Carbon nanotube compared with carbon black: effects on bacterial survival against grazing by ciliates and antimicrobial treatments

Tiffany S. Y. Chan¹†, Fatima Nasser¹†, Christine H. St-Denis², Himadri S. Mandal¹, Parnian Ghafari¹, Nacima Hadjout-Rabi², Niels C. Bols², & Xiaowu (Shirley) Tang¹

¹Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada and ²Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Abstract
The ingestion and digestion of Escherichia coli by the ciliated protozoan, Tetrahymena thermophila, was investigated after an initial exposure to either water-soluble single-walled carbon nanotubes (SWNT) or to carbon black (CB). Both SWNT and CB were internalised and visible in food vacuoles of ciliates. When presented with E. coli expressing green-fluorescent protein (GFP), these ciliates internalised bacteria as well. However, ciliates that had first internalised SWNT but not CB subsequently externalised or egested vesicle-like structures with fluorescent bacteria inside. These egested bacteria were viable and less susceptible than planktonic E. coli to killing either by the antibiotic, chloramphenicol or the disinfectant, glutaraldehyde. These results suggest that SWNT can alter the intracellular trafficking of vesicles within ciliates, leading to bacterial prey being packaged externally and protected for a time from environmental killing, which could have implications for sewage treatment and for public health.

Keywords: Carbon nanotube, carbon black, protozoa, bacterial survival, toxicity, ecotoxicology

Introduction
Technological development and applications of carbon nanotubes (CNT) is set for explosive growth. CNT have been incorporated into a wide range of commercial products and many others are in the stage of fast industrialisation. It is crucial to better understand the fate and toxicity of CNT in various environmental compartments, air, water and soil, before the production and disposal of CNT hikes up significantly. So far, the majority of toxicity studies are on mammalian cell lines and small animals (Johnston et al. 2010; Tsuji et al. 2006; Helland et al. 2007). Recent findings show that airborne CNT are more toxic than other carbon allotropes such as carbon black (CB), quartz and graphite, which has raised the concern that CNT might be a serious occupational and public health hazard (Schulte et al. 2010; Lam et al., 2004, 2006). The toxicity of water-soluble CNT is generally much less pronounced (Johnston et al. 2010). However, CNT is a class of highly biodurable nanomaterials, which suggest long-term accumulation and interactions with diverse organisms in the environment. Studies on aquatic organisms have been reported showing that both soluble and insoluble CNT can be consumed by organisms and move up the food chain to higher trophic levels (Oberdorster et al. 2005; Templeton et al. 2006; Smith et al. 2007; Baun et al. 2008; Kang et al. 2009).

However, the ecological effects of CNT are still poorly understood. More research is needed to better understand the impact of CNT on a microscale involving simple interactions between a few microorganisms as well as on a macroscale involving ecosystems (Oberdorster et al. 2005). An example of a simple interaction would be the one between bacteria, such as Escherichia coli, and ciliates, such as Tetrahymena thermophila. T. thermophila belongs to the ecologically important group, the grazing protists, which contribute to the regulation of bacterial population through bacterivory, which is feeding on bacteria via phagocytosis. Previously we found that SWNT at concentrations above 7 µg/ml completely blocked the bacterivory of T. thermophila, whereas at lower concentrations the bacteria and ciliates remained viable and bacterivory did eventually proceed (Ghafari et al. 2008). These lower or sub-lethal SWNT concentrations have been examined further in the current study, where impact on bacterial survival resulted from ciliate exposure to SWNT and bacteria-ciliate interactions were investigated. Surprisingly, the prior exposure to SWNT caused Tetrahymena to expel viable bacteria inside vesicles where they were more resistant to antibacterial treatments, which is an observation with potential ecological and health implications.

