EFFECTS OF MULTI WALLED CARBON NANOTUBES AND SEDIMENT ON THE
TOXICITY AND BIOAVAILABILITY OF DIPHENHYDRAMINE

by

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Under the Direction of

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ABSTRACT

The effects of multi-walled carbon nanotube addition to sediment on the
toxicity of diphenhydramine were investigated using invertebrate and fish models.
Sediment elutriate exposures were undertaken with the freshwater crustacean
*Ceriodaphnia dubia* to compare the toxic effects of diphenhydramine in the presence
and absence of sediment and multi-walled carbon nanotubes. In both sediment and
solution-only treatments, addition of 0.318 mg/g of functionalized multi-walled
carbon nanotubes significantly decreased overall 48-hour mortality relative to the
positive control. In a subsequent study, juvenile fathead minnows (*P. promelas*)
were exposed to sublethal concentrations of diphenhydramine in the presence of
natural sediment, with some treatments receiving MWCNTs. Addition of MWCNTs
did not have a protective effect upon DPH-related growth inhibition, and did not
reduce the whole-body burden of DPH in exposed fish. Mass-balance calculations
indicated that significant amounts of DPH were adsorbed to MWCNTs, and DPH
concentrations in water and sediment were commensurately reduced.
INDEX WORDS: Multi-walled carbon nanotubes, toxicity, diphenhydramine, bioaccumulation, bioconcentration, adsorption, desorption, sediment, fathead minnows, *Ceriodaphnia dubia.*
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DEDICATION

To the great researchers who have come before me and laid the groundwork of science, to my contemporaries and colleagues pushing the boundary of knowledge ever further, and most of all to those in the future who will take humanity to a summit now unknown.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>2 MULTI-WALLED CARBON NANOTUBES REDUCE TOXICITY TO CERIODAPHNIA DUBIA IN WATER AND SEDIMENT EXPOSURES</td>
<td>27</td>
</tr>
<tr>
<td>3 EFFECTS OF MULTI-WALLED CARBON NANOTUBES AND SEDIMENT ON THE BIOACCUMULATION OF DIPHENHYDRAMINE IN PIMEPHALES</td>
<td>44</td>
</tr>
<tr>
<td>4 CONCLUDING REMARKS</td>
<td>79</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1: Characteristics of Sediment

Page 41
LIST OF FIGURES

Page

Figure 2.1: Log-logistic fitted dose-response curve for diphenhydramine acute toxicity ..........................................................39

Figure 2.2: *C. dubia* mortality in acute (48h) elutriate exposures ........................................40

Figure 3.1a: Mean dry mass of 5-fish composite sample by day and treatment ........68

Figure 3.1b: Mean body length by treatment and day ..........................................................68

Figure 3.2: Concentrations of diphenhydramine in water (μg/ L) by treatment and day .................................................................69

Figure 3.3: Concentrations of diphenhydramine in sediment (ng/g) on Day 10........70

Figure 3.4: Mean body burdens of DPH by day and treatment ........................................71

Figure 3.5a: Bioconcentration factor (BCF) of diphenhydramine by treatment at Day 1 and Day 10 ..........................................................72

Figure 3.5b: Biota-sediment accumulation factor (BSAF) of diphenhydramine by treatment at Day 10 ..........................................................72

Figure 3.6: Mass balance of diphenhydramine after 10 days of exposure ................73

Supplemental Figure 3.1: Experimental pH by treatment over time .........................74

Supplemental Figure 3.2: Standard curve used for determination of MWCNT concentration in water ..........................................................75

Figure 4.1: Conceptual model of organic contaminant exposure in the presence of MWCNTs and sediment .........................................................83
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Toxicology is a science of interactions. At its most fundamental level, observing the interaction between a poison and its victim formed the historical foundation of our discipline. In the intervening centuries since Paracelsus’ famous axiom, scientists have made great strides in understanding the fine points of toxicity, extending our knowledge beyond single organism interactions to ecotoxicity, mechanistic toxicology, population toxicology, and more. With the advent of “omic” techniques, we now have the ability to understand the effects of individual toxicants in minute detail. However, outside of the laboratory exposure to only one toxicant at a time is exceedingly rare, or perhaps even impossible. The disconnect between laboratory results and the reality of toxicant exposure provides an impetus to cross over the next horizon of toxicology: the interaction between multiple toxicants in an environmental context.

The Chemical Abstracts Service Registry (CAS-R) currently catalogues over 88 million distinct compounds, and in the United States alone, the US Environmental Protection Agency’s (USEPA) Toxic Substances Control Act inventory receives over 1000 new registrations per year. The rate of increase in the design of new chemicals has followed an exponential curve since records began in the late 19th century, and alarmingly, the most conservative estimates indicate that even rudimentary acute
toxicity data is known for less than 13% of all compounds (Binetti et al., 2008). Resources do not exist to conduct toxicity testing on even a small fraction of known compounds, so toxicologists and policymakers must choose the ones that are perceived to pose the most imminent and probable threat. These substances are characterized by the USEPA as contaminants of emerging concern (CECs).

Among the substances classified by the USEPA as CECs are pharmaceuticals and personal care products (PPCPs) and carbon nanomaterials. Pharmaceuticals are released into the environment mainly through excretion by humans and livestock and subsequent wastewater discharge, and to a smaller extent via improper disposal and factory waste (Heberer, 2002). They are not commonly detected in concentrations that are known to cause toxicity (with the exception of certain hormonally-active compounds), however due to the principles of partitioning/fate and biomagnification, organisms may nevertheless receive an effective dose.

Carbon nanomaterials are a class of carbon-only macromolecules that have properties unlike those of elemental carbon. There are many types, the most commonly produced being carbon nanotubes, fullerenes (C60 or buckyballs), and graphene. These nanomaterials have promising applications in high-tech industries such as resilient materials fabrication, electronics, and biosensing. As their use progresses beyond research and into consumer goods, there is the potential for nanomaterial release into the environment through both disposal of goods and point source accidental dumping. Carbon nanotubes are a particular focus of interaction toxicology work, as they have a high surface area and adsorptive capacity for organic compounds. There is concern that with the increased use and
disposal of carbon nanotubes, there may be an effect upon the fate of pharmaceutical and other organic contaminants in the aquatic environment and they may act as a mobilizing and/or concentrating agent.

The purpose of this research is to investigate the combined toxicity of multi-walled carbon nanotubes and diphenhydramine, a commonly detected environmental pharmaceutical used here as a model compound. In order to maintain environmental relevance, natural sediment was included as a component in both experiments. A model freshwater aquatic invertebrate, Ceriodaphnia dubia, and a model freshwater fish, Pimephales promelas, were exposed to diphenhydramine with a multi-walled carbon nanotube spiked sediment substrate. Mortality, morbidity, and physical development were recorded, and metrics of bioaccumulation such as Biota-Sediment Accumulation Factor and Bioaccumulation Factor were calculated. We hypothesize that the presence of multi-walled carbon nanotubes will sequester diphenhydramine in the sediment fraction, rendering it less available to organisms that have little or no sediment-phase interaction.

Literature Review

Properties of Carbon Nanotubes

Carbon nanotubes (CNTs) are a novel material that have seen increased use in research, medicine, and industry in the two decades since their first synthesis (De Volder et al., 2013). Some recent developments in CNT use include addition to paints to repel barnacles from forming on ship hulls (Beigbeder et al., 2008) and allow any surface to become a solar-energy storing battery (Cho et al., 2014),
construction of nanoscale sensors to detect everything from trace oxygen levels (Llobet et al., 2008) to the binding of proteins to ligands (Münzer et al., 2014), and creating flexible, fiber-shaped batteries for use in wearable electronics (Lin et al., 2014). CNTs have 19 to 56 times the tensile strength of steel wire, can conduct electricity over long distances with essentially no heat discharge, and can be used to create mechanical actuators which discharge more than 100 times the force of natural muscle fibers (Baughman et al., 2002).

While CNTs are a useful tool in developing high-strength and low-weight materials (Ajayan et al., 2001), they have been shown to have toxic effects at high concentrations in a variety of aquatic organisms including invertebrates and fish (Cheng et al., 2009; Jackson et al., 2013). As carbon nanotubes, especially the cheaper multi-walled variety (MWCNTs) enter mass production for commercial use, their deposition into the aquatic environment via accidental discharge or intentional disposal becomes increasingly likely. MWCNTs are a particular cause for concern, as there is evidence that they can be spontaneously generated due to the combustion of propane or natural gas in a source as innocuous as a kitchen stove. (Bang et al., 2004; Murr et al., 2004). In a particularly alarming case, carbon nanotubes were found in the lungs of first responders to the 9/11 World Trade Center attacks, and were tentatively linked to high incidence of lung disease among those patients (M. Wu et al., 2010).

As with many solid contaminants, sediment is the ultimate compartment of deposition for MWCNTs, with modeled deposition rates in the United States predicted to range from 40 to 229 ng/kg annually, based on (what year?)
production levels. (Gottschalk et al., 2009). The same modeling experiment predicted far lower concentrations suspended in water, from 6.6 to 18 ng/L in the United States. Settling experiments have concluded that MWCNTs fall out of suspension and sink to sediments within 120 minutes in water, rendering them less available to pelagic species (Kennedy et al., 2008). While MWCNTs alone do not pose a likely ecotoxicological threat due to their low environmental concentrations relative to effective toxic doses (Mueller et al., 2008), there are several components of sediment that have the potential to interact with MWCNTs including natural organic matter (NOM) and anthropogenic compounds with affinity for binding to organic carbon.

**Carbon Nanotubes as a Toxic Substance**

Considering the wide range of current and potential applications for carbon nanomaterials, their potential to cause adverse health effects has been widely studied. Several routes of exposure are postulated, including inhalation in terrestrial organisms, uptake through the gills and skin of aquatic organisms, ingestion, and direct administration to humans and animals as a method of drug delivery.

A 7-day inhalation study in rats resulted in granuloma formation and increased expression of inflammatory factors beginning at 0.5 mg/ m³ MWCNTs (Ma-Hock et al., 2013). For comparison, carbon black was tested at concentrations as high as 10 mg/m³ and resulted in no observed lung toxicity. In a longer-term, 90-day study, direct installation of a single dose of 0.5 mg of single-walled carbon nanotubes (SWNT) into the tracheas of mice resulted in formation of epithelioid
granulomas within 7 days, which persisted in number and severity over the full 90 days of the exposure period (Lam et al., 2004). While these concentrations/dosages are much higher than can be expected in an environmental setting, it is probable that workers synthesizing, preparing, and transporting carbon nanotubes are at risk if proper respiratory protection is not used.

The majority of aquatic MWCNT toxicity data has been collected using aquatic invertebrate models, due to their standardization in acute toxicity and uptake experiments. It has been shown that the sediment-dwelling invertebrate *Lumbriculus variegatus* does not avoid MWCNTs when ingesting soil, and that due to the comparatively large diameter of MWCNTs relative to intestinal capillary villi they pass through the gut tract and are excreted (Petersen et al., 2008). In contrast, the water-dwelling invertebrate *Daphnia magna* was found to retain MWCNTs after a 24-hour exposure in a 0.4 mg/L solution, with MWCNT comprising on average 6.3% of total body mass. Light microscopy indicated that MWCNTs were retained in the gut tract and were unable to be excreted (Petersen et al., 2009). While no acute toxicity was observed in either organism, blockage of the intestinal tract may result in long-term health issues relating to impaired digestion of food, backup of undigested matter, and sepsis.

