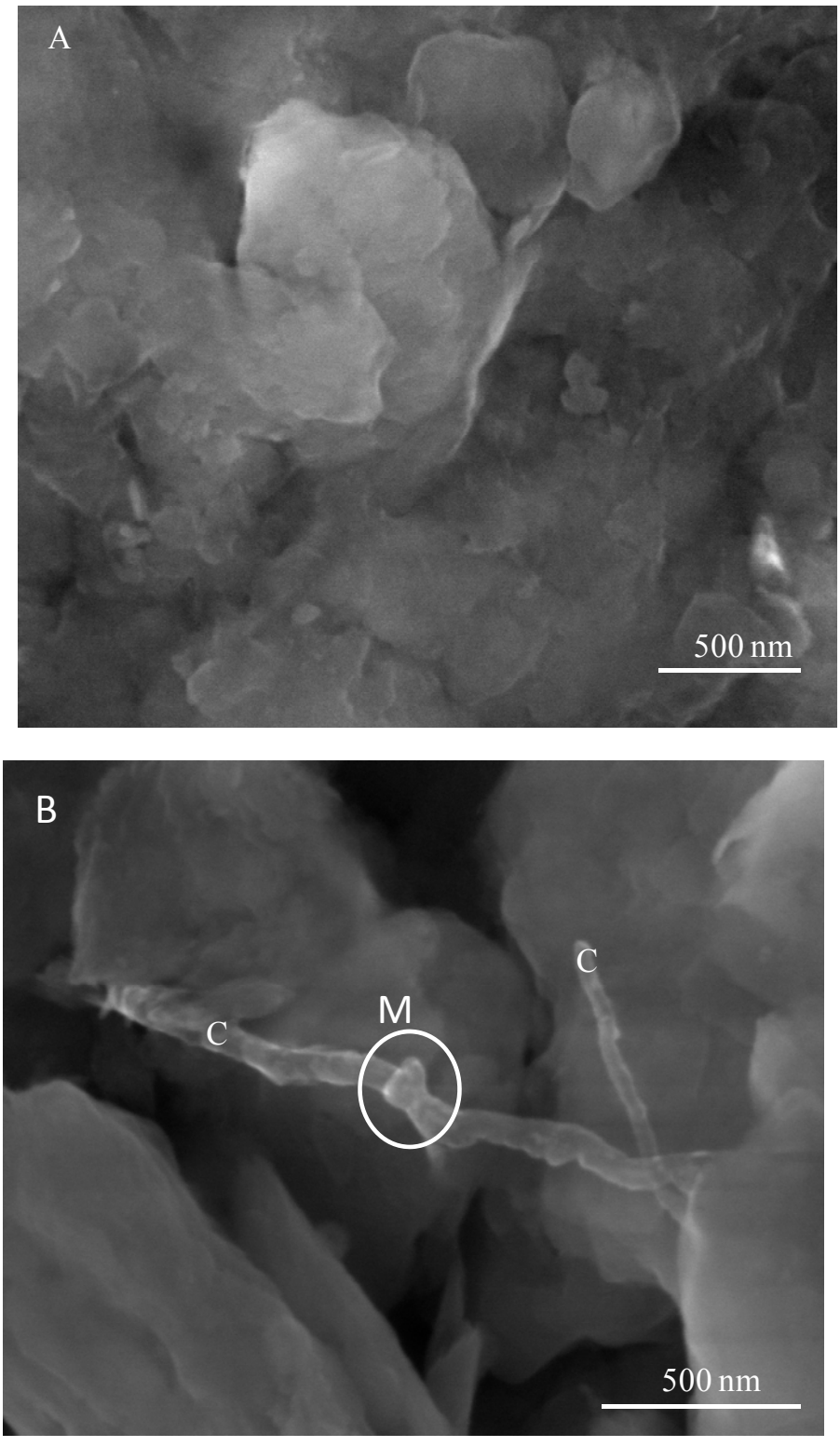

Figure 4.3. Scanning electron microscopy of a whole sediment sample at the top layer on day 0 showing: (A) Control sediment and (B) Sediment spiked with 1% multi-wall carbon nanotubes marked with C and metal catalyst attached to a carbon nanotube.

However, it was observed that there was an apparent separation and layering of CNT in sediments that were initially well homogenized in the sediment (Figure 4.4, 4.5, and 4.6).

