ON THE INTERACTIONS BETWEEN SINGLE WALLED CARBON NANOTUBES AND A MODEL AQUATIC ORGANISM: BIOLOGICAL RESPONSES AND BIOACCUMULATION POTENTIAL

By

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To my family

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AFM	Atomic force microscopy
BDL	Below detection limit
CCC	Critical coagulation concentration
CNT	Carbon nanotubes
Co	Cobalt
Cu	Copper
CVD	Chemical vapor deposition
D	Defect band
DCF	2',7'–dichlorofluorescein
DCFH-DA	2',7'–dichlorofluoresceindiacetate
DDT	Dichlorodiphenyltrichloroethane
DI	Deionized
DLVO	Derjaguin, Landau, Verwey, and Overbeek
DNA	Deoxyribonucleic acid
DOC	Dissoloved organic carbon
DOM	Dissoloved organic matter
E.coli	Escherichia coli
EDL	Electron double layer
EPA	United States environmental protection agency
EU	European united
FA	Fulvic acid
Fe	Iron
G	Graphite band
GA	Gum Arabic

GSH	Glutathione
HA	Humic acid
HiPCO	High pressure carbon monoxide
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
K_{ow}	Octanol water partition coefficient
MN	Manufactured nanomaterial
Mo	Molybdenum
MWNT	Multi walled carbon nanotube
Ni	Nickel
NIR	Near infrared
NOM	Natural organic matter
OM	Organic matter
P.subcapitata	*Pseudokirchneriella subcapitata*
PAAP	Preliminary algal assay procedure
PBS	Phosphate buffered saline
PF	Pluronic acid F108
PL	Photoluminescence
PVP	Polyvinyl pyrrolidone
RBM	Radio breathing mode
REACH	Registration, evaluation, authorization and restriction of chemical substances
ROS	Reactive oxygen species
RPM	Rotation per minute
SC	Sodium cholate
SDBS	Sodium dodecylbenzene sulfonate
SDS	Sodium dodecyl sulfate

SR	Suwannee river
SUVA	Specific ultra violet absorbance
SWNT	Single walled carbon nanotubes
TA	Tannic acid
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TSCA	Toxic substances control act
UV	Ulatra violet

Abstract of Dissertation Presented to the Graduate School
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The rapid growth of nanoscience and nanotechnology, and the resulting widespread production and use of manufactured nanomaterials (MNs) have stimulated research on their environmental fate and potential implications. Among the most commonly used and studied MNs, carbon nanotubes (CNTs) have attracted the attention of both engineers and scientists, and this is due primarily to their unique physical, chemical, and electrical properties. The anticipated applications of CNTs are multiple and they are expected to radically improve the rate of success in biomedical, optical, electronic, and environmental research; to name a few. However, large scale production and use of CNTs would undoubtedly lead to the introduction of this new class of hazardous materials into the environment, with potential adverse effects on human health and ecosystem functions.

The above concerns have led to the development of “nanotoxicology”, an emerging research field that focuses on the biological implications of MNs. In the past decade, most nanotoxicity studies have produced rather conflicting conclusions, due primarily to an overall failure to adequately address the diversity in the physicochemical

properties of CNTs, and how such variability could be linked to the observed impacts on cells and organisms.

To narrow the knowledge gap in our current understanding of the environmental implications of CNTs, this study was designed to investigate the interaction between single walled carbon nanotubes (SWNTs) and aquatic organisms, using the freshwater algae, *Pseudokirchneriella subcapitata*, as a model aquatic primary producer. Besides the physicochemical characterization of SWNTs and produced SWNT-suspensions, the study focused primarily on the biological responses of *P. subcapitata* when exposed to SWNTs under different environmental scenarios. The potential for SWNTs bioaccumulation was also assessed as a function of water chemical composition and SWNT suspension manufacturing methods.

SWNTs used in this study were obtained from commercial sources and SWNT suspensions were produced in our laboratory by a non-covalent functionalization method that uses aqueous surfactant solutions and mechanical energy. The biological responses of *P. subcapitata* exposed to SWNTs were investigated using typical 96-hour toxicity assays and long-term exposure studies. In these experiments, changes in algal biomass were monitored using biochemical tracers (e.g. chlorophyll a); while changes in cell morphology (using transmission electron microscopy), the occurrence of oxidative stress (by measuring changes in GSH levels), and the degree of aggregation of SWNTs (using a nanospectrolyzer) were tracked over time to investigate the mechanisms of observed biological responses. Finally, phase distribution studies were conducted using a modified shake-flask method. This set of laboratory studies emphasized the role of dissolved organic matter (quantity and type) through the traditional octanol-water

distribution approach to asses the bioaccumulation potential of SWNTs as a function of the aqueous chemistry of SWNT-suspensions. The major findings from the above studies can be summarized as follows.

Aqueous suspensions of SWNTs stabilized with non-toxic surfactants can still be toxic to *P. subcapitata*. However, the magnitude of the observed toxic effects can be reduced by manipulating the suspension manufacturing process. Although this conclusion has been reached based on studies that rely on a single test organism (*P. subcapitata*), the results suggest that the development of a green approach which eliminates the toxicity of SWNTs during the manufacturing process is possible. Accordingly, a wide variety of model organisms (e.g. bacteria, invertebrates, and vertebrates) should be used in future studies to account for biochemical differences and SWNT-sensitivity specific to each organism type.

Aqueous suspensions of SWNTs prepared in either natural river water collected from the Suwannee River (SR) or synthetic water (DI-water) spiked with different types of organic matter isolates (e.g. humic acid, fulvic acid, and tannic acid) were used in phase distribution experiments aimed at determining the octanol-water distribution coefficients (K_{ow}) of SWNTs. The results show the following.

SWNTs suspended directly into synthetic surfactant such as gum Arabic (GA) and sodium dodecyl sulfonate (SDS) do not partition between aqueous and organic phases. SWNTs become trapped at the water-octanol interface even when high energy homogenization techniques are used.

While the dispersion of the highly hydrophobic SWNTs is nearly impossible in water, the presence of high levels of dissolved organic matter in tested waters produced

SWNT suspensions with a dispersion degree and stability that are quite similar to those obtained with the ideal and commonly used synthetic surfactants. Our findings suggest that SWNTs introduced into organic-rich waters could remain well dispersed, and therefore, prone to long-range transport/dispersal while exhibiting an increased probability of interaction with pelagic organisms.

The chemical composition of the water, namely the type and quantity of organic matter present in the water sample in which SWNTs are suspended controls the degree of SWNT suspension and stability. For instance, a complex mixture of organic compounds dominated by humic and fulvic acids (e.g. SR water) produced stable SWNT suspensions with a higher degree of dispersion than suspensions obtained with solutions dominated with tannic acid or algal and bacterial extracts.

When SWNTs suspended in organic-rich waters are used in K_{ow} experiments for determination of bioaccumulation tendency, it was found that SR water would favor the transfer of SWNTs from water to biological tissues.

Dose-exposure studies using SWNT suspended in organic-rich waters showed little to no toxicity to *P. subcapitata* based on 96 hour toxicity studies. While the organic coating of SWNTs favors their transfer from aqueous solution to organic phase, it appears that the same coating would eliminate the bio-aggressivity of SWNTs.

Overall, findings from this study point to SWNT surface modifications by different stabilizing agents as main criteria for adverse biological effects. Studies on a wide variety of organisms and the investigation of the potential for SWNT bio-magnification throughout a model food chain are significant avenues for future research.

CHAPTER 1
INTRODUCTION AND PROBLEM STATEMENT

Manufactured nanoparticles (MN) find use in a wide variety of human activities, from medicine and electronics, to environmental/agricultural research and applications. Accordingly, nanoscience and nanotechnologies are currently generating an extraordinary amount of interest and are anticipated to have a substantial impact on several anthropogenic activities (Gwinn and Vallyathan 2006). According to the National Institute for Occupational Safety and Health, the U.S. government invested between $432 million and $1,240 million per year from 1997 to 2005 and on the global scale, the investment is expected to reach $1 trillion by 2015 (Roco 2003).

Carbon nanotubes (CNT), consisting of carbon atoms arranged into the shape of a hollow tube, may be one of the most useful MNs owing to its many unique properties such as high surface area, superior mechanical strength to mass ratio, and excellent electronic properties. Since the discovery of these distinctive materials, researchers have investigated many potential uses including bio- and environmental applications. Therefore, methods for their synthesis and industrial scale production have been extensively studied. Thus, in the near future, a significant amount of multiple (MWNT) and single (SWNT) walled carbon nanotubes will likely be introduced into aquatic systems either through intentional (e.g. environmental remediation) or non-intentional (e.g. accidental spillage) discharges. With the recognition of their future application in the medical and environmental fields, their toxicity and fate in the environment must be investigated.

Research on the toxicity of CNTs is still in its infancy and published data are often controversial and difficult to compare because various competing factors (e.g.

production methods, physical properties, surface chemistry, dispersion states and impurities) collectively determine the reactivity and toxicity of CNTs. However, published data indicate that CNTs, once inside living organisms, could be difficult to remove and could induce significant damage because of their physical resemblance to asbestos (Donaldson and Tran 2002). For example, similar to the biological effects of asbestos, Mutlu et al. reported significant granuloma formations in lung airways of mice (Mutlu et al. 2010). In addition, CNT toxicity has been demonstrated in several in-vivo (Blaise et al. 2008; Cheng et al. 2009; Lam et al. 2004) and in-vitro studies (Jia et al. 2005; Manna et al. 2005; Shvedova et al. 2003).

There is now plenty of evidence exhibiting the potential threats of CNTs. Toxicological investigations focusing on CNTs begun just a few years ago, but a significant number of toxicological studies have already been published in a rather short period. So far, several of the studies have resulted in controversial results due primarily to lack of understanding of the physical properties of SWNTs. In spite of the current progress, a knowledge gap persists, mostly with regard to the potential mechanisms of toxicity of CNTs. For instance, while the hydrophobicity of CNTs tends to suggest a great potential for CNT lipo-solubility, and therefore bioaccumulation, research on bio-accumulation and bio-magnification potentials remain very limited and inconclusive. Overall, further studies are required to elucidate (i) the fate and behavior of CNTs in natural aquatic systems, and (ii) the potential for CNT bioaccumulation and transfer through different trophic levels. Questions are also being raised about the suitability of current research methods, which often fail to adequately characterize CNTs used in environmental studies.

Researchers are facing challenges in studying the environmental impact of SWNTs. The hydrophobic nature of SWNTs and the van der Waals forces that dominate their interactions lead to aggregation, which is followed by sedimentation of bundled SWNTs (Oberdorster et al. 2005). But certain anticipated uses of CNTs may require specifically processed CNTs such as surface functionalized nanotubes (Moore et al. 2003). In addition to such surface modifications, natural organic matter (NOM) present in aquatic systems could stabilize CNTs suspensions (Hyung et al. 2007). These surface modifications would enhance the dispersion of CNTs in aqueous solutions and as a result, extend their residence time in aquatic systems. Clearly, surface modifications of CNTs would impact their interactions with both living organisms and dissolved organic matter. However, not enough studies have been conducted to examine the stability of CNTs and their transport and fate in aquatic environments.

This dissertation focuses on the interactions between single walled carbon nanotubes (SWNTs) and a model aquatic organism, the freshwater green algae *Pseudokirchneriella subcapitata*. The study emphasizes the biological response of the above primary producer to exposure to SWNTs, and investigates the bioaccumulation potential of SWNTs in aquatic systems. To achieve these objectives, a research approach was developed as outlined below.

Following an introduction section which articulates the research problem statement (Chapter 1), a review of relevant literature on SWNTs in the environment is provided. This second chapter points out the gaps and weaknesses in our current understanding of the physiscochemical properties of CNTs and their implications on the

environment. The review also addresses the issue of conflicting toxicity results when CNTs are used as toxicants in bioassays.

The impacts of SWNTs suspended in selected surfactants, namely gum Arabic (GA), sodium cholate (SC) and pluronic acid F108 (PF), towards a model organism are investigated and the results reported in Chapter 3. The above synthetic surfactants were selected for their biocompatibility (no observable toxicity to *P. subcapitata* even for concentrations in excess of 1000mg/L) and their ability to produce stable and well dispersed SWNT suspensions. Additionally, small scale bioassays are used for the determination of potential biological effects on aquatic organisms.

Chapter 4 presents the results of laboratory studies focusing on the distribution of SWNTs between aqueous and organic phases as a proxy for determination of bioaccumulation capability under environmentally relevant conditions. The study compares the ability of SWNTs to transfer from aqueous solutions stabilized with synthetic (GA, SC. and PF) or natural (humic acid (HA), fulvic acid (FA), tannic acid (TA), and a non-identified mixture of organic compounds present in a natural water sample) surfactants to an octanol phase. The bioaccumulation potential is determined by assessing the distribution of SWNTs between octanol and water phases in the traditional shake-flask method. The effects of some key environmental factors (pH, ionic strength, and organic matter concentration) are also investigated.

Finally, Chapter 5 is a general conclusion summarizing the key findings of the study and stating how such findings lay the ground work for future research avenues.

CHAPTER 2
CARBON NANOTUBES: PRODUCTION, PHYSICOCHEMICAL PROPERTIES AND BIOLOGICAL IMPLICATIONS

2.1 Production and Use of CNTs

Carbon nanotubes (CNT) are single or multiple layers of graphene sheets rolled up into hollow tubes with diameters of around 1nanometer (nm) and lengths that can extend to a few micrometers (μm). Their first appearance in peer reviewed literature dates back to 1952 (Radushkevich and Lukyanovich 1952). However, a true recognition of the importance of CNTs was not achieved until 1991 (Iijima 1991). The structure of CNTs is described by a chiral vector, which is the direction of the rolling, and the chiral angle (Figure 2-1). SWNTs can be classified as either metallic or semi-conducting based on their electronic properties, which may be predicted from the chiral vector (n,m) (O'Connell et al. 2002).

The synthesis of CNTs has been the subject of several studies, and the following are the three primary synthesis methods: (1) electric arc, (2) laser ablation, and (3) chemical vapor deposition (CVD) (Lam et al. 2007). These methods have been extensively reviewed (Baddour and Briens 2005; Chen and Zhang 2009; Dresselhaus et al. 2008; Lam et al. 2006; Oncel and Yurum 2006), and can be briefly summarized as follows. In the electric arc method, a carbon electrode is sublimated in the reactor by arc discharge which increases the temperature to 6000°C. Carbon atoms are vaporized from the first electrode and then deposited on the opposing carbon electrode. The laser ablation method is similar to the electric arc method, but carbon sources are vaporized using powerful laser in the furnace (1200°C). Ejected carbon atoms are carried by inert gas flow and then deposited on the water cooled collector at the end of the furnace. These two synthesis methods, developed in earlier stages of CNT research, have been

studied under various conditions with modifications in carbon sources, choice of inert gas, metal catalysts, power of arc and laser, temperature, and pressure. There are some reports which conflict in terms of the purity and structural integrity of the CNTs produced using these methods, but the general disadvantages are costly equipment, additional sorting procedures required due to tangled CNTs, and low production yield.

In CVD methods, carbon sources are applied in either liquid or gas phases as opposed to the electric arc and laser ablation methods. Carbon atoms are vaporized from a gaseous or volatile compounds of carbon in the reactor (600~1000°C), and then absorbed onto the metal catalysts anchored on the substrate. The metal catalysts serve as nucleation sites for the growth of CNTs. It has also been reported that CVD methods provide more controls over structure due to the support of the substrates. Additionally, CVD methods are easily scaled up to mass production and are thus the most important commercial methods for SWNT production (Cassell et al. 1999). Among several different types of CVD methods, the high pressure catalytic decomposition of carbon monoxide (HiPCO) process developed by the Smalley research group is a method for the preparation of SWNTs in a high pressure environment using carbon monooxide as the carbon source. It is the only practical process capable of producing SWNTs at the kilogram scale. Indeed, CNTs used in many recent toxicological studies have been produced by HiPCO process (Cherukuri et al. 2004; Kagan et al. 2006; Shvedova et al. 2003).

The CNT production methods mentioned above are similar in that carbon atoms are thermally generated from carbon sources in a furnace or reactor filled with an inert gas under controlled temperature and pressure conditions. Additionally, the presence of

metal catalysts is required to produce SWNTs in all methods. Commonly used metal catalysts are iron, nickel, cobalt, molybdenum, yttrium, and alloys of these metals, and they play a critical role in controlling physical properties and yield of SWNTs (Dresselhaus et al. 2008). Many aspects of the growth of SWNTs growth on metal catalysts remain in question and it is believed that more than one mechanism may be responsible for their development (Harris 2009). Briefly, it is believed that carbon and metal atoms are vaporized and then condensed into a liquid metal carbide. As more carbon atoms are fed into this mixture, they become supersaturated and begin to grow on the surface. It is obvious that metal catalysts are integrated into the structure of SWNTs and therefore difficult to remove. Such metal impurities in SWNTs could be either intrinsically toxic or involved in reactions inducing toxicity.

It did not take a long time for researchers to discover that these materials have highly desirable mechanical, thermal, photochemical, and electrical properties. SWNTs are not only strong and stiff, but also light and flexible (Baughman et al. 2002). They are the best known electrical conductors with twice the conductivity of copper (Lam et al. 2007). It is obvious that CNTs would attract the attention of both engineers and scientists because of these unique properties and their anticipated widespread commercial and industrial applications. It is estimated that the global CNT market will be between 900 billion and one trillion dollars in 2011(Roco and Bainbridge 2005). SWNT production on the global scale is estimated to exceed 1000 tones by 2011 (Lekas 2005). CNTs and their derivatives are used in plastics, catalysts, batteries and fuel cells, super capacitors, conductive coatings, adhesives and nano-composites, sensors, air craft, and car industries (Klaine et al. 2008). In addition, CNTs have properties which make them

good candidates for medical and environmental applications. CNTs have been studied for drug delivery due to their ability to penetrate through cell membranes, which will maximize the bioavailability of a drug to target organs. Near-infrared photoluminescence of semi-conducting SWNTs makes them potential cellular imaging devices (Cherukuri et al. 2006). CNTs have received a lot of attention for their possible use in environmental clean-up for organic compounds (Chen et al. 2007) and CNT are considered excellent sorbents for environmental contaminants compared to conventional carbon based materials. This is primarily due to their large surface area and extremely hydrophobic nature. A number of studies have reported antimicrobial properties in the environment (Kang et al. 2008; Liu et al. 2009) and they may also be applicable for water treatment and distribution systems.

While the novel properties of CNTs make them useful for so many exciting applications, like a double edged sword, it is uncertain how these properties will affect human health and ecosystem functions. Some of the application of CNTs in the medical, pharmaceutical and environmental fields involve direct introduction into the human body and the environment, and therefore a precautionary approach with an assessment of the risk to human health and implications for the environment must be adopted. Recent studies indicate that some of the toxicological properties of CNTs might not be benign to biological targets (Alpatova et al. 2010; Mutlu et al. 2010; Simon-Decker et al. 2009) (Alpatova et al. 2010; Mutlu et al. 2010; Simon-Deckers et al. 2009). The uncertainties and potential threats of CNTs are of serious concern.

2.2 Potential Toxicity and Environmental Behavior of CNTs

The discovery of CNTs and their properties has contributed immensely to the advance of nanotechnology. Numerous studies have been conducted concerning the

development of economical large scale production methods of CNTs. Increased production of CNTs, however, will result in an increased potential for either deliberate or accidental release into the environment. Therefore, CNTs are also the subject of concerns owing to potential environmental hazards and human health risks. In addition to the toxicity issues common to nanosized materials, the very properties of SWNTs that make them attractive have lead to current concerns. For instance, the physical character of needle shaped SWNTs brings about potential applications in medical areas, such as for nano-syringes (Rivera and Starr 2010) or antimicrobials (Kang et al. 2008). But this specific property can be a potential threat to human health due to the similarity with asbestos (Kostarelos 2008; Sargent et al. 2009). Accordingly, SWNTs pose new challenges for engineers and scientists and collaborative studies are needed to address the currently unknown aspects of this novel material. EPA reports that CNTs regulated under Toxic Substances Control Act (TSCA) may be new chemicals which have significantly different properties from the traditional graphite or other carbon substances already listed under the above mentioned TSCA (EPA 2008).

The literature is now abundant with papers dealing with both the synthesis and use of CNTs, but current knowledge on the environmental fate and impact of MNs including CNTs remains very limited despite of the increasing interest on the impact of nanoscience and nanotechnology. In an effort to fill this gap, the federal research and development funding for investigations on the environmental implication of MNs has been significantly increased since 2004 (Guzman et al. 2006), and experimental data on environmental fate and toxicity of SWNTs are being published at a rapidly increasing

rate as well. Some of the findings are summarized in Table 2-1 and briefly discussed below.

Ever since oxidative damage in fish brains induced by fullerene was observed (Oberdorster 2004), interest in the toxicity of carbon based MNs has rapidly increased. Earlier studies had focused on lung or skin tissue because they were indicated as the main exposure routes of CNTs (Huczko and Lange 2001; Lam et al. 2004). Although certain test methods used in these earlier studies have been critisized as inappropriate (e.g. intratracheal instillation, inhomogeneous CNT suspensions) (Lam et al. 2007), disturbing results obtained in these experiments demonstrate the potential risks of CNTs. Most CNT toxicity studies have focused on cytotoxicity and they have revealed that SWNTs can cause damage to cells (Jia et al. 2005; Shvedova et al. 2003). Additionally, primary producers and secondary consumers in aquatic systems are negatively affected by CNTs (Blaise et al. 2008). However, data have also been published on the lack of toxicity of CNTs on several model organisms (Cherukuri et al. 2004; Cherukuri et al. 2006; Pulskamp et al. 2007). These conflicting findings point to gaps in our current understanding of the potential implications of SWNTs. CNTs are generally complicated mixtures, and the lack of general agreement on toxicity data could be attributed to variations in the samples. In fact, several hypotheses have been suggested to explain the toxicity of CNTs.

2.2.1 Physical Characteristics (e.g. Shape, Length, Surface Area, Speciation)

As mentioned previously, CNTs are physically similar to asbestos fibers or other types of mineral fibers. This resemblance has raised concerns about the toxicity that would be anticipated from the inhalation of long, thin non-biodegradable fibers. In earlier stages of CNT toxicity studies, CNTs containing soot were tested on guinea pigs using

an intratracheal installation method and no significant toxicity was observed (Huczko and Lange 2001). Recent studies have shed more light on the relevance of CNTs' physical properties on biological targets. In a study comparing different length distribution of CNTs, CNTs suspended in bovine serum albumin were injected into mice, and it was found that granuloma lesion formations occurred more frequently with longer CNTs (Poland et al. 2008). The authors proposed that this phenomenon may be attributed to the incomplete phagocytosis of the longer CNTs. Moreover, it seems that the CNTs shorter than <10 μm do not influence the viability of human lung cells (Simon-Deckers et al. 2008). In a similar study, investigators discovered that while short (<300 nm) SWNTs presented no sign of toxicity, and were excreted outside of the body, long SWNTs (>10 μm) induced granuloma formation (Kolosnjaj-Tabi et al. 2010). Comparable observations have also been reported for multi-walled nanotubes (MWNTs) (Takagi et al. 2008). The biological responses of SWNTs and MWNTs are often compared in terms of physical differences. When *E.coli* has been used as a test organism, stronger antibacterial effects were demonstrated from larger surface area and smaller diameter SWNTs (Kang et al. 2008). Interestingly, it was claimed that the smaller surface area was responsible for the negative impact on human fibroblast cells, with SWNTs eliciting a stronger toxic response than MWNTs (Tian et al. 2006). While larger surface area was hypothesized to provide more cell-CNTs contacts and thus cause more membrane damage, others claimed that a CNT with less surface area would more easily penetrate into cells. Another important physicochemical property of SWNTs is electronic structure (i.e. metallic and semi-conducing SWNTs) as mentioned above. As-produced SWNTs are mixtures of these two species consisting of

approximately one third metallic and two thirds semi-conducting SWNTs (Saito et al. 1992). The difference in chemical reactivity between two SWNT species may be important in toxicological studies but it has been hampered by the lack of good separation method. Recently, Vecitis et al. (Vecitis et al. 2010) reported that the viability of *E.coli* was reduced significantly by increasing the concentration of metallic SWNTs. Therefore, how speciation of SWNTs impacts the toxicity has much research potential in terms of characterization of separated SWNTs.

2.2.2 Toxic Impurities

CNTs synthesis involves the use of catalysts such as Co, Fe, Ni, and Mo, and the alloys of these metals as well (Lam et al. 2006). Such metals are considered as impurities in that they are not necessary for the application of CNTs, and additionally, they are known to impact living organisms. In CNT toxicity studies, damage to tested organisms in comparison studies between purified and unpurified CNTs was attributed to the presence of the above metals (Cheng et al. 2009; Chlopek et al. 2006; Koyama et al. 2009; Murray et al. 2009; Porter et al. 2009; Vittorio et al. 2009; Walker et al. 2009). Purified SWNTs were found to be non-toxic to human muscle cells but slight changes in cell morphology were observed (Garibaldi et al. 2006). It has been demonstrated that significant oxidative stress in RAW 264.7 macrophages was probably caused by unpurified SWNTs (Kagan et al. 2006). Toxicity of unpurified SWNTs was also observed in living organisms such as estuarine copepod (Templeton et al. 2006), and the purification process could improve the biocompatibility of CNTs in zebra fish (Cheng et al. 2009). Although removal of impurities from CNTs seemingly enhances their biocompatibility, there are still a few limitations to make definite conclusions. Various purification methods of CNTs have been developed in the last decade (Chen and Zhang

2009; Hou et al. 2008). The researchers usually adopt the developed methods or, in some cases, accept the manufacturer's description about the purification process of CNTs which is often lack of necessary details. In addition of the fact that different methods most likely yield different purity of CNTs, they also modify physical characteristics of CNTs. This aspect of CNTs will be discussed further later herein.

2.2.3 Dispersion (i.e. Degree and Methods of Dispersion)

Because of their morphology and hydrophobic surface, as-produced CNTs tend to aggregate into bundles (Hilding et al. 2003). Extensive efforts have been made to obtain stable and individually separated CNT suspensions because their widespread application has been impeded by CNT's low solubility in polar liquids and their tendency to aggregate (Tasis et al. 2006). The use of organic solvents and surfactants during dispersion processes could be toxic as shown in the case of tetrahydrofuran (THF) with fullerene (Fortner et al. 2005). Better dispersed CNTs could provide more contacts between organisms and CNTs. Therefore, toxicity of CNTs was studied with respect to the manner and degree of dispersion. Suspended SWNTs in polyoxyethylene sorbitan monooleate were less cytotoxic to human MSTO-211H cells than bundled SWNTs and the authors emphasize the need for thorough material characterization prior to the experiment and demonstrated the role of aggregation in the toxicity of CNTs (Wick et al. 2007). Qu et al. tested purified CNTs suspended using carboxyl groups and CNTs with different degrees of aggregation in mice (Qu et al. 2009). Although no significant toxicity was observed, poorly dispersed CNTs remained in organs longer, unlike seemingly easily eliminated CNTs with higher degree of dispersion quality. In a more recent study, SWNTs suspended in Pluronic F108 (PF) were applied to mice by intratracheal injection (Mutlu et al. 2010).The authors observed granuloma formations in the airways of mice

treated with aggregated SWNTs which were absent when treated with well dispersed SWNTs. In terms of dispersion methods, only a handful of studies are available about their effects on the biocompatibility of CNTs. In vitro assessment of the toxicity of functionalized SWNTs and surfactant stabilized SWNTs shows that SWNTs with more functional groups are less cytotoxic than ones with less functional groups or surfactant stabilized SWNTs (Sayes et al. 2006). SWNTs dispersed using different surfactants show differences in cytotoxicites, which are due to the toxicity of surfactant molecules attached on the nanotubes' surfaces (Dong et al. 2008).