In fish, potential routes of nanotube exposure include ingestion, and absorption through gills or skin. A 10-day exposure of river trout (*Oncorhynchus mykiss*) to 0.1 – 0.5 mg/L single-walled carbon nanotubes (SWCNT) resulted in a dose-dependent rise in various inflammation-related gill pathologies and mucus secretion, as well as nanotube precipitation on gill mucus (Smith et al., 2007). In the
same study, examination of the gut showed adhesion of SWNTs to the intestinal lumen, as well as inflammation-related gut pathology. Fish studies with MWCNTs are less conclusive; few have been conducted and only *Danio rerio* (zebrafish) have been investigated. Zebrafish are a common test organism used to evaluate teratogenicity, as the stages of their embryonic development are well understood. MWCNTs were shown to cause no developmental problems to embryos injected with 2 ng of functionalized MWCNTs; however after rearing and breeding the exposed fish, the subsequent generation experienced increased mortality relative to controls (Cheng et al., 2009). A more recent study found that 21-day exposure to very low concentrations of MWCNTs, 0.1 to 0.001 mg/L, induced changes in *D. rerio* brain and gonadal lipid levels. No associated morbidity was observed, and MWCNTs were not observed to cross the blood-brain barrier (Li et al., 2015). While there are few studies investigating the toxic effects of MWCNTs alone, more information is available on the more environmentally relevant combined effects of MWCNTs and natural organic matter.

**Natural Organic Matter**

Natural organic matter (NOM), a large organic molecule consisting primarily of humic and fulvic acids, is a primary mediator of the interaction between sediment and MWCNTs (Hoon Hyung et al., 2008). NOM is formed through the decomposition of plant and animal matter in water bodies, and is a major nonliving organic component of sediments. Due to the large number of variables involved in NOM formation, it characteristically contains many functional groups including
polyphenols, ketones, carboxylic acids, alcohols, aromatic rings, and methoxyl
groups (Chen et al., 2002). This abundance of functional groups renders NOM highly
reactive, and as a result it is known to conjugate with metal cations (Kinniburgh et
al., 1999), aqueous anions such as OCl\textsuperscript{-} and OBr\textsuperscript{-} (Westerhoff et al., 2004), and even
the cell surface of bacteria (Johnson et al., 1996).

While carbon nanomaterials are inherently hydrophobic and do not readily
form stable suspensions in water, this same hydrophobicity lends a strong
adsorptive capacity for organic molecules, especially those with aromatic ring
structures (Yang et al 2007, Lin et al 2008). This binding affinity for carbon rings is
likely related to pi-pi stacking interactions between ring-containing organic
compounds and the six-membered carbon rings in the walls of CNTs (Saikia et al.,
2013). When bound to NOM, the ability of MWCNTs to form a stable suspension in
water and hence become available to pelagic organisms is greatly increased (H.
Hyung et al., 2007).

Further, the presence of NOM has been shown to increase the toxicity and
bioavailability of MWCNTs to the aquatic invertebrates Daphnia magna and
Ceriodaphnia dubia (Edgington et al., 2010). In that study, a dose-dependent
relationship was found between mortality and MWCNTs in the presence of Suwanee
River NOM for D. magna, with LC50 ranging from 1.90 to 2.48 mg/L. Lethal
concentrations were not affected by the amount of NOM present, although a range
from 2.0 to 18.8 mg/L NOM was tested. This indicates that there may be a
“saturation effect” at work similar to that seen in the Michaelis-Menten kinetics of
enzymes, in which increasing levels of a substrate have no effect beyond a certain level due to saturation of all available binding points.

**Sediment and Toxicity**

Sediments are present in any natural water body, and are often the final compartment of deposition for aquatic toxicants. As such, sediment may act as a sink for contaminants, mitigating the exposure of organisms that dwell in the water column. Conversely, sediment-dwelling and benthic organisms will have greater exposure to contaminants that partition to sediment. Compounds with a high octanol:water partition coefficient ($K_{ow}$) are more likely to partition in sediment with high levels of NOM, potentially affecting exposure. In a study examining the effects of sediment composition on the toxicity of polyaromatic hydrocarbons (PAHs), a highly hydrophobic class of compounds, mortality to Japanese medaka embryos was mitigated in sediments containing higher amounts of NOM. Upon investigation, bioavailability of PAHs was concurrently lowered, implying that NOM in sediment sequestered the contaminants (Perrichon et al., 2014). Similarly, the presence of sediment was found to decrease the observed toxicity of parathion, an organophosphate pesticide, to the aquatic midge larva *Chironomus riparius*. Percentage composition of NOM was determined to be the primary factor in determining the magnitude of effect (Lydy et al., 1990). Other factors that may influence sediment-mediated toxicity include sediment composition (i.e. clay, silt and sand content) as well as particle size. Hydrophobic contaminants tend to adsorb most readily to the soil fraction that contains the most NOM, however it has been
reported that the quality of NOM has an effect upon adsorption capacity. A study examining PAH levels in Boston Harbor sediments found that NOM resulting from charcoal and plant detritus that associated with the sand and silt fractions of sediment, tend to adsorb more PAHs than the NOM associated with the clay fraction (X.-C. Wang et al., 2001). Conversely, a study comparing the sediment adsorption of three classes of compounds including an estrogenic pharmaceutical (17α-ethinyl estradiol), a diphenyl compound (bisphenol A), and a PAH (phenanthrene) found that while bisphenol A and phenanthrene conformed to previous assumptions, 17α-ethinyl estradiol (EE2) was adsorbed to a greater extent in the clay fraction of sampled sediments. EE2 has a lower $K_{OC}$ value than the other two compounds, which may have caused it to sorb by other mechanisms to the fraction with less NOM (Sun et al., 2012). These conflicting results indicate that, as with many studies examining the interactions of natural systems, all possible factors need to be taken into account in order to arrive at an accurate model.

The effect of sediment type alone upon test organisms is an often-overlooked factor in toxicity testing. Even without the presence of a xenobiotic, it has been shown that sediment composition affects commonly measured toxicity endpoints. Feeding rate and reproductive rate of *Lumbriculus variegatus*, both standard nonlethal endpoints, were significantly effected by sediment composition in a study examining two aquatic sediments with different levels of NOM (Leppänen et al., 1998). This result highlights the importance of including adequate controls in the design of toxicological experiments. For example, in a study involving sediment it is imperative to include a comparison of sediment-only exposure to a non-sediment
water control. Toxicity testing of sediment alone (with no added chemicals) is especially important when using natural sediment collected from the field, as it is often impractical to analyze the complete chemical composition of every sample. A rapid, standardized bioassay for sediment toxicity such as the USEPA 48-hour *Ceriodaphnia dubia* sediment elutriate test provides an inexpensive and reproducible method for evaluating the toxicity of sediment samples with or without added chemicals.

**Pharmaceuticals in Surface Water**

Pharmaceuticals and their metabolites are found in surface waters of the United States that receive municipal wastewater effluent or are downstream from animal feeding operations (Reif et al., 2012). While wastewater treatment plants are efficient at removing biosolids and most nutrients from influent, they are much less effective when it comes to the removal of pharmaceuticals. Removal efficiency is dependent upon the specific pharmaceutical compound to be removed and the treatment method, with the traditional biological systems that make up the vast majority of municipal methods (activated sludge, trickle filtering, anaerobic film, etc.) ranking among the least effective (Du et al., 2014). While technologies are available that remove pharmaceuticals at high efficiencies (including UV ozonolysis and reverse osmosis filtering), they are expensive, infrequently adopted, and are usually limited in application to on-site treatment of pharmaceutical and chemical company effluents (Deegan et al., 2011). The impact of pharmaceuticals on fish, invertebrates, and other aquatic animals is a subject of current study, and there are
indications that levels in some water bodies are high enough to cause adverse chronic effects (Halling-Sørensen et al., 1998; Sanderson et al., 2004). Especially worrying in the immediate sense are pharmaceuticals with an MOA that involves modification of the endocrine system such as ethinylestradiol, commonly found in birth control medications (Lyons, 2008). These compounds mimic hormones, and as such can have effective concentrations as low as a few ng/L. Other pharmaceuticals are generally not present in levels that can cause acute harm, but there is potential for chronic toxicity, adverse reproductive effects, and bioaccumulation/biomagnification to toxic levels.

A field study investigating bioaccumulation of pharmaceuticals and personal care products (PPCPs) by San Francisco Bay mussels detected 71 compounds using a combination of SPME, POCIS, and mussel tissue sampling. Diphenhydramine, an over-the-counter antihistamine drug, was the only compound found in both tissue and water samples, and bioaccumulation was observed (Alvarez et al., 2014). Compounds like DPH with a log Kow of greater than 3 are considered to have the potential to bioaccumulate due to their affinity for lipid tissues. As might be expected, many PPCPs have also been detected in sediments. A study conducted in Puget Sound, Washington, USA detected 14 pharmaceutical compounds in bay area sediments, with diphenhydramine and triclosan being the most commonly found (Long et al., 2013). PPCPs are classified by the USEPA as contaminants of emerging concern due to their release into surface waters, and further research on their effects and mitigation/prevention methods are forthcoming.
**MWCNT Binding of Pharmaceuticals**

Considering the prevalence of carbon ring structures in pharmaceutical compounds, it is unsurprising that many pharmaceuticals will bind readily to MWCNTs. The binding of hydrophobic organic compounds to MWCNTs is an area of intense study, as MWCNTs and other carbon nanomaterials show promise in remediation of contaminated water (Upadhyayula et al., 2009). Pseudo-first and second-order kinetic models seem to fit the observed adsorption/desorption rates of ring-containing compounds to MWCNTs, with very fast initial adsorption and a rate of desorption dependent upon a large variety of factors including nanotube functionalization, pH, temperature, pore volume, and compound structure (Bohdziewicz et al., 2013; Oleszczuk et al., 2009). In some cases, adsorptive equilibrium was reached in as little as 40 minutes to 6 hours (Liao et al., 2008; Peng et al., 2003), although more conservative experiments allow a mixing time of 5 days to ensure equilibrium is reached (Oleszczuk et al., 2011; Yang et al., 2006).

It remains uncertain whether MWCNT-adsorbed pharmaceutical compounds will remain bioavailable. Reports are contradictory, with some results showing desorption of organic compounds in the gut tract of exposed animals (Shen et al., 2014) and in simulated gastrointestinal environments (Z. Wang et al., 2011), and others finding that compounds were excreted without desorption (Linard et al., 2015). Wang et al. (date) showed that in the presence of bile salts and pepsin the solubility of CNT-adsorbed phenanthrene was increased three- to thirty-fold, which resulted in the release of 43 to 69% of the compound in simulated gut fluid.
It is known that a wide variety of pharmaceuticals bind readily to MWCNTs, and that these compounds may have negative health effects in fish and aquatic invertebrates. Additionally, NOM (such as is present in natural sediment) has a potentiating effect on MWCNT suspension, allowing nanotubes to remain stable in the water column long enough to adsorb cyclic organic contaminants present and potentially be consumed by aquatic life. Synthesizing these two ideas, a third question arises: can the presence of MWCNTs in a NOM-rich natural environment, by virtue of their adsorptive ability for pharmaceuticals, affect the levels of toxicity experienced by aquatic organisms? MWCNTs may deposit quickly into sediment and be subsumed by benthic mixing, rendering adsorbed compounds less available. Alternatively, MWCNTs may remain in suspension long enough to be consumed by organisms, due to their association with soluble NOM, increasing toxin exposure.

