Impact of Single-Walled Carbon Nanotubes on Ciliated Protozoa & Bacteria

By

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

As pointed out more and more frequently in the literature, there is a pressing need for research into the health and environmental impact of nanoparticles. This work represents a joint effort between scientists in nanotechnology, chemistry and biology to answer this call and to investigate the environmental effects of carbon nantoubes (CNTs) from a brand new aspect.

The results showed clearly the dose-dependent effects of single-walled carbon nanotubes (SWNTs) on the ingestion and digestion of bacteria by *Tetrahymena thermophila*, a ciliated protozoan, propagated to its prey bacteria, *Escherichia coli*. Investigated by confocal microscopy *Tetrahymena* were able to internalize large quantities of SWNTs and then excrete SWNTs and undigested bacteria in aggregates. Inhibition of ciliate bacterivory measured by Ciliate Bacterivory assay was evident at far below lethal concentrations. At high tube concentrations (above 6.8 $\mu g \cdot ml^{-1}$), cell viability was affected. In addition, explored by fluorescence microscopy and scanning electron microscopy, SWNTs stimulated *Tetrahymena* to abnormally egest viable bacteria inside membrane protected structures, which enhanced bacterial survival during antimicrobial treatments, bacteriostatic or bacteriocidal. This phenomenon may have important implications to public health. In general, research on toxicity of nanoparticles is in a very early stage with most studies on direct fatality (kill or not to kill) of a single organism or certain type of cells. This work is believed to be among the first few investigating extrapolated effects. Hopefully, this wok will stimulate a line of research towards better understanding of the effects of nanomaterials on diverse organisms, and stimulate not only toxicology but also ecotoxicology studies.

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Special thanks are due to my parents, brother, Mohammad, and my numerous friends who endured this long process with me, offering me endless support and love.

DEDICATION

To my loved ones

In the name of God, the Most Gracious, the Most Merciful

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LIST OF ILLUSTRATIONS

AB	**Alamar Blue**
AFM	**Atomic Force Microscopy**
APTES	**Aminopropyltriethoxysilane**
CB	**Ciliate Bacteriovory**
CF	**5-CarboxyFluorescein**
CFDA-AM	**5-CarboxyFluorescein Diacetate AcetoxyMethyl ester**
GFP	**Green Fluorescence Protein**
CFU	**Colony Forming Units**
CNTs	**Carbon Nanotubes**
CVD	**Chemical Vapor Deposition**
EAD	**Electric Arc Discharge**
E. coli	***Escherichia coli***
EDAX	**Energy Dispersive Analytical X-ray**
LA	**Laser Ablation**
MWNTs	**Multi Walled Carbon Nanotubes**
NIR	**Near-Infrared Red**
PBS	**Phosphate Buffer Saline**
PI	**Propidium Iodide**
PNEC	**Predicted No Effect Concentration**
PPYE	**Proteose Peptone Yeast Extract**
RF	**Fluorescence Units**
RFU	**Relative Fluorescence Units**
SEM	**Scanning Electron Microscope**
SWNTs	**Single Walled Carbon Nanotubes**
TSA	**Tryptone Soy Agar**
TSB	**Tryptone Soy Broth**
T. thermophila	***Tetrahymena thermophila***
UV	**Ultra-Violet**

CHAPTER 1: INTRODUCTION

1.1 Discovery of Carbon Nanotubes & Nanotechnology

The serendipitous discovery of Single Walled Carbon Nanotubes is one of the landmarks in the history of nanotechnology. Whether the history of its discovery goes back to 1958 as Hillert and Lange stated that "Filamentary growth of graphite has recently been discovered again" or to Baker's group note of "Interest in catalytic decomposition of hydrocarbons on metallic surfaces… has recently become more active" in 1973 or even 1991 Iijima's paper in *Nature*, from a scientific point of view all teams are acknowledged the credit for the discovery of SWNTs that revolutionized a wide range of industries.[1]

The historical perspective of nanotechnology, similar to SWNTs, is indefinite and in fact "the rudiments of the science of nanotechnology evolved from research in a variety of endeavors"[2]; however, the famous speech by the renowned physicist and later Noble Prize Winner in Physics, Richard Feynman is considered to be the foundation of nanotechnology. The term "nanotechnology" was first introduced by Taniguchi in 1974 and later on popularized by Drexler's famous book entitled "Engines of Creation" in 1986. Less than fifty years ago, Feynman articulated the concept of nanotechnology in his lecture "There's plenty of room at the bottom"[2]. Today nanotechnology is a rapidly expanding field defined as the science and technology that deals with nanoparticles, substances with one or more external dimensions, or an internal structure, on the nanoscale[3]. As nanoparticles may exert toxic effects, nanotoxicology emerged as one category of nanotechnology that is defined by its focus on gaps in knowledge and possible undesirable effects of nanoparticles[4,5].

1.2 Physical Characteristics, Properties, Appearance and Synthesis of Carbon Nanotubes

Among the category of insoluble nanoparticles, carbon nanotubes are one of the most widely studied and used. Two nanostructures, shown in Figure 1, single-walled and multi-walled carbon nanotubes (SWNTs and MWNTs) are two forms of CNTs. CNTs are well ordered, high aspect ratio allotropes of carbon with ultra-light weight that possess high tensile strength as well. They have excellent chemical and thermal stability and superior electronic properties.[6] SWNTs have diameters on the order of 1 nm, about half the diameter of the average DNA helix and lengths in the range of 20-100 nm; while MWNTs depending on the number of walls in their structure have diameters that range from several nanometers to tens of nanometers and lengths from 1 to several um[7]. CNTs are fabricated using electric arc discharge (EAD), laser ablation (LA), chemical vapor deposition (CVD), or combustion processes[8].

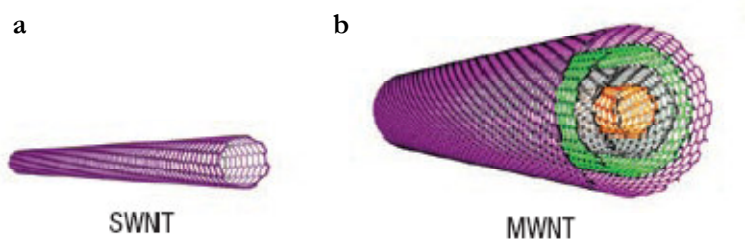


Figure **1** Diagram representation of single walled carbon nanotubes (**a**) multi walled carbon nanotubes (**b**)[9].

Pristine CNT (as prepared, non-functionalized) are inherently hydrophobic. Therefore the main obstacle in the utilization of CNT in biology and medicinal chemistry is their lack of solubility in most solvents compatible with biological milieu. To overcome this problem, surface modification of CNTs (functionalization) can be achieved by covalent and noncovalent sidewall functionalization with surfactants and polymers (Fig. 2). In addition, endohedral functionalization (Fig. 2d) is possible and may be used for capsulation of functional molecules or synthesis of hybrid structures (e.g.

magnetic CNT). Through different modifications, the water solubility of CNT is improved, their biocompatibility profile completely transformed, propensity to cross cell membranes increased and possibilities offered for introducing biologically active moieties. Moreover, the bundling/aggregation of individual tubes through van der Waals and hydrophobic forces is also reduced by the functionalization of their surface.

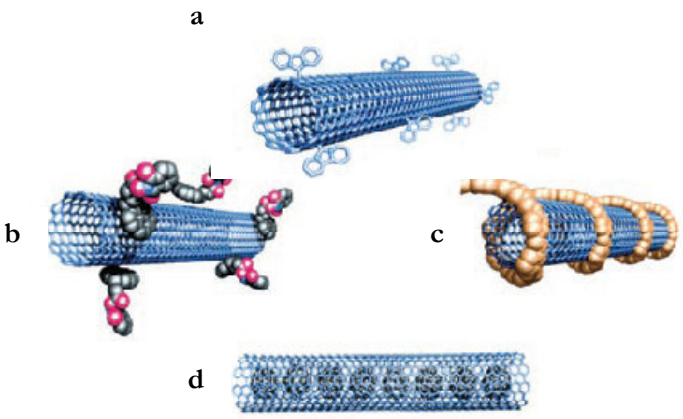


Figure **2** Functionalization possibilities for SWNTs: covalent sidewall functionalization (**a**) noncovalent exohedral functionalization with surfactants (**b**) noncovalent exohedral functionalization with polymers (**c**) endohedral functionalization with, for example, C_{60}(**d**). (Modified from: Hersam, 2008[9])

1.3 Potential Wide Applications of Carbon Nanotubes

"As of April 2008, it is estimated that there are currently greater than 610 available consumer products utilizing nanomaterials"[10]. The superior combination of properties of carbon nanotubes have made them particularly promising nanomaterials for industrial use and, therefore one can easily imagine that they become a more frequent component of consumer products and their production will continue to increase in the future. "Carbon nanotubes are often considered to epitomize the

field of nanotechnology"[11]. The size, geometry, and surface characteristics of these structures make them appealing for diverse applications in biomedicine, electronics and environmental protection. In medical field, SWNTs have been proposed as chemical sensors for gaseous molecules, vectors for drug delivery, and photothermal therapy for cancer[12].

Approximately sixty five tons of CNTs and fibers were produced in 2004, and mass production is predicted to grow annually over the next few years by well over 60%.[13] By 2010, easy access to CNTs will allow extensive usage of them in a wide variety of applications and the global market for CNTs is projected to reach multi-billion dollars by 2014[11]. Meanwhile, widespread concerns have been raised about the hazards that nanoparticles can have on human and environmental health.

1.4 Environmental Health Issues of Carbon Nanotubes

With the larger scale production of CNTs in the future, it is inevitable that its products and by-products will release in different environmental media[14], including waterways and aquatic systems despite any safeguards. Consequently, as accidental spillages or permitted release of industrial effluents increase, direct as well as indirect exposure to nanoscale products and wastes of humans will also increase. Direct exposure could arise from skin contact, inhalation of aerosols and direct ingestion of contaminated drinking water or particles adsorbed on vegetables or other foodstuffs. On the other hand, indirect exposure may result from ingestion of aquatic organisms, such as fish, as part of the human diet[15].

1.5 Nanotoxicology of Carbon Nanotubes

CNT, in the context of nanotoxicology, can be continued together with nanofibers due to the fact they share characteristics within both categories; therefore unexpected toxicological effects

upon contact with biological system may be induced[6]. In particular, CNT resemble carcinogenic asbestos fibers in size, shape and cellular persistance[16]. In fact CNTs introduced into the abdominal cavity of mice have shown asbestos-like pathogenicity[11]. CNTs that are long, thin (few nanometers in diameter) and insoluble contribute to fibre toxicity in lungs.[17]Also Environmental toxicologists are challenged by the fact that living organisms were not encountered by such materials during the course of biological evolution.. Consequently, it is expected to be "a little or no selection pressure for defensive or protective systems to counter any adverse properties that such particles may present beyond those already naturally occurring"[15] ones. Additionally, CNTs like many nanoparticles have natural propensity to bind transition metals and organic chemical pollutants that could enhance the toxicity of either. Furthermore, CNTs ability to penetrate the cellular membrane provides potential routes for the delivery of other toxic pollutants when they are conjugated to CNTs to sites where they would normally unable to go[15].