2.2.4 Oxidative Stress by Reactive Oxygen Species (ROS)

Due to their physical resemblances, CNTs are often compared with fibrous materials and viewed under the toxicological paradigm of fibers. The ability of fibers to cause inflammation via oxidative stress has been extensively reviewed (Donaldson and Tran 2002), and the same mechanism can, a priori, be used to explain the adverse effect of CNTs. In general, oxidative stress is caused by the generation of reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals produced in cells (Stohs and Bagchi 1995). One of the defense mechanisms of cells toward foreign substances is the production of ROS at the site of invasion (Figure 2-2A) (Apostol et al. 1989). These are neutralized by cellular defense mechanisms under usual circumstances (Blokhina et al. 2003). The intensity of the cellular response, however, depends on properties of the MNs digested, and the defense system can be overwhelmed as it reaches its threshold (Nel et al. 2006).

Another ROS producing mechanism is the interaction between electron donor or acceptor sites on the surface of MNs and oxygen (Nel et al. 2006). Pure CNTs are supposed to be significantly inert (Zhang et al. 2002) but defective sites can be

introduced to the sidewall either intentionally or unintentionally (Tasis et al. 2006). Superoxide radicals are produced by electron reduction and these can serve as precursors to additional ROS in the presence of transition metals through Fenton chemistry (Figure 2-2B). Several CNT studies report the generation of ROS due to metal impurities such as Fe in cells (Kagan et al. 2006; Manna et al. 2005; Pulskamp et al. 2007) and in mammals (Shvedova et al. 2007). After metal impurities are removed via the purification process, CNTs can induce oxidative stress in fish (Smith et al. 2007) and human lung cells (Choi et al. 2009). Conversely, purified CNTs are reported to not induce oxidative stress in biological targets nor to generate free radicals in aqueous solutions (Fenoglio et al. 2006).

2.2.5 The Combination of Any of These Factors

In some cases, the toxicological properties are difficult to address as a function of a single factor. As mentioned above, heavy metal impurities in CNTs are potential pollutants and also often involved in the generation of harmful ROS. In addition to the complications caused by heavy metals, physical properties of CNTs can be significantly affected during the dispersion and purification process, generating further ambiguity. For instance, purified CNTs usually become shorter than as-produced ones due to the treatment conditions. Defective sites on the side walls and biologically malignant surfactants can be introduced during the dispersion process. This could have important toxicological consequences. These mechanisms collaborate to stimulate adverse effects, but one may provide resistance or reduce the negative impact of another. While most studies have concluded that purified CNTs are more benign than unpurified CNTs, it has also been reported that the physical characteristics (e.g. surface area) play a more dominant role in determining toxicity than impurities (Tian et al. 2006). Moreover,

the study using CNTs suspended in gum Arabic reported that metal impurities did not influence cytotoxicity (Simon-Deckers et al. 2008; Simon-Deckers et al. 2009).

2.3 Fate and Behavior of CNTs in Aquatic Systems

As stated earlier, the unique properties of CNTs and their anticipated multiple applications will eventually result in large mass production, raising questions on the potential implications for both human health and the environment. This is because the introduction of CNTs to the environment could result in serious risks with adverse impacts on ecosystem functions as well as human health. While most research on the potential implications of CNTs has focused on toxicity aspects, studies on their environmental fate, with emphasis on physicochemical transformations and distribution in the environment remain very limited and highly challenging.

The environmental behavior of CNTs is likely analogous to that of colloidal particles found in aquatic and terrestrial systems. This assumption is in part due to the fact CNTs are not truly soluble in water and their size distribution range fits within the 1 to 1000nm range given for colloids (Holbrook et al. 2010; Lead and Wilkinson 2006; Moore 2006). Therefore, CNTs could be prone to phase distribution between water, sediments, and biota (Klaine et al. 2008). This partition process would be controlled by certain key environmental parameters such as pH, ionic strength, and presence and types of dissolved organic matter (Handy et al. 2008). For instance, the adsorption of humic substances onto colloidal particles can lead to changes in surface properties resulting in negatively charge surfaces and reduced aggregation of colloids (Lead and Wilkinson 2006). This behavior was observed in the aggregation trend of CNTs in the presence of Suwannee River humic substances (Hyung et al. 2007), resulting in a long residence time as suspended particles in aqueous solution. This could also result in an

extended contact time with aquatic organisms such as algae. Since NOM is ubiquitous in any aquatic systems, their interaction with CNTs may alter the surface properties of CNTs and influence their fate and their biological effects.

In the natural environment, even well stabilized colloids eventually aggregate over time and this is a key removal mechanism in colloidal systems (Buffle and Leppard 1995). Aggregation may occur when two particles transported by mechanisms such as Brownian motion come into contact and then stay attached (Handy et al. 2008). The likelihood of attachment is determined by the competition between van der Waals (vdW) forces and electron double layer (EDL) forces according to Derjaguin, Landeau, Verwey, and Overbeek (DLVO) theory (Derjaguin and Landau 1993; Verwey 1945). As two adjacent particles approach, the repulsive forces of EDL increase, while the attractive forces of vdW predominate once they pass the energy barrier. The sum of these two forces determine whether the interaction between two particles will be repulsive (i.e. stable) or attractive (i.e. aggregated).

The DLVO theory may be used to understand the effects of various water chemistry parameters on CNT aggregation. As mentioned earlier, NOMs may introduce a negative charge on the surface of particles which results in steric repulsion and natural river water enriched with NOMs could stabilize CNTs (Hyung et al. 2007). In an effort to better understand interactions between NOM and CNTs, chemicals with simpler structures such as tannic acid (TA) (Lin et al. 2009a; Lin and Xing 2008) and standard humic substance (Saleh et al. 2010) were used as surrogates for NOM in dispersion tests of CNTs. Dispersed MWNTs concentrations showed a strong correlation with increasing TA and pH. The presence of ions and size of MWNTs also affected the

quantity of dispersed MWNTs (Lin et al. 2009b). In addition, a higher input of organic matter impeded coagulation of MWNTs in a model water treatment facility (Holbrook et al. 2010). Conversely, an increase in ion concentrations may promote aggregation due to the compression of EDL. The concentration of electrolyte ions at which the transition from stable dispersion to aggregation occurs is known as the critical coagulation concentration (CCC). The CCC for various ions were evaluated using both SWNTs (Sano et al. 2001)(Na^+: 37 mM) and MWNTs (Lin et al. 2009b; Saleh et al. 2010)(Na^+: 6.9 mM and 25 mM, respectively). These results cannot be directly compared due to differences in experimental conditions (i.e. type of CNTs and methods of dispersion) and there are too few data to make draw any decisive conclusions.

In addition, there is a need to assess the potential for bioaccumulation. There is no evidence that CNTs would behave as organic pollutants such as polychlorinated biphenyl or dichlorodiphenyltrichloroethane (DDT), but the lipophilicity of CNTs has been well recognized in their potential use as remediation tools along with their large surface area (Chen et al. 2007). Moreover, several reports have noted that CNTs are taken up by living cells (Smart et al. 2006). Certain CNTs can cross cell membranes and accumulate inside living organisms (Zhu et al. 2006a). This provides the basis for potential bioaccumulation in living organisms and contamination of different trophic levels through diet. There have been some criticisms about the rather higher concentration of MNs tested in the lab, compared with proposed MN concentrations in natural systems. However, for certain xenobiotics, the total ambient concentrations in water gives very little information of potential impacts to living organisms, particularly for compounds with strong bioaccumulation abilities. In addition to bioaccumulation, the

concentration of such xenobiotics could increase in tissues as they are passed to other organisms through diet (Mackay and Fraser 2000). This is usually favored by the low acute toxicity of such compounds, which allows organisms accumulating them to survive long enough and pass the contamination to higher trophic level organisms.

Only a few studies have been conduced to examine bioaccumulation of CNTs. The Biological uptake of CNTs and depuration process were observed in estuarine copepods (Templeton et al. 2006) and daphnia (Kennedy et al. 2008), but a quantitative analysis was not performed. During the ingestion and depuration processes, aquatic organisms modified the surface coatings of SWNTs and changed the stability of their dispersed state (Roberts et al. 2007). Beside qualitative studies, ingestion and depuration levels of ^{14}C-labeled CNTs were quantitatively determined using earth worms (Petersen et al. 2008). Peterson et al. reported that uptake of CNTs was significantly lower than that of pyrene and that they were not bioaccumulated in target organisms. In a terrestrial study, the results must be carefully interpreted because homogeneous mixing of CNTs in soil can be cumbersome (Klaine et al. 2008). The study of bioaccumulation of CNTs has been hampered due to the lack of an assessment method for xenobiotics inside target organisms. Electron microscopic imaging technique and elemental composition analysis are not favorable because of the strong background signal coming from other carbon sources in organic materials. Photoluminescence of SWNTs has been applied in living organisms (Cherukuri et al. 2004; Cherukuri et al. 2006), but the change of aggregation state under the surrounding environment and detection failure due to the presence of metallic SWNTs are limitations of this method. Additionally, the attachment of tracing agents on the CNT surface (Kam

et al. 2004; Petersen et al. 2008; Singh et al. 2006) could alter their physicochemical properties, or the integrity of tracing agents could be damaged during experimental periods. Therefore, there is a plenty of research potential in establishing reliable and simple procedures for estimating bioaccumulation potentials of CNTs.

2.4 Dispersion of CNTs

SWNTs are prone to aggregation in aqueous suspension due to their hydrophobic nature and insolubility, and hence expected to settle within a rather short time in aquatic systems (Oberdorster et al. 2005). It is noted that CNT in natural systems could encounter physical or chemical processes such as water waves, NOMs, or detergents, and the stability of CNTs in the environment will be affected by these environmental factors as well. Moreover, CNTs may not be released in their as-produced form because many useful properties of CNTs are only accessible when they are modified to be individually suspended. For example, water soluble SWNTs are being developed as contrast agents in medical imaging (Ziegler 2005) and for the delivery of therapeutically active molecules to target cells (Lacerda et al. 2006). In this modification process, SWNTs are dispersed in water with the aid of functional groups and the behavior and fate of functionalized SWNTs will be completely different from that of non-functionalized SWNTs in aquatic systems. Despite its significance, the impact of the modification on SWNTs toxicity remains largely unknown.

The extremely hydrophobic nature and strong van der Waals interactions between tubes causes CNTs to bundle as they are synthesized (Girifalco et al. 2000; Hertel et al. 1998). Therefore, one of the biggest challenges to achieving the promise of CNTs is separating tubes from each other, and thus, SWNT suspension is one of the major research areas in the study of CNTs. There are two major ways to achieve the

dispersion of CNTs in solution, covalent and non-covalent functionalization (Hilding et al. 2003). The covalent method involves bounding solublizing functional groups on the sidewall of SWNTs. Unfortunately, intrinsic sp^2 bonds of graphene sheet consisting SWNTs sidewall are often destroyed in the process because of the introduction of sp^3 hybridized carbon sites on the SWNTs sidewall (Garg and Sinnott 1998). The breakage of sp^2 bonds is undesirable, since it reduces the phenomenal strength of SWNTs which is important for applications (Garg and Sinnott 1998). Compared with the covalent method, non-covalent functionalization using dispersing agents has been proven to disperse and maintain individual SWNTs separated while preserving electrical properties and mechanical integrity. Furthermore, mechanical and electrical properties of SWNT nanocomposites have been significantly improved using surfactants (Tasis et al. 2006). Therefore, noncovalent functionalization is favorable for many applications.

Surfactants, amphiphilic molecules having both a polar and non-polar group, can accumulate on surfaces or interfaces between immiscible media, reduce surface tension, and thus provide stable dispersions. Similarly, surfactants and CNT interactions in aqueous solutions are primarily based on the adsorption of the hydrophobic tail of the surfactant molecule on the hydrophobic surface of SWNT bundles, together with attraction of the hydrophilic head to the ambient water. In addition, efficient surfactant coating onto CNT surfaces induces electrostatic (Strano et al. 2003) or steric repulsion (Bandyopadhyaya et al. 2002) that could neutralize van der Waals forces between tubes. In a typical dispersion process, high shear homogenization and ultrasonication may follow after surfactants have been introduced. This mechanical exfoliation of the bundles assists the infiltration of surfactant molecules into the inner circle of CNT

bundles in order to increase the efficiency of dispersion. Various surfactants have been studied due to the advantages of non-covalent functionalization. A few well known examples are sodium dodecylbenzene sulfonate (SDBS), sodium dodecyl sulfate (SDS) cetyltrimethylammonium bromide, Tween, and Pluronic (Moore et al. 2003). In addition, naturally existing substances, including NOMs (Hyung et al. 2007), DNA (Enyashin et al. 2007), and GA (Bandyopadhyaya et al. 2002), have been evaluated as dispersants for CNTs.

It is obvious that surfactants used to disperse CNTs will play an important role not only in determining the toxicity of CNTs, but also in deciding their fate in the environment. Living organisms could actively ingest coating substances used on SWNTs' surfaces and resultantly change their behavior in the aquatic system (Roberts et al. 2007). Owing to possible medical applications, surfactants are at the center of interest as the need to enhance the biocompatibility of CNTs increases. Although the toxic effects of surfactants on various aquatic organisms have been well studied, their general effects on living organisms are often found to be ambiguous (Ostroumov 2006). For example, anionic surfactants can bind and interfere with bioactive molecule such as peptides, enzyme, and DNA. While this may cause serious environmental pollution, it can also promote the decomposition of other pollutants (Cserhati et al. 2002). Another problem is that, the amphiphilic property of surfactants may be responsible for bioaccumulation in aquatic organisms (Tolls et al. 1994). Non-ionic surfactants show antimicrobial activity by interfering with the permeability of the cell membrane and this may cause cell death or damage through the loss of ions or amino acids (Schubert et al. 1986). Therefore, using surfactant-suspended CNTs in toxicology studies may

complicate the interpretation of data. Moreover, this may raise a question as to whether the observed biological responses are not just artificial toxicity from surfactants or modifications of the surface chemistry. For example, previous investigations on the toxicity of fullerenes have used THF as a dispersing agent (Oberdorster 2004). It was later suggested that residual THF in unique fullerene structures may be responsible for elevated toxicities (Fortner et al. 2005; Oberdorster et al. 2006). Stable dispersion of CNTs, however, must be achieved in studying their impact and fate in aquatic systems. First, only stable CNTs provide sufficient interaction time with aquatic organisms to produce a threat of potential uptake or chronic toxic effects. Secondly, unexpected aggregations during toxicological experiments complicate the interpretation of results. Thirdly, it is reported that CNTs can be stabilized through interactions with NOMs and thus stay in aquatic system longer than expected. Fourthly, since CNTs are expected to be separated and dispersed in suspensions for most of their applications, more realistic scenarios of exposure and discharge should account for these modified CNTs rather than just the as-synthesized versions.

The biological impact of surfactants and their effects on CNTs' physicochemistry make the choice of surfactant used one of the most important factors involved in CNT suspensions. Surfactants used in toxicity test must be as benign to targets as possible so that artificial effects are minimized. In recognition of these factors and taking into account preliminary lab studies, GA, sodium cholate (SC), and pluronic acid F 108 (PF) have been suggested. Basic information, toxicity reviews, and CNTs suspensions using these surfactants are discussed in next chapter.

2.5 Concluding Remarks

Nanotoxicology is still in its infancy, and in general, studies focusing on the environmental fate and impacts of NMs remain highly challenging. Numerous toxicological studies on the effects of NMs have been published in a rather short time, but many of these reports have been controversial due to a lack of understanding of the physicochemical properties of CNTs. Additionally, the potential mechanisms of observed adverse biological effects have only been explored in a skin-deep manner, and many questions remain unanswered. In fact, studies on the environmental fate and behavior of CNTs in the environment are only beginning to receive due attention as the initial research efforts focused mostly on implications on human health.

Based on the above review, CNTs' size, shape, degree of dispersion, and the presence of metal impurities could all result in toxic responses. However, the discrepancy in reported results seems to be linked primarily to the lack of a good understanding of CNT characterization prior to use in and during bioassays. For instance, several authors rely simply on information provided by manufacturers. The evaluation of the toxicity potential of NMs is therefore made difficult without clear links between specific physicochemical characteristics and biological responses. Used dispersion processes may also add another level of complexity in that some of the phycochemical characteristics of pristine NMs as provided by the manufacturers may no longer be relevant following changes induced by surface modification techniques.

CNTs are new materials with many undiscovered properties, and consequently, with unexpected impacts on organisms. Only with appropriately validated analytical methods and carefully designed experiments can we gain insight into the mechanisms of environmental fate and toxicity of CNTs.

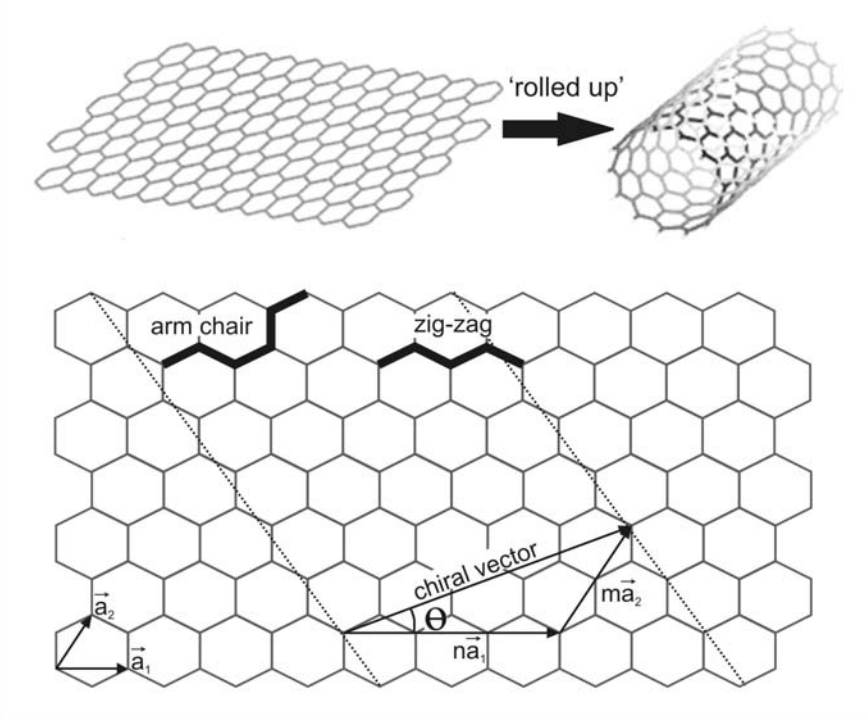

Figure 2-1. Schematic diagram showing how a hexagonal sheet of graphene is rolled up to single walled carbon nanotubes (SWNT). The various ways of rolling graphene into tubes are described by the tube chirality as defined by chiral vector = $n\vec{a}_1 + m\vec{a}_2$, where the integers (n,m) are the number of steps along the unit vector ($\vec{a}_1$ and $\vec{a}_2$) of the hexagonal lattice. Adopted from O'Connell et al. 2002.

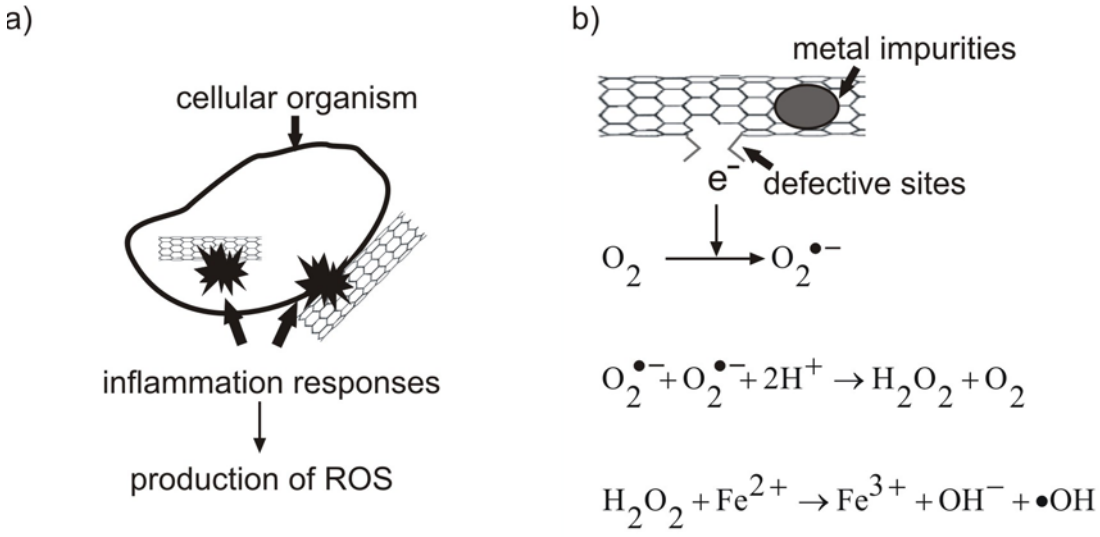

Figure 2-2. Possible mechanisms of reactive oxygen species (ROS) production in the presence of single walled carbon nanotubes (SWNTs). Examples illustrate that SWNT can participate in production of ROS via (a) inflammation responses of cells that may cause oxyradical release, and (b) electron release from reactive sites and subsequent Fenton reaction by transition metals.

Table 2-1. Toxic effects of carbon nanotubes as reported in the literature

Target organism	CNT type	Production method	Surfactants	Effects and mechanisms	Reference
Guinea pigs		Arc discharge	TWEEN	no	(Huczko et al. 2001)
Human skin cell	SWNT	HiPCO		toxic (ROS)	(Shvedova et al. 2003)
Mice	SWNT	HiPCO Arc discharge	mice serum	toxic	(Lam et al. 2004)
Macrophage	SWNT	HiPCO	PF	no	(Cherukuri et al. 2004)
Human kidney cell	SWNT			toxic	(Cui et al. 2005)
Macrophage	SWNT MWNT	Arc discharge CVD		toxic	(Jia et al. 2005)
Macrophage	SWNT MWNT		Dimethyl sulfoxide	toxic	(Murr et al. 2005)
Human skin cell	SWNT		Dimethyl formamide	toxic (ROS)	(Manna et al. 2005)
Human skin cell	MWNT	CVD		toxic	(Monteiro-Riviere et al. 2005)
Macrophage	SWNT MWNT		Dimethyl sulfoxide	toxic	(Soto et al. 2005)
Human muscle cell	SWNT			moderate	(Garibaldi et al. 2006)
Macrophages	SWNT	HiPCO		toxic (ROS)	(Kagan et al. 2006)
Human fibroblasts	SWNT MWNT			toxic(surface area)	(Tian et al. 2006)
Estuarine copepod	SWNT	Arc discharge		toxic (impurity)	(Templeton et al. 2006)
Rabbit	SWNT	HiPCO (Rice)	PF	no	(Cherukuri et al. 2006)
Human fibroblasts/osteoblastic cell	MWNT	CVD		toxic (metal impurities)	(Chlopek et al. 2006)
Human skin cell	SWNT	HiPCO (Rice)		toxic (dispersion method)	(Sayes et al. 2006)
Rat macrophage human lung cell	SWNT	CVD	SDS	no	(Pulskamp et al. 2007)
human MSTO-211H	SWNT	Arc discharge	TWEEN80	toxic	(Wick et al. 2007)

Table 2-1. Continued.

Target organism	CNT type	Production method	Surfactants	Effects and mechanisms	Reference
Human skin cell	SWNT	HiPCO	PF(127)	toxic (dispersion method)	(Zhang et al. 2007)
Daphnia magna	SWNT	HiPCO	lipid coating	minimum	(Roberts et al. 2007)
Human lung cell	SWNT	HiPCO	serum	minimum	(Davoren et al. 2007)
Rainbow trout	SWNT		SDS	toxic (ROS)	(Smith et al. 2007)
Mice	SWNT	HiPCO		toxic (ROS)	(Shvedova et al. 2007)
Human leukaemia cell	SWNT MWNT	Arc discharge / CVD		moderate	(De Nicola et al. 2007)
mice lung cell	SWNT	HiPCO		toxic (DNA damage)	(Kisin et al. 2007)
mice lung cell	SWNT	HiPCO		toxic	(Saxena et al. 2007)
E.coli	SWNT	HiPCO		toxic (size)	(Kang et al. 2007)
Human osteoblast cell	SWNT	HiPCO Arc discharge Laser ablation		biocompatible	(Kalbacova et al. 2007)
human lung cell	SWNT	HiPCO Arc discharge	serum	toxic (nutrient depletion by SWNT)	(Casey et al. 2008)
mice	MWNT	CVD	serum	toxic (size and shape)	(Poland et al. 2008)
mice	MWNT		TritonX100	toxic (size and shape)	(Takagi et al. 2008)
human astrocytoma cells	SWNT			toxic (dispersion method)	(Dong et al. 2008)
mice lung cell	SWNT			toxic (ROS)	(Jacobsen et al. 2008)
algae,daphnia,fish	SWNT			toxic	(Blaise et al. 2008)
human lung cell	MWNT	CVD	GA	toxic (independent of size)	(Simon-Deckers et al. 2008)
E.coli	SWNT MWNT	HiPCO CVD		toxic(size)	(Kang et al. 2008)
mice/macrophage	SWNT		PF(68)	toxic(ROS)	(Chou et al. 2008)
zebra fish	MWNT			toxic (impurity and method)	(Cheng et al. 2009)

Table 2-1. Continued.

Target organism	CNT type	Production method	Surfactants	Effects and mechanisms	Reference
human lung cell	SWNT			toxic (ROS)	(Choi et al. 2009)
human skin cell	SWNT	HiPCO		toxic (ROS)	(Murray et al. 2009)
human lung cell	SWNT			toxic (surface oxidation)	(Panessa-Warren et al. 2009)
mouse	MWNT	floating reactant method		toxic (impurity and ROS)	(Koyama et al. 2009)
human aortic endothelial cell	SWNT MWNT	CVD floating reactant method		toxic	(Walker et al. 2009)
human lung cell	SWNT	HiPCO Arc discharge	bovine serum	toxic (dispersion method)	(Herzog et al. 2009)
human bronchial epithelial cell	SWNT			toxic (DNA damage)	(Lindberg et al. 2009)
human lung cell	SWNT	HiPCO Arc discharge	bovine serum	toxic (dispersion method)	(Herzog et al. 2009)
human bronchial epithelial cell	SWNT			toxic (DNA damage)	(Lindberg et al. 2009)
mice	SWNT MWNT	CVD	mice serum	toxic (allergic reaction)	(Nygaard et al. 2009)
mice, bone marrow dendritic cells	MWNT	CVD	Tween 80	toxic (allergic reaction)	(Inoue et al. 2009)
osteoblastic cells	SWNT	HiPCO	SDS	toxic	(Tutak et al. 2009)
human macrophage	SWNT	HiPCO		no	(Porter et al. 2009)
mice organ	MWNT	CVD	PBS / PBS +1%Tween 80	toxic (dispersion method)	(Qu et al. 2009)
human mocyte	MWNT	Arc discharge		toxic	(De Nicola et al. 2009)
lung cell	SWNT	HiPCO		toxic (DNA damage)	(Sargent et al. 2009)
human astrocytoma cell	SWNT		DNA /SC/SDBS/ SDS	toxic (dispersion method)	(Dong et al. 2009)

Table 2-1. Continued.

