EFFECTS OF SUSPENDED MULTI-WALLED CARBON NANOTUBES ON

DAPHNID GROWTH AND REPRODUCTION

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Multi-walled carbon nanotube aggregates can be suspended in the aqueous phase by natural organic matter. These aggregates are ingested by filter feeding zooplankton. Ingested aggregates result in decreased growth and decreased reproduction. These effects may be caused by reduction in energy input from normal feeding behavior.

pH alters natural organic matter structure through changes in electrostatic repulsion. Altered natural organic matter structure changes multi-walled carbon nanotube aggregate size. This size variation with variation in pH is significant, but not large enough a change in size to alter toxicity, as the aggregate size range remains well within the particle size selection of the organisms.

ACKNOWLEDGEMNTS

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INTRODUCTION

Carbon Nanotubes

The US National Nanotechnology Initiative (NNI) defines nanoparticle as any particle that has at least one dimension within the range of 1 nanometer to 100 nanometers (Russell et al. 2000). Carbon nanotubes (CNT) are hollow nano-scale tube structures composed of carbon rings with very strong sp^2 bonds. Although the first description of "graphitic carbon fibers" was by two Russian scientists (Radushkevich and Lukyanovich 1952), it is mostly due to timing and new resolutions in transmission electron microscopy (TEM) imaging, credit for the discovery of CNTs has most commonly been given to Sumio Iijima (Iijima 1991). CNTs are relatively new to materials science and the full breadth of their application is still in early development.

CNTs are divided into two categories: single-walled (SWNT) and multi-walled (MWNT). The former varies by diameter, length and chirality. The latter consists of smaller diameter single-walled tubes inside larger diameter tubes and may vary from a double-walled nanotube to as many as fifty concentric tubes (Yamabe 1995). The interwall space between concentric tubes is 0.34 nm (Ajayan 1999).

Single and multi-walled nanotubes have a large amount of surface area in relation to mass. All chemical interaction occurs on this surface, giving CNTs a

great deal of potential for bonding and reaction (Helland et al. 2007, Oberdörster et al. 2005).

CNTs are classified as artificial materials even though they can be produced by natural processes, including volcanic events (Velasco-Santos et al. 2003). CNTs have been found in ice core samples (Esquivel and Murr, 2004), deep rock formations, and crude oil in ultra-trace concentrations (Velasco-Santos et al. 2003). CNTs also form in less extreme conditions such as incinerators (Murr et al. 2004, 2005). However, the size, purity, quality, and quantity of CNTs produced by such processes are far below what is needed for laboratory testing or composite material applications. For the foreseeable future, the only major source of CNTs is laboratory and industrial production. The annual SWNT production, as of 2004, was 9,000 kg/yr (Templeton et al. 2006). A single German company, Bayer, increased MWNT production capacity to 60 metric tons per year in 2007 (Rakov 2008). The same company announced plans to increase further to 200 tons per year by 2009 and 3,000 tons per year by 2012 (Rakov 2008).

Diameters of CNTs range from one to several nanometers, while lengths in excess of one centimeter have been achieved (Hyung 2008). Depending on the diameter and chirality, the tubes may have electrochemical properties similar to metals (Ouyang et al. 2001), or as semiconductors (Itkis et al. 2002). Their variability in electrochemical behavior makes them attractive to microelectronic applications (Avouris 2007). Single-walled nanotubes (SWNTs) have already

been used in microtransistors and experimental processor architecture (Derycke 2001, 2009). Others have proposed using CNTs in medicine as carrier-mediated delivery vehicles for biofunctional molecules, as targets for biophysical treatments, and as templates for tissue regeneration (Foldvari and Bagonluri, 2008). Electronic circuits could be scaled down by several orders of magnitude by using conductive CNTs in the place of traditional conductive materials. SWNTs have already been used as field effect transistors (Dercyke et al. 2001). MWNTs are viewed as attractive new materials for processor architecture beyond the 22 nm node (Naeemi et al. 2005). A ring oscillator using a single SWNT was tested in 2005 (Chen et al. 2006). Applications for CNTs range from integration into bulk materials, to circuits measuring less than 90 μm^2. Material engineers have designed composite materials using CNTs with many times the strength and durability, yet a fraction of the weight and stiffness of current materials. The proposed uses of CNTs range from domestic to military applications. Military applications include sorbative filters, use as visual obscurants on the battlefield, simple paints, and hybrid polymer structural materials for vehicles and buildings (Kennedy et al. 2008).

In 2005, worldwide funding for nanotechnology was estimated to be 9.6 billion USD (Lux Research Inc. 2006). Projections of the nanomaterials industry reach as high as 1 trillion USD by 2015 (Nel et al. 2006). These projections reflect a wide range of industrial sources of CNTs, and widespread CNT use.

This supposition of widespread manufacture and use of CNTs presents a variety of environmental routes of exposure, as both large quantities of CNTs will be used in mass production of bulk composite materials, and very small quantities will be used in microprocessor architecture, but with a potential to be as ubiquitous as cell phones and notebook computers. Potential widespread use of CNT composites may lead to an unavoidable pervasiveness of CNTs in post-consumer waste streams (Figure 1).
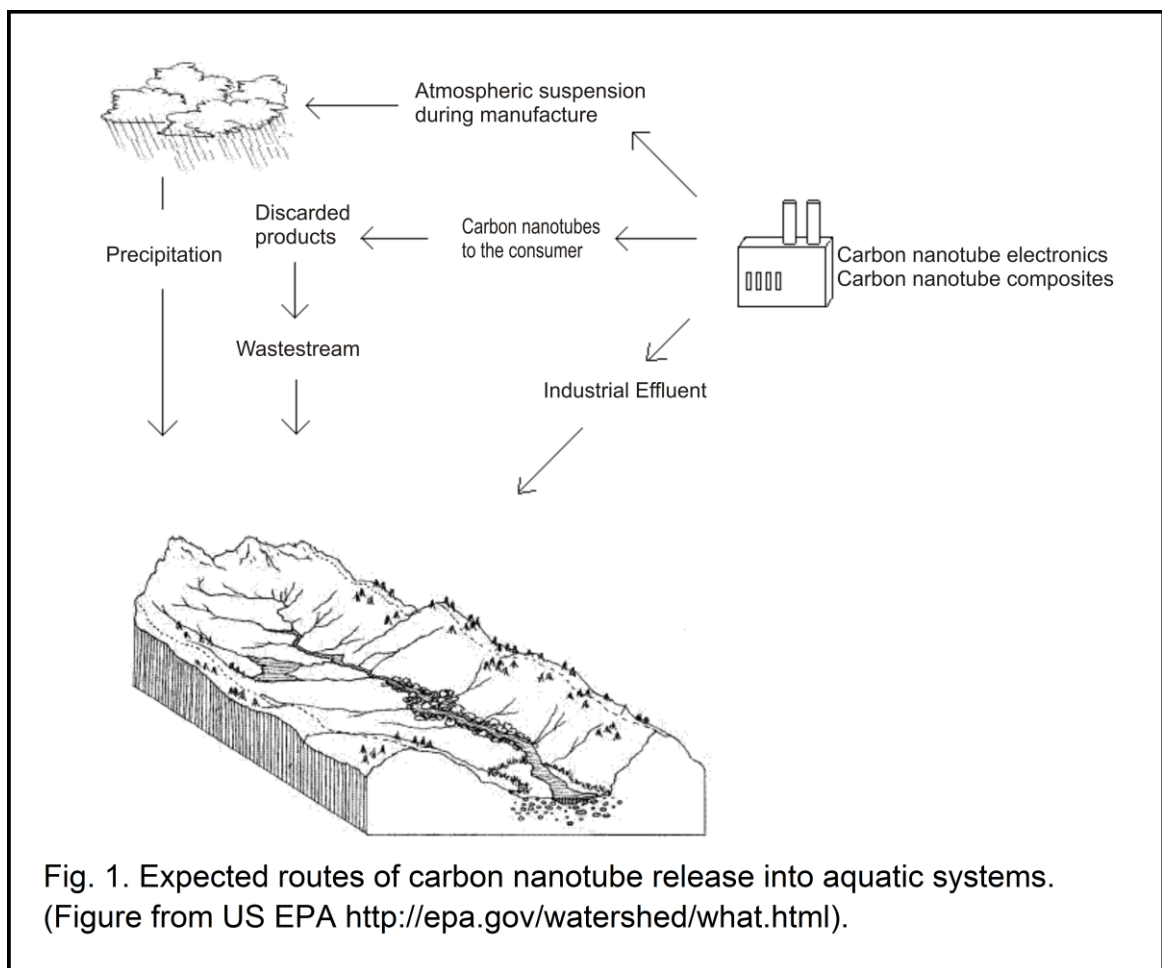

Fig. 1. Expected routes of carbon nanotube release into aquatic systems. (Figure from US EPA http://epa.gov/watershed/what.html).

<center>Toxicity</center>

The toxicity of CNTs includes macro-level effects on organisms, such as granuloma formation in alveoli in lungs of Cavia porcellus (Grubek-Jaworska et al. 2006). The authors conducted inhalation studies and reported that CNT aggregates often become too large to pass directly through the alveolar walls, and instead build up and block alveolar space. The same study showed a litany of lung pathologies associated with MWNTs from a variety of purities and origins. Pathologies included: perivascular, peribronchial and interstitial infiltration of inflammatory cells, central and peripheral atelectasis (lung collapse) and emphysema (destruction of alveolar support structures) and alveolar exudation (oozing fluid). Smith et al. (2007) reported increasing gill mucous and dose dependent decrease of glutathione in the liver of Oncorhynchus mykiss. Tu et al. (2009) showed DNA sequences selectively binding to SWNTs in vitro, though the ability of CNTs to enter cells and their nucli have not been substantially supported in the literature.

Much of the toxicological study of CNTs has centered on airborne aggregates. This has been driven by the need for data to ensure occupational safety for those involved in the manufacture of CNTs and other nanomaterials. Maynard et al. (2004) studied CNT deposits on protective gloves used by workers producing and processing the nanomaterials. They found that even careful handling still resulted in both the release of aggregates into the air (less than 53 $\mu g/m^3$), which stayed suspended for prolonged periods of time (as long

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as 1 hr 30 min), and deposits on gloves ranging from 0.2 mg per hand to 6.0 mg per hand (Maynard et al. 2004).

Studies have been done on the lungs in rodents as well as lung cell lines. Huczko et al. (2001) exposed C. porcellus to CNTs containing carbon soot as a preliminary study seeking to emulate workplace exposure occurring during refining and purification of CNTs. The authors did not find any significant differences in intertidal volume, respiration rates, and resistance to tidal flow between the CNTs and soot exposures and soot without CNTs controls. Neither did the authors find any significant differences among treatments in bronchoalveolar lavage examinations of macrophage counts, total protein, polymorphonulcear leukocytes, lymphocytes, or losinophils (sic) (Huczko et al. 2001). Shvedova et al. (2005) exposed mice to CNTs through pharyngeal aspiration. They observed that within the first three days there was a dose-dependent increase in protein, lactate dehydrogenase, and glutamyl transferase activities in bronchoalveolar tissues, as well as a depletion of glutathione. By day three, they observed lymphocyte influx. Proinflammatory cytokines were seen to increase from day one and peak on day seven. The primary morphologies observed were hypertrophied epithelial cells surrounding SWNT aggregates and diffuse interstitial fibrosis with alveolar wall thickening (Shvedova et al. 2005). Intratracheal instillation into rats showed temporary inflammation and multifocal granulomas around SWNT aggregates (Warheit et al. 2004). Lam et al. (2004) observed epitheliod granuloma lesions after a single instillation into mice.