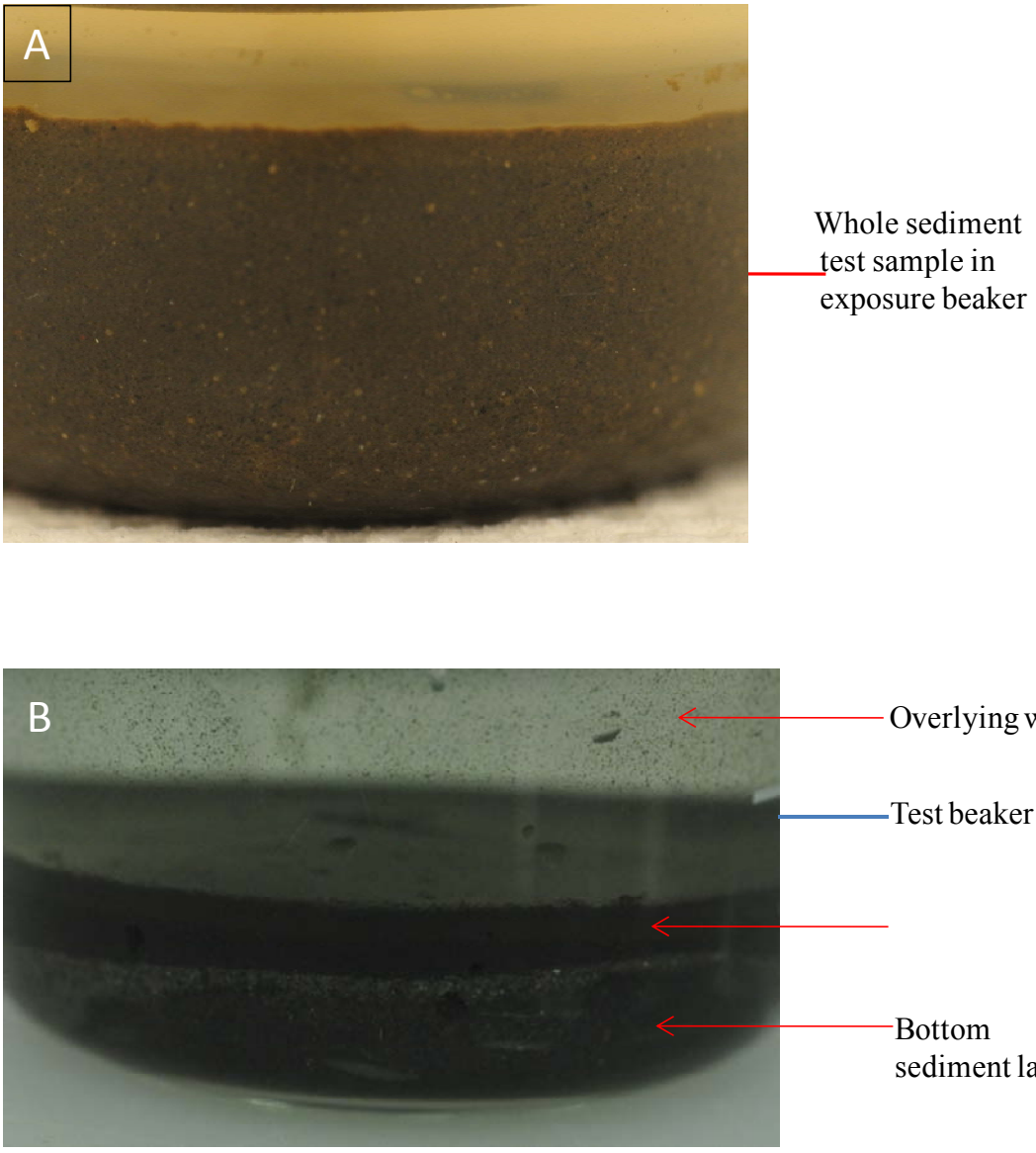

Figure 4.4. Side view of Raisin River whole sediment treatments (in test beakers) (A) Control sediment with consistent coloration and (B) Sediment spiked with 1% multi-wall carbon nanotubes on test day 0. The darker color of the top sediment layer indicates relatively more CNT than the bottom sediment layer with lighter color.

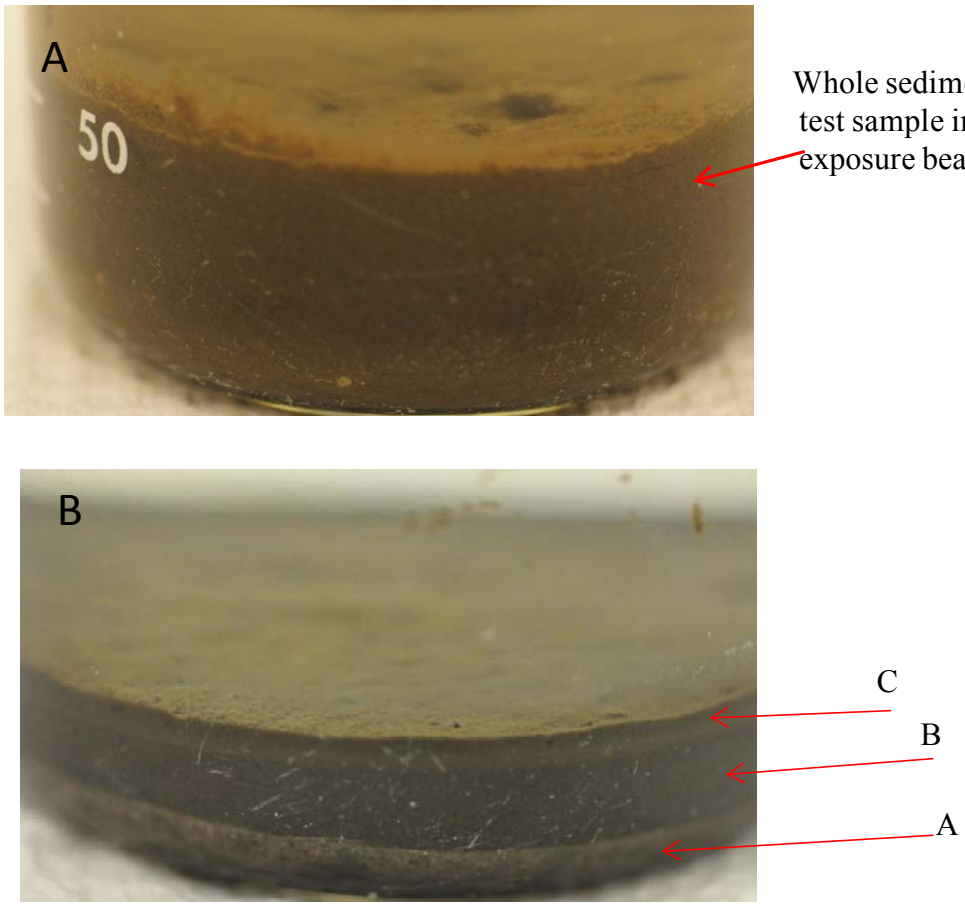

Figure 4.5. Side view of Dow Creek whole sediment test samples on day 0: (A). Control (sediment only) with consistent coloration in whole depth and (B) Spiked with 1% multi-wall carbon nanotubes . The layers A, B, C with contrasting colors indicates layering of spiked sediment probably with different amounts of CNT between layers.