Target organism	CNT type	Production method	Surfactants	Effects and mechanisms	Reference
mice fibroblast cell	SWNT	HiPCO	SDBS/ PF(68)/ PF(127)/ Peptide	toxic (dispersion method)	(Bakota et al. 2009)
E.coli	MWNT	CVD	GA	toxic (target species. impurity irrelevant)	(Simon-Deckers et al. 2009)
E.coli	SWNT	HiPCO	Tween60	toxic (shape and dispersion method)	(Liu et al. 2009)
Daphnia magna	SWNT + Cu		lipid coating	Cu uptake increased with CNTs	(Kim et al. 2010)
Mice	SWNT	HiPCO	Tween60	toxic (size)	(Kolosnjaj-Tabi et al. 2010)
Mice	SWNT	HiPCO	PF	toxic (size /aggregation state)	(Mutlu et al. 2010)
human liver cancer cell	MWNT	CVD	PF hydroxypropylcellulose	(dispersion method)	(Piret et al. 2010)
P12 cell (neuron)	SWNT	CVD		toxic (ROS)	(Zhang et al. 2010)
E.coli/rat liver cell	SWNT	CVD	GA / PVP/ Triton X100/ SR NOM	toxic (dispersion method)	(Alpatova et al. 2010)
E.coli	SWNT	HiPCO	SDS/SC	toxic (CNTs speciation)	(Vecitis et al. 2010)
Daphnia magna	MWNT	CVD	commercial NOM	toxic	(Edgington et al. 2010)
rat	MWNT			toxic (size and dispersion method)	(Wako et al. 2010)
mice	MWNT			no	(Kobayashi et al. 2010)
Daphnia magna	MWNT			toxic (size and dispersion method)	(Petersen et al. 2011)

**Blank space represents lack of description in the paper.*

CHAPTER 3
MITIGATION OF THE IMPACT OF SINGLE-WALLED CARBON NANOTUBES ON A FRESHWATER GREEN ALGAE: *PSEUDOKIRCHNERIELLA SUBCAPITATA*

3.1 Introductory Remarks

Single-walled carbon nanotubes (SWNTs) have attracted the attention of both engineers and scientists because of their anticipated widespread commercial and industrial applications (Baughman et al. 2002; Dresselhaus et al. 2003). This interest stems primarily from their unique size-related characteristics, such as strength, elasticity, high adsorption capacity, and controllable conductivity, which has led to their introduction into a wide variety of commercial products. However, the potential danger of introducing of SWNTs into the environment during manufacturing stages and during both use and disposal of SWNT-containing products could grow with the economic success of manufactured nanomaterials (MN). Accordingly, SWNTs entering waste streams would likely interact with ecological systems, and ultimately impact the biosphere. In fact, aquatic systems, such as rivers and lakes, behave as primary environmental sinks by integrating pollutants from atmospheric deposition, terrestrial surface runoffs, and groundwater discharges, raising concerns on the potential implications of SWNTs that might accumulate in these systems (Gao et al. 2009).

Recent research on the biological implications of SWNTs has produced rather conflicting results, in that both toxic and non-toxic effects are reported (Chen et al. 2007; Chen et al. 2006; Cherukuri et al. 2004; Chou et al. 2008; Federici et al. 2007; Griffitt et al. 2007; Lam et al. 2004; Lecoanet et al. 2004; Shvedova et al. 2005; Smith et al. 2007; Warheit et al. 2004). In addition to studies focusing on the environmental aspects, the anticipated use of SWNTs as biosensors and in medicine (Chen et al. 2003; Federici et al. 2007; Mattson et al. 2000; Trommer and Neubert 2005) has also stimulated research

on the investigation of the effects of MNs on humans. In the latter case, preliminary results have raised concerns about the biological effects of SWNTs at both the cell and organism levels (Fenoglio et al. 2006; Lam et al. 2004; Shvedova et al. 2005; Warheit et al. 2004). In fact, the currently observed toxic effects of SWNTs have been attributed to several parameters, including the levels and types of metal impurities (Kagan et al. 2006; Koyama et al. 2009; Shvedova et al. 2003); the degree and kind of aggregation in produced SWNTs (Choi et al. 2009; Kostarelos et al. 2007), physical characteristics, such as shape and surface area (Flahaut et al. 2006; Kang et al. 2007), the type and role of surfactants used to disperse them (Dong et al. 2008; Monteiro-Riviere et al. 2005; Sayes et al. 2006; Simon-Deckers et al. 2008), and more likely a combination of two or more of the above parameters.

The significance of the toxic role of used surfactants stems from the fact that ideal SWNT-suspensions are currently obtained by means of a wide variety of aqueous surfactant solutions, with different chemical properties. Commonly used surfactants include gum Arabic (GA) (Bandyopadhyaya et al. 2002), sodium dodecyl sulfate (SDS) (Moore et al. 2003; O'Connell et al. 2002; Regev et al. 2004; Smith et al. 2007; Vigolo et al. 2000), sodium dodecylbenzene sulfonate (SDBS) (Attal et al. 2006; Cognet et al. 2007; Moore et al. 2003), and sodium cholate (SC) (Moore et al. 2003). Some preliminary studies have also indicated that dissolved natural organic matter could help enhance nanoparticle suspension in aqueous solutions (Hyung et al. 2007). While these surfactants are used to enhance the dispersion/suspension of SWNTs, some can also affect the inherent properties of SWNTs (Garg and Sinnott 1998; Silvera-Batista et al. 2009), and potentially the interactions between SWNTs and living organisms in toxicity

studies (Dong et al. 2008). Our preliminary studies on the effects of surfactant types and SWNTs toxicity to *P. subcapitata* and *Ceriodaphnia dubia* have shown that certain surfactants (e.g., SDBS, SDS, SC, Triton X-15 and Triton X-100) are toxic to these two model organisms (Gao 2008). Therefore, toxicity studies based on the above model organisms and using SWNTs suspended in such surfactants could not provide an accurate assessment of the effects of pristine SWNTs. In contrast, other surfactants, such as GA (Alpatova et al. 2010; Simon-Deckers et al. 2008), pluronic acid F108 (PF) (Cherukuri et al. 2004; Mutlu et al. 2010), have been found to be non-toxic at environmental relevant concentrations.

Efforts to reduce or eliminate the risks associated with SWNTs or any other ENMs would require an understanding of the nanoparticle characteristics responsible for toxicity, as well as the mechanisms of interactions between nanoparticles and organisms. In this study, a model aquatic organism, the freshwater green algae *Pseudokirchneriella subcapitata*, also known as *Selenastrum capricornutum* (US-EPA 2002), was used in a series of laboratory bioassays to investigate the biological effects of SWNTs as a function of dispersing agent types and algal growth conditions. Quantitative (algal biomass) and qualitative (morphology) changes associated with specific SWNT-treatments were monitored as a function of time, while the mechanisms of biological responses were investigated using a combination of biochemical and spectroscopic methods.

3.2 Materials and Methods

3.2.1 Preparation and Characterization of SWNT Suspensions.

Surfactants used to suspend SWNTs in this study were selected based on the results of bioassays conducted by our research group and published in peer reviewed

literature. Using criteria such as biocompatibility with respect to target test organism and quality of SWNT suspensions obtained, three surfactants (i.e. GA, SC, and PF) were selected. Their properties and use in toxicity investigations are summarized briefly below.

3.2.1.1 Gum Arabic (GA)

GA is a natural polysaccharide approved for use as a food additive by the US Food and Drug Administration (USDA) and has been used as an emulsifier in the food and pharmaceutical industry (Anderson et al. 1982). GA has been used successfully to disperse aggregated CNTs in aqueous suspensions. In fact, GA compounds are basically carbohydrates that are fundamental components of living organisms. This is particularly important in terms of biocompatibility and of potential bio-applications of MNs in general (Lin et al. 2004). In toxicity tests using the combination of GA and CNTs, GA has not expressed any adverse effects on target organisms (Alpatova et al. 2010; Simon-Deckers et al. 2008).

3.2.1.2 Sodium cholate (SC)

SC ($C_{24}H_{39}NaO_5.xH_2O$), commonly known as bile salts, is an anionic surfactant with a molecular weight of 430.55 g/mol. It was used as early as 1932 in biomedical studies to enhance the absorption of drugs (Duchateau et al. 1986) by taking advantage of its biocompatibility and solubilzing properties (Hofmann and Roda 1984). SC has been proven to be very effective as a surfactant for the suspension of CNTs (Wenseleers et al. 2004), but it has shown contrasting toxicity results. For instance, no adverse impacts were detected in tested organisms in a study by Dong et al. (Dong et al. 2008). On the other hand, adverse biological effects were reported on certain

bacteria used as model organisms with (Treyer et al. 2002) or without carbon nanotubes (Liu et al. 2009).

3.2.1.3 Pluronic acid F108 (PF)

The pluronic group of compounds have been used as drug delivery agents (Kabanov et al. 2002). PF is a nonionic surfactant with an average molecular weight of 14,600 g/mol. It is a good surfactant for the preparation of aqueous suspensions of SWNTs (Moore et al. 2003). The use of PF suspended CNTs in bioassays has shown a good biocompatibility (Cherukuri et al. 2004; Mutlu et al. 2010), but excessive use of this surfactant may lead to potential toxic impacts (Sayes et al. 2006).

SWNT suspensions used in this study were prepared by suspending approximately 40 mg of raw SWNTs (obtained from Rice University. Rice HPR 145.1) into 200 mL of aqueous (1%, m/v) solution of surfactant. To facilitate the dispersion process, the mixture was homogenized using a high-shear IKA T-25 Ultra-Turrax mixer for about 1.5 h followed by ultrasonication using a Misonix S3000 for 10 min. The mixture was then ultra-centrifuged at 20,000 rpm (Beckman Coulter Optima L-80 K) for 4 h, and the supernatant carefully separated from the aggregated SWNTs at the bottom of the centrifuge tube. The obtained suspensions were spin-coated onto mica to be characterized using atomic force microscopy (AFM, Digital Instruments D3100; Veeco Co., Plainview, NY). Metal impurities in raw SWNTs were measured by monitoring loss of nanotubes weight up to 800°C for 3.5 h using thermo-gravimetric analysis (TGA, Mettler Toledo TGA, Mettler-Toledo International Inc., Columbus, OH)). The elemental analysis of acid digested SWNT suspensions was conducted after an overnight digestion with concentrated HCl at 100°C, and then followed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis.

3.2.2 Algal Growth in SWNT-Containing Culture Media and SWNT Aggregation State in Algal Suspensions.

Pure cultures of *P. subcapitata* were obtained from Hydrosphere Research (Alachua, FL) and cultivated under constant light exposure at room temperature. The algal growth procedure used in this study was adapted from Environmental Protection Agency (US-EPA)'s *P. subcapitata* 96-h growth inhibition method 1003.0 (US-EPA 2002). In dose-exposure studies, 50 mL of a preliminary algal assay procedure (or PAAP) culture medium (US-EPA 2002) were transferred into 150 mL Erlenmeyer flasks and sterilized by autoclaving. Growth experiments were conducted under continuous illumination (~86 $\mu E \cdot m^{-2} s^{-1}$) and both control and SWNT-treated samples were prepared in triplicates, with each experiment repeated at least twice. Changes in chlorophyll ***a*** concentrations were used as an indicator of general biological responses and were monitored over time using a Turner Quantech Digital-Filter Fluorometer. Algal growth rates determined from control samples were averaged, assigned a value of 100%, and used as a reference value to report the growth rates determined from SWNT-treated culture media. To investigate the effects of SWNTs on algal growth, the following experiments were conducted.

3.2.2.1 Effects of increasing SWNT concentrations with fixed concentration of surfactants to *P. subcapitata*.

In this set of experiments, sterilized culture media were first spiked with aliquot volumes of a concentrated SWNT-suspension to produce growth media with various concentrations. The addition of different volumes of the SWNT-suspension to culture media resulted in different final concentrations of surfactants, which was then corrected by adding needed volumes of a plain surfactant-solution to obtain uniform final surfactants concentrations in all growth media. Based on this approach, algal exposure

studies were conducted at two different final surfactant concentrations (Table 3-1). Following the addition of SWNTs and after vigorous mixing, culture media were inoculated with 1 mL of a pure algal suspension.

3.2.2.2 Long-term exposure of algal cells to a pre-identified toxic combination of GA-SWNTs and SC-SWNTs.

In addition to the above short-term (96 h) experiments, the pre-identified most toxic combination of GA (0.023%)-SWNT (0.5 ppm) and SC (0.048%)-SWNT (0.8 ppm) concentrations were used in a long-term (2 week) exposure study. In this set of experiments, aliquot volumes of SWNTs suspended in GA and SC were added to growing algal suspensions either at the start or at different points along the algal S-growth curve (i.e., additions during lag, exponential, and plateau phases). Algal growth conditions (e.g. culture media, light exposure) and analytical methods used in these long-term experiments were similar to those described in 'Effects of exposure to increasing SWNT concentrations in algal cultures with a fixed concentration of surfactants.

3.2.3 Determination of Glutathione and Reactive Oxygen Species as Indicator Oxidative Stress

To investigate the biological responses of our model organism, concentrations of reduced glutathione (GSH) were determined using culture media spiked with a fixed amount of SWNTs and increasing GA levels in 96-h growth experiments. The GSH assay was adapted from methods described by Hissin and Hilf (Hissin and Hilf 1976) and Cohn and Lyle (Cohn and Lyle 1966). Briefly, algal suspensions were first centrifuged at 4°C and 2000 rpm for 15 min. The supernatant was discarded and the pellet was re-suspended in 5 mL of a sodium phosphate buffer (0.1 M sodium phosphate - 0.005 M ethylenediaminetetraacetic acid, pH 8). After 10 min of incubation

on ice, the mixture was transferred into a vial and sonicated within an ice bath with a Cell Disruptor W-375 (Heat Systems-Ultrasonics INC, Farmingdale, NY). 500 µL of the sonicated suspension were then vigorously mixed with 500 µL of *o*-phthalaldehyde and 4.5 mL of the buffer solution and allowed to react at room temperature for 20 min. The fluorescence of GSH was finally measured with a Turner Quantech Digital-Filter Fluorometer (with emission and excitation wavelengths set at 420 and 350 nm, respectively). Assessment of reactive oxygen species (ROS) was adapted from acute toxicity assay described by Kobayashi (Kobayashi 2000) and Yoshida et al. (Yoshida et al. 2003). Briefly, 10 ml of algal suspensions were incubated with 2',7'–dichlorofluoresceindiacetate DCFH-DA (20 uM in methanol) in the dark for 2 hrs and then washed 2 times with CM. The pellets, re-suspended in CM were incubated for 1hr and then the fluorescence was measured with the fluorometer (with emission and excitation wave lengths set at 530 and 488 nm, respectively). DCFH-DA, a cell permeable indicator of oxidation, was oxidized in the presence of ROS to fluorescent 2',7'–dichlorofluorescein (DCF) in the cell.

For all of the above algal growth experiments, statistical differences (a<0.05) in observed biological responses amongst treatments were determined using a single factor analysis of variance.

3.2.4 Determination of the Aggregation State of SWNTs in Algal Culture Media

The aggregation state of SWNTs within the culture media at both the initial time (t_0) and after 96 h (t_f) was probed using a combination of the following techniques. The vis-NIR (near infrared) absorbance and NIR fluorescence spectra of both controls and SWNT-treated algal suspensions were recorded using a Nanospectralyzer (Applied NanoFluorescence, Houston, TX), with excitation from a 662 nm diode laser. The

concentration of SWNTs in all suspensions was determined using Beer-Lambert's Law and the absorbance measured at 763 nm, similar to prior studies (Moore 2005; Parra-Vasquez et al. 2007). Raman spectra were also recorded using a Renishaw Invia Bio Raman with excitation from a 785 nm diode laser. The relative intentisites of Raman peaks at 267 cm^{-1} are good indicator of CNTs aggregation state (Heller et al. 2004).

3.2.5 Transmission Eectron Microscopy Studies

TEM imaging was conducted at the University of Florida's Interdisciplinary Center for Biotechnology Research, using a Hitachi H-7000 TEM (Hitachi High Technologies America, Inc. Schaumburg, IL). Samples were prepared using methods adopted from the literature (Ellis 2006), and digital images were acquired with a Veleta camera (Veleta- Olympus Soft-Imaging Solutions Corp, Lakewood, CO) and iTEM software. Briefly, algal cultures were centrifuged at 1300 RPM for 10 min and then fixed with Trumps (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer), which was purchased from Electron Microscopy Sciences, Hatfield, PA. Fixed cells were stored at 4°C overnight and processed with a Pelco BioWave Microwave (Ted Pella, Redding, CA) to improve fixation. Samples were next washed in 0.1 M sodium cacodylate (pH 7.24), post-fixed with 2% buffered osmium tetroxide, water washed and dehydrated in a graded ethanol series of 25%, 50%, 75%, 95%, and 100%, followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurr's epoxy resin and cured at 60°C. Cured resin blocks were trimmed, cut into thin sections and collected on formvar copper slot grids. Samples were post-stained with 2% aqueous uranyl acetate and Reynold's lead citrate to improve contrast. Sections were then examined with TEM. Digital images were acquired with a Veleta camera (Veleta-Olympus Soft-Imaging Solutions Corp, Lakewood, CO) and iTEM software.

3.3 Results and Discussions

3.3.1 Characterization of Raw SWNT and Their Suspensions

For the raw SWNTs used in this study, the load of impurities amounted to about 13.4 weight % as measured by TGA, and the elemental analysis by ICP-AES detected iron as the main metal impurity (Table 3-2). An example image collected with AFM and determined nanotube length distribution from it are shown in Figure 3-2. GA-suspended SWNTs exhibited a length distribution ranging from 300 to 1200 nm. AFM images of SC-SWNTs and PF-SWNTs were not obtained. However, the length distribution is expected to be similar to that of GA-SWNTs because it is mainly determined by sonication time (Simon-Deckers et al. 2008) which was identical for all suspensions prepared in this study. For suspensions prepared in our laboratory, stability was evaluated for the duration of the experiments using spectroscopic techniques. The absorption spectra of freshly prepared GA, SC, and PF-SWNT suspensions (prepared in triplicates) were measured as a function of time to assess the stability of the suspension. As shown in Figures 3-3 and 3-4, the absorption spectra showed multiple peaks associated with the electronic structure of different (the chirality vector *n,m*; Figure 2-1) types within the SWNT suspension. These results show that absorbance of SWNTs remains unchanged over two weeks, the period used for long-term studies reported in this work. The lack of changes in days 1, 5, 9, and 14 in absorption trends/peaks indicates stability of GA-SWNTs (Figure 3-3). In fact, changes due primarily to nanotubes aggregation would yield either red-shifted or broadened peaks due to the energy transfer between bundled tubes (O'Connell et al. 2002). As a colloidal suspension, SWNT suspensions may experience aggregation process over period of time which would impact the long term experiments in particular. The aggregation

process in nano-size systems drastically modifies the overall physical characteristics of nanomaterials such as size and surface area. These physical parameters are critical factors determining their toxicity. The insets show the absorbance at 763 nm as a function of time and confirms that the aggregation state has not changed in a significant manner during the course of the experiments (Figures 3-3 and 3-4). Therefore, SWNT suspensions used in this study are considered stable for the duration of the bioassays.

3.3.2 Effect of Increasing SWNT Concentrations on *P. subcapitata* Growth

Figure 3-5 shows the growth response of *P. subcapitata* to increasing concentrations of SWNTs in culture media containing fixed GA levels (0.023% and 0.046% v/v) in a series of 96-h growth experiments. The dotted horizontal lines represent the average algal growth rates determined from control cultures. SWNT-treated media containing a final GA concentration of 0.023% show no growth inhibition effects for SWNT concentrations ranging from 0.01 to 0.05 ppm. However, a negative impact on algal growth, and therefore, toxicity is observed for SWNT concentrations >0.05 ppm (Figure 3-5A). In contrast, when the same SWNT-concentration gradient is used with increased final GA-concentration from 0.023 to 0.046% v/v, SWNT-toxicity disappears and a tendency for growth bio-stimulation is observed (Figure 3-5B).

Figure 3-6 exhibits the growth response of *P. subcapitata* to increasing concentrations of SWNTs in culture media containing fixed SC concentrations (0.05% or 0.1% v/v) in a 96-h growth experiments. Additionally, a series of oxidative stress evaluation experiments were performed. The dotted horizontal lines represent the average of algal growth rates determined from control cultures free of either SC or SWNTs. Vessel control is the culture medium containing an amount of SC that is equivalent to other SWNTs treated culture media. Culture media treated with SWNTs

having a final SC concentration of 0.05% did not express an adverse effect on algal growth within 0.2 and 0.4 ppm, but toxicity was observed at SWNT concentration of 0.8 ppm. Notably, it is observed that algal growth in culture media containing SWNTs at a final concentration of 0.8 ppm was significantly reduced when final SC concentration was increased from 0.05 to 0.1%.

The effects of PF-SWNTs on *P. subcapitata* after 96 hours are shown in Figure 3-7. The growth of *P. subcapitata* in culture media containing fixed PF concentrations (0.24% or 0.56%) showed significant stimulation when compared to control (i.e. horizontal line). The growth of algae exposed to increasing gradient of SWNT with final PF concentration of 0.24% were significantly decreased with 5 and 7 ppm of SWNT, indicating a dose-dependent toxicity of SWNT. The negative impact of SWNT on algal growth was significantly reduced upon increasing final PF concentration to 0.56%. With increased final PF concentration, SWNT concentrations up to 7 ppm showed no toxicity.

Overall, SWNT suspensions prepared in different surfactants (1% wt.) showed significant algal growth inhibition for concentration ranging from 0.5 to 7 ppm. This concentration range is comparable to the result of Blaise et al. which reported toxic concentration from 1~10 ppm using *P. subcapitata* (Blaise et al. 2008). The negative impact of carbon nanotubes was significantly mitigated when the final concentration of each surfactant in culture media was increased about 2 times. In other words, for the tested range of SWNT concentrations, the increase in levels of a non-toxic surfactant for the model organism alters the toxicity of SWNTs relative to this model organism. The potential mechanisms are discussed later herein. In addition, the growth stimulation was observed in culture media with SC and PF. The growth stimulation of *P. subcapitata*

induced by surfactants had been observed in previous studies; various anionic and nonionic surfactants were shown to increase growth of algae up to 250% as compared to control growth experiments (Nyberg 1988). Proof of biodegradation of certain types of surfactants by green algae was demonstrated in earlier literatures and this could have an effect on the biological responses of algae (Ernst et al. 1983). In other words, *P. subcapitata* used in this study may be able to utilize surfactants in culture media and this may explain the increased growth effects observed.

3.3.3 Interactions of P. subcapitata with SWNTs Introduced into Culture Media at Different Points along the Growth Curve.

In this set of experiments, aliquot volumes of SWNT-suspensions were added to algal growth media eitherprior to the inoculation with pure algal suspension (Figure 3-8A) or subsequently after an initial growth period (Figures 3-8B, 3-8C, and 3-8D). These experiments focused on the effect of the above determined toxic combination of 0.5 ppm of SWNTs and 0.023% of GA (Figure 3-5A) and 0.8 ppm of SWNTs and 0.05% of SC (Figure 3-6A) on different growth stages of *P. subcapitata*.

3.3.3.1 GA-SWNT

The results show that when SWNT is added prior to inoculation of the culture medium with algal cells, the growth inhibition is pronounced in the first 150 h (% growth decreased below ~60% of growth observed in control samples). However, this inhibition appears to be reversible as the algal growth recovers over time, reaching growth rate levels similar to that of the control after 200 h (Figure 3-8A). The addition of SWNTs several hours after the inoculation of culture media with algal cells showed different behaviors. First, SWNT addition to culture media 24 h after the initiation of the algal growth (Figure 3-8B) had a less inhibition effect than SWNT introduction during the

exponential growth phase (96 h, Figure 3-8C). For the former (Figure 3-8B), algal growth rebounds from the initial inhibition following the introduction of SWNTs, and increases to reach levels that exceed growth rates in control samples. In contrast, the addition of SWNTs after 96h had a pronounced inhibition effect, and although a general trend towards recovery is observed over time, growth rates in these culture media did not return to levels measured in control samples (Figure 3-8C). Finally, the addition of SWNTs at the end of the exponential growth phase or plateau (Figure 3-8D) leads to a drop in growth without recovery due to the limited nutrients in these batch cultures. Overall, in these long-term growth experiments, the tested toxic mixture of GA-SWNT would delay algal growth, but would not result in a complete growth inhibition of *P. subcapitata.* Our preliminary experiments using SWNTs suspended in SDS as surfactant (data not shown) resulted in total algal growth inhibition.

3.3.3.2 SC-SWNT

The results show that the introduction of SWNTs prior to inoculation of the culture medium with algal cells exhibited the most profound impact (Figure 3-8E). Algal growth was inhibited below 60 % of that of control for first 3 days and then sharply increased up to nearly 90% from 4th day. However, the growths were never recovered to the level of control during the experiment unlike reversible inhibition observed previously in certain culture media impacted with GA-SWNTs. Clearly, the change of the surfactant leads to different growth behaviors. First, SC-SWNT addition to culture media 1 day after the initiation of the algal growth (Figure 3-8F) had a more inhibitory effect than SC-SWNT introduction during the exponential growth phase (4th day, Figure 3-8G). In both cases, algal growth have been stabilized to the end point once they are impacted SC-SWNTs. Magnitude of the impact during the exponential growth phase (Figure 3-8G) are less

pronounce than that of GA-SWNTs. The Exposure of SC-SWNTs at the plateau decrease growth of algae but subsequent recovery was observed. In case of GA-SWNTs, drop of the growth at the plateau phase did not lead to recovery due to the depletion of nutrients. Utilization of the surfactants may be responsible for this observed difference and it will be discussed more in next experiment. In general, the tested toxic concentration of SC-SWNTs may adversely impacted algal growth, but the impact would be compensated up to certain level.

3.3.4 Potential Mechanisms of Observed Biological Responses of P. subcapitata Exposed to SWNT Suspensions

Several physicochemical characteristics of SWNTs can be linked to the biological responses of *P. subcapitata*. Such parameters would include (i) the generation of reactive oxygen species (ROS) and associated oxidative stress (Kagan et al. 2006; Shvedova et al. 2003), (ii) direct contact between cell and SWNTs resulting in cell membrane deformation, piercing, or uptake through passive or assisted transport (Kang et al. 2007; Liu et al. 2009), (iii) the levels and types of impurities associated with SWNT matrices (Kagan et al. 2006; Koyama et al. 2009; Shvedova et al. 2003), and (iv) the degree of SWNT aggregation (Kostarelos et al. 2007). The roles of some of these parameters in the biological responses observed in this study are discussed below.

3.3.4.1 Potential ROS activity and impact on P. subcapitata growth

In this study, GSH, which is an important biomolecule for defense against heavy metals and oxidative stress in plants, was used as a specific toxicity indicator (Noctor and Foyer 1998). GSH can be considered as a representative of nucleophilic target biomolecules in algal cells. Since the enzymes glutathione-S-transferase and glutathione-synthetase are inducible by electrophilic compounds, such as SWNTs, the

cellular GSH concentration would then be modulated by GSH-induction as well as by GSH-depletion caused by the presence of reactive toxicants. Therefore, disturbance of GSH metabolism is considered a toxicity parameter indicating cell damage as well as providing information about molecular modes of toxic action.

GA. Levels of GSH were investigated in 96-h algal standard growth exposure assays (Figure 3-9). The final GA concentrations were varied from 0.04 to 0.17% in two sets of algal culture media containing either 0.5 or 1 ppm of SWNTs. The results showed that the increase of GA concentrations above the initially tested toxic combination of 0.023% GA and 0.5 ppm of SWNTs resulted in biomass production similar or in excess to that measured in control cultures (Figure 3-9A). Although, the algal growth in culture media containing 1 ppm of SWNTs showed a slight inhibition for GA concentrations up to 0.1%, this toxicity is eliminated and the algal growth rate catches up with levels in control media as GA concentration reaches 0.17%.