**MWCNTs, NOM, and Organic Contaminant Toxicity**

The ability of NOM to suspend MWCNTs along with the adsorptive properties of both compounds has led to an interest in MWCNT/NOM modulation of organic contaminant toxicity. The protective effects of MWCNTs in aqueous exposures has been documented. A study investigating coexposure of MWCNTs and triclocarban, an antibacterial agent, to the aquatic invertebrate *Daphnia magna* found that addition of 1 mg/L MWCNTs to exposures containing triclocarban reduced observed mortality significantly (Simon et al., 2015). The authors hypothesized that MWCNTs bound triclocarban through a mixture of pi-pi interactions, Van der Waals forces,
and ionic interactions, resulting in a high degree of adsorption that rendered triclocarban unavailable even when MWCNTs were ingested by *D. magna.* Shen et al (2014) conducted a study investigating the effect on polyaromatic hydrocarbon (PAH) accumulation due to sediment amendment with MWCNTs at a range of NOM concentrations. This study found a decrease of 63.8% to 96.8% in the biota-sediment accumulation factor (BSAF) in the aquatic worm *Chironomus plumosus* in MWCNT/NOM treatments, with addition of NOM contributing a significantly greater reduction than MWCNTs alone. They concluded that the combined adsorptive affinity of MWCNTs and NOM reduced the concentration of PAHs available in the sediment, which was supported by GC/MS verification of sediment PAH concentrations. However, in the same study, there was a nearly 1.5-log increase in water bioaccumulation factor (BAF, normalized to tissue lipid content) with the addition of MWCNTs to the sediment. The larvae, overall, accumulated more PAHs in the presence of MWCNT/NOM than they did in control sediments spiked with PAHs but without added MWCNTs and NOM. This discrepancy between BSAF and BAF reflects a difference in their calculation: the BSAF is the ratio of PAH found in tissue to PAH found in sediment, while BAF is the ratio of PAH in tissue to PAH found in water. Synthesizing these two measurements to explain the seemingly contradictory findings, it becomes apparent that MWCNTs had the effect of decreasing the amount of PAH available in the water while the larvae in MWCNT-amended sediments accumulated more PAHs overall, which increased their body burden and led to an increase in BAF. The authors suggested that this may be due to larvae ingesting PAH-laden MWCNTs from the sediment and
incorporating them into their lipid tissues, removing them from sequestration in sediments (Shen et al., 2014).

Only one study to date has examined the effects of MWCNT amendment on the toxicity of an adsorbed organic compound in fish. Linard et al. (2015) used a fluorescence microplate reader to quantify adsorbed and bioaccumulated fluoranthrene, a polycyclic aromatic compound, in the fathead minnow *P. promelas*. It is generally difficult to analyze adsorption/desorption kinetics of MWCNT-adsorbed compounds due to both the strong binding affinity of MWCNTs and their tendency to pass through even very fine filters and clog chromatograph columns. However, this study took advantage of fluoranthrene’s eponymous fluorescence at 280/440 nm to measure concentrations in both fish and water samples. Addition of 2 mg/L MWCNTs and 10 mg/L NOM to solutions of 1, 15, and 25 μg/L fluoranthrene resulted in a 60-90% reduction in bioaccumulation of fluoranthrene. Also of note, fluorescence examination of the sacrificed fish revealed that fluoranthrene-containing MWCNTs were present in the gut tract of all samples, but fluoranthrene was present only at greatly reduced concentrations in gallbladder fluids. This indicates that the gut environment of *P. promelas* was insufficient to desorb the polyaromatic compound from the MWCNTs (Linard et al., 2015).

**Diphenhydramine**

In order to investigate the question of pharmaceutical toxicity in the presence of sediment and MWCNTs, the antihistamine compound diphenhydramine was chosen as a model. Diphenhydramine is commonly sold over-the-counter as
Benadryl® in the United States, and as Unisom®, Dytuss®, Dramamine®, or many other brand names worldwide. It is an H-1 receptor antagonist that works by blocking the effects of histamine, and is used to treat seasonal allergies, cough, insomnia, and motion sickness (Couper et al., 2014). Diphenhydramine is classified pharmacologically as an ethanolamine ether, and contains an amine group at one terminus and two phenyl groups at the opposite. These two aromatic rings offer a pair of binding sites for MWCNTs and make diphenhydramine a good candidate for a nanotube adsorption study. Additionally, with log $K_{ow}$ and $K_{oc}$ values of 3.27 and 3.80 respectively, diphenhydramine has a moderately high affinity for lipids and a high affinity for organic carbon. Due to its terminal amine group, diphenhydramine is a weak base and has a pKA of 8.98. In basic conditions, it will exist almost entirely in neutral form (i.e. the terminal amine will exist as NH$_3$ rather than NH$_4^+$). Conversely, in neutral to acidic conditions diphenhydramine will remain ionized, increasing its solubility in water. The neutral form of diphenhydramine is the more toxic (Berninger et al., 2011). Because of high $K_{oc}$ and potential for cation-exchange and covalent based interactions with sediments and NOM (Weber et al., 2001), diphenhydramine will likely adsorb to NOM and other organic components in sediment at environmental pH.

Diphenhydramine has been detected in surface waters that receive wastewater treatment plant effluent, likely due to its near-ubiquitous use as an over-the-counter allergy medicine. A 2006 study of pharmaceutical concentrations downstream of wastewater treatment plants (Reif et al., 2012) detected diphenhydramine at all locations studied, with concentrations ranging from 7 – 135
ng/L. Diphenhydramine was also detected in all influent and effluent samples in a recent study conducted at the Baylor Wastewater Research Program site in Waco, Texas, with influent concentrations ranging from 160 – 600 ng/L (Du et al., 2014). In the latter study, diphenhydramine removal efficiency using municipal activated sludge technology ranged from 68-69%, with the remaining DPH was released unchanged. Diphenhydramine has also been detected in ocean sediments. A recent study found DPH in 87.5% of samples, with mean concentrations of 1.68 ng/g wet weight (Long et al., 2013). Additionally, diphenhydramine has been found to be persistent in sediments, with no significant change in concentration during a 70-day dissipation study under anaerobic conditions (C. Wu et al., 2010).

There is a wide range of environmental toxicity data available for diphenhydramine, ranging from human and mammalian studies to fish, invertebrates, and microfauna. A recent study (Berninger et al., 2011) examined acute and subchronic endpoints for diphenhydramine exposure to a range of model organisms: the fathead minnow, *Pimephales promelas*, a freshwater microcrustacean, *Daphnia magna*, and in duckweed, *Lemna gibba*. Acute studies were conducted using standard USEPA methods over a time span of 48 hours. *P. promelas* experienced an LC50 of 2.09 mg/L at pH 8.5 and reduced toxicity (LC50 = 59.28 mg/L) at pH 6.5, indicating a strong correlation between ionization ratio and diphenhydramine toxicity. Monitoring pH during every step of toxicity testing involving diphenhydramine is therefore extremely important, as estimates of toxicity may need to be adjusted to account for differences in dose-response relationship at differing ionization ratios. *D. magna* was far more sensitive to
diphenhydramine, with an LC50 of 0.37 mg/L at pH 8.5. The plant *L. gibba* showed no response to even the highest doses of diphenhydramine.

Subchronic toxicity occurred at far lower concentrations of diphenhydramine, especially to *D. magna*. Reproductive toxicity was assessed by measuring the concentration at which there was a statistically significant reduction in the number of neonates produced. In a 10-day study, the LOEC and NOEC values for reproductive inhibition were 3.4 and 0.8 μg/L respectively. For *P. promelas*, subchronic endpoints were survival, growth inhibition, and feeding rate, measured by counting the number of brine shrimp nauplii eaten by three randomly selected fish over a course of 15 minutes. The LOEC for survival was 836.7 μg/L, while corresponding values for growth and feeding rate were 49.1 and 5.6 μg/L respectively.

If diphenhydramine bioconcentrates in animal tissue (and it may, as indicated by its high K<sub>ow</sub> value) these toxic concentrations may be attained over long-term exposure at environmentally relevant levels. A study investigating detection of PPCPs in birds and fish from effluent-dominated streams in Japan found that diphenhydramine had a mean brain-to-plasma concentration ratio in fish of 12, indicating that it concentrates in the brain of exposed organisms. The authors hypothesized that the brain's high proportion of lipids was responsible for the preferential concentration (Tanoue et al., 2014).

Further highlighting the need for strict pH control when testing ionizable compounds, a recent study of bioconcentration of diphenhydramine in fathead minnows found that whole-body bioconcentration factor (BCF) was highly
influenced by changes in pH across a conservative range of values from 6.7 to 8.7. The observed changes in BCF were dramatic, with a range of 4.2 at pH 6.7 to 53.3 at pH 8.7. By contrast, whole body to plasma concentration ratios remained constant, around 3.0, for all pH values tested. This suggests that despite changes in diphenhydramine's ionization in the surrounding environment, speciation within the organism was unchanged. A model was fitted, taking into account localized acidification at the fish gill surface, and found that the relationship between pH and BCF was nonlinear. Toxicity was not assessed, however the authors hypothesized that diphenhydramine would be more toxic at higher pH, a conclusion that is supported by similar research (Nichols et al., 2015).
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CHAPTER 2

MULTI-WALLED CARBON NANOTUBES REDUCE TOXICITY OF DIPHENHYDRAMINE

TO CERIODAPHNIA DUBIA IN WATER AND SEDIMENT EXPOSURES

\[^1\]Myer, M.H. and M.C. Black. To be submitted to *Environmental Toxicology and Chemistry.*
Abstract

Sediment elutriate exposures were undertaken with the freshwater crustacean *Ceriodaphnia dubia* to compare the toxic effects of diphenhydramine in the presence and absence of sediment and multi-walled carbon nanotubes. In both sediment and solution-only treatments, addition of 0.318 mg/g of functionalized multi-walled carbon nanotubes significantly decreased overall 48-hour mortality relative to the positive control (p < 0.01), with a 78.7-90.1% reduction in treatments with nanotube-amended sediment and 40.7-53.3% reduction in nanotube-amended water exposures.

Introduction

Multi-walled carbon nanotubes (MWCNTs) are a class of carbon nanomaterial that has seen a recent increase in interest for applications in a wide variety of fields. They are comprised of overlapping planar sheets of single atom thick six-membered carbon rings, which “roll” along their long axis to form a multilayered tube. MWCNTs have extraordinary properties in comparison to elemental carbon, including an extreme surface area to volume ratio, thermal and electrical conductivity greater than that of silicon or diamond, and a strength-to-weight ratio greater than steel (Baughman et al., 2002). These properties make them the focus of intense scrutiny in the area of research and development, where they are used in preparing highly corrosion and impact resistant coatings and composite materials, high-capacitance electronics, and biosensors among many other applications (De Volder et al., 2013). With the increased use and production of
MWCNTs, it is imperative that their impact on the health of humans, animals, and ecosystems is evaluated. The environmental crises of the early 20th century provide a stark warning of the folly of determining chemical toxicity on a post-hoc basis, and as such research on the potential environmental impact of MWCNTs is progressing rapidly.