There is some question as to the method of instillation used being the cause of the granulomas rather than the CNTs themselves (Helland et al. 2007). Monteiro-Riviere et al. (2005) observed well dispersed MWNTs were able to enter cultured human epidermal keratinocytes and elicit an inflammatory response. They reported MWNTs alter protein Interleukin-8 release in keratinocytes. Huczko and Lange (2001) exposed the skins of forty human volunteers to fullerene soot containing CNTs in an in vivo patch test, and four albino rabbits were subjected to eye exposure. The authors reported no significant response and concluded that dermal exposure to CNTs is not associated with any risks.

Few studies can be found in the literature examining CNT toxicity to aquatic biota. Roberts et al. (2007) observed that lipid coated carbon nanomaterials may not be highly stable in aquatic environments due to biotic interactions. The authors reported that zooplankton (D. magna) not only ingested CNT aggregates from the water column, also but altered the lipid coating. Toxicity was observed only at very high concentrations. Other investigators have observed ingestion of suspended MWNT aggregates in a fine-mesh filter feeder organism, Ceriodaphnia dubia (Kennedy et al. 2008).

Fullerenes, another carbon nanomaterials, have been shown to interact differently with biota depending on surface functionalization. Suspended C_{60} fullerenes have been reported to induce lipid peroxidation in the brains of juvenile bass (Micropterus salmoides) (Oberdörster, 2004). However, others have

reported that interactions between the CNTs and assay reagents are responsible for false positives (Worle-Knirsch et al. 2006), or that metal catalyst impurities are the cause of confirmed reactive oxygen species generation (Pulskamp et al. 2006). The solvent tetrahydrofuran (THF) has been used to solubilize C_{60} in bioassays (Zhu et al. 2006, Oberdörster, 2004). It has been reported that toxic endpoints observed in many of those assays could be attributed to THF decomposition products (Henry et al. 2007). In their D. magna bioassays with nanoparticulate TiO_2 and C_{60}, Lovern and Klaper (2006) used THF, but constructed their method to evaporate THF before organism exposure. In their statistical analysis, there was no significant difference between toxicity of TiO_2 solutions that had once contained THF and solutions that never had. However, sonicated C_{60}, that was not treated with THF, did not follow a trend of increasing concentration leading to increased D. magna mortality indicating that THF may have been responsible for toxicity.

The properties of CNTs can vary greatly depending on the tube's diameter, and chirality, thus presenting problems when comparing toxicity data. For example, a toxicological assay may have been conducted using tubes of similar dimensions (e.g. single-walled carbon nanotubes) from different manufacturing sources can result in different outcomes. A change of one in the (n,m) index of a tube's chirality, for example; comparing an (8,6) tube to an (8,7) (Figure 2), the absorption spectra is shifted into the near infrared by about 100 nm (Tu et al. 2009). By the same token, two tubes of the same chirality but

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differing length or diameter may also react differently to assay conditions. Very

short tubes may be able to be phagocytosed, while longer tubes might not

(Cheng 2009). This can further be complicated by surface functional groups.

Kennedy et al. (2009) exposed C. dubia over 96 hours to (1) pristine, (2) alkyl

functionalized, (3) amine functionalized, and (4) hydroxylated MWNTs (Table 1).

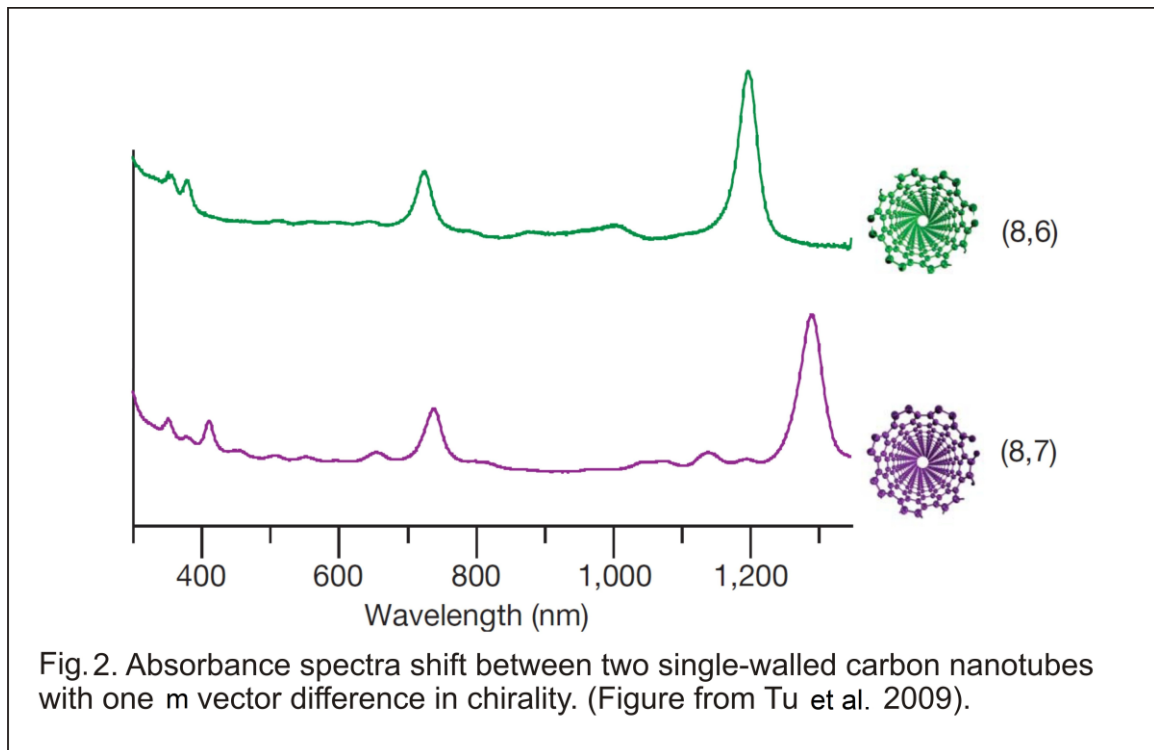Surface functionalization greatly influenced toxicity.

Fig. 2. Absorbance spectra shift between two single-walled carbon nanotubes with one m vector difference in chirality. (Figure from Tu et al. 2009).

TABLE 1. Toxicity of multi-walled carbon nanotubes to Ceriodaphnia dubia influenced by surface functionalization (Table from Kennedy et al. 2009).

	Percent Survival with Standard Deviation	Exposure Concentration
MWNT	75 ± 19	16ppm
MWNT	25 ± 19	26ppm
MWNT-NH$_2$	0 ±0	2ppm
MWNT-C$_8$	0 ±0	15ppm
MWNT-OH	93 ± 12	48ppm

Carbon Nanotubes in Aquatic Ecosystems

MWNTs can be suspended in the water column by means of natural organic matter (NOM), a ubiquitous constituent of natural aquatic systems (Hyung et al. 2008). Despite their natural hydrophobicity, interactions with NOM may stabilize MWNTs in aqueous suspension thus increasing the risk of exposure to pelagic organisms. NOM forms from the decomposition of plant and animal biomass. It consists of complex polyelectrolytes that can have a variety of molecular weights depending on their specific origin (Hyung et al. 2008). NOM typically has a negative charge due to carboxyl and phenol functional groups attached throughout the molecule (Hyung et al. 2008). Before publications demonstrating NOM adsorption behavior to CNTs, the only model known to suggest that NOM would readily adsorb to CNT surfaces was NOM behavior with activated carbon (Summers and Roberts 1988). NOM structure is affected by the ionic strength, and the pH of the water it is dissolved in (Hong and Elimelech 1997). Those parameters change the charge and molecular configuration of NOM by altering electrostatic repulsion within the molecule. In activated carbon it

has been shown that NOM adsorption increases as ionic strength increases, but decreases as pH increases (Hong and Elimelech 1997).

In general, Hyung and Kim (2008) showed that with increasing hydrophobicity, adsorption capacity to CNT increases. This means that the more aromatic NOM varieties, such as humic acid, have greater intrinsic affinity for CNTs. Diameter may play a part in determining the degree to which NOM acids can stabilize tubes. Lin and Xing (2008) tested adsorption and stability of tannic acid to SWNTs and MWNTs of mean outer diameters from about 9 nm to 70 nm (Table 2). They found that the SWNTs –having a mean outer diameter mean of 0.4 nm- had very low stability with tannic acid, as well as the MWNTs with mean diameters under 40 nm (Figure 3).

TABLE 2. Table of the purity and measured diameters with standard deviation of the carbon nanotubes used in a tannic acid suspension study (table from Lin and Xing 2008).

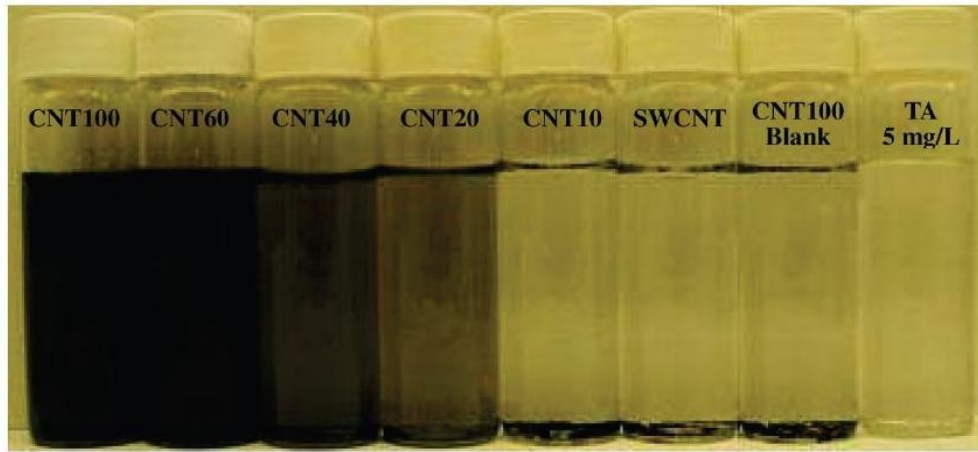| CNTs	purity %	length (μm)	outer diameter (nm)
SWCNT	>90	5- 15	1.4 ± 0.4
MWCNT10	>95	1- 2	9.4 ± 1.8
MWCNT20	>95	1- 2	20.9 ± 3.0
MWCNT40	>95	1- 2	27.8 ± 6.0
MWCNT60	>95	1- 2	42.7 ± 6.4
MWCNT100	>95	1- 2	70.1 ± 9.5

Fig. 3. Stability of single-walled carbon nanotubes and five diameter classes, 10 nm, 20 nm, 40 nm, 60 nm, and 100 nm multi-walled carbon nanotubes in tannic acid. (Figure from Lin and Xing 2008).

Obligate fine mesh grazing zooplankton, such as in the case of two cladocerans, Ceriodaphnia dubia and Daphnia magna, have been shown to ingest suspended CNT aggregates (Roberts et al. 2007, Petersen et al. 2009, Kennedy et al. 2009). In the pelagic zone, grazing zooplankton are the main primary consumer. Trophic cascades, indirect effects at other trophic positions due to changes at lower levels, have been triggered in experimental lakes by manipulating the zooplankton population either by food source or predation pressure changes (Carpenter et al. 2001) The authors observed population trends in phytoplankton, zooplankton, planktivorous fish, and piscivorous fish, in lakes with nutrient additions. They reported that the presence or absence of predation manipulated the body size of dominant zooplankton populations, and that the primary production rate influenced the biomass production in all

consumer levels. Thus, a food shortage or inhibition in feeding activities caused

by a suspended material, would lower zooplankton populations, which in turn,

would deprive the planktivorous species of food. Similar feeding inhibitions by

suspended particles have already been described in the literature (Kirk 1990,

1992). Grazing limits the biomass of the zooplankton, which determines the

possible biomass of the higher trophic positions. Carbon nanotubes, as a

suspended material, may act in a similar manner as other suspended materials,

by adversely affecting zooplankton growth and reproduction, and thus causing a

cascade of energy deficiency up the food web.