Measurements of GSH (Figure 3-9B) in these culture media showed an accumulation of GSH levels, regardless of SWNT concentrations and an overall increasing trend with increasing GA concentrations. Elevated levels of GSH in SWNT-treated algal media as compared to control samples indicate the induction of GSH stimulated by oxidative stress (Lei et al. 2006), caused by SWNTs in this case. In fact, the toxicity of SWNTs has been attributed primarily to the formation of ROS and the resulting oxidative stress to living cells (Chou et al. 2008; Kagan et al. 2006; Manna et al. 2005; Shvedova et al. 2003). In the experiments using GA-SWNTs in this study, the determination of ROS was omitted but toxicological studies focusing on oxidative stress have used exclusively the glutathione assay by either assessing the concentration of

glutathione (Niederer et al. 2004; Vecitis et al. 2010), or observing the mitigation of oxidative damage through the introduction of an antioxidant-glutathione (Zhu et al. 2007). Moreover, the observed progressive build up in GSH levels with increasing concentrations of GA, which is considered a sign of oxidative stress induction (Gupta et al. 1991; Madamanchi et al. 1994), is also suggestive of the fact that GSH synthesis exceeds GSH depletion as GA concentrations increase in the culture media.

These results suggest that the presence of SWNTs stimulates the algal defense mechanisms as indicated by increased GSH levels. At the same time, the increasing GA levels enhance GSH-synthesis with production rates that are higher than rates of GSH depletion through reaction with generated ROS. The overall result is a protective effect of the algal cells, and mechanisms specific to this process would include the previously reported antioxidant potential of GA in pharmaceutical studies (Abd-Allah et al. 2002; Trommer and Neubert 2005). In this case, GA in much higher concentration than GSH in culture media would behave as the primary antioxidant, allowing the accumulation of GSH produced by algal cells.

SC-SWNT. Levels of GSH and the formation of intracellular ROS were determined using final SC concentration of 0.05 and 0.1% in algal culture media containing varied concentrations of SWNTs (Figure 3-6). The results showed that production of ROS was increased with SWNTs concentration in culture media. Levels of GSH in algae treated with the combination of SWNTs (i.e. 0.2 and 0.4 ppm) and 0.05% SC were maintained near control but the depletion of GSH was observed at the toxic concentration of 0.8 ppm showing the maximum ROS production (Figure 3-6B). Meanwhile, increasing trends of ROS was also observed in culture media treated with

SWNTs and 0.1% of SC but GSH level was still above that of control (i.e. above the horizontal line). It shows that oxidative stress exceeded the anti-oxidant defense system and subsequently caused negative biological impacts on algal growth when they were exposed to 0.8 ppm of SWNT with 0.05% of SC. On the contrary, the cellular anti-oxidative system of algae in culture media treated with 0.8 ppm of SWNT with 0.1% SC was still in tact.

PF-SWNT. Evaluations of oxidative stress induced by PF-SWNTs were conducted using ROS and GSH analysis (Figure 3-7). Overall trend of ROS and GSH levels in algae was identical between two different final concentrations of PF. ROS levels were slightly increased as SWNTs were introduced into the culture media and concentration of GSH was neither accumulated nor depleted in entire treated culture media. This implies that cellular defensive mechanisms of algae were not overwhelmed the ROS production induced by SWNTs when they were exposed to PF-SWNTs. Although oxidative stress induced by PF-SWNTs might partially contribute to growth inhibition, it seemed that different mechanisms were involved with growth inhibition observed in culture media treated with PF-SWNTs (Figure 3-7A) as compared with GA and SC-SWNTs. Other than oxidative stress, aggregation of state and physical characteristics of carbon nanotubes havebeen regarded as some of the major mechanisms of toxicity (Kang et al. 2008; Liu et al. 2009). Specifically, strong anti-microbial activities of carbon nanotubes have been explored: physical frictions and piercing by individually separated nanotubes can induce cell death. It is already shown that carbon nanotubes suspended in PF were well separated and their dispersion qualities had not been changed during algal bioassays (discussed later in ‘State of

aggregation of suspended SWNTs and impacts on algal biological responses'). Therefore, direct physical contacts between algal cells and PF-SWNTs are the plausible mechanism for negative impacts. The toxic impact of PF-SWNTs was significantly reduced in culture media with increased PF concentration of 0.56%. Increase of surfactant concentration can drastically modify adsorption of surfactant on sidewall of nanotubes (Blanch et al. 2010), and subsequently impact the aggregation state of SWNT suspensions (Angelikopoulos et al. 2010). Most of the nanotubes are exposed to the surrounding environment at a low concentration of surfactant while the absorption of surfactant on the sidewall of carbon nanotubes is rapidly increased with increasing concentration of surfactant (Figure 3-10). This 'coating' formed by increased surfactant concentration can prevent direct contacts between nanotubes and living organism and exposure of harmful catalyst metals. In fact, the coating of MNs is a common technique in order to obtain desirable properties such as biocompatibility and solubility (Hardman 2006).

3.3.5 State of Aggregation of Suspended SWNTs and Impacts on Algal Biological Responses

The analysis of the physical state of SWNTs in algal suspensions was conducted at the initial (t_0) and final (t_f) times of the algal growth experiments to gain insight into the potential mechanisms of observed cell-SWNT interactions. The sensitivity of NIR-fluorescence and Raman spectra to environmental effects can be used to assess changes due to SWNT aggregation and quenching mechanisms during algal growth.

3.3.5.1 GA-SWNT

Figure 3-11 shows different emission spectra obtained for sub-samples of algal suspensions containing different concentrations of GA and SWNTs collected at times t_0

and t_f. Since only individual and well-dispersed semiconducting SWNTs emit fluorescence (O'Connell et al. 2002), these spectra are evidence that most of the SWNTs remain suspended and well-dispersed in the culture media throughout these algal exposure experiments. However, the intensity of the spectra decreased slightly for each of the tested concentrations between times t_0 and t_f. These changes correlate to the relative ratio of surfactant to SWNTs; photoluminescence (PL) intensity changes are more significant as this ratio decreases. The observed decrease in emission intensities suggest that one or more of the following could occur to SWNTs during algal growth: (1) covalent or non-covalent sidewall reactions that quench SWNT-fluorescence; (2) aggregation in the suspension; or (3) removal of SWNTs by sedimentation of either SWNTs or algal cells interacting with SWNTs.

Decreases in fluorescence intensity can occur when the environment surrounding the SWNTs changes due to the replacement of surfactant molecules (GA in this case) by polymers, such as proteins (Cherukuri et al. 2004; Cherukuri et al. 2006) and polysaccharides released in the culture media by growing algal cells. Therefore, it is possible that the production of dissolved organic matter (DOM) or organic exudates during algal growth could result in the formation of DOM-coated SWNTs. However, surfactant replacement is typically accompanied by characteristic solvatochromic peak shifts associated with the new environment surrounding the nanotubes (Cherukuri et al. 2004; Cherukuri et al. 2006; Moore et al. 2003). In this study, only a 1 to 2 nm solvatochromic shift in peak position was observed. This small difference indicates that only a very small amount of algal produced DOM could possibly replace GA molecules surrounding the SWNTs. These changes could indicate some changes to the surface

coverage of the surfactant, which reduces the ability of the layer to prevent fluorescence quenching mechanisms (Silvera-Batista et al. 2009; Wang et al. 2008b). However, any changes to the surfactant structure must be minimal since exposure to higher dielectric environments are expected to decrease the PL intensity dramatically (Silvera-Batista et al. 2010).

It is also possible that the sidewalls of the nanotubes become functionalized during algal growth, reducing the PL intensity. However, Raman spectra would indicate structural changes to the carbon atoms on the sidewalls of SWNTs associated with covalent functionalization. The D-peak (at about 1300 cm^{-1}) and G-peak (at about 1600 cm^{-1}) on such spectra correspond to phonon modes associated with sp^3 and sp^2 carbon atoms of SWNTs, respectively. In this study, spectra obtained for different GA concentrations (0.023% and 0.046% with 0.5 ppm SWNTs) at both t_0 and t_f exhibit nearly identical D/G peak ratios (Figure 3-12), suggesting that nanotubes do not undergo significant chemical changes, such as oxidation or sidewall functionalization. This is because high D/G ratios are obtained only when covalent bonds are formed on SWNT surfaces causing the phonon modes of sp^2 carbon atoms to shift to sp^3 phonon modes (Dresselhaus et al. 2003).

The decrease in the intensity of the PL (Figure 3-11) and G-peak between t_0 and t_f (Figure 3-12) indicate that some SWNTs could be removed from the algal suspensions. Such sedimentation or removal of SWNTs could occur following the aggregation of a small fraction of SWNTs present in the suspension. This is important as previous exposure studies investigating the effects of the degree and kind of aggregation of SWNTs on cells have linked these parameters to biological responses in human MSTO-

21 cells (Kostarelos et al. 2007). Although the above discussion suggests that complete exchange of the surfactant with DOM on SWNT surfaces is unlikely, the adsorption of even a small amount of DOM could alter the surfactant structure around the nanotubes (Wang et al. 2008b) or change the surface charges (Myklestad 1995), disrupting the mechanism that disperses SWNTs. The induced progressive aggregation of SWNTs in such cases would promote removal from solution by sedimentation and possibly alter the biological response of the tested model organisms as well. However, considerable aggregation of SWNTs would be expected to have significant decreases in PL intensity, which is not observed.

One may therefore conclude from the spectroscopy data suggest that the SWNTs in the suspension are similar in regards to surfactant coverage and aggregation state throughout algal growth. However, the fact that the PL intensity changes are correlated to the relative ratio of surfactant to SWNTs indicates that both of these factors could occur to a limited extent during algal growth. Although aggregation and surfactant changes cannot be ruled out, it is clear that these effects have minimal influence on the SWNTs during algal growth at all SWNT concentrations. Therefore, our study suggests that well-dispersed SWNTs in aqueous suspensions can result in negative biological effects at concentrations >1 ppm, which is in contrast to previous studies that found that aggregation was responsible for toxicity (Kostarelos et al. 2007).

3.3.5.2 SC-SWNT

The dispersion state of SC-SWNTs used in this study was monitored using NIR-fluorescence spectra and Raman spectroscopy before and after the 96 hours algal growth experiments. Photoluminescence spectra obtained from culture media treated with SC-SWNTs are shown in Figure 3-13. Fluorescence emitted from SWNTs at the

beginning (i.e. black line) was significantly reduced at the end of the experiment (i.e. dotted line) for SWNTs concentrations of both 0.05% and 0.1% SC. As discussed earlier, decreases in fluorescence intensity are mainly caused by sidewall modifications, changes in the interaction between surfactant and nanotubes, or aggregation in the suspension and subsequent removal of SWNTs. Lack of decreases in absorbance and identical Raman D/G-peak ratio between before and after the experiment indicate that removal of SWNTs and side wall functionalizations are unlikely the cause of changes in PL intensity (data not shown). Changes in intensity of Raman aggregation peak show signs of nanotubes aggregation in final SC concentration of 0.05%, but no significant changes are observed in 0.1% (Figure 3-14). Biological modification of SWNTs that target living organisms consumed coating materials of carbon nanotubes was reported (Roberts et al. 2007). If surfactants used to disperse SWNTs were degraded and utilized by algae in the culture media, it would disturb stability of the suspension and then result in aggregation of SWNTs. This aggregation induced by algae was not observed in higher SC concentration because there is enough SC in culture media to replace exposed sidewalls of carbon nanotubes.

3.3.5.3 PF-SWNT

Fluorescence spectra changes were also monitored in the experiment using PF-SWNTs (Figure 3-15) Emission spectra observed in this experiment shows that quenching of fluorescence does occur but in relatively small magnitude. This suggests that most of the SWNTs remained suspended and well dispersed in culture media. The existence of stable nanotubes suspensions during algal growth are also confirmed in monitoring the aggregation peak in Raman spectra (insets of Figure 3-16). Additional modifications that nanotubes may under go during algal growth are the surfactant

replacement and functionalization of the sidewalls. PF-SWNTs in culture media did not undergo significant shifts of peak position in fluorescence and Raman spectra (Figures 3-15 and 3-16), implying that surfactant replacement is unlikely to occur during the experiment. Finally, functionalization of sidewalls could occur by introducing defected sites on the sidewalls of nanotubes. The degree of the functionalization has been shown to influence toxic response of living organisms (Cheng et al. 2009; Choi et al. 2009; Sayes et al. 2006). The degree of functionalization can be assessed from two distinctive peaks of Raman spectra; the D-band (i.e. defect- induced) and G- band (i.e. graphite-related) (Dresselhaus et al. 2008). D and G peak obtained for different SWNT concentration before and after exhibit insignificant changes (Figure 3-16), indicating that sidewall structures have been intact without chemical changes such as oxidation.

3.3.6 TEM Observations of *P. subcapitata* Cells Grown in the Presence and Absence (control) of GA-SWNTs

A previous study on SWNT-organism interactions showed that cell death could be attributed to the physical piercing of cell membranes by individual SWNTs (Kang et al. 2007). TEM observations of cells from the different treatments used in this study (Figure 3-17) showed no detectable physical cell-SWNT interactions. This could be due to the lack of sufficient contrast to distinguish between carbon-based background cell components and the carbon nanotubes. However, a look at cells exposed to the previously identified toxic combination of 0.023% GA and 0.5 ppm SWNTs (Figure 3-17B) in comparison with cells from control samples (Figure 3-17A) showed significant changes in the morphology of cell membranes. In these dividing cells, SWNT-treated cells have reduced size and deformed cell membranes (Figure 3-17B). These impacts could be attributable to the destructive effects of ROS due to the fact that membrane

integrity is a primary target (Cabiscol et al. 2000), but one may not exclude the potential physical interaction between cell and SWNT particles, even though our TEM images do not show such occurrences. Finally, the lack of physical impacts on either controls (e.g., Figure 3-17A) or other samples obtained from culture media treated with GA concentrations >0.023% (data not shown) supports the finding that GA does mitigate the toxicity of SWNTs to *P. subcapitata*.

3.3.7 Impacts of SWNT-impurities on Algal Growth

The effective concentration of SWNTs impacting algal growth was different between GA, SC, and PF-SWNT in this study. Approximately 50% growth inhibitions were observed in culture media with SWNT concentration of 0.5, 0.8, and 5 ppm for GA, SC, and PF-SWNT, respectively (Figures 3-5A, 3-6A, and 3-7A). Analysis of oxidative stress showed that GA and SC-SWNTs exhibited more oxidative stress than PF-SWNTs used in this study. This biological response of organisms exposed to SWNTs can also be affected by the load of impurities common to carbon nanotubes (Kagan et al. 2006; Koyama et al. 2009; Shvedova et al. 2003; Smart et al. 2006). For raw SWNTs used in this study, the load of impurities amounted to 13.4 weight % as measured by TGA, and ICP-AES analyses detected only iron in levels above the instrument's detection limits (ca. 2.8 ppm) in SWNT suspensions. Previous research has linked Fe-impurities to toxicity due to its role in the formation of free radicals and ROS (Nel et al. 2006; Shvedova et al. 2003), and the activity of ROS tends to be proportional to the concentration of Fe impurities in SWNT suspension (Kagan et al. 2006). Therefore, higher Fe concentrations were found in GA and SC-SWNTs and they yielded more severe oxidative stress than PF-SWNTs. Moreover, the continuous exposure of algal cultures to light certainly favors different photochemical reactions, such as the Fenton

reaction. With regard to the experimental results obtained in this study, ROS production induced by Fe-impurities is possible, and if so, the previously reported antioxidant potential of GA (Abd-Allah et al. 2002; Trommer and Neubert 2005) could play a role in the observed decreasing toxicity trend with increasing GA concentrations in culture media.

3.4 Concluding Remarks

As aquatic systems behave as terminal sinks for most environmental pollutants including waste NMs, aquatic organisms would likely become exposed to these emerging pollutants. This is not only in their pristine forms, but also as surface modified materials due to surfactant coatings as well as to surface transformations induced by the interaction with water chemistry parameters. In this study, the biological responses of the freshwater green algae, *P. subcatpitata* exposed to SWNTs suspended in aqueous surfactant solutions (GA, SC, and PF) of different chemical composition were investigated. GA, SC and PF-SWNT suspended in aqueous solutions resulted in stable suspensions for the duration of the experiments. In growth inhibition experiments, the adverse biological impact was dependent on concentrations of SWNTs and both type and amount of surfactant in algal culture media. Mechanistic studies point to oxidative stress as the major cause of algal growth inhibition. *P. subcapitata* appeared to stand higher concentration of SWNTs in culture media when nanotubes were suspended in PF as compared to either GA or SC.

Increasing the concentrations of used surfactants in culture media reduced the toxic effects of SWNTs. In the case of GA and SC-SWNT suspensions, *P. subcapitata* showed less oxidative stress in culture media with higher concentrations of surfactant. The following mechanisms were responsible for the observed mitigation of SWNT

toxicity: (i) the antioxidant activity of GA neutralizes the formation of ROS and this is supported by the measured levels of GSH which showed an accumulation over time, and (ii) higher concentration of SC would reduce the exposure of SWNT sidewalls due to biodegradation of surfactant as observed in culture media with low concentration of SC, which resulted in increased oxidative stress. In the case of PF-SWNTs, increased concentrations of the surfactant provided a more complete coating of nanotubes, preventing physical contacts between the nanotubes and the cells.

In terms of the toxicity of SWNTs, the dispersion state, the average diameter, and surface area should be known as they are key factors controlling the negative impacts of the naotubes. A significant finding of this study is the potential for mitigation of the toxic impact of carbon nanotubes. This could be beneficial toward biocompatibility research. Non-covalent functionalization using surfactants is simple method, non-destructive for intrinsic structure of nanotubes, and optimizable by simply switching appropriate surfactants for specific target organisms.

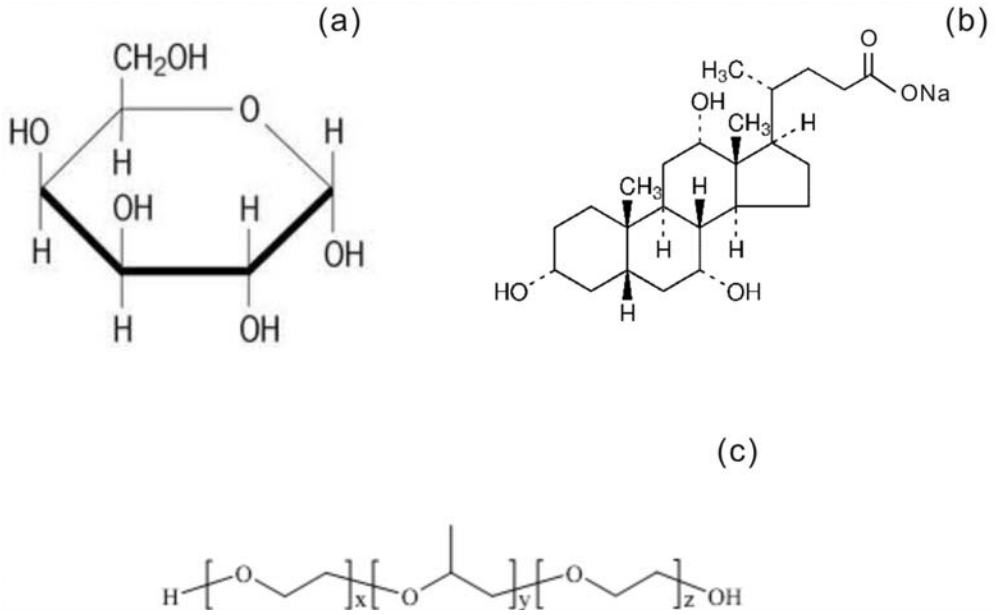

Figure 3-1. Molecular structures of selected surfactants (a) basic unit of gum Arabic (GA), (b) sodium cholate (SC), and (c) pluronic acid F108 (PF).

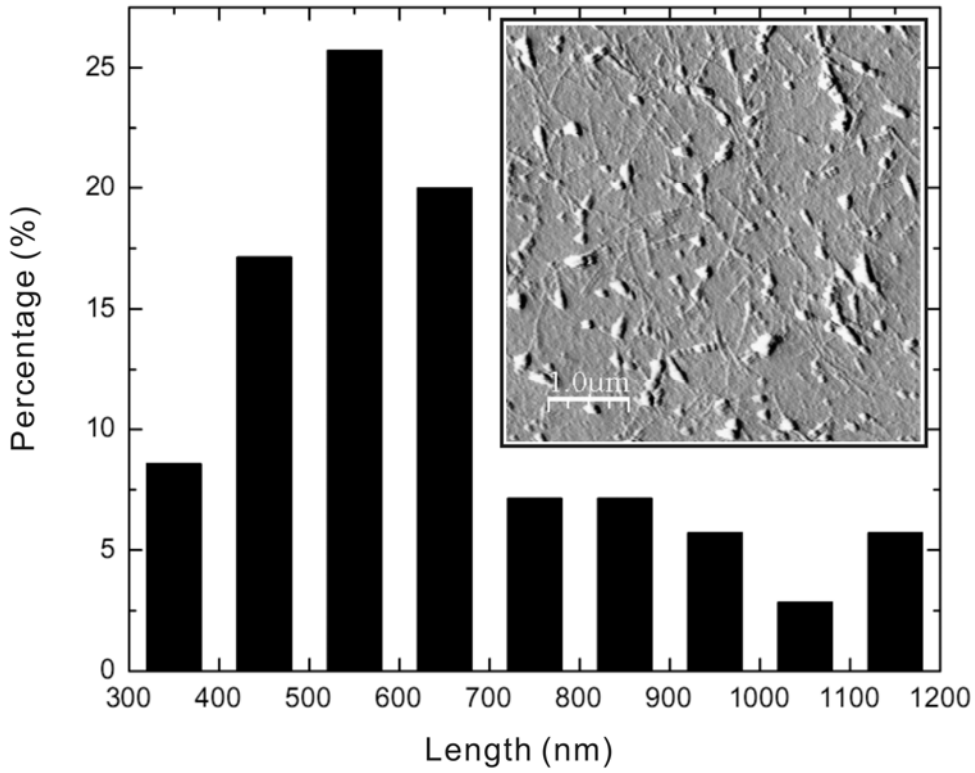

Figure 3-2. Length distributions of individual SWNTs suspended in 1 % GA determined by analysis of AFM image (inset).

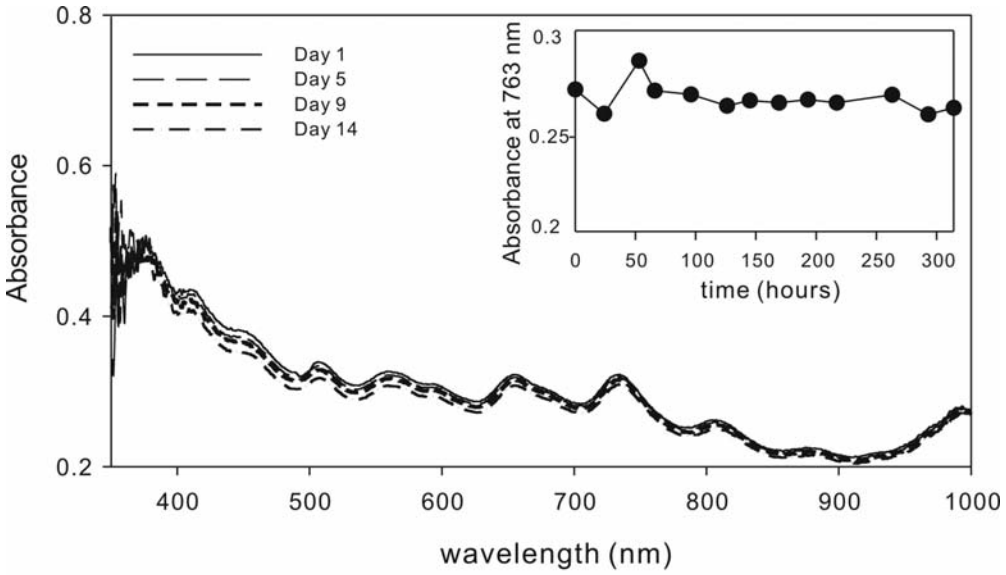

Figure 3-3. Absorption spectra of freshly prepared SWNTs in gum Arabic (GA-SWNT). The inserted figure shows measurements of absorbance of GA-SWNT at 763 nm for 14 days.

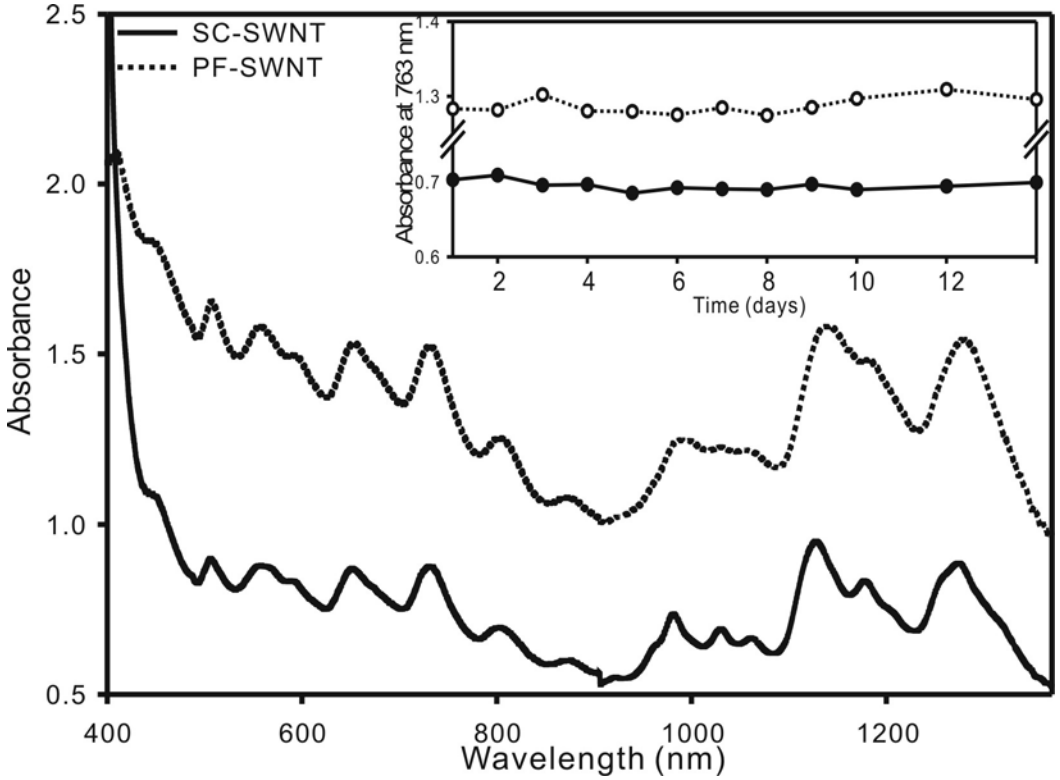

Figure 3-4. Absorption spectra of freshly prepared SWNTs in sodium cholate (SC-SWNT) and pluronic acid F108 (PF-SWNT). The inserted figure shows measurements of absorbance of SC-SWNT and PF-SWNT at 763 nm for 14 days.