Generally, the harmful effects of CNT arise from the combination of various factors, three of which are particularly important[6]:

1. structural characteristics including the high surface area and dimension
2. biopersistence and the retention time
3. the intrinsic history (the toxicity of the chemical component of CNT)

As the degree of sidewall functionalization increases, the nanotube sample becomes less toxic[18]. Further, sidewall functionalized nanotube samples are substantially less toxic than surfactant stabilized nanotube. However, the intrinsic toxicity of CNT does not only depend on the degree of surface functionalization and the different toxicity of functional groups.[18] Impurities in pristine CNTs such as amorphous carbon and metallic nanoparticles (catalysts: Co, Fe, Ni and Mo) can also be the source of severe toxic effects.

1.6 Current Status of Toxicity of Carbon Nanotubes

Currently, the main route of exposure of the general public to nanoparticles is by air pollution; since from a terrestrial organisms' toxicology standpoint or specifically human toxicology, significant exposures result from direct exposure via dermal or inhalational exposure. But this is not the only route. CNTs can be introduced by other means such as oral and parental methods that are most likely be from the use of nanotechnology in a medical setting. For aquatic animals there may be other routes of entry such as direct passage across gill and other external surface epithelia. Aquatic organisms are particularly susceptible to pollutants due to their large, fragile respiratory epithelium[10]. The fate and transport of CNTs in various environmental media including air, water, and soil are largely lacking from literature. A review by Heland et al[19] in 2007 suggests, there were as few as 50 studies focusing on the impact of CNT on human health and environment, with the majority of them from *in vitro* studies on mammalian cell lines. Even fewer studies could be found on ecotoxicology where the effects of CNTs on only a small range of aquatic organisms have been reported. Mammalian studies have raised concerns about the toxicity of CNTs, but there is very limited data on ecotoxicity to aquatic life. Our knowledge of the harmful effects of nanoparticles is very limited and is almost non-existent in aquatic animals.

Despite the increasing effort made to identify the cytotoxicity of CNTs, the toxicological evidence for CNTs is sparse, fragmentary, mammalian based and sometimes contradictory. Uptake of carbon nanomaterials including SWNTs by aquatic organisms following exposure in the water has been documented. The study by Zhu et al[20], shows that the viability of *Stylonychia mytilus* exposed to MWNTs is concentration dependent. Concentration higher than 1.0 $\mu g.ml^{-1}$ induced a dose-independent growth inhibition to the cells, whereas, concentration lower than 1 $\mu g.mL^{-1}$ stimulated the cell growth. Another study by the same group shows MWNTs can be either toxic or nontoxic, depending on the medium used to cultivate *Tetrahymena pyriformis*. MWNTs stimulated growth of the

cells cultured in proteose peptone yeast extract (PPYE), but inhibited growth in FPW (stand for?).[21] Another study on MWNTs by Asharani et al[22], shows concentration-dependent toxicity of MWNTs on Zebrafish embryos. . At concentration above 60 μg mL^{-1}, such as significant phenotypic defects slimy mucus like coating was observed around the zebrafish embryo. At high concentrations, MWNTs was found to cause the apoptosis, delayed hatching and formation of abnormal spinal chords. Recently, a study by Blaise et al[23], shows the SWNTs are capable of eliciting toxicity to one or more of the aquatic taxonomic groups at concentrations lower than 5 mg L^{-1}. Furthermore, Smith et al[24] concluded aqueous SWNTs are a respiratory toxicant in trout. SWNT exposure was found to cause a dose dependent rise in ventilation rate, gill pathologies and mucus secretion with SWNT precipitation on the gill mucus; however, no major haematological disturbances were observed. SWNT interaction with *E. coli* studied by Raja et al[25] should significant morphological changes of *E. coli*, including elongation was observed. Interaction of SWNTs with *E. coli* was also investigated by Elimelech et al[26], and their group also observed strong antimicrobial activity by SWNTs. Cell membrane damage as a result of direct contact with SWNT aggregates was considered to be the most likely underlying mechanism.

1.7 Research Objectives

These results regarding the toxicology of SWNTs suggest that both water soluble and insoluble SWNTs could be consumed and transferred by organisms at different levels in the aquatic food chain. Carbon nanotubes are probably the least biodegradable class of nanomaterials, suggesting serious issues with its long-term accumulation and interaction with diverse organisms in the environment. Therefore, the potential for their exposure and health effects needs to be considered. This makes it imperative that we have effective risk assessment procedures in place as soon as possible to deal with potential hazards.

The ciliated protozoan *Tetrahymena thermophila* has been widely studied by ecotoxicologists because it has long been used as a model of eukaryotic cell in basic research as well as in toxicological and health risk assessments[27]. The genome of *T. Thermophila* has recently been sequenced, which enhances the value of the ciliate as a research organism[28]. Furthermore, *Tetrahymena* is representative of an ecologically important group, the grazing protists. Bacterivory by grazing protists, which is the phagocytosis and digestion of bacteria, is a major mechanism in regulating the microbial population composition in both natural and artificial aqueous environments. In particular, it is an important organism in wastewater treatment and an indicator of sewage effluent quality. *Tetrahymena* are ciliated protozoa that ingest bacteria by phagocytosis and sequester them within food vacuoles or phagosomes, which eventually fuse with the cytoproct before being released from the protozoa. The entire process, commonly termed bacterivory, occurs over a period of 1-2 h at 30ºC. Phagotropic protists contribute to aquatic ecology at several levels[29, 30]. They transfer energy from the base of food webs to higher tropic levels by grazing on microbes and then by themselves being eaten by multicellular organisms. Additionally, grazing can change the phylogenetic composition of bacterial assemblages. Although the importance of grazing protists to the environment and public health is well known, few reports can be found on exposure of such organisms to carbon nanotubes, partly because it has been thought that CNTs do not dissolve in water. However, this belief has been challenged by a recent report showing stable CNT suspension in natural surface water over a long period of time.

This study is the first detailed investigation of the ecotoxicity of SWNTs on two common organisms on adjacent trophic levels of the freshwater food chain. In detail the impact of SWNTs was studied on bacterivory by *Tetrahymena thermophila* and through *T. thermophila -Eschericia coli interaction,* its effect on bacterial survival. *Tetrahymena* was exposed to a wide range of single-walled carbon nanotube concentrations (0-17.2 $\mu g\ ml^{-1}$) to fully demonstrate the different modes of impact,

from behavior change to cell death. The results extend the current knowledge on CNTs and microorganisms, and help define critical paramteres such as predicted no effect concentrations (PNEC) for environmental risk assessment models.

CHAPTER 2: RESEARCH METHODOLOGY AND APPROACH

2.1 SWNT Preparation & Characterization

The SWNTs were produced by acid oxidation, which is a widely used method to render purified, shortened and water-soluble CNTs[31], and characterized using several techniques. Atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to characterize nanotube morphology, and their chemical purity was determined by elemental analysis using energy-dispersive analytical X-ray (EDAX). In brief, SWNTs (raw HipCo tube, Carbon Nanotechnologies) were refluxed in 6M HNO_3 for a 20-h period. The resulting mixture was then filtered through a polycarbonate filter with a pore size of 100 nm, rinsed thoroughly, and resuspended in deionized water with cup-horn sonication for 1h. Centrifugation (22,000 g, 5h) removed larger unreacted impurities from the solution to afford a stable suspension of acid oxidized nanotubes. For AFM imaging, SWNTs were deposited onto a 3-aminopropyltriethoxysilane (APTES) treated silicon substrate. For incubation of *T. thermophila* and *E. coli,* stock solution of SWNTs was serially diluted in Osterhout`s minimal salts medium (5.2 g NaCl, 0.997 g $MgCl_2 \cdot 6\ H_2O$, 0.2 g $MgSO_3$, 0.115 g KCl, 0.066 g $CaCl_2 \cdot H_2O$, in 100 ml of distilled water) or PPYE medium. UV-visible-NIR spectroscopy (Cary) was used to characterize SWNT concentration, based on Beer's law, absorbance at 808 nm and molar extinction coefficient 7.9 x $10^6\ M^{-1}\ cm^{-1}$. [32].

2.2 Microorganisms

2.2.1. T. thermophila Culture

The ciliated *T. thermophila* were cultured axenically at room temperature in 10 ml of PPYE medium. To prepare the cultures for assays in brief, from the stock culture, 1 ml was aseptically transferred into 50 ml sterile PPYE in untreated 75 cm^2 tissue-culture flasks (Falcon, VWR) and grown for 36 h at room temperature on an orbital shaker at 50 rpm. The culture was then

centrifuged (450 g, 5 min) and washed three times, and resuspended in 50 ml Osterhout's solution. Cells were counted using a Coulter Z2 particle counter and adjusted to a cell density of 10^6 cells ml^{-1} (±10%) using Osterhout's solution.

2.2.2. Bacterial Strains

The green fluorescent protein (GFP) expressing *E. coli* we used in this study is *E. coli* XL-1, previously transformed with the recombinant plasmid containing the expression pET vector (Stratagene, La Jolla, CA) into which has been ligated a red shifted gfp construct with an excitation maximum at 490 nm and was observed to produce a stronger fluorescence than that of wild-type (provided by Ian Macarra, Center for Cell Signaling, University of Virginia). The *E. coli* XL-1 (*pET*-gfp) was maintained on Tryptone Soy agar (TSA) (Difco, Toronto, ON) plates supplemented with 100 μg ml^{-1} ampicillin grown overnight at 37ºC and then stored at 4ºC. In order to obtain fresh cultures for the ciliate bacterivory (CB) assay, single, isolated colonies from previous plates were picked and then re-streaked onto new TSA plates which contained ampicillin to create lawns of bacteria expressing gfp. The plates were incubated as previously mentioned. The fully saturated cultures were harvested off of the TSA plates and resuspended to the desired concentration in Osterhout's solution. The cells were then collected by centrifugation (3000 x *g*, 10 min), and washed three times with Osterhout's solution. The washed cells could be maintained at 4ºC for 1 week with no loss in fluorescence or viability.

The non-gfp expressing *E. coli* used in this study is *E. coli* B63 (ATCC 11202E). Similar to fluorescing *E.coli*, this strain was also maintained on TSA plates but without ampicillin. To obtain fresh cultures, colonies were picked from streaked TSA plates, which had been incubated at 37ºC for 24 h, and 20 ml of tryptone soy broth (TSB) (Difco) were inoculated with several colonies from the plates and incubated with several colonies from the plates and incubated at 37ºC at 200 rpm for 18

to 24 h. The cultures were harvested by centrifugation (3000 g, 10 min), washed and resuspended to the desired concentration in Osterhout's solution.

