5-2014

Characterizing the Effect of Suspended Carbon Nanotubes on the Bioavailablility of Adsorbed Fluoranthene to P. Promelas

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CHARACTERIZING THE EFFECT OF SUSPENDED CARBON NANOTUBES ON THE BIOAVAILABILITY OF ADSORBED FLUORANTHENE TO P. PROMELAS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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by
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May 2014

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ABSTRACT

The introduction of carbon nanomaterials into the environment has increased exponentially in the last decade, causing environmental as well as health concerns. One concern is the interaction that such nanomaterials have with the biota in the aquatic ecosystem and the direct and indirect toxic effects that may result. Previous research has documented a positive influence of natural organic matter (NOM) on the stability of carbon nanotube (CNTs) suspensions in surface waters. Further, research has quantified the ability of these carbon nanomaterials to adsorb aquatic contaminants such as polycyclic aromatic hydrocarbons (PAHs). Though both CNTs and PAHs can co-occur in wastewater treatment effluents few studies have investigated the bioavailability of these adsorbed PAHs to fish. The goal of this research was to characterize the bioavailability of fluoranthene (FLU) adsorbed to suspended muliwalled-carbon nanotubes (MWNTs) in a solution containing NOM. Results indicated that while NOM was critical for producing stable MWNT suspensions, it did not influence the bioavailability of FLU to P. promelas in the absence of MWNTs. Adsorption isotherms indicated that NOM significantly influenced the adsorption of FLU to MWNTs. P. promelas were exposed for 16 hrs in moderately hard water (MHW) containing only FLU, FLU in the presence of different concentrations of NOM, and FLU adsorbed to MWNTs in the presence of NOM. Bioavailable FLU was quantified in each exposure through bile analysis using a fluorescence microplate reader. Results indicated that 2 mg/L NOM as dissolved organic carbon (DOC) were sufficient to produce a stable MWNT suspension. The bioavailability of FLU was significantly reduced in the presence

of this suspension. Through comparing the concentration of FLU metabolites in the bile to the concentration of FLU added to MWNT and DOC solutions we were able to quantify the relative bioavailability of FLU adsorbed to MWNTs. Results indicate that approximately 60-90% of the FLU was adsorbed to the MWNTs and that adsorbed FLU was not bioavailable to P. promelas.

DEDICATION

I would like to dedicate this work to my parents, Karen and Terry Robinson, for their tremendous amount of support and encouragement.

ACKNOWLEDGMENTS

I would like to express my sincerest appreciation to my advisor, Dr. Stephen Klaine, and co-advisor, Dr. Peter van den Hurk, for taking a chance on an ecology-based student and the impact they have had collectively on my professional growth. I'd also like to thank my committee member Dr. Tanju Karanfil, for his valuable insight and input during this wide scoping study.

Special thanks to Ron Gossett for his assistance in constructing my exposure apparatus and maintaining the Challenge room, where a majority of my work was done. Thank you to Dr. Ben Sharp for all his help with the statistical analysis and the efficiency in which he worked with me remotely. Thanks to all my ENTOX colleagues and various friends, who have helped me in various measures with this project. Funding for this study was provided by Clemson Public Service Activities.

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CHAPTER ONE

LITERATURE REVIEW

1.1 PAHs in the Environment

Polycyclic aromatic hydrocarbons (PAHs) are one of the most well-known classes of organic pollutants present in the environment. This extensive group of toxicants are formed by the incomplete combustion of organic materials and as a result enter the environment from a number of anthropogenic as well as natural sources [1]. The largest source of PAH emissions are biofuels, wildfires, consumer product and oil production with the total global atmospheric emissions estimated to be 530 Gg/y in 2004 [2]. The U.S. alone has been estimated to produce 11000 metric tons of PAHs annually [21]. Because of the effect on both human and environmental health, much concern and research is devoted to investigating various aspects of these contaminants.

As semivolatile compounds, nearly all PAHs are initially emitted into the atmosphere during formation. After chemical and physical interactions take place, PAHs will be deposited in soil and water sometimes long distances from the original source. Once deposited in the environment, water becomes the main means of transportation, largely by means of runoff, streams and wastewater effluent [3, 4]. The aromatic structure of the toxicants and lack of nonpolar substituents make them highly lipophilic, which consequentially results in low water solubility and high association with sediment or colloids present [21]. In the aquatic environment, sorption of PAHs with natural organic materials (NOM) can greatly influence the ultimate fate of PAHs [5, 4].

NOM may be present in particulate or dissolved form known as dissolved organic carbon (DOC). Regardless, all water systems contain some concentration of NOM, generally in the range of 0.5 mg C/L to 10 mg C/L in freshwater systems [6]. NOM are complex structures with both hydrophobic and hydrophilic fractions. The particular chemical makeup of NOM is varied depending on the source, which greatly influences the sorbing and partitioning behavior of PAHs [7]. A variety of water chemistry parameters such as pH, temperature and ionic strength can further influence the association of PAHs with NOM [8]. Inevitably, much research has been devoted to understanding the effect on NOM on the degradation, bioavailability and fate of PAHs, often resulting in contradictory conclusions. A substantial reduction in freely dissolved PAHs in the water phase has been observed when PAHs partition or sorb to NOM [9]. While this reduces the bioavailability and bioaccumulation of PAHs in aquatic organisms, it can also slow degradation. Partitioning into NOM reduces the bioaccessibility of PAHs to abiotic and biotic factors, resulting in the increased stability and persistence of PAHs in the environment [10, 11]. In contrast, studies also concluded that the association with the hydrophobic portion of NOM solubilizes PAHs and increased biodegradation [4, 5]. The partitioning coefficients of PAHs with organic matter vary drastically depending on the hydrophobicity, the form (colloidal or dissolved), and the chemical characteristics of NOM as well as the physicochemical properties of the contaminant.

In the aquatic environment, organisms considered most at risk for PAH exposure are the bottom feeders, filter feeders and other organisms that are closely associated with

sediment and organic material where PAHs accumulate [12]. The two main routes of PAH exposure are considered to be either ingestion of PAH-contaminated particles or through exposure across the gills [13]. The uptake of PAHs across the gills is largely moderated by the compound's water solubility; studies found that compounds with log Kow values between 3 and 6 are readily taken up from the water, but uptake rates greatly decline when log Kow values are above 6, largely because there is less of the compound remaining in aqueous form [81]. Acute toxicity from PAH exposure is largely a consequence of oxidative stress, where the production of reactive free radicals is occurring faster than the body can detoxify or repair the damage. The resulting toxic response may be just mild irritation, but can be more severe. Oxidative stress can lead to the interruption of ATP production and other cell functions that can ultimately result in necrosis [62]. The presence of UV light can further enhance the toxicity of PAHs; inducing lipid peroxidation and the degradation of cell membrane function, which has been shown to lead to acute mortality in fish and other aquatic organisms [16]. Chronic exposure to PAHs can lead to a number of other detrimental effects such as immunological, developmental and reproductive alteration as well as mortality [14, 63]. Further, a number of PAHs are known carcinogens and though many are not genotoxic or mutagenic in parent form, once metabolized, the reactive intermediate species can form DNA adducts or react with proteins [21].

There are hundreds of PAHs in existence and the toxicity of a particular PAH compound to an organism greatly depends on the mechanism in which the PAH is metabolized [58]. Like most vertebrates, the metabolism of PAHs occurs readily in the

liver of fish, largely by cytochrome P-450 enzymes followed by Phase II conjugation. The hydrophilic metabolites are then excreted from the body through either the bile or urine, both of which can be monitored as exposure biomarkers [15]. Most vertebrates and invertebrates can efficiently metabolize and excrete PAHs with little bioaccumulation occurring; consequentially, analysis of tissue concentrations of PAHs is often an inadequate measure of PAH exposure [3, 64]. The parent compounds and metabolites of PAHs are highly fluorescent and can be accurately measured in both water and bile via fluorescence spectrometry [67, 68]. Relative fluorescent intensity is directly proportional to PAH metabolite concentration in bile and is dose-dependent of PAH exposure. Several studies have concluded that bile analysis is a sensitive biomarker to assess the uptake and bioavailability of PAHs [68, 65, 66].

1.1.1 Fluoranthene: A model PAH

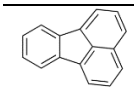
The 16 most ubiquitous PAHs, including fluoranthene (FLU), are listed on the EPA's priority pollutant list. FLU is one of the highest contributors to total PAH concentrations in the environment, detected in most PAH contaminated sites. Concentrations as high as 59 mg/kg have been reported in polluted sediments and although typical waste water concentrations are no higher than 500 μg/L, some industries produce FLU waste in the mg/L range [76, 1]. In most surface waters FLU is generally found in the ng/L to low μg/L range; storm runoff can greatly increase concentrations with reports as high as 130μg/L [17-20]. Aquatic environments near urban or industrial areas are particularly affected with elevated levels of FLU in both the sediment and water [19]. Concentrations of FLU above the water solubility limit, 0.26 mg/L, will readily sorb

to sediment or particulate matter once entering the water column. Freely dissolved FLU has a half-life of a few days, but sediment-associated FLU is quite stable and will accumulate [17]. Studies indicate that FLU may persist for years in sediment, as desorption is slow. Further, the desorbing fraction of FLU is bioavailable to aquatic organisms present [70].

A majority of studies have concluded FLU is not carcinogenic, but it can cause immunosuppression, growth arrest and tumors in the lungs and liver [69, 77]. For many aquatic species, FLU is not lethal within its water solubility limit, although studies have shown LC50s of 187 to 500 µg/L FLU for fresh and salt water species, respectively [18]. The LC50 for juvenile fish is estimated to be 0.0077 mg/L at a duration of 96 hours, while the EC50 (immobility) in daphinds was found to be 0.19 mg/L [72]. Further, it is well documented that the presence of UV-light increases the sensitivity and acute toxicity of FLU to numerous aquatic organisms by several orders of magnitude [73]. For most PAHs, activation of the AhR receptor elicits toxic responses either by "metabolic activation" or by altering gene expression that effect cell function; FLU has a low affinity for the AhR receptor [80, 77]. The metabolism of FLU is largely mediated by epoxide hydrolase, rather than by the induction of CYP1A and most toxic modes of action are independent of the AhR receptor. The parent form of FLU is not particularly reactive, while the epoxide intermediates and predominant metabolites are [69]. Studies have found that both FLU and FLU metabolites can be easily detected via fluorescence spectrometry in bile, making bile analysis an ideal FLU exposure biomarker [69, 76].

In terms of most physicochemical properties, FLU encompasses characteristics that are comparable to a wide range of PAHs; being of median weight, water solubility, hydrophobicity, density and photo-toxicity [71] (Table 1.1). The abundance of FLU in the environment and the ease of detection in bile, make FLU a good model PAH to use in investigations concerning bioavailability.

Table 1.1 Selected physicochemical properties of FLU

Structure	Planarity	MF[a]	MW[b] (g/mol)	Density (g/cm^3)	S_w[c] (mg/L)	log K_{ow}[d]	log K_{doc}[e]	LOD_{FL}[f] (µg/L)
	Planar	$C_{16}H_{10}$	202.26	1.25	0.26	5.23	3.80-4.40	0.39

[a] molecular formula; [b] molecular weight; [c] water solubility [71]; [d] cited from [75]; [e] cited from [74]; limit of detection for Molecular Devices Gemini fluorescence spectrometer.

1.2 Nanotechnology: Carbon nanotubes

Although nanomaterials have existed in our environment from both natural and anthropogenic causes, in the recent decades there has been an exponential increase in the production of manufactured and synthetic nanomaterials [22]. The high surface area to volume ratio of nanomaterials cause them to behave differently than their bulk counterparts; the materials are uniquely reactive, highly hydrophobic and have very high sorption capacities [23]. To be defined as nano, the material must have at least one dimension <100nm in size [24]. Carbon nanomaterials, or materials composed of graphite, first debuted in 1985, with the discovery of fullerenes; later followed in 1991, by the discovery of multi-walled carbon nanotubes (MWNT). Of the carboneous nanomaterials, carbon nanotubes (CNT) are known to be one of the strongest and most

resilient materials that exist [25]. CNTs are essentially graphene sheets rolled into cylinders that are produced as either individual graphene cylinders that can be capped or uncapped, known as single walled nanotubes (SWCNTs), or as concentric open-ended graphene cylinders, known as multi-walled carbon nanotube (MWNTs) [26]. Besides extraordinary mechanical properties, they also possess exceptional electrical and thermal properties leading to widespread application in numerous fields [28]. The increasing investment in such materials, linked with the capability to efficiently produce and apply CNTs in various industries, has led to a growth of production [27]. Concerns on the widespread production of CNTs, focus on the behavior of CNTs once released into the environment (Figure 1.1).

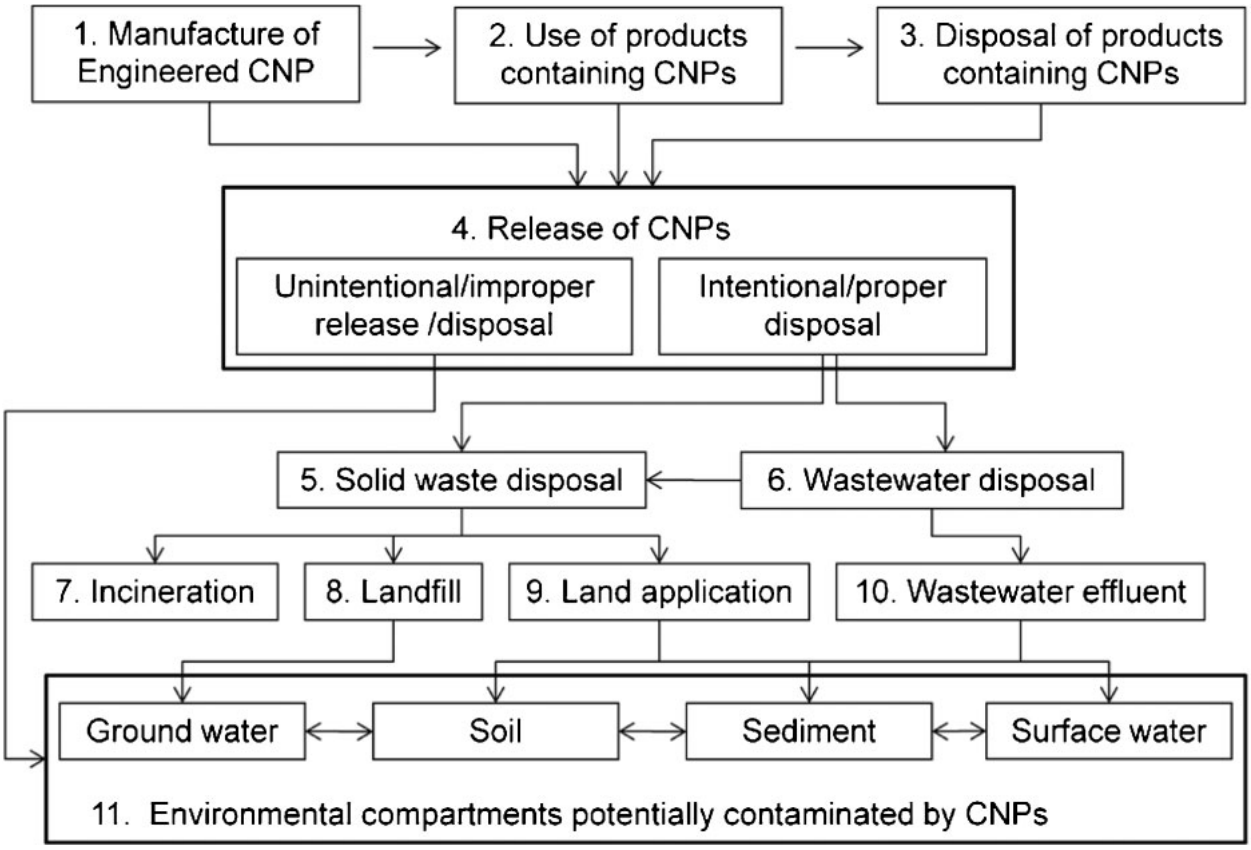


Figure 1.1 Diagram of carbon nanoparticle (CNP) sources and pathways that would elicit CNPs to enter various environmental compartments [61]. The presented study focuses on contamination of the aquatic environment.