GOALS AND HYPOTHESES

The goal of this research is to investigate the effects of suspended multi-walled carbon nanotube (MWNT) aggregates on zooplankton growth and reproduction. Carbon nanotubes (CNTs) are hydrophobic nanomaterials that are largely insoluble in water. However, interactions with surfacewater constituents such as natural organic matter (NOM) can result in relatively stable suspensions of CNTs. Previous research examining the toxicity of CNTs to zooplankton, observed toxicity (mortality) only at the highest test concentrations (Roberts et al. 2007). Other studies (Kirk 1990, 1992) have shown effects of suspended clays on daphnid growth. These findings lead to the following hypotheses:

Hypothesis 1: Suspended CNTs result in decreased growth and reproduction in grazing zooplankton.

Ingested CNTs inhibit normal grazing and assimilation of food in the digestive tract. The resulting energetic cost affects growth of the organism. Decrease in body size ultimately reduces the fitness of the organism by reducing reproductive potential. My first hypothesis was tested using a series of chronic and acute toxicity tests in which <u>Daphnia magna</u> and <u>Ceriodaphnia dubia</u> were exposed to varying concentrations of MWNTs suspended in NOM.

Hypothesis 2: Variation in pH has a significant effect on MWNT toxicity.

NOM changes structure with strong variation in pH (Hong and Elimelech 1997). This, in turn, could change adsorption of NOM to MWNTs. A change in

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NOM adsorption might change the behavior of MWNT aggregates in freshwater, which might alter toxicity to grazing zooplankton. My second hypothesis was tested using a series of chronic and acute toxicity tests in which D. magna and C. dubia were exposed to varying concentrations of MWNTs suspended in NOM in freshwater of different pHs.

METHODS

Material Preparation

Water preparation reagents were obtained from Fisher Scientific (Pittsburg, Pennsylvania, USA). Multi-walled carbon nanotubes (MWNTs) were obtained from NanoAmor (Houston, Texas, USA). Because natural organic matter (NOM) in natural surface waters varies widely in composition, for this study Suwannee River natural organic matter, a natural mix uncontrolled for NOM species composition, was used (International Humic Substances Society, St. Paul, Minnesota, USA).

Reconstituted moderately hard water (RHW) was prepared to the Environmental Protection Agency (EPA) standards (Table 3) (US EPA 2002). Pure water was obtained by recirculation of reverse osmosis deionized water through a system consisting of approximately four liters of granulated activated carbon, and a MilliQ purifier with another carbon column and two ion exchange columns. Water was allowed to recirculate through the system for at least 24 hours before being pumped into a 50 L carboy with the appropriate amounts of dissolved salts.

NOM was mixed into the RHW to a concentration of 15 mg NOM/L. This was the concentration used as water for all MWNT suspensions referred to hereafter.

TABLE 3. Moderately hard water preparation and quality parameters.

Reagent added in mg per L				Expressed as mg CaCO₃ per L		
NaHCO$_3$	CaSO$_4$·H$_2$O	MgSO$_4$	KCl	pH	Hardness	Alkalinity
96	60	60	4	7.4 -7.8	80-100	57-64

To make MWNT test suspensions, the desired amount of MWNTs was weighed using a Cahn C-31 microbalance to one tenth of a microgram. The weighed amount of MWNTs was placed into a 100mL borosilicate glass centrifuge tube with 100 mL of NOM solution. The MWNTs were sonicated with a Fisher model 500 sonic dismemberator for thirty minutes at an average of 100 watts of power. This prepared a stock suspension used to make exposure suspensions by dilution using NOM solution adjusted to the desired pH.

All test chambers, pipettes, flasks, and volumetric glassware used were borosilicate glass to avoid possible hydrophobic reactions with plastics. Aluminum foil was used for weighing boats in the preparation of NOM solutions and CNT stock suspensions.

Material Characterization

Stock Characteristics

NanoAmor reported the purity of MWNTs purchased to be greater than 95%, the outer diameter to be within the range of 20 nm to 30 nm, and the length to be between 0.5 µm to 2 µm.

S.E.M. Characterization

MWNTs were dispersed by the methods described above, except MilliQ water was used instead of RHW to reduce the number and size of salt crystals in a dry sample. Samples were prepared at 5ppm MWNTs in suspensions adjusted to pH 6, 7, and 8 to image aggregate formation at those pHs. Drop-wise aliquots were placed on cleaned glass cover slips and allowed to dry in a low humidity environment with Petri dish covers to minimize contamination of the samples by dust. All samples were sputter coated with a gold-palladium alloy and imaged (Nova Field Emission Gun Scanning Electron Microscope, FEI North America, Hillsboro, Oregon USA). Aggregate diameter means was determined by measuring each aggregate along four angles and calculating the mean as the aggregate diameter.

Dynamic Light Scattering

Mean aggregate size in aqueous phase suspension was measured using dynamic light scattering on a Malvern instruments Zeta Sizer (Worcestershire, U.K.) Nano Series model ZS with a DTS1060C clear disposable zeta cell. Each

sample was prepared with RHW and NOM concentrations as described earlier. All three pH adjustments (6, 7, and 8) were analyzed at 5ppm MWNT.

Zeta Potentials

Zeta potential was measured using a Malvern instruments Zeta Sizer (Worcestershire, U.K.) Nano Series model ZS with a DTS1060C clear disposable zeta cell. A refractive index of 1.12 and absorbtion coefficient of 39.92 was used to calculate surface charge. Each sample was prepared with RHW and NOM concentrations as described earlier. All three pH adjustments (6, 7, and 8) were analyzed at 5ppm MWNT.

Test Organisms

Ceriodaphnia dubia

C. dubia is a model organism firmly established in aquatic toxicity testing literature. It has been used in US EPA protocols for at least 24 years, having been described in US EPA publications in 1986, but in the literature for about 116 years (Richard 1894). It has a recognized distribution across most surface waters.

C. dubia is a small bodied cladoceran (adult length rarely exceeds 0.88 mm) and has a short generation time. It is a parthenogenetic species and does not produce males under normal circumstances. A variety of predators prey upon C. dubia, including the mysids, Chaoborus larvae, and copepods. In the wild, an individual organism rarely survives beyond its third brood, thus making its total

reproduction of those three broods the most ecologically important (USEPA 1986).

Like other daphnia, C. dubia utilize a fair amount of phenotypic plasticity to react to predation and other stressors. They have been observed to alter the mean time to first brood, brood sizes, and even offspring size in reaction to various pressures (Lynch 1979). Under standard testing conditions of a 16:8 photoperiod, 24 °C, and ad libitum feeding, numbers of neonates per brood ranges from 6 to 10, but individual broods greater than twenty are not unheard of (USEPA 1986). Standard EPA test protocol requires a 3-brood mean of at least 15.

The EPA standard chronic toxicity bioassay for C. dubia is 7 days, with three broods expected from controls in that time. USEPA testing protocol calls for a control survival of 80% or greater and a mean brood size of 15 or more to have valid tests.

Daphnia magna

D. magna is another model organism extensively utilized in aquatic toxicity work. It is much larger than C. dubia, growing as large as 6.0 mm as an adult instar. Like C. dubia, D. magna has been known to the literature for more than one hundred years (Straus 1820). Its distribution is also multi-continental (USEPA 1986).

D. magna parthenogenetic reproduction is quite similar to C. dubia, but on a longer time scale. In 21 days an individual can be expected to be born, grow,

and produce 3 broods totaling more than 40 neonates. Average brood sizes vary from 10 to 15, but large individuals producing as many as 57 neonates in a single, huge, brood has been documented (US EPA 1986).

The life cycle of D. magna is parallel to C. dubia in that the first three broods are the most ecologically important even though the organism can live in a controlled environment long enough to produce as many as 22 broods.

The EPA standard chronic toxicity bioassay for D. magna is 21 days, with three broods expected from controls in that time. EPA testing protocol calls for a control survival of 80% or greater and a mean brood size of 40 or more to have valid tests.

D. magna and C. dubia neonates used in all tests were obtained from in-house cultures maintained in RHW, prepared as described by the EPA. Stock cultures were fed a diet of Selenastrum capricornutum and Cerophyll.

S. capricornutum was cultured in RHW under constant light with algal growth nutrients added. Nutrients were separated from algae by centrifugation, supernatant extraction, and resuspension in RHW without nutrients added. The Cerophyll was prepared by homogenizing 2 grams standardized alfalfa with 250mL RHW, settling overnight, and then extracting the supernatant.

Bioassays

Acute Tests

Three acute tests were run for each test organism. A test at pH 7.0 ± 0.2 for all exposure and control waters, and two similar tests with pH varied to 6.0 ±

0.2 and to 8.0 ± 0.2 by means of additions of hydrochloric acid, and sodium hydroxide respectively. All tests used EPA standard RHW, and all exposure waters had an addition of NOM to 15 mg/L.

All tests were performed in a climate controlled environmental chamber with a photoperiod of 16hr light, 8hr dark, with a constant temperature of 22.5ºC. Solutions were renewed daily, and food (100µL Cerophyll, 200µL S. capricornutum algae) was added after survival had been counted. For the C. dubia tests, three hundred neonates per test were used. Treatments and controls consisted of five replicates of ten individual neonates per replicate (Figure 4).There were two controls: a RHW without NOM control and a NOM solution control. There were four exposure treatments with concentrations of 0.5, 1.0, 2.0, and 4.0 ppm MWNT for pH 7, and 1, 2, 4 and 10ppm for pH 6 and 8 C. dubia tests.

For D. magna acute tests, controls and treatments consisted of five replicates of ten neonates each (Figure 5). Exposure concentrations for D. magna were 1 and 10ppm MWNT. Additionally, the D. magna pH 7 test was limited to only 5 individual organisms per replicate instead of the 10 per replicate used in all of the other tests.

The logistical challenges in both sample handling, and measurements of C. dubia dry weights precluded C. dubia growth study. D. magna, even after only 96 hrs, typically grows far larger than any adult C. dubia and handling of freeze-

dried organisms for mass analysis can be performed without unacceptable damage to samples during handling.