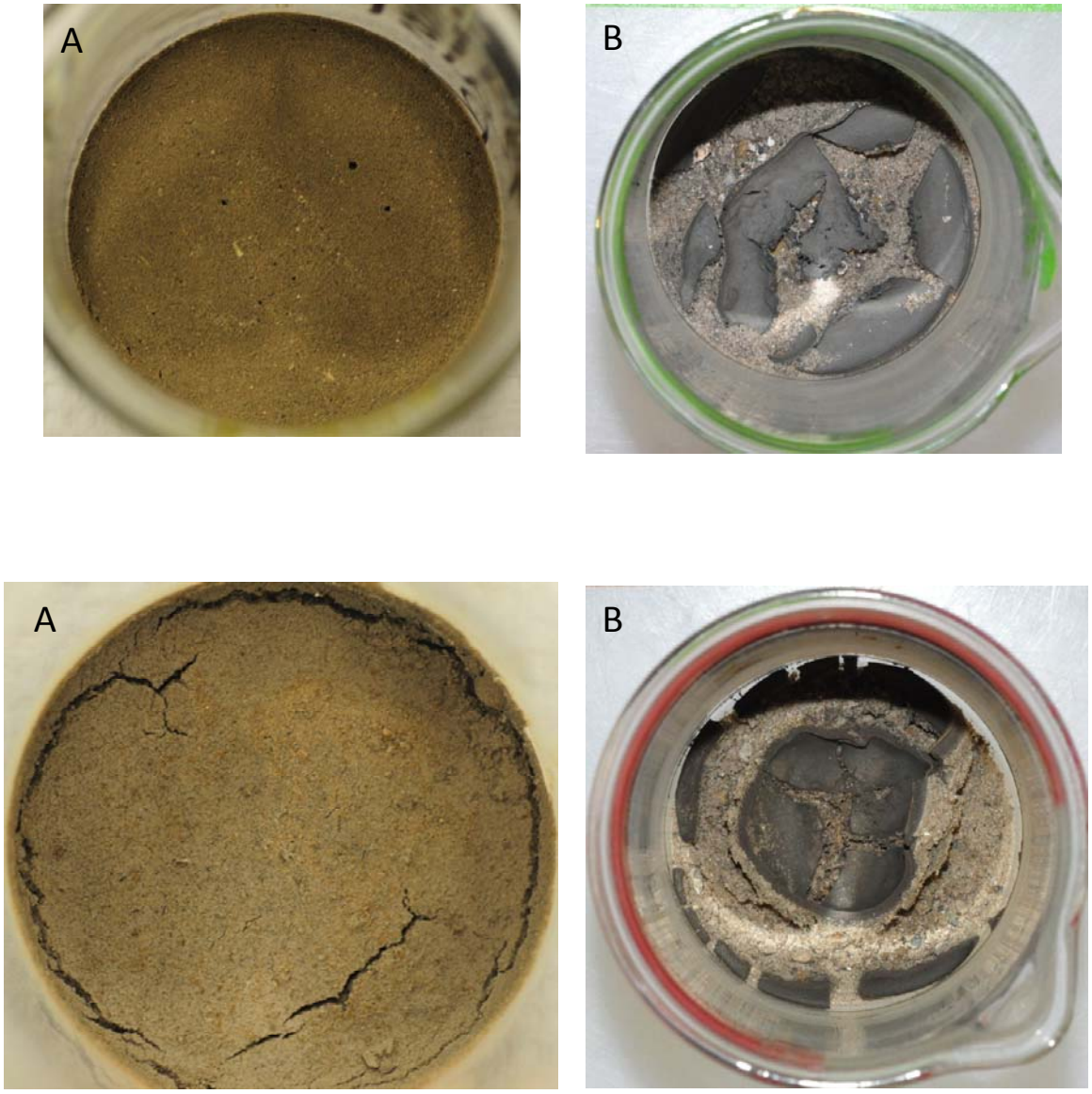

Figure 4.6. Dried post 14-d whole sediment toxicity test samples showing 1. Raisin River (A) Control (B) Spiked with 1% CNT and 2. Dow Creek (A) Control (B) Spiked with 1% CNT. The black crusts on (B) shows peeled off thin sediment layer with high concentration of CNT on what was the water-sediment interface.

DISCUSSION

The survival of amphipods exposed for 14d to 1% CNT in various sediments tested is significantly reduced with the silica sand and Florissant soil, but not with the other sediments. The biomass, however, shows more significant decrease in six out of eight sediments relative to the respective controls. Also with Florissant soil, the impact of 0.1% CNT on the growth is found statistically significant and even with 0.01% CNT, there is 30% reduction in the amphipod biomass. The effect of CNT on the growth is more apparent in the 28-d tests.

A number of factors may have affected the observed toxicity of CNT on amphipods including properties of CNTand sediment characteristics. The amphipod is relatively tolerant to a wide range in sediment properties such as grain size and total organic carbon (ASTM 2009, USEPA 2000). The toxicity differences observed for various sediments were not likely caused by the physical characteristics of the sediments (Table 4.1), but due to the CNT in the sediments and CNT interactions with the sediments (Table 4.2, Figure 4.1). The CNT spiked in sediments with < 1.0% TOC or about 0.1 μmole AVS/g dry weight (dw) was toxic to amphipods compared to sediments with > 2.0% TOC or > 1.4 μmole AVS/g dw which were not toxic (Figure 4.1A, 4.1B). For example, mean biomass with 1% CNT in the sediments was reduced by only 0.8% in West Bearskin (AVS= 42 μmol/g dw, TOC=11%) and by 9% in Mill Creek sediment (AVS=13 μmol/g dw, TOC=11%). In contrast, the mean biomass with Florissant soil (AVS=0.1 μmol/g dw, TOC=1%) was reduced by 50 to 56% and by 31% in Raisin River (AVS=0.6 μmol/g dw, TOC=1%).