1.2.1 CNTs in Natural Waters

Although naturally produced CNTs have been detected in ice-cores, engineered CNTs are often released into the environment as a result of the degradation of the product matrix they were embedded in [29]. Consequently, there is little information available on the actual concentrations of CNTs present in the environment. Estimations currently available rely on predictive models that use information such as worldwide production volume and the flow coefficients of CNTs in various environmental compartments. In 2007 it was estimated that about 350 tons/ year of CNTs were released worldwide, but

with the increase in investment and production, the input in the environment today is significantly higher [30]. A recent study by Velzebor et al. [31] estimated that environmentally relevant concentrations of MWNTs were in the low parts per million range.

Ingestion is the main route of CNT exposure to aquatic organisms, although interactions at the gills can also occur [32]. Studies using a wide range of test organisms from D. magna to various fish species have largely concluded that ingested CNTs do not cross intestinal cells and are not absorbed into tissues. Observed toxicity has largely been attributed to the physical interaction with CNTs, such as gut impaction or blockage at the gills [33, 34]. Toxicity of CNT has been found to range substantially depending on the type of CNT (single or multi-walled), functionalization or coating of the CNT, and the degree of dispersion. Typical effects in aquatic vertebrates are induced when exposed to a range of 10-240 mg/L CNT [32].

The risk of CNT presence in the environment not only encompasses the direct toxicity of CNTs themselves, but also the indirect toxicity that may arise as a result of adsorption of other compounds, such as metals and organic contaminants already present in the environment. Research has concluded that many organic contaminants preferential adsorb to carbon nanomaterials over most natural sediments, soils or colloids [35]. Adsorption to CNTs is driven largely by π-π bonding, hydrophobic interactions, hydrogen-bonding or electrostatic interactions [36]. CNTs high carbon content, reactivity and overall structure give the material a particularly high affinity for adsorption of aromatic organic contaminants, such as PAHs [37]. For aromatic compounds, adsorption

is mainly a mechanism of π-electron coupling between the surface of the nanotube and the compound, where an increase in contact between the two surfaces result in increased adsorption [37]. Although some PAHs can conform to the surface curvature of the nanotubes, adsorption is stronger when PAHs are more linear and have more contact with the surface of the CNT [38]. In instances where CNTs aggregate, PAHs have access to the outer surface of the nanotube as well as interstitial channels and grooves associated with the aggregate bundles. Most adsorption will occur on the outer surface of dispersed CNTs, as the inner layers are generally too condensed for molecules to access [36]. Studies have found that at low concentrations, PAHs will adsorb as a monolayer on CNTs, but with higher concentrations multilayer adsorption has been observed [39]. With CNTs capacity for adsorption of PAHs being approximately 2 orders of magnitude higher than that of natural sediments/soils, there is substantial concern that CNTs presence in the environment will drastically alter the fate and bioavailability of such contaminants [39]. While CNTs have a particularly high capacity for PAH adsorption there has been little indication of hysteresis in water; the near to complete reversal of PAH adsorption implies that desorption of PAHs from CNTs may occur in the aquatic environment after having been transported long distances or being taken up by an organism [40]. As PAHs are often present in waste water effluent and already ubiquitous in the environment, there is a high likely hood that CNTs will either enter the environment with PAHs already adsorbed or quickly interact with PAHs upon release.

1.3 Implications of PAHs adsorbed to CNTs in the Environment

CNTs are highly prone to aggregation and were initially predicted to remain for only a short time in the water column before the aggregates would settle [41]. This being stated, a number of studies have found that in the presence of NOM, CNTs will not only de-bundle and disperse individually, but they can also remain stably suspended in the water column [42, 43]. Electrostatic and steric repulsion are largely responsible for dispersing CNT, while the addition of NOM on the surface of CNTs provide a more thermodynamically favorable surface in water [43]. The stability of suspensions is further affected by water chemistry and NOM characteristics, such as aromaticity and size [44]. It is inevitable that CNTs entering the environment will interact with NOM to some degree and some fraction will become suspended in the aquatic environment. The increased mobility of suspended CNTs is an environmental issue, as fate and exposure of CNTs is greatly altered [42].

The influence of NOM on the adsorption of contaminants to CNTs can vary significantly. The ability of NOM to help disperse CNTs, particularly MWNTs, helps to create more surface area on individual nanotubes. Although, dispersion of CNTs can result in the loss of other adsorption sites in terms of interstitial channels and grooves present in aggregates [45]. The adsorption of NOM molecules can also act as a physical blockage to available adsorption sites or directly compete for the same sites. Depending on the structure of the contaminant and CNT, the results vary as to whether or not pore blockage has a significant influence on the reduction of CNTs adsorption capacity [46-48]. Other factors such as the length of contact time between NOM and CNT, prior to the

adsorption of a contaminant can drive the extent of adsorption as well. For instance, Sun et al. [49] found that although a short time period of NOM and CNT contact lead to a suppression in PAH adsorption, a longer contact time actually resulted in NOM molecules transferring into the interstitial space of the CNT, allowing adsorption sites to reopen.

Depending on what environmental compartment CNTs are released into, the influence on adsorbed contaminant mobility, biodegradation and bioavailability vary. Studies have shown that CNTs reduce the mobility of PAHs in soil even with high flow rates of ground water, whereas in aquatic environments the suspension of CNTs by NOM increase the transport of adsorbed contaminants [50, 51]. In the absence of CNTs, PAH biodegradation and removal from soils and sediments is largely mediated by microbial communities [52]. The effect of CNTs on biodegradation is ultimately a function of the bioaccessibily of the PAH, although factors such as presence of organic carbon, aging time and PAH structure are also influential. Studies largely have found that the presence of CNTs in soil reduce the biodegradation and mineralization efficiency of PAHs, resulting in a longer retention time of PAHs in soil [24]. Even though CNTs appear to reduce the readily bioavailable fraction of PAHs, microbes are still able to access some of the adsorbed portion; the presence of organic carbon further increases PAH degradation [53]. In contrast, increased aging time can also reduce the portion of bioavailable PAH to bacteria, attributed to the movement of PAH molecules from the outside surface to the internal area of the CNT [54].

Baun et al. [59] found that, depending on the contaminant, carbon nanomaterials could act as "facilitated transporters", increasing toxicity to an exposed organism. Where it was found that C60 fullerenes diminished the toxicity of pentachlorophenol to D. magna, the toxicity of the PAH phenanthrene, increased by 60% [59]. Similarly, the toxicity of the herbicide, diuron, to green algae increased when adsorbed to CNTs [56]. In terms of vertebrates, Su et al. [57] recently found that the bioaccumulation of phenanthrene in the tissue of Japanese medaka increased when adsorbed to SWCNTs prior to exposure. On the contrary, the addition of CNTs to soil spiked with pyrene, substantially decreased pyrene uptake by earthworms [55]. Further, though the presence of MWNTs increased the bioaccumulation of pentachlorophenol in carp, the presence of suspended particulate matter actually elicited a higher extent of bioaccumulation of pentachlorophenol. This was attributed to the higher strength of adsorption of pentachlorophenol to MWNTs over particulate matter and the resulting slower rate of desorption in digestive fluids [60].

The presence of NOM can further influence the effect CNTs have on the bioavailability of adsorbed contaminants. Shen et al. [79] demonstrated that the addition of NOM to sediment spiked with MWNT and pyrene greatly reduced the bioaccumulation of pyrene in C. plumosus. The presence of NOM, a preferred food source, reduced C. plumosus ingestion of pyrene-MWNT complexes. Other research showed that while NOM typically sequesters Cu, the adsorption of NOM to MWNT reduced the number of Cu-binding ligands in NOM and increased the bioavailability of Cu [78]. Such studies have demonstrated contradictory results in terms of the effect of

NOM on the bioavailability of contaminants adsorbed to CNTs. It is therefore imperative to consider the influence that NOM may have on the contaminant itself as well as on the adsorption of contaminant to CNT.

1.4 Research Goals and Objectives

Overall, the understanding of how CNTs effect the bioavailability of organic contaminants is still largely unknown and can be quite varied depending on a number of factors, including, but not limited to, the structure of CNT, the presence of organic carbon, the contaminant structure, and exposed species. There is a need for additional research in this area, particularly studies that incorporate an understanding of the interactions that will occur in the natural environment after the release of CNTs. The present study aims to provide a stronger assessment of the effect of CNTs on bioavailability of PAHs by focusing on three main objectives:

1. ***Characterize the effect of NOM and concentration of MWNTs on the adsorption behavior of FLU.*** This was achieved by developing adsorption isotherms of FLU in the presence of three different NOM concentrations while also varying the concentration of MWNT within a single NOM concentration. Results were further analyzed for differences in adsorption capacity and surface heterogeneity.

2. ***Investigate the effect of NOM on the bioavailability of FLU to fish. Adult fathead minnows (P. promelas) were exposed to FLU in the presence and absence of varying concentrations of NOM.*** The response in terms of bile fluorescence was analyzed for significant reduction or enhancement when NOM was also present in the water.

3. ***Determine the bioavailability of FLU adsorbed to suspended MWNT to fish. Adult P. promelas were exposed to solutions of MWNTs with a known concentration of adsorbed FLU.*** The responses were compared with responses of fish exposed to identical concentrations of un-absorbed FLU, providing quantitative results on the bioavailability of adsorbed FLU.

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CHAPTER TWO

INTRODUCTION

Applications for carbon nanotubes (CNTs) in industrial, consumer and medical products have increased exponentially, resulting in increased production and release into the environment. It is currently estimated that concentrations of CNTs in the environment range from ng/kg to the low mg/kg range in sediment and occur in water in the low μg/L range (Velzebor et al., 2013; Mueller and Nowack, 2008). CNTs have many interesting physicochemical properties, including high surface-to-volume ratio and a very hydrophobic surface that make them unstable in aqueous environments. Hyung et al. (2007) reported that MWNTs formed stable suspensions in NOM, and Edgington et al (2010) demonstrated that MWNTs suspended in 2 mg/L NOM as DOC were available and caused acute and chronic effects to daphnids. Even in the presence of NOM, the highly hydrophobic surface area of CNTs can serve as adsorption sites for organic contaminants, like polycyclic aromatic hydrocarbons (PAHs) (Zhang et al., 2011). This phenomenon can greatly alter the transport and bioavailability of such contaminants (Yang et al., 2006). There is a significant concern that CNTs will behave as contaminant-transporters and that PAHs will desorb once ingested by an organism (Pan and Xing, 2008); this is supported in part by a recent study that found that phenanthrene would desorb from CNTs in simulated human digestive fluids (Wang et al., 2013).

Although some studies have examined the influence of CNTs on the bioaccumulation of adsorbed organic contaminants in various organisms, results are contradictory and sparse. CNTs present in soil have been found to decrease the

bioavailability of pyrene to earthworms (Peterson et al., 2011); however Shen et al. found that while CNTs sequestered PAHs from the pore water in sediment, when the PAH-CNT complex was ingested the bioaccumulation of the PAH increased (2012). Even fewer studies have investigated how stably suspended CNTs influence the bioavailability of contaminants to organisms in the water column in spite of the fact that CNTs are often processed in an aqueous suspension and have the potential to be released into the environment in this form. Furthermore, surfactants or NOM present in the environment can disperse and suspend CNTs, while simultaneously PAHs are adsorbing to the CNTs (Schwyzer et al., 2011). Recently a study found that phenanthrene sorbed to CNTs had contrasting effects on bioaccumulation of phenanthrene in Japanese Madeka, depending on how stably suspended the CNTs were. The more stably suspended CNTs did not have a significant effect while the less stable suspensions significantly increased the whole-body burden of the fish by 72 hours, potentially because the more stably suspended solutions was more easily eliminated (Su et al. 2013).

The adsorption of NOM to CNTs is efficient at stabilizing CNTs and can have a significant effect on the adsorption of PAHs. The presence of NOM can alter the adsorption capacity and strength of PAHs to CNTs, which may influence the degree to which CNTs affect bioavailability of the adsorbed PAHs (Wang et al., 2008). While PAH adsorption to CNTs in pure water is completely reversible, studies have found that in the presence of NOM, adsorption of PAHs on various other materials was no longer reversible (Yang & Xing, 2007; Sun et al., 2003). Considering that both NOM and PAHs are ubiquitous in the natural environment, the bioavailability of PAHs adsorbed to CNTs

cannot be accurately quantified without accounting for the influence of NOM. Kim et al. demonstrated that the presence of MWNTs increased the bioavailability and toxicity of Cu in NOM solutions (2009). The adsorption of NOM to MWNTs reduced the number of Cu-binding ligands in NOM thereby reducing the capacity of NOM to sequester the Cu. While a few studies have examined the interactions between NOM, PAHs, and MWNT in soil and sediment, to our knowledge no study has yet quantified the bioavailability of CNT adsorbed PAHs to fish in the presence of NOM.

Fluoranthene (FLU) is one of the most prominent PAHs in the environment with concentrations in the low µg/L range, reaching as high as 130 µg/L in storm runoff (Manoli & Samara, 1999). Few studies have examined the adsorption of FLU to CNTs and none have quantified the bioavailability of FLU adsorbed to CNTs. FLU is a good model PAH for this study as FLU can easily be analyzed in fish bile (Ariese et al. 1993).

The goal of this research was to quantify the bioavailability of FLU adsorbed to NOM-stabilized suspensions of MWNTs to *P. promelas*. Adsorption isotherms were developed to describe the interaction between FLU and MWNTs in the presence of NOM; the influence of NOM on the bioavailability of FLU to *P. promelas* was quantified; and the bioavailability of FLU adsorbed to NOM-stabilized MWNTs to *P. promelas* was characterized. The testable hypothesis of this current study was that FLU adsorbed to NOM-stabilized CNTs would not be bioavailable to *P. promelas*.