An acute survival test was conducted on <u>D. magna</u> using the same methods as described above, but at the termination of the test the organisms were depurated in clean RHW for 4 hrs before freezing at -80ºC. Frozen organisms were lyophilized (Freezone 6, Labconco, Kansas City, Missouri, USA) before measuring dry weights using a Cahn C-31 microbalance.
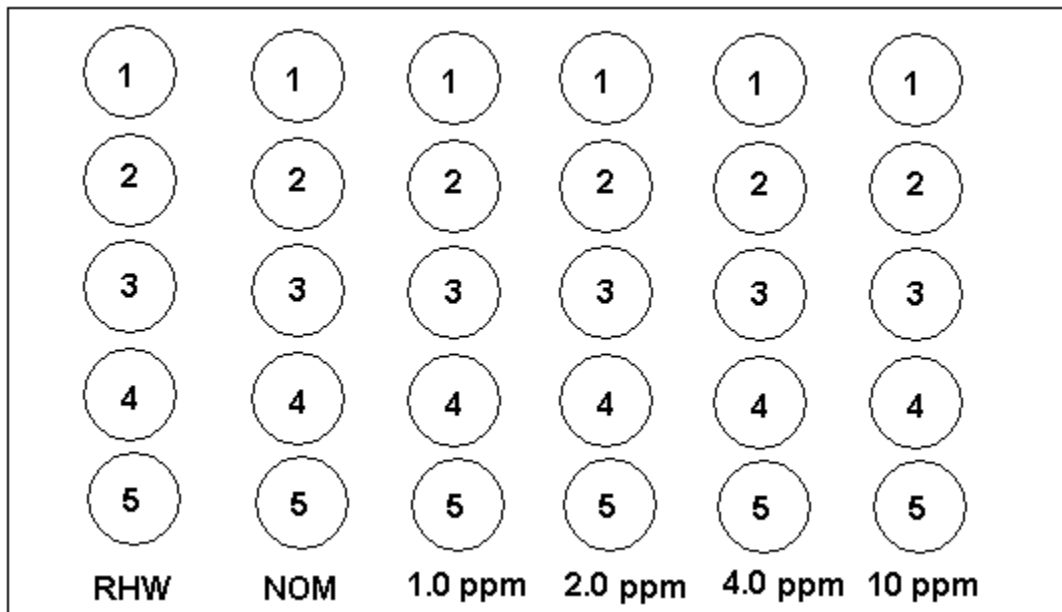

Fig. 4. <u>Ceriodaphnia dubia</u> test board arrangement for 96 hr acute exposure survival tests.
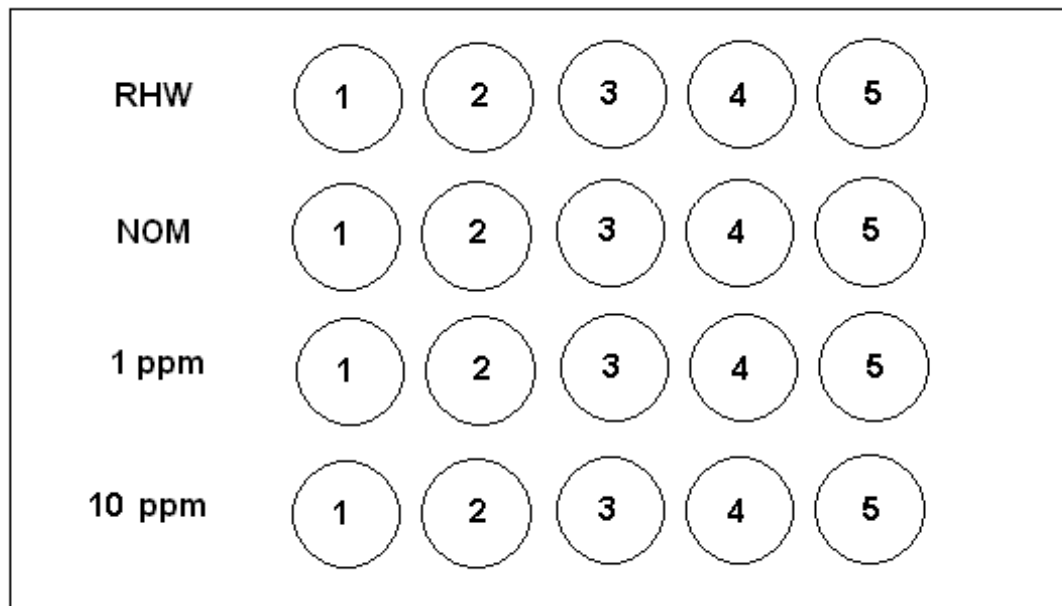
Fig. 5. <u>Daphnia magna</u> test board arrangement for 96 hr acute exposure survival tests and 96 hr growth tests.

Chronic Tests

Neonates were divided up into a moderately hard water control group of 10 replicates, and a NOM control group of 10 replicates with 4 different exposure groups of 10 replicates (Figure 6). Each individual test organism was held in 15 mL of exposure media in 30 mL glass beaker.
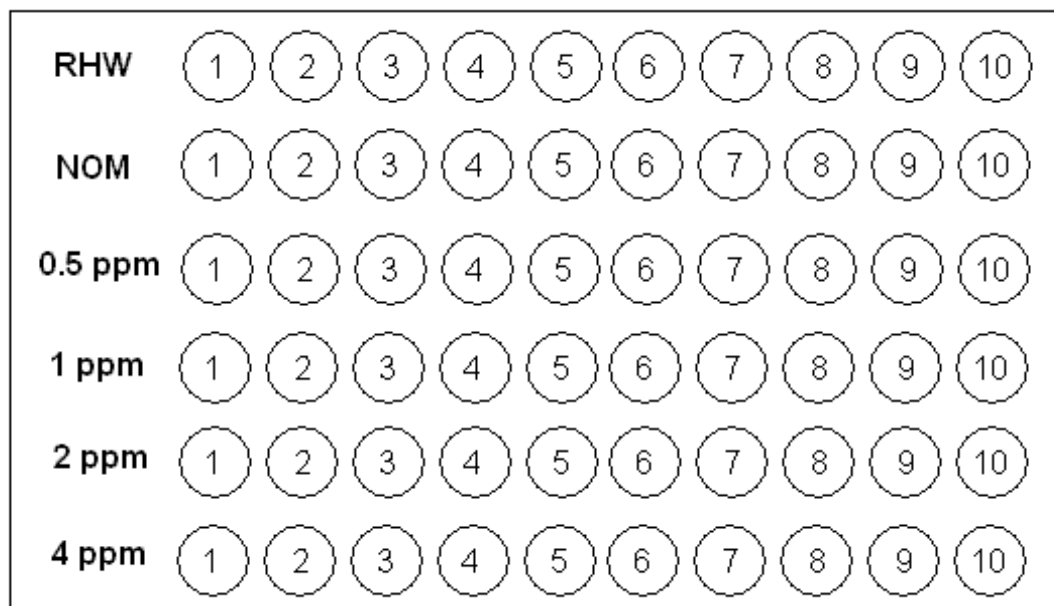
Fig. 6. Chronic test board arrangement for both Daphnia magna (21 day chronic) and Ceriodaphnia dubia (7 day chronic).

Test suspensions were renewed daily along with counts of mortality, and reproduction. After renewal and counts, the organisms were fed 200 µL 2.0×10^5 cells/ml of S. capricornutum and 100 µL Cerophyll. Daily renewal continued until all controls reached their third brood, about 7 days for C. dubia and 21 days for D. magna.

Data Analysis

All data analyses were run on SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA) and all tests for significance used an alpha value of 0.05. Before any multisample test was run, Grubb's test was used to check for outliers.

Acute survival was analyzed as percent of control survival with arcsine transformation used to meet analysis of variance (ANOVA) assumption of homogeneity of variances. A one factor, exposure concentration, ANOVA using survival expressed as a percentage of control survival with arcsine transformation, followed by a Tukey's post-hoc test was used to group any differences in survival. A two-factor, pH and exposure concentration, ANOVA using survival as percentage of control with arcsine transformation, followed by a Tukey's post-hoc test was used when comparing acute tests across pHs.

Chronic reproduction was analyzed as percent of control mean reproduction. The percentage data was arcsine transformed to meet ANOVA assumptions of homogeneity of variances. A one-factor, exposure concentration, ANOVA using percentage of control reproduction with arcsine transformation, followed by a Tukey's post-hoc test was used to group differences in reproduction. Two-factor, pH and exposure concentration, ANOVA followed by Tukey's post-hoc test was used when comparing chronic tests across pHs. In all tests, an alpha value of 0.05 was used in determining significance.

RESULTS

Material Characterization

Scanning Electron Microscope

The mean aggregate diameter for multi-walled carbon nanotubes

(MWNTs) at pH 7 was 6.5263 μm with a standard deviation of 2.3741 μm. The

mean for pH 6 was 9.1725 μm ± 6.7533 μm, and pH 8 was 2.2557 μm ± 0.9635

μm (Table 4, Figure 7).

Dynamic Light Scattering

The mean aggregate diameter for MWNTs in pH 7 NOM solution was

149.2 nm. The mean aggregate diameters in pH 6 and pH 8 were 129.1 nm and

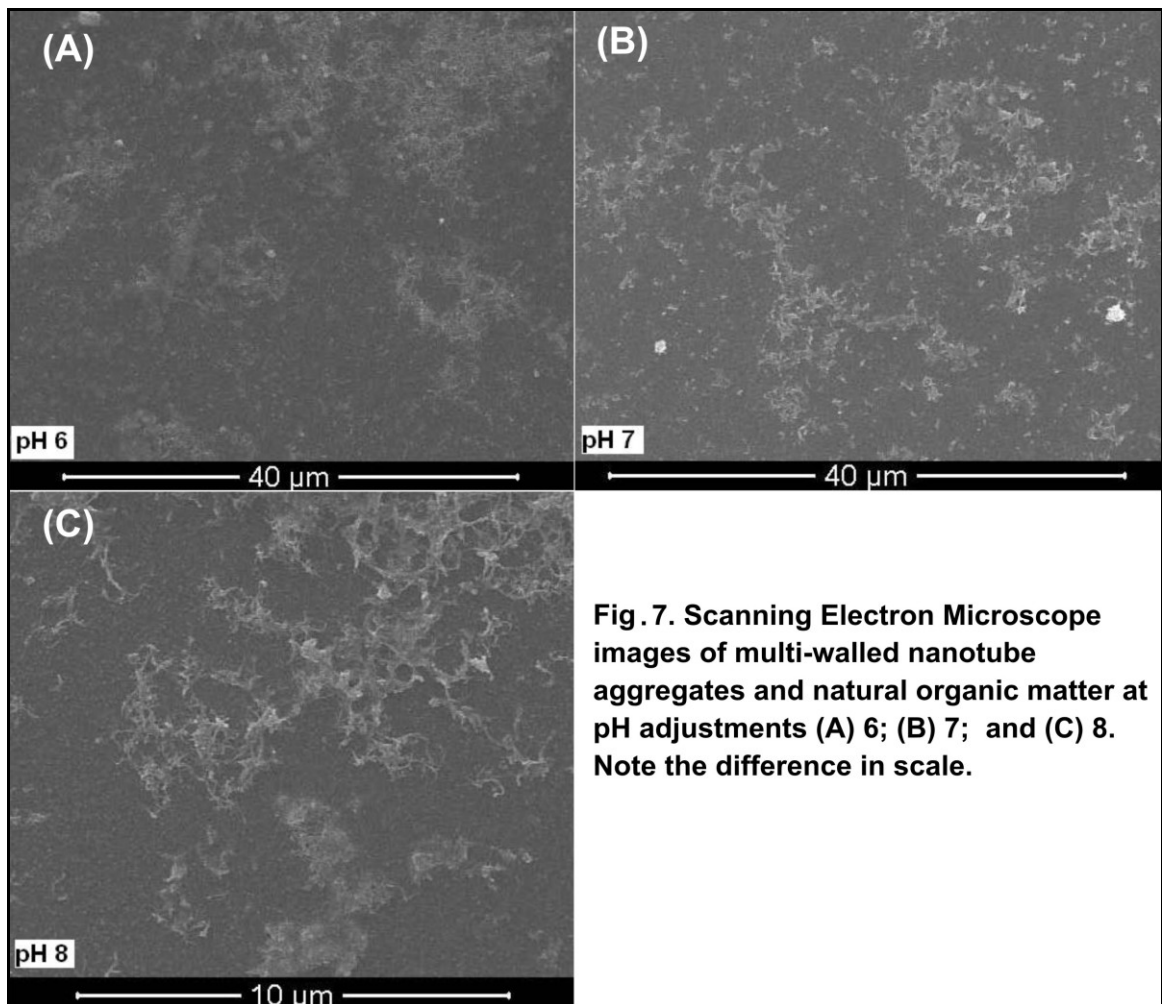142.4 nm, respectively (Table 4).