Current estimates of MWCNT concentrations in North American surface waters range from 6.6 - 18 ng/L. However, as they are denser than water and are unstable in aqueous solution, sediment is the ultimate compartment for MWCNT deposition, with modeled deposition rates between 40 – 229 ng/kg yearly (Gottschalk et al., 2009). Natural organic matter (NOM), a component of surface waters resulting from the decomposition of plants and animals, has the ability to suspend MWCNTs in aqueous solution (Hyung et al., 2007). The high surface area, sp² surface hybridization, and hydrophobic character of MWCNTs make them highly adsorptive to organic contaminants, especially those containing carbon ring structures (Saikia et al., 2013). This has raised concern that MWCNTs may adsorb and transport co-occurring organic contaminants within aquatic environments.

Although the combined toxicity of nanomaterials and organic contaminants is a relatively young science, some studies have examined organic contaminant uptake in the presence of MWCNTs. A 21-day chronic exposure found that addition of 1 mg/L MWCNTs to exposure solutions of triclocarban reduced mortality in Daphnia magna (Simon et al., 2015). A coexposure with MWCNTs and two polycyclic aromatic hydrocarbons (PAHs) with the sediment-dwelling invertebrate Chironomus plumosus found that addition of MWCNTs to sediment reduced free
concentrations of both PAHs, yet increased their bioaccumulation factors (Shen et al., 2014). Exposures of earthworms to pyrene in MWCNT-amended soil resulted in a significantly increased rate of pyrene elimination compared to non-amended soil, with body burdens of pyrene significantly reduced by the addition of MWCNTs at 6 days (Petersen et al., 2009).

In order to investigate the effects of natural sediment on the combined toxicity of MWCNTs and organic contaminants, this study used the common antihistamine medication diphenhydramine and acid-functionalized MWCNTs to conduct 48-hour acute toxicity exposures with the aquatic invertebrate Ceriodaphnia dubia. Sediment-elutriate solutions were prepared by mixing MWCNTs, sediment, and diphenhydramine solution, with exposure occurring after removal of the solid phase. Two different types of sediment were used in order to determine whether sediment composition and NOM content had an effect upon diphenhydramine-MWCNT binding. Comparisons of mortality were used as a surrogate measure to investigate the amount of diphenhydramine removed from the aqueous system by the addition of MWCNTs. We hypothesize that addition of MWCNTs and sediment will reduce the observed toxicity of DPH to C. dubia by rendering DPH unavailable for uptake.

**Materials and Methods**

*Experimental Animals*

Neonatal C. dubia were obtained from an in-house culture, and were used at less than 24 hours of age. Cultures were maintained in 30 mL plastic cups containing
15 mL moderately hard water prepared according to the US Environmental Protection Agency (EPA) method for the preparation of synthetic water (USEPA, 2004). Cups were arrayed in a 6 row x 10 column configuration across polyurethane foam boards. Each cup contained one adult *C. dubia*. Culture boards were kept in a Percival Scientific I-36VL incubator at 25°C with a 16 hours light/8 hours dark photoperiod. Water changes and feeding of 0.1 mL each algae (*Psuedokirschneriella subcapitata*, $3 \times 10^7$ cells/mL) and YTC (yeast/cerophyll/digested trout chow (Aquatic Biosystems, Ft. Collins, CO) were conducted daily, along with a daily count of neonates. After the third round of reproduction, adult *C. dubia* were discarded and the <24-hour old third brood neonates were used for toxicity testing.

**Chemicals and Reagents**

Diphenhydramine HCl was purchased at >98% purity from Sigma-Aldrich. Suwannee River natural organic matter (NOM) was purchased from the International Humic Substances Society and is certified to contain at least 52.63% carbon by mass. Multi-walled carbon nanotubes were purchased in pristine form from Cheap Tubes Inc. (Cambridgeport, VT). The MWCNTs had an outer diameter of 30-50 nm, length of 10-20 μm, and were characterized as >95% purity, containing <1.5% fly ash. The MWCNTs were functionalized by acid carboxylation, using a modification of a method known to induce nanotube surface functionalization (Liu et al., 1998) and described briefly as follows. One gram of pristine MWCNTs was added to 100 mL of concentrated trace metal grade H$_2$SO$_4$/HNO$_3$ (aqua regia) prepared in a 3:1 ratio. The resulting slurry was sonicated for 20 minutes in a
Branson B5510-MT bath sonicator, then transferred to a fume hood and filtered through a 0.45 μm pore size Millipore PTFE LCR membrane in a 1:10 ratio of slurry to boiling Milli-Q water. The semi-dry cake of functionalized MWCNTs was transferred to a clean watch glass and cut into small pieces using a stainless steel scoop, then dried at 100°C for 8 hours. The dried MWCNTs were ground to a fine powder in a porcelain mortar and weighed before transfer to an acid-washed glass scintillation vial.

*Sediment*

All sediment used in this study was collected from a pond at the United States Department of Agriculture J. Phil Campbell Sr. Natural Resource Conservation Center in Watkinsville, Georgia, USA (33°52'14" N 83°25'31" W). Sediment was collected at a minimum of 3 meters distance from the shore of the pond, to avoid leaves and other debris. After collection by shovel, sediment was sieved (0.5 mm stainless steel sieve) to remove gravel and debris and placed in an acid-washed 10-gallon plastic bucket. Each sediment was aged under refrigeration at 4°C for 28 days. Physical component characterization of the two sediment samples revealed that they differed significantly in composition (Table 1).

*Acute Toxicity Assays*

To investigate the effects of MWCNT addition to sediment on the toxicity of diphenhydramine to *C. dubia*, 48-hour acute toxicity tests were performed. Before selecting a concentration of diphenhydramine for sediment exposures, three 48-
hour acute toxicity experiments were conducted to determine the sensitivity of in-house culture *C. dubia* to DPH. All 48-hour acute toxicity assays were conducted according to US EPA standard *C. dubia* testing protocols (USEPA, 2004), with a range of diphenhydramine concentrations from 0.5 – 4 mg/L. The initial 48-hour acute exposures resulted in an observed LC50 for diphenhydramine in *C. dubia* of 1.81 ± 0.34 mg/L (95% CI). A fitted log-logistic curve (Figure 1) was used to estimate the LC90, which was chosen as the experimental concentration of diphenhydramine for sediment/MWCNT exposures to ensure that most, but not all of the positive control animals would experience mortality, and that sediment and MWCNT treatments would not reduce mortality rates entirely to zero.

For MWCNT/sediment/DPH exposures, five treatment mixtures were prepared in acid-washed 250 mL amber glass bottles. Treatment mixtures consisted of MHW as a control, 3 mg/L diphenhydramine, 3 mg/L diphenhydramine and 318 μg/g MWCNTs, 3 mg/L diphenhydramine and 25 g sediment, and 3 mg/L diphenhydramine plus 318 μg/g MWCNTs and 25 g sediment. The bottles were then placed on a rocker and mixed for 5 days in the dark to discourage photodegradation of DPH. After equilibration on the rocker, bottles were removed and each solution was filtered through a Whatman #1 qualitative-grade 11 μm pore size filter to remove MWCNTs and sediment from suspension (Shen et al. 2014). Remaining suspended solids were removed by centrifugation at 2500 RPM for 20 minutes. The supernatant (elutriate) was pipetted using a clean glass pipet into an acid-washed 125 mL Erlenmeyer flask and wrapped with aluminum foil to exclude light. By filtering and centrifuging all elutriate solutions, diphenhydramine adsorbed to sediment or MWCNTs was removed prior to *C.
*dubia* exposure. Forty-eight hour acute toxicity exposures were conducted in a Percival Scientific I-36VL incubator at 25°C with a 16:8 light/dark photoperiod. For each of the five treatments, three 30 mL plastic cups were placed on a polyurethane foam board, for a total of three replicates per treatment and fifteen cups per test. Treatment solutions (15 mL) were pipetted into each cup using a sterile disposable repipetter. Five neonatal *C. dubia* were randomly selected from the stock culture and placed into each cup. Food was not provided for the duration of the test. At 24 and 48 hours, mortality was recorded for each cup. The test was repeated seven times for each sediment type to ensure reproducibility and statistical power.

**Statistical Analysis**

The dose-response curve for the initial 48-hour acute exposures was fitted using CurveExpert Professional 2.2.0 2013. A log-logistic regression was applied upon initial examination of the data in keeping with standard methods for determining dose-response relationships. Goodness-of-fit was assessed with the correlation coefficient, $r^2$. For all comparisons, homogeneity of variance was assessed using Bartlett’s test of homogeneity, and normality was assessed using the Shapiro-Wilk test for all comparisons. Comparisons between treatments within each sediment type and between similar treatments among sediment types were conducted with one-way ANOVA, using Rstudio 0.97.551. Significance for all tests was set at $p = 0.05$. 
Results

Addition of MWCNTs to water without sediment reduced acute mortality by 53.3% (ANOVA, p<0.001, n = 7) in Experiment 1 (Figure 2a) and by 40.7% (ANOVA, p<0.001, n=7) in Experiment 2 (Figure 2b). The inclusion of sediment 1, which had a higher percent organic matter content and is classified as sandy clay loam, reduced acute mortality by 68.6% (ANOVA, p<0.001, n = 7). Mortality was reduced by 69.3% (ANOVA, p<0.001, n = 7) using sediment 2, which was lower in organic matter and is classified as sand. There were no statistically significant differences in mortality reduction between the two different types of sediment (ANOVA, p=0.136, n=7). Addition of MWCNTs to sediment treatments reduced acute mortality exposures in Sediment 1 exposures by 90.1% (ANOVA, p<0.001, n = 7) relative to the DPH-only treatment, and by 78.7% relative to the DPH-only treatment (ANOVA, p<0.001, n = 7) with Sediment 2. Comparing sediment treatments without MWCNTs to sediment with the addition of MWCNTs, mortality was reduced by 10.4% (ANOVA, p=0.005, n=7) with Sediment 1 and by 9.4% in sediment 2, although with this sediment type the change in mortality was not statistically significant.

Discussion

The results of this study demonstrate that MWCNTs are effective in reducing diphenhydramine exposures in a model aquatic invertebrate, both in water exposures and in the presence of sediment. The greatest degree of reduction in DPH toxicity occurred in exposures with MWCNTs in water with sediment not present.
This is consistent with other studies that have shown that organic contaminants readily adsorb to MWCNTs in an aqueous environment (Liao et al., 2008; Lin et al., 2010; Linard et al., 2015). While the degree of toxicity reduction was statistically significant for exposures with Sediment 1, which was classified as sandy clay loam and had a higher percent organic matter, the combined effect of MWCNTs and sediment was less pronounced and not statistically significant for Sediment 2, which had less than half the organic matter and was comprised mainly of sand. This may be due to high response variability in the sand-dominated sediment exposures without MWCNT, resulting in reduced statistical power. Although sediment was blended thoroughly before dispensing into exposure vessels, there may have been individual variation in NOM content among sediment aliquots. Additionally, the dose-response curve for diphenhydramine is logistic in shape, and consequently additional reduction in available diphenhydramine concentration has a diminishing effect upon observed mortality as it approaches control values. Considering that the sand-dominated sediment contained approximately half as much organic carbon by mass than the sandy clay loam, the discrepancy in DPH toxicity reduction may be attributed to the combinatory effects of MWCNTs and organic carbon in sediment, which in aquatic bodies is present as NOM. Studies have shown that the presence of NOM increases the ability of MWCNTs to form a colloidal aqueous suspension (Hyung et al., 2007), and can increase the ability of MWNTs to adsorb organic contaminants, although this effect is highly dependent upon nanotube pore size (Zhang et al., 2011). Others reported that the presence of NOM can modify the toxicity of MWCNTs and co-occurring contaminants such as PAHs (Shen et al.,
These results indicate that sediment NOM increased the ability of MWCNTs to adsorb DPH, reducing mortality. Concentrations of MWCNTs used in this study are unlikely to occur in the environment, barring a point exposure such as a container spill. However, the results do indicate that MWCNTs have the potential to adsorb organic compounds, especially in the presence of natural NOM found in sediments, and provide evidence that NOM and MWCNTs have an additive effect upon reduction in observed diphenhydramine mortality in aqueous exposures.