MATERIALS AND METHODS

Chemicals and Particles

Multi-walled carbon nanotubes (MWNT) (25nm diameter X 50µm length) with greater than 95% purity, were synthesized using the thermal chemical vapor deposition

method at Clemson University (Andrews et al., 2002). Fluoranthene (FLU) with a 98% purity was purchased from Ultra Scientific and stock solutions were prepared in methanol (HPLC grade). Natural organic matter (NOM) was collected from Suwannee River Visitor's Center (Fargo, GA) and vacuum filtered with a 0.45 μm Nylon filter, to give an aqueous stock of only the dissolved organic carbon (DOC) fraction. NOM was quantified as DOC using a Shimitzu total organic carbon (TOC) analyzer; Suwannee River stock water contained 70 mg C/L as DOC. Specific ultraviolet absorbance ($SUVA_{254}$) was used to characterize NOM; the SUVA254 value of the collected NOM in this study was 3.8 L/mg-m, a similar value to purchased Suwannee River NOM and indicative of a moderately hydrophobic and aromatic nature. All solutions were made in MHW which was prepared using reagent grade chemicals following the U.S. EPA recipe; 96mg/L $NaHCO_3$, 60mg/L $CaSO_4$, 60mg/L $MgSO_4$, 4mg/L KCl in 18 mega-ohm. MILLIPORE MILLI-Q water.

Suspension of MWNT

MWNTs were suspended in three different concentrations of NOM: 2, 5, and 10 mg C/L following published methods (Edgington et al., 2010). MWNTs were weighed on aluminum weigh paper and transferred to a 100mL glass centrifuge tube in a transfer hepafilter hood to which 100mL of a NOM solution was added. Solutions were then sonicated with a Branson model 450 digital sonifier with a 0.5 inch microtip (Branson) for 10 min with an output of 60 watts. Solutions were allowed to settle for approximately 24 hrs before the supernatant (stable suspension) was removed with a glass pipette. Sedimented MWNTs or those that settled out of solution after the 24 hr settling period,

were weighed using a pre-rinsed, oven dried and weighed 0.45μm nylon filter. The concentration of the stable MWNT suspension was estimated by mass difference between initial and sedimented MWNTs divided by the volume of solution. The concentration of stably suspended MWNT was confirmed by visible light absorbance at 800 nm using a Molecular Series Spectramax 190 microplate spectrophotometer (Hyung and Kim, 2008). MWNT suspensions were diluted to the desired concentrations with the same NOM solution that was used to produce the suspension.

Adsorption Isotherms

Adsorption of FLU to MWNTs was characterized using a full factorial experimental design with three concentrations of MWNT suspensions and three concentrations of NOM giving nine treatments. All treatments were run in triplicate, and adsorption isotherms of FLU to MWNTs were measured by a batch approach via centrifugation. For each treatment a 10mL glass centrifuge tube was filled with MWNT suspension leaving minimal head room and then spiked with a predetermined concentration of FLU. Initial concentrations of FLU were controlled to range over three orders of magnitude with methanol levels in each vial controlled to 0.1% volume fraction to avoid a co-solvent effect. Controls were spiked with only methanol. Once the tubes were sealed with aluminum-foil-lined Teflon screw caps they were placed on a rotary tumbler at room temperature (23ºC± 1°C) for 5 days. Previous studies indicated that sorption equilibrium was reached within 4 days (Wang et al. 2008; Yang et al., 2006). After tumbling, the samples were centrifuged at approximately 1500 g for 30 min; preliminary tests showed this was sufficient to sediment MWNTs out of the stable

suspension. The supernatant was analyzed for FLU in black polystrene 96-well plates with a Molecular Devices Gemini fluorescence microplate reader at 280/ 440 nm excitation/emission. Preliminary tests showed FLU concentration can accurately be quantified in the media by examining the extent of the fluorescence or relative fluorescent units (RFUs). Centrifuge tubes without MWNT suspensions and only FLU spiked DOC solutions served as positive controls. Analysis of a methanol rinse of the centrifuge tubes indicated negligible loss of FLU sorption to the centrifuge tube walls, therefore sorbed FLU to MWNTs could be calculated directly by concentration differences from the positive controls. The pH of the solutions from the beginning and end of the experiments reflected no difference, nor did a significant difference exist across the different treatments.

Isotherm Modeling

Experimental data was transformed and fit to the linearized form of the Freundlich and Langmuir models (Yang et al., 2006). The goodness of fit was analyzed by comparing the model constants, the correlation coefficients (R^2) and residual root mean square error (RMSE). In both models q_e is the equilibrium adsorbed concentration of FLU (ug FLU/ mg MWNT) and C_e is the equilibrium concentration of FLU left in liquid phase.

Freundlich model (FM):

$$\log q_e = \log K_f + n \log C_e$$

where K_f ((ug FLU/mg MWNT)/(ug FLU/L)) represents the Freundlich constant and adsorption capacity of the absorbent while *n* (dimensionless) represents the Freundlich exponent and is an indicator of surface heterogeneity.

Langmuir model (LM):

$$q_e = q_m - \{(1/K_L)*(q_e /C_e)\}$$

where q_m (ug FLU/ mg MWNT) is the saturation capacity of a single monolayer of sorbed FLU and K_L is the Langmuir constant indicative of adsorption affinity.

Organisms

Pimphales promelas were cultured at the Clemson University Institute of Environmental Toxicology (CU-ENTOX) in a flow-through system with a water turnover rate of 3-4 times a day (hardness = 87 mg/L as $CaCO_3$; alkalinity = 56 as $CaCO_3$; pH = 8). Temperature was maintained at 25°C ± 1°C with a 16/8 hr light/dark photoperiod. Once reaching maturity and a size greater than 45mm total length, *P. promelas* were transferred to a wet lab at Clemson Cherry Farm Fish Facility for holding and experiments. Holding aquaria were supplied with filtered water from Lake Hartwell on a flow-through system with the same previously described parameters (hardness = 15.84 mg/L as $CaCO_3$; alkalinity = 12 mg/L as $CaCO_3$; pH = 7.5). Fish were fed Tetramin mix to satiation daily during culturing and holding periods. Test organisms were acclimated for at least 7 days in holding aquaria and starved 24 hrs prior to and during exposures.

Experimental Design

Bioavailability of FLU in MHW

Exposures were static, non-aerated, and conducted for a total of 16 hrs with full water changes every 2.5 hrs. Water samples were taken at the beginning and end of each time point to verify aqueous FLU concentration. Six liters of MWH was spiked directly with FLU stock resulting in water concentrations of FLU ranging from 5-125 μg/L and stocked with *P. promelas* not to exceed 1 g biomass/L. Controls were spiked with only methanol. 65 female and 50 male fish were used in the exposure with total length ranging from of 45-65 mm and weight range of 1.5-3.5 g wet weight. Dissolved oxygen and ammonia concentrations were monitored during exposures to ensure it did not drop below 5 mg/L or exceed 0.5 mg/L, respectively. Temperature was maintained to ± 2°C of the temperature at the start of an exposure, generally in the range of 20-25ºC. At end of an exposure, fish were euthanized with 1.5 g/L of MS-222 (tricaine methanesulfonate) buffered with $CaCO_3$ (pH 7.0-7.5) and the gallbladder was harvested. Bile fluoresence was measured as a function of aqueous FLU concentration and used to construct a dose-response relationship for FLU exposure.

Effect of NOM on the Bioavailability of FLU

NOM solutions were prepared by diluting stock NOM to 2, 5, and 10 mg C/L with MHW and placed in separate 20 L glass containers. MHW only was used as the positive control. The solutions of 0, 2, 5, and 10 mg/L DOC were directly spiked with identical concentration of FLU then the holding glass containers were capped, and mixed for 24 hours allowing for equilibrium to be reached. Resulting aqueous concentrations of FLU ranged from 10-30 μg/L in each of the NOM solutions. After equilibrium was reached, 6 L of the prepared solution was placed in exposure aquaria where *P. promelas*

were stocked so as not to exceed 1g biomass/L. Every 2.5 hrs for 16 hrs the exposure water was fully renewed and water samples were taken at the beginning and end of each time point to verify aqueous FLU concentrations. Fish were similarly sized to those used in the dose response exposures and parameters such as temperature, DO and ammonia were maintained in the same manner. A total of 43 female and 39 male fish were exposed, with approximately 10 fish per treatment. At the end of the exposure fish were euthanized, the gallbladder harvested and the response to FLU exposure was determined via bile fluorescence.

Effect of MWNT on the Bioavailability of FLU

MWNT exposure solutions were similarly prepared to those for adsorption isotherms, with the following differences. MWNT was combined with a 5 mg C/L solution in 100 mL glass centrifuge tubes and sonicated for 30 mins at an output of 60 watts; preliminary experiments found these parameters to produce the highest concentration of stable MWNT suspension. After a 24 hr settling period the stably suspended MWNT suspensions were combined in a 20 L glass container. The initial concentration of this combined suspension was determined via UV-vis at 800nm and then diluted to the desired concentration of MWNT using a combination of 2 mg C/L and MHW so as to result in 20 L of MWNT suspension in a 2 mg C/L solution. The MWNT solution was then spiked with FLU and tumbled on a rotary tumbler in amber glass containers for 4.5 days.

After equilibrium was reached, *P. promelas* were exposed to the solution in 16 hrs static renewal tests with full water renewals occurring every 4 hrs. As in the previous

exposures discussed the same water quality and fish stocking parameters were maintained. Mortality occurred rarely and randomly among treatments and controls, indicating that MWNT alone had no adverse effect on the organism. At the end of the exposure series the organisms were euthanized, the gallbladder harvested and the response to the bioavailable concentration of FLU was determined via bile fluorescence.

In the initial exposure series controls were prepared with and without ~1.5 mg/L MWNT in 2 mg C/L solution, and spiked with only methanol. Positive controls were prepared with 2 mg/L C without MWNT and spiked with 25, 10 and 1 μg/L of FLU. Treatments containing ~1.5 mg/L MWNT were spiked with 25 and 10 μg/L FLU, estimated via the prepared adsorption isotherm to leave approximately 9 and 3 μg/L of un-adsorbed FLU. A total of 64 male fish were exposed with approximately 9 fish per treatments and control groups, although the MWNT treatment spiked with 25 μg/L FLU contained only 5 fish. Water samples were collected at the beginning and end of each renewal time point in all treatments to determine the concentration of both the freely dissolved FLU and adsorbed FLU. Bile fluorescence of fish, a function of metabolite concentrations, were compared across treatments with and without MWNT in a categorical manner.

In the secondary exposure series, the developed adsorption isotherm models were used to prepare solutions with a continuous range of freely dissolved (or un-adsorbed) FLU. MWNT suspensions of 0, 0.4, 0.6, 1.3, 1.7 and 2.5 mg/L were prepared and spiked with 25 μg/L FLU. A total of 13 male fish were exposed. Water samples taken from the beginning and end of each renewal were analyzed to establish the concentration of freely

dissolved FLU at each MWNT concentration. Bile fluorescence was plotted as a function of freely dissolved FLU and compared to the dose-response of male *P. promelas* to FLU in 2 mg/L NOM solutions.

Bile Analysis

Gallbladders harvested from *P. promelas* were stored in dark 1.5 ml centrifuge tubes in an -80ºC freezer until processed. After defrosting, the bile was drained from the gallbladder. 140µl of MILLIPORE MILLI-Q water was added to the microcentrifuge tube, the tube was vortexed then centrifuged for 2 mins at 14000 rpm. 25µl of the diluted bile was placed in a 0.5ml plastic micro centrifuge tube for a protein assay while 80µl were further diluted with 180µl of a 50:50 MeOH:H_2O solution resulting in an overall dilution of the bile to 1:50. Diluted bile fluorescence was analyzed in a black microplate at wavelengths 280/440nm using a Molecular Devices Gemini fluorescence microplate reader. Protein content was determined with the Pierce™ bicinchoniaic acid (BCA) protein assay, using Molecular Devices Spectramax 190 UV microplate reader to measure absorbance and bovine serum albumin (BSA) to prepare the standard curve. Bile fluorescence data was given without normalization, as normalization to protein content increased the coefficient of variance. Similar to other studies, while important to record protein content, normalization is not as necessary when feeding status was not varied among exposures (Vuorinen et al., 2006).

Statistical Analysis

Statistical tests were performed with the use of JMP® software, version 10, SAS Institute Inc. The linearized form of the adsorption isotherms were fit with linear

regression and compared across different CNT and NOM treatments using analysis of covariance (ANCOVA). All fish samples were analyzed for outliers using box and whisker plots on biliary protein content. Dose-response of fish to FLU exposure were also fit with linear regression and goodness of fit was determined by the correlation coefficient (R^2). The influence of gender on response was analyzed using ANCOVA. Response of fish to FLU in treatments containing NOM and also containing MWNT were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and linear squared contrasts to further investigate significance among treatments. Linear regression lines of the dose-response of fish in 2 mg C/L and fish exposed to 25 μg/L FLU with varying MWNT concentrations were compared using ANCOVA. Homogeneity of variance was tested for using the Brown and Forsythe test and normality was tested using Shapiro-Wilk's test. The level of significance for all tests was set at $p < 0.05$.

RESULTS AND DISCUSSION

FLU adsorption to MWNTs

When q_e vs C_e values for each treatment were plotted on linear coordinates, all adsorption isotherms were found to be nonlinear. Presented in Table 2.1 is the analysis of data from all the reps combined for each treatment. The variation among reps lowered the R^2 values when analyzed together and although separate analysis of each rep provided higher R^2 values, either way the results indicated that the data fit the FM better than LM throughout all treatments. Experimental data were therefore further analyzed when log transformed.

Regression analysis of the log transformed data provided values for the intercept and slope (K_f and *n*, respectively) which correlated with adsorption capacity and surface heterogeneity of adsorption to MWNT. An ANCOVA indicated that in the presence of 2 mg C/L NOM, there was no significant difference in K_f and *n* between the MWNT dosage of 1 and 2 mg/L, while 3.5 mg/L MWNT treatment had significantly lower K_f ($p = 0.0003$) and higher *n* ($p < 0.0001$) values (Fig. 2.1.A). In the presence of 5 mg C/L NOM there was a significant decrease of K_f ($p < 0.0001$) and increase in *n* ($p < 0.0001$) between the dosage of 1.5 and 6 mg/L MWNT (Fig. 2.1.B). In the presence of 10 mg C/L NOM, the increase of MWNT dosage from 1.5 mg/L to both 3 and 6 mg/L resulted in a significant decrease in K_f ($p = 0.005$ and $p < 0.0001$, respectively) and a significant increase in *n* ($p = 0.0025$ and $p < 0.0001$, respectively), although there was no significant difference between the dosage of 3 and 6 mg/L MWNT (Fig. 2.1.C.). Overall within all concentrations of NOM, the general trend observed with an increasing MWNT dose was a reduction in adsorption capacity (K_f) and a decrease in surface heterogeneity (*n*) (Table 2.1). Studies have suggested that this occurrence is consequential of entanglement and aggregate formation with increased concentrations of MWNT in solution, ultimately resulting in the loss of surface area and adsorption sites (Wu, 2007; Zhang et al., 2009). The degree to which aggregation may play a role in the results observed warrants more research as the presence of NOM in all treatments not only suspended and dispersed the MWNTs, but also further influenced the adsorption of FLU to MWNTs.