2.3 Microscopy

The interactions of SWNTs and *Tetrahymena* were monitored with a video camera in a phase contrast microscope and by confocal microscopy (Zeiss LSM510). For the latter, cells were seeded into eight-well chambered cover slides. Immediately before imaging, 0.01% neutral formalin buffer was added as a fixative. For all SWNT concentrations, cell densities were kept the same, that is, 5 x 10^5 cells ml^{-1} for *T. thermophila* and 5 x 10^8 c.f.u. ml^{-1} for *E. coli*. *Tetrahymena* nuclei were stained red using DRAQ5 (Biostatus Ltd.) The yellow appearance of *E. coli*-gfp is due to overlapping of green (gfp) and red (stained by DRAQ5).

To investigate the surface structures of *T. thermophila* and *E. coli* with SWNTs incubation, SEM was carried out using LEO FESEM 1530 field emission scanning electron microscope. After incubation of *E. coli* and *Tetrahymena* independently with 12.41 μg ml^{-1} SWNT for 6 hours, the samples were fixed for one hour in 2.5% glutaraldehyde in Osterhout's solution, gradually medium exchanged into acetone, dehydrated through critical point drying and finally coated with 30 nm gold. There are SWNTs attached to the outer surface of both *E. coli* and *Tetrahymena*, which is expected because the samples were not washed prior to fixation.

2.4 Bioassays

2.4.1. Ciliate Bacterivory Assay & Colony Forming Assay

A schematic of the ciliate bacterivory assay is presented in Figure 12. The assay was carried out in 96-well plates, usually with 3 or 6 wells per treatment. Each well contained ciliates (shown in three of the wells in the schematic; 5×10^5 cells ml^{-1}) and E. coli (5×10^5 c.f.u. ml^{-1}) expressing gfp. For a typical assay without treatment (control), the bacteria (green rods) are nearly all outside the

ciliates shortly after initiation of the assay (1), reduced in number and mostly inside ciliate food vacuoles after 1 h (2), and largely destroyed by 4 h (3). The assays were begun by either the exposure of *Tetrahymena* to SWNTs concurrently *(protocol 1)* or 4 hours incubation prior (*protocol 2)* adding *E. coli*-gfp, and was monitored with a fluorescence plate reader (Victor V, Perk and Elmer) that repeatedly measured over time the relative fluorescence units (RFUs) per well as described previously[33]. The results were expressed as a percentage of the starting RFUs. Two types of experiments were carried out with only SWNTs and *E. coli*-gfp together. First, the effect of different SWNT concentrations on the fluorescence of *E. coli*-gfp alone (6 wells for each concentration) was monitored over time and expressed as a percentage of starting RFU values. The starting RFUs were 196,908 ± 706 (control), 152,822 ± 8,660 (0.9 mg ml^{-1}), 133,084 ± 11,756 (1.8 mg ml^{-1}), 130,763 ± 441 (3.6 mg ml^{-1}), 87,660 ± 2,578 (7.3 mg ml^{-1}) and 54,309 ± 4,997 (14.6 mg ml^{-1}). Second, the effect of SWNTs on colony formation was examined. Several dilutions of the samples were prepared by the addition of sterile Osterhout's and, subsequently, dilutions were plated on TSA ampicillin plates and incubated at 37ºC overnight. Colonies were counted and expressed as colony forming units c.f.u. ml^{-1}.

2.4.2 Bacterial viability and proliferation Assays

The growth of pET-gfp was assessed in two different ways. One was to directly observe the fluorescence from GFP. After pET-gfp were inoculated onto a TSA plate, fluorescence images (Olympus BX41) of the plate were taken (at the same location) after incubation at 37ºC for 0, 12, and 24 hours. For a more quantitative assessment of bacterial growth rate, as described before, pET-gfp were cultured in TSB in 96-well plates with 3-6 replicates, of which relative fluorescence level was measured using a plate reader every 2 hours over a period of 26 hours at 37ºC.

The Live/Dead BacLight kit (Invitrogen) was used to evaluate bacterial survival after anti-microbial treatments. The kit includes two nucleic acid staining dyes, green-fluorescent SYTO 9 (Abs/Em 490/500 nm) and red-fluorescent propidium iodide (PI) (Abs/Em 490/635 nm). Staining condition was optimized to 1:2 ratio of SYTO 9 and PI in 3:1000 dilution. STYO 9 labels live bacteria with intact membrane, while PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO 9 fluorescence when both dyes are present. To avoid overlapping in fluorescence with GFP (Abs/Em 480/530 nm), the non-gfp expressing *E. coli* strain was used. In the presence of antibiotics or disinfectant, SYTO 9/PI mixture was introduced right after administration of those compounds into bacteria cultures and green fluorescence level (i.e. live bacteria population) was monitored by fluorescence microscopy and the fluorescence plate reader over the period of 24 hours. The intensities of green/red fluorescence are well correlated to live/dead bacteria populations.

2.4.3. Alamar Blue (AB) & 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA) Assays

Similar to CB assay, AB and CFDA assays were carried out in a filter bottom 96-well plates, usually with 3 wells per treatment. Each well contained ciliates (5×10^5 cells ml^{-1}) and for treatment study were exposed to SWNTs for 4 hours. After the 4-h exposure of *T. thermophila* to SWNTs had been terminated by vacuum suction, the cells were resuspended in working solution of the fluorescent indicator dyes, which was prepared. The cells were incubated at room temperature for 60 min in the solution of AB and CFDA. Afterward the fluorescence as fluorescent units (FUs) was quantified with the microplate reader at the excitation and emission wavelengths of 530 and 596 nm.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Characterization of Single Walled Carbon Nanotubes

The oxidization approach was to render SWNTs water soluble by standard refluxing in nitric acid and sonication, which is known to produce oxidized tubes with oxygen-containing groups (e.g. –COOH) along the sidewalls and at the ends (Fig. 3a)[31]. These functional groups impart hydrophilicity to the nanotubes and make them stable in water without apparent aggregation. Furthermore, oxidized SWNTs were used so that the impact of the nanotubes could be studied without interference from surfactants; especially certain surfactants are known to contribute to cytotoxicity[34]. SWNTs were characterized structurally by AFM in a simple solution (Osterhout's) and in a complex ciliate growth medium, PPYE. Solubilized SWNTs were mostly individual or in small bundles (Fig. 3b) with length predominantly <500 nm (based on manual count on AFM software) and diameters ranging from 2 to 10 nm (based on AFM software cross-section examination) (Fig. 3e) in Osterhout's, but appeared as micrometer-size complexes in PPYE (Fig. 3c). SEM of SWNT aggregates after 24 h with in Osterhout's revealed amorphous tangles (Fig. 3d). Iron contamination was successfully removed by our nanotube preparation procedure (Fig. 3f). UV-visible-NIR spectroscopy was used to characterize SWNT concentration (Fig. 3g).

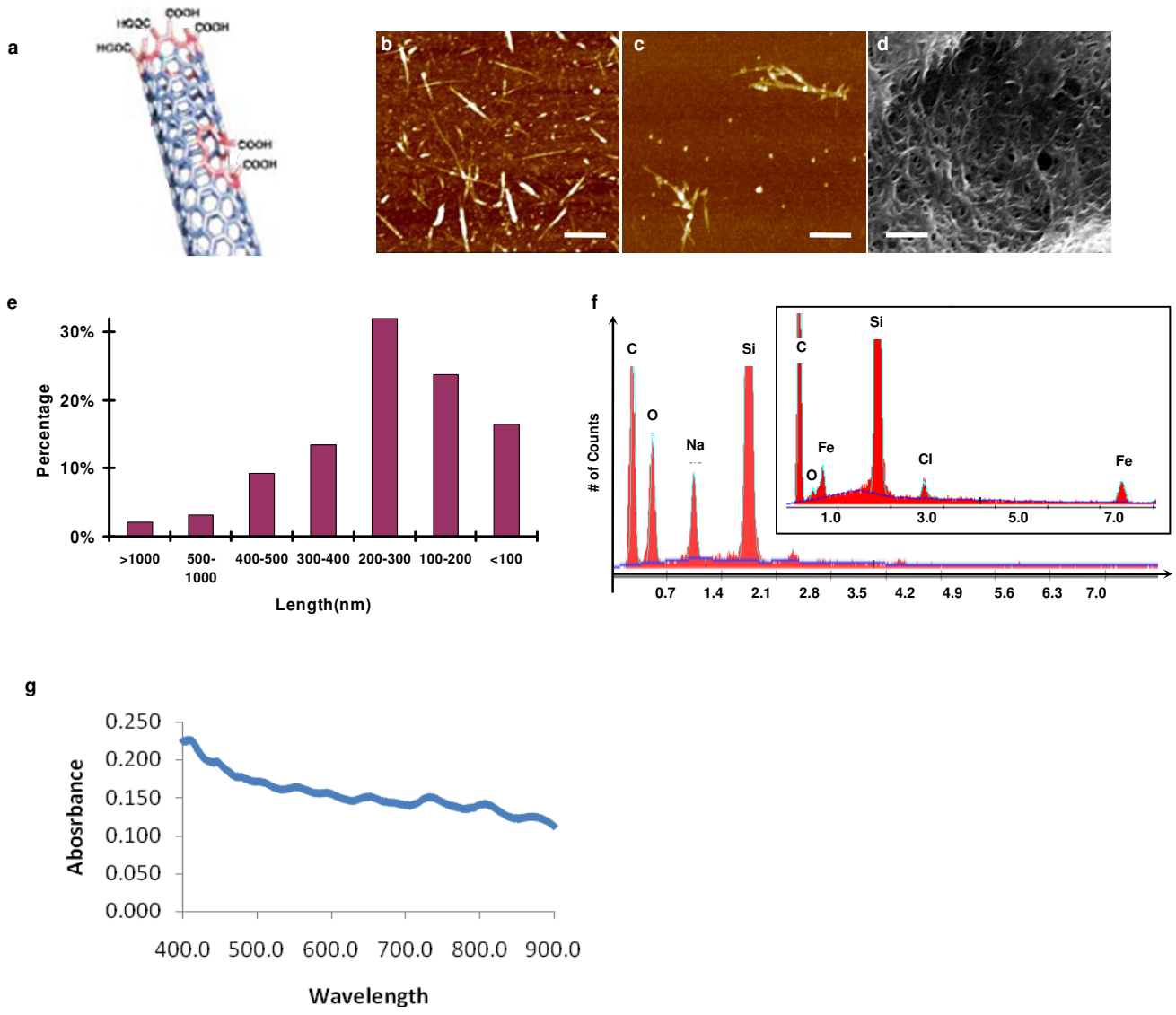


Figure **3** Characterization of SWNTs. **a**, schematic representation of oxidized SWNT. , **c**, AFM images showing the morphology of SWNTs in Osterhaut's solution (**b**) and PPYE growth medium (**c**). Scale bars, 500 nm. **d**, SEM image of SWNT aggregates collected from a *T. thermophila* culture. Scale bar, 200 nm. **e**, Distribution of nanotube length obtained from **a**. **f**, EDAX spectrum of nanotubes. Oxidized SWNTs presented to *T. thermophila* do not contain iron. Raw nanotubes show the presence of iron (inset). The two spectra are normalized against the carbon peak intensity. **g**, UV-vis spectrum of solutions of individual SWNTs functionalized via oxidation.