Zeta Potential

The zeta potential of MWNTs in pH 7 NOM solution was measured to be -

23.3 mV. The zeta potentials of pH 6 and pH 8 were -21.7 mV and -25.8 mV,

respectively (Table 4).

TABLE 4. Variation of mean multi-walled nanotube aggregate size as measured by Scanning Electron Microscope, and Dynamic Light Scattering. Change in Zeta Potential among three pH adjustments.

	Mean diameter measured by SEM		DLS	Zeta Potential
pH 6	$9.17245 \pm 6.7533\mu m$ *	n = 32	129.1nm	-21.7mV
pH 7	$6.52635 \pm 2.3741\mu m$ *	n = 40	149.2nm	-23.3mV
pH 8	$2.25577 \pm 0.9634\mu m$ *	n = 24	142.2nm	-25.8mV

* Significant at an alpha = 0.05

Fig. 7. Scanning Electron Microscope images of multi-walled nanotube aggregates and natural organic matter at pH adjustments (A) 6; (B) 7; and (C) 8. Note the difference in scale.
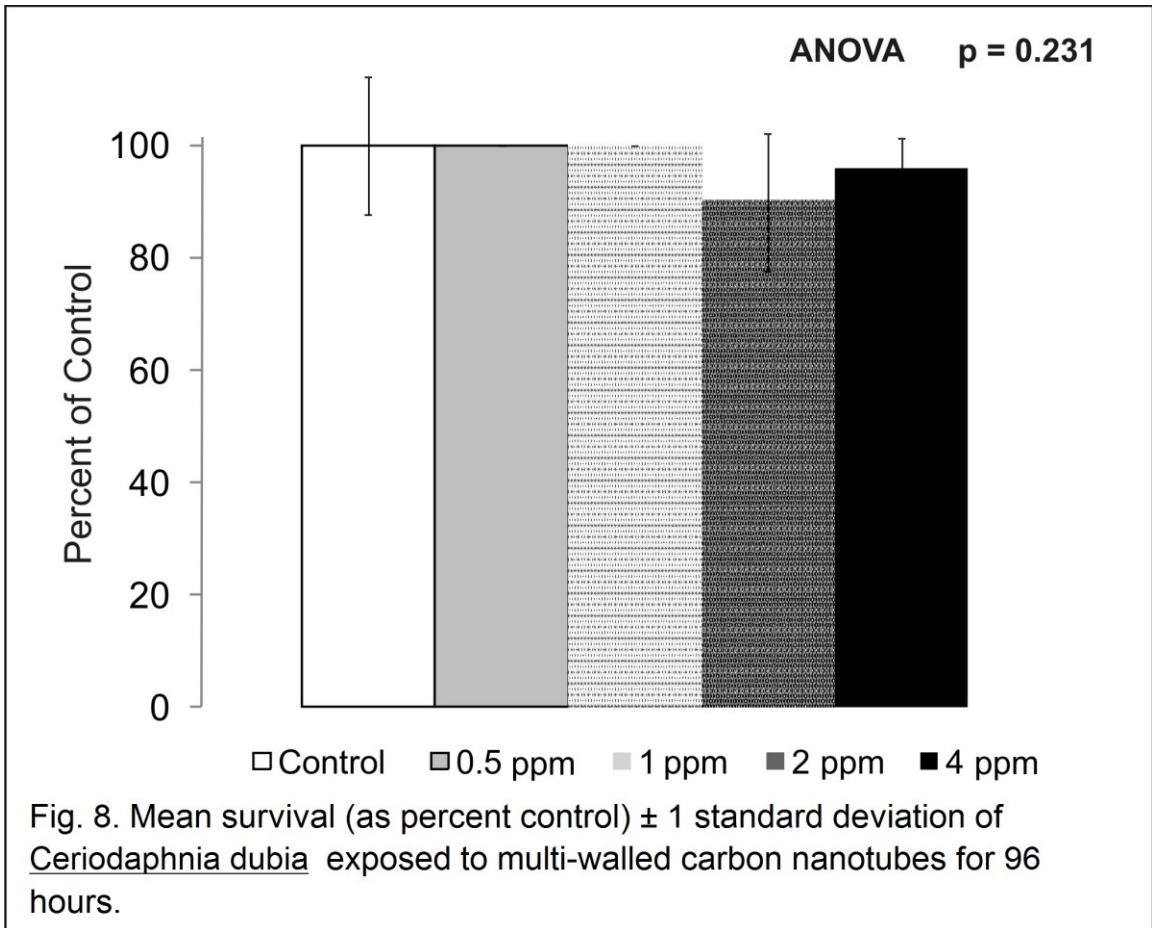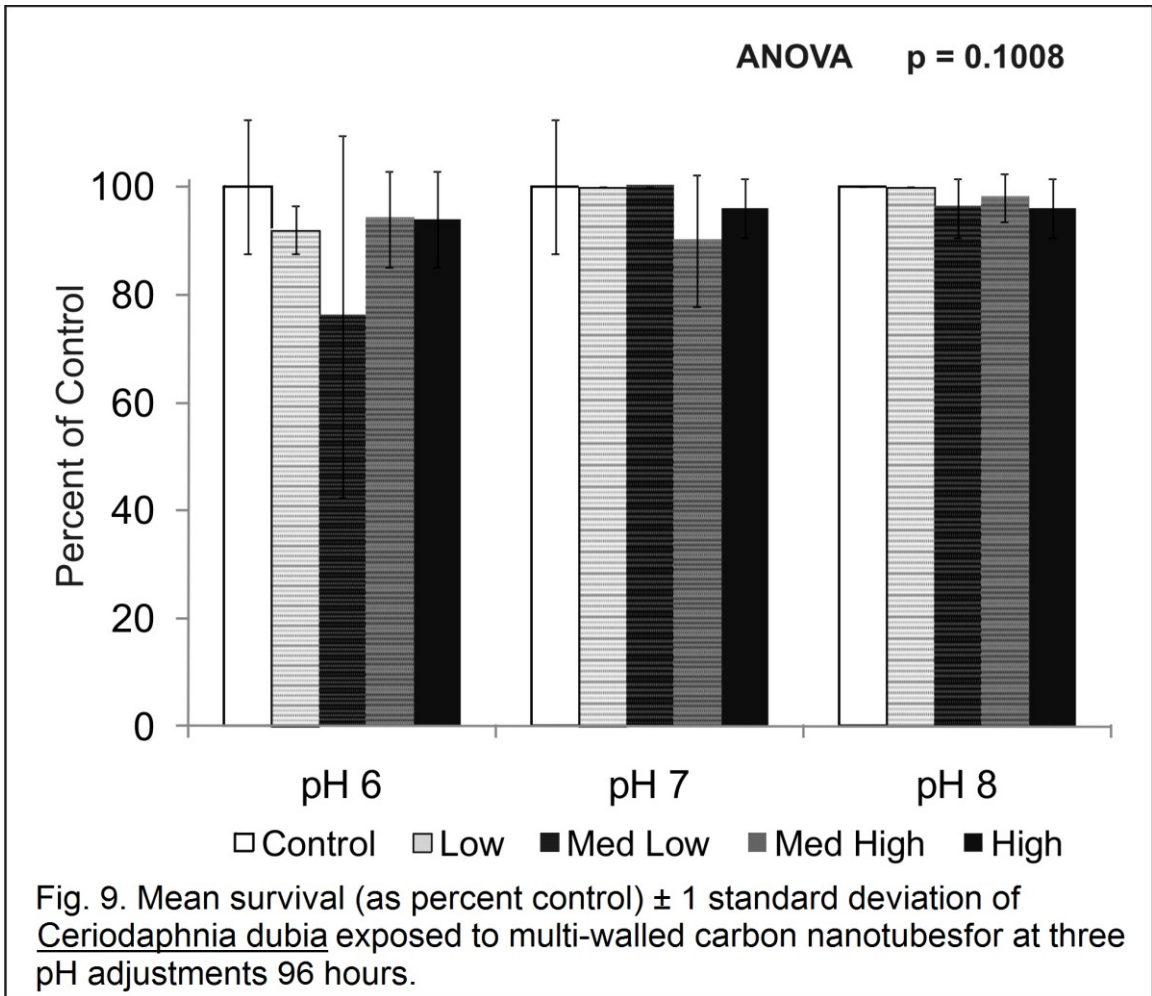
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Survival

There was no significant difference in survival over the observed 96hrs in the Ceriodaphnia dubia acute test at pH 7 (ANOVA F =1.49 p =0.231)(Figure 8, Table 5). Mean transformed percent survival across the test was 84.65 ± 9.97. C. dubia survival was not different with pH adjustments (ANOVA F_{pH6} =1.47 p_{pH6} =0.235; F_{pH8} =1.45 p_{pH8}= 0.243). When run as a two-factor ANOVA using pH and exposure as factors the overall model is not significant (ANOVA F=1.70 p=0.1008)(Figure 9, Table 4), indicating that there was no significant interactive effect between pH and exposure concentration on survival. The mean transformed percent survival across the test was 82.03 ± 11.79. Among the three C. dubia tests, pH, as a factor by itself, did significantly influence survival in the observed time period (ANOVA p =0.0430).

There was complete survival in the Daphnia magna pH 7, 96 hr acute test, no statistics were used since there was no variance in that test. In the other D. magna tests there was also a lack of significant difference between survival over 96 hrs from exposure groups and control groups, though not as dramatic, (ANOVA F_{pH6} =1.0 p_{pH6}=0.3466; F_{pH8} =2.67 p_{pH8} =0.1411)(Figure 10, Table 5). When run as a two-factor ANOVA using pH and exposure as factors the overall model is not significant (ANOVA F=1.88 p=0.1346). The mean transformed percent survival across the test was 88.29 ± 4.89. Among the three D. magna tests, pH did not significantly influence survival in the observed time period (ANOVA p =0.2568).

Fig. 8. Mean survival (as percent control) ± 1 standard deviation of Ceriodaphnia dubia exposed to multi-walled carbon nanotubes for 96 hours.

Fig. 9. Mean survival (as percent control) ± 1 standard deviation of <u>Ceriodaphnia dubia</u> exposed to multi-walled carbon nanotubesfor at three pH adjustments 96 hours.