Considering that the concentration of MWNT had a significant effect on both K_f and *n* values, the effect of NOM was analyzed across similar MWNT concentrations

(~1.5, ~3 and 6 mg/L MWNT). At the concentration of ~1.5 mg/L MWNT, no significant difference was found in terms of K_f values while there were significant differences among *n* values ($p < 0.0001$) across all NOM concentrations, indicating that while the available adsorption sites had changed, the same amount still existed (Fig. 2.2.A). At the concentration of ~3 mg/L MWNT, there were significant differences among the *n* values ($p < 0.0001$) across all NOM concentrations and also a significantly reduced K_f value when in the presence of 10 mg C/L NOM compared to 2 and 5 mg C/L NOM ($p = 0.015$, $p = 0.014$, respectively). At 6 mg/L MWNT, adsorption isotherms were only obtained in 5 and 10 mg C/L NOM where both the *n* and K_f values were significantly different ($p = 0.0196$, $p = 0.032$, respectively).

In general at all doses of MWNT, surface heterogeneity (*n*) significantly increased in the following order of the concentration of NOM present in the water: 2<10<5. Also at all doses of MWNT, adsorption capacity was reduced when in the presence of 10 mg C/L NOM, although this reduction was not found to be statistically significant at ~1.5 mg/L MWNT. Based on these results adsorption capacity was most effected in the presence of 10 mg C/L NOM, perhaps because this concentration of NOM was two orders of magnitude higher than the concentration of FLU. Zhang et al found that NOM had less of an effect on adsorption when the concentration of the PAH and NOM were at similar levels, such is the case with the lower concentrations of NOM in the present study (2011). The lack of an ordinal trend in the surface heterogeneity suggests that the adsorption of NOM may also be altering the surface of MWNT (Wang et al., 2008). This would explain the significantly different *n* across NOM concentrations when there were

not significant changes in adsorption capacity. Further, previous studies have found the adsorption of other planar PAHs, like FLU, to MWNTs to be less affected by NOM as NOM lacks site selectivity on MWNT and therefore does not necessarily occupy the same sites (Zhang et al., 2011). Again indicating the adsorption of NOM may change surface heterogeneity, but not adsorption capacity until a higher NOM: PAH ratio occurred as is the case in 10 mg C/L. Data was also analyzed in terms of the ratio of NOM to CNT concentrations, but no trends were observed. This suggested that the effect of NOM on FLU adsorption may be a consequence of competition or other structural factors of the various components involved, rather than just a ratio effect of NOM and CNT. Because the highest adsorption capacities and R^2 values existed in the presence of 2 mg C/L NOM, exposures of MWNTs to organisms occurred only in the presence of 2 mg C/L NOM.

Table 2.1. Nonlinear model fits of adsorption of FLU on MWNTs in various concentrations of NOM

		Freundlich Model (FM)				Langmuir Model (LM)			
NOM	MWNT	*n*	K_f	R^2	RMSE	q_m	K_L	R^2	RMSE
2	1	0.50±0.02	3.48±0.19	0.968	0.07	23.26±4.49	0.21±0.06	0.780	0.04
	2	0.52±0.04	3.37±0.24	0.962	0.08	23.27±3.94	0.24±0.1	0.809	0.07
	3.5	0.72±0.04	2.29±0.27	0.925	0.14	54.75±10.25	0.04±0.001	0.735	0.16
5	1.5	0.18±0.01	3.25±0.18	0.588	0.12	6.44±0.8	1.9±1.08	0.470	0.07
	3	0.35±0.04	2.3±0.33	0.806	0.14	7.74±0.47	0.62±0.20	0.454	0.18
	6	0.82±0.11	0.57±0.16	0.715	0.27	119.38±92.6	0.04±0.01	0.453	0.60
10	1.5	0.41±0.07	2.47±0.6	0.717	0.19	9.77±1.05	0.47±0.25	0.347	0.14
	3	0.72±0.03	1.17±0.07	0.919	0.15	11.04±1.36	0.11±0.03	0.895	0.18
	6	1.56±0.13	0.13±0.06	0.758	0.35	2.59±1.56	0.068±0.01	0.462	2.50

NOM (mg/L): concentration of NOM present in treatment; MWNT (mg/L): concentration of MWNT present in treatment; *n* (dimensionless): index of nonlinearity; K_f (μg/mg)/(μg/L): adsorption capacity parameter for FM; q_m (μg/mg): maximum adsorption capacity; K_L (L/μg): adsorption affinity coefficient for LM; R^2: coefficient of determination; RMSE: residual root mean square error. Data represented as mean±95% confidence.

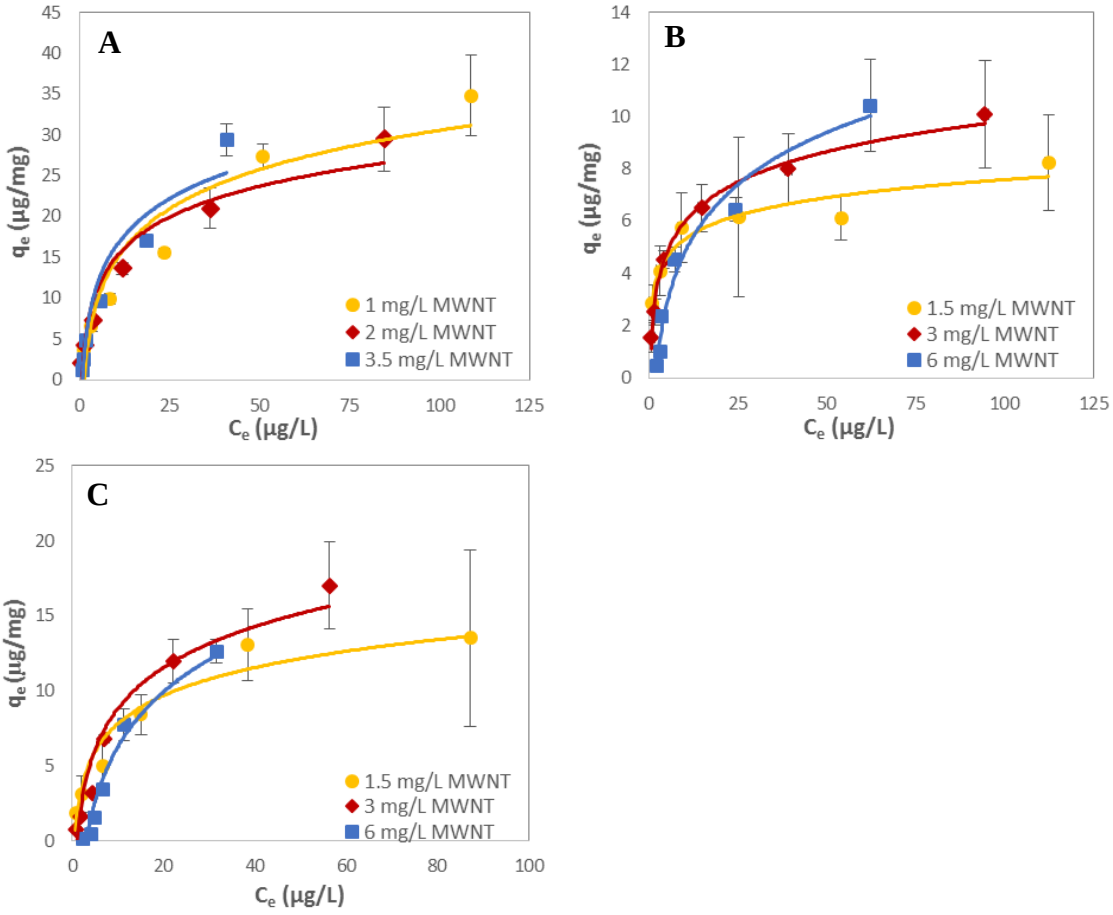

Figure 2.1. Adsorption isotherms of FLU to MWNT when MWNT dosage concentration is varied within the same concentration of NOM (A) 2 mg C/L (B) 5 mg C/L (C) 10 mg C/L. Error bars represent standard deviation among replicates.

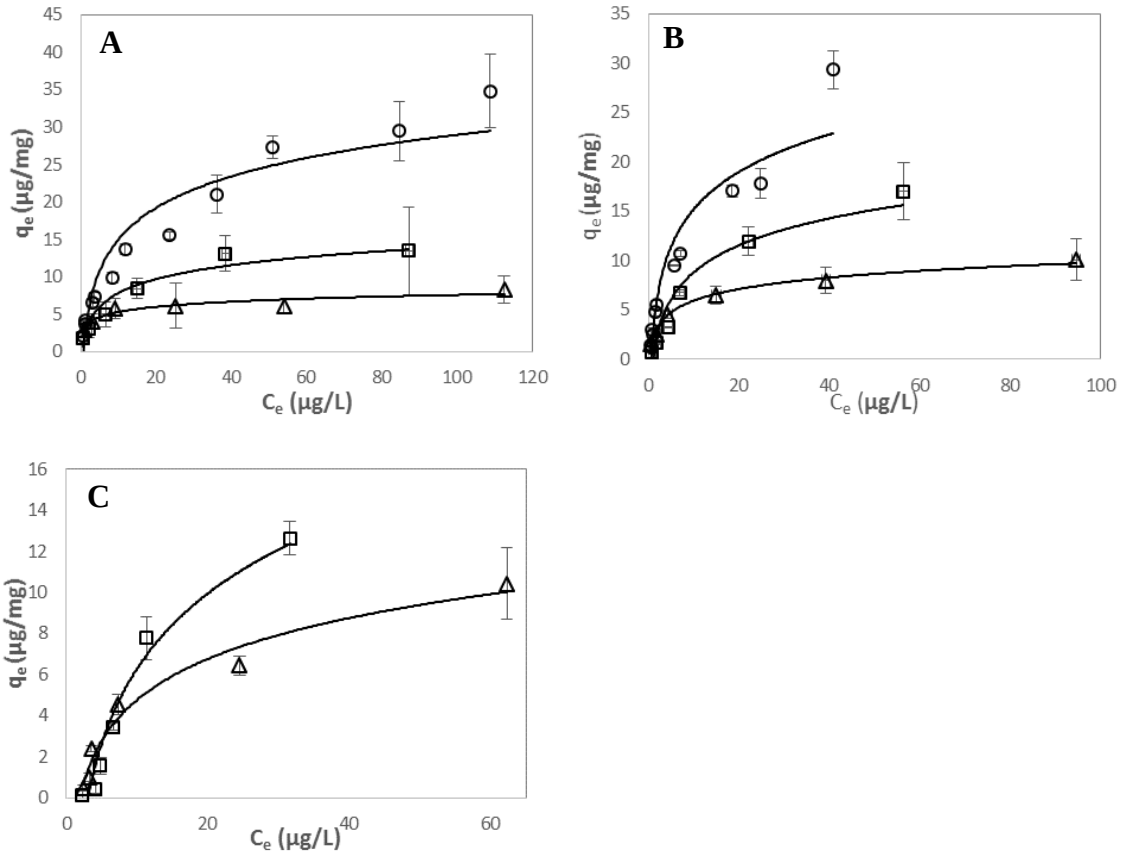

Figure 2.2. Comparison of adsorption isotherms of FLU to MWNT in the presence of 2 (○), 5(Δ) and 10 (□) mg C/L NOM at MWNT concentrations of (A) ~1.5 mg/L (B) ~3 mg/L (C) ~6 mg/L. Error bars represent standard deviation among replicates.

Bioavailability of FLU to *P. promelas*

Multiple studies have shown that in fish, bile fluorescence is directly proportional to the concentration of PAH metabolites indicating that bile analysis is a sensitive method to quantify the amount of PAH that is bioavailable (Ariese et al., 1993; Güngördü, 2011). The optimal wavelength pair for both the parent compound and metabolites of FLU was found to be 280/440 nm, similar to wavelength pairs stated in the literature (Ariese et al., 1993). As FLU in water exhibits a strong linear fluorescence concentration dependence (Schwarz and Wasik, 1976), the response of fish in terms of fluorescent intensity was plotted as a function of aqueous FLU exposure (µg/L) and analyzed via linear regression.

Exposure to aqueous FLU showed a dose-dependent increase of bile fluorescence in *P. promelas,* although the response in males was significantly lower than in females ($p < 0.0001$) (Fig. 2.3). Therefore, data were plotted separately for gender and analyzed using general linear regression ($R^2 = 0.65$ and $R^2 = 0.42$ for females and males respectively). Gender differences exist in fish in terms of biotransformation and metabolic rates; this can vary among species. In perch, eelpout and flounder, biliary fluorescence resulting from exposure to 4-ring PAH compounds was significantly higher in males than in females believed to be from higher CYP1A activity in males (Vuorinen et al. 2006). It is important to note that FLU is a known inhibitor of CYP1A (Willett et al., 1998); this may partially explain the contrasting results in the present study where the higher response and metabolite concentrations are observed in the females over the males. Further a different pathway than CYP1A may be responsible for the biotransformation of FLU. Not only have recent investigations found that PAHs can

activate PXR and induce CYP3A4 (Luckert et al. 2013), but Crago and Klaper found that expression of PXR and CYP3A4 in *P. promelas* was significantly higher in females than males and higher in unfed fish than fed fish when exposed to a range of xenobiotics (2011). Although, in the present study the fish were starved prior to exposure therefore feeding regime did not seem to have a significant influence on the response. Further, the response of both male and female fish in the controls exhibited the same degree of baseline bile fluorescence. Only once exposed to FLU did gender-differentiate responses appear, suggesting that a more likely explanation is the difference in the biotransformation of FLU between sexes. To account for gender differences in the remainder of experiments in the present study, males and females were analyzed separately.