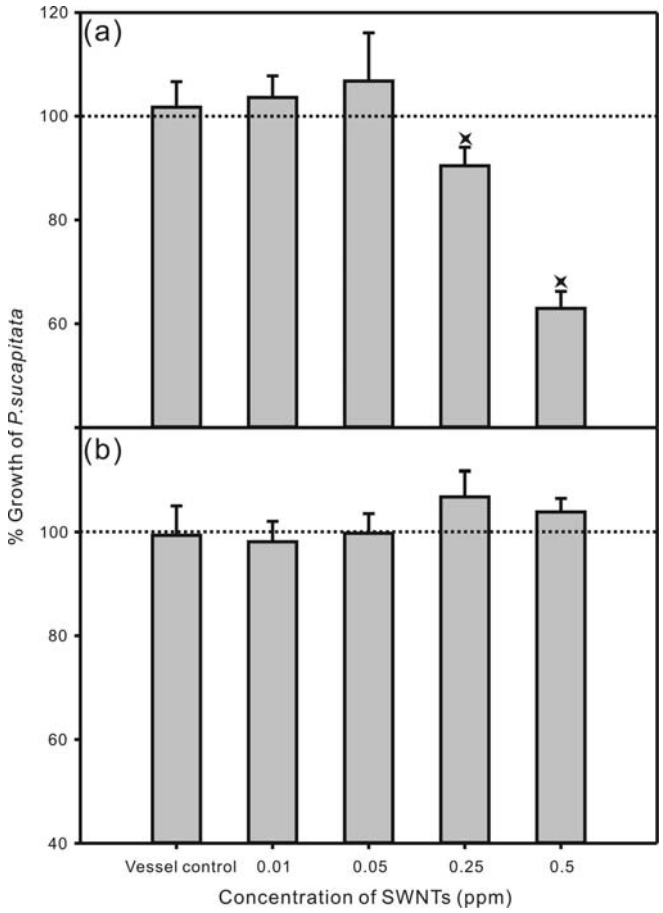

Figure 3-5. Effect of increasing concentrations of single-walled carbon nanotubes (SWNTs) on the growth of *P. subcapitata* in a standard 96-hour chronic algal assay. The final concentration of gum Arabic (GA) used as surfactant to suspend SWNTs was adjusted in all culture media to a final concentration of 0.023% (a), and 0.046% (b). The horizontal line represents the growth of P. subcapitata in control culture media (i.e. plain culture media containing neither GA nor SWNTs). Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control. (*) indicates a significant difference as compared to controls (α<0.05)

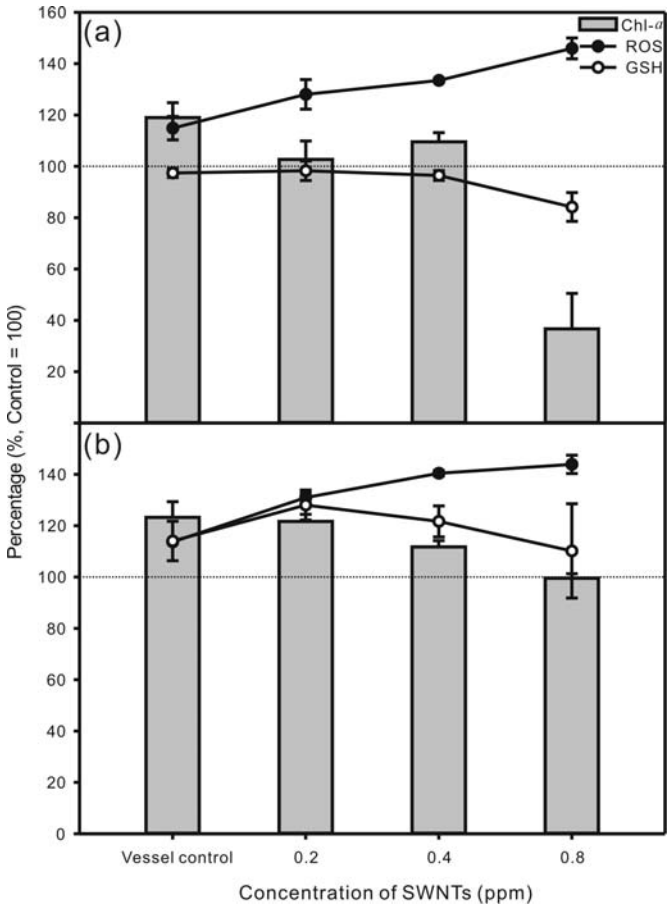

Figure 3-6. Effect of increasing concentrations of SWNTs on the growth of *P. subcapitata* in a standard 96-hour chronic algal assay. The final concentration of sodium cholate (SC) used as surfactant to suspend SWNTs was adjusted in all culture media to a final concentration of 0.05% (a), and 0.1% (b). Changes in biomass measured as chlorophyll-a are shown as gray bars, and trends of glutathione (GSH) and reactive oxygen species (ROS) are illustrated as black (●) and open dots (○), respectively. The dotted horizontal line represents the growth of *P. subcapitata*, GSH, and ROS in control culture media (i.e. plain culture media) and vessel control contains only SC. Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control.

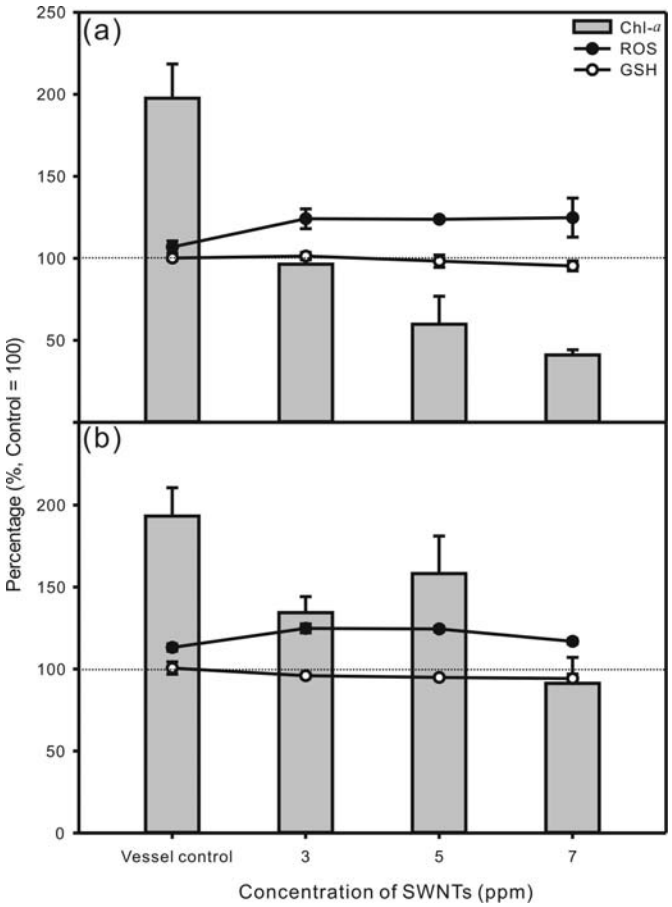

Figure 3-7. Effect of increasing concentrations of SWNTs on the growth of *P. subcapitata* in a standard 96-hour chronic algal assay. The final concentration of pluronic acid F108 (PF) used as surfactant to suspend SWNTs was adjusted in all culture media to a final concentration of 0.24% (a), and 0.56% (b). Changes in biomass measured as chlorophyll-*a* are shown as gray bars, and GSH and ROS are illustrated as black (●) and open dots (○), respectively. The dotted horizontal line represents the growth of P. subcapitata in control culture media (i.e. plain culture media) and vessel control contains only PF. Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control.

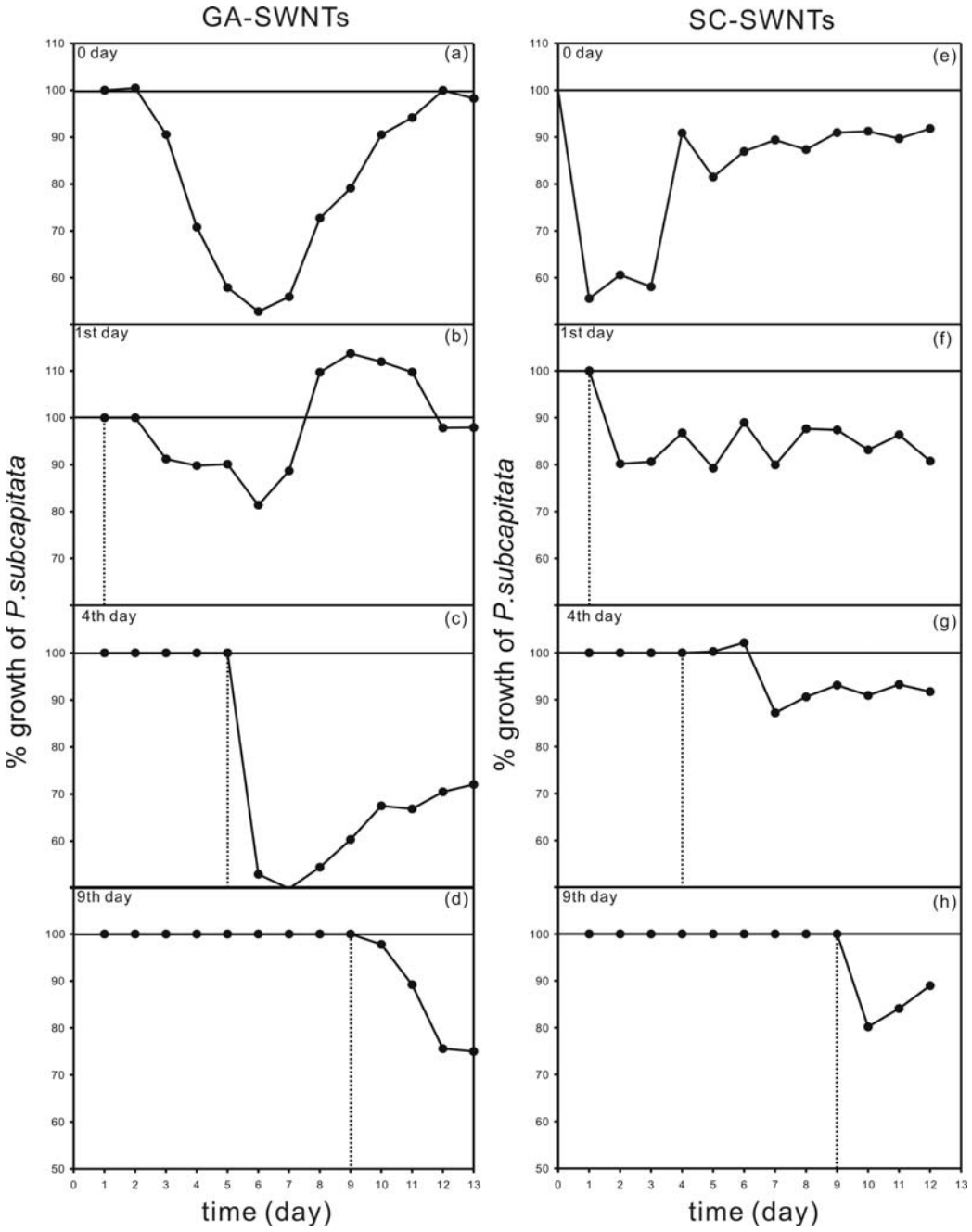

Figure 3-8. Biological response of *P. subcapitata* to GA-SWNT and SC-SWNT introduction to the culture medium at different algal growth phases. (A and E) SWNTs added prior to inoculating with the algal cells time t_0; (b and f) SWNT addition after 24 hours of growth; (C and G) SWNT addition after 96-hours and during the exponential growth phase, and (D and H) SWNT addition after 216 hours of growth (near plateau). The horizontal lines represent the percentage of algal growth in control culture media (i.e. culture media containing only surfactants but no SWNTs), which have been assigned values of 100%, and the observed effects in treated samples are normalized to the

control. Vertical dotted lines indicate the point of SWNT addition to the culture media.

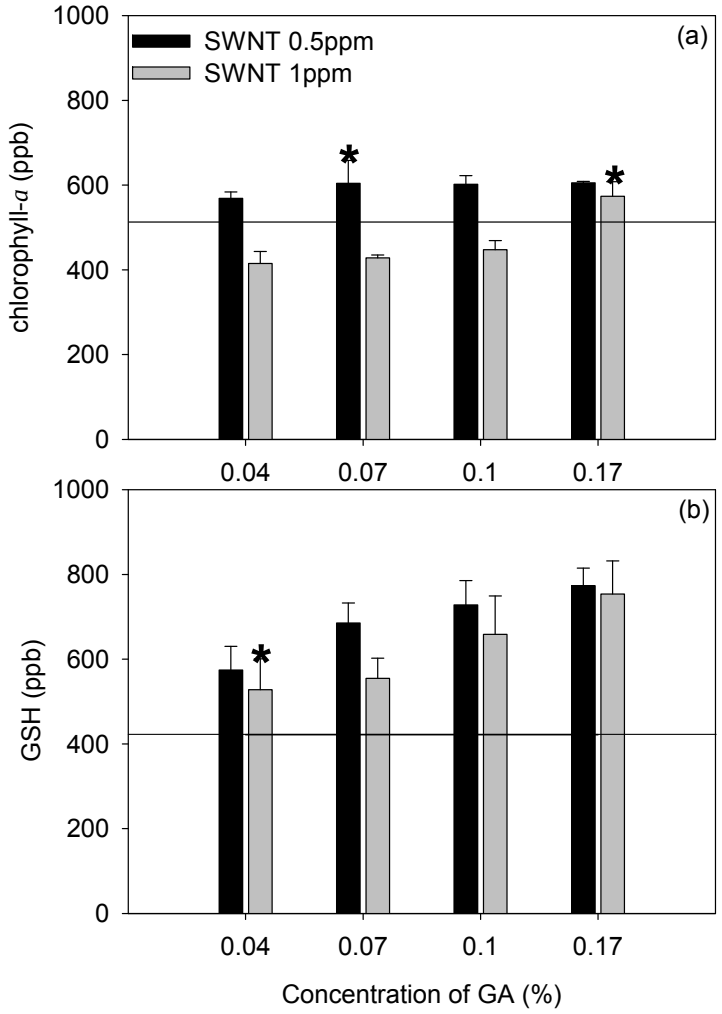

Figure 3-9. Effect of increasing concentrations of GA on *P. subcapitata* growing in culture media (standard 96-hour chronic algal assay) containing fixed SWNT concentrations of 0.5 and 1 ppm. Figure 9A shows changes in biomass measured as chlorophyll-a, and Figure 9B illustrates the trends of GSH in culture media. The horizontal line represents the growth of *P. subcapitata* in control culture media (i.e. culture media containing neither GA nor SWNTs). Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control. (*) indicates the lack of significant differences as compared to controls (a>0.05)

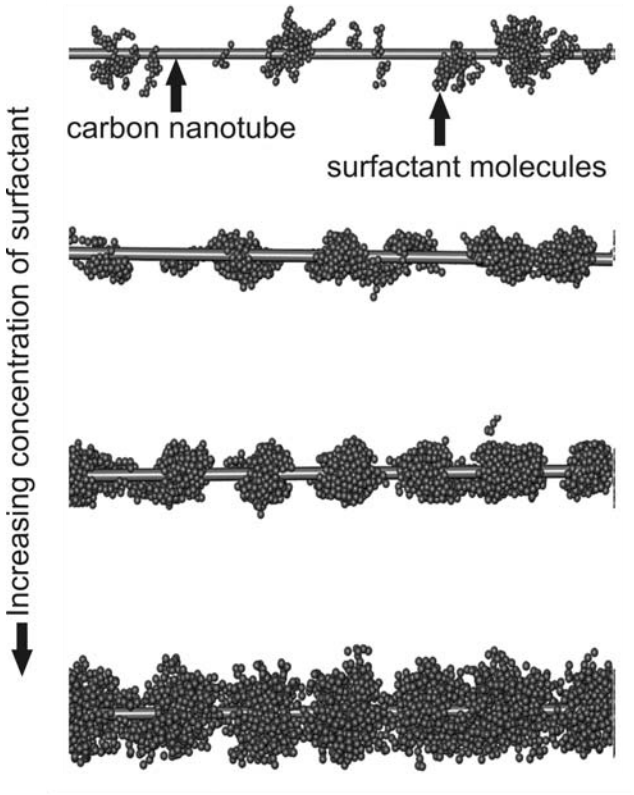

Figure 3-10. Diagram of the relationship between carbon nanotubes exposure and concentration of surfactant. Adopted from Angelikopoulos. et al. (Angelikopoulos et al. 2010)

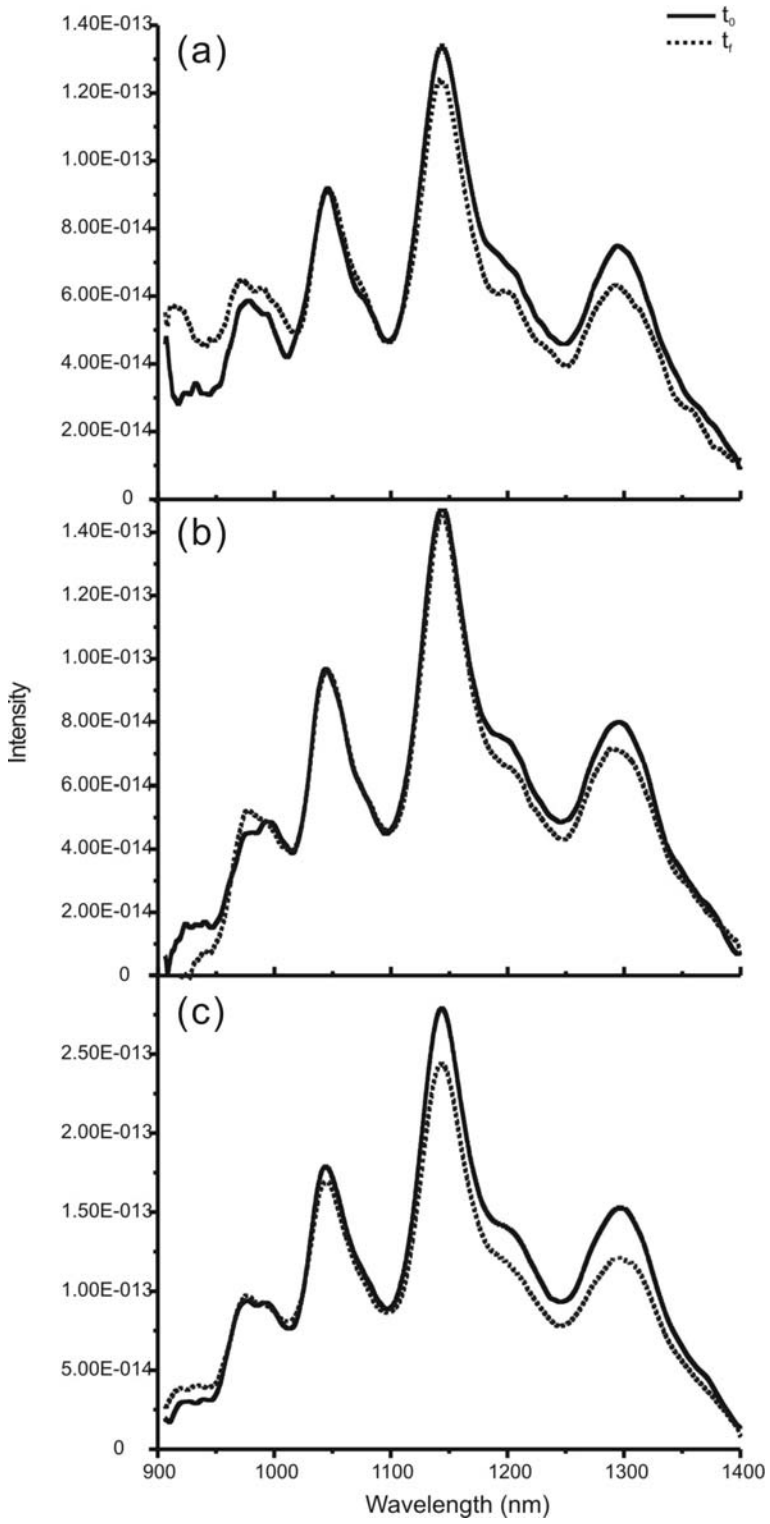

Figure 3-11. Near infrared fluorescence spectra of GA-SWNTs in algal suspensions with GA concentrations of (a) 0.023% with 0.5 mg SWNTs/L, (b) 0.046% with 0.5 mg SWNTs/L, and (c) 0.046% with 1mg SWNTs/L. Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments.

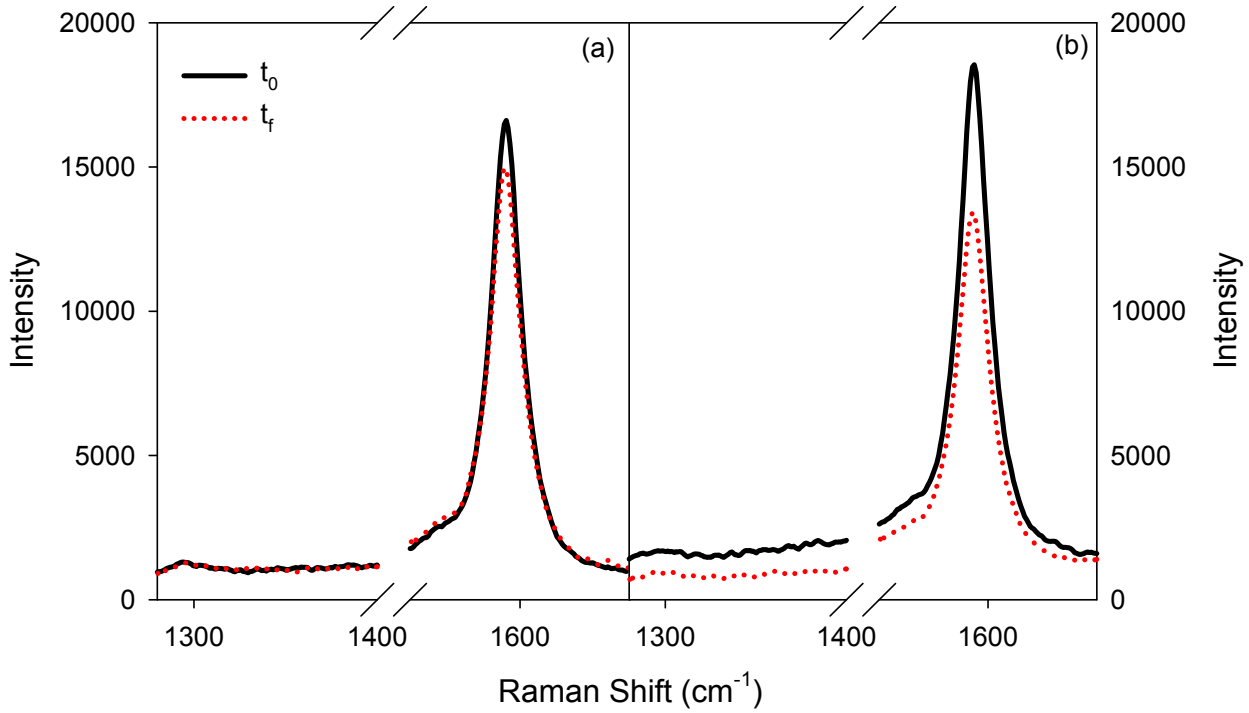

Figure 3-12. Raman spectra of GA-coated SWNTs (0.5 ppm) in algal suspensions measured on the first (t_0) and fourth (t_f) day of algal exposure experiments. (a) 0.023% GA; (b) 0.046% GA.

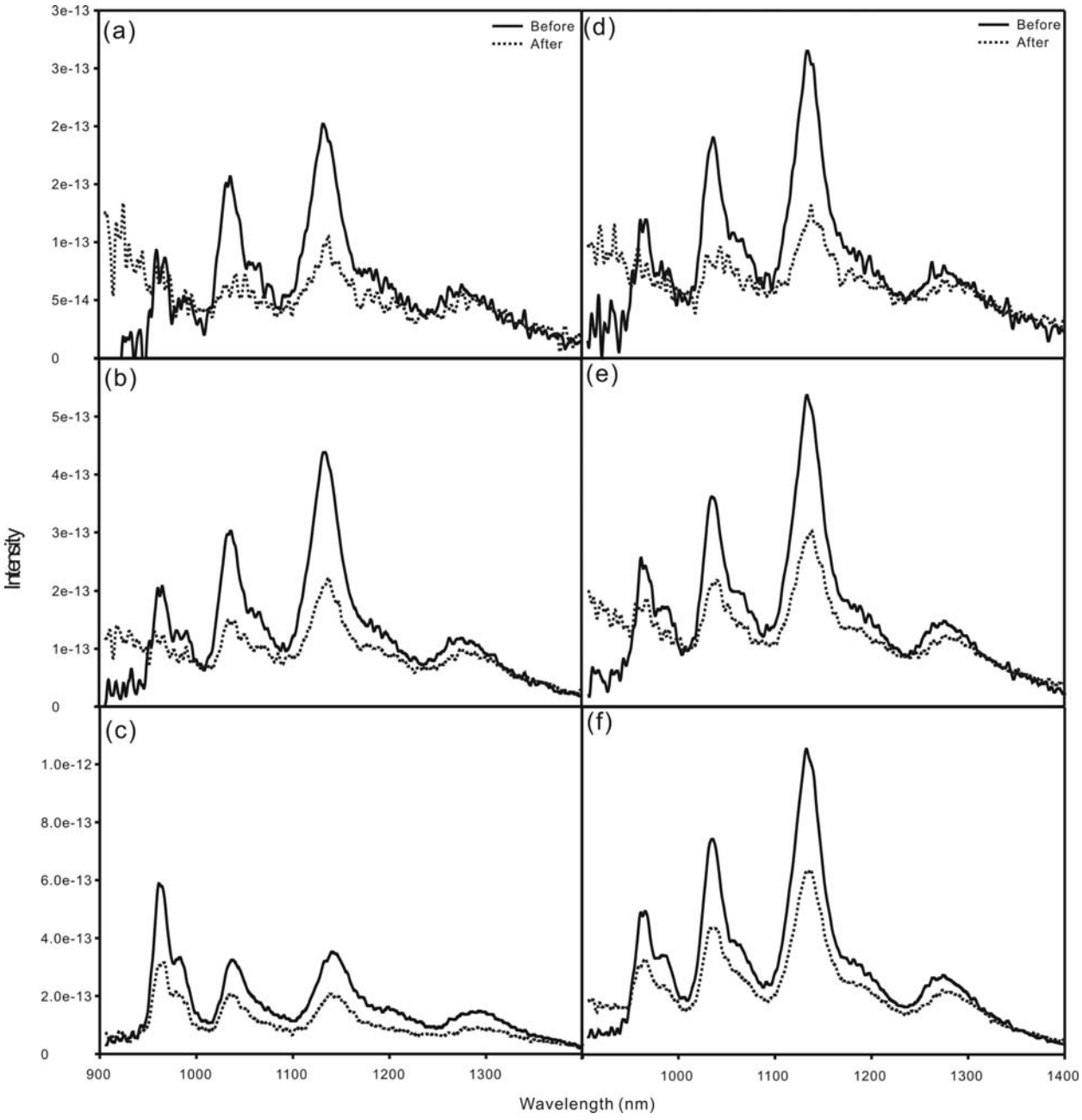

Figure 3-13. Near infrared fluorescence spectra of single walled carbon nanotubes suspended in sodium cholate (SC-SWNTs) in algal suspensions with SC concentrations of 0.05% with 0.2, 0.4, and 0.8 mg SWNTs/L (A, B, and C) and 0.1% with 0.2, 0.4, and 0.8 mg SWNTs/L (D, E, and F). Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments.