**Conclusions**

Addition of MWCNTs was shown to reduce the toxicity of a model pharmaceutical compound to *Ceriodaphnia dubia* in sediment-elutriate toxicity assays. As both trace pharmaceuticals and carbon nanotubes are compounds of emerging concern, it is encouraging that the release of MWCNTs may have an unintentional protective effect upon pharmaceutical toxicity to pelagic organisms in the environment. The degree of protection may be dependent upon individual contaminant characteristics and how they interact with sediment composition and concentration of organic matter in sediment, among other characteristics such as pH and suspended solids. Further, by showing that MWCNTs show significant adsorption capacity for a commonly-detected pharmaceutical compound, these results indicate that MWCNTs have potential for use in water filtration systems for the removal of pharmaceuticals, which are typically not well-removed by traditional wastewater treatment systems (Upadhyayula et al., 2009).
Future work will focus on quantifying reduction in bioavailable diphenhydramine using LC/MS-MS to determine its distribution in water, sediment, and biota pre- and post-addition of MWCNTs in longer-term exposures conducted using model fish species. Further investigation into applying MWCNT filtration to remediation of wastewater and pharmaceutical-contaminated surface waters should be conducted, with the aim of engineering technologies that will allow safe removal and disposal of contaminants without the risk of MWCNT release into the environment.

Acknowledgements

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Figure 2.1. Log-logistic fitted dose-response curve for diphenhydramine acute toxicity. n=3 exposures were used to create this curve, with three averaged replicates at each concentration per exposure. $r^2 = 0.997$. 
Figure 2.2. C. dubia mortality in acute (48h) elutriate exposures with (A) sediment 1 (sandy clay loam, high %OC). and (B) sediment 2 (Sand, low %OC). Error bars represent 95% CI. Differences between groups denoted by different letters were determined by Tukey’s HSD (* = p<0.05, ** = p<0.01)
Table 1. Characteristics of Sediment

<table>
<thead>
<tr>
<th></th>
<th>Equiv. water pH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Organic Matter&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Sand</th>
<th>% Silt</th>
<th>% Clay</th>
<th>Soil Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment 1</td>
<td>6.70</td>
<td>4.00</td>
<td>63.9</td>
<td>16.0</td>
<td>20.1</td>
<td>Sandy Clay Loam</td>
</tr>
<tr>
<td>Sediment 2</td>
<td>5.24</td>
<td>1.90</td>
<td>94.0</td>
<td>4.0</td>
<td>2.0</td>
<td>Sand</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sediment characterization was performed at the University of Georgia Soil, Plant and Water Analysis Lab.<br><sup>b</sup>pH was measured in a 1:1 ratio of soil to 0.01M CaCl<sub>2</sub> with subsequent correction for equivalent pH in pure water.<br><sup>c</sup>Measured via loss on ignition for 3 hours at 360°C, expressed as % by weight.
References


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CHAPTER 3
EFFECTS OF MULTI-WALLED CARBON NANOTUBES ON THE BIOAVAILABILITY AND TOXICITY OF DIPHENHYDRAMINE TO P. PROMELAS IN SEDIMENT EXPOSURES

1Myer, M.H., W.M. Henderson, and M.C. Black. To be submitted to Environmental Toxicology and Chemistry.
Abstract

Multi-walled carbon nanotubes (MWCNTs) and pharmaceutical compounds are classified by the US Environmental Protection Agency as contaminants of emerging concern, with significant research devoted to determining their potential environmental and toxicological effects. MWCNTs are known to have a high adsorptive capacity for organic contaminants, and there is concern that coexposure with MWCNTs may alter the bioavailability of trace organic compounds. Extant studies investigating MWCNT/organic contaminant coexposures have had conflicting results, and no study to date has examined the combined effects of MWCNTs and a common pharmaceutical. In this study, juvenile fathead minnows (P. promelas) were exposed to sublethal concentrations of the over-the-counter antihistamine diphenhydramine (DPH) in the presence of natural sediment for 10 days, with some treatments receiving MWCNTs. Addition of MWCNTs did not have a protective effect upon DPH-related growth inhibition, and did not reduce the whole-body burden of DPH in exposed fish. Mass-balance calculations indicated that significant amounts of DPH were adsorbed to MWCNTs, and DPH concentrations in water and sediment were commensurately reduced. Bioconcentration factor and biota-sediment accumulation factor increased in the presence of MWCNTs, indicating that P. promelas accumulates DPH adsorbed to MWCNTs in sediment, likely by coingestion of MWCNTs during feeding from the sediment surface.
Introduction

Multi-walled carbon nanotubes (MWCNTs) are contaminants of emerging concern, with uses in high-tech industries including biotechnology, materials science, and medicine (De Volder et al., 2013; Upadhyayula et al., 2009). They are composed of overlapping sheets of graphene “rolled” along their long axis, with carbon atoms arranged in a repeating pattern of six-membered rings. Due to an extremely high surface area to volume ratio and novel electrochemical properties relating to their repeating ring structure, MWCNTs are a target of intense scrutiny for nanoscience and nanotechnology applications. In particular, MWCNTs are known to have an adsorptive capacity for cyclic compounds far in excess of traditional sorbents such as activated carbon or diatomaceous earth (Oleszczuk et al., 2009). As they are adopted for increasing use in consumer products and materials, there is potential for MWCNTs to enter the environment through both intentional disposal and accidental discharge. Because MWCNTs are more dense than water and unstable in aqueous suspension, sediments are thought to be the ultimate compartment for their deposition. The most recent MWCNT fate models predict that current water concentrations are between 6.6 – 18 ng/L, with an annual sediment deposition rate of 40 – 229 ng/kg in the United States (Gottschalk et al. 2008).

Multi walled carbon nanotubes are highly sorptive towards organic compounds, and their adsorptive capacity is potentiated in the presence of natural organic matter (NOM) found in sediments. Natural organic matter is a complex macromolecule comprised mainly of humic and fulvic acids, containing a panoply of
functional groups including polyphenols, ketones, carboxylic acids, alcohols, aromatic rings, and methoxyl groups (Chen et al., 2002). NOM has been shown to increase the ability of MWCNTs to form a stable colloid-like suspension in water, and is the primary mediator of sediment-MWCNT interaction (Hyung et al., 2007). The increased stability of NOM-MWCNT colloids increases the ability of MWCNTs to adsorb both organic compounds and inorganic ions including PAHs, metals, and pharmaceuticals (Oleszczuk et al., 2009; Yang et al., 2006; Zhang et al., 2011). This has raised concerns that MWCNTs may transport or increase the bioavailability of contaminants in an aquatic environment.

Pharmaceutical compounds are recognized as contaminants of emerging concern in the United States, with hundreds of compounds detected in wastewater-receiving surface waters and sediments (Alvarez et al., 2014; Long et al., 2013; Reif et al., 2012). Traditional wastewater treatment methods including trickling-filter and activated sludge processes do not adequately remove excreted pharmaceuticals (Du et al., 2014), and while more effective technologies such as UV ozonolysis are available, they are expensive and typically limited in application to pharmaceutical production and industrial effluents (Deegan et al., 2011). While detected concentrations of pharmaceuticals are typically well below the threshold for an effective acute dose, bioaccumulation and biomagnification may allow organisms to build up effective concentrations within their bodies. MWCNTs may exacerbate this problem by concentrating organic compounds on their surface, potentially exposing organisms that ingest or respire water containing MWCNTs. Shen et al. (2013) found that addition of MWCNTs to sediment decreased observed sediment and
water concentrations of polycyclic aromatic hydrocarbon (PAH) compounds, yet led to a nearly hundred-fold increase in bioaccumulation factor in the sediment-dwelling worm *Lumbriculus plumosus*. Conversely, Linard et al. (2015) found that addition of MWCNTs to aquatic sediment reduced body burdens of the PAH fluoranthrene, in Japanese medaka (*Oryzias latipes*), sequestering it in sediment and rendering it unavailable. These results demonstrate the importance of considering routes of exposure in evaluating potential toxicity of nanotube-bound contaminants. When organisms have access to and interact with sediment, there is potential for uptake of nanotubes and their adsorbed compounds. There is a need for additional research on the combinatorial effects of MWCNTs and organic contaminants under natural environmental conditions, so that the potential risk can be understood before MWCNT contamination becomes widespread.

In this experiment, we investigated the effect that deposition of MWCNTs onto sediment may have on the bioavailability and adverse health effects of pharmaceuticals in fish, using diphenhydramine as a model. Diphenhydramine (DPH) is sold over-the-counter as Benadryl® in the United States, and is a commonly used antihistamine and sleep aid. It can be found in surface waters, sediments, and wastewater effluent with near-ubiquity (Du et al., 2014; Long et al., 2013; Reif et al., 2012). DPH was chosen as a model pharmaceutical compound due to its high frequency of detection in environmental samples, its relatively well-studied toxicity to fish used in standard toxicity testing, and because it contains two phenyl groups as well as a charged terminal amine, rendering it a prime candidate for adsorption to both MWCNTs and NOM. The model freshwater test fish
Pimephales promelas (fathead minnow) was used for this study, as relatively detailed information is available on its sensitivity to DPH. In subchronic exposures to fathead minnows, DPH has been shown to cause growth inhibition at a LOEC of 49.1 μg/L, and mortality at an LC50 of 59.28 mg/L (Berninger et al., 2011).

We hypothesized that MWCNTs would, by adsorbing DPH from water and sediment and reducing bioavailability, decrease the exposure and subsequent body burden of DPH in exposed fish, and concurrently reduce toxic effects associated with diphenhydramine exposure.

**Materials and Methods**

*Experimental Animals*

Fathead minnows (*P. promelas*) were obtained from in-house culture at the University of Georgia Aquaponics Research Laboratory. Eggs were collected daily from breeding tanks and incubated in 250 mL glass beakers until hatching at 25°C with gentle aeration, with a 16:8 light/dark photoperiod. Hatched fry were transferred to 60-liter glass tanks. Holding water and all water used in subsequent exposures conformed to United States Environmental Protection Agency standards for Moderately Hard Synthetic Water (USEPA, 2004). Larvae were fed 5 mL neonatal brine shrimp twice daily. Daily monitoring of pH, temperature, and free ammonia nitrogen was conducted. Cleaning and 50% water renewal was conducted once weekly to reduce ammonia buildup. At three weeks of age (21 days), six hundred fish were transferred to the University of Georgia Aquatic Organism Lab and acclimated for three days prior to being randomly allocated to treatment jars. All
Animal Use Protocols (AUP) involving *P. promelas* were approved by the University of Georgia Institutional Animal Care and Use Committee (permit no. A2014 04-014-Y1-A0).