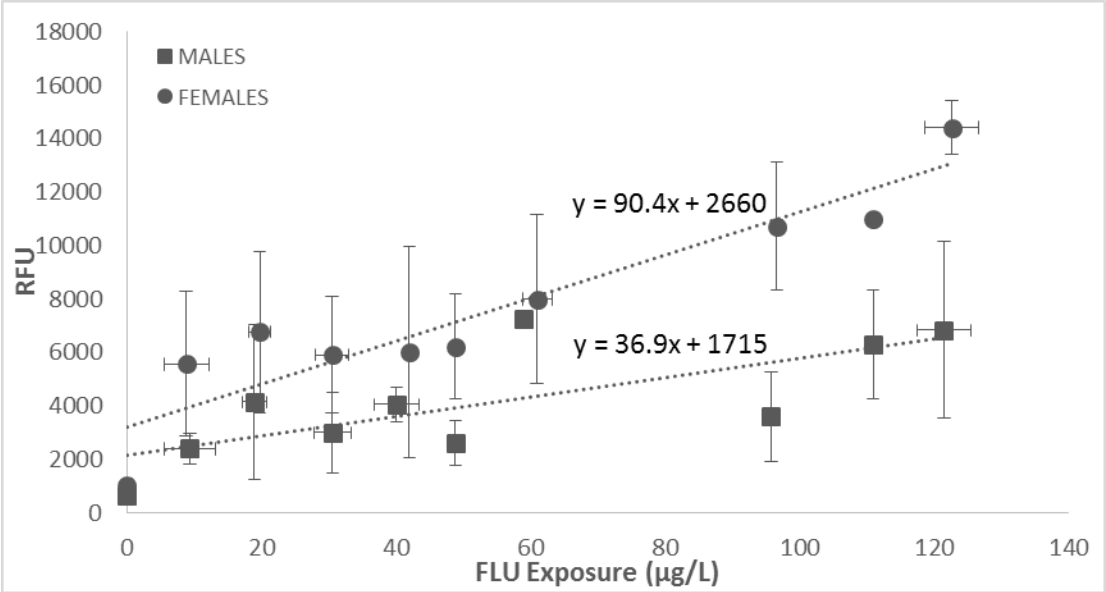

Figure 2.3. Dose-response relationship between aqueous FLU exposure to *P. promelas* and bile fluorescence (RFUs). Data points represent the mean bile fluorescence of individuals with error bars represented by standard deviation. Lines fitted by least-square regression model indicate a positive dose-dependent response observed in both sexes (R^2=0.42 and R^2=0.65, males and females respectively).

Effect of NOM on bioavailability of FLU

Aqueous measurements during exposures confirmed that actual exposure concentrations in each treatment were similar with an overall mean of 19 ± 7 µg/L FLU. An ANCOVA of the data showed that while gender did have a significant effect on bile fluorescence ($p < 0.0001$), there was no significant effect of NOM concentration or interactions between gender and NOM concentrations. Although males exhibited a lower response than females in terms of bile fluorescence, the response in both genders was statistically the same across the range of NOM concentrations indicating that NOM up to 10 mg C/L did not have an effect on the bioavailability of FLU (Fig.2.4).

Although it has been largely concluded that the presence of NOM will decrease the bioavailability and/or uptake of PAHs, the contradiction in the presented results can largely be explained by the hydrophobicity of the NOM used. The $SUVA_{254}$ of the NOM in this study, 3.8 L/mg-m, indicated that it was moderately hydrophobic, but not particularly rich in aromatic content, similar to other aquatic NOM sources (Weishaar et al., 2003). Studies that used NOM with greater hydrophobicity and aromaticity, as is characteristic of commercial humic acid, have partition coefficients for PAHs that are 5-7 times greater than that of typical aquatic NOM; use of which can greatly overestimate the effect of NOM on the bioavailability of PAHs (Weinstein and Oris, 1999; Chiou et al, 1986). Our results are in agreement with past studies that found no effect of aquatic NOM on the bioaccumulation of FLU in *D. magna*, although commercial humic acid did greatly reduce bioaccumulation (Akkanen et al., 2012; Gourlay et al., 2003). Because we found that NOM did not influence bioavailability of FLU to *P. promelas*, it is assumed in

exposures containing MWNTs, that the effects on bioavailability were directly linked to the presence of MWNTs.

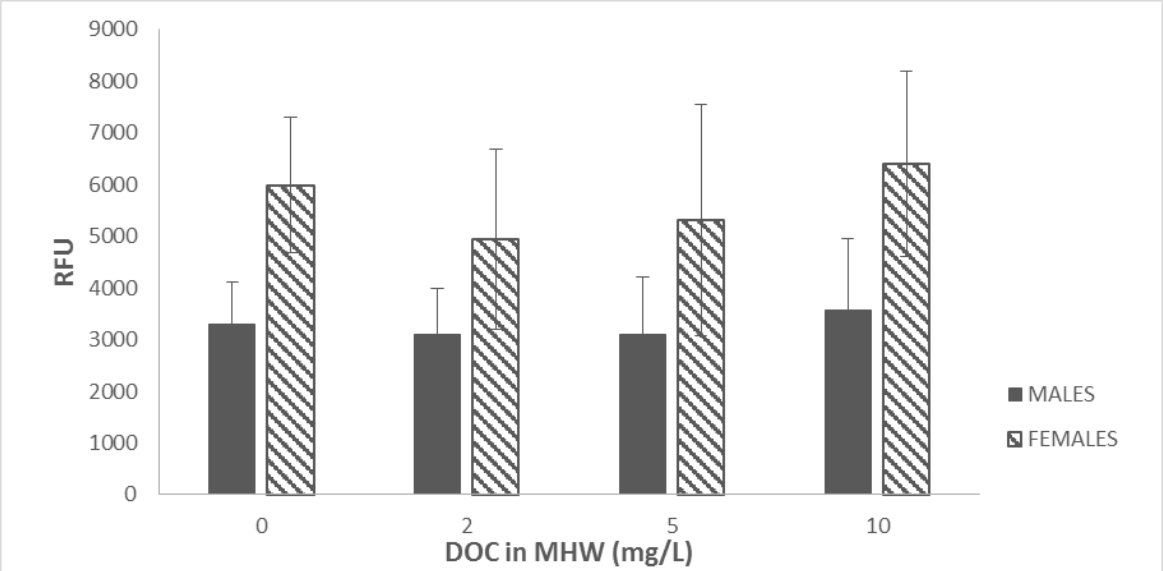

Figure 2.4. Effect of NOM on bile fluorescence of *P. promelas* exposed to 19 ± 7 µg/L FLU across varying NOM concentrations. Error bars represent the 95% confidence level determined from the standard error of means.

Effect of MWNT of the bioavailability of FLU

A two-way T test performed on the controls that were not spiked with FLU found that there was no significant difference in response of fish in treatments with or without MWNT. Therefore MWNTs did not have an effect on bile fluorescence and the measured response in FLU spiked treatments was directly related to the amount of bioavailable FLU. The response of fish in treatments that were spiked with 1, 10 and 25µg/L FLU were compared to the response of fish in the treatment with ~1.5 mg/L MWNT spiked with 10 and 25 µg/L FLU. Adsorption isotherms indicated that in the presence of ~1.5 mg/L MWNT, ~3 and ~9 µg/L FLU would remain un-adsorbed when spiked with 10 and 25 µg/L FLU, respectively (Table 2.2). An ANOVA followed by a Tukey's post hoc test indicated that the response of fish in treatments spiked with 1, 10 and 25µg/L of FLU were significantly different from the controls ($p = 0.0285$, $p < 0.0001$, $p < 0.0001$ respectively) and while the responses to 1 µg/L FLU were significantly different from the responses to 10 and 25 µg/L FLU ($p < 0.0001$), the responses to the higher two concentrations were not significantly different (Fig. 2.5). The response of fish in MWNT treatments were not significantly different from one another nor significantly different from the response of fish exposed to 1 µg/L FLU. Further, least squares contrast indicated that while the response to 25 µg/L FLU with MWNT was significantly lower than the response to just 25 µg/L without MWNT ($p = 0.0112$) it was not significantly different from the response to treatments spiked with 10 µg/L FLU. The results indicate that the presence of MWNTs significantly reduced the bioavailability of FLU to *P.*

promelas. Computations based on the adsorption isotherms suggested that only the un-adsorbed fraction of FLU in the MWNT treatments was available; observed bile fluorescence of fish exposed to 10 and 25 μg/L FLU in the presence of MWNTs were similar to the predicted bile fluorescence of fish exposed to 3 and 9 μg/L FLU, respectively (Table 2.2). Regression analysis of predicted vs observed bile fluorescence indicated a fairly good fit in both treatments with and without MWNT, indicating that fish were responding to just the unadsorbed fraction of FLU (Fig. A.1).

Table 2.2. Comparison of the predicted and observed response of fish expressed as bile fluorescence as a function of predicted and actual FLU exposure.

		Predicted		**Observed**	
MWNT	FLU Dose	Ce	Response to Ce	Ce	Response to Ce
0	0	0	1384	0	949±142
0	1	1	1488	1±0.6	1453±337
0	10	10	2424	5±0.5	2346±744
0	25	25	3984	16±3	2529±453
~1.5	0	0	1384	0	870±364
~1.5	10	3	1696	0.3±0.5	1643±501
~1.5	25	9	2320	9±3	1817±525

MWNT (mg/L): concentration of MWNT present in treatment; FLU Dose (μg/L): concentration of FLU spiked into treatments; Predicted Ce (μg/L): concentration of FLU predicted to remain un-adsorbed, determined by adsorption isotherm; Predicted Response to Ce (RFUs): predicted bile fluorescence in fish when exposed to predicted Ce, determined from dose-response prediction line Y=104X+1384; Observed Ce (μg/L) and response (RFUs): actual concentration of FLU measured in water and actual bile fluorescence observed in treatments. Data represented as mean ± standard deviation.

To further clarify if the response of fish was dose-dependent of CNT concentrations, *P. promelas* were also exposed to varying MWNTs concentrations all spiked with 25 μg/L of FLU, resulting in a range of concentrations of un-adsorbed FLU. ANCOVA was used to compare the response of fish as a function of the un-adsorbed concentration of FLU in MWNTs treatments to the dose-response of fish to FLU in 2 mg C/L NOM. The regression line from this exposure series was concurrent with the

regression line observed in the dose-response, indicating a dose-dependence on MWNT concentrations. This further supports our findings from the previous exposure series that only the un-adsorbed FLU in the water was bioavailable and metabolized by *P. promelas* (Fig. 2.6).

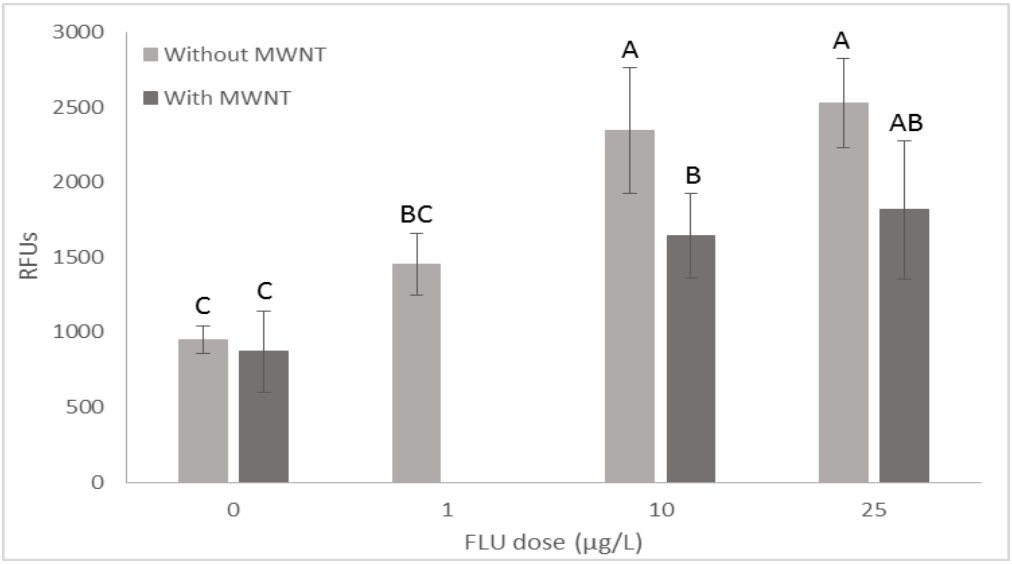

Figure 2.5. Response of male *P. promelas* in treatments with and without MWNT in 2 mg C/L NOM expressed in terms of the spiked FLU concentration. Error bars are represented by 95% confidence intervals determined from the standard error of means. Levels not connected by the same letters are significantly different based off Tukey's Post hoc test (p-value < 0.05).

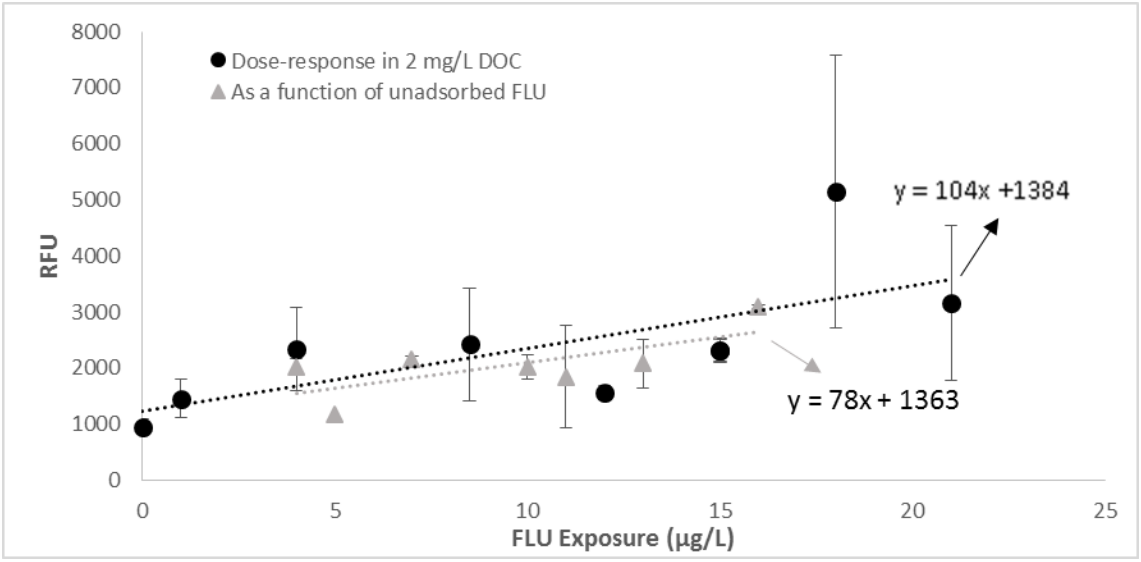

Figure 2.6. Regression lines for the correlation of bile fluorescence as a function of the un-adsorbed portion of FLU in solutions with MWNT (R^2 = 0.34) and for the dose-response of male *P. promelas* to FLU exposure in 2 mg C/L NOM (R^2 = 0.39). No significant difference between lines. Error bars are represented by standard deviation.

Though studies have shown the uptake of carbon nanotubes across the gills in fish exposed to CNT suspensions, the main route of exposure in the present study was through ingestion where *P. promelas* consumed the FLU-MWNT complexes (Smith et al., 2007). A comparable study by Su et al., also found that SWCNTS were primarily found in the digestive tract of the fish species, Japense madeka, when exposed to treatments of SWCNT and phenanthrene, indicating that exposure to PAHs associated with CNTs primarily occurred in the gut tract (2013). Studies have found that while the uptake of PAHs with similar water solubilities to FLU are readily taken up across the gills, association of these PAHs with particles such as sediment reduced the uptake of the PAH; a consequence of a reduction of the free fraction of the PAH left dissolved in the water (Kolok et al., 1996). Our results suggest a similar mode of action, where FLU adsorbed to MWNTs becomes unavailable to *P. promelas*, while the remaining fraction of free FLU in the water is still bioavailable for uptake across the gills and gut tract.