Fig. 10. Mean <u>Daphnia magna</u> survival (as percent control) ± 1 standard deviation across three pH adjustments exposed to multi-walled carbon nanotubesfor 96 hours.
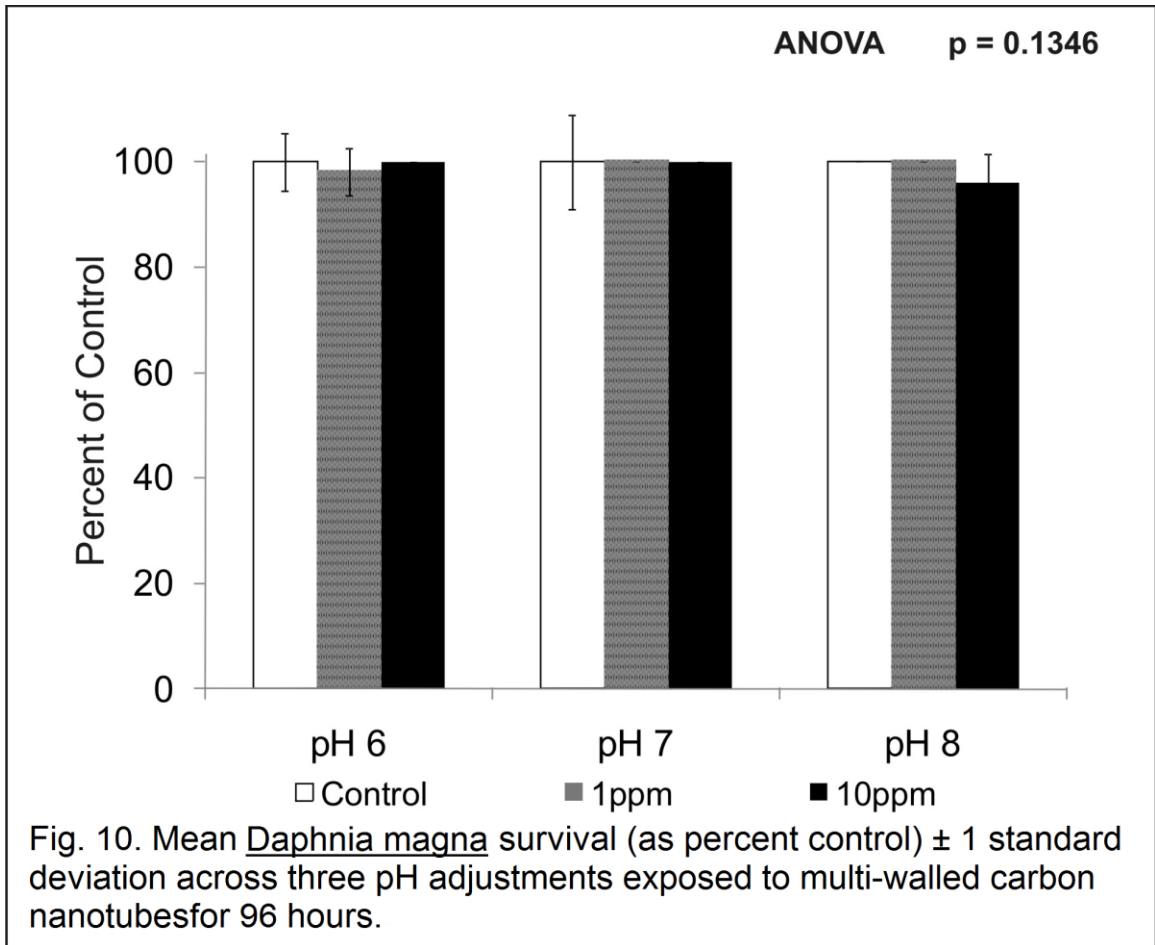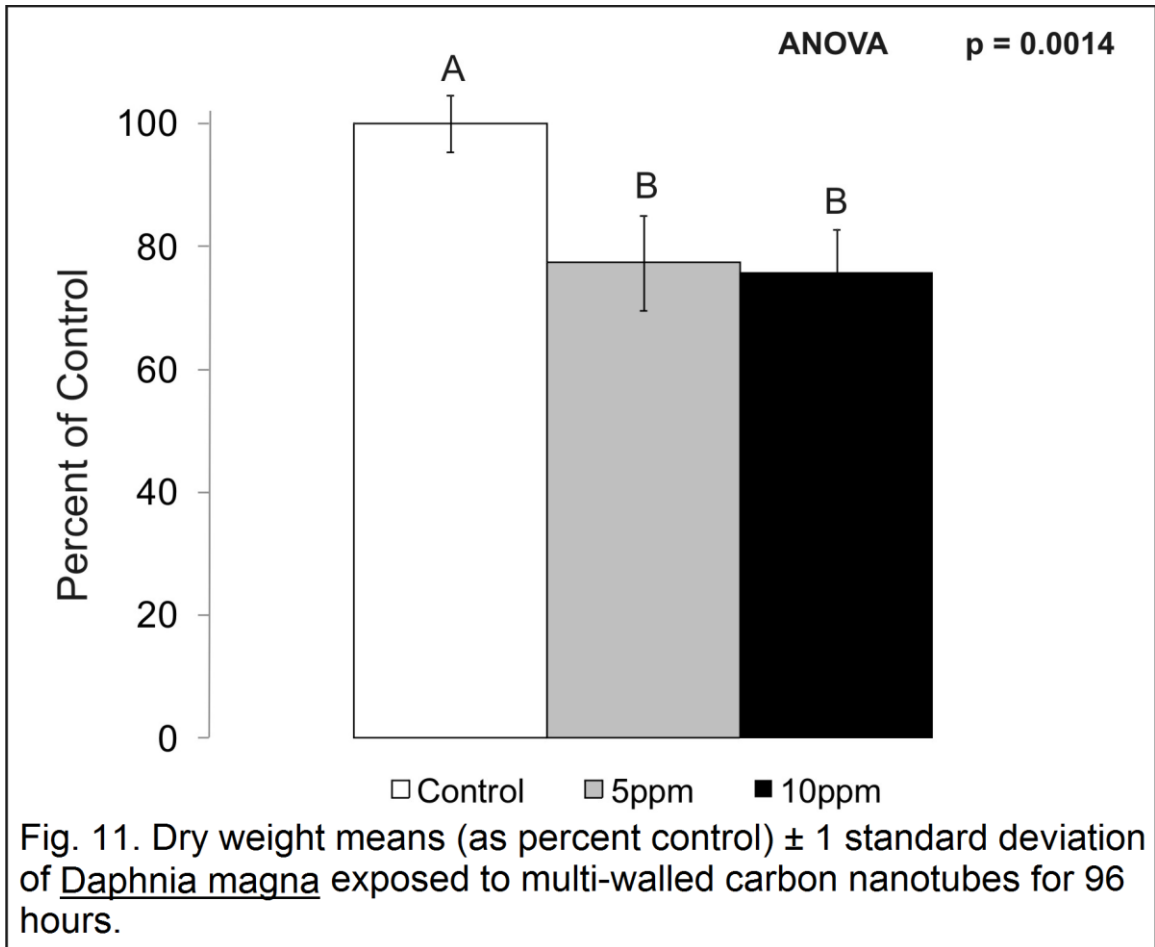
T<small>ABLE</small> 5. 96 hr Acute survival test means (as percent control) ± 1 standard deviation.

Mean Percent of Control with Standard Deviation				
Ceriodaphnia dubia				
	0.5ppm	1ppm	2ppm	4ppm
pH 7	100 ± 0	100 ± 0	90 ± 12.24	96 ± 5.48
	1ppm	2ppm	4ppm	10ppm
pH 6	92 ± 4.47	76 ± 33.61	94 ± 8.94	94 ± 8.94
ph 8	100 ± 0	96 ± 5.48	98 ± 4.47	96 ± 5.48
Daphnia magna				
	1ppm	10ppm		
pH 6	98 ± 4.47	100 ± 0		
pH 7	100 ± 0	100 ± 0		
pH 8	100 ± 0	96 ± 5.47		

Growth

There was a significant difference between mean dry body masses of exposed D. magna relative to controls (ANOVA F= 12.71 p= 0.0014).The mean dry weight was 36.04 ± 3.48µg. Relative to control the 5ppm MWNT exposure showed a 21.55% reduction in dry mass, and the 10ppm MWNT exposure showed a 23.06% reduction in dry mass. Tukey's post-hoc grouping placed both MWNT exposures together (Figure 11, Table 6).

Across three pH adjustments, there was a significant difference between the dry weights of D. magna (ANOVA F=7.5 p=0.0003). Individually, both pH (p=0.0004) and exposure concentration (p=0.0033) proved significant in the ANOVA, but there was no significant observed interactive effect between pH and exposure concentration (p=0.1659). Tukey's post-hoc test grouped pH 6 and pH 8 together, but placed pH 7 into its own group (Figure12, Table 6).
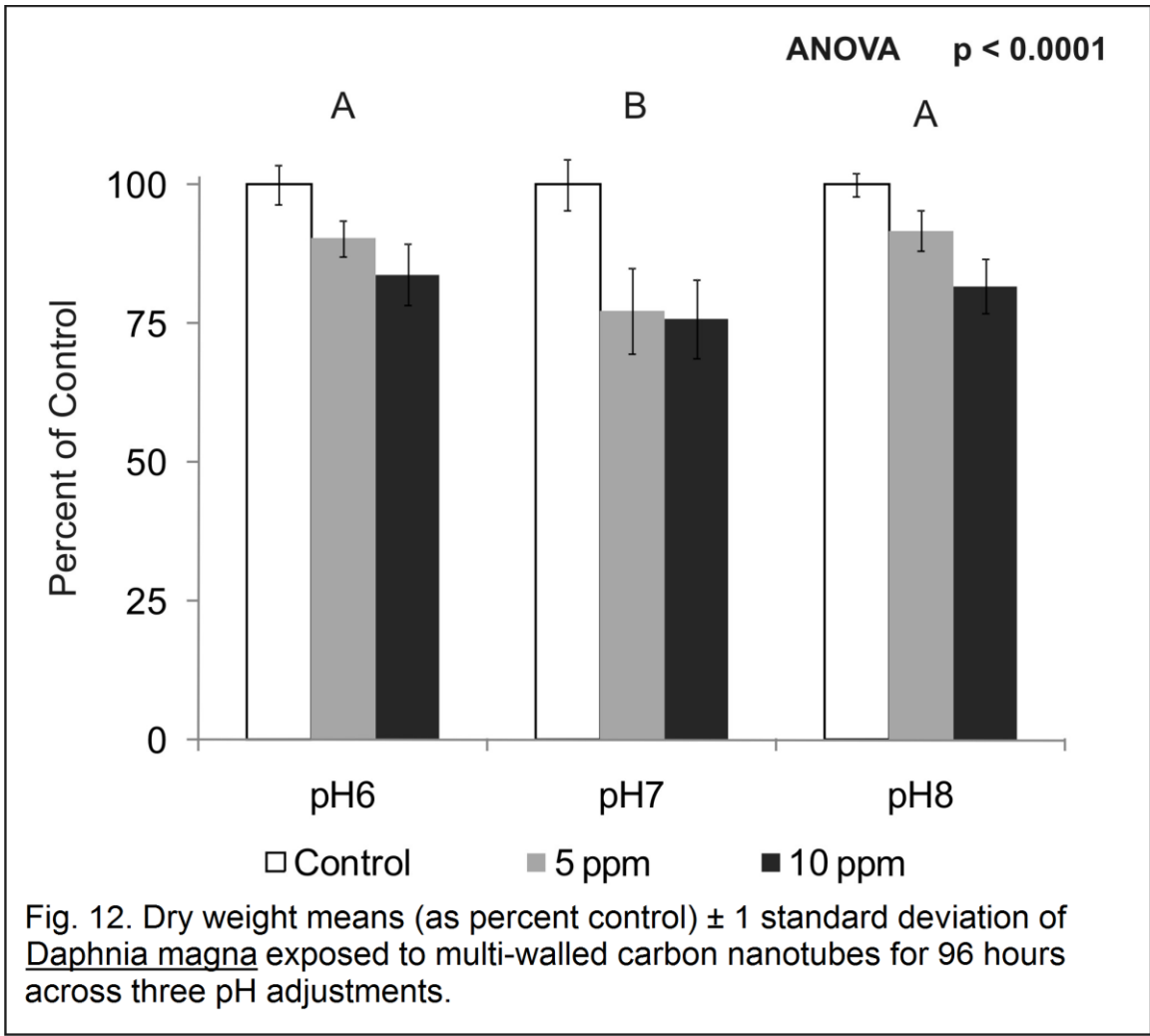
Fig. 11. Dry weight means (as percent control) ± 1 standard deviation of <u>Daphnia magna</u> exposed to multi-walled carbon nanotubes for 96 hours.