*Chemicals and Reagents*

Diphenhydramine HCl was purchased at >98% purity from Sigma-Aldrich. Suwannee River NOM was purchased from the International Humic Substances Society, and is certified to contain at least 52.63% carbon by mass. Multi-walled carbon nanotubes were purchased in pristine form from Cheap Tubes, Inc. (www.cheaptubes.com). The MWCNTs had an outer diameter of 30-50 nm, length of 10-20 μm, and were obtained at >95% purity, containing <1.5% fly ash. MWCNTs were functionalized by acid carboxylation, using a modification of a method known to induce nanotube surface functionalization (Liu et al., 1998). Pristine MWCNTs (1.0g) were added to 100 mL of concentrated trace metal grade \( \text{H}_2\text{SO}_4/\text{HNO}_3 \) (aqua regia) prepared in a 3:1 ratio. The resulting slurry was sonicated for 20 minutes in a Branson B5510-MT bath sonicator, then transferred to a fume hood and filtered through a 0.45 μm pore size Millipore PTFE LCR membrane to remove acid and associated metal impurities, in a 1:10 ratio of slurry to boiling Milli-Q water. The semi-dry functionalized MWCNTs were then transferred to a clean watch glass and cut into small pieces using a stainless steel scoop and dried at 100°C for 8 hours. The dried MWCNTs were then ground to a fine powder in a porcelain mortar and transferred to an acid-washed glass scintillation vial.
Sediment

All sediment used in this study was collected from a pond at the United States Department of Agriculture J. Phil Campbell Sr. Natural Resource Conservation Center in Watkinsville, Georgia, USA (33°52’14” N 83°25’31” W). Sediment was collected at a minimum of 3 meters distance from the shore of the pond, to avoid leaves and other debris. After collection, sediment was sieved (0.5 mm stainless steel sieve) to remove gravel and debris and placed in an acid-washed 10 gallon plastic bucket. The sediment was aged under refrigeration at 4°C for 28 days. The sediment used in this study had an organic material content of 2.70%, measured by loss on ignition for 3 hours at 360°C. The water-equivalent pH was 5.03, determined by measurement in a 1:1 ratio of dried sediment to 0.01 M CaCl₂ with subsequent correction for equivalent pH in pure water (Kissel et al., 2009). Mechanical analysis determined that the sediment was characterized as loamy sand and was comprised of 83.8% sand, 6.2% silt, and 9.9% clay.

Experimental Design

Ten-day exposures with fathead minnows were conducted in 3.7-L acid-washed glass jars containing sediment (±MWCNTs) and overlying water (±DPH) at a 4:1 ratio of water to sediment. Four treatments were prepared: a control containing MHW and sediment, a MWCNT positive control containing MHW and sediments amended with MWCNTs, a DPH treatment, containing DPH dissolved in MHW and sediment; and a DPH-MWCNT treatment, containing DPH dissolved in MHW and sediment amended with MWCNTs. The MHW control functioned as both a mortality
control and as a benchmark against which to compare morphological endpoints. The MWCNT positive control served to determine whether MWCNTs alone had an effect upon mortality or morphological endpoints. Five exposure vessels were prepared for each treatment, for a total of twenty jars. All jars received 500 g of wet premixed sediment, and 2 liters of overlying solution. Treatments with DPH received 500 μg/L diphenhydramine dissolved in MHW, a concentration known to cause growth inhibition in *P. promelas* at our experimental pH (Berninger et al., 2011). Treatments with MWCNTs received sediment containing 318 μg/g acid-carboxylated MWCNTs. One additional jar received only 2 L of 500 μg/L diphenhydramine in MHW with no sediment or MWCNTs, designated as a diphenhydramine blank for use in correcting for percent recovery. The locations of the jars in the incubator were randomized by randomly labeling each jar with a number from 1 to 27 and placing them on a numbered 3x3 grid assigned to each of the three incubator shelves. Jars were not moved for the duration of the exposure. Jars with sediment and test solutions were acclimated for 24 hours to allow settling of sediment and partitioning of diphenhydramine between water and sediment fractions. Once settling was complete, water samples were taken from each jar, then thirty fish were added to each jar at random by netting from a common source. Nets were washed with acetone and rinsed well with Milli-Q water after each transfer to avoid cross-contamination of diphenhydramine.

Throughout the exposure, vessels were maintained at 25°C with a 16h light/8h dark photoperiod in a Percival Scientific I-36VL incubator with gentle aeration to keep dissolved oxygen above 5 ppm. Fish in each jar received 2 mL of
neonatal brine shrimp twice daily, and daily measurements were taken of pH, water temperature, and total ammonia nitrogen. The pH of overlying test solutions was maintained at a range of 6.0-7.0 (Supplemental Figure 3.1), temperature was maintained at a range of 22-25°C, and TAN did not exceed 2 ppm at any time point.

**Sampling Protocol**

After 1, 4, 7, and 10 days of exposure, five fish were sampled from each jar by netting and euthanized in 25 mL glass beakers containing 15 mL of buffered MS-222 (tricaine methanesulfonate) solution in MHW (1 g/L, pH 7.0 – 7.5). Fish were measured from tail to snout with an acid and acetone-washed stainless steel digital caliper. Each fish was weighed while wet, then dried for 12 h at 60°C. Dried fish were weighed on a pre-dried and weighed aluminum weigh boat, placed in 20 mL acid-washed glass scintillation vials, and stored at -20°C for subsequent LC-MS determination of whole body DPH burden. At 1 and 10 days, water samples were taken via pipet and stored in 20 mL glass scintillation vials at -20°C, and at 10 days, the top 1 cm of sediment was collected from each jar using an acid-washed stainless steel spatula and stored in 250 mL amber jars at -20°C.

**Determination of MWCNTs in water**

A Shimadzu UV-1601 UV-Visible spectrophotometer was used in photometric mode at 800 nm to prepare a calibration curve for a range of MWCNT concentrations from 0.049 to 100 mg/L dispersed in a solution of 10 mg/L natural organic matter in MHW as a substitute for organic matter present in sediment (Supplemental Figure
3.2). During the experiment MWCNT concentrations in overlying solutions were determined by measuring the absorbance at 800 nm of 1 mL water samples collected daily from each jar. This measurement was compared to a previously-prepared calibration curve to determine the concentration of MWCNTs remaining suspended in the water column, with recalibration using a MHW blank after each sample (Linard et al., 2015). Due to the relatively low organic matter content of the sediment used in the experiment, and the lack of sediment agitation in the exposure, MWCNTs were not expected to remain suspended in the water column during the exposure period.

Sample Analysis

Diphenhydramine was extracted from all samples with Solid Phase Extraction (SPE) using procedures modified from the United States Environmental Protection Agency Method 1694 (USEPA, 2007).

Water Extraction. Water samples were centrifuged for 20 minutes at 2500 RPM to remove suspended solids including MWCNTs (Linard et al., 2015), and the supernatant was pipetted off into a clean glass test tube. Sep-Pak HLB SPE cartridges were then conditioned with 4 mL of 100% methanol followed by 4 mL of Milli-Q water on a vacuum manifold. Water samples were then passed through the conditioned SPE cartridges at a rate of 1 mL/min. Cartridges were eluted with 6 mL of 100% methanol, and the resulting solution was blown dry under ultrapure nitrogen gas. The dried concentrate was reconstituted in 1 mL of 10% acetonitrile for LC/MS analysis. The DPH concentrations were corrected for percent recovery by
comparison with a diphenhydramine treatment (containing no fish or sediment) held in the same conditions as all exposure vessels, and sampled concurrently. As MWCNTs were removed via centrifugation prior to sample elution, analyzed concentrations did not include DPH sorbed to MWCNTs; this was intentional and intended to allow estimation of MWCNT-sorbed DPH by calculating a mass-balance for all other compartments (water, sediment, and biota) and subtracting the known DPH mass from a measured initial concentration.

*Sediment Extraction.* Sediment samples (10 g) were placed into 50 mL plastic centrifuge vials and centrifuged for 20 minutes at 1500 RPM to remove sediment from suspension. Each sample then received 24 mL of 100% methanol, and was vortexed for 10 seconds to resuspend and mix the sediment. Samples were then sonicated in an ice bath for 30 minutes and centrifuged for 20 minutes at 2500 RPM. The supernatant was then decanted into a clean glass test tube, and dried to a volume of 0.5 mL under a stream of ultrapure nitrogen. The concentrated methanol solution was diluted with 18 mL of Milli-Q water, and passed through conditioned Sep-Pak HLB SPE cartridges at a rate of 1 mL per minute. Each cartridge was then eluted with 8 mL of 100% methanol and the resulting solution was blown to dryness under a stream of ultrapure nitrogen. The dried concentrate was reconstituted in 1 mL of 10% acetonitrile for LC/MS analysis. This extraction method was modified to remove DPH from sediment and pore water, but leave DPH adsorbed to MWCNTs in order to estimate the MWCNT-adsorbed fraction through mass-balance calculation. DPH is not sufficiently soluble in methanol to break the pi-pi interaction of DPH to MWCNTs (Xiaojun Chang, personal communication, June 17 2015).
Fish Extraction. Each fish sample for body burden analysis was comprised of five individual fish, compounded to ensure sufficient mass for accurate estimation of DPH concentration. Fish samples were thawed for 1 hour, then pulverized in a ceramic mortar with approximately 10 mL of liquid nitrogen and decanted into clean 20 mL glass test tubes. After allowing the nitrogen to boil off, pulverized samples were reconstituted in 2 mL of a 3:3:1 ratio of acetonitrile/methyl-tert-butyl ether/methanol and mixed on a rocker for 20 minutes. Samples were then centrifuged for 20 minutes at 1500 RPM and the supernatant was decanted into a new clean 20 mL glass test tube. Solutions were centrifuged for an additional 5 min at 1500 RPM to ensure that no fish tissue remained in suspension. The clarified supernatant was then dried under ultrapure nitrogen until approximately 0.5 mL remained, then diluted with 10 mL of HPLC-grade water. Diluted solutions were passed through Oasis Florisil SPE cartridges on a vacuum manifold, and cartridges were subsequently eluted with 6 mL of 100% methanol. The resulting solutions were dried under a stream of ultrapure nitrogen and reconstituted in 250 μL of 10% acetonitrile for LC/MS analysis.

LC/MS Analysis

Water, sediment, and fish tissue samples were analyzed on a Varian 1200L triple quadrupole mass spectrometer interfaced with a Varian ProStar HPLC (Agilent Technologies). Chromatographic separation was achieved on a Kinetex C18 column (2.6 μm particle size, 2.1 x 150 mm, Phenomenex). The initial mobile phase was 85% water with 0.1% formic acid (A) and 15% acetonitrile with 0.1% formic
acid (B) and a flow rate of 250 µL/min was maintained. Initial conditions were held for 0.5 minutes, then ramped to 95% B over 14.5 minutes, held for 1 minute, then returned to starting conditions, and equilibrated for 9 minutes. Total run time was 24 minutes. Diphenhydramine was detected in positive mode using electrospray ionization, with m/z transitions at 256>167 (-8V), and 256>152 (-30V).

**Bioaccumulation Factors**

Bioconcentration factor (BCF) was calculated using the following formula:

\[
BCF = \frac{C(\text{tissue})}{C(\text{water})}
\]  

(1)

where \(C(\text{tissue})\) is the concentration of DPH in whole-body fish tissues in \(\mu g/g\) and \(C(\text{water})\) is the concentration of DPH in water samples in mg/L. Biota-sediment accumulation factor (BSAF) was calculated using the following formula:

\[
BSAF = \frac{C(\text{tissue})}{C(\text{sediment})}
\]  

(2)

where \(C(\text{tissue})\) is the concentration of DPH in whole-body fish tissue in \(\mu g/g\) and \(C(\text{sediment})\) is the concentration of DPH in sediment in \(\mu g/g\).