Such findings are in accordance with Peterson et al., who reported a reduction in the bioaccumulation of pyrene in earthworms that consumed soil with MWNTs present (2009). In contrast, the ingestion of SWCNT- associated phenanthrene, by Japanese madeka, showed an increase in phenanthrene bioaccumulation. This was largely attributed to the degree to which SWCNTs aggregated in the gut tract, which directly correlated with increased retention time of phenanthrene. Treatments with more stably suspended SWCNTs were quickly taken up and expelled from the body, resulting in low phenanthrene accumulation (Su et al., 2013). Studies have concluded that not only are suspensions of MWNTs more stable than those of SWCNTs, as well as MWNTs are less

prone to aggregation, but MWNTs have been found to be eliminated at a significantly higher rate than SWCNTs from the body (Vaisman et al., 2006; Peterson et al., 2013). In the present study, the use of stably suspended MWNTs rather than SWCNTs may partially explain why adsorbed FLU was not bioavailable when ingested by *P. promelas*.

The use of NOM to enhance the stability of MWNTs potentially influenced the bioavailability of FLU as well. A recent study found that while the presence of MWNT in sediment reduced the bioaccumulation of pyrene to some degree, the presence of NOM further enhanced this reduction (Shen et al., 2013). In the presence of humic acid, it has been found that PAH adsorption to various materials is no longer reversible. PAHs can partition into already adsorbed humic acid matrices or become entrapped in nanopores, resulting in desorption hysteresis (Sun et al., 2003). The adsorption-desorption process of PAHs from CNTs seems to play a key role in the bioavailability of PAHs. The adsorption of phenanthrene to SWCNTs in the study by Su et al. was reversible in the treatment conditions and also found to be bioavailable once consumed by the fish, though the influence of NOM was not accounted for in this study (2013). Considering that adsorption of NOM to MWNTs is not completely reversible (Su and Lu, 2014) and that NOM can cause irreversibility of PAH adsorption to various other adsorbents, the influence of NOM on FLU desorption from MWNT may explain the contrasting results of the present study.

Studies have also suggested that conditions in the digestive fluids can influence desorption of PAHs from CNTs; high concentrations of bile salts, in simulate human gastrointestinal fluids, significantly increased desorption of phenanthrene from SWCNTs

and MWNTs (Wang et al., 2011). Though in the present study, *P. promelas* did have elevated levels of bile salts as a result of a starvation period prior to exposure, the digestive conditions of a fish are very different than that of a human or mammal; retention time in the digestive tract and dietary efficiency or adsorption is significantly lower in fish, which are influential on the uptake of organic contaminants (Kelly et al., 2004). Wang et al. did note that the structure of CNTs had an effect on how bioaccessible phenanthrene was, resulting in 50-80% total desorption of phenanthrene after 6 hours in simulated digestive fluids, a much longer time than would occur in *P. promelas*. Further, adsorbed pepsin, a biomolecule with both hydrophilic and hydrophobic properties, suppressed desorption of phenanthrene; the adsorption of NOM may have acted similarly to suppress desorption of FLU from MWNT in the present study.

Environmentally, CNTs have often been considered in terms of their environmental pollution management potential. They have a higher adsorption capacity for many organic contaminants in comparison to a number of adsorbents, such as activated carbon, that are already used remedially for contaminated water. Further, not only can CNTs adsorb organics and metals ions efficiently, but studies have shown that by altering the structure and the surface of CNTs, even higher adsorption capacities can be reached (Ren et al., 2011). To date, though, many studies have concluded CNTs to act as contaminant carriers in the aquatic environment, largely as a result of studies finding a lack of desorption hysteresis in pure water and in turn predicting increased bioaccumulation of contaminants in aquatic organisms. Though some studies have investigated the bioavailability and bioaccumulation of contaminants adsorbed to CNTs,

most have failed to incorporate the influence of NOM, although NOM is present in all natural aquatic systems. A handful of studies, including the present one, have found a decrease in the bioavailability of PAHs adsorbed to CNTs when in the presence of NOM, to a range of organisms. Such findings are promising for the use of CNTs as a remedial tool that could potentially tie up contaminants without adverse effects to the aquatic organisms present. Though further work is required, future studies should focus on how CNTs effect the bioavailability of a range of contaminants when in the natural environment.

In conclusion, MWNT reduced the bioavailability of FLU to *P.promelas.* This was due to adsorption of FLU to MWNT and results indicated that this fraction of FLU was completely un-available. NOM did not have an effect on the bioavailability of FLU in water although it did significantly influence the surface heterogeneity and adsorption capacity of MWNT for FLU. The effect of NOM may not have been limited to just adsorption; rather it may have had an influence on the desorption process of FLU from MWNT further reducing bioavailability of the bound fraction. The influence of NOM on the adsorption-desorption processes of PAHs to CNTs should be further studied as desorption of PAHs from CNTs seems to be the main driver in bioavailability of the PAH to aquatic organisms.

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CHAPTER THREE

CONCLUSIONS AND FUTURE STUDIES

As far as we are aware no other study has examined the bioavailability of an adsorbed PAH on MWNTs when in the presence of NOM to fish, although this would simulate a likely interaction to occur in the natural environment. The present study aimed to test the hypothesis that adsorbed FLU to MWNTs was not bioavailable in the presence of NOM, by also examining the influence of NOM on the adsorption behavior of FLU and the on the bioavailability of FLU to *P. promelas*. Results indicated that NOM had a significant effect on surface heterogeneity at all concentrations of NOM and had the potential to reduce adsorption capacity of FLU when present at 10 mg/L DOC. Although NOM had a significant effect on adsorption, it had no effect on the bioavailability of FLU exposure in water, where responses of *P. promelas* were similar across all concentrations of NOM. The presence of MWNTs significantly reduced the amount of FLU metabolites present in the bile of *P. promelas,* providing strong evidence that MWNTs reduced the bioavailability of FLU. Further, results indicated that *P. promelas* only responded to the free concentration of FLU remaining in the water therefore adsorbed FLU was not bioavailable to *P. promelas*, confirming our hypothesis.

The ubiquity of NOM and PAHs in the environment leads to a number of interactions to occur with the entrance of CNTs including the suspension of CNTs, the adsorption of both PAHs and NOM, and the exposure of aquatic organisms to these complexes. The importance of such a study as the one conducted, is that those relevant interactions were all taken into account therefore the results are applicable to what is

occurring in the environment. Not only did this study provide insight on the bioavailability of FLU adsorbed to suspended MWNTs in the presence of NOM, but also insight on the adsorption behavior of FLU to MWNT in varying conditions. From the results of this study it can be concluded that MWNT in the presence of 2 mg/L NOM does not act as a contaminant carrier for FLU. Further research is needed to understand the mechanisms behind this.

Future studies should investigate the potential change in the bioavailability of adsorbed FLU to *P. promelas* in a range of concentrations of NOM, as well as in the presence of NOM of different hydrophobicities. Considering that NOM influenced the surface heterogeneity and adsorption capacity of MWNT for FLU, there may be a relationship between these adsorption parameters and the associated bioavailability of adsorbed FLU. Characterization of the effect of different NOM sources on adsorption of FLU to CNTs would provide information on the associated risk of adsorbed FLU throughout varying conditions and may provide a good model for other PAHs. Further investigations should also focus on the impact of NOM on the desorption-adsorption process of FLU from MWNT, as this may provide insight into the mechanism in which NOM influences the adsorption of FLU and the ultimate influence on bioavailability. This could be done by comparing desorption of FLU from MWNTS in solutions with a range of NOM concentrations and in solutions with different water chemistry. Considering pH change and ionic strength can have a large effect on the interaction of NOM and CNTs, it is important to incorporate changes in such water chemistry parameters to provide a more complete understanding of the influence of NOM on PAH

adsorption to CNTs. The use of microscopy techniques to help visualize the surface of MWNTs when in the presence of varying concentrations of NOM may be able to provide insight to whether PAHs are becoming trapped or sequestered. There is also great potential to expand this study to investigate the bioavailability of other contaminants adsorbed to CNTs in a simulated natural environment. Ultimately, characterizing the water chemistry parameters that influence the bioavailability of adsorbed contaminants, may lead to the ability to model and predict bioavailability based off adsorption-desorption isotherms.

APPENDIX

Table A.1. Raw data; C_e and q_e for adsorption isotherms in 2, 5 and 10 mg/L NOM

NOM (mg/L)	CNT (mg/L)	Spiked FLU (µg/L)	rep 1		rep 2		rep 3		AVG	
			Ce	qe	Ce	qe	Ce	qe	Ce	qe
2	0	0	0.00		0.00		0.00		0.00	
2	0	5	4.77		4.74		4.99		4.83	
2	0	11	10.01		9.46		9.89		9.79	
2	0	22	19.20		18.63		17.09		18.31	
2	0	44	39.63		38.61		39.19		39.15	
2	0	83	81.29		76.44		77.10		78.27	
2	0	147	148.47		141.29		141.15		143.63	
2	3	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	3	5	0.33	1.48	0.71	1.35	0.55	1.48	0.53	1.44
2	3	11	0.64	3.12	1.17	2.76	0.56	3.11	0.79	3.00
2	3	22	1.42	5.93	2.03	5.53	1.96	5.04	1.80	5.50
2	3	44	7.21	10.81	5.77	10.95	8.09	10.37	7.03	10.71
2	3	83	22.69	19.53	25.40	17.01	26.26	16.95	24.78	17.83
2	1	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	1	5	1.69	3.09	1.02	3.72	0.90	4.09	1.20	3.63
2	1	11	2.82	7.19	3.40	6.06	3.56	6.32	3.26	6.52
2	1	22	8.92	10.28	8.35	10.28	7.99	9.10	8.42	9.89
2	1	44	23.61	16.01	23.03	15.59	24.16	15.03	23.60	15.54
2	1	83	55.24	26.04	49.52	26.91	48.06	29.04	50.94	27.33
2	1	147	108.56	39.91	111.25	30.03	106.63	34.52	108.81	34.82
2	2	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	2	5	0.80	1.99	0.64	2.05	0.39	2.30	0.61	2.11
2	2	11	1.73	4.14	0.97	4.24	1.29	4.30	1.33	4.23
2	2	22	3.96	7.62	3.45	7.59	3.84	6.63	3.75	7.28
2	2	44	12.11	13.76	12.83	12.89	10.64	14.28	11.86	13.64
2	2	83	33.52	23.88	37.82	19.31	37.35	19.87	36.23	21.02
2	2	147	80.37	34.05	87.27	27.01	86.31	27.42	84.65	29.49
2	3.5	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	3.5	5	0.82	1.13	0.44	1.23	0.99	1.14	0.75	1.17
2	3.5	11	1.32	2.49	0.76	2.49	1.20	2.48	1.09	2.48
2	3.5	22	1.99	4.92	1.19	4.98	1.74	4.39	1.64	4.76
2	3.5	44	5.94	9.63	5.25	9.53	5.90	9.51	5.70	9.56
2	3.5	83	19.93	17.53	19.13	16.37	16.37	17.35	18.48	17.08

2	3.5	147	37.79	31.62	43.17	28.03	41.66	28.43	40.87	29.36
5	0	0	0.00		0.00		0.00		0.00	
5	0	4	5.12		6.74		3.84		5.24	
5	0	10	8.46		10.04		9.28		9.26	
5	0	18	16.38		18.93		18.11		17.81	
5	0	35	31.61		35.44		36.41		34.49	
5	0	64	66.96		65.26		57.34		63.19	
5	0	136	123.43		121.30		129.83		124.85	
5	1.5	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1.5	4	0.44	3.12	1.62	3.42	0.70	2.09	0.92	2.88
5	1.5	10	3.96	2.99	3.12	4.61	2.28	4.66	3.12	4.09
5	1.5	18	10.08	4.20	9.23	6.47	8.25	6.57	9.18	5.75
5	1.5	35	26.43	3.45	27.16	5.52	22.18	9.49	25.26	6.15
5	1.5	64	58.82	5.42	54.72	7.03	48.50	5.90	54.01	6.12
5	1.5	136	107.95	10.32	109.99	7.53	119.50	6.89	112.48	8.25
5	3	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	3	4	0.44	1.56	0.40	2.11	0.87	0.99	0.57	1.56
5	3	10	0.54	2.64	1.15	2.97	3.39	1.96	1.69	2.52
5	3	18	2.72	4.55	6.36	4.19	3.57	4.84	4.22	4.53
5	3	35	15.19	5.47	14.54	6.97	15.09	7.11	14.94	6.52
5	3	64	40.79	8.72	38.83	8.81	38.00	6.45	39.21	7.99
5	3	136	99.81	7.87	85.46	11.94	98.33	10.50	94.54	10.11
5	6	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	6	4	1.36	0.63	4.30	0.41	1.43	0.40	2.36	0.48
5	6	10	1.75	1.12	5.53	0.75	2.62	1.11	3.30	0.99
5	6	18	2.34	2.34	5.51	2.24	2.83	2.55	3.56	2.37
5	6	35	7.07	4.09	8.68	4.46	6.08	5.05	7.28	4.53
5	6	64	28.87	6.35	23.50	6.96	21.09	6.04	24.49	6.45
5	6	136	71.44	8.67	58.64	10.44	56.82	12.17	62.30	10.43
10	0	0	0.00		0.00		0.00		0.00	
10	0	4	1.95		3.11		3.95		3.01	
10	0	8	6.76		7.31		6.19		6.75	
10	0	15	11.86		15.25		15.28		14.13	
10	0	30	26.69		28.67		26.71		27.36	
10	0	61	55.33		61.69		57.03		58.02	
10	0	111	105.76		114.82		101.80		107.46	
10	1.5	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	1.5	4			0.21	1.93	1.20	1.83	0.71	1.88
10	1.5	8	2.61	2.77	0.55	4.51	2.93	2.17	2.03	3.15
10	1.5	15	7.13	3.15	5.83	6.28	6.89	5.60	6.62	5.01

10	1.5	30			17.45	7.48	12.68	9.35	15.06	8.42
10	1.5	61	39.82	10.34	40.24	14.30	35.18	14.57	38.41	13.07
10	1.5	111	95.08	7.12	86.93	18.59	79.53	14.85	87.18	13.52
10	3	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	3	4	0.56	0.46	0.65	0.82	1.07	0.96	0.76	0.75
10	3	8	1.38	1.79	2.85	1.49	1.18	1.67	1.80	1.65
10	3	15	2.02	3.28	5.40	3.28	5.92	3.12	4.45	3.23
10	3	30	6.18	6.84	7.43	7.08	7.25	6.49	6.95	6.80
10	3	61	21.24	11.36	20.82	13.63	24.31	10.91	22.12	11.96
10	3	111	55.99	16.59	54.50	20.10	58.66	14.38	56.38	17.02
10	6	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	6	4	1.50	0.07	1.20	0.32	4.17	0.00	2.29	0.13
10	6	8	3.75	0.50	3.67	0.61	5.04	0.19	4.15	0.43
10	6	15	5.25	1.10	3.92	1.89	5.24	1.67	4.80	1.55
10	6	30	6.73	3.33	6.62	3.67	6.51	3.37	6.62	3.46
10	6	61	13.79	6.92	7.91	8.96	12.35	7.45	11.35	7.78
10	6	111	32.86	12.15	33.29	13.59	28.56	12.21	31.57	12.65