DOI: 10.3109/17435390.2011.652205
ISSN: 1743-5390 print / 1743-5404 online
© 2013 Informa UK, Ltd.
Nanotoxicology, May 2013; 7(3):251–258
© 2013 Informa UK, Ltd.
ISSN: 1743-5390 print / 1743-5404 online
DOI: 10.3109/17435390.2011.652205
Nanotoxicology Downloaded from informahealthcare.com by University of Waterloo on 04/10/13

Correspondence: Xiaowu (Shirley) Tang, Department of Chemistry, University of Waterloo, 200 University Ave West, Waterloo, Ontario N2L 3G1, Canada.
Tel: +519 888 4567 ext. 8037. Fax: +519 746 0435. E-mail: tangxw@uwaterloo.ca
†These authors contributed equally to this work.
(Received 8 May 2011; accepted 30 November 2011)
Materials and methods

Microorganisms

*T. thermophila* was maintained routinely as described previously (Orias et al. 2000). Growth was done at room temperature in proteose peptone yeast extract (PPYE), which includes 0.125% dextrose, 0.5% protease peptone and 0.5% yeast extract dissolved in Millipore water. During carbon material exposure and bacteria feeding, Osterhout’s solution was used, which is a saline solution with 5.2 g NaCl, 0.907 g MgCl₂·6H₂O, 0.2 g MgSO₄, 0.115 g KCl, 0.066 g CaCl₂·2H₂O, per 100 ml of distilled water. Both media were autoclaved before usage.

Two *E. coli* strains were used as model bacteria. The first was *E. coli* XL-1, which expressed green-fluorescent protein (gfp), previously transformed with an 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 Macara, Center for Cell Signaling, University of Virginia). The *E. coli* XL-1 (pET-gfp) was maintained on Tryptic Soy Agar (TSA) (Difco, Toronto, ON) plates supplemented with 100 μg/ml ampicillin (AMP) grown overnight at 37°C and then stored at 4°C. In order to obtain fresh cultures, colonies were picked from the plates and restreaked on to fresh TSA + AMP plates to create lawns of bacteria and incubated again at a similar temperature and duration. The lawns of bacteria were harvested by saturating the plates with 10 ml of Osterhout’s solution. The collected cell suspension was centrifuged (3000 × g, 10 min), washed and resuspended to the desired concentration in Osterhout’s solution. The washed cells could be maintained at 4°C for 1 week with no loss in fluorescence or viability. The second *E. coli*, B63 (ATCC 11303E) strain, was non-gfp expressing. The non-fluorescing *E. coli* 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 37°C for 24 h, and 20 ml of Tryptic Soy Broth (TSB) (Difco) were inoculated with several colonies from the plates and incubated at 37°C 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.

Preparation and characterisation of SWNT and CB particles

SWNT powder (HipCo tube, Carbon Nanotechnologies, Inc) was acid oxidised, purified and made water soluble without surfactants. In brief, SWNT purity was confirmed by elemental analysis using energy-dispersive analytical X-ray (EDAX). SWNT concentration and morphology in various media were characterised by UV–Vis spectroscopy (extinction coefficient of 0.0079 mM⁻¹cm⁻¹ at 808 nm) (Kam et al. 2005), atomic force microscopy (AFM) and scanning electron microscopy (SEM). Majority of SWNT in water and Osterhout’s solution were found to be individual with a length less than 500 nm. Small bundles of SWNT in micrometre size were often found in PPYE. Detailed protocol and characterisation results were reported previously (Ghafari et al. 2008).

India ink (Speedball) containing 100% CB pigments at 50 mg/ml concentration was administered/diluted as is into PPYE or Osterhout’s solution. The CB pigments or particles were characterised by SEM (Supplementary Material, Figure S1) to be irregularly shaped and majority of the particles were between 100 nm to 2 μm with small aspect ratios.

*T. thermophila* growth curve

Growth curves of *T. thermophila* were constructed over a period of 96 h. At the beginning of the experiment, 5 ml of *T. thermophila* stock was added to 20 ml of freshly made PPYE medium in each 75-cm² non-tissue culture-treated flask. Stock solutions of SWNT and CB were directly drop diluted into *T. thermophila* cultures. The flasks were kept on an orbital shaker over 4 days. Every 24 h, 500 μl was taken out of each flask, put into a new vial and 500 μl formalin fixative was added to immobilise the ciliates. Cell counts were then taken using a haemocytometer to obtain cell density (cells/ml) of the *T. thermophila* cultures.