3.2 Effect of Single Walled Carbon Nanotubes on Tetrahymena Thermophila Viability

Tetrahymena cultures in Osterhout's (control) and with SWNTs (11.9 $\mu g.ml^{-1}$) were monitored by phase contrast video microscopy over 3 days. Still images (Fig. 5) and movies were taken at various time points. Control cultures remained healthy for 72 h, as judged by the continued motility of the ciliate and no apparent change in their morphology (Fig. 4a). In contrast, the response of *Tetrahymena* to SWNTs exposure was complex. Concentration-dependent effects of SWNTs on cell mobility and viability were evident. SWNTs elicited four interrelated responses: diminished mobility, cell aggregation, matrix accumulation and cell death. These occurred sequentially in three distinct stages: (1) initial aggregation and loss of mobility (0–3 h), (2) recovery of mobility by some cells and their movement out of aggregates (3–12 h) and (3) increased visibility of the matrix associated with the persisting aggregates and appearance of dead cells (12–72 h).

In the first phase, upon addition of SWNTs (11.9 $\mu g.ml^{-1}$), almost immediately all cells bunched together in groups of approximately 5 to 50 cells with slight or no mobility (Fig. 4b). The size of *Tetrahymena* aggregates persistently grew larger and reached its maximum after about 1 hour (Fig. 4c). A few single cells were also present and all cells showed little or no mobility during roughly the first 3 hours. It was suspected that aggregated *Tetrahymena* were unable to ingest either bacteria or SWNTs, which well explained the impeded bacterivory as shown in results from CB assay (*protocol **1***).

After 3 hours, in the second stage, a stage of recovery was observed. Some cells began to break free from the aggregates, and the number of motile single cells increased gradually over time. The observed recovery of ciliates is mainly responsible for the less profound impact found in CB assay (protocol ***2***). At this stage, freed *Tetrahymena* would be able to internalize SWNTs and/or bacteria as supported by confocal images. Examination after 24 hours found that a small portion of the ciliates regained mobility and appeared healthy. Cellular division could be spotted occasionally.

In the third stage, the aggregates persisted and a large number of cells remained trapped in a matrix along with small portion of the ciliates that remained motile and appeared healthy. Over time, the matrix grew darker and became progressively more obvious as being responsible for holding cells together (Fig. 4d). The dark matrix could arise from the release of DNA and proteins from dead cells or from exudates from living cells including SWNT aggregates. The two possibilities cannot yet be distinguished but the latter is favored, because the matrix was formed while majority of cells were obviously viable (as shown in the movies). Continued monitoring showed obvious cell death in the matrix after 24 hours, and the ciliates regained mobility and assumed bacterivory earlier (during the 3 to 24 hours time frame) lost viability later. Very few live cells could be found in the culture after 3 days.

In contrast, in cultures with SWNTs concentrations < 6.8 $\mu g.ml^{-1}$ no loss of cell viability was observed after 3 days, even though initial aggregation was observed. Because the responses at low concentrations and the early responses at all concentrations do not appear to be from SWNTs quickly killing the cells, releasing DNA and proteins that immobilize and aggregate the ciliates, the prior favored assumption is valid. In general, aggregate size, loss of mobility and cell death increased with increasing SWNTs concentrations. This trend is evident in the videos taken from cultures with 1.6 $\mu g.ml^{-1}$ (the lowest concentration with visible initial aggregation) and 11.9 $\mu g.ml^{-1}$ SWNTs.

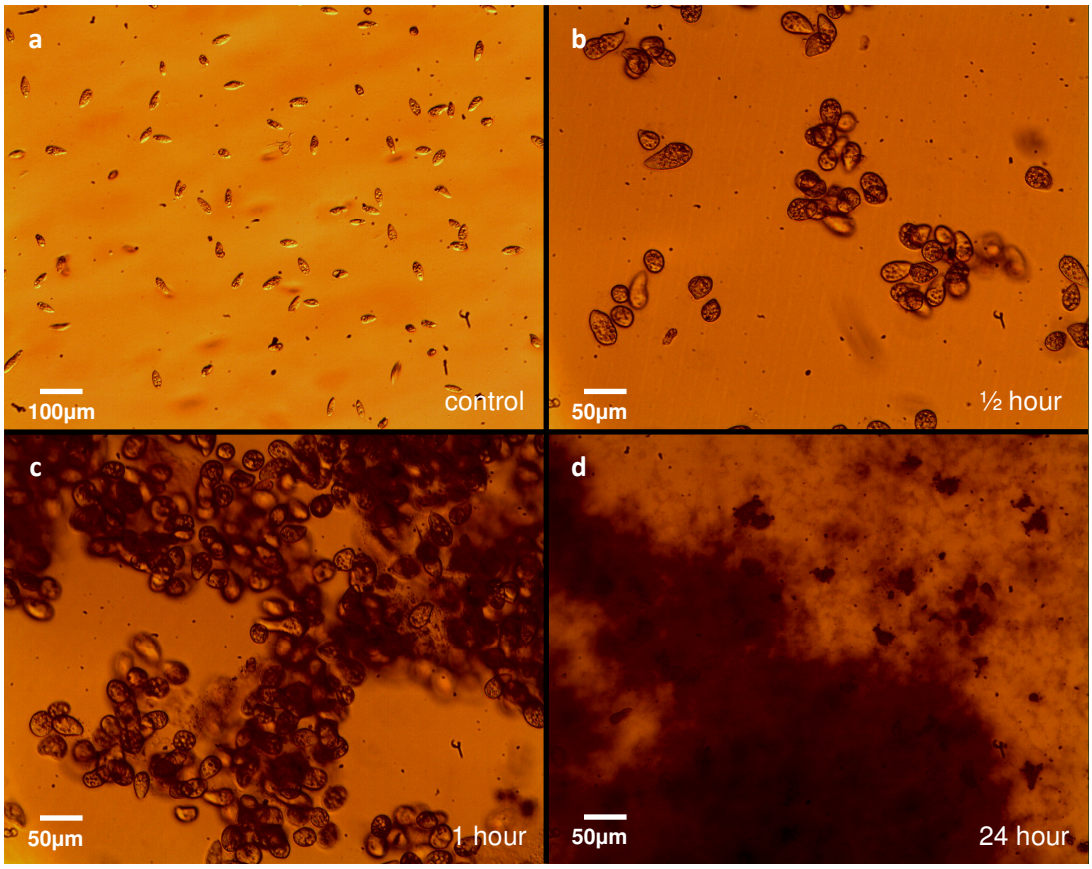


Figure **4** Phase contrast micrographs of *T. thermophila* after addition of SWNTs. **a – d**, Still images of control cultures (**a**) and 0.5 h (**b**), 1 h (**c**), 24 h (**d**) after addition of 11.9 µg.ml^{-1} SWNTs. Dueto the high mobility of the control cells, image **a** was taken at a lower magnification. Scale bar for **a**, 100 um; scale bars for **b – d**, 50 µm.

When SWNT-treated cultures were examined more closely by confocal microscopy at cellular level, five interactions between SWNTs and *Tetrahymena* were seen. First, a faint matrix was seen to surround some individual cells shortly after SWNT exposure (Fig. 5a, white arrows). These resemble the capsules shed upon exposure of *Tetrahymena* to Alcian blue[35]. With Alcian blue, the capsular material originates from the discharge of mucocysts located just beneath the cell surface, and cells slow and aggregate, but eventually swim away from capsules.[4, 36] Therefore, the induction of capsule formation could be responsible for the decreased motility and early aggregation of *Tetrahymena* with SWNTs. Thesimilarity of the phenomena induced by SWNT and Alcian blue will require further investigation, but structurally the inducers are very different. For long-term exposures at high SWNT concentrations, continued stimulation of mucocyst discharge might have led to excessive loss of membrane and cell death.

Second, *Tetrahymena* internalized the SWNTs. A comparison of *Tetrahymena* cultures (no SWNTs) and exposed to SWNTs clearly demonstrated the internalization of SWNTs by the ciliate and the accumulation of SWNTs inside. When *Tetrahymena* were examined ½ hour after being fed *E. coli*-gfp, but no SWNTs, as the bacteria were fluorescent green, ingested *E. coli*-gfp were visible within the cytoplasm as large fluorescent (green or yellow) spheres, which are interpreted to be clumps of bacteria within phagosomes or food vacuoles (Fig. 5a inset, 5b). Control ciliates were able to intake large quantities of *E. coli-gfp* within ½ hour and effectively digest and destroy the fluorescent bacteria within 4 hours (Fig.5d). After incubation of *Tetrahymena* with SWNTs solution for roughly 2 h, dark structures appeared within ciliates that were interpreted to be internalized SWNTs (Fig. 5a). The internalized SWNTs appeared as dark aggregates of a similar size and shape as the fluorescent bacteria clumps, which is an indication like bacteria; SWNTs were engulfed or phagocytized into food vacuoles. This observation was supported by comparison of *Tetrahymena* being fed *E. coli*-gfp,

but no SWNTs and *Tetrahymena* in SWNTs solution (Fig. 5a inset, 5a). Exposure of *Tetrahymena* to *E. coli*-gfp and SWNTs led to vacuoles that contained both SWNTs and the bacteria (Fig. 5b).

Third, *Tetrahymena* egested SWNT. Under continuous microscopic observation, some ciliates (after a few hours in SWNTs solution) excreted SWNT globules through their posterior ends while swimming around with no obvious signs of toxicity. After 4 hours, the control ciliates were able to effectively digest and destroy the large quantities of fluorescent bacteria that were taken in (Fig. 5d). In contrast, SWNTs-treated *Tetrahymena* excreted SWNT aggregates, along with fluorescent spheres, which were observed externally (Fig. 5e). The latter are interpreted to be patches of partially digested or undigested *E. coli*-gfp. SWNTs egestion was observed with (Fig. 5e) or without (Fig. 5a) the presence of *E. coli*, suggesting that this behavior is triggered solely by SWNTs. Others have previously observed the food vacuole contents of *Tetrahymena* being egested from the cytoproct in defecation balls[37,38,39]. For example, India ink was egested as carbon-containing faecal pellets without membranes[36]. The possible egestion of SWNT aggregates raises the further possibility that these aggregates might be ingested again.