**Statistical Methods**

Homogeneity of variance was assessed with Bartlett’s test, and normality was assessed with the Shapiro-Wilk test for all comparisons. Comparisons of morphological endpoints and measured DPH concentrations were conducted using RStudio 0.97.551. One-way ANOVA was used to compare all treatments, with post-hoc application of Tukey’s Honestly Significant Difference (HSD) test.
Bioaccumulation and biota-sediment accumulation factors for different treatments were compared with one-way ANOVA at each measured time point. Significance for all tests was set at $p = 0.05$. No data transformations were applied, and no deviations from normality or homogeneity of variance were detected. Outliers were determined, where applicable, by assessing Cook’s distance by treatment and removing data points with Cook’s $D$ greater than $4/n$. For all means noted in the text, error is expressed as a 95% confidence interval for the mean.

**Results and Discussion**

*Spectrophotometric Determination of MWCNTs in Water*

No MWCNTs were detected in water samples at any point during exposure, suggesting that MWCNTs deposited in sediment within 24 hours of addition to exposure vessels. Fathead minnows are known to disturb sediment during feeding (McCarthy et al., 2003), however the small size of fish used and the relatively low degree of sediment perturbation observed was apparently not sufficient to resuspend measurable quantities of MWCNTs into the overlying water.

*Morphological Endpoints*

Tail-to-snout body length and dry mass were used as indicators of growth inhibition in exposed fish. Dry mass (Figure 3.1a) did not differ significantly among treatments until 10 days of exposure, at which point all fish exposed to DPH, with or without MWCNTs, had lower mean dry mass compared to controls (Tukey’s HSD, $p=0.007$, $n=25$), resulting in nearly identical inhibition of growth (ANOVA, $F(1,9)$,
Dry mass measured in the MWCNT positive control treatment was not significantly lower, although the mean value was somewhat lower compared to the control. Body length, like dry mass, did not differ significantly until after 10 days of exposure (Figure 3.1b), at which point all DPH treatments (± MWCNTs) had reduced body lengths compared to the controls (Tukey’s HSD, p=0.006, n=25). Regardless of addition of MWCNTs, both treatments resulted in nearly identical reduction in body length (ANOVA, F(1,9), p=0.003, n=10). The MWCNT positive control (no DPH) treatment did not have a significantly different body length from either the MHW Control or DPH-containing treatments, lying at an intermediate between the two. These results were contrary to our hypothesis, and demonstrate that addition of MWCNTs had not reduced diphenhydramine’s growth-inhibiting effect.

**Diphenhydramine in Water and Sediment**

All treatments were analyzed for DPH content in water at 24 h and 10 days of exposure (Figure 3.2). As expected, the MHW control and MWCNT positive control (no DPH) treatments had no detectable concentration of diphenhydramine at any time point, and are not shown. There was no significant difference in DPH concentration between DPH treatments with or without MWCNTs at 24 hours (ANOVA, F(1,9), p=0.25, n=10). After 10 days of exposure, significantly less diphenhydramine was detected in MWCNT-containing treatments (ANOVA, F(1,9), p=0.009, n=10). In treatments with and without MWCNTs, significantly less diphenhydramine was detected at day 10 than at 24 hours (Tukey’s HSD, p=0.001,
These results support the hypothesis that MWCNTs can adsorb a significant amount of diphenhydramine from the water fraction.

The ability of MWCNTs to adsorb organic contaminants is well documented (Liao et al., 2008; Lin et al., 2008; Oleszczuk et al., 2009), so it is not surprising that addition of MWCNTs reduced the amount of free DPH in the water column. However, Oleszczuk et al. (2009) found that the MWCNT adsorption kinetics of the cyclic pharmaceutical compounds carbamazepine and oxytetracycline were quite fast, and that equilibrium was achieved within 24 hours. In the present study, DPH from the water column continued to sorb to MWCNTs beyond 24 hours as evidenced by the large reduction in waterborne DPH between the day 1 and day 10 samples. This may be due to the mixing of MWCNTs with sediment, in that not all of the MWCNTs were immediately exposed to the water column. Percolation of water into the pore water of sediment is a slow process, and combined with slight mixing of the sediment by foraging fish, MWCNTs may have gradually been exposed during the experimental timeframe.

Sediment samples were only collected at day 10 (Figure 3.3) to avoid perturbation of diphenhydramine-laden sediment, which would disturb the equilibrium of diphenhydramine partitioning within the exposure system. At day 10, significantly less diphenhydramine was detected in MWCNT-containing treatments (ANOVA, F(1,9), p=0.009, n=10). As seen in the water-column measurements, MWCNTs reduced the amount of DPH bound to sediment, likely by binding DPH more tightly than sand/silt particles in sediment. These results demonstrate the ability of MWCNTs to adsorb diphenhydramine from both water and sediment
compartments, when MWCNTs have fully settled in sediment and are not detectable in the water column. It is therefore apparent that MWCNTs, if deposited onto the sediment fraction of a water body, can sequester organic contaminants from both water and sediment fractions, suppressing detectable concentrations of those contaminants in the aquatic environment depending on the detection procedures used. In the present experiment, our extraction method for sediment was designed to remove diphenhydramine from the sediment fraction only, leaving MWCNT-bound diphenhydramine behind. However, some extraction procedures using stronger solvents may be able to remove diphenhydramine from MWCNTs and sediment, which would increase the DPH detected in the solid (sediment + MWCNT) fraction.

**Bioaccumulation and Mass Balance of Diphenhydramine in Sediment Exposures**

Aggregate body burdens of DPH in fathead minnows were analyzed for DPH-containing treatments after 1, 4, 7, and 10 days of exposure (Figure 3.4). In both treatments (±MWCNTs), a U-shaped accumulation curve was observed. At 24 h fish in the DPH treatment appeared to have a higher mean body burden than the DPH-MWCNT treatment, although due to high variability the difference was not statistically significant. At Day 7, body burdens of DPH were slightly higher in the DPH treatment than in the DPH-MWCNT treatment (ANOVA, F(1,9), p=0.05, n=10). At the conclusion of the exposure, body burdens in both treatments had returned to an intermediate point relative to initial 24-hour concentrations, and were not significantly different. The similarities in body burden seen in both DPH treatments,
regardless of addition of MWCNTs, underscore the similar levels of growth inhibition seen in the two DPH treatments and indicate that addition of MWCNTs to sediment was insufficient to reduce the physiological effects of DPH despite reducing detectable concentrations in both water and sediment.

The initial decrease in DPH body burden and subsequent return to an equilibrium level is possibly due to the induction of CYP450 enzymes, responsible for the metabolism of DPH in humans (Akutsu et al., 2007). While the details of DPH metabolism in fish remain unknown, CYP450 are a family of enzymes that are common to all living organisms. Michaelis-Menten enzyme saturation kinetics accurately modeled the accumulation and depuration of DPH in mosquitofish ($r^2 = 0.9521$), which is in agreement with the kinetics of CYP450 DPH metabolism in mammals (Wang et al., 2013). In the present experiment, initial DPH concentrations in fish reached a maximum within 24 hours, then began to decrease, perhaps as CYP450 was induced. The eventual return to equality between the two treatments may represent saturation of the CYP450 complex.

Bioconcentration factors were calculated at day 1 and day 10 (Figure 3.5a). Among DPH treatments ($\pm$MWCNTs), BCF was not significantly different at Day 1, with similar values implying similar initial uptake rates. However, by day 10 a significant increase in BCF was noted for the MWCNT-containing treatment (ANOVA, $F(1,9), p=0.0008, n=10$), while values for the DPH treatment with no MWCNTs did not change over the timeframe of the experiment.

Biota-Sediment Accumulation Factors (BSAF) were calculated at Day 10, the only day that sediment DPH was measured, as perturbing the sediment during the
exposure may have disturbed the equilibrium of diphenhydramine partitioning (Figure 3.5b). Curiously, the MWCNT-containing treatment had a significantly higher BSAF compared to the DPH treatment (ANOVA, F(1,9), p=0.04, n=10).

Mass-balance calculations were undertaken in order to confirm that the addition of MWCNTs to sediment resulted in significant DPH adsorption (Figure 3.6). As the concentrations of DPH added to each treatment and subsequent DPH concentrations in water, sediment, and fish were measured, estimates were made assuming that the fraction of DPH remaining unaccounted-for was a combination of DPH adsorbed to MWCNTs, DPH adsorbed to the walls of the exposure vessel, and DPH lost through microbial metabolism and photodegradation. It is likely that MWCNTs (presumably with sorbed DPH) were either removed during SPE sample cleanup prior to analysis in the case of water samples, or were not extracted in the case of sediment samples. The exposure vessels were acid and acetone-washed before the outset of the exposure, and all vessels used were the same size and volume. All treatments received sediment from a common source, and light levels were held constant throughout the exposure. Supporting the conclusion that MWCNTs adsorbed a significant amount of DPH, MWCNT-containing treatments had significantly higher amounts of unaccounted-for DPH (manifested as lower overall DPH concentrations in sediment) compared to DPH treatments with no MWCNTs (ANOVA, F(1,9), p=0.0001, n=10).

While BCF and BSAF were elevated at Day 10 in the DPH-MWCNT exposures, the mean body burden of DPH in both treatments (±MWCNTs) remained the same. At the same time detectable DPH decreased in water and sediment for these
MWCNT-containing treatments. The BSAF was artificially elevated in DPH+MWCNT treatments because our extraction process excluded DPH bound to MWCNTs, resulting in a lower detectable concentration of DPH in sediment than was actually present if DPH adsorbed to MWCNTs was included. As body burdens of DPH and toxicity were similar between the two DPH-containing treatments, it is probable that fish in the DPH+MWCNT treatments accumulated the unaccounted-for DPH from the MWCNT fraction of sediment. As there is evidence that much of the remaining DPH was adsorbed to MWCNTs, it is likely that fish in the MWCNT-containing treatment ingested MWCNTs from the sediment and that DPH desorbed during digestion, accounting for the comparable body burdens and observed toxicity. The similar inhibition of growth seen in DPH treatments with or without MWCNTs supports the conclusion that DPH exposure remained the same between the two treatments, regardless of addition of MWCNTs. In previous sediment elutriate exposures using the aquatic invertebrate Ceriodaphnia dubia (Myer et al., Chapter 2), addition of MWCNTs to sediment resulted in a decrease in DPH toxicity. However, in C. dubia exposures, sediments and MWCNTs were removed from the aqueous exposure media before the organisms were introduced. Isolation of Ceriodaphnia from the sediment (and MWCNT) fraction resulted in decreased DPH exposure, with a commensurate decrease in toxicity. These results imply that a significant portion of DPH was adsorbed to MWCNTs in sediment, as seen in the present study.

There is evidence that P. promelas will consume sediment in search of food, with co-ingestion of MWCNTs (Linard et al., 2015), however, it is unknown whether
the gut environment of fathead minnows can desorb DPH from MWCNTs.

Considering that DPH is a weak base (pKa = 8.9), the basic environment of the fish intestinal tract (Hlophe et al., 2014; Montgomery et al., 1988) may have caused some adsorbed DPH to revert to its neutral form and accumulate in the lipid-rich tissues of the body. It has been shown that bile salts and pepsin in a simulated gut environment can cause the cyclic organic compound phenanthrene, which is un-ionized and carries no charge, to desorb from MWCNTs through acting as a surfactant and increasing phenanthrene’s solubility. These mechanisms may also be responsible for similar desorption and accumulation of un-ionized DPH (Wang et al., 2011).