The transition metals released from CNT appear to playa role in the toxicity. With 1% CNT, the metal concentrations measured in the overlying water were typically comparable across the sediments except in silica sand where Ni and Mo were much more elevated relative to the controls or in the other sediment tests (Table 4.4). As described earlier, the tests with CNT in the silica sand showed the highest toxicity to amphipods with 0% survivals in 14-d.

Nickel was reported as the main metal that could be solubilized from the CNT sample (Hua et al. 2008). A significant amount of Ni was also observed in water-only tests using the same CNT material (Chapter 3). In this study, Ni concentration in the silica sand test exceeded published effect concentrations including the criteria maximum concentration (CMC; 470 µg Ni/L) for the protection of aquatic community in freshwater (USEPA 2009), the *H. azteca* 7-d median lethal concentration at 50% mortality (LC50; 147 µg Ni/L, Borgmann et al. 2005), and the 14-d effective concentration at 20% (EC20; 61 µg Ni/L, Keithly et al 2004) under similar test conditions. Studies have reported that exposure of Ni to amphipods adversely affected the regulation of calcium, magnesium and sodium ions (Keithly et al. 2004). Therefore, Ni likely contributed to the toxicity of amphipods in the silica sand spiked with CNT in this study as reported in other studies that toxic metals solubilized from CNT can be one of the mechanisms causing toxicity to aquatic organisms (Helland et al. 2007, Strydom et al. 2006).

It is known that AVS and natural organic matters in sediments can form metal sulfides or metal-organic complexes with divalent transition metals including Ni (USEPA 2005) that reduce metal bioavailability to aquatic organisms including amphipods (Doig and Liber 2006, Ankley et al. 1996).

It has also been reported that clay minerals in the sediment provide metal binding sites and reduce their concentrations in overlying water (Hassan et al. 1996). The metal concentrations across sediment treatments were typically below reported effect concentrations for amphipods, which could be partially due to the bindings between toxic metals and sediments. Sequestration of toxic metals by such bindings could partially explain the lower toxicity of CNT in sediments with higher AVS and TOC. The metal binding capacities of the sediments tested are sufficient to decrease the toxic metal concentrations and hence bioavailability except in silica sand test where minimal metal sequestration is expected.

In addition to toxic metals associated with CNT, other mechanisms for the toxicity of CNT to amphipods likely include coating of respiratory surfaces or blocking digestive tract (Chatterjee 2008, Smith et al. 2007), and physical smothering through coating of the body (Zhu et al. 2009) have been proposed to explain the toxicity of CNTs to various aquatic organisms.

The degree of CNT aggregation and distribution in sediments is another dimension of complexity that may affect its toxicity to aquatic organisms. TEM and SEM images showed the CNT can be dispersed relatively well in the sediments except in silica sand treatment where mixing was poor. It is known that natural organic matter (NOM) enhances CNT dispersion in the aqueous solution (Hyung et al. 2007). The CNT have negative zeta potential at pH >7 because of oxygen-containing functional groups on the surface (Han et al. 2008). The CNT sample tested in this study has 12% (wt) oxygen, indicating the presence of substantial number of oxygen atoms on the surface.

The NOM molecules enhance the zeta potential thus increasing electrostatic repulsion between the nanotubes (Smith et al. 2009). Aggregation and separation of CNT mixed into the sediment, however, can occur with time. For example, at the end of 14-d testing, the black colored substance, presumably CNT, visibly accumulate at the sediment/water interface, and upon drying, a black crust formed in the sediment spiked with MWCT (Figure 4.5). Apparently, separation of CNT occurred in the testing sediments that were originally quite homogenous. The densities of CNT (1.3 -1.4 g/cm^3) (Collins and Avouris 2000) is much lower that sand grains (2.6 g/cm^3) and even graphite (2.1 g/cm^3). We hypothesize that under the influence of physical and/or biological disturbance in shallow sediments, CNT that are physically mixed but not bound to sediment particles could accumulate at the sediment/water interface, and such accumulation/concentration might impact the toxicity to benthic aquatic organisms. Past studies have reported that amphipods spend a lot of time in direct contact with sediment and burrow into the upper layer of sediment during exposures (Ingersoll et al. 2000). Due to such concentration of CNT at the water/sediment interfaces, amphipods dwelling at the interface of water and sediment may have experienced a level of exposure that can be significantly higher than what is indicated by the average CNT concentration in the sediment or in the aqueous phase.

In summary, CNT mixed in some sediments can be toxic to amphipods at concentration as low as 0.01 g CNT/g sediment, although sediments with high AVS and TOC appear to decrease the degree of toxicity. Growth is a better end point for the toxicity testing, and long term testing (i.e., 28-d or 42-d) is preferred because of its higher sensitivity.

Binding of toxic metals by sediment components is one of the mechanisms for the decreased toxicity. Since not all sediments have strong metal binding capacity, removing toxic metals from CNT prior to its application and release is recommended. Similar recommendations are made by other researchers (Plata 2009, Hull et al. 2009). However, eliminating toxic metals alone may not be sufficient to eliminate toxicity of CNT to benthic aquatic organisms. Other mechanisms such as blockage of digestive track and physical smothering of the organisms could contribute to the toxic effect.