C_e (μg/L): Unadsorbed FLU remaining in aqueous form after equilibrium has been reached; q_e (μg/mg): FLU adsorbed per mg of MWNT after equilibrium has been reached

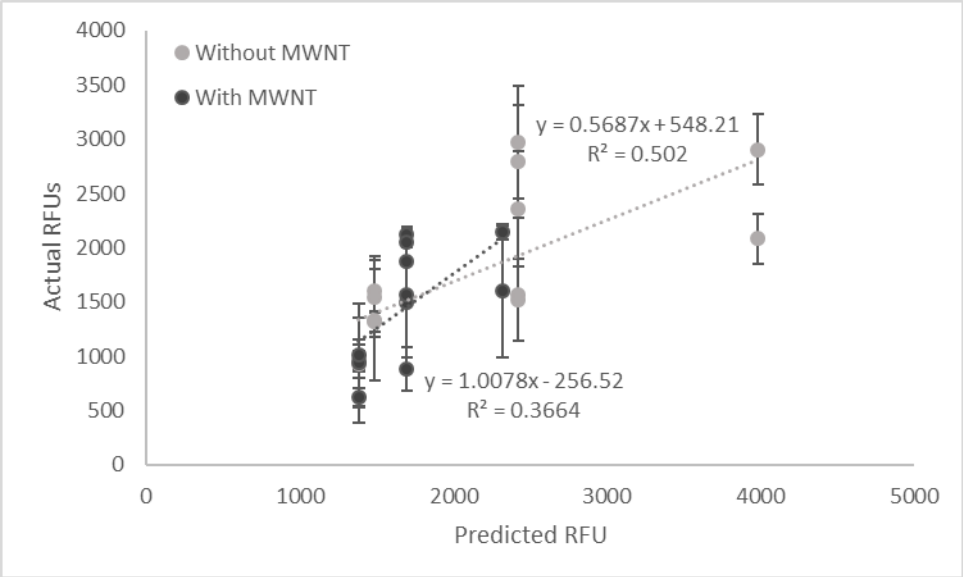

Figure A.1. Regression analysis of predicted bile fluorescence vs. actual bile fluorescence in treatments with and without ~1.5 mg/L MWNT and a range of FLU concentrations. Data represented is average bile fluorescence of all fish from an individual experiment and error bars represent standard deviation among individual fish. There was a significant correlation between predicated and actual response in treatments without and with MWNT ($p<0.0001$ and $p=0.0085$, respectively)

Table A.2. Raw data; bile fluorescence and protein content of *P. promelas* for dose-response to FLU exposure in MHW

Date	Sample Id	Aqueous FLU (µg/L)	Sex	Bile Notes	Blank Corrected RFU	Protein Content (mg/mL)	Normalized RFU to Protein
8_19	1_2_C	0	f	lime	1017.0	0.9	1105.0
8_19	1_3_C	0	f	lime	741.9	1.2	614.2
8_19	1_4_C	0	f	lime	954.8	0.8	1217.7
8_19	1_2_50	12	f	lime	7671.2	1.3	5758.7
8_19	1_4_50	12	f	lime	11300.0	1.4	7824.8
8_19	1_1_100	42	f	lime	3210.4	1.2	2644.4
8_19	1_4_100	42	f	lime	8795.9	1.0	8724.7
8_19	1_2_175	95	f	lime	9650.2	1.3	7195.3
8_19	1_4_250	118	f	lime	13319.0	1.7	7772.6
8_19	1_1_C	0	m	lime	654.4	1.6	418.9
8_19	1_1_50	12	m	lime	2239.1	1.3	1769.4
8_19	1_3_50	12	m	lime	2803.9	2.4	1159.1

8_19	1_2_100	42	m	clear	2325.0	1.8	1265.2
8_19	1_3_100	42	m	lime	5835.7	2.7	2165.8
8_19	1_1_175	95	m	lime	2749.8	0.9	3142.8
8_19	1_3_175	95	m	lime	1984.4	2.7	725.1
8_19	1_4_175	95	m	lime	2792.6	2.4	1154.5
8_19	1_1_250	118	m	lime	6762.0	1.3	5064.7
8_19	1_3_250	118	m	lime	11043.0	2.4	4682.4
8_22	2_1_C	0	f	lime	1228.3	1.0	1256.1
8_22	2_2_C	0	f	lime	966.2	1.7	579.2
8_22	2_3_C	0	f	lime	825.5	0.8	980.8
8_22	2_4_C	0	f	lime	1092.1	0.7	1528.5
8_22	2_1_50	21	f	lime	10501.2	0.9	11298.3
8_22	2_2_50	21	f	lime	8886.2	0.7	12873.4
8_22	2_4_50	21	f	lime/small	6936.2	0.4	16723.0
8_22	2_1_100	59	f	lime	7298.1	1.1	6809.6
8_22	2_2_100	59	f	lime/small	9670.5	0.8	12477.3
8_22	2_3_100	59	f	lime	3235.6	0.3	13033.3
8_22	2_1_175	97	f	lime	7724.9	0.7	10950.9
8_22	2_2_175	97	f	lime	12676.2	2.1	5939.0
8_22	2_2_250	111	f	lime	10984.2	1.2	9302.9
8_22	2_3_50	21	m	lime	7480.8	1.7	4373.3
8_22	2_4_100	59	m	lime	7244.5	1.6	4575.3
8_22	2_3_175	97	m	lime	4301.0	2.1	2044.1
8_22	2_4_175	97	m	lime	6179.3	1.9	3204.1
8_22	2_1_250	111	m	lime	7690.1	1.8	4305.2
8_22	2_3_250	111	m	lime	7211.9	2.9	2526.1
8_22	2_4_250	111	m	lime	3976.2	1.8	2256.6
8_23	3_2_C	0	f	lime	1208.5	1.2	997.9
8_23	3_4_C	0	f	lime/tiny	968.1	0.7	1402.5
8_23	3_3_50	26	f	lime	3952.8	1.2	3200.0
8_23	3_4_50	26	f	lime	3728.2	1.6	2363.6
8_23	3_1_100	63	f	lime	12729.9	1.3	10011.2
8_23	3_2_100	63	f	lime	8400.8	1.3	6513.6
8_23	3_3_100	63	f	lime	6632.3	0.7	9165.9
8_23	3_2_175	97	f	lime	9382.7	1.5	6416.4
8_23	3_3_175	97	f	lime	14274.9	1.9	7448.7
8_23	3_4_175	97	f	lime	10630.9	1.3	8340.6
8_23	3_1_250	125	f	lime	15242.9	0.9	16399.9
8_23	3_2_250	125	f	lime	14670.9	1.2	11994.6
8_23	3_1_C	0	m	lime	485.5	0.9	529.2

8_23	3_3_C	0	m	lime	612.5	1.9	326.9
8_23	3_1_50	26	m	lime	3663.6	0.9	4047.1
8_23	3_2_50	26	m	lime	3075.5	2.3	1352.7
8_23	3_4_100	63	m	lime	7453.7	4.2	1775.0
8_23	3_1_175	97	m	clear	4130.0	3.6	1154.1
8_23	3_3_250	125	m	lime	6564.5	2.2	2986.6
8_23	3_4_250	125	m	lime	2977.1	1.0	3082.6
1_14	0.1f	11	f	lime	5001.5	2.5	1992.8
1_14	0.2f	11	f	lime	2726.8	2.0	1357.0
1_14	0.3f	11	f	lime	5483.4	1.5	3672.7
1_14	0.4f	11	f	lime	5542.1	1.3	4184.5
1_14	0.1m	13	m	lime	3006.9	6.9	438.0
1_14	0.2m	13	m	lime	3268.8	1.6	2032.8
1_14	0.3m	13	m	grey	2870.6	2.0	1436.2
1_14	0.4m	13	m	lime	2463.4	2.9	850.1
1_21	0F.1	0	f	lime	1030.5	0.9	1210.9
1_21	0F.2	0	f	lime	1048.3	0.8	1254.3
1_21	0F.3	0	f	lime	753.0	0.6	1255.0
1_21	0F.4	0	f	lime	1854.6	1.3	1383.2
1_21	0F.5	0	f	lime/empty	525.6	0.6	948.5
1_21	50F.2	5	f	clear/large	3451.2	0.7	5243.6
1_21	50F.3	5	f	lime/tiny	4774.3	0.8	5630.3
1_21	50F.4	5	f	lime/tiny	2833.8	0.4	7120.7
1_21	50F.5	5	f	lime	6282.8	0.6	11339.0
1_21	0F.1	0	m	lime	617.6	1.3	459.6
1_21	0F.2	0	m	clear	1138.5	1.5	755.9
1_21	0F.3	0	m	lime	513.1	1.3	399.0
1_21	0F.4	0	m	clear	673.7	1.3	538.1
1_21	0F.5	0	m	lime	839.3	4.4	189.6
1_21	50F.1	5	m	clear	1592.9	0.7	2409.0
1_21	50F.2	5	m	lime	2663.2	1.2	2142.8
1_21	50F.3	5	m	lime	1480.6	1.8	831.0
1_21	50F.4	5	m	clear/large	1357.7	4.7	286.5
1_21	50F.5	5	m	lime	2160.1	1.7	1266.8
1_23	175F.1	31	f	lime	6543.8	1.4	4538.5
1_23	175F.2	31	f	lime	3181.7	0.8	4108.1
1_23	175F.3	31	f	lime	4892.7	2.3	2159.8
1_23	175F.4	31	f	lime	5547.4	1.4	4063.1
1_23	175F.5	31	f	lime	3390.9	0.7	4615.3
1_23	250F.1	49	f	lime	4760.9	2.2	2172.1

1_23	250F.2	49	f	lime	9469.6	1.2	8161.9
1_23	250F.3	49	f	lime/small	5715.2	1.0	5691.9
1_23	250F.4	49	f	lime/tiny	4658.2	1.0	4488.7
1_23	250F.5	49	f	lime	6420.2	1.4	4500.5
1_23	175F.1	31	m	lime	2824.5	1.8	1530.1
1_23	175F.2	31	m	lime/tiny	492.3	0.3	1675.0
1_23	175F.3	31	m	lime	1811.8	1.8	1031.1
1_23	175F.4	31	m	lime	3505.0	1.2	2943.3
1_23	175F.5	31	m	lime	2338.4	1.6	1466.1
1_23	250F.2	49	m	lime	3077.8	1.5	2094.6
1_23	250F.3	49	m	lime	1636.7	1.9	847.3
1_23	250F.4	49	m	lime	3142.3	1.4	2301.5
10_3	4_0NF_2	30	f	lime	8876.7	1.6	5638.4
10_3	4_0NF_3	30	f	lime	8785.8	1.2	7557.2
10_3	4_0NF_4	30	f	lime	9170.1	1.0	8934.8
10_3	4_0NF_1	30	m	lime/tiny	6319.1	0.9	6711.3
10_7	5_0NF_1_3	34	f	lime	6987.8	2.0	3497.1
10_7	5_0NF_1_4	34	f	lime	5773.4	1.4	4145.6
10_7	5_0NF_1_1	34	m	lime/tiny	2747.7	1.7	1641.2
10_7	5_0NF_1_2	34	m	lime	3160.5	2.3	1350.5
10_7	50NF_2_2	36	m	lime	3983.1	1.6	2515.5
3_12	0_0_2	0	f	lime	1047.0	1.7	612.5
3_12	0_0_3	0	f	lime	1042.7	1.3	831.3
3_12	0_30_4	18	f	lime	3755.1	1.7	2211.5
3_12	0_30_5	18	f	lime	3763.7	1.8	2074.1
3_12	0_0_1	0	m	clear	448.5	0.8	577.7
3_12	0_30_1	18	m	lime	2307.6	0.6	4160.7
3_12	0_30_2	18	m	lime	2647.8	1.5	1786.9

Red-highlighted rows signify data that was considered outliers based on the boxplot of protein content; genders analyzed separately.

Table A.3. Raw data; bile fluorescence and protein content for *P. promelas* exposed to FLU in MHW in the presence of 0, 2, 5, and 10 mg/L NOM

Date	Sex	Sample Id	NOM (mg/L)	Aqueous FLU (µg/L)	Bile Notes	Blank Corrected RFU	Protein Content (mg/mL)	Normalized RFU to Protein
1_14	m	0.1m	0	13	lime	3007	6.9	438
1_14	m	0.2m	0	13	lime	3269	1.6	2033
1_14	m	0.3m	0	13	grey	2871	2.0	1436

1_14	m	0.4m	0	13	olive	2463	2.9	850
1_14	f	0.1f	0	11	lime	5001	2.5	1993
1_14	f	0.2f	0	11	lime	2727	2.0	1357
1_14	f	0.3f	0	11	lime	5483	1.5	3673
1_14	f	0.4f	0	11	lime	5542	1.3	4184
1_14	m	2.1m	2	9	olive	2488	2.1	1212
1_14	m	2.2m	2	9	clear	1080	2.5	433
1_14	m	2.3m	2	9	lime	3500	2.1	1646
1_14	m	2.4m	2	9	lime	2615	1.2	2102
1_14	f	2.1f	2	8	lime	1494	2.6	569
1_14	f	2.2f	2	8	lime	5420	1.0	5181
1_14	f	2.3f	2	8	clear	1877	4.7	398
1_14	f	2.4f	2	8	lime	7259	1.0	7557
1_2	m	1-2NF(1/2)	2	12	lime	1645	2.0	823
1_2	m	2-2NF(1/2)	2	12	clear	1476	2.4	608
1_2	m	1-5NF(1/2)	5	12	olive	2198	3.5	633
1_2	f	2-5NF(1/2)	5	12	lime	5022	1.7	2883
1_2	f	3-5NF(1/2)	5	12	lime	3550	1.6	2249
1_2	m	1-10NF(1/2)	10	18	lime	5522	1.7	3250
1_2	f	2-10NF(1/2)	10	18	lime	2429	1.5	1650
1_2	m	3-10NF(1/2)	10	18	clear	2922	2.1	1416
1_2	f	4-10NF(1/2)	10	18	lime	2148	2.5	868
10_3	m	4_0NF_1	0	30	olive/ small	6319	0.9	6711
10_3	f	4_0NF_2	0	30	lime	8877	1.6	5638
10_3	f	4_0NF_3	0	30	lime	8786	1.2	7557
10_3	f	4_0NF_4	0	30	lime	9170	1.0	8935
10_3	m	4_10NF_1	10	23	lime/popped	3821	0.8	4657
10_3	f	4_10NF_2	10	23	lime	7427	1.5	4966
10_3	f	4_10NF_3	10	23	lime	8266	1.4	5910
10_3	f	4_10NF_4	10	23	lime/small	10839	0.9	12562
10_7	m	5_0NF_1/1	0	34	lime/small	2748	1.7	1641
10_7	m	5_0NF_1/2	0	34	lime	3160	2.3	1350
10_7	f	5_0NF_1/3	0	34	lime	6988	2.0	3497
10_7	f	5_0NF_1/4	0	34	lime	5773	1.4	4146
10_7	m	5_0NF_2/2	0	36	lime	3983	1.6	2516
10_7	f	5_10NF_2	10	35	lime/small	3572	2.1	1708
10_7	m	5_10NF_3	10	35	lime	6433	2.6	2434
10_7	f	5_10NF_4	10	35	lime	8083	1.2	6496
12_31	m	1-2NF (12/31)	2	14	clear	2105	3.8	552
12_31	m	2-2NF (12/31)	2	14	lime	2610	2.6	998