Conclusions

Addition of MWCNTs reduced the detectable levels of DPH in both sediment and water, yet did not have an effect upon either fish body burdens of DPH or protect fish from toxic effects resulting from DPH exposure. Growth of *P. promelas* was inhibited to a similar degree in sediment treatments with or without MWCNTs, and body burdens were the same after 10 days of exposure, indicating that while MWCNTs adsorb a significant amount of DPH from water and sediment, exposure to fish remains the same. We propose that exposed fish ingested MWCNTs from the sediment while foraging for food, and that DPH was desorbed from MWCNTs in the gut tract. Our model of diphenhydramine accumulation from MWCNTs shows similar results to those seen in exposures with sediment-dwelling worms, decreasing concentrations in sediment and water yet increasing bioaccumulation
factors (Shen et al., 2014). Conversely, our results show the opposite trend as a study which found a decrease in fluoranthene accumulation with the addition of MWCNTs, and a lack of desorption of PAHs in the *P. promelas* gut (Linard et al., 2015). However, there are no extant studies examining fish accumulation of a non-PAH compound from MWCNTs in the presence of sediment or desorption of pharmaceuticals from MWCNTs in vivo, and the mechanisms of uptake and desorption are likely different for diphenhydramine. As fluoranthene has a much higher affinity for organic carbon than diphenhydramine (log $K_{oc} \approx 4.58$ for fluoranthene and 2.58 for diphenhydramine), it is likely that fluoranthene binds much more tightly to MWCNTs (which are comprised entirely of carbon) than DPH (Couper et al., 2014; Pan et al., 2007). Additionally, fluoranthene is a neutral compound that does not ionize, while diphenhydramine is a weak base. Ionized compounds have electrochemical interactions with functionalized MWCNTs that are absent with un-ionizable compounds, complicating their binding behavior. In a gut environment that is unable to break the strong binding of fluoranthene to MWCNTs, the bound complex may be excreted, reducing its toxicity, as found by Petersen et al. (2009) in earthworms exposed to pyrene-contaminated soils amended with MWCNTs. Conversely the less hydrophobic DPH may be desorbed from MWCNTs in the gut, resulting in DPH accumulation and toxicity.

In conclusion, addition of MWCNTs to sediment is insufficient to reduce uptake or toxicity of diphenhydramine from either sediment or water in bottom-foraging fish. However, the remedial use of MWCNTs should not be discounted entirely, as studies have shown that MWCNT addition reduces exposure to neutral
hydrophobic compounds such as the PAHs fluroanthene and pyrene and the antibacterial agent triclocarban in fish and invertebrates (Linard et al., 2015; Petersen et al., 2009; Simon et al., 2015). More study is needed in order to understand the mechanisms of ionic compound sorption and desorption to MWCNTs in both aquatic and intra-organism environments, which may have a moderating or potentiating effect upon uptake depending on the organism-specific digestive environment and feeding habits.

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Figure 3.1(a). Mean dry mass of 5-fish composite sample by day and treatment. At Day 10, DPH (No-MWCNT) and DPH+MWCNT treatments had significantly less mass than controls (p=0.007, Tukey’s HSD, n=25). (b). Mean body length by treatment and day. At Day 10, No MWCNT and MWCNT treatments had significantly lower body length than controls (p=0.006, Tukey’s HSD, n=25. Error bars represent 95% CI.
Figure 3.2. Concentrations of diphenhydramine in water (µg / L) by treatment and day. Initial concentrations of DPH were not statistically different. After 10 days of exposure, addition of MWCNTs reduced available diphenhydramine in water by 55.43% (p=0.009, ANOVA, n=10). Error bars represent 95% CI.
Figure 3.3. Concentrations of diphenhydramine in sediment (ng/g) on Day 10. Addition of MWCNTs reduced available diphenhydramine by 71.48% (ANOVA, F(1,9), p=0.009, n=10). However, with correction for estimated DPH bound to MWCNTs, there was no significant difference in sediment DPH concentration. Error bars represent 95% CI.
Figure 3.4. Mean body burdens of DPH by day and treatment. At Day 7, the DPH (no MWCNT) treatment had a significantly higher mean body burden (ANOVA, F(1,9), p=0.05, n=10) than the DPH+MWCNT treatment. Body burdens were not significantly different at Day 1, 4, or 10, although the mean body burden was higher on Day 1 in the No-MWCNT treatment. Error bars represent SE.
Figure 3.5(a). Bioconcentration factor (BCF) of diphenhydramine by treatment at Day 1 and Day 10. Treatments without MWCNTs (No MWNCTs) experienced no significant change in BCF over the course of the exposure, while BCF in MWCNT-containing treatments were significantly higher at Day 10 (ANOVA, F(1,9), p=0.0008, n=10). (b). Biota-sediment accumulation factor (BSAF) of diphenhydramine by treatment at Day 10. MWCNT-containing treatments showed a significantly higher BSAF at Day 10 (ANOVA, F(1,9), p=0.04, n=10). Error bars represent 95% CI.
Figure 3.6. Mass balance of diphenhydramine after 10 days of exposure. The amount of DPH detected in fish tissue is not visible due to the small mass of experimental fish compared to other compartments, but were statistically similar for both treatments. Total added DPH was verified by LC/MS. Addition of MWCNTs resulted in a 50.6% increase in unaccounted-for DPH. MWCNT-containing treatments had significantly greater unaccounted-for DPH (ANOVA, F(1,9), p=0.0001, n=10) implying that a significant amount of DPH was adsorbed to MWCNTs.
Supplemental Figure 3.1. Experimental pH by treatment over time. As DPH is a weak base, maintaining pH in a narrow range is important to avoid significant changes in ionization, which affect toxicity and accumulation. At day 8, the DPH treatments had a significantly higher pH than the DPH+MWCNTs treatments (Tukey's HSD, p=0.03, n=20). By day 10 the pH difference had been eliminated. DPH remained over 99% ionized at all time points measured.
Supplemental Figure 3.2. Standard curve used for determination of MWCNT concentration in water. Nominal concentrations used ranged from 0.049 to 100 mg/L. \( r^2 = 99.85\% \). Regression equation: Absorbance(@800nm) = 0.060466 + 0.00272512 \((\text{MWCNT concentration, mg/L})\).
References


USEPA. (2007). Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS.


CHAPTER 4
CONCLUDING REMARKS

Carbon nanotubes have enjoyed considerable attention from academia and engineers since their discovery, with new and exciting applications announced, promised, and researched at a rapid pace. While nanocarbon has been lauded in popular science media as a revolutionary class of material, the reception from the toxicology community has been somewhat more skeptical. Toxicology research involving carbon nanotubes overwhelmingly focuses on their potential negative effects on organisms and the environment (Cheng et al., 2009; Jackson et al., 2013; Lam et al., 2004; Smith et al., 2007; Su et al., 2013). This is perhaps unsurprising, as toxicologists traditionally study the potential harm of substances; if they studied helpful substances they would be pharmacologists! However, there are occasions when toxicologists have an opportunity to reach less grim conclusions about the substances they study. In the case of carbon nanotubes, promising research has recently investigated their potential to protect aquatic organisms from the harmful effects of other anthropogenic substances released into the environment, by adsorbing and sequestering contaminants where they are unavailable to biota (Ferguson et al., 2008; Linard et al., 2015; Shen et al., 2014). In the present study, we investigated addition of multi-walled carbon nanotubes (MWCNTs) to sediment containing diphenhydramine (DPH), used as a model pharmaceutical contaminant.
We hypothesized that amendment of MWNCTs to sediment would result in a protective effect similar to that seen in previous studies.

Our first experiment used sediment-elutriate toxicity evaluation procedures developed by the United States EPA (USEPA, 2004) to determine the effects of MWCNT sediment amendment on the toxicity of DPH to the aquatic invertebrate *Ceriodaphnia dubia*. Our results were promising, indicating that addition of MWCNTs to sediment resulted in a mean 78.7-90.1% reduction in observed mortality compared to DPH positive controls. Simply adding MWCNTs to exposure solution containing DPH reduced mortality as well, by 40.7-53.3%. These results compare favorably with a recent study that found that adding MWCNTs to exposure water containing triclocarban, an antibacterial compound found commonly as an environmental contaminant, resulted in a reduction in mortality to the larger aquatic invertebrate *Daphnia magna* (Simon et al., 2015). Significantly, in this first experiment, MWCNTs and sediment were removed from the aqueous exposure system before organisms were added. This may be the crucial step in applying MWCNT-based pollution mitigation, as evidenced by the results of our second experiment.

In our second experiment, we endeavored to increase the environmental relevance of our exposures by leaving natural sediment and MWCNTs in the exposure media. We exposed the model freshwater fish *Pimephales promelas* (fathead minnow) to DPH at a concentration that is known to cause growth inhibition at our experimental pH (Berninger et al., 2011; Nichols et al., 2015) in exposure vessels containing natural sediment amended with MWCNTs. The results
from this experiment were markedly different from those seen in *C. dubia* exposures. Although addition of MWCNTs reduced concentrations of DPH in the water column by over 50%, fish body burdens of DPH were unaffected by the addition of MWNCTs. Growth inhibition was similarly unaffected, with nearly identical toxicity seen in treatments with and without MWCNT amendment.

This result was surprising, as a similar study found that addition of MWCNTs reduced the accumulation of fluoranthene, a PAH compound, in fathead minnows (Linard et al., 2015). In order to investigate how minnows in treatments with and without MWCNTs accumulated similar amounts of DPH despite a significant MWCNT-mediated reduction in DPH water concentration, we calculated mass-balance to determine the fate of DPH in both treatments. We found that in MWCNT-containing treatments, the missing DPH from the water column was adsorbed to MWCNTs in the sediment fraction. This indicates that minnows in the MWCNT-containing treatments accumulated the “missing” DPH from MWCNTs in the sediment. Similar conclusions have been found in exposures investigating accumulation of PAH compounds in aquatic invertebrates, in which organisms ingested MWCNT-adsorbed PAHs, leading to increased exposure (Shen et al., 2014). We developed a conceptual model for DPH accumulation in the presence of MWCNTs that helps to make the potential modes of uptake clear (Figure 4.1).

Although the kinetics of DPH desorption from MWCNTs during digestion by fathead minnows are unclear, it seems likely that sediment-feeding fish are able to accumulate DPH from ingested MWCNTs, thus negating any protective effect that MWCNT sorption and sequestration may have had.
In conclusion, the seemingly contradictory results of our two experiments point to a common practical result: if MWCNTs are to be used as a low-cost method to remove organic contaminants from water, as has been proposed in the past (Ajayan et al., 2001; De Volder et al., 2013; Upadhyayula et al., 2009), they must be contained so that they can be effectively removed. Otherwise, their effectiveness in mitigating toxicity can be compromised by ingestion and desorption of adsorbed contaminants by sediment-feeding and dwelling organisms. While pelagic organisms may enjoy a protective effect from dispersion of MWCNTs in organic compound-contaminated environments, benthic organisms may not be so lucky. We therefore recommend that investigation into carbon nanotube pollution mitigation should employ a particular focus on containment and safe disposal of nanotubes, in order to extend protective benefit to the greatest number of organisms.
Figure 4.1. Conceptual model of organic contaminant exposure in the presence of MWCNTs and sediment.
References


85