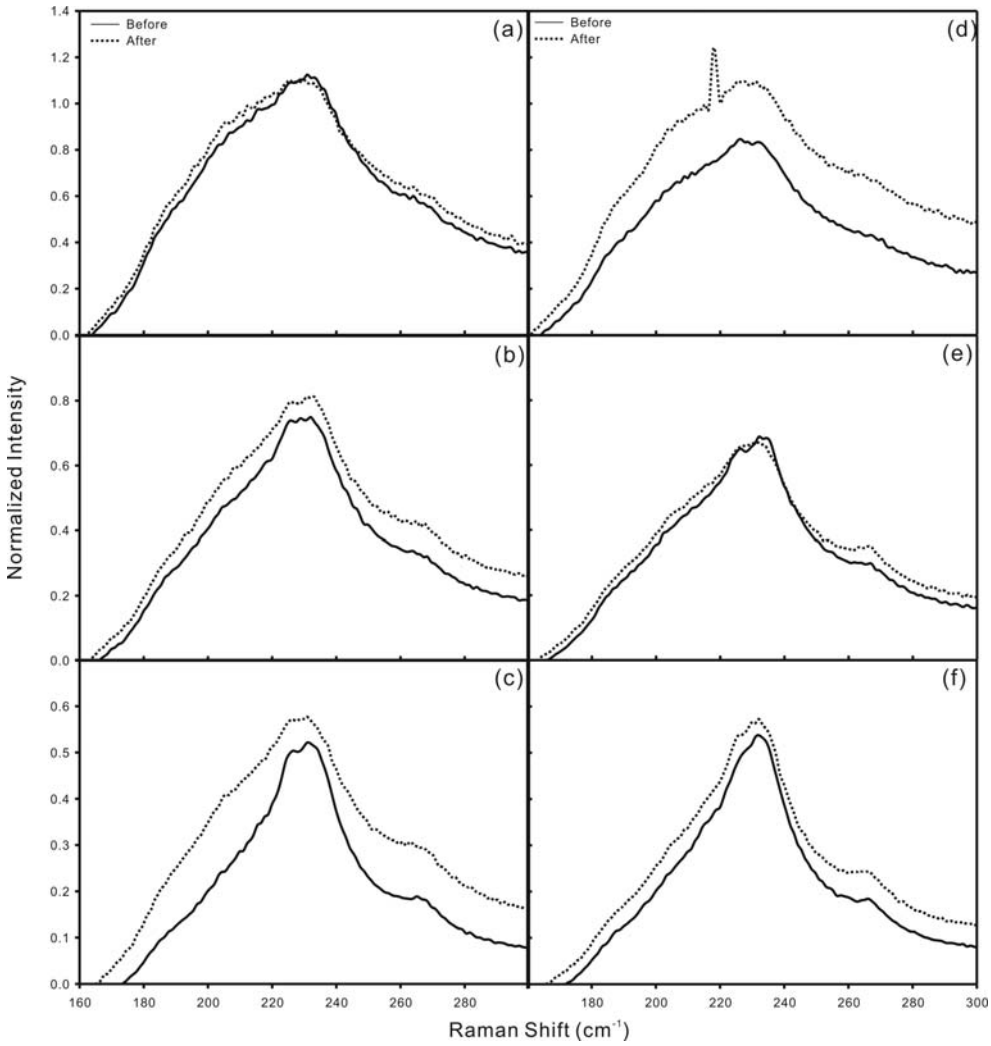

Figure 3-14. Raman spectra of single walled carbon nanotubes suspended in sodium cholate (SC-SWNTs) in algal suspensions with SC concentrations of 0.05% with 0.2, 0.4, and 0.8 mg SWNTs/L (A, B, and C) and 0.1% with 0.2, 0.4, and 0.8 mg SWNTs/L (D, E, and F). Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments.

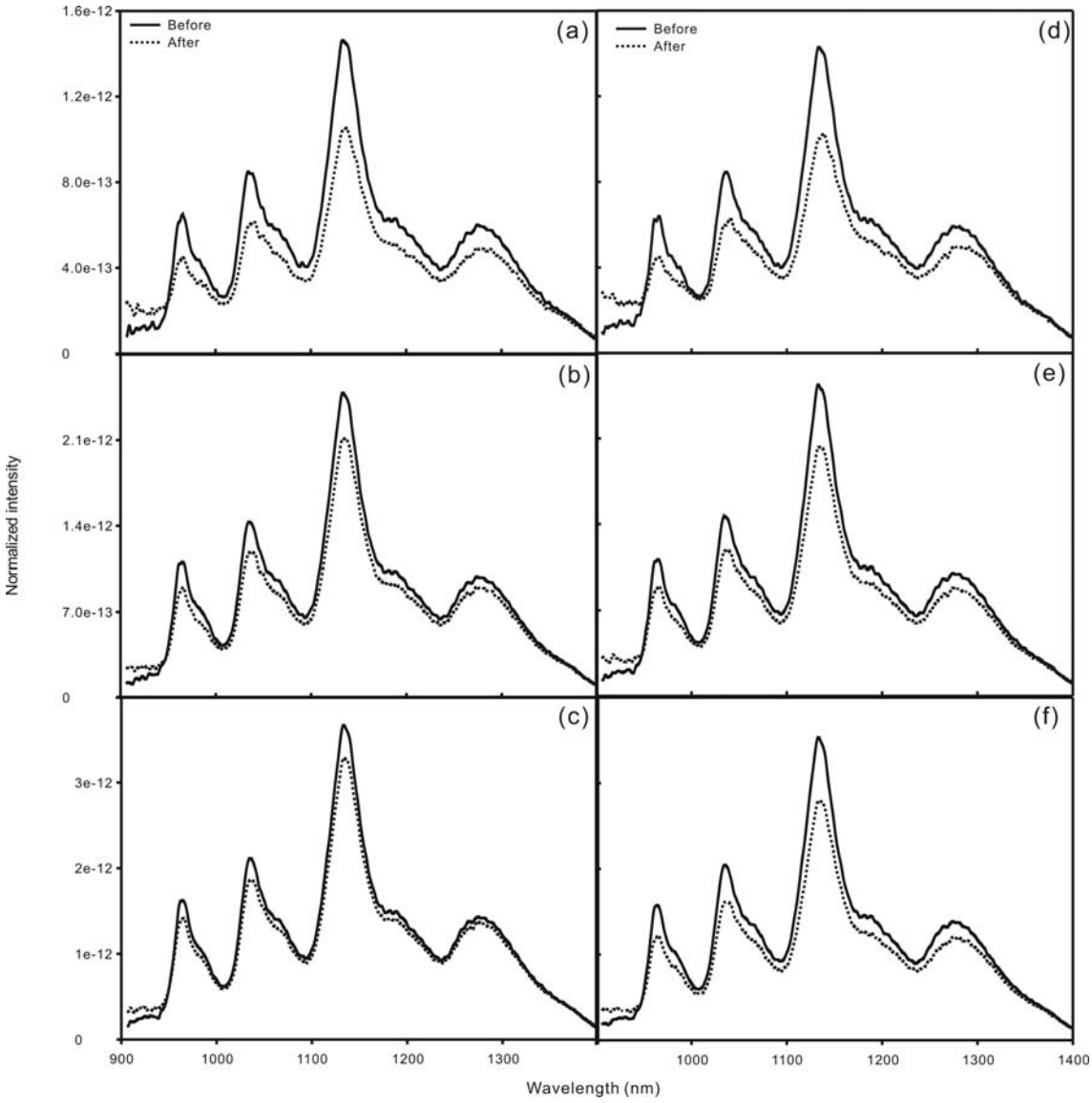

Figure 3-15. Near infrared fluorescence spectra of PF-SWNTs in algal suspensions with PF concentrations of 0.24% with 3, 5, and 7 mg SWNTs/L (A, B, and C) and 0.56% with 3, 5, and 7 mg SWNTs/L (D, E, and F). Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments.

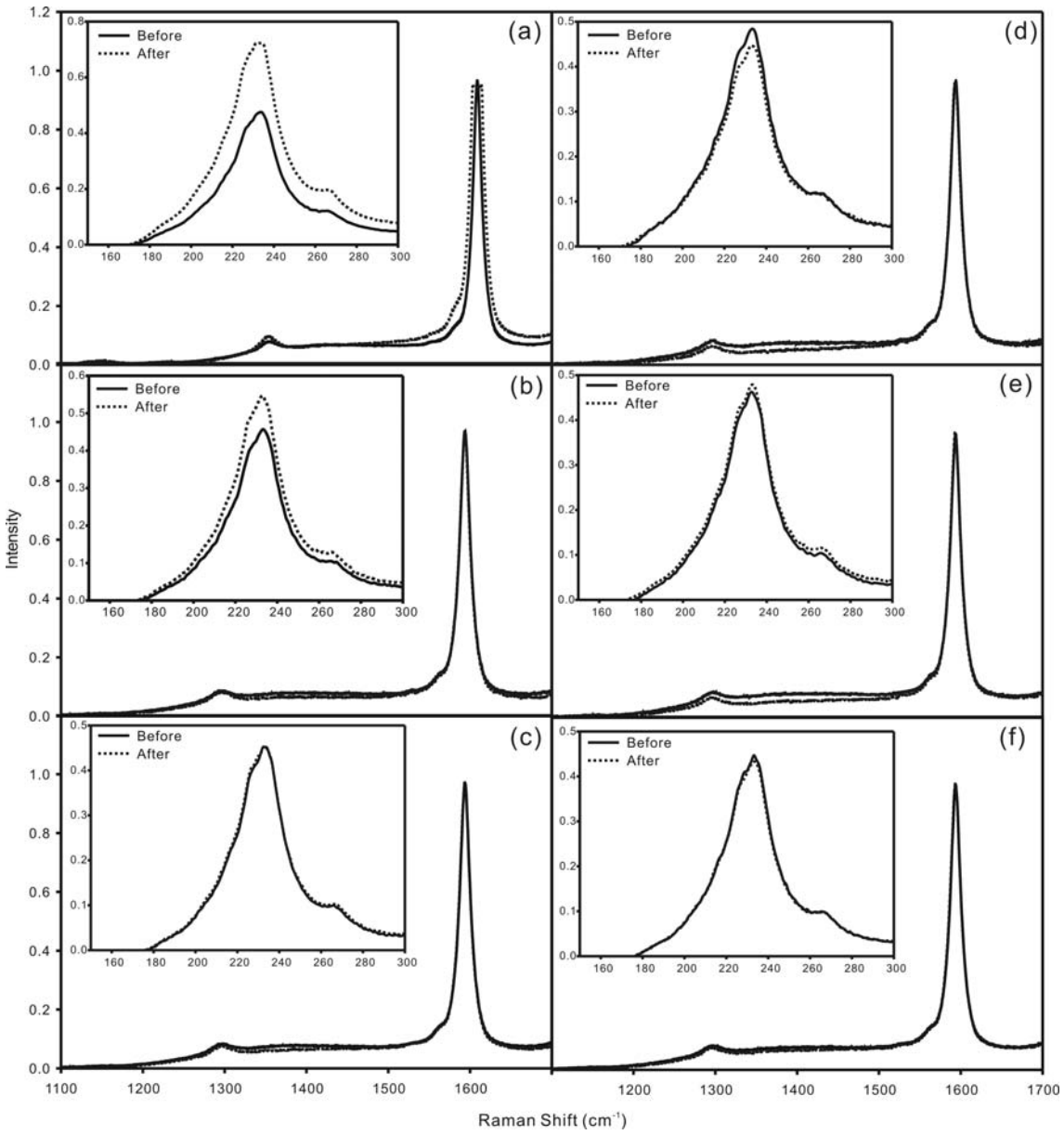

Figure 3-16. Raman spectra of SWNTs suspended with PF in algal suspensions with PF concentrations of 0.24% with 3, 5, and 7 mg SWNTs/L (A, B, and C) and 0.56% with 3, 5, and 7 mg SWNTs/L (D, E, and F). Inserted figures shows radial breathing mode (RBM) and aggregation peak at 267 cm^{-1}. Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments

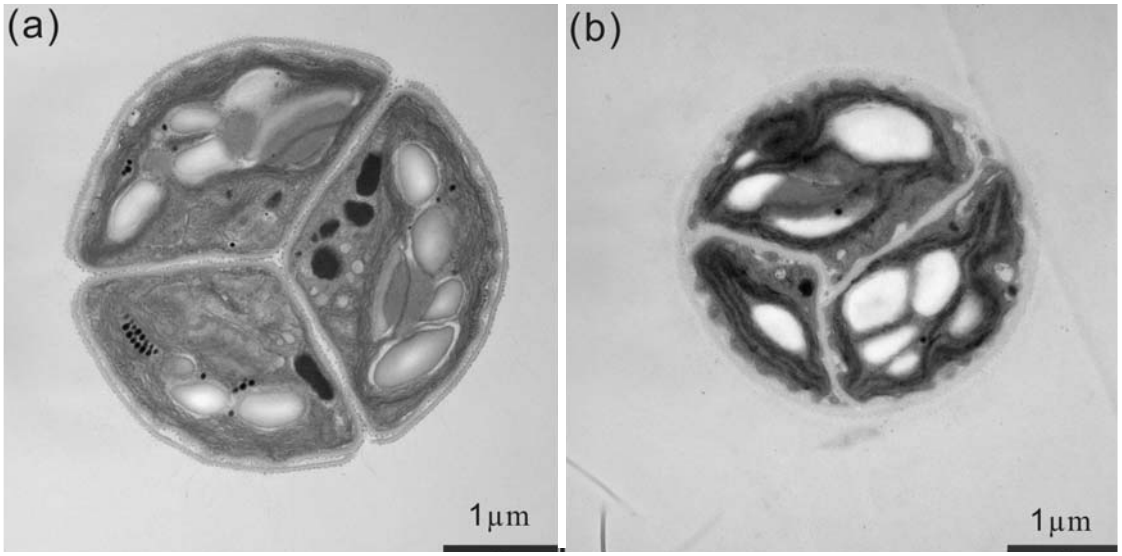

Figure 3-17. TEM images of *P. subcapitata* from a control sample (a) and from a culture medium containing final concentrations of 0.5 ppm and 0.023% (v/v) for SWNTs and GA (b).

Table 3-1. Final surfactant concentrations in algal culture media

	Final surfactant concentration in culture media (%, v/v)	
	Low	High
GA	0.023	0.048
SC	0.050	0.100
PF	0.240	0.560

Table 3-2. Detected metal impurities in SWNT suspensions

Suspension types	Metal concentrations (ppm)		
	Fe	Cu	Mg
GA-SWNT	2.8	BDL*	BDL
SC-SWNT	3.6	0.2	0.2
PF-SWNT	1.2	0.2	0.4

*BDL=Below detection limit.

CHAPTER 4
ROLE OF DISSOLVED ORGANIC MATTER IN THE PARTITIONING OF SINGLE WALLED CARBON NANOTUBES BETWEEN AQUEOUS AND ORGANIC PHASES: IMPLICATIONS FOR BIOACCUMULATION

4.1 Introductory Remarks

The ongoing mass production and anticipated widespread use of carbon nanotubes (CNT) in various industrial activities, including biomedical and environmental fields, are raising concerns about the potential risks for both the environment and human health (US-EPA 2007). The toxicity of CNTs has been studied quite extensively in the last few years and several of these studies have reported adverse biological effects on cells and test model organisms (Blaise et al. 2008; Jia et al. 2005; Shvedova et al. 2003). From these pioneering studies, the physical properties (e.g. size, surface area and speciation) (Kolosnjaj-Tabi et al. 2010; Poland et al. 2008; Simon-Deckers et al. 2008; Tian et al. 2006), the presence of metal impurities (Cheng et al. 2009; Chlopek et al. 2006; Koyama et al. 2009; Murray et al. 2009; Porter et al. 2009; Vittorio et al. 2009; Walker et al. 2009) as well as the degree of dispersion of CNTs in prepared suspensions have been linked to observed adverse biological impacts (Mutlu et al. 2010; Qu et al. 2009; Wick et al. 2007). The toxic effects of CNTs have been associated mainly with the direct contact between cell membranes and the nanotubes and to the interactions of CNT-generated reactive oxygen species (ROS) with cell components (Kang et al. 2007; Rivera and Starr 2010; Shvedova et al. 2003). Under natural conditions, the magnitude of any potential biological response to CNT exposure would likely be due not only to the effects of the intrinsic physicochemical properties of the nanotubes, but also to the secondary characteristics acquired through environmental transformations.

One of the most important properties affecting CNTs ability to interact with microorganisms is its state of dispersion. For industrial applications, the dispersion state of CNTs can be altered via processes such as wall-functionalization, and in natural aquatic systems, interactions with dissolved organic matter (e.g. humic substances) could also play a role (Hyung et al. 2007). To better understand these interactions, the aggregation behavior of CNTs has been modeled based on rather well established principles of colloidal behavior, such as Derajaguin, Landau, Verwey, and Overbeek (DLVO) theory (Holbrook et al. 2010; Lead and Wilkinson 2006; Moore 2006). The comparison to colloids stems primarily from the overlapping size range of engineered nanomaterials (at least one dimension between 1 and 100 nm) and colloidal particles (up to 10 μm). According to DLVO theory, for two adjacent particles separated by a given distance, either repulsive forces of the electrical double layer (EDL) or attractive forces due to van der Waals (vdW) interactions will dominate (Israelachvili 1985). While vdW interactions are fairly universal forces, EDL forces are easily affected by solution's chemistry (Handy et al. 2008; Israelachvili 1985). For instance, increased ionic strength caused by addition of salts induces compression of the EDL via screening of the surface charges, leading to dominance of attractive forces. This, in turn, brings particles together, which is the beginning of the aggregation process. Therefore, key environmental factors such as pH, ionic strength, and quantity and quality of dissolved organic matter affect colloidal stability (Cosgrove 2005). One might naturally conclude that the same would occur for CNTs, resulting in the predictions illustrated in Figure 4-1.

The effect of different electrolytes on the stability of SWNT suspensions prepared by the strong acid oxidization process has been studied in (Sano et al. 2001). As the

ionic strength of the solution increased, a disturbance of the suspensions occurred, resulting in CNT aggregation. This trend was observed in studies using SWNT (Saleh et al. 2010) and MWNT (Lin et al. 2009b; Smith et al. 2009) suspensions prepared by use of extensive sonication followed by centrifugation. In addition to ionic strength, pH has also been found to influence the dispersion state of CNTs, in that the decrease of pH leads to aggregation of CNTs (Lin et al. 2009b; Shieh et al. 2007; Smith et al. 2009; Zhao et al. 2002).

Besides pH and ionic strength, NOM is another factor affecting aquatic stability of CNTs. Presence of NOM is known to facilitate both the transport and sorption of different types of contaminants (Chiou et al. 1986; Yamamoto et al. 2003). Recent studies showed that NOM could lead to a rather uniform functionalization of CNTs, (Domingos et al. 2009; Hyung et al. 2007), with implications on the fate of CNTs in aquatic systems. The chemical composition of NOM is highly complex and variable owing to its diverse sources, and therefore, the interactions between CNTs and NOM are simulated by use of simple surrogates such as fulvic acid (FA) (Yang and Xing 2009), tannic acid (TA) (Lin et al. 2009b), and humic acid (HA) (Saleh et al. 2010) isolates. The adsorption of NOM onto CNTs is believed to be the result of π - π interactions or stackings of aromatic fractions of NOM on graphene surfaces of CNTs (Hyung and Kim 2008; Lin and Xing 2008). Obviously, the magnitude and stability of CNT-NOM complexes are dependent on the chemical composition of NOM, as well as the physicochemical parameters of natural waters. Due to the above mentioned complexity and uncertainty (e.g. structure of NOM), understanding the stability of CNTs suspended in NOM-rich solutions remains a challenge.

CNTs can enter aquatic systems through either intentional or unintentional discharges. Although, the behavior of CNTs in natural aquatic systems is still poorly understood, one can hypothesize based on current knowledge that CNTs stabilized by NOM in aquatic systems are more susceptible to enter the food chain. Aggregated and subsequently sedimented CNTs may also re-enter the water column through bioturbation, or they may be consumed by bottom dwellers. For instance, *Daphnia magna*, a low level consumer, was capable of accumulating and transferring SWNTs via absorption onto its carapace (Lam et al. 2007), or consumption of lipid-coated SWNTs (Roberts et al. 2007). However, this aspect of SWNT research has been hampered mainly because of a lack of adequate analytical methods for detecting and quantifying CNTs in complex biological systems. Therefore, there is a need to establish reliable, and ideally simple, procedures for estimating the bioaccumulation potential of CNTs.

One common metric is the octanol-water coefficient (K_{ow}), which has been used widely to predict the distribution of organic contaminants between aqueous and organic phases and to estimate the bioaccumulation potentials (Mackay and Fraser 2000; Neely et al. 1974). The principle of K_{ow} mimics the interaction between xenobiotics and fatty tissues of cell membranes having chemical similarities with octanol. Previous studies have shown that experimentally determined values of K_{ow} were positively correlated with actual values of bioconcentration for a diverse range of hydrophobic compounds (Neely et al. 1974) and several regression equations have been developed since the 1970's as predictive tools based on K_{ow} values (Mackay 1982). In fact, regulatory programs such as the EU's "registration, evaluation, and authorization of chemicals" or 'REACH' for short, have used the above approach to identify the bioaccumulation potential through

bioconcentration factor predictions (European-Commission 2007). In the US, the EPA includes K_{ow} as one of the most important chemical properties that needs to be determined for emerging MNs (US-EPA 2007).

Nevertheless, K_{ow} remains a rather primitive tool, as research on fate and potential bioaccumulation of SWNTs in natural waters is still in its infancy. SWNTs, once exposed to natural aquatic systems, will undergo physicochemical alterations controlled by water chemistry parameters. In such cases, the study of octanol water partitioning behavior of SWNTs suspended by typical dispersion method (e.g. synthetic surfactants) may not be the appropriate scenario because SWNTs in natural aquatic systems could be significantly modified before contacting aquatic organisms. Therefore, one may only adequately assess the potential of SWNT bioaccumulation by use of experimental conditions that closely mimic natural conditions.

In this chapter, SWNTs suspensions were prepared using natural and synthetic waters to help gain insight into how water chemistry controls the fate of SWNTs. In addition, the octanol-water phase distribution percentages of SWNT were determined using waters with different chemical compositions to assess the impact on the bioaccumulation potential of SWNTs.

4.2 Materials and Methods

4.2.1 Aqueous Organic-rich Solutions Used to Prepare SWNT Suspensions

In order to simulate the interaction between SWNTs and NOM, natural water and synthetic solutions spiked with commercially available organic compounds were used. NOM is a mixture of various compounds with diverse molecular sizes and chemical compositions (Leenheer and Croue 2003). Due to the complex nature of NOM, the use of simple and well characterized surrogates for NOM has provided an understanding of

the interaction between hydrophobic pollutants and NOM (Chin et al. 1997; Chiou et al. 1987; Yamamoto et al. 2003).

Natural water samples used in this study were collected from the upper portion of the Suwannee River (SR) watershed (Fig. 2). Water samples were collected in pre-washed polyethylene containers after rinsing with site water. Collected samples were placed in a cooler packed with ice and transported back to the laboratory. In the laboratory, the samples were immediately filtered (0.45μm) and analyzed for major ion concentrations by ion chromatography and pH.

Organic-rich waters containing various well-characterized species of dissolved organic matter were prepared by use of commercially available HA, FA, and TA. HA was purchased from Sigma-Aldrich, FA from international humic substances society, and TA from Alfa Aesar. Organic-rich aqueous solutions were prepared by dissolving a pre-weighed amount of any one of the above listed compounds into Nanopure® water, followed by filtration (0.45μm). The concentration of dissolved organic carbon (DOC) in filtered natural water and laboratory prepared solutions were determined by a Tekmar Dohrmann-Apollo-9000.

For the experiments conducted in this study, solutions with DOC concentration gradients were prepared from mother solutions, and the effect of the DOC gradient on solution's pH was corrected by use of either 0.1M NaOH or 0.1M HCl.

4.2.2 Preparation and Characterization of SWNT Suspensions

SWNT Suspensions used in this study were prepared using three different dispersing agents. First, SWNT suspensions were prepared by suspending approximately 10 mg of raw SWNTs (Rice HPR 145.1) into 100mL of DI-water

containing either SR, HA, TA, or FA. These mixtures were homogenized using a high-shear IKA T-25 Ultra-Turrax mixer for about 1.5h followed by ultrasonication using a Misonix S3000 for 10 min. The mixtures were then ultra-centrifuged at 14,000 rpm (Beckman Coulter Optima L-80 K) for 4h, after which, the supernatant was carefully separated from the aggregated SWNTs at the bottom of the centrifuge tube, and then filtered through a 20 μm membrane. The obtained suspensions were characterized using Bio-Raman with excitation from a 785 nm diode laser while visible absorbance spectra and fluorescence were recorded with a Nano-Fluorescence Nanospectralyzer (Applied NanoFluorescence, Houston, TX). Second, SWNT suspensions were prepared using conventional surfactants (i.e. sodium cholate (SC), gum Arabic (GA), pluronic acid F108 (PF), and sodium dodecyl sulfate (SDS). Preparation and characterization methods have been published elsewhere (Youn et al. 2011). Third, organic matter generated by a primary producer, *Psuedokirchneriella subcatpiata*, was harvested and used to prepare SWNT suspensions. A culture of *P. subcapitata* was cultivated as described in the US-EPA's *P. subcapitata* 96-h growth inhibition method 1003.0. After 7 days from the time of the inoculation of the culture media with seed algal cells, 1L of algal culture media was centrifuged at 1200 RPM for 10 min and then the supernatant was discarded. The pellet was next resuspended in Nanopure® water and centrifuged as described above. This washing process was repeated twice. The pellet was then resuspended in 500 mL of Nanopure water and sonicated within an ice bath with a Cell disuptor W-375 (Heat Systems-Ulatrasonics INC, Farmingdale, NY.). The obtained mixture was analyzed for organic carbon content and used to prepare SWNT suspensions as described by Youn et al (Youn et al. 2011).

4.2.3 Octanol-water Partitioning Experiments

Experiments on phase distribution of SWNTs between 1-octanol and water were conducted using a modified “shake-flask” method. Briefly, for each of the SWNT suspension types, 5 mL were combined with an equal volume of 1-octanol in a 20 mL scintillation vial and then mixed at 300 RPM for 24h using a New Brunswick G24 horizontal shaker (Edison, NJ). The mixtures were next stabilized at room temperature until complete separation of the two phases was achieved. Aliquots of water and octanol phases were removed and SWNT concentrations in each phase determined by absorbance measurements at 763 nm using a Nano-Fluorescence Nanospectralyzer.

4.2.4 Algal Growth in NOM-SWNT Containing Culture Media

The 96hr *P. subcapitata* chronic toxicity assay was used (US-EPA method 1003.0). Briefly, 50 mL of the preliminary algal assay procedure (PAAP) culture medium were transferred into pre-autoclaved 150 mL Erlenmeyer flasks and then the effects of NOM suspended SWNTs were investigated by injecting a pre-determined toxic concentration of SWNTs into the *P. subcapitata* (e.g. 0.5 ppm) culture medium. Control experiments consisted of reactors containing an equivalent amount of the tested NOM solution, but without SWNT addition. All algal growth experiments were conducted under continuous illumination and all treatments were prepared in triplicates.

4.3 Results and Discussion

4.3.1 Characterization of SWNT Suspensions in Aqueous Solutions with Different Types of Organic Matter

Table 4-1 presents the physicochemical characteristics of the various organic rich solutions as well as the concentrations of SWNTs suspended in each of such solutions. For comparison purposes, the concentration of DOC of each organic-rich solution was

adjusted to match the level of DOC naturally occurring in the SR water sample used in this study. The results show that the degree of SWNT dispersion is dependent on the type or chemical makeup of organic matter present in the water. Earlier studies on CNT-organic matter interactions showed that the adsorption of hydrophobic organic contaminants onto CNTs was influenced by the physicochemical properties of NOM present in water (Chin et al. 1997; Chiou et al. 1987; Yamamoto et al. 2003). Also, the presence of benzene rings in surfactants used to suspend CNTs produces highly dispersed and stable CNT suspensions (Islam et al. 2003). Overall, a strong correlation is observed between adsorption coefficient and both the molecular weight and the aromaticity of organic compounds present in water. Aromaticity can be determined by specific UV absorbance at 254 nm ($SUVA_{254}$) of organic-rich solutions divided by the DOC concentration of the solution; and $SUVA_{254}$ is strongly correlated with the degree of aromaticity as determined by ^{13}C-nuclear magnetic resonance (Weishaar et al. 2003).