Exposure of *Tetrahymena* to SWNT or CB

A concentrated SWNT or CB solution was directly dropped into *Tetrahymena* cultures to achieve a desired final concentration with a typical dilution factor of 1:5. The SWNT concentration (6.8 μg/ml or equivalently 40 nM) was chosen so that there were no short-term (less than 24 h) or long-term (one week) viability effects on *Tetrahymena* judging from ciliate mobility and reproduction. The impact of SWNT at higher concentrations was reported in a previous study. CB concentration of 7 mg/ml was achieved by diluting India ink at 14% v/v, which was chosen so that CB concentration was significantly higher than SWNT concentration (about 1000 times) and yet the cultures were not too dark for optical microscopy. No toxicity was observed up to 50 mg/ml of CB.

All results presented in this report were from 4-h exposure. During exposure, the *Tetrahymena* cultures were maintained at 5 × 10⁵ cells/ml density in Osterhout’s solution. To end exposure, whether SWNT or CB, aliquots of *Tetrahymena* cultures were gently centrifuged (450 g, 10 min) to first separate solution-phase carbon particles in culture medium (supernatant) from ciliates and aggregates (pellet). The pellet was then resuspended in fresh Osterhout’s solution and centrifuged (450 g, 5 min). The centrifuge tube was allowed to sit for 15 min before the supernatant was collected. During this time, aggregates of carbon particles remained in the pellet, while the ciliates swam out of the pellet into the supernatant. This process was usually repeated multiple times to collect free swimming SWNT-fed or CB-fed *Tetrahymena* in supernatant.

*T. thermophila* feeding on bacteria

*E. coli* (either pET-gfp or non-gfp-expressing *E. coli*) were introduced to *Tetrahymena* cultures in Osterhout’s solution, which contains no nutrients. Cell densities were always maintained the same with 5 × 10⁵ cells/ml *Tetrahymena* and 1 × 10⁶ cfu/ml *E. coli*. To study the effects of SWNT and CB on feeding, *Tetrahymena* was pre-exposed to SWNT or CB for 4 h. Then SWNT-fed and CB-fed *Tetrahymena* were collected and re-suspended in...
Osterhout’s solution at desired cell densities prior to adding *E. coli*. With this experiment design, direct interactions between *E. coli* and carbon particles in solutions were minimised. The fate of pET-gfp after ingestion by carbon-fed *Tetrahymena* was monitored by confocal fluorescence microscopy (Zeiss LSM 510). Drops of 0.01% neutral buffered formalin were used to fix cells immediately prior to imaging. CellMask Deep Red plasma membrane fluorescent dye (Invitrogen) was used to identify membranes surrounding food vacuoles inside *Tetrahymena*, and after egestion the excretes, including egested food vacuoles/vesicles and carbon pellets, were isolated and examined by high-resolution confocal imaging and SEM. To isolate the excretes, 1 ml of *Tetrahymena* culture was taken and centrifuged (450 g, 15 min) and was allowed to sit for 20 min allowing ciliates to swim out into the supernatant and be removed. The precipitate was resuspended in Osterhout solution and the above process was repeated four times. The final precipitate was resuspended in 1 ml of Osterhout’s solution, which was visually examined to exclude free ciliates. Very small number of bacteria was often found though attaching to excretes. For counting vesicles and pellets, the 1-ml precipitate solution was mixed with 9-ml isoton and then placed into a Z2 coulter counter. The upper and lower boundaries of the counted structures were set to 1.6 and 4.8 μm respectively, which were the dimensions of a typical vesicle or carbon pellet.

**Bacterial viability and proliferation assay**

The Live/Dead BacLight kit (Invitrogen) was used to evaluate bacterial viability and 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 PI (Abs/Em 490/635 nm). Staining condition was optimised to 1:2 ratio of SYTO 9 and PI in 3:1000 dilution. SYTO 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 (Olympus BX41) and the fluorescence plate reader (Victor V, Perka and Elmer). The intensities of green/red fluorescence are well correlated to live/dead bacteria populations (Stocks 2004).