Fourth, SWNTs caused a dark matrix to build up in cultures treated for 24 h or more with nanotubes (Figs 4d, 5f). An SEM image of a sample taken from the dark matrix shows heavily coated SWNT amorphous tangles (Fig. d). Multiple processes likely contributed to this. Two of these are the apparent induction by SWNTs of capsule secretion, and the egestion of SWNT aggregates (Fig. 5e). Capsular material might provide mats to which the egested SWNT aggregates stick, as well as SWNTs that had not been internalized. Also, over the long term some ciliates appear to die and lyse, and the lysate could be added to the matrix or provide an additional matrix framework.

Finally, two observations suggest that SWNTs could be inhibiting bacterivory. In particular, when *E. coli*-gfp was presented together with high SWNT concentrations (>7.3 $\mu g.ml^{-1}$), fewer ciliates were seen to have incorporated *E. coli*-gfp (Fig. 5), suggesting that phagocytosis was impaired. Also, at a concentration of 11.9 $\mu g\ ml^{-1}$, more round fluorescent aggregates were seen outside the ciliates than in control cultures (Fig. 5e, 5f). These might be egested digestion remnants or undigested viable bacteria, which would suggest that SWNTs interfered with the digestion process. The egested SWNTs and bacteria appeared to be contained inside vesicle structures surrounded by a membrane. *Tetrahymena* have been shown to release viable bacteria within vesicles.[40] Alternatively, the fluorescent aggregates might have been individual bacteria that aggregated as a result of matrix build-up.

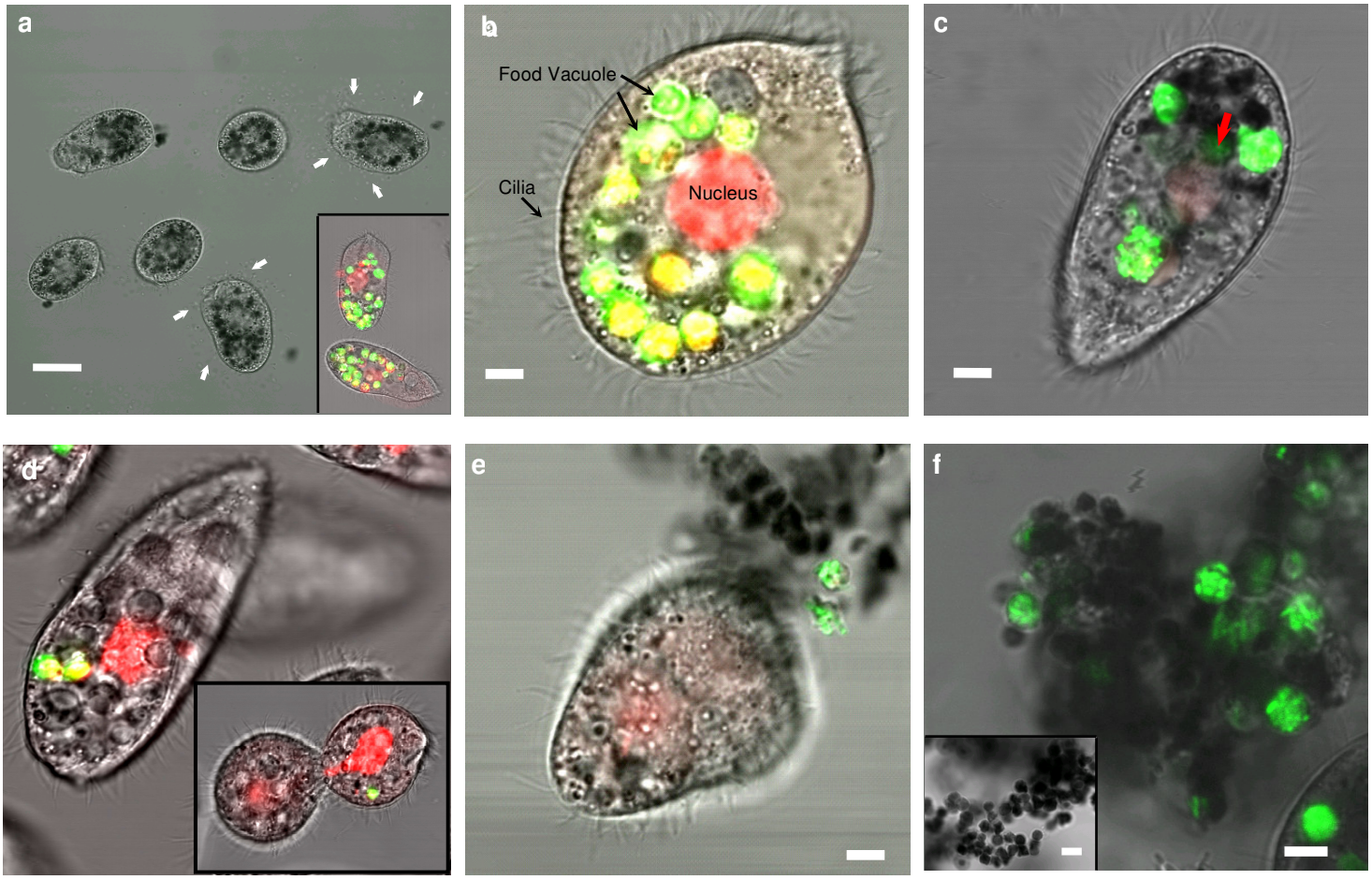


Figure **5** Confocal and white light images of *T. thermophila*. **a**, ingestion of SWNTs (black granules) stimulated the release of mucus-like substance (white arrow). Internalized SWNTs are then egested as aggregates. Inset shows ingestion of *E. coli*-gfp alone (green-yellow). **b**, Control cells ½ hour after co-culture showing *E. coli*-gfp in food vacuoles and DRAQ5 stained nuclei (red). **c**, Ingestion of SWNTs and *E. coli*-gfp inside the same (red arrow) or different food vacuoles. **d**, Control cells remain healthy and could still proliferate by going under cellular division shown in inset. **e,f**, *T. thermophila* egesting SWNT aggregates and remnant or viable *E. coli*-gfp after 4 h (**e**) and 24 h (**f**). Scale bar for **a**, 20 μm; scale bar for **b-f**, 5 μm.

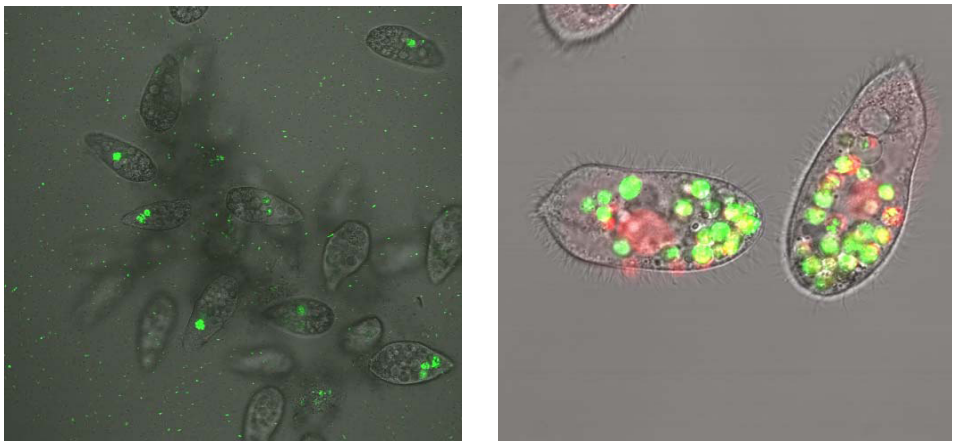


Figure **6** Confocal fluorescence images of *Tetrahymena* ½ hour after feeding *E. coli-gfp*. This set of images demonstrates that fewer ciliates were seen to incorporate *E. coli*-gfp when *E. coli*-gfp were presented together with high concentrations of SWNT (11.9 $\mu g.ml^{-1}$). The images were taken with the same *Tetrahymena* and *E. coli*-gfp cell densities. There are much less fluorescent food vacuoles shown inside *Tetrahymena* with SWNT (**a**) compared to the control (**b**). **a**, Also shows abundant *E. coli*-gfp in culture (green fluorescent specks). The control ciliates were also stained by DRAQ5 (red).

3.3 Effect of Single-Walled Carbon Nanotubes on *Escherichia Coli* Viability

To investigate further the egested vesicles, CellMask Deep Red fluorescent dye was used to stain the vesicles. In control cultures, vesicles stained by the red fluorescent dye, were seen almost exclusively inside the ciliates (Fig. 7a). They were approximately the same size as the vesicles that stained green as a result of the engulfment of *E. coli*-gfp (Fig. 7a), hence interpreted to be food vacuoles. A few small red speckles were seen outside the ciliates and are believed to be bits of membrane debris. On the other hand, cultures that had been pre-incubated with SWNT prior to being fed *E. coli*-gfp, many vesicles were seen outside the ciliates (Fig. 7b). These vesicles were also

visualized with red fluorescent dye and had a similar feature to food vacuoles normally inside *Tetrahymena* with a 2.8 – 4.8 μm diameter range. Many of the vesicles were with black granules of SWNT and some also had fluorescent green regions, which are attributed to *E. coli*-gfp. A three dimensional image of a single red fluorescent vesicle reconstructed from a Z-stack of high-resolution confocal images (the middle slice is shown in (Fig. 7c) shows a well-defined membrane (red) enclosed structure with green fluorescent *E. coli*-gfp inside. Surface examination of vesicles by scanning electron microscopy confirmed the continuous membrane enclosure (*E. coli* inside are not visible) with no SWNT or bacteria attached to the outer surface (Fig. 7d). Periodic microscopic inspection shows the ciliates do not appear to lyse and remain intact and during the course of experiment. Therefore, these results suggest that SWNT cause the ciliates to abnormally egest food vacuole-sized vesicles that can contain live *E. coli*-gfp.