Fig. 12. Dry weight means (as percent control) ± 1 standard deviation of Daphnia magna exposed to multi-walled carbon nanotubes for 96 hours across three pH adjustments.

TABLE 6. Dry weigth means (as percent control with arcsine transformation) ± 1 standard deviation of Daphnia magna exposed to MWNTs for 96 hrs with three pH adjustments.

| | Mean dry weight with SD | |
	Low Exposure	High Exposure
pH 6	72.07 ± 3.01	66.51 ± 4.29
pH 7	61.81 ± 5.18	60.70 ± 4.89
pH 8	73.60 ± 3.93	64.86 ± 3.71

Reproduction

There was a significant difference in C. dubia reproduction exposed to three concentrations of MWNTs (ANOVA F=9.21 p= 0.0002). The exposure concentrations were 0.5 ppm, 2.5ppm, 5ppm MWNT. Only the highest exposure was significantly different from the control (Figure 13, Table 7).
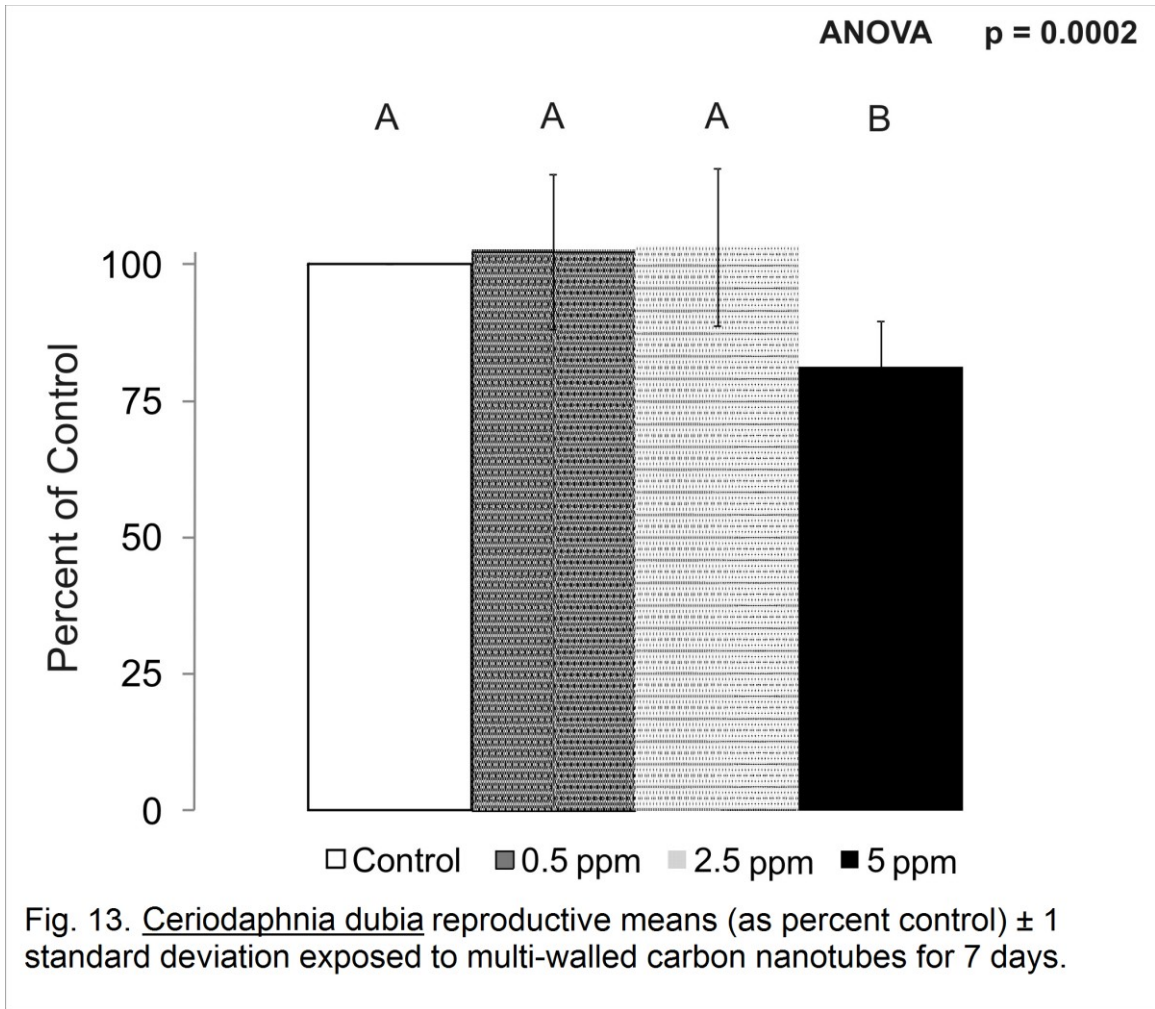
There was also a significant difference in D. magna reproduction when exposed to varying concentrations of MWNTs for three broods (ANOVA F=6.61 p=0.0005). Exposure concentrations were: 0.125ppm, 0.25ppm, 0.5ppm, and 1.0ppm MWNTs. The lowest exposure's mean reproduction was not significantly different from the control (Figure 14, Table 7). The control was significantly different from the three highest exposures, however, none of the exposures were significantly different from each other.
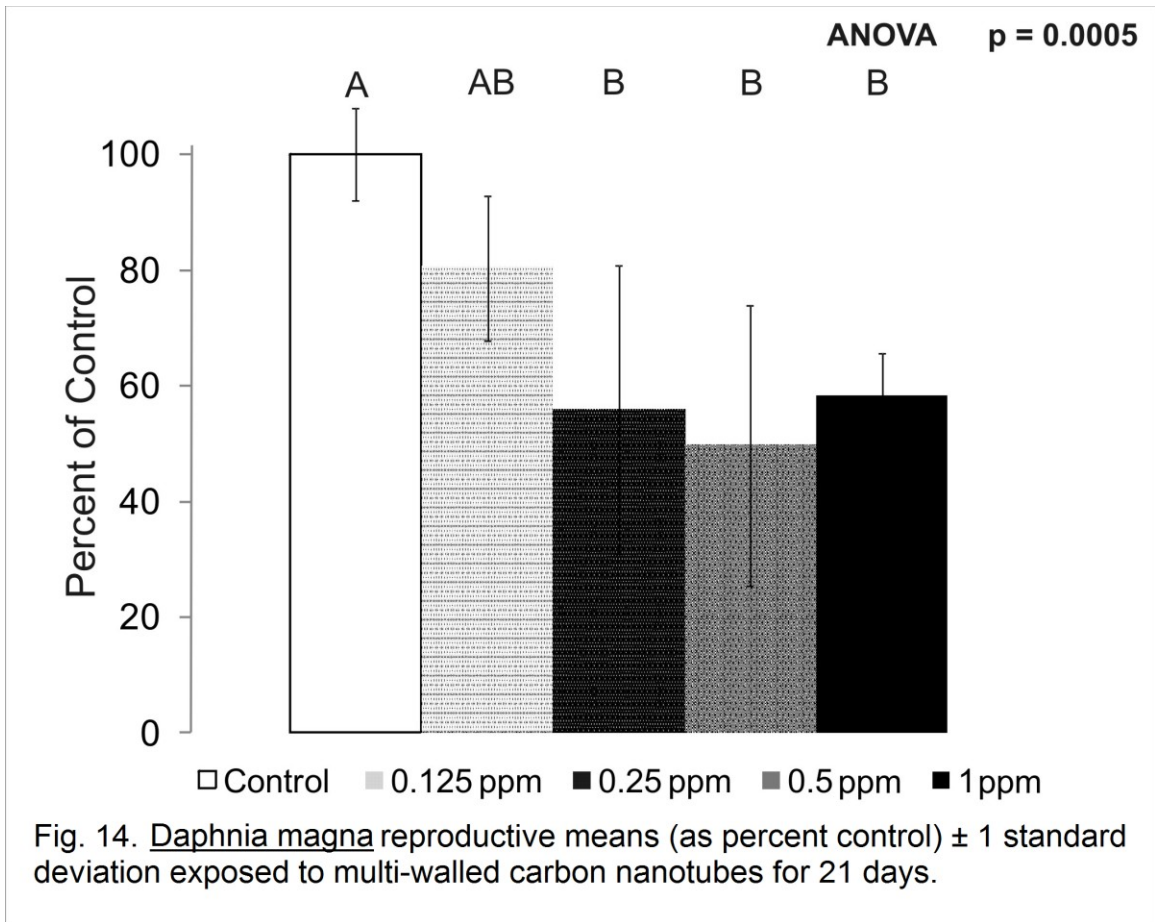
There was no difference in reproduction between C. dubia controls and D. magna controls (Welch's Approximate t test p = 0.2014). There was, however, a significant difference between the reproduction of C. dubia exposed to 0.5ppm MWNTs and D. magna exposed to 0.5ppm MWNTs (Student's t test p = 0.002).

Reproduction with Variation in pH

Among C. dubia chronic exposures to the same concentrations of MWNT, but among pH adjustments to pH 6, pH 7, and pH 8, there was a significant difference among treatments (ANOVA F=10.81 p<0.0001). There was a significant difference among pHs (p=0.0018) and among exposures (p=0.0001),

but no observed significant interaction between MWNTs and pH variation

(p=0.1791) (Figure 15, Table 7).

Fig. 13. <u>Ceriodaphnia dubia</u> reproductive means (as percent control) ± 1 standard deviation exposed to multi-walled carbon nanotubes for 7 days.

Fig. 14. <u>Daphnia magna</u> reproductive means (as percent control) ± 1 standard deviation exposed to multi-walled carbon nanotubes for 21 days.

Fig. 15. <u>Ceriodaphnia dubia</u> reproductive means (as percent control) ± 1 standard deviation exposed to multi-walled carbon nanotubes at three pH adjustments for 7 days.

TABLE 7. Reproductive means (as percent control) ± 1 standard deviation.

Mean reproduction with SD			
Ceriodaphnia dubia			
7 day	**0.5ppm**	**2.5ppm**	**5ppm**
pH 7	102.35 ± 14.25	103.27 ± 14.41	81.23 ± 8.38 *
pH 6	87.95 ± 12.65	77.68 ± 11.88 *	62.86 ± 13.49 *
pH 8	73.36 ± 23.30	78.79 ± 22.78 *	75.17 ± 6.55 *

Daphnia magna				
21 day	**0.125ppm**	**0.25ppm**	**0.5ppm**	**1ppm**
	80.37 ± 12.53	55.83 ± 24.95 *	49.69 ± 24.18 *	58.28 ± 7.30 *

* Significantly different at an alpha = 0.05

DISCUSSION

Survival

Significant mortality was not found in exposures up to 10ppm multi-walled carbon nanotube (MWNT). Kennedy et al. (2009) did not report acute mortality in Ceriodaphnia dubia with non-functionalized MWNTs until 16ppm (Table 1). The highest exposure used in acute toxicity assays (10ppm) may not have been concentrated enough for significant toxicity to be observed. In a cladoceran lifetable suspended solids toxicity test, Kirk and Gilbert (1991) did not report significant juvenile C. dubia mortality at 10ppm suspended clay, but total juvenile mortality at 50ppm suspended clay, but the same 50ppm exposure concentration caused 80% morality in juvenile Daphnia ambigua and 44% in Daphnia pulex. The lower exposure, 10ppm suspended clay, did not induce significant mortality among any of the three cladocerans tested (Kirk and Gilbert 1991).