**Results**

**No direct toxicity effect on Tetrahymena**

The impact of SWNT and CB on *Tetrahymena* viability was investigated first. The growth of *Tetrahymena* in PPYE alone (control), and in PPYE dosed with 6.8 μg/ml SWNT, and in PPYE with CB (7 mg/ml), was monitored over 4 days by counting the cell number every 24 h. Average of two separate trials were plotted in Figure 1A & 1B, along with error bars showing standard deviation. Cultures treated with SWNT and CB both showed slightly increases than the control culture, but otherwise normal growth. Microscopic examination of the cultures at 24 h (Figure 1C & 1D) revealed that ciliates ingested and egested SWNT and CB particles. In both cases dark granules inside ciliates were observed and were interpreted as food vacuoles containing SWNT or CB. The ciliates appeared to be incapable of digesting either SWNT or CB particles because extracellular dark pellets, which are SWNT or CB aggregates egested by *Tetrahymena*, were observed to accumulate in the cultures. The ciliates, nevertheless, remained healthy as judged by the morphology and continued motility. In summary, growth curves together with microscopic examination confirmed that acid-oxidised SWNT at concentrations ≤6.8 μg/ml showed no direct toxicity to *Tetrahymena*. This result is consistent with our previous findings, where the toxicity of SWNT was shown to be concentration dependent, and cell death was not observed at this concentration and lower (Ghafari et al. 2008). It is also consistent with literature reports that CB or India ink has no significant impairment to ciliates and has widely reported as a benign particulate substance that is commonly used for staining food vesicles of protozoa for microscopy.

**Effect on Tetrahymena bacterivory**

Next, we studied the effects of SWNT and CB on the ability of *Tetrahymena* to ingest and digest bacteria, a process commonly termed bacterivory. Through bacterivory, *Tetrahymena* plays an important role in the regulation of microbial populations in freshwater and in wastewater treatments. The use of green fluorescent protein (gfp)-expressing *E. coli* XL-1 (pET-gfp) has permitted us to monitor the bacterivory process by fluorescence, phase contrast and confocal microscopy. To observe the co-localisation of plasma membranes and pET-gfp, a dye (CellMask Deep Red, Invitrogen) was used to stain membranes red in confocal images. Three cultures, *Tetrahymena* (control), *Tetrahymena* pre-exposed to SWNT and *Tetrahymena* pre-exposed to CB, were allowed to feed on pET-gfp over 2 days and confocal fluorescent images were taken at various time points. The conditions for SWNT and CB pre-exposure are described in the Materials and Methods section. The cell densities of *Tetrahymena* and *E. coli* in all three cultures were kept the same. Starting at half-an-hour after feeding, green granules inside *Tetrahymena* were observed, which are interpreted as pET-gfp sequestered inside food vacuoles enclosed by plasma membranes (red), as shown in Figure 2A, which has been observed and suggested previously (Power et al. 2006; Ghafari et al. 2008). Neither SWNT nor CB showed any obvious effect on bacteria intake, since *Tetrahymena* were observed to continually ingest pET-gfp in all three cultures. However, many extracellular fluorescent-red vesicles with the diameters 2.8–4.8 μm, in addition to SWNT or CB pellets, were shown in the SWNT and CB pre-exposed *Tetrahymena* cultures (Figure 2), which were not observed in the control culture (Figure 2A inset). It appeared that SWNT and CB, in the same way, stimulated *Tetrahymena* to excrete an excess number of vesicles with
the size and shape of food vacuoles, which are a normal feature of ciliates and can be seen inside *Tetrahymena* in Figure 2A as green and red structures.

We quantitatively studied the rate of excretion. In a 24-h period, 1 ml of each culture was collected every hour, from which the extracellular vesicles and black pellets were isolated and counted (see Materials and Methods section for the procedure). Plotted in Figure 3 is the average of two separate trials. Error bars, which are ± standard deviation, are shown for every four data points to avoid overcrowding. Up to 18 h, control *Tetrahymena* cultures (without SWNT or CB) were free of extracellular vesicles or pellets. The small count after 18 h is attributed to aggregated debris as confirmed by visual examination of the cultures. In the SWNT- and CB-treated cultures, however, the number of extracellular structures started to increase rapidly at about 2 h. Without *E. coli*,

![Figure 1](image1)

Figure 1. Growth curves of *Tetrahymena* exposed to (A) single-walled carbon nanotubes (SWNT) and (B) carbon black (CB) show no obvious difference compared with *Tetrahymena* in PPYE (control). Optical images of *Tetrahymena* cultures show that the ciliates ingest and egest (C) SWNT and (D) CB in a similar fashion.