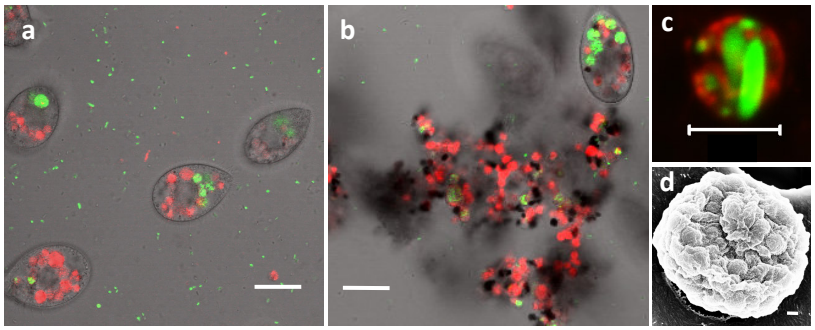


Figure **7** Confocal and SEM images of SWNT stimulated egestion of membrane enclosed vesicle structures. **a**, Control cells. **b**, SWNT-treated cultures show vesicles (red & green) inside and outside *Tetrahymena*. **c**, A single vesicle reveals two intact pET-gfp (green) enclosed by a membrane (red). **d**, Surface of a vesicle. Scale bars for **a-b**, 25 μm; **c**, 5 μm and **d**, 200 nm.

Viability and proliferation capability of *E. coli*-gfp egested in vesicles from SWNT-fed were further confirmed by monitoring the change in fluorescence of isolated vesicles with two different fluorescence based studies. Vesicles isolated from SWNT-fed ciliate cultures were seeded on a TSA plate and incubated at 37 ºC were examined with a fluorescence microscope at times 0 h , 12 h and 24 h. At 0 h, which corresponds to immediate seeding, the *E. coli*-gfp were seen as clusters within well defined spherical boundaries (Fig. 8a). After 12 h some bacteria appeared outside these spherical boundaries or vesicles (Fig. 8b). This observation is attributed to excessive bacterial proliferation within vesicles that might have caused some vesicles to break and release individual or small groups of bacteria. After additional 12 h, the *E. coli*-gfp appeared as a galaxy of fluorescent particles (Fig. 8c), because those bacteria that were free from the vesicles were able to spread and grow over the agar surface.

This sequence is supported by the findings of the second fluorescence study. Once again vesicles isolated from SWNT-fed ciliate cultures were incubated in wells of 96-well plates in TSB at 37 ºC were examined with a fluorescence plate reader. Vesicle free *E. coli*-gfp were also incubated in other wells as control for sake of comparison. When RFUs, which are linearly correlated with the number of *E. coli*-gfp[10], were recorded over a 26 h period and expressed as a percentage of the starting RFUs, the egested and the control bacteria showed different patterns and magnitude of change (Fig. 8d). In the control case, RFUs remained constant for a period (0-6 h lag phase) followed by a phase (6-26 h) in which RFUs increased in approximately a linear manner. By contrast, for vesicle bound bacteria, RFUs remained constant for a longer period (0-10 h) before increasing linearly, but only to about half that observed from control. The delay in the lag phase is due to the confinement of bacteria in vesicles, which might have restricted the growth of bacteria. The second delay is when the bacteria break free of vesicles and grow faster but not as fast as the control bacteria, which could

be from not all the bacteria breaking free of vesicles and possibly some bacteria dying as a result of having been partially digested by the ciliates.

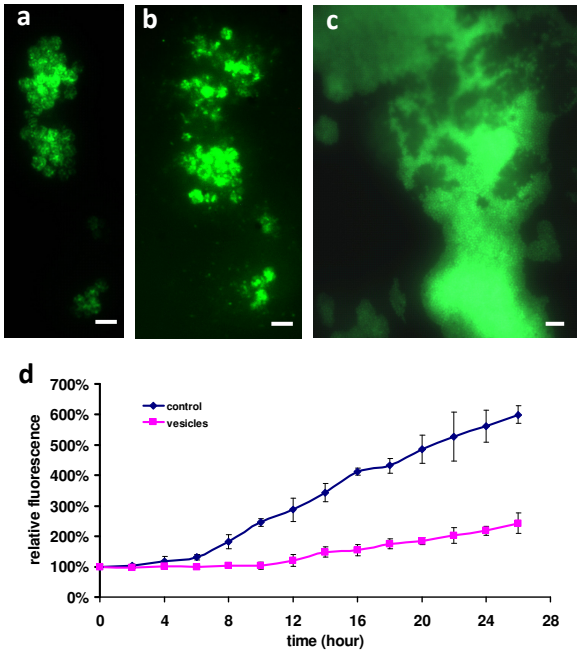


Figure **8** pET-gfp inside egested vesicles are viable and able to proliferate. **a-c**, Fluorescence images of vesicles right after seeded on agar plate (**a**) 12 h after (**b**) 24 h after (**c**). Scale bars for **a-c**, 20 μm. **d**, Proliferation assay on free (control) and vesicle-enclosed pET-gfp.

Confinement of bacteria in vesicles creates a potential survival advantage over those bacteria that remain free in an aquatic environment. The possible protection of bacteria egested in vesicles from SWNT-fed ciliates against killing by antibiotics and disinfectants was assessed with the LIVE/DEAD Baclight bacterial viability kit. The kit consisted of two stains, SYTO 9 (green) and Propidium Iodide (PI, red). In order to avoid overlapping of fluorescence of *E. coli*-gfp with the green stain of the kit, a non-gfp expressing *E. coli* strain (B63) was tested using the same protocols. Chloramphenicol is an antibiotic that kills bacteria indirectly by inhibiting peptide bond formation in

ribosome. Prior to 50 μg.ml^{-1} chloramphenicol treatment, the majority of free *E. coli* and *E. coli* contained in vesicles were stained green by SYTO 9 (Fig. 9a, c) and lacked red fluorescence from PI (Fig. 9e, g), indicating that they were alive with intact cell membranes. After 12 h exposure to the antibiotic, chloramphenicol, most free *E. coli* stained strongly red due to PI (Fig. 9f), which indicates the cells were dead. In contrast, *E. coli* in egested vesicles were green and thus alive (Fig. 9f). Similarly, free *E. coli* and egested *E. coli* showed different response when they were exposed to disinfectant; egested *E. coli* survived 0.25% glutaraldehyde better than free *E. coli*. Despite differences in mechanisms of killing, the vesicles offered protection for at least 12 h against both compounds.

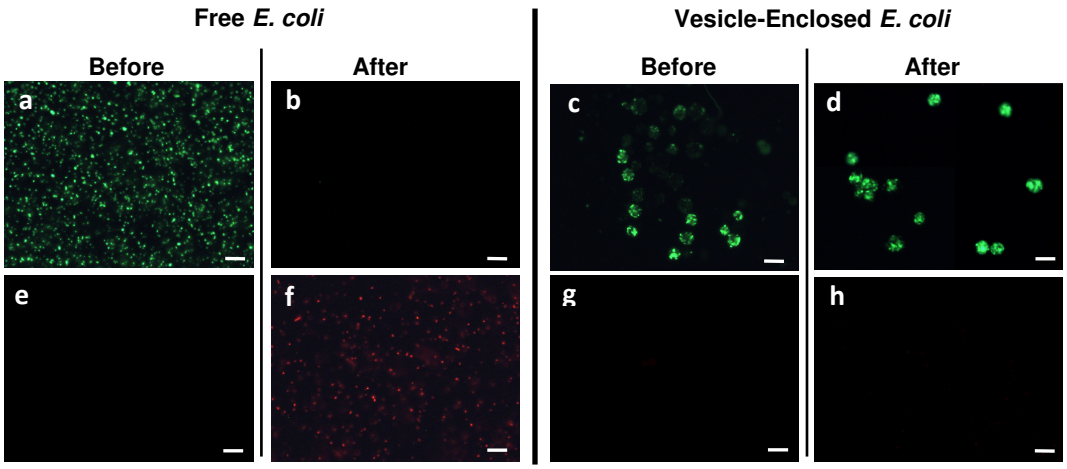


Figure **9** Images of *E. coli* cultures before and after antibiotic treatment show enhanced survival of *E. coli* (non-gfp) in vesicles egested by SWNT-treated *Tetrahymena*. **a-d**, Images showing live cells stained by green fluorescent SYTO 9. **e-h**, Images of dead cells stained by red fluorescent PI. Scale bars **a-h**, 10 μm.

The duration of protection was evaluated by following over 24 h changes in SYTO 9 staining with a fluorescence plate reader as RFUs (Fig. 10). RFUs of control bacteria declined steadily after

adding chloramphenicol and rapidly after glutaraldehyde treatment, whereas, for egested bacteria, a rapid but small drop in RFUs was seen in the first few hours, for approximately next 8 hours a relatively constant plateau period and ultimately slow decline with chloramphenicol and rapid one with glutaraldhyde. The initial drop is attributed to the killing of a small number of free bacteria presented among the egested vesicles and the remaining constant RFUs suggest the bacteria within vesicles are being protected. Furthermore, microscopic examination during the plateau period revealed many intact vesicles but upon longer treatment these were disrupted, indicating that integrity of vesicle membrane is necessary to the protection of bacteria against antimicrobial treatments. This interpretation is further supported by the work of Brand et al, who found that *Salmonella enteria* released from *Tetrahymena* inside vesicles were protected against low concentrations of calcium hypochlorite, a common disinfectant.

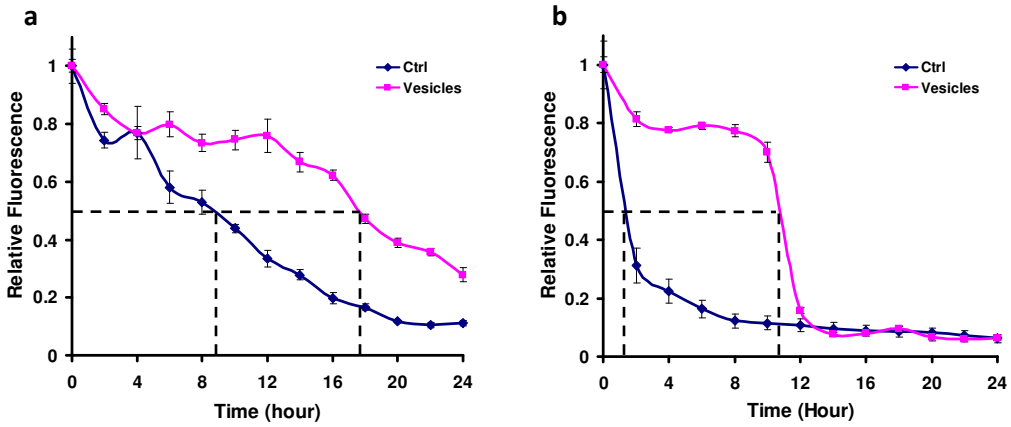


Figure **10** Enhanced survival of egest vesicle-enclosed *E. coli* against antibiotic and disinfectant. Fluorescent assay detects the gradual disappearance of green fluorescence (STYO 9), which is proportional to live bacteria population after chloraphenicol (**a**) and glutaraldehyde (**b**) treatment over a period of 24 hours. The dashed lines indicate 50% fatality.