Since the dispersion of SWNTs in aqueous solutions is affected by the adsorption efficiency of NOM on the sidewalls of CNTs, the degree of aromaticity of organic compounds present in solutions used in this study can explain the observed differences in CNT's dispersion behaviors. In HA and TA containing solutions, the ability of dissolved organic matter to disperse SWNTs is related to the degree of aromaticity. The aromaticity of used HA (i.e. 0.076 $Lmg^{-1}m^{-1}$) was found to be the highest, and the amount of SWNTs suspended in HA-containing aqueous solution was also the highest. In contrast, TA suspensions, with a low degree of aromaticity, exhibited to the lowest concentration of suspended SWNTs. This is primarily due to π - π interactions between aromatic moities of organic compounds on CNT surfaces (Hyung and Kim 2008; Lin and

Xing 2008). SR water samples and FA-containing solutions showed a similar degree of aromaticity, but the SR water exhibited a higher ability to suspend SWNTs, thus resulting in a higher concentration of suspended CNTs. In fact, the aromatic content of SR is nearly half that of HA, yet the concentration of SWNTs in SR water is close to that determined in HA-containing solutions. It should be noted that SR water sample is obtained directly from the Suwannee River, and therefore, its molecular characteristics are different from the commercially purchased HA, FA, and TA isolates (Malcolm and Maccarthy 1986).

In Figure 4-3, the four different NOM-SWNT suspension types are compared by use of spectra obtained by sample excitation at λ = 785nm, and using radial breathing mode and D/G peak ratio mode for characterization. Previous work revealed that the intensity of the 267 cm^{-1} peak increased as CNTs were aggregated (Heller et al. 2004). Therefore, Raman spectra in this range can be used to explore the aggregation state of SWNTs in aqueous suspensions. Variability of the peak intensity shown in the inserted figure implies that different organic compounds impacted the separation process by different amounts, resulting in different degrees of SWNT aggregation in prepared suspensions. As seen in Table 4-1, the D/G peak ratio changes with the type of organic matter used as surfactant, with FA being the least aggregated suspension, followed by TA, SR, and then HA. In addition, the Raman spectra of the D-peak (at about 1300 cm^{-1}) and G-peak (at about 1600 cm^{-1}) can provide insight into the structure of carbon atoms on the side walls of SWNTs. The values of the D/G peak ratios obtained in this study are not significantly different when any two tested organic-rich aqueous solutions are compared (Table 4-1).

4.3.2 Impact of NOM Concentrations and pH on SWNT Suspension

Concentrations of SWNTs suspended in aqueous solutions containing the different commercially available organic compounds are shown in Figure 4-4. SWNT concentrations varied with the type of organic compound and concentration range (50 to 800 mg/L). The obtained SWNT suspensions were stable for at least 7 days as determined by the lack of changes in absorbance measurements over time. The concentrations of SWNTs in HA-containing solutions increased with increasing HA concentrations. However, the concentrations of SWNTs suspended in TA and FA containing solutions increased only in solutions with TA and FA concentrations below 200 and 400 ppm, respectively. This increase was then followed by a decrease in SWNT suspension ability (Figure 4-4). The decrease in the concentration of SWNTs suspended in TA and FA containing solutions could be due to decreasing pH. The impact of pH on the stability of CNT suspensions has been investigated and it was shown that decreased pH leads to the decreased stability due to decreased electrophoretic mobility of CNTs (Lin et al. 2009b; Shieh et al. 2007; Smith et al. 2009; Zhao et al. 2002). In order to investigate the effect of pH on the ability of tested waters to suspend SWNTs, organic-rich aqueous solutions were prepared with the pH of the solutions adjusted to 5 and 4 for TA and FA, respectively (Figures 4-4B and 4-4C). Each pH is the one measured from TA and FA final concentration of 50 mg/L. The results showed that the increase in SWNT suspension induced by pH adjustment was not significant and the concentrations of suspended SWNTs were even reduced in the case of FA-containing water samples. The decrease in SWNT suspension ability at higher dissolved organic compound concentrations could be due to attractive depletion interactions, which is a re-aggregation phenomenon of SWNTs caused by micelle

formations (Blanch et al. 2010). Specifically, the pressure from surfactant micelles builds up as surfactant concentration increases and then begins to push SWNTs together, thus inducing aggregation.

Overall, it is obvious that SWNTs remained in the supernatant after centrifugation, and the degree of aggregation and non-covalent functionalization of SWNTs obtained with the tested organic compounds could be significantly altered. These findings are particularly relevant in regards to the potential toxicity and fate of SWNTs, because these factors have been proposed as toxicity mechanisms in previous studies (Sayes et al. 2006; Wick et al. 2007) .

4.3.3 Octanol-water Partitioning Behaviors of SWNTs

Based on the hydrophobic nature of carbon nanotubes, it is expected that CNTs would easily transfer across the boundary between octanol and water phases. For instance, fullerene, another carbon based nanomaterial, exhibits a high octanol-water partition coefficient that is close to those of well known hydrophobic pollutants such as DDT (Jafvert and Kulkarni 2008). Currently, there are only two published papers in peer reviewed literature containing experimental data on CNT partitioning behavior (Petersen et al. 2010; Wang et al. 2008a). This lack of experimental data could be due to analytical difficulties in assessing CNT concentrations in complex organic matrices. In addition, the transfer of CNTs to octanol from a water phase does not occur as easily as one would expect from the trends of other hydrophobic compounds. Experiments conducted in this study using several organic solvents (e.g. toluene, methylene chloride, hexane, and olive oil) failed to yield clear extractions of SWNTs when initially suspended into SR water. Other researchers have claimed a poor partition of SWNTs between aqueous and organic liquid phases (Wang et al. 2008a), yet they reported

octanol-water partitioning coefficient of $10^{5.18}$; a value that is close to that of the well known hydrophobic substances such as DDT (i.e. $10^{5.75}$ (Mackay 1982)). This inconsistency raises questions about the accuracy of the reported K_{ow} number. Additionally, multi-walled carbon nanotubes (MWNTs) suspended in water by acid functionalization were unable to transfer to octanol phase (Petersen et al. 2010).

4.3.3.1 SWNT suspensions in synthetic surfactants and algal organic matter and determination of octanol-water partitioning coefficients.

As shown in the previous chapter, GA, SC, and PF have proven to yield stable suspensions, and SDS has been widely used as an ideal surfactant to prepare CNT suspensions. In this set of experiments, SWNT suspensions were prepared in GA, SC, PF, SDS, and algal organic matter. The obtained suspensions were then used in octanol-water partitioning experiments. The results are presented in Figure 4-5.

The results of octanol-water partitioning experiments indicate that traditional compound 'transfer between two immiscible liquids' based methods may not be appropriate for SWNTs suspended in surfactants such as GA, SC, PF, and SDS (Figures 4-5A, 4-5B, 4-5C and 4-5D). In the case of GA-SWNTs, the majority of SWNTs were trapped in micelles between octanol and water phases (Figure 4-5A). These micelles were extremely robust and were stable for at least 4 weeks. The other SWNT suspensions prepared in synthetic surfactants mixed with octanol phase could not be completely separated between the two phases, making the determination of the K_{ow} partition coefficient impossible (Figures 4-5B, 4-5C, and 4-5D). Only SWNT suspensions prepared in solution containing algal extracted organic matter showed CNT transfer to the octanol phase. Unfortunately, CNT concentrations in both aqueous and organic phases could not be determined by the analytical technique used in this study

due to high degrees of aggregation. Overall, surfactants tested in this study did not lead to a clear transfer of SWNTs from aqueous solutions to organic phase.

It would be reasonable to assume that the aggregation of CNTs observed in this study suggests that the introduction of surfactant coated CNTs to natural waters would likely lead to sedimentation and deposition onto surface sediments. Therefore, such aggregated CNTs could enter the food chain through ingestion by bottom feeders. With regard to algal organic matter**,** a previous study showed that algal exudates could impact the stability of colloidal suspensions (Koukal et al. 2007). Additionally, the presence of biochemical compounds such as polysaccharides in the algal organic matter mixture (Buffle and Leppard 1995), could point to some similarly with GA, and therefore a potential to produce relatively stable SWNT suspensions. However, algal organic matter was not able to successfully suspend SWNTs to the extent of suspension quality obtained with GA (Figure 4-5E). The DOC concentration of algal extract used in this study (i.e. 52 ppm) was closed to that of SR water (i.e. 62 ppm), but the measured $SUVA_{254}$ of 1.92 $\times 10^{-4}$ $Lmg^{-1}m^{-1}$ was much lower than that of the SR water (0.041 $Lmg^{-1}m^{-1}$). This is an indication of clear differences in the chemical makeup of compounds present in these aqueous solutions.

4.3.3.2 SWNT suspensions prepared in natural SR water and organic-rich synthetic solutions and use in octanol-water partitioning experiments

In this experiment, migration of SWNTs into the octanol phase was observed for SWNTs initially suspended in both SR water and synthetic solutions (Figure 4-6). Synthetic solutions were prepared in such a way that the final DOC concentrations were similar to that naturally occurring in SR water samples. At the end of the experiment, the % of SWNTs transferred from water to octanol were 38.3, 31.5, 32.1 and 17.2 for SR,

HA, TA and FA-SWNT, respectively (Table 4-2). The concentrations of SWNTs in aqueous phases (i.e. SR, HA, TA, and FA solutions) were below the instrument's analytical detection limit (<0.5 ppm). Unlike the results obtained from SWNT-surfactant suspensions discussed earlier, SWNTs suspended in organic rich waters appear to be prone to transfer to the octanol phase, and therefore, potentially able to cross cell membrane and bioaccumulate.

An additional set of experiments was conducted and in which SWNTs suspended in SR waters were first centrifuged at different speeds (from 0 to 14,000 RPM for 30 minutes). Second, following the separation of the supernatant from the pellet, the former was used in phase partitioning experiments. These experiments were designed to investigate the impact of the size of SWNTs on phase-partitioning behavior. The results showed increased SWNT concentrations in octanol phases with increased centrifugation speed (Table 4-2), indicating that well-dispersed and small size SWNTs transfer better to the octanol phase than the larger SWNT bundles.

4.3.3.3 Impacts of increasing concentration of organic matter and pH on SWNT distribution between water and octanol

In addition to SWNTs suspended in waters containing environmentally relevant concentration of DOC, the octanol-water partitioning behavior of SWNTs with different concentrations of commercially available organic matter isolates are shown in Table 4-3.

The transfer of SWNTs occurred in suspensions containing 50 and 100 ppm of HA and 50 ppm of TA. The transfer of SWNTs using FA containing solutions was observed for FA concentrations starting from 100 mg/L and the amount of transferred nanotubes decreased gradually with increasing FA concentrations. The amount of SWNTs

partitioned to octanol phase seems to be related to the concentration of dissolved organic carbon present in the suspension. In general, SWNTs do not transfer to octanol phase when concentrations of OM in aqueous phase are >300 mg/L). As the concentrations of organic matter in prepared suspensions increased, persistent emulsion was observed between the octanol and water (Figure 4-7A), and the transfer of SWNTs to octanol phase seemed to be hampered by bubbles formed at the interface.

The impact of adjusting the pH of SWNT suspensions on SWNT transfer between phases can vary markedly as shown in Table 4-4. In comparison with the transfer experiment without pH adjustment as shown in Table 4-3, the trend of TA-SWNT transfer did not change as the concentration of organic carbon increased in the range between 200mg and 800 mg of TA/L. However, enhanced FA-SWNT transfer percentage was observed in the same range when SWNTs were suspended in pH adjusted FA solutions.

4.3.4 Mechanisms of SWNT Phase Partitioning

There are 2 notable findings from the above octanol-water partitioning studies. First, unlike SWNTs suspended into synthetic surfactants, SWNT suspension in the natural SR water sample and water containing isolates of humic and fulvic acids transfer from aqueous solutions to the octanol phase using the simple shake-flask technique. Second, the size and concentration of SWNTs as well as solution pH and organic carbon concentration impact the octanol-water distribution behavior of SWNTs.

Previous studies found no partitioning of CNTs between water and octanol (Petersen et al. 2010; Wang et al. 2008a); results that differ from observations made in this study. This may be due to differences in physical characteristics of carbon nanotubes either inherited in production process or derived from the preparation method

used in each study. For instance, Peterson et al (2010) used MWNTs which in general have bigger diameter than SWNTs and made them hydrophilic through strong acid-derived functionalization, a process that introduces defective sites on the sidewalls. Moreover, the ultra-centrifugation process used in this study removes SWNT aggregates, resulting in well dispersed suspensions. For most nanoparticles including CNTs, the diameter, length, and contact angle of the nanoparticles at the octanol-water interface would influence their behavior. Specifically, the partitioning of SWNTs at the interface between octanol and water can be explained by changes in free energy of the interface, and the change in energy induced by inserting the CNTs into either phase depends on both the diameter and the length of CNTs. For a SWNT particle of the radius R and the length L placed at the octanol-water interface with the contact angle (θ) (Figure 4-7), the area of contact between the SWNT and water is

$$A_{\text{SWNT-water}} = 2\pi RL\frac{2\theta}{360^{\circ}} \tag{1}$$

The planar area of the octanol-water interface removed by the SWNT is

$$A_{\text{oil-water removed by the SWNT}} = 2R\sin\theta \times L \tag{2}$$

If we can assume that individual SWNT is small enough so the effect of gravity is negligible and the interface is planar, the energy change required to remove the SWNT from the interface into the octanol phase can be described as (Binks and Lumsdon 2000),

$$\Delta E = 2\pi RL\frac{2\theta}{360^{\circ}}(\gamma_{\text{S-o}} - \gamma_{\text{S-w}}) + 2RL\sin\theta \times \gamma_{\text{o-w}} \tag{3}$$

where γ refers to the interfacial tension between SWNT, octanol and water shown as the subscript S, o and w, respectively. The interfacial tensions are related with the

contact angle through Young's equation (Binks and Clint 2002) (Figure 4-7A), $\gamma_{S\text{-}o} - \gamma_{S\text{-}w} = \gamma_{o\text{-}w} \times \cos\theta$, and the equation 3 can be simplified as,

$$\Delta E = 2RL\gamma_{o-w}\left[\frac{\pi\theta}{180^\circ}\cos\theta + \sin\theta\right] \quad (4)$$

The required energy for the transfer to the octanol phase is increased when the size of CNTs is bigger (equation 4) and subsequently they are stabilized at the interface rather than transferred. The impact of the size of SWNTs on the partitioning behavior was also confirmed with the SR-SWNTs prepared using a different centrifugation time. As seen in Table 4-2, a higher octanol transfer of SWNTs was observed in the suspension with smaller size SWNTs.

In addition to the impact of the physical characteristics of CNTs, the presence and type of surfactants are other key factors influencing CNT's behavior at the oil–water boundary (Binks 2002). The observed emulsion with SWNTs residing at the interface between the immiscible fluids was reported previously (Figure 4-8B) (Hobbie et al. 2005; Wang and Hobbie 2003) and the self-assembly of SWNTs on hydrophobic substances has been used to remove SWNTs bundles (Wang et al. 2008c). The emulsion occurs with the SWNTs themselves, but the addition of a surfactant yields more stable and larger volume emulsions (Hobbie et al. 2005). The observed formation of bubbles between octanol and water phases is due to octanol droplets surrounded by organic matter-SWNT complexes. The transfer of SWNTs from aqueous solution to the octanol phase was thus hampered with increasing organic matter concentration, due primarily to the interfacial trapping phenomenon. Note that when the concentration of organic carbon in solution prepared using isolates obtained from commercial sources was equivalent to DOC concentration naturally occurring in the SR water sample used;

the formation of droplets at the octanol-water interface was significantly lower (Figure 4-6). This observation suggests that the concentration of dissolved organic matter is a crucial factor in the interfacial trapping of SWNTs. Other key factors influencing the interfacial trapping are the concentrations of SWNT and pH as illustrated by data presented in Table 4-4.

Besides the physical characteristics (e.g. diameter and length) and surfactant type, the contact angle of SWNTs which SWNTs make with the octanol-water interface can influence the observed interfacial trapping. As illustrated in Figure 4-8C, the position of a nanotube in the suspension depends on the contact angle (θ) with the oil-water interface. When the contact angle is more than 90°, SWNTs are transferred more easily into the organic phase. The contact angle of nanoparticles is determined by their hydrophobicity (Binks 2002) and can be manipulated through nanoparticle's surface modifications (Duan et al. 2004; Shen and Resasco 2009). Dissolved organic compounds can effectively increase the contact angle of SWNTs at the interface of octanol-water by modifying the sidewalls of SWNTs. As a result, the complex organic matter-SWNT can be transported across the interfacial boundaries between octanol and water.

Although a significant migration of SWNTs into the octanol phase was observed, it was still very difficult to accurately measure the partition coefficient (K_{ow}), which requires the concentration of a compound in both phases. In most cases, the SWNTs remaining in the water phase after the mixing process was below detection limit (<0.5 ppm), as determined by UV-vis-NIR spectroscopy in this study. In addition, the determination of the amount of SWNTs in the octanol phase was hampered by

aggregation. Therefore, it was more convenient to represent the results in terms of transfer percentage, which is based purely upon the fraction of SWNTs leaving the water phase rather than K_{ow}. This allows one to exclude issues that may arise from the aggregation of SWNTS trapped at the water-octanol interface. Finally, following the transfer to the octanol phase of SWNTs previously suspended in aqueous solutions containing either HA, FA, or TA or suspended directly in natural SR water; SWNTs became unstable and gradually aggregated and deposited near the water-octanol interface. This is probably due to the drastic changes of the coverage of the sidewalls of nanotubes by NOMs, and additionally, changes of the surrounding environment upon moving from water to octanol. Upon transfer to the octanol phase, SWNTs would experience enhanced charge screening due to the lower dielectric constant of octanol (e.g. dielectric constants of 1-octanol and water are 10.3 and 80, respectively)(Equation 5). Subsequently, the repulsive force between SWNTs would be diminished. The Coulomb force F is given by

$$F = \frac{z_1 z_2 e^2}{4\pi\varepsilon_\circ \varepsilon r^2} \quad (5)$$

where z is the ionic valency, e is the magnitude of their electrical charge (i.e. 1.6×10^{-19}C), ε is the relative permittivity or dielectric constant, and r is the distance between two charges. Once nanotubes begin to aggregate each other, the deposition process is accelerated in the less dense octanol phase compared to the water phase.

4.3.5 Relationship between SWNT Transfer in Octanol Phase and Algal Growth Inhibition

Figure 4-9 shows the 96hr growth response of *P. subcapitata* in culture media containing fixed SWNTs levels (0.5 ppm), pre-suspended in aqueous solutions containing the above discussed organic matter isolates or in SR water. The 0.5 ppm

was selected as concentration of SWNTs to be tested due to the significant adverse biological impacts it induces based on previous studies (Youn et al. 2011). The horizontal line in Figure 4-8 represents the average algal growth rates determined from control cultures. Culture media treated with FA-SWNTs showed a slight increase in growth of *P. subcapitata* compared to the control. A severely negative impact on algal growth was observed with TA suspended SWNTs. The negative biological responses observed in this case are mainly due to TA itself as shown by data obtained from algal growth in vessels containing only TA and no SWNTs (Figure 4-9). In fact, TA has well known anti-algal properties (Pillinger et al. 1994). In contrast, HA- and SR-suspended SWNTs showed no toxicity, and significant growth stimulation was observed.

The investigation of the mechanisms driving the observed biological responses of algae treated with HA- and SR-suspended SWNTs was not attempted due to resource limitation. However, it is known that natural dissolved organic matter can protect aquatic organisms from heavy metal toxicity (Koukal et al. 2007) and metal impurities do contribute to the toxicity of CNTs (Cheng et al. 2009; Chlopek et al. 2006; Kagan et al. 2006; Shvedova et al. 2003). Moreover, the adsorption of dissolved organic matter on the surface of the algal cells (Campbell et al. 1997) may reduce the probability of direct contact between cell surfaces and SWNTs, hence eliminating the toxicity attributable to cell piercing. It is therefore postulated that some of the tested organic isolates and organic compounds present in SR water could provide protection against the adverse effects of SWNTs.

In comparison with the octanol-water distribution of NOM-SWNTs (Table 4-2), a higher algal growth stimulation was observed in culture media treated with HA- and SR-

suspended SWNTs. However, these two organic-rich aqueous solutions favored a higher transfer of SWNTs to the octanol phase. The octanol-water partition approach determines the tendency for bioaccumulation of target substances by measuring the level of their affinity to octanol. Therefore, a high transfer of SWNTs from water to octanol implies higher affinity of SWNTs for the model organism. With this concept in consideration, the bio-stimulation observed in this study is opposite to the anticipated trend, likely because the higher affinity of nanotubes for the algal cells would lead to more physical contacts between them. Certainly, several factors are involved in the biological responses observed. There are a few studies reporting positive responses such as growth stimulation of living systems caused by nanotubes (Leeuw et al. 2007; Roberts et al. 2007; Templeton et al. 2006; Zhu et al. 2006b). Interestingly, positive responses of target organisms were related with adsorption of nutrients onto the large surface area of nanotubes and then subsequent ingestion (Roberts et al. 2007; Zhu et al. 2006b). If similar nutrient concentration and delivery process occurred in this experiment, then higher affinity of SWNT toward cell surface would benefit the growth of *P. subcapitata*.

4.4 Concluding Remarks

In this chapter, SWNTs suspended in either a natural water sample collected from the Suwannee River or synthetic solutions containing either HA, FA, or TA were used in lab experiments for the determination of the octanol-water partition percentage of SWNTs. The use of organic-rich natural water resulted in well dispersed and stable SWNT suspensions, suggesting that naturally occurring dissolved organic matter could behave as surfactant for suspension of SWNTs in aqueous solutions. The ecological implication is that SWNTs could potentially be well dispersed and well suspended in

organic rich aquatic environments. This would then impact the transport and fate of SWNTs, as well as their interaction with biological membranes. Moreover, SWNTs suspended in SR water and in different solutions produced by dissolving natural organic compound isolates (e.g. HA and FA) were easily transferred from aqueous solution to organic phase in experiments using the conventional shake-flask method. In contrast, SWNTs suspended in synthetic surfactants (GA, SC, and PF) formed large aggregates that accumulated in the water-octanol interface. These results suggest that organic matter induced changes of SWNT surface properties drives the partition of these CNTs between water and octanol. There is therefore a need for further investigations focusing on characterization of SWNTs and on surface property changes induced by natural dissolved organic carbon. Toxicity studies using SWNTs suspended in aqueous solutions containing natural dissolved organic matter show that the chemical makeup of the dispersing agent (the different organic compounds used in this study) play a critical role in determining the biological responses of *P. subcapitata*. Additionally, the concentration of SWNTs found to be toxic in previous studies did not produce adverse biological impacts on *P. subcapitata,* except for TA, which is already toxic to *P. subcapitata* in the absence of SWNTs. Finally, SWNTs coated by HA, FA, and organic matter present in the SR samples stimulated algal growth in a significant manner, indicating a potential nutrient delivery mechanism.

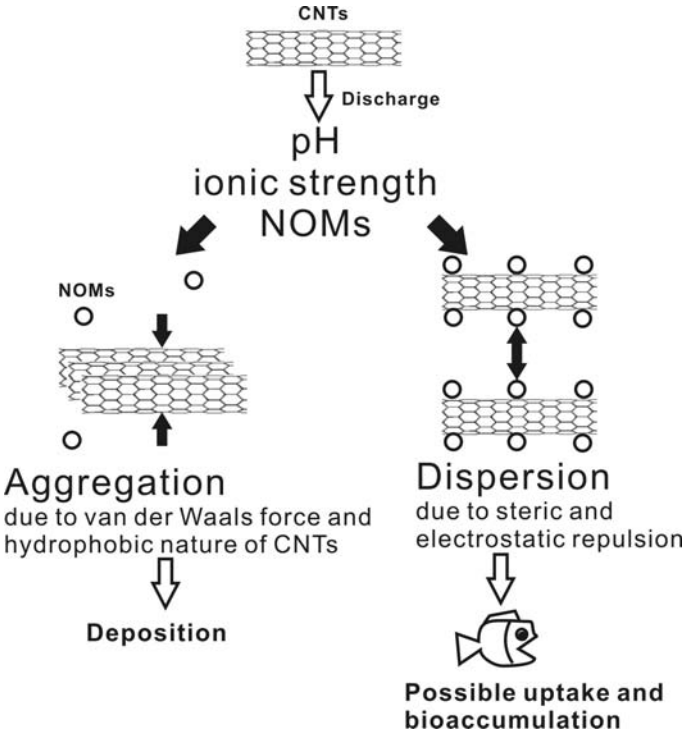

Figure 4-1. Potential behavior and fate of carbon nanotubes in natural aquatic systems as a function of key environmental conditions such as pH, ionic strength, and dissolved natural organic matter.

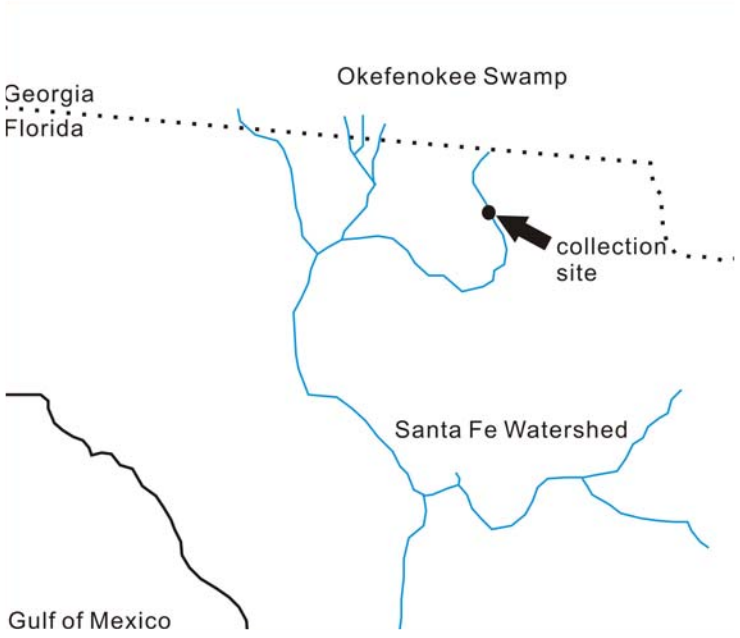

Figure 4-2. Map of the Suwannee River watershed showing the sampling location.

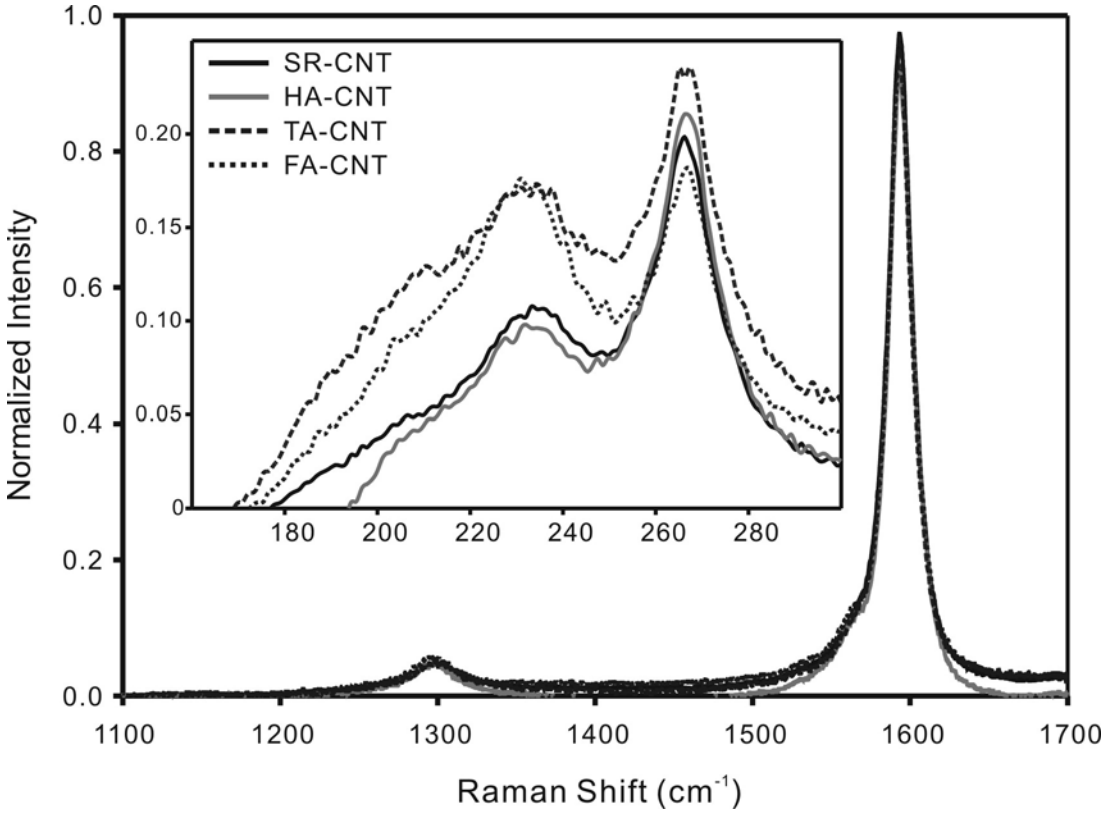

Figure 4-3. Raman spectra of single walled carbon nanotubes (SWNTs) suspended in Suwannee River water (SR-SWNT), fulvic acid (FA-SWNT), tannic acid (TA-SWNT), and humic acid (HA-SWNT). The inserted figure shows radial breathing mode (RBM) and aggregation peak at 267 cm^{-1}.