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SUPPORTING INFORMATION

Table S4.1. **C**onditions for conducting 14- and 28-d whole sediment toxicity tests with as-produced multi-wall carbon nanotubes (CNTs) with the amphipod *Hyalella azteca* under static renewal conditions using procedures adapted from test methods by ASTM (2009) and USEPA (2000)

1. Test type:	Whole-sediment exposure with renewal of overlying water
2. Test material:	CNTs (Shenzhen Nanotech Port Inc., China)
3. Test duration:	14 to 28 d
4. Temperature:	23 ± 1°C
5. Light quality:	Ambient laboratory illumination
6. Light intensity:	Wide-spectrum fluorescent lights at 200 lux
7. Photoperiod:	16L: 8D
8. Test chamber:	300-ml glass beaker
9. Overlying water volume:	About 180 ml
10. Water renewal:	About 100 ml (50% of water renewal) on Monday (M), Wednesday (W), and Friday (F)
11. Organisms/age:	7-d old amphipods
12. Organisms/chamber:	10
13. Replicates/treatment:	4
14. Feeding:	0.5 ml YCT (1800 mg/L suspension of stock solution) on MWF after water renewal
15. Aeration:	3-5 air bubbles /second from the bottom of the test beakers
16. Overlying water:	Diluted well water (Hardness of 100 mg/L as $CaCO_3$)
17. Test concentration/beaker:	1-2. 200 mg CNT (dry weight, dw) + 20-g Florissant soil or silica sand (dw), and 180 ml overlying water; and 20-g Florissant soil (dw) or 20-g silica sand (dw) with 180 ml overlying water as negative controls 3. 216 mg CNT (dw) + 20-ml Dow Creek (DCr) wet sediment with 180 ml overlying water; and 20-ml DCr as control with 180-ml overlying water 4. 238 mg CNT (dw) + 20-ml Raisin River (RR) wet sediment with 180 ml overlying water; and 20-ml RR wet sediment as negative control with 180 ml overlying water 5. 173 mg CNT (dw) + 20-ml St Joseph River (SJR.) wet sediment with 180 ml overlying water; and 20-ml SJR wet sediment with 180 ml overlying water as control

Table S4.1 continued

17. Test concentration/beaker:	6. 143 mg CNT (dw) + 20-ml Mill Creek (MC) wet sediment with 180 ml overlying water; and 20-ml MC wet sediment with 180 ml overlying water as control 7. 33 mg CNT (dw) + 20 ml West Bearskin (WB) wet sediment with 180 ml overlying water; and 20 ml WB wet sediment with overlying water as control 8. 137 mg CNT (dw) + 20 ml Spring River (SR) wet sediment with 180 ml overlying water; and 20 ml SR wet sediment with 180 ml overlying water as control
18. Mixing conditions:	1. Tumble dry sediment treatments on rolling mill for 1 h at about 40 revolutions/minute 2. Tumble wet sediment treatments on rolling mill for 1 h at about 20 revolutions/minute 3. Held for 7 days at 23 ± 1°C before the start of the exposures
19. Dilution factor:	None
20. Chemical residues:	Ni, Fe, Co, Mo analyzed in composited overlying water weekly
21. Water quality:	Dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia measured in Overlying water weekly
22. Endpoints:	Survival, growth and biomass
23. Test acceptability:	≥ 80% survival in control

Table S4.2. Mean water quality characterisitcs measured on days 0, 7 and 14 in whole sediment 14-d toxicity tests with amphipods (*Hyalella azteca*) exposed to nine sediments with and without addition of multi-wall carbon nanotubes (MWCNT). Standard deviation in parenthesis, n=3

Test Number	Treatment	Dissolved oxygen (mg/L)	Conductivity (µS/cm)	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Total ammonia (mg/L)
1	Silica sand (control)	8.0 (0.8)	221 (12)	8.3 (0.1)	82 (3.5)	108 (12)	0.3 (0.1)
	Silica sand +1% MWCNT	7.9 (0.7)	229 (12)	8.3 (0.1)	81 (2.3)	116 (18)	0.4 (0.4)
2	Florissant soil (control)	8.0 (0.8)	239 (28)	8.2 (0.1)	82 (2.0)	120 (17)	0.2 (0.1)
	Florissant soil + 1% MWCNT	7.9 (0.8)	238 (6.4)	8.2 (0.1)	82 (2.9)	125 (17)	0.2 (0.1)
3	Florissant soil (control)	6.8 (1.1)	288 (33)	8.1 (0.2)	81 (6.8)	113 (22)	0.6 (0.5)
	Florissant soil + 1% MWCNT	6.8 (1.0)	299 (42)	8.1 (0.2)	82 (1.4)	101 (38)	0.4 (0.3)
	Florissant soil + 0.1% MWCNT	6.5 (1.0)	288 (35)	8.2 (0.2)	83 (3.8)	96 (44)	0.5 (0.5)
	Florissant soil + 0.01% MWCNT	6.6 (1.0)	282 (29)	8.2 (0.2)	81 (2.6)	108 (14)	0.5 (0.4)
4	Spring River (control)	6.8 (1.1)	300 (63)	8.1 (0.3)	87 (2.9)	104 (3.2)	0.2 (0.2)
	Spring River +1% MWCNT	6.7 (1.0)	277 (15)	8.2 (0.2)	89 (3.6)	116 (8.0)	0.2 (0.3)
5	West Bearskin (control)	6.8 (1.0)	240 (13)	7.9 (0.1)	42 (4.3)	78 (15)	0.3 (0.3)
	West Bearskin + 1% MWCNT	6.6 (0.9)	275 (45)	7.6 (0.2)	34 (7.4)	91 (15)	0.6 (0.6)
6	DOW Creek (control)	7.0 (0.7)	275 (18)	8.3 (0.1)	93 (11)	107 (9.2)	0.4 (0.5)
	DOW Creek +1% MWCNT	7.1 (0.8)	271 (8.1)	8.3 (0.1)	96 (13)	109 (3.6)	0.3 (0.4)
7	St. Joseph River (control)	6.9 (0.7)	314 (9.0)	8.3 (0.1)	113 (10)	139 (7.9)	0.3 (0.4)
	St. Joseph River +1% MWCNT	6.9 (0.7)	306 (5.7)	8.3 (0.2)	109 (3.6)	139 (19)	0.2 (0.3)
8	Raisin River (control)	7.1 (0.7)	288 (8.1)	8.4 (0.1)	110 (7.2)	133 (4.2)	0.6 (0.9)
	Raisin River +1% MWCNT	7.2 (0.8)	291 (6.9)	8.3 (0.3)	107 (3.1)	122 (9.5)	0.6 (0.9)
9	Mill Creek (control)	7.0 (0.7)	340 (20)	8.4 (0.2)	119 (16)	156 (5.3)	0.7 (0.7)
	Mill Creek +1% MWCNT	7.1 (0.8)	337 (18)	8.4 (0.2)	125 (20)	156 (17)	0.7 (0.6)