Growth

Significant reduction in growth as compared to controls was observed following exposures to 5ppm and 10ppm MWNT. This is in agreement with preliminary data collected by Taylor et al. (2007). The growth deficit may be due to an inhibition of nutrient uptake. It is established in the literature that daphnia readily ingest carbon nanotube (CNT) aggregates (Roberts et al. 2007, Kennedy et al. 2009, Figure 16). These aggregates likely displace digestible material in the daphnid gut tract and thus change the ratio of energy expended in feeding to

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energy derived by the organism. This energy deficit in turn changes the partitioning of energy for activity, growth and reproduction (Kirk 1991).
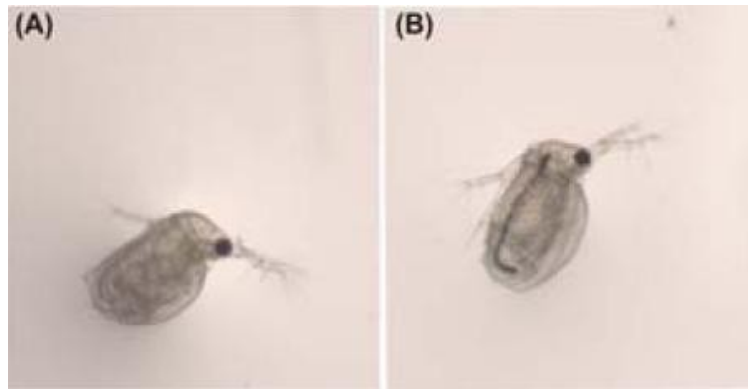

Fig. 16. Cerodaphnia dubia, (A), in natural organic matter solution without multi-walled carbon nanotubes, and (B), after exposure to multi-walled carbon nanotubes.

This mode of inhibition has been established for other inorganic suspensions, such as clays, with daphnia (Arruda et al. 1983, McCabe and O'Brien 1982, Hart 1986, Kirk 1991). The actual mechanism at work could be reduction in algal ingestion per unit volume, reductions in the assimilation of ingested algae, or a combination of the two. Kirk and Gilbert (1990) and Kirk (1991) conducted long-term feeding experiments with a variety of cladocerans to determine population effects of feeding inhibition resulting from exposure to suspended solids. Kirk (1991) reported that 50ppm suspended solids greater than 2 μm in size reduce cladoceran algae intake by 13-83%. Kirk (1991) stated that greater reductions in algal ingestion rate will result in less energy and

nutrients being available for the individual's use. Reduced energy and nutrients result in reduced body size.

Effect of pH on Growth and Survival

Mean aggregate size was altered by adjustment in pH (Table 4). Dynamic light scattering (DLS) data showed modest variation in hydrodynamic size. However, the scanning electron microscope (SEM) imaging showed aggregates with means an order of magnitude larger than predicted by DLS measurements. This may be due to a limitation of DLS measurements. Particle size measurement is limited by the wavelength of the laser beam on the DLS unit, in this case 633 nm. Once aggregates surpass this wavelength (particles >600 nm in diameter), the DLS measures the size of the sub-units of the actual aggregate (D'Souza personal communication 2010). Thus, for the remainder of this thesis, aggregate measurements referred to are those measured by SEM.

Zeta potential measurements indicate there was pH-dependent variation in surface charge which alters the electrostatic repulsion in the natural organic matter (NOM) coating the MWNTs. This changes the folding of the NOM molecules which determines the amount which can fit onto the MWNT surface. The difference in the amount of NOM coating changes the stability of the suspension as the NOM acts like a surfactant in stabilizing an otherwise very hydrophobic molecule in aqueous media. The range of zeta potential change achieved, although small, (Table 4) was enough to alter aggregate size as measured by SEM. A change of ± 10 mV would be the most expected from the

pH range tested as reported in the literature (Lin et al. 2009, Figure 17). This is similar to what was measured in my samples.

However, the data demonstrate that the changes in mean aggregate size did not affect growth or survival. The lack of effect of aggregate size on toxicity likely means that, while pH affected nanomaterial behavior, the differences in aggregate size were not enough to cause differences in the biological response. D. magna are able to ingest particles ranging in size from 0.1 μm to >30 μm (Porter et al. 1983). The SEM data indicate the MWNTs in this experiment changed +/- 3.5 μm with pH adjustment. Thus, this relatively small change in particle size compared to the range of ingestible particle sizes may be negligible for the organism. Perhaps a more broad range of pH adjustment would alter aggregate size sufficiently, but such pH extremes themselves may have an effect on the test organisms.
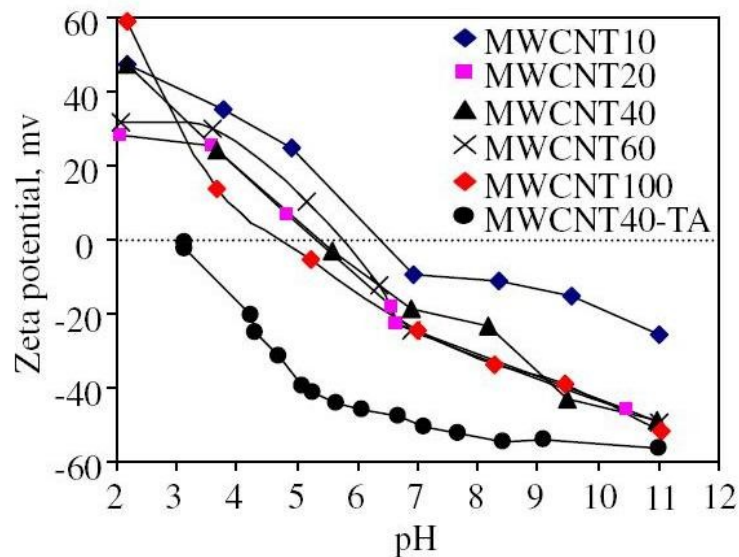
Fig. 17. Zeta potentials of multi-walled carbon nanotubes in deionized water and multi-walled nanotubes outer diameter of 40 nanometers in 20ppm tannic acid solution at a range of pHs (Figure from Lin et al. 2009).

Reproduction

There was a deleterious effect of exposure to MWNTs on daphnid reproduction. There is no published literature on the reproductive effects of carbonaceous nanoparticles on cladocerans. However, in lifetable and population growth experiments, Kirk and Gilbert (1990) demonstrated declines in the populations of four cladoceran species (Bosmina, Ceriodaphnia, and two Daphnia) exposed to suspended clay particles (<2 µm). They reported decreased population growth rates in the presence of suspended solids ranging between 1 µm and 3 µm, and stated that particles of that size range in concentrations greater than 50ppm would likely suppress cladoceran reproduction rates (Kirk

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and Gilbert 1990). Suspended clay has been reported to reduce the assimilation efficiency of ingested algal food (Arruda et al. 1983). Arruda et al. (1983) reported assimilation rates in two cladocerans, D. pulex and D. parvula, by approximately 20% when exposed to 10ppm suspended clay, and reduction approximating 85% at 100ppm suspended clay. If suspended MWNTs behave similarly in cladocerans once ingested the mechanism of reproductive depression could be twofold; feeding inhibition combined with reduction of ingested nutrient assimilation.

Based on reproductive endpoints, Daphnia magna were more sensitive to MWNT exposure than C. dubia (Table 7). D. magna showed approximately a 50% reproductive inhibition at 0.5ppm MWNT exposure, while C. dubia did not show significant reproductive inhibition at the same exposure concentration and pH. This could be explained by differences between the test organisms. Lynch (1978) reported that Ceriodaphnia still exhibited high growth rates in periods of low food compared to Daphnia pulex sharing the same diet. Porter et al. (1983) reported that, per unit mass, C. dubia filters approximately twice the volume of water that D. magna per hour (64.66 ± 4.42 µl/µg/h C. dubia vs. 29.77 ± 0.61 µl/µg/h D. magna). This combination of D. magna being more famine sensitive and C. dubia having a greater mass based filtration rate could be the cause of the difference in reproductive means between the two test organisms

There was no interactive effect of pH and MWNT concentration on reproduction. As discussed previously, the relatively small differences pH had on particle size were not significant for the organism.

Conclusions

MWNTs affected grazing zooplankton growth and reproduction at the tested exposure concentrations. The suspected mode of action of this toxicity is feeding inhibition, leading to a deficit of nutrient intake. Variation of pH did not significantly alter observed toxicity in acute or chronic tests despite minor alterations in aggregate size.

This investigation has shown that MWNTs can interact with NOM to form stable suspensions which result in toxicity. With current knowledge, there is no easy estimate of an environmentally relevant exposure concentration thus determination of potential risk posed by MWNTs to aquatic ecosystems is difficult. However, MWNTs are an emergent contaminant as both applications and manufacture continue to increase. It is the conclusion of this investigation that MWNTs exhibit little potential for acute toxicity to grazing zooplankton but chronic toxicity could present concerns.

Future Directions

Feeding Inhibition

The next step in this research should be to test the suspected mode of observed toxicity. Provided that it is qualified as being a feeding inhibition the further advancement would be developing an assay to quantify feeding inhibition.

An experiment similar to the uptake assays was reported by Petersen et al.
(2009), using radiolabeled tubes and perhaps radiolabeled algae. This would
allow a dose-response curve relating exposure concentration to the amount of
interference in nutrient uptake. This would facilitate the creation of energetics
models. Such models could be incorporated into risk assessments of
nanomaterials.

<div align="center">Comparative Toxicity</div>

Toxicity of MWNTs as varied by MWNT dimensions is poorly understood.
The size of aggregates, rate of aggregate formation, and the stability of
suspension as varied by the number of concentric tubes and overall length is
poorly understood. Changes in the dimension of MWNTs have been shown in the
literature to alter some forms of chemical behavior (Chen 2007). MWNTs
released into the environment as a part of waste streams will not be uniform
tubes of identical measure.

Functionalization of nanomaterials and MWNTs in particular has been
shown to alter chemical behavior and in CNTs, their toxicity by orders of
magnitude (Kenndey et al. 2009). Studies of how suspension in freshwater and
other waters changes surface chemistry of already functionalized MWNTs are
needed. Futhermore, there is a lack of basic toxicity data on identical MWNTs
with different surface functionalizations. Already published studies (Kennedy et
al. 2009) show that hydroxylated or pristine MWNTs are acutely toxic to daphnids
in concentrations tens of parts per million greater than amine or alkyl

functionalized tubes of the same dimensional characteristics (Table 1). Changes in surface chemistry as exposed to the conditions found in surface waters are critical to further understanding of MWNT aquatic toxicity as well as risk assessment of carbon nanomaterials as a group.

Trophic Transfer

Preliminary tests have shown MWNTs in the digestive tracts of fish (Danio rerio) after ingestion of daphnia exposed to MWNTs without time for the daphnia to depurate. The fish were never placed into water with suspended MWNT aggregates, exposure was dietary. This leads to the question; could the presence of MWNTs impede nutrient uptake by the fish, leading to a similar chronically toxic effect in the next trophic level?

Multi-walled Nanotube Cycling

As a nanomaterial, MWNTs do not strictly follow the behavior of a normal molecule or of a normal bulk material. Thus predictions on how MWNTs should partition between sediment and pelagic suspension needs to be empirically tested. Complex substrates and biotic activity could interact to move MWNTs out of suspension and into sediment even in solutions with adequate NOM concentrations. Conversely, biotic activity, such as benthic macroinvertebrates' feeding and burrowing activity, could force a certain amount of MWNTs to remain in the pelagic zone. Careful study of likely scenarios with biotic interactions as a component is crucial for future risk assessment of MWNTs in aquatic environments.

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