![Figure 2](image2)

Figure 2. Single-walled carbon nanotubes (SWNT) and carbon black (CB) stimulated *Tetrahymena* to egest vesicle-like structures. Confocal images revealed a large number of extracellular vesicles in (A) SWNT- and (B) CB-treated cultures, but very few in control cultures (inset).
Figure 3. Counts of excreted vesicle structures and pellets over a 24-h period in Tetrahymena cultures.

dark pellets (with some vesicles) were counted to be about the same, 28 million/ml, at the end of the 24-h period. With E. coli, the total number of vesicles and dark pellets was about 40 million/ml at 24 h in the SWNT pre-exposed cultures. The cell density of Tetrahymena cultures was maintained at $5 \times 10^5$ cells/ml. Therefore, the rate of excretion by Tetrahymena is estimated to be 80 SWNT pellets and 24 vesicles per cell per 24 h. In the same way, we estimated that Tetrahymena excreted 80 CB pellets and 16 vesicles per cell per 24 h. Statistically, the ciliates packed SWNT and CB into pellets at about the same rate. But SWNT stimulated the ciliates to excrete 50% more vesicles than CB.

Compared with CB, SWNT caused Tetrahymena to excrete vesicles not only at a higher rate but also with a strikingly different structure. At high magnification, confocal images of egested vesicles in the SWNT-exposed cultures showed that almost all of them had a fluorescent green centre region enclosed or embedded in red (Figure 4A), whereas those in the CB-exposed cultures were red only (Figure 4B). A three-dimensional image of a single vesicle reconstructed from a Z-stack of high-resolution confocal images (the middle slice is shown in Figure 4A inset) shows a well-defined membrane (red) enclosed structure with green fluorescent pET-gfp inside. The fact that the bacteria still express GFP suggests that they had avoided digestion and were alive. Results with a commercial kit for assaying bacterial viability supported this conclusion and can be seen in Figure 6C & 6D. On the other hand, the egested vesicles in CB-treated cultures (red only) do not contain live bacteria and are believed to consist of decomposed bacteria debris or membranes. Therefore, SWNT impaired the ability of Tetrahymena to digest bacteria, which was not observed even with CB at 1000 times higher weight concentration.

SEM was used to further exam the excreted vesicles in SWNT-fed Tetrahymena cultures. SEM images showed that the vesicles or vesicle-like structures have a distinctively different appearance compared with E. coli self-aggregates (Figure 5A & 5B). Close-up examination of the vesicle surface suggested a continuous but rough membrane enclosure (Figure 5C). Along with the light and confocal microscopy examination, this observation further suggests that Tetrahymena packaged E. coli into vesicle-like structures. These were not simply aggregated E. coli in medium because they appeared only when the ciliates were present.

Indirect effect on bacterial survival against antimicrobial treatments

Viability of egested vesicle-enclosed bacteria by SWNT-fed Tetrahymena and their ability to resist antimicrobial treatments were assessed with LIVE/DEAD Baclight bacterial viability kit, which consists of two stains, SYTO 9 (green) and Propidium Iodide (PI, red). In order to avoid overlapping of fluorescence interfering with the viability kit, a non-gfp-expressing E. coli strain (B63) was used. All experimental procedures and conditions, including SWNT exposure, E. coli feeding and vesicle isolation, were identical to that used for the gfp-expressing strain (XL-1). Free or planktonic E. coli were used as control, in comparison with vesicle-enclosed E. coli excreted by SWNT-fed Tetrahymena. Prior to antibiotic treatment, majority of free E. coli (control) and E. coli contained in vesicles were stained green by SYTO 9 (Figure 6A & 6C) and lacked red fluorescence from PI, indicating that they were alive with intact cell membranes.
hymena cocultures survive antibiotic treatment better than free
Viability was evaluated by enclosed bacteria were dead. By contrast, egested vesicle-red (Figure 6B), which was due to PI and indicates that the
Figure 6. Escherichia coli
After 12-h exposure to the antibiotic, chloramphenicol (50 µg/ml), most free or planktonic E. coli stained strongly red (Figure 6B), which was due to PI and indicates that the bacteria were dead. By contrast, egested vesicle-enclosed E. coli continued to be fluorescent green and thus alive (Figure 6D). Similar results were obtained with a disinfectant: E. coli in vesicles survived 0.25% glutaraldehyde better than free E. coli (images not shown). This result suggested that i) SWNT stimulated Tetrahymena to egest live E. coli inside vesicles and ii) the vesicle enclosure offered protection for E. coli to survive both antibiotic and disinfectant treatments.