12_31	m	1-5NF (12/31)	5	15	lime	1310	6.7	195
12_31	f	2-5NF(12/31)	5	15	lime	3469	1.9	1866
12_31	m	3-5NF(12/31)	5	15	clear	2439	2.4	1010
12_31	f	1-10NF(12/31)	10	17		6005	1.4	4392
12_31	m	2-10NF(12/31)	10	17	orange	2730	1.7	1654
12_31	f	3-10NF(12/31)	10	17	orange	6443	0.5	11920
12_31	m	4-10NF(12/31)	10	17	orange	961	2.8	342
3_12	m	B-1	0	0	clear	446	0.8	574
3_12	m	0.30.1	0	18	lime	2305	0.6	4156
3_12	m	0.30.2	0	18	lime	2645	1.5	1785
3_12	m	2.30.1	2	16	lime	2213	1.8	1251
3_12	m	2.30.2	2	16	lime	2302	3.6	644
3_12	m	5.30.1	5	16	lime	2414	1.9	1265
3_12	m	5.30.2	5	16	olive	1585	2.2	718
3_12	f	B-2	0	0	lime	1045	1.7	611
3_12	f	B-3	0	0	lime	1040	1.3	829
3_12	f	0.30.4	0	18	lime	3753	1.7	2210
3_12	f	0.30.5	0	18	lime	3761	1.8	2073
3_12	f	2.30.3	2	16	lime	1822	2.5	717
3_12	f	2.30.4	2	16	lime	3331	1.1	3098
3_12	f	2.30.5	2	16	lime	3557	1.5	2350
3_12	f	5.30.4	5	16	lime	2124	2.7	781
3_12	f	5.30.5	5	16	lime	3079	4.1	748
9_16	m	1_1_NF/2	2	17	dark/large	2337	3.2	728
9_16	m	1_2_NF/2	2	17	lime	6582	1.2	5303
9_16	f	1_3_NF/2	2	17	lime	5872	1.2	5038
9_16	m	1_1_NF/5	5	15	dark	2888	2.5	1149
9_16	f	1_2_NF/5	5	15	lime/popped	1893	0.3	7445
9_16	m	1_3_NF/5	5	15	lime/large	5610	1.6	3543
9_16	f	1_1_NF/10	10	14	clear/large	2633	5.2	504
9_16	f	1_2_NF/10	10	14	lime	8763	1.1	8085
9_16	m	1_3_NF/10	10	14	clear/large	2555	2.6	970
9_22	f	2_2NF_1	2	22	lime	3390	0.6	5802
9_22	m	2_2NF_2	2	22	olive	5833	1.2	4890
9_22	m	2_2NF_3	2	22	olive	3230	1.1	2931
9_22	f	2_2NF_4	2	22	mottled	1556	2.1	732
9_22	f	2_5NF_1	5	21	olive	10027	1.1	8785
9_22	f	2_5NF_2	5	21	olive	6051	3.3	1859
9_22	m	2_5NF_3	5	21	olive	5688	3.4	1660
9_22	f	2_5NF_4	5	21	clear/small	768	1.5	520

9_27	f	3_2NF_1	2	19	lime	10575	0.9	11528
9_27	f	3_2NF_2	2	19	lime	10052	1.5	6641
9_27	m	3_2NF_3	2	19	lime	6526	1.7	3870
9_27	f	3_2NF_4	2	19	lime/large	7956	0.9	8532
9_27	m	3_5NF_1	5	31	lime/small	1923	1.4	1421
9_27	f	3_5NF_2	5	31	lime	9961	0.8	13213
9_27	f	3_5NF_4	5	31	lime	10163	1.0	10329

Red-highlighted rows signify data that was considered outliers based on the boxplot of protein content; genders analyzed separately.

Table A.4. Raw data; bile fluorescence of male *P. promelas* exposed to FLU in the presence and absence of ~1.5 mg/L MWNT

Date	Sex	Bile Notes	Sample ID	Blank Corrected RFU	CNT (mg/L)	Spiked FLU (µg/L)	Ce (µg/L)
10_1	m	lime	C_CNT_1	1317	1.5	0	0.0
10_1	m	lime	C_CNT_2	502	1.5	0	0.0
10_1	m	lime	C_CNT_3	1011	1.5	0	0.0
10_6	m	clear	C_CNT_1	679	1.5	0	0.0
10_6	m	clear	C_CNT_2	1342	1.5	0	0.0
9_12	m	lime	C-CNT_1	787	1.7	0	0.0
9_12	m	lime	C-CNT_2	452	1.7	0	0.0
10_6	m	clear	CNT_1	1680	1.5	10	0.0
10_1	m	lime	CNT_2	2080	1.5	10	-0.3
10_1	m	lime	CNT_3	2157	1.5	10	-0.3
10_6	m	lime	CNT_2	2247	1.5	10	0.0
10_6	m	lime	CNT_3	1683	1.5	10	0.0
10_11	m	lime	CNT_1	2077	1.5	10	0.1
10_11	m	forest	CNT_2	1246	1.5	10	0.1
10_11	m	clear	CNT_3	1161	1.5	10	0.1
10_16	m	lime	CNT_1	1029	1.5	10	0.9
10_16	m	lime	CNT_2	742	1.5	10	0.9
9_8	m	lime	CNT_3	2045	1.7	10	0.3
9_12	m	lime	CNT_1	1565	1.7	10	0.8
9_4	m	lime	CNT_2	1175	1.7	20	5.0
11_3	m	lime	1.6CNT_2	2092	1.3	25	6.9
11_3	m	lime	1.6CNT_3	2196	1.3	25	6.9
12_13	m	lime	1.2CNT_1	1100	1.2	35	11.1

12_13	m	lime	1.2CNT_2	2275	1.2	35	11.1
12_13	m	olive	1.2CNT_3	1420	1.2	35	11.1
10_1	m	clear	C_DOC_1	1024	0	0	0.0
10_1	m	lime	C_DOC_2	874	0	0	0.0
10_1	m	lime	C_DOC_3	990	0	0	0.0
10_6	m	clear	C_DOC_1	1191	0	0	0.0
10_6	m	lime	C_DOC_2	803	0	0	0.0
10_6	m	lime	C_DOC_3	802	0	0	0.0
10_11	m	clear	C_DOC_1	799	0	0	0.0
10_11	m	clear	C_DOC_2	1108	0	0	0.0
10_11	m	lime	C_DOC_3	950	0	0	0.0
10_16	m	lime	1_DOC_1	1282	0	1	0.4
10_16	m	lime	1_DOC_2	1811	0	1	0.4
10_1	m	lime	1DOC_1	1383	0	1	0.5
10_1	m	lime	1DOC_2	1660	0	1	0.5
10_1	m	lime	1DOC_3	1766	0	1	0.5
10_6	m	clear	1DOC_1	687	0	1	1.3
10_6	m	lime	1DOC_2	1604	0	1	1.3
10_6	m	lime	1DOC_3	1701	0	1	1.3
10_11	m	lime	1DOC_2	1252	0	1	1.7
10_11	m	clear	1DOC_3	1382	0	1	1.7
10_11	m	lime	10DOC_1	1843	0	10	3.3
10_11	m	lime	10DOC_2	1609	0	10	3.3
10_11	m	clear	10DOC_3	1108	0	10	3.3
9_12	m	lime	10DOC_1	2733	0	10	3.8
9_12	m	clear	10DOC_2	1985	0	10	3.8
10_6	m	lime	10DOC_1	3564	0	10	3.8
10_6	m	lime	10DOC_2	2710	0	10	3.8
10_6	m	lime	10DOC_3	2641	0	10	3.8
10_16	m	clear	10_DOC_2	1564	0	10	4.0
10_1	m	cloudy	10DOC_1	2241	0	10	4.3
10_1	m	lime	10DOC_2	3275	0	10	4.3
10_1	m	lime	10DOC_3	2879	0	10	4.3
10_22	m	lime	DOC_1	2244	0	25	12.9
10_22	m	lime	DOC_2	1918	0	25	12.9
11_11	m	lime	DOC_1	2533	0	25	15.7
11_11	m	lime	DOC_2	3073	0	25	15.7
11_11	m	lime	DOC_3	3113	0	25	15.7
12_13	m	lime	DOC_4	2804	0	35	20.4
12_13	m	lime	DOC_1	1893	0	35	20.4

12_13	m	lime	DOC_2	2421	0	35	20.4
12_13	m	lime	DOC_3	2767	0	35	20.4

Table A.5. Raw data; bile fluorescence and protein content of male *P. promelas* in a dose-response to FLU exposure in the presence of 2 mg/L NOM

Date	Sex	Bile Notes	Sample Id	Blank Corrected RFUs	Protein Content (mg/L)	Aqueous FLU (µg/L)
10_1	m	clear	C_DOC_1	1024	0.83	0
10_1	m	lime	C_DOC_2	874	1.14	0
10_1	m	lime	C_DOC_3	990	1.01	0
10_6	m	clear	C_DOC_1	1191	2.15	0
10_6	m	lime	C_DOC_2	803	1.01	0
10_6	m	lime	C_DOC_3	802	0.74	0
10_11	m	clear	C_DOC_1	799	3.14	0
10_11	m	clear	C_DOC_2	1108	3.17	0
10_11	m	lime	C_DOC_3	950	1.14	0
10_16	m	lime	1_DOC_1	1282	0.91	0
10_16	m	lime	1_DOC_2	1811	1.32	0
10_1	m	lime	1DOC_1	1383	0.96	0
10_1	m	lime	1DOC_2	1660	0.97	0
10_1	m	lime	1DOC_3	1766	1.35	0
10_6	m	clear	1DOC_1	687	0.82	1
10_6	m	lime	1DOC_2	1604	1.05	1
10_6	m	lime	1DOC_3	1701	1.19	1
10_11	m	lime	1DOC_2	1252	1.06	2
10_11	m	clear	1DOC_3	1382	3.09	2
10_11	m	lime	10DOC_1	1843	0.40	3
10_11	m	lime	10DOC_2	1609	2.29	3
10_11	m	clear	10DOC_3	1108	1.46	3
9_12	m	lime	10DOC_1	2733	1.22	4
9_12	m	clear	10DOC_2	1985	1.86	4
10_6	m	lime	10DOC_1	3564	0.64	4
10_6	m	lime	10DOC_2	2710	0.71	4
10_6	m	lime	10DOC_3	2641	0.91	4
10_16	m	clear	10_DOC_2	1564	4.28	4
10_1	m	cloudy	10DOC_1	2241	1.89	4

10_1	m	lime	10DOC_2	3275	0.61	4
10_1	m	lime	10DOC_3	2879	2.47	4
1_14	m	olive	2.1m	2488	2.05	9
1_14	m	clear	2.2m	1080	2.50	9
1_14	m	lime	2.3m	3500	2.13	9
1_14	m	lime	2.4m	2615	1.24	9
1_2	m	lime	1-2NF(1/2)	1645	2.00	12
1_2	m	clear	2-2NF(1/2)	1476	2.43	12
12_31	m	clear	1-2NF (12/31)	2105	3.82	14
12_31	m	lime	2-2NF (12/31)	2610	2.61	14
3_12	m	lime	2.30.1	2213	1.77	16
3_12	m	lime	2.30.2	2302	3.57	16
9_16	m	dark/large	1_1_NF/2	2337	3.21	17
9_16	m	lime	1_2_NF/2	6582	1.24	17
9_27	m	lime	3_2NF_3	6526	1.69	19
12_13	m	lime	DOC_4	2804	1.34	20
12_13	m	lime	DOC_1	1893	1.30	20
12_13	m	lime	DOC_2	2421	4.02	20
12_13	m	lime	DOC_3	2767	4.12	20
9_22	m	olive	2_2NF_2	5833	1.19	22
9_22	m	olive	2_2NF_3	3230	1.10	22

Table A.6. Raw data; bile fluorescence and protein content of *P. promelas* when exposed to spiked concentrations of 25 µg/L in solutions with varying MWNTs concentrations in the presence of 2 mg/L NOM

Date	Sex	Bile Notes	Sample ID	Blank Corrected RFU	Protein Content (mg/L)	CNT (mg/L)	Ce (µg/L)
9_4	f	lime	DOC_1	4208	0.81	0	11
9_4	f	lime	DOC_2	1900	0.30	0	11
10_22	f	clear	DOC_3	1649	0.90	0	13
10_22	f	lime	.4CNT_1	2695	2.11	0.4	10
11_3	f	clear	.8CNT_3	1640	0.92	0.6	11
11_3	f	lime	1.6CNT_1	2208	1.48	1.3	7
9_4	f	lime	CNT_1	2764	0.99	1.7	5
9_4	f	lime	CNT_3	3475	0.57	1.7	5
10_22	m	lime	DOC_1	2244	2.14	0	13
10_22	m	lime	DOC_2	1918	2.42	0	13

11_11	m	lime	DOC_1	2533	2.83	0	16
11_11	m	lime	DOC_2	3073	3.48	0	16
11_11	m	lime	DOC_3	3113	1.63	0	16
12_13	m	lime	DOC_4	2804	1.34	0	20
12_13	m	lime	DOC_1	1893	1.30	0	20
12_13	m	lime	DOC_2	2421	4.02	0	20
12_13	m	lime	DOC_3	2767	4.12	0	20
12_13	m	lime	.2CNT_1	1575	3.01	0.13	13
12_13	m	lime	.2CNT_3	1327	3.08	0.13	13
10_22	m	lime	.4CNT_2	1853	1.54	0.4	10
10_22	m	clear	.4CNT_3	2160	2.94	0.4	10
11_3	m	lime	.8CNT_1	2498	2.15	0.6	11
11_3	m	clear	.8CNT_2	1193	2.94	0.6	11
12_13	m	lime	1.2CNT_1	1100	1.95	1.2	11
12_13	m	lime	1.2CNT_2	2275	1.84	1.2	11
12_13	m	olive	1.2CNT_3	1420	2.33	1.2	11
11_3	m	lime	1.6CNT_2	2092	0.98	1.3	7
11_3	m	lime	1.6CNT_3	2196	2.87	1.3	7
9_4	m	lime	CNT_2	1175	1.01	1.7	5
11_11	m	lime	2.4CNT_2	2123	1.04	2.5	4
11_11	m	clear	2.4CNT_3	1922	2.73	2.5	4