Table S4.3. Mean water quality characterisitcs measured in 14-d and 28-d whole sediment toxicity tests with amphipods (*Hyalella azteca*) exposed to two sediments with and without addition of multi-wall carbon nanotube (CNT). Standard deviation in parenthesis

Treatment	Dissolved oxygen (mg/L)	Conductivity (µS/cm)	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Total ammonia, NH_3 (mg/L)
			Day 14 (n=3)			
Dow Creek (control)	8.3 (1.1)	303 (21)	8.4 (0.2)	94 (13)	120 (0)	0.2 (0.2)
Dow Creek +1% MWCNT	8.2 (0.8)	323 (19)	8.7 (0.2)	111 (5.2)	114 (1.2)	0.2 (0.3)
Raisin River (control)	8.1 (1.0)	315 (4.0)	8.6 (0.1)	105 (15)	131 (1.4)	0.3 (0.4)
Raisin River +1% MWCNT	8.0 (0.8)	321 (17)	8.8 (0.2)	115 (2.9)	122 (5.8)	0.7 (0.6)
			Day 28 (n=5)			
Dow Creek (control)	8.2 (1.1)	302 (21)	8.1 (0.2)	94 (13)	120 (0.4)	0.2 (0.2)
Dow Creek +1% MWCNT	8.2 (0.8)	323 (19)	8.6 (0.2)	110 (5.2)	114 (1.4)	0.2 (0.3)
Raisin River (control)	8.1 (1.0)	315 (4.0)	8.6 (0.1)	105 (15)	130 (1.4)	0.3 (0.4)
Raisin River + 1% MWCNT	8.0 (0.8)	321 (17)	8.8 (0.2)	115 (2.5)	122 (63)	0.6 (0.6)

Table S4.4. Concentrations (µg/L) of nickel (Ni), molybdenum (Mo), cobalt (Co) and iron (Fe) measured on days 0, 7 and 14 in overlying water of whole sediment toxicity tests with and without multi-wall carbon nanotubes (MWCNT) spiked into nine sediments testing amphipods (*Hyalella azteca*)

Test			Test day			
Number	Treatment	Element	0	7	14	Mean
1	Silica sand (control)	Ni	29	<2.1[a]	<2.1	10 (16)
		Mo	85	<2.0[a]	<2.0	29 (49)
		Co	<1.2[a]	<1.2	<1.2	0.6 (0)
		Fe	16	11	7	11 (4.5)
	Silica sand + 1% MWCNT	Ni	2495[b,d,e]	368[c,d,e]	172[c,d,e]	1012[b,d,e]
		Mo	1588	1046	241	958 (678)
		Co	26	22	<1.2	16 (14)
		Fe	39	26	13	26 (13)
2	Florissant soil (control)	Ni	23	19	<2.1	14 (12)
		Mo	131	<2.0	<2.0	44 (75)
		Co	13	10	<1.2	7.9 (6.5)
		Fe	892	173	65	377 (450)
	Florissant soil +1% MWCNT	Ni	32	8.2	6.8	16 (14)
		Mo	376	356	362	365 (10)
		Co	11	<1.2	<1.2	4.1 (6.0)
		Fe	522	159	35	239 (253)
3	Florissant soil (control)	Ni	16	6.4	<2.1	7.8 (7.6)
		Mo	161	101	<2.0	88 (81)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	978	372	39	463 (476)
	Florissant soil + 0.01% MWCNT	Ni	28	5.1	<2.1	11 (15)
		Mo	45	<2.0	<2.0	16 (25)
		Co	8.2	<1.2	<1.2	3.1 (4.4)
		Fe	801	163	35	333 (410)
	Florissant soil + 0.1% MWCNT	Ni	22	10	<2.1	11 (11)
		Mo	119	36	<2.0	52 (61)
		Co	13	5.3	<1.2	6.3 (6.3)
		Fe	186	155	14	118 (92)
	Florissant soil + 1% MWCNT	Ni	59	19	<2.1	26 (30)
		Mo	182	170	148	167 (17)
		Co	32	18	4.8	18 (14)
		Fe	190	91	49	110 (72)
4	Spring River (control)	Ni	4.0	<2.1	<2.1	2.0 (1.7)
		Mo	<2.0	<2.0	<2.0	1.0 (0)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	998	512	327	612 (347)
	Spring River +1% MWCNT	Ni	51	33	<2.1	28 (25)
		Mo	159	60	16	78 (73)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	760	891	789	813 (69)