The duration of protection was evaluated quantitatively by following over 24-h changes in SYTO 9 staining with a fluorescence plate reader as RFUs (Figure 7), which are linearly correlated to live bacteria population. Glutaraldehyde (0.25%) was able to kill free E. coli rapidly as indicated by the rapid decreasing of RFUs (Figure 7A). For egested bacteria collected in vesicle form from SWNT-fed Tetrahymena cultures, a rapid but small drop in RFUs was seen in the first few hours. After this initial decline, the RFUs remained relatively constant (plateau period) for approximately 10 h, a period during which the vesicle-enclosed bacteria are being protected. Ultimately, the RFUs declined again. Microscopic examination during the plateau period revealed many intact vesicles, but upon longer treatment these were disrupted, indicating that integrity of vesicle enclosure is necessary to the protection of bacteria against antimicrobial treatments. For the vesicles collected from CB-fed Tetrahymena cultures, the profile of RFUs resembles that of free E. coli (Figure 7B). It is consistent with our confocal results that CB stimulated Tetrahymena to egest vesicles containing no viable bacteria. The RFU reading at the beginning was from free E. coli attached to excreta, which could not be removed completely using our vesicle isolation procedure, as observed under an optical microscope. Data plotted in Figure 7 was collected from two separate trials, with at least three replicas of each culture per trial. Error bars show the ± standard deviations in all readings.

Discussion

We compared the effects of two carbon allotropes, SWNT and CB, on a ciliated protozoan T. thermophila, which show two contrasting actions. First, when the current results are considered together with our previous studies (Ghafari et al. 2008), SWNT is established to be much more acutely toxic than CB. Ciliate death was observed at SWNT concentrations beyond 10 µg/ml, while CB up to 7 mg/ml remained non-toxic. Second, at sub-lethal concentrations, which are the focus of this paper, SWNT has a complex action that is not observed with CB. Exposure to SWNT prior to the addition of bacteria causes T. thermophila to release vesicles with viable bacteria enclosed.

Tetrahymena consumed SWNT, CB and E. coli essentially as described in previous studies on the interactions between ciliates and either inert particles or living bacteria. After being internalised by phagocytosis in the cytosome, a portion of the CB, SWNT and E. coli was expelled at the cytoproct. In previous studies, indigestible inert material, such as CB (Elliott & Clemmons 1966) and SWNT (Ghafari et al. 2008) was observed being expelled at the cytoproct. By contrast, ingested bacteria have been observed to have two possible fates. The bacteria can be partially digested inside food vacuoles with the indigestible material being released at the cytoproct (Nilsson 1987) or in some circumstances they can be released alive either inside vesicles (Brandl et al. 2005; Gourabathini et al. 2008;...
bacteria in vesicle-like structures and the enhanced bacterial egestion of vesicle-like structures or SWNT aggregates excreted by SWNT, but not CB, allows bacteria to avoid digestion and be expelled instead is unclear.