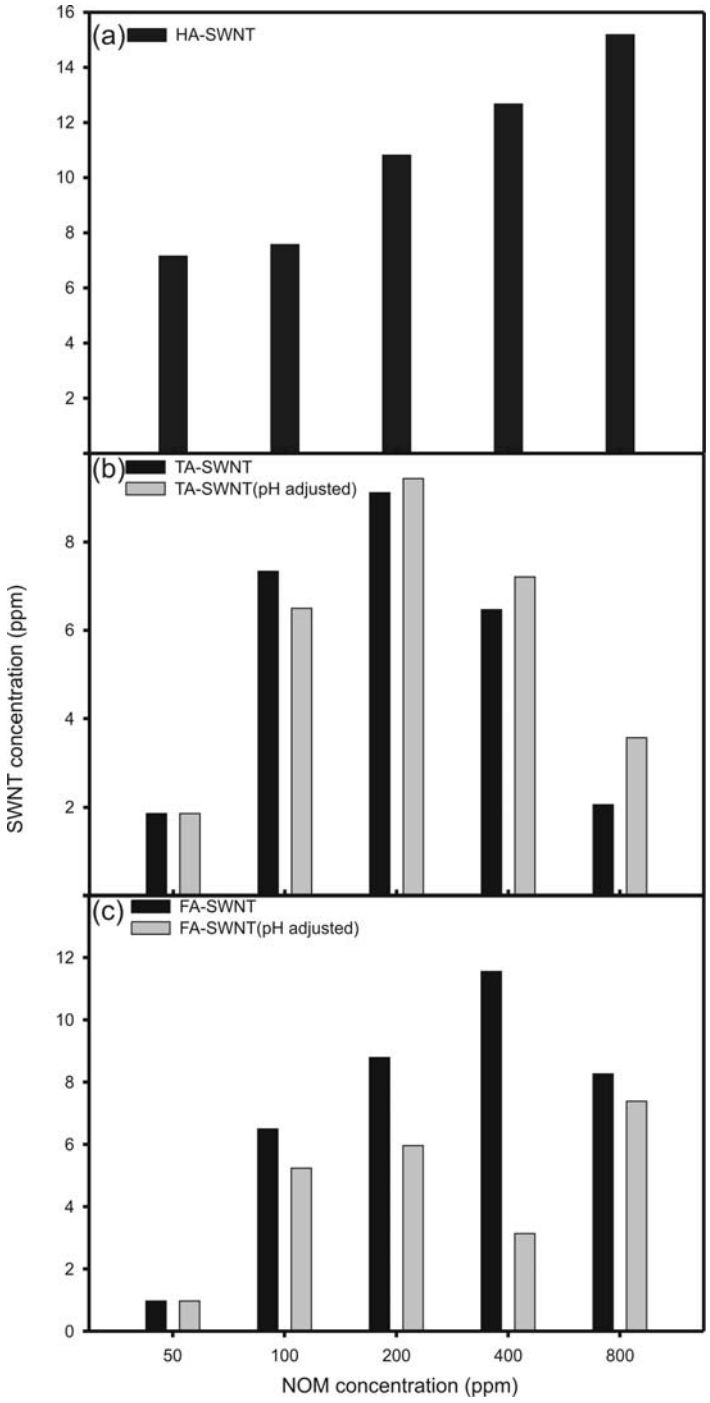

Figure 4-4. Concentration of single walled carbon nanotubes (SWNTs) suspended in synthetic organic matter containing waters. (a) humic acid (HA)-SWNT (b) tannic acid (TA)-SWNT and (c) fulvic acid (FA)- SWNT. Black and grey bars represent SWNT suspensions prepared with as-prepared organic matter solutions and solutions whose pH's were adjusted to the level of organic matter at a concentration of 50 mg/L, which is 5 (i.e. TA) and 4 (i.e. FA), respectively.

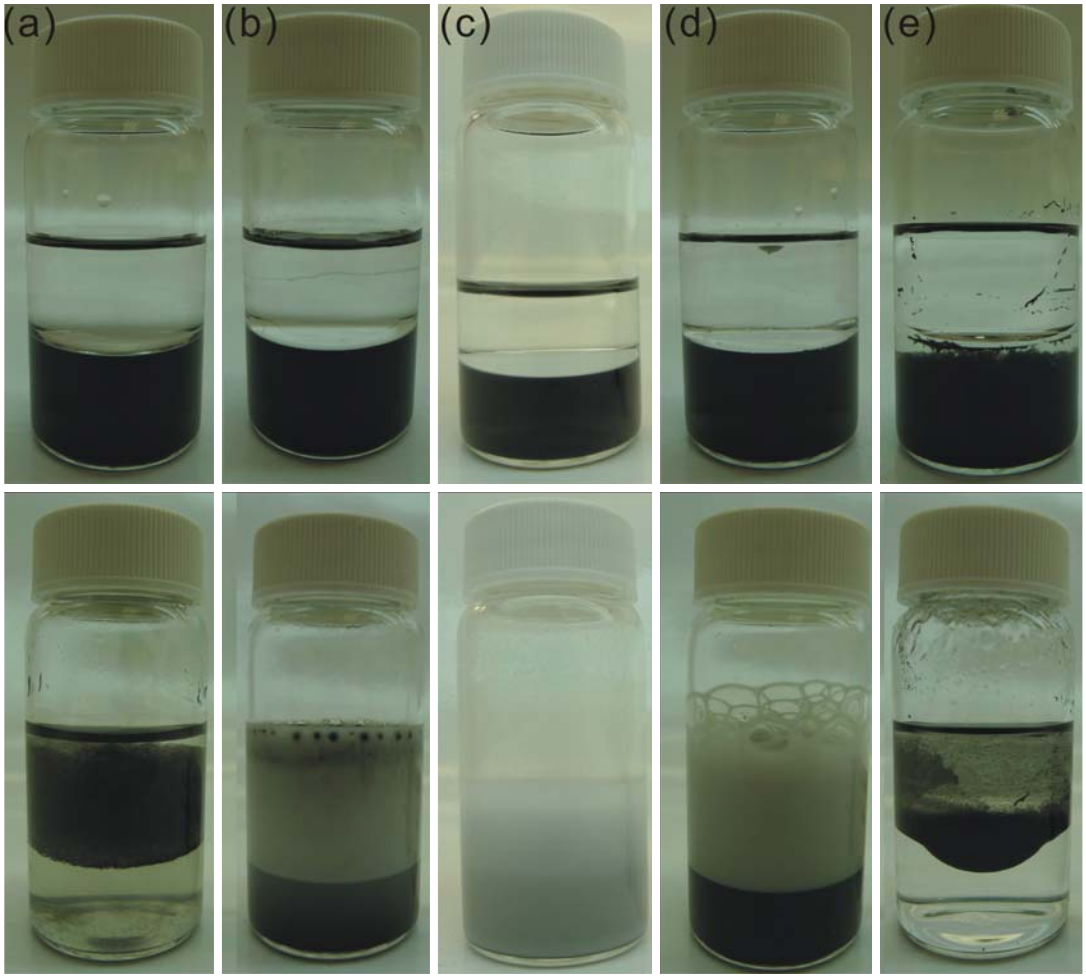

Figure 4-5. Octanol-water partitioning of single-walled carbon nanotubes (SWNTs). SWNTs were first suspended in aqueous solutions containing either gum Arabic (GA), sodium cholate (SC), pluronic acid F108 (PF), sodium dodecyl sulfate (SDS), or algal organic matter. Upper and lower images were taken before mixing and after 24 hours of stabilization, respectively. (a) GA, (b) SC, (c) PF, (d) SDS, and (e) algal organic matter.

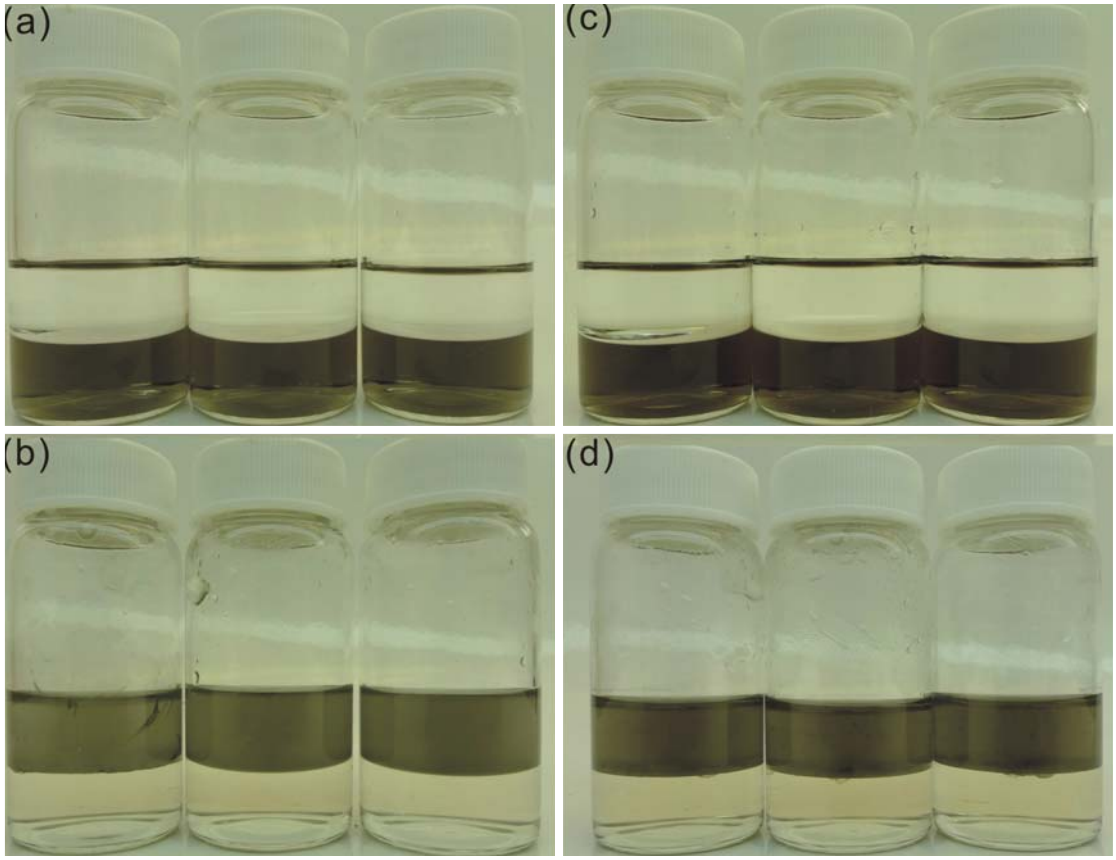

Figure 4-6. Octanol-water partitioning behavior of natural organic matter suspended single walled carbon nanotubes (NOM-SWNTs) using the conventional 'shake flask' method. (a) Suwannee River water suspended SWNT (SR-SWNT) before. (b) SR-SWNT after. (c) Humic acid suspended SWNT (HA-SWNT) before. (d) HA-SWNT after. Tests were run in triplicates.

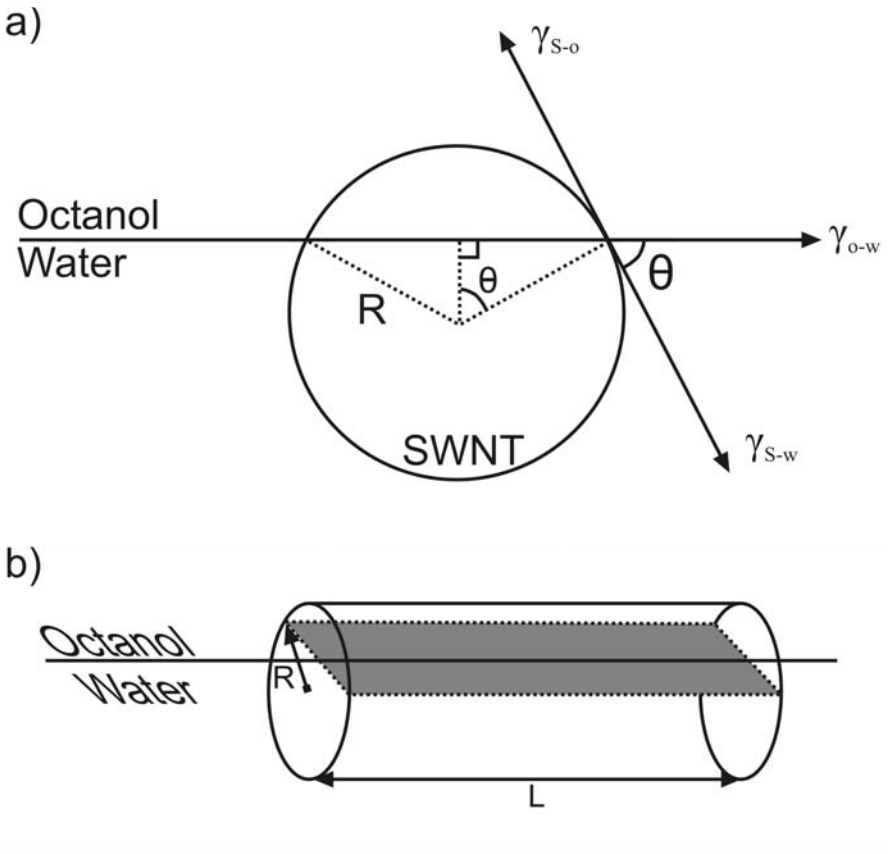

Figure 4-7. Schematic diagram of a SWNT at an octanol-water interface showing (a) the various inter facial energies and the contact angle and (b) the shaded area represents the planar area of the octanol-water interface removed by the SWNT

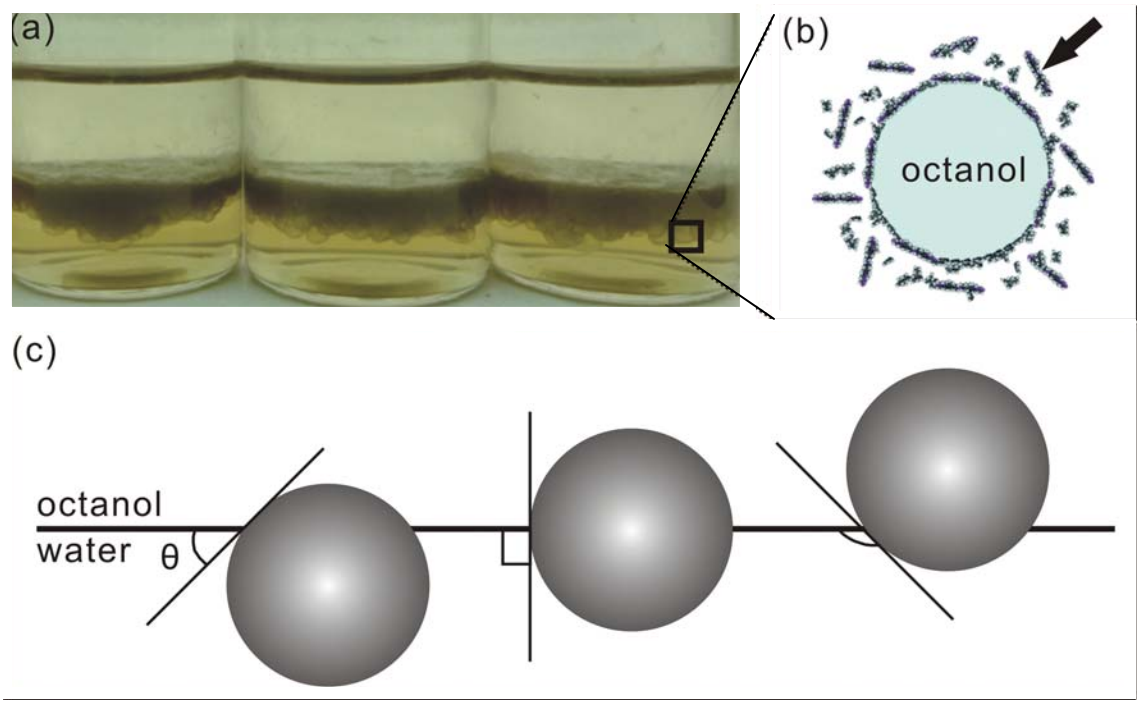

Figure 4-8. Interfacial behavior of SWNTs between octanol and water phases. (a) Formation of the emulsion between octanol and water phases at the end of octanol-water partitioning experiment. The concentration of HA in the solution was 200 mg/L. (b) Diagram showing the emulsion of octanol droplets stabilized by HA-SWNTs. Black arrow indicated SWNTs suspended by HA. Adopted from Hobbie et al. (Hobbie et al. 2009) (c) Diagram of SWNT position at a planar octanol-water interface with a contact angle less than 90° (left), equal to 90° (center), and greater than 90° (right). Adopted from Binks (2002).

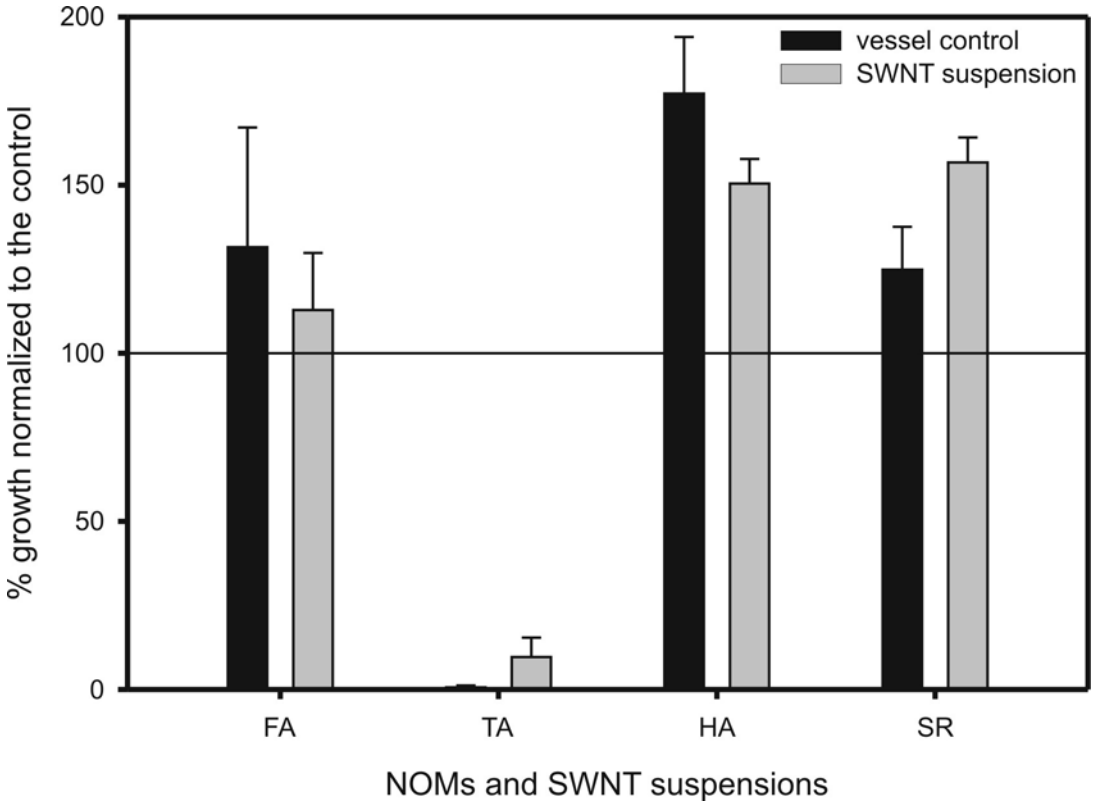

Figure 4-9. Effect of NOM-SWNTs on the growth of *P. subcapitata* in a standard 96 hour chronic algal assay. The final concentration of SWNTs was 0.5 ppm. The horizontal line represents the growth of *P. subcapitata* in control culture media (i.e. plain culture media containing neither NOMs nor SWNTs). Vessel control contains only each NOM but no SWNTs. Growth rates obtained from controls were assigned to a value of 100% and other growth rates from treated samples normalized to the control. All data points show significant differences compared to controls. (α<0.05)

Table 4-1. Dissolved organic carbon (DOC) and specific UV absorption (SUVA) of Suwannee River water (SR), humic acid (HA), tannic acid (TA), and fulvic acid (FA) and dispersion quality comparison of aqueous single walled carbon nanotubes (SWNTs) suspensions.

Parameters	SR	HA	TA	FA
DOC (mg/L)	62	58	61	65
$SUVA_{254}$($Lmg^{-1}m^{-1}$)	0.041	0.076	0.008	0.043
SWNTs concentration (ppm)	8.0	9.7	2.8	4.9
Fluorescence intensity	weak	weak	weak	weak
Absorbance features	significant	significant	weak	significant
Raman aggregation ratio*	1.81	2.18	1.36	1.05
D/G peak ratio**	0.048	0.052	0.052	0.059
Stability (1 week)***	stable	stable	stable	stable

*Calculation based on intensity from Raman peaks (i.e. ratio 234 cm^{-1} to 267cm^{-1})
**Calculation based on Raman spectra of D-peak (at about 1300 cm^{-1}) and G-peak (at about 1600 cm^{-1})
***Monitoring absorbance changes at 763 nm over 1 week.

Table 4-2. Octanol-water partitioning experiment using both OM isolate-SWNT and SR-SWNT suspensions and increased centrifugation speed. The percent of SWNTs transferred from aqueous solutions to octanol is calculated based on SWNT concentrations determined from the octanol phase at the end of the partition experiment.

Types of SWNT suspensions in solutions with different types of organic compounds	% of SWNTs transferred from aqueous solution to organic phase
SR-SWNT	38.3
HA-SWNT	31.5
TA-SWNT	32.1
FA-SWNT	17.2
SR-SWNT :centrifugation speed (RPM)	
0 (no centrifugation)	48.5
4000	57.8
8000	62.9
14000	68.0

Table 4-3. Octanol-water distribution of SWNT as a function of dissolved organic concentrations. The percentage of SWNTs transferred from water to octanol is determined based on SWNT concentrations in octanol phase at the end of the experiment.

Concentration of organic carbon (mg/L)	Percentage (%) of SWNTs transferred from water to octanol		
	Humic acid	Tannic acid	Fulvic acid
50	58.7	60.9	*BDL
100	68.0	BDL	95.2
200	BDL	BDL	22.3
400	BDL	BDL	11.6
800	BDL	BDL	BDL

*BDL = below detection limit

Table 4-4. Octanol-water partition of SWNT from aqueous suspensions prepared in pH adjusted to 5 and 4 for TA and FA solutions, respectively. Octanol transfer percent is calculated from measured SWNT concentrations in octanol phase at the end of the experiment.

Concentration of organic carbon (mg C/L)	Percentage of SWNTs transferred to the octanol phase	
	TA	FA
100	27.2	65.9
200	BDL	82.3
400	BDL	30.9
800	BDL	38.1

*BDL: below detection limit

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Progress in nano-science has resulted in the rapid development of nanotechnology and production of new nanosized materials such as carbon nanotubes (CNTs), which are likely to be mass produced and introduced to the environment. Currently, the impact and fate of CNTs are largely unknown. This study has investigated the interaction between single walled carbon nanotubes' (SWNTs) and an aquatic organism while assessing the bioaccumulation potential of SWNTs in aquatic biota.

The biological responses of *Pseudokirchneriella subcapitata* to exposure to SWNTs suspended in gum Arabic (GA), sodium cholate (SC), and pluronic acid F108 (PF) was investigated using typical 96-hour algal bioassays and long-term growth studies. Changes in algal biomass and cell morphology associated with specific SWNT-treatments were monitored and the biological response mechanisms were studied through a combination of biochemical and spectroscopic methods. The main findings can be summarized as follows.

Results from short-term bioassays show significant growth inhibition in culture media containing SWNT concentrations of >0.5 mg, 0.8 mg and 5 mg /L for GA, SC, and PF, respectively. However, the observed toxicity was significantly mitigated when the surfactant concentrations were nearly doubled while SWNT concentrations were maintained constant in culture media. On the other hand, long-term bioassays using toxic combinations of either SWNTs-GA or SWNT-SC show that *P. subcapitata* would easily recover from an initial growth inhibition effect but at different rates depending on the surfactant. These results suggest that the toxicity of SWNTs can be mitigated, and potentially eliminated at the manufacturing stage. However, these conclusions are

obtained using a single model aquatic organism. The study should be extended to other organisms such as bacteria (Gram negative and positive), invertebrates (e.g. daphnids), and vertebrates (e.g. fish).

Oxidative stress appears to be the primary mechanism of observed adverse biological impacts based on the detection of reactive oxygen species and measurements of glutathione concentrations in culture media. This is supported by (1) the fact that used SWNTs exhibited high levels of iron impurities, high enough to induce "Fenton-like" reaction and ROS production; and (2) the lack of physical damages to cells as determined through transmission electron microscopy (TEM) observations.

The mitigation of SWNT's toxicity is also likely related to the properties of used surfactants. For instance, GA has antioxidative properties as illustrated by GSH accumulation in algal culture media treated with GA only. In the case of SC, the exposure of the SWNT sidewall (which leads to increased oxidative stress) due to the degradation of SC is reduced when there is ample SC in culture media to replace degraded SC. In the case of PF, increased coverage provided by higher concentrations of PF is suggested as a mechanism to help protect against physical contact between SWNTs and *P. subcapitata*.These findings point to the possibility of using the surfactants to mitigate the toxicity of SWNTs, making them not only ideal dispersing agents but also toxicity inhibitors, provided that they do not alter the performance sought from these nanotubes.

Dispersion of SWNTs in natural Suwannee River (SR) water and synthetic solutions of organic matter (OM) isolates (e.g. humic acid (HA), tannic acid (TA), and fulvic acid (FA)) demonstrate that the naturally occurring organic compounds yield

nanotube suspensions which are as stable as those produced with synthetic surfactants. Thus, naturally occurring OM could enhance SWNT transport and hence, impact the fate of SWNTs beyond what is currently known.

SWNTs suspended in SR water and aqueous synthetic solutions of HA, FA and TA are transferred to the organic phase in the traditional shake-flask separation method. This phenomenon is not observed when SWNTs are dispersed first in synthetic surfactants such as GA or SC. This suggests that surface property modifications of SWNTs caused by naturally occurring OM are more favorable for the transfer process than those induced by synthetic surfactants.

From the environmental perspective, changes in SWNT properties produced after they are released into organic-rich water systems may have a greater impact on potential interactions with biological membranes. Further investigation is therefore required in order to fully characterize SWNT surface property changes induced by OM.

The toxicity of SWNTs is altered when suspended directly into organic rich waters, while their transfer from the aqueous solution to the octanol phase is increased. This discrepancy calls for further studies with emphasis on bioaccumulation and biomagnifications. The use of other model organisms representing different trophic levels in an aquatic system should be considered.

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BIOGRAPHICAL SKETCH

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