3.4 Effect of Single-Walled Carbon Nanotubes on *Tetrahymena Thermophila* Bacterivory

Fluorescence based ciliate bacterivory (CB) assay was used to further investigate the impact of SWNT on CB, which is the ability to ingest and digest bacteria, even before affecting ciliate viability. It is known that *Tetrahymena* ingest bacteria by phagocytosis and sequester within food vacuoles or phagosomes[32, 36]. Eventually, the phagosomes fuse with the cytoproct at the posterior end of the ciliate, releasing their residual contents. The entire process occurs over a period of 1-2 hours at 30°C[41]. The CB assay, which is newly established by Bols group, were able to monitor this dynamic process in real-time. The assay was done using multi-well plates and the fluorescence level of each well, containing a co-culture of *Tetrahymena* and *E. coli-gfp*, was measured in relative fluorescent unit (RFU) by scanning the plates with a multi-titre plate fluorimeter (Fig. 11a). Since the fluorescence of *E. coli-gfp* engulfed into food vacuoles of *Tetrahymena* would be destroyed by subsequent acidification and activation of digestion, the capacity of *Tetrahymena* to reduce fluorescence over time is a direct measure of phagocytosis or bacterivory by the ciliate. This correlation is illustrated in figure 11a & 11b with three corresponding points labeled at 0, 1, and 4 hours. The cell densities of *Tetrahymena* (5×10^5 cells ml^{-1}) and *E. coli-gfp* (5×10^8 cfu ml^{-1}) were optimized and maintained the same in all CB assays. All assays were done in Osterhout's solution with *E. coli-gfp* as the sole food source for the ciliate.

The fluorescence level from control (no SWNT) wells with healthy *Tetrahymena* declined rapidly over 2 h due to the engulfment and digestion of bacteria by *Tetrahymena* (Fig. 11b). When different concentrations of SWNTs were added concurrently with *E. coli-gfp* into the *Tetrahymena* culture (procedure ***1***), bacterivory was impeded in a dose-dependent manner (Fig. 11b). We observed a clear transition from nearly no effect at low SWNT concentrations (0.9 and 1.8 $\mu g.ml^{-1}$), to obvious slowing down (3.6 $\mu g.ml^{-1}$), and to complete impairment at high SWNT concentrations (7.2 and 14.6

$\mu g.ml^{-1}$). To make sure this result is the true representation of SWNT's impact on bacterivory, control experiments were carried out to rule out 1) the possible effect of SWNTs on bacterial viability and 2) the possible interference of SWNTs on fluorescence. With *E. coli*-gfp and SWNTs only (without *Tetrahymena*), the fluorescence level or bacterial count from each well, with or without SWNTs, remained roughly the same over the monitored period of 8 hours (Fig. 11c). *E. coli-gfp* collected after 4 and 24 hours of treatment in a SWNT solution (7.8 $\mu g.ml^{-1}$ in Osterhout's) showed normal growth in our colony forming assay (Fig. 11d). This result was expected since at the cellular level, unlike *Tetrahymena* that have highly developed processes for the cellular internalization of particles (endocytosis and phagocytosis), *E. coli* do not have mechanisms for the bulk transport of suspended particles across their cell walls. Therefore, in general, prokaryotes in comparison to eukaryotes will be largely protected against the uptake of many types of nanomaterials. Although nanotubes did not appear to have any inhibitory effect on the *E.coli* growth, but in presence of nanotubes they showed change in morphology that included elongation (Fig. 13), which is consistent with work of Raja et al reference. It is known that similar morphological changes happen in *E. coli* in response to extreme temperature, pressure, chemical agents and quantum dots. Therefore, lowered RFU readings from wells with SWNTs were simply due to optical absorption by SWNTs, which should not alter the kinetics of RFU reduction caused by ciliate bacterivory. The almost constant RFU readings for each SWNT concentration also indicate that SWNT did not stimulate or depress GFP expression over time. Expressing CB assay data relative to starting RFU for each SWNT concentration corrects the interference by SWNT on fluorescence readings and also the small variations in cell densities.

The CB assay was conducted with a slightly different protocol this time, where SWNTs were added to *Tetrahymena* culture 4 hours prior to *E.coli-gfp (protocol **2**)*. Interestingly, although the result demonstrated similar dose-dependent impact of SWNTs on bacterivory (i.e. curves with tails showing slower RFU reduction); a distinctive initial stimulation of RFUs was observed for all concentrations of SWNT, except the control (Fig. 12). The rate of RFU rising was also concentration dependent. Plate counts of *E. coli*-gfp at 0 and 1 hour are almost the same, suggesting that the rise in RFUs is not due to bacterial proliferation. A similar trend was present in all type ***2*** CB assays (appendices).

With these two different experimental procedures (***1*** & ***2***), CB assays likely detected the impact of SWNT at different stages. When using ***1***, SWNT and bacteria were added concurrently and the ingestion of bacteria appeared to be partially or completely blocked depending on SWNT concentration. We suspect that, in the case of ***2***, our assay monitored the gradual recovery of *Tetrahymena*. The initial stimulation of RFUs was likely due to removal of SWNTs partially from solution phase, and the rate is an indication of the rate of recovery which is slower at higher SWNT concentrations. At later hours, bacterivory was restored and the destruction of *E. coli-gfp* became more dominant and the reduction of RFUs was then closely correlated to ciliate bacterivory. At this point, is not clear the mechanisms behind SWNT removal and why it's not observed in protocol ***1***. According to our confocal images excretion of SWNT aggregates by *Tetrahymena* could be a reason. Since SWNT affected both ingestion and digestion of bacteria by *Tetrahymena*, bacterivory might only be restored after SWNTs were egested.

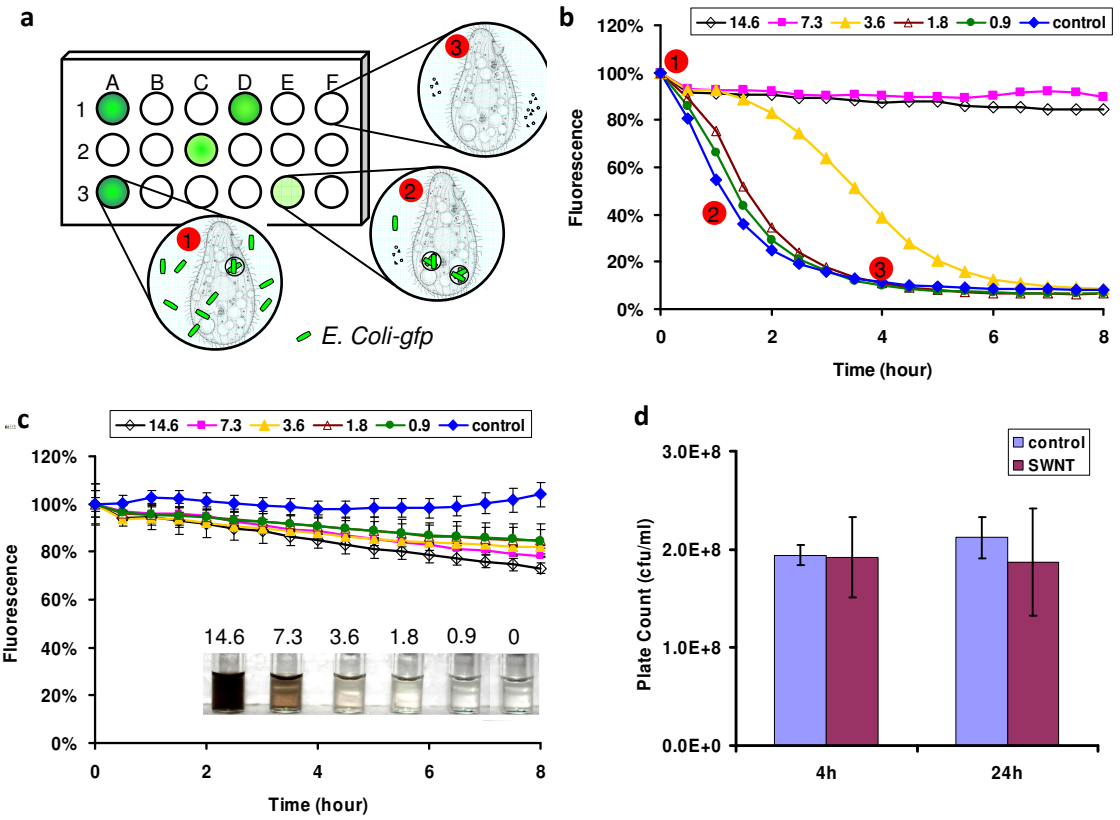


Figure **11** Effect of SWNTs on bacterivory of *T. thermophila*. **a**, CB assay detects the gradual disappearance of fluorescence upon exposure (1), ingestion (2) and eventual destruction (3) of ingested *E. coli*-gfp by *T. thermophila*. **b**, Various concentrations of SWNTs (final concentration in μg. ml^{-1}) were added concurrently with *E. coli*-gfp to microwells containing *T. thermophila*. High concentrations of SWNTs (7.3 and 14.6 $\mu g.ml^{-1}$) blocked bacterivory. **c**, CB assay on *E. coli*-gfp alone (no *T. thermophila)* shows SWNTs have little effect on *E. coli*-gfp viability over time. Fluorescence is expressed as a percentage of starting RFU values. Inset shows SWNT solutions. **d**, Plate counts from a colony forming assay of *E. coli*-gfp with and without SWNTs show no difference.

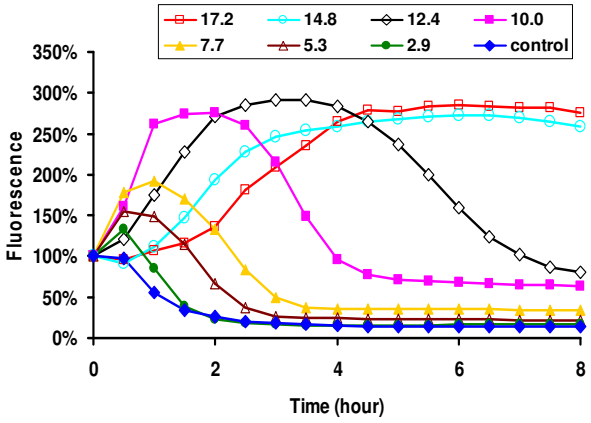


Figure **12** CB assay result from *Tetrahymena* pre-treated with various concentrations (μg.ml^{-1}) of SWNT solutions for 4 hours prior to adding *E. coli*-gfp.

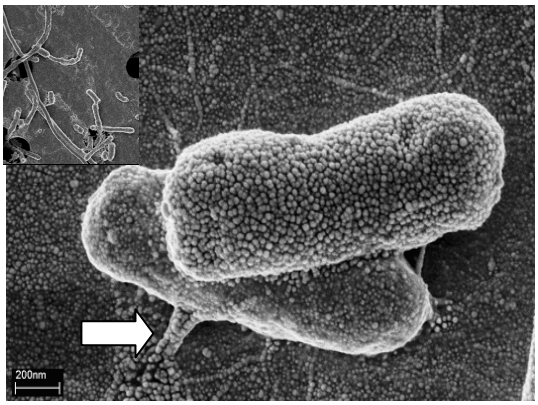


Figure **13** SEM micrograph of *E. coli*-gfp pre-incubated with SWNTs. Inset shows bacterial elongation.