Table S4.4 continued

5	West Bearskin (control)	Ni	22	<2.1	<2.1	8.0 (12)
		Mo	<2.0	<2.0	<2.0	1.0 (0)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	53	41	35	43 (9.2)
	West Bearskin + 1% MWCNT	Ni	126	37	<2.1	55 (64)[c]
		Mo	101	<2.0	<2.0	34 (58)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	34	22	5.4	21 (14)
6	DOW Creek (control)	Ni	10	3.6	<2.1	4.9 (4.6)
		Mo	9.2	<2.0	<2.0	3.7 (4.7)
		Co	<1.2	<1.2	77	26 (44)
		Fe	87	54	74	72 (17)
	DOW Creek + 1% MWCNT	Ni	120	4.4	<2.1	42 (68)
		Mo	697	354	137	396 (282)
		Co	<1.2	12	80	31 (43)
		Fe	<0.9[a]	<0.9	27	9.6 (15)
7	St. Joseph River (control)	Ni	37	16	6.5	20 (16)
		Mo	<2.0	<2.0	<2.0	1.0 (0)
		Co	<1.2	<1.2	<1.2	0.6 (0)
		Fe	353	42	52	149 (177)
	St. Joseph River +1% MWCNT	Ni	43	31	11	28 (61)
		Mo	268	171	85	175 (92)
		Co	<1.2	15	78	31 (41)
		Fe	349	72	48	156 (167)
8	Raisin River (control)	Ni	33	19	<2.1	18 (16)
		Mo	8.6	<2.0	<2.0	3.5 (4.4)
		Co	16	<1.2	3.8	6.8 (8.1)
		Fe	<0.9	<0.9	<0.9	0.5 (0)
	Raisin River +1% MWCNT	Ni	45	35	<2.1	27 (23)
		Mo	690	410	132	411 (279)
		Co	<1.2	<1.2	10	3.7 (5.4)
		Fe	<0.9	<0.9	8.5	3.1 (4.7)
9	Mill Creek (control)	Ni	47	8.8	<2.1	19 (25)
		Mo	8.7	7.3	5.2	7.1 (1.8)
		Co	<1.2	<1.2	30	10 (17)
		Fe	13	<0.9	15	9.5 (7.9)
	Mill Creek +1% MWCNT	Ni	128	5.1	<2.1	45 (72)
		Mo	377	281	104	254 (139)
		Co	<1.2	<1.2	85	29 (49)
		Fe	422	8.3	4.7	145 (240)

[a]Less than detection limit.

[b]Criteria maximum concentration (CMC) of 470 µg Ni /L for the protection of aquatic community in freshwater in the U.S. Environmental protection Agency (US EPA) water quality criteria (WQC) (water hardness 100 mg/L as $CaCO_3$).

[c]Criteria continous concentration (CCC) of 52 µg Ni/L in WQC (water hardness 100 mg/L as $CaCO_3$) (USEPA 2009).

[d]Borgmann et al. (2005) for *H. azteca* 7-d median lethal concentration at 50% mortality (LC50) of 147 µg Ni/L, > 3,150 µg /L for Mo or Fe, and 89 µg Co /L (water hardness 124 mg/Las $CaCO_3$, alkalinity 84 mg/L as $CaCO_3$) .

[e]Keithly et al. (2004) 14-d 20% effective concentration (EC20) for *H. azteca* of 61 µg Ni/L (water hardness 98 mg/L as $CaCO_3$).

CHAPTER 5

SUMMARY, CONCLUSIONS AND ADDITIONALRESEARCH NEEDS

SUMMARY

This study evaluated the toxicity of one-dimensional CNMs represented by commercially acquired CNTs and laboratory synthesized SiCNWs to amphipods, midge, oligochaetes and mussels as representative aquatic test organisms. Eight reference sediments including sand, Florissant soil, West Bearskin, Spring River, Dow Creek, St. Joseph River, Raisin River and Mill Creek with different physical chemical properties were used for the tests with CNTs. The West Bearskin sediment was used in the tests with SiCNW tests. The study hypothesized that CNMs in water and sediment are toxic to aquatic organisms and toxicity is due to: (1) the CNMs with or without metals, (2) metals solubilized from the CNMs upon contact with water or sediment, and (3) other factors affecting toxicity include type and sources of CNMs, sonication and sediment characteristics including total organic carbon and acid volatile sulfide.

Characterization and analysis of test samples

The characterization data show that SiCNWs the elemental composition (percent weight) of the SiCNWs is silicon (57.3%), carbon (40.3%) and oxygen (2.4%) and no heavy metals within a 0.1% detection limit. The morphology of the CNTs samples show entangled rope-like bundles with scattered dark spots.

The elemental compositions of the SWCNT sample is carbon (93.4%), Fe (0.3%), Co (4.9%), and Mo (1.5%), with no oxygen, the composition of the two MWCNT samples is carbon (70.9 - 89.7%), oxygen (11.8 - 15.3%), Si (0.1 - 0.9), Fe (10.1%), Co (0.2 - 2.5%), Mo (0.6 - 1.2%), and Ni (0.5 - 3.0%). The MWCNT modified by 3.0 M nitric acid were free of metal impurities except Fe at 0.4%. The other elements present are carbon (86.1 %) and oxygen (13.5 %). The metals in the CNTs could have undesirable environmental consequences acting as either toxic substances or additions to other contaminants in the environment (Plata 2009).

Test methods

The toxicity tests were conducted using procedures adapted from test methods developed by the (EPA (2000) and the ASTM (2009). The toxicity tests satisfied the criteria of minimum control survival for amphipods, midge and mussels and biomass for oligochaetes. In addition, a high quality of overlying water, specifically dissolved oxygen in exposure chambers, was maintained during tests as recommended in ASTM (2009) and EPA (2000). These approaches could be suitable for toxicity testing of other types of nanomaterials in water and sediment and it is recommended that they be adopted as a step towards the standardization of toxicity tests with nanomaterials.

Toxicity tests

The toxicity tests show: (1) non-sonicated SiCNWs in water were not acutely toxic to amphipods, (2) sonicated SiCNWs in water were acutely toxic to amphipods but not toxic to midge, oligochaetes or mussels, and (3) sonicated SiCNWs mixed in

sediment or layered on sediment surface were chronically toxic to amphipods. Sonication enhanced the toxicity of the SiCNW to the amphipods.