The unique observation of this study is that exposure to the nanoparticles before exposure to bacteria appeared to stimulate *Tetrahymena* to release more vesicles with viable bacteria inside of them. The solution-phase SWNT were removed prior to introducing *E. coli*, although the presence of SWNT during *E. coli* feeding would not alter production of vesicle-like structures or *E. coli* growth (Ghafari et al. 2008). This experimental design makes it clear that egestion of bacteria in vesicle-like structures and the enhanced bacterial survival is solely due to the impact of SWNT on *Tetrahymena*. Direct interactions between *E. coli* and SWNT are believed to be minimal, except possible attachment of *E. coli* to the SWNT aggregates excreted by *Tetrahymena*, which normally accumulate at the bottom of the culture vessel. Optical images of the overall bacterivory process suggest that the digestion stage is interrupted, but how the exposure of *Tetrahymena* to SWNT, but not CB, allows bacteria to avoid digestion and be expelled instead is unclear.

Possibly the prior internalisation of so much indigestible SWNT overwhelms the internal membrane trafficking system that is needed to complete digestion and the default mode is to externalise subsequently ingested bacteria without digestion. A recent study with *T. pyriformis* supports this idea (Thurman et al. 2010). When the number of prey bacteria was above six per food vacuole, digestion was less efficient and intact bacterial cells were egested from the ciliate. As well, the packaging of viable bacteria in some cases has been found to be more evident at a high bacterium/ciliate ratio (Berk et al. 2008). However, if it were merely a matter of prior ingestion of inert material, CB also might be expected to stimulate egestion of bacteria but this was not the case. The reason for this difference is currently unexplained but one line of speculation focuses on particle size and autophagy. The most prominent difference between the two kinds of particles is likely the size. Their surfaces would be expected to be similar, with each decorated predominantly with carboxylic acid and hydroxyl functional groups. In a separate study, preliminary results showed that Au nanoparticles triggered *Tetrahymena* to egest viable bacteria (Supplementary Material, Figure S2) similar to SWNT despite their drastically different surface property and morphology, which suggests that size could be a key factor. For mammalian cells, fullerene C60 nanoparticles have been shown recently to stimulate autophagy in a size-dependent manner (Wei et al. 2010; Zhan et al. 2010). Autophagy leads to the formation of autophagosomes that fuse with lysosomes to form autolysosomes. Perhaps SWNT induce autophagy in *Tetrahymena*, leading to fewer lysosomes being available to digest bacteria so viable *E. coli* are released.

Regardless of the mechanism behind the stimulation by SWNT, the release of *E. coli* inside vesicles afforded the bacteria protection against antimicrobial treatments, bacteriostatic or bactericidal, for an extended period. SWNT are thought not to directly protect bacteria and not to influence bacteria growth. Protection is attributed to the vesicles because vesicles were isolated from *Tetrahymena* cultures and resuspended in Osterhout’s solution free of SWNT during antimicrobial treatments. This interpretation is supported by the work of Brandl et al. (2005), who found that *Salmonella enterica* released from *Tetrahymena* inside vesicles were protected against low concentrations of calcium hypochlorite, a common disinfectant. The results have broad implications. By altering the bacteria/ciliate interaction, SWNT have the potential to promote the environmental survival of bacteria, which could impact the microbial ecology of natural and manmade aquatic systems. Already disease outbreaks have been attributed to water systems in which pathogenic bacteria inside protozoa or in biofilms have been protected from disinfection (Barker & Brown 1994; Berk et al. 1998; Snelling et al. 2006). SWNT and ciliates together provide another route to bacterial protection and could be a risk to human health.

In summary, SWNT exhibited more acute toxicity than CB at weight concentrations on the order of 10 µg/ml or above. At sub-lethal level, SWNT promoted bacteria survival directly by impairing the ciliates ability to digest bacteria and indirectly by enhancing survival of packaged bacteria against killing by antimicrobial treatments. This study highlights the need for more extended and systematic evaluations of environmental and health risks of nanoparticles, which could be...
significantly different from their bulk or micrometre-sized allotropes even at minute levels devoid of acute toxicity.

Acknowledgements

The authors would like to thank D. Weber for his assistance in confocal microscopy and SEM sample preparation. This work is supported by a Leaders Opportunity Fund from the Canada Foundation of Innovation (CFI) and Ontario Research Foundation (ORF) to Dr. Tang, and a Strategic grant from the Natural Science and Engineering Research Council (NSERC) of Canada to Drs Bols and Tang.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


Supplementary material available online

Supplementary Figures S1, S2.