3.5 Effect of Single-Walled Carbon Nanotubes on *Tetrahymena Thermophila* Further Assessed by Common Cell Viability Indicatory Dyes

Studies by Casey et al[42], Davoren et al[43], Hurt et al[44] and Worle-Knirsch et al[45] strongly suggest that in order to assess the cytotoxicity of carbon nanomaterials at least two or more independent test systems are required. The SWNTs were found to interfere with a number of the dyes used in cytotoxicity assessment. Davoren group concluded that among the multiple cytotoxicity assays they used, the Alamar Blue (AB) assay was found the most sensitive and reproducible. Furthermore, removal of SWNTs from the tested medium prior to addition of dyes makes it possible to employ the assays with fewer complications. Cytotoxicity of SWNTs was further evaluated based on cell viability indicatory dyes, AB and CFDA. AB is a commercial preparation of the dye resazurin and providesa very simple and versatile way of measuring cell proliferation and cytotoxicity. AB is nontoxic to cells and does not necessitate killing the cells to obtain measurements. There is a direct correlation between the reduction of AB in the growth media and the quantity/proliferation of living organisms; resazurin is reduced to a fluorescent form resorufin by viable cells and an impairment of cellular metabolism will diminish the reduction. Another convenient, rapid and inexpensive methodology to evaluate the viability of the ciliates CFDA. Carboxyfluorescein diacetate acetoxymehtyl ester diffuse into cells and is converted by the nonspecific esterases of living cells from a nonpolar, nonfluorescent dye into a polar, fluorescent dye (5-carboxyfluorescein, CF), which diffuses out of cells slowly. A decline in fluorescence readings is interpreted as a loss of plasma membrane integrity. SWNTs induced a dose dependent lethal and sublethal toxicity when introduced to *Tetrahymena* (Fig. 14). Also similar to the findings from the above investigations, AB and CFDA reported different levels of toxicity, possibly due to interaction of SWNTs with the organic indicatory dyes.

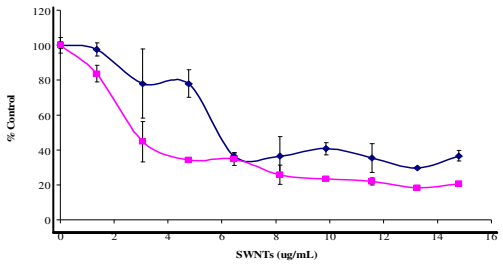


Figure **14** Viability and functionality of the ciliate *T. thermophila* upon exposure to SWNTs for 4 h in Osterhout's buffer. Cell viability and functionality was measured with fluorescent indicator dyes: AB (blue) and CFDA-AM (pink). Results are expressed as a percentage of the readings in control wells exposed to Osterhout's buffer alone. The data points with error bars represent the mean and standard deviation of 3 wells per treatment.

CHAPTER 4: CONCLUSIONS AND INTO THE FUTURE

The toxicity of carbon nanotubes is the subject of ongoing debate. This study using *Tetrahymena thermophila* shows that they may be safe within a certain concentration, but a more rigorous set of studies with different types of carbon nanotubes is necessary. SWNTs are shown capable of entering the ciliated protozoan, *Tetrahymena thermophila*. *Tetrahymena* ingested SWNTs and bacteria with no apparent discrimination. Impact of SWNT on ciliate bacterivory, but not cell viability, was evident at fairly low concentrations (3.6 $\mu g.ml^{-1}$ from CB assays and 1.6 $\mu g.ml^{-1}$ from phase contrast optical microscopy). *Tetrahymena* immobilization and recovery were observed first after SWNT exposure. *Tetrahymena* is known to secrete mucous and form a capsule in response to mechanical and chemical stimuli[46]. A matrix generated by *Tetrahymena* aggregates was observed which consists of obvious SWNT aggregates and possibly other excudates such as mucous, DNA, proteins, and membrane fragments. At high concentrations, there was obvious loss of cell viability. Immobilization and membrane interference by internalized SWNTs are suspected to be the toxicity mechanisms.

Furthermore, SWNT stimulated*Tetrahymena* to abnormally egest viable bacteria inside membrane protected structures, which enhanced bacterial survival during antimicrobial treatments, bacteriostatic or bacteriocidal. The exact cellular mechanisms leading to SWNT-induced vesicle egestion awaits further investigation. One possibility is that the internalization of SWNT disrupts vesicle trafficiking within the cell so that engulfed bacteria are transported out of the cell in vesicles rather than being sent to fuse with primary lysosmes for digestion. Abnormal egestion of food vacuoles by ciliates has been reported in the literature. Direct interactions between *E. coli* and SWNT are believed to be minimal, except possible attachment of *E. coli* to the SWNT aggregates expelled by *Tetrahymena* which normally precipitate to the bottom of the culture dish.

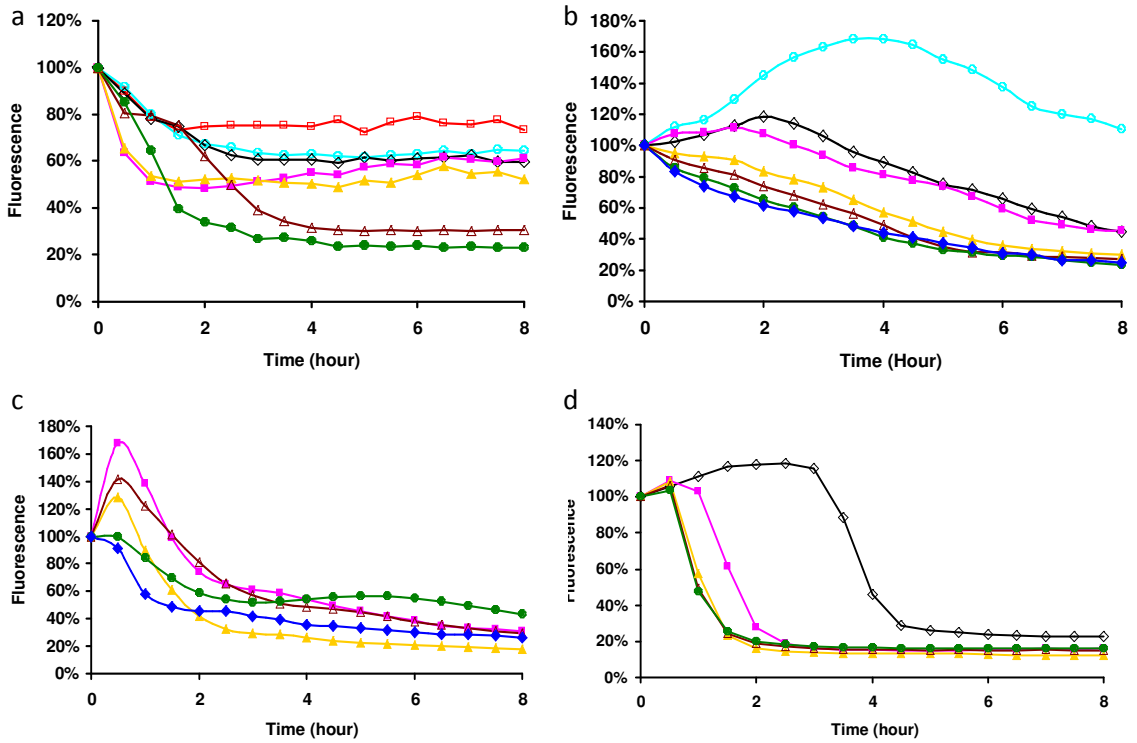
SWNT internalization by *Tetrahymena* and SWNT inhibition of ciliate bacterivory have several ecotoxicological implications. Internalization within ciliates followed by consumption of the ciliates by multicellular animals could be a route for SWNT to move up food chains. Additionally, the ciliates appeared to contribute to SWNT aggregation through exudates and/or the egestion of SWNT clumps. As aggregates, the SWNT possibly can be considered as another form of detritus, which is dead particulate organic matter that contributes to food webs by providing microhabitats for colonizing bacteria[47]. SWNT may impact microbial ecosystems multi-fold. First, the inhibition of ciliate bacterivory could lead to stimulated bacterial population. Second, the bacteria aggregates (possibly enclosed in a membrane) excreted by SWNT-exposed *Tetrahymena* may enhance bacteria survival when exposed to inhibiting compounds and other types of stresses[48]. Third, an underestimation of actual pathogen population during surveys or studies could be introduced by the packaging of bacteria as high density clusters.

Further investigations are needed to better understand the internalization routes of SWNT and the mechanisms leading to *Tetrahymena* immobilization/aggregation. Both soluble and insoluble SWNT could contribute to the microscopically visible aggregates within the *Tetrahymena* cytoplasm. Soluble SWNT may internalize through macropinocytosis with vesicles approximately 1 μm in diameter[49,50,51], micropinocytosis with vesicles less than 200 nm in diameter [32,50,52] , endocytosis[53], and also simple penetration of cell membrane without the use of vesicles. SWNT aggregates greater than 0.5 μm and SWNTs attached to bacteria surfaces could be internalized by phagocytosis. In the future TEM can be done to distinguish between these possibilities. *Tetrahymena* has long been known to phagocytosis inert particles[36], and interestingly *Tetrahymena* showed less discrimination towards India ink (carbon pigment) than to colloidal gold. Whether ciliates will be generally non-specific to carbon nanoparticles remains to be studied. The overall effect of SWNTs, and other engineered nanomaterials in general, on environmental ecology remains to be a field largely unexplored.

Whether *Tetrahymena* or grazing protists will show similar susceptibility to other classes of nanomaterials is unknown. The fact that common biological assays can be used should make comparisons easier.

APPENDICIES

Additional CB assay trials consistently demonstrated 1) the dose-dependent effect of SWNTs on bacterivory by *Tetrahymena*, 2) the initial stimulation of RFUs when *Tetrahymena* were incubated in SWNT solutions 4 hours prior to adding bacteria, and 3) the more pronounced impact at lower concentrations when SWNTs and bacteria were added concurrently.



CB assay results from multiple trials of adding SWNTs either concurrently with (a, procedure ***1***) or 4 hours prior to (b,c,d, procedure ***2***) adding bacteria (*E. coli-gfp*). The curves are color coded from control (♦) to higher concentrations in the order of (●), (Δ), (▲), (■), (◊), (○), (□). The typical range of concentrations is from 0 to 15 μg.ml^{-1}. Exact concentration of each curve in the figures varies slightly.

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