The CNTs water-only tests indicate (1) non-sonicated or sonicated as produced CNTs (SWCNT and MWCNT) are toxic to amphipods, midge, oligochaetes and mussels, (2) MWCNTs modified with the addition of EDTA or with nitric acid reduces toxicity to the amphipods, and (3) exposures of nickel in water to amphipods was toxic. Toxicity tests with modified CNTs (acid cleaned or with the addition of EDTA) show that post synthesis modifications could alter but not eliminate toxicity of the CNTs to aquatic organisms.

The as-produced 1% (dry weight) MWCNTs spiked into two sediments (sand and Florissant soil) of the total eight reference sediments tested were toxic to the amphipods in 14-d exposures. Also, 0.1% MWCNTs spiked into Florissant soil was toxic to amphipods while the difference in final biomass between the 0.01% MWCNTs spiked into the Florissant soil and the control was 30%, but that is not statistically significant to the control. The 1% MWCNTs spiked in two other reference sediments (Raisin River and Dow Creek) significantly reduced growth of amphipods in 28-d exposures but did not reduce the growth significantly in 14-d whole sediment exposures indicating growth was a more sensitive endpoint than survival.

The sediments tested appear to bind metals dissolved from MWCNTs and reduce their bioavailability to amphipods except with sand, where the metal concentrations in the overlying water were elevated to levels potentially toxic to the amphipods.

The risks from metal toxicity from the MWCNTs, therefore, would be low in sediments containing substantial amounts of binding agents. However, sediments containing low metal binding capacities (e.g., sand) would be at risk of contamination with metals from the as-produced MWCNTs. The potential for toxicity of CNTs (SWCNTs or MWCNTs) in the environment due to the metal impurities could be prevented by the production of CNTs without the metals potentially toxic in aquatic environments as recommended in other studies (Plata 2009, Hull et al. 2009).

The MWCNTs, when mixed with sediment, appear to separate and become distributed on the surface of the sediment. It is likely sediments with lower total organic carbon (e.g., <1% TOC) and other parameters (e.g., higher proportions of sand) may contribute to the separation of MWCNT spiked into the sediment. For example, Hyung et al. (2007) reported that aqueous natural organic matter disperses MWCNT aggregates and keeps them suspended in water.

Species sensitivity

The SiCNWs and CNTs 14-d water-only toxicity tests showed that amphipods were the most sensitive compared to midge, oligochaetes and mussels. Both water-only and whole-sediment toxicity tests showed that growth was a more sensitive indicator of toxicity than survival for the amphipods.

The results in general showed that the duration of exposures, test endpoints, properties of the materials, preparation of the test materials (e.g., sonication, acid rinsing) and test media (e.g., water or sediment type) influenced toxicity to the test organisms.

Mechanisms of toxicity

The amphipods, midge, and oligochaetes ingested the test materials during exposures and these materials also coated the body surfaces of these test organisms including the shells of the mussels. This suggests that the mechanism of toxicity was likely due to factors including physical smothering of the organism, impairment of respiration through surface coating of vital organs (e.g. gills) or blockage or injury of the digestive tract. Also, the dissolved metals from the CNT tests likely contributed to the observed toxicity. However, the modes of toxicity need to be investigated further. Also, further studies should be conducted over a broad range of water quality conditions to better determine if water quality influences the toxicity of CNMs.

CONCLUSIONS

1. The SiCNWs were toxic to amphipods in water and sediments while CNTs were toxic to the amphipods, midge, oligochaetes and mussels in water and sediment. The treatments applied to the test materials (sonication or non sonication), modifications (acid cleaning or the addition of EDTA) altered the toxicity of the CNTs.

2. The factors contributing to the toxicity of the CNTs include type, source, state (i.e., whether pre-treated or not). For example, metals dissolved from the CNTs caused toxicity to the aquatic organisms while acid cleaned CNTs were reduced in toxicity to the amphipods. The tests showed that nitric acid treatment of CNTs can remove significant amounts of soluble toxic metals and should be practiced prior to potential applications, if possible.

3. The adapted procedures for conducting toxicity tests with SiCNWs and CNTs were suitable and could be used in toxicity studies with other nanomaterials.

4. Laboratories should develop safety procedures to be observed while conducting toxicity tests with nanomaterials to protect staff from potential contact or inhalation. These measures should include, for example, weighing dry samples in a fume hood and wetting the samples before manipulation (e.g., stirring).

5. The whole-sediment toxicity tests showed that sediments could potentially bind metals from CNTs and reduce their bioavailability to aquatic organisms. However, this could result in the build-up of metals in the sediments with an accumulation of CNTs in the environment. In the environment, sediments could already be contaminated and therefore their binding capacity reduced. Post-synthesis removal of metal impurities in CNTs supplied to the market would eliminate the potential toxicity of metals associated with CNTs (as recommended in other studies: Plata 2009, Guo et al. 2007).

ADDITIONAL RESEARCH NEEDS

Due to variability in properties of commercial CNMs, especially CNTs, and the range of physical characteristics of sediments, more whole-sediment toxicity studies are necessary to provide data for use in the management or applications of CNTs in the environment.

Specifically the research should focus on the following areas:

1. Long term exposures (>28 d) with dilutions of commercial CNTs (as produced and modified (e.g., surface coated)) in reference sediments with different properties with growth and or reproduction as endpoints with amphipods, midge and mussels should be undertaken to determine dose responses and safe concentrations for these materials in sediment.
2. Tests with mixtures of CNTs (as produced or modified) in the market with other dilutions of contaminants in sediments (e.g., organics) to determine the likely patterns of toxicity of the mixtures in the environment.

The data from such tests would validate the suitability of the test procedures developed in this study.

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