

Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa

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Research on the toxicity of carbon nanotubes has focused on human health risks¹, and little is known about their impact on natural ecosystems². The ciliated protozoan *Tetrahymena thermophila* has been widely studied^{3,4} by ecotoxicologists because of its role in the regulation of microbial populations through the ingestion and digestion of bacteria^{5–7}, and because it is an important organism in wastewater treatment and an indicator of sewage effluent quality⁸. Here we show that single-walled carbon nanotubes are internalized by *T. thermophila*, possibly allowing the nanotubes to move up the food chain. The internalization also causes the protozoa to aggregate, which impedes their ability to ingest and digest their prey bacteria species, although it might also be possible to use nanotubes to improve the efficiency of wastewater treatment^{8–16}.

Tetrahymena belongs to an ecologically important group, the grazing protists, which contribute to aquatic ecology at several levels^{7,9}. *Tetrahymena* are ciliated protozoa that ingest bacteria by phagocytosis and sequester them within food vacuoles or phagosomes^{10,11}, 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 30 °C (ref. 3). 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 (CNTs)¹², 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 time¹³. Here, we expose *Tetrahymena* to a wide range of single-walled carbon nanotube (SWNT) concentrations (0–17.2 µg ml⁻¹) to fully demonstrate the different modes of impact, from behaviour change to cell death. The results extend current knowledge on CNTs and microorganisms, and help define critical parameters such as predicted no effect concentrations (PNEC) for environmental risk assessment models¹⁴.

Oxidized SWNTs were used so that the impact of the nanotubes could be studied without interference from surfactants and were characterized by atomic force microscopy (AFM) in a simple solution (Osterhout's) and in a complex ciliate growth medium, proteose peptone extract (PPYE). Solubilized SWNTs were

mostly individual or in small bundles (Fig. 1a) with length predominantly <500 nm and diameters ranging from 2 to 10 nm (Fig. 1d) in Osterhout's, but appeared as micrometer-size complexes in PPYE (Fig. 1b). Scanning electron microscopy (SEM) of SWNT aggregates after 24 h with *Tetrahymena* in Osterhout's revealed amorphous tangles (Fig. 1c). Iron contamination was successfully removed by our nanotube preparation procedure (Fig. 1e).

Tetrahymena cultures in Osterhout's (control) and with SWNTs (11.9 µg ml⁻¹) were monitored by phase contrast video microscopy over 3 days (Fig. 2; see also Supplementary Information, movies). Control cultures remained healthy for 72 h, as judged by the continued motility of the ciliates (Fig. 2a). However, SWNTs elicited four interrelated responses: diminished mobility, cell aggregation, matrix accumulation and cell death. These occurred 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, all cells immediately showed slight or no mobility and most cells bunched together in groups of approximately 5 to 50 cells that grew larger and reached a maximum ~1 h after the addition of SWNTs (Fig. 2b,c). After ~3 h, recovery started and some cells began to break free from the aggregates, the number of motile single cells increasing gradually over time. In the third stage the matrix grew darker and became progressively more obvious (Fig. 2d). A small portion of the ciliates remained motile and appeared healthy, but obvious cell death was observed in the culture (data not shown). In contrast, in cultures with SWNT concentrations <6.8 µg ml⁻¹ no loss of cell viability was observed after 3 days, even though initial aggregation was observed. Thus 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. In general, aggregate size, loss of mobility and cell death increased with increasing SWNT concentrations. This trend is evident in the videos taken from cultures with 1.6 µg ml⁻¹ (the lowest concentration with visible initial aggregation) and 11.9 µg ml⁻¹ SWNTs (see Supplementary

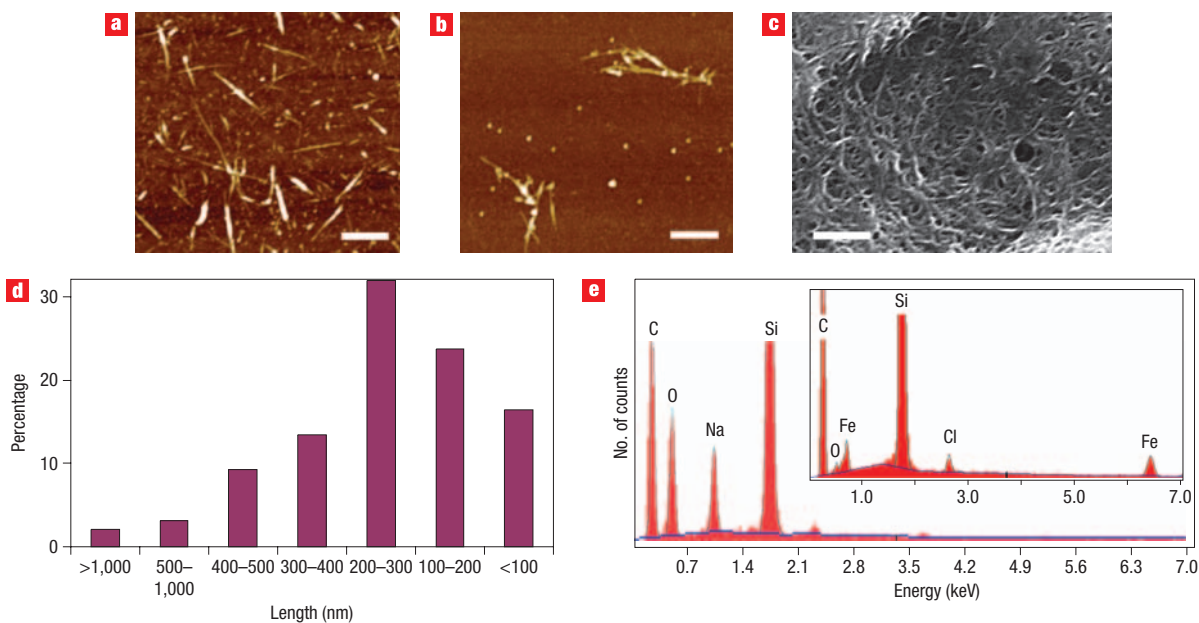


Figure 1 Characterization of SWNTs. **a, b**, AFM images showing the morphology of SWNTs in Osterhaut's solution (**a**) and PPYE growth medium (**b**). Scale bars, 500 nm. **c**, SEM image of SWNT aggregates collected from a *T. thermophila* culture. Scale bar, 200 nm. **d**, Distribution of nanotube length obtained from **a**. **e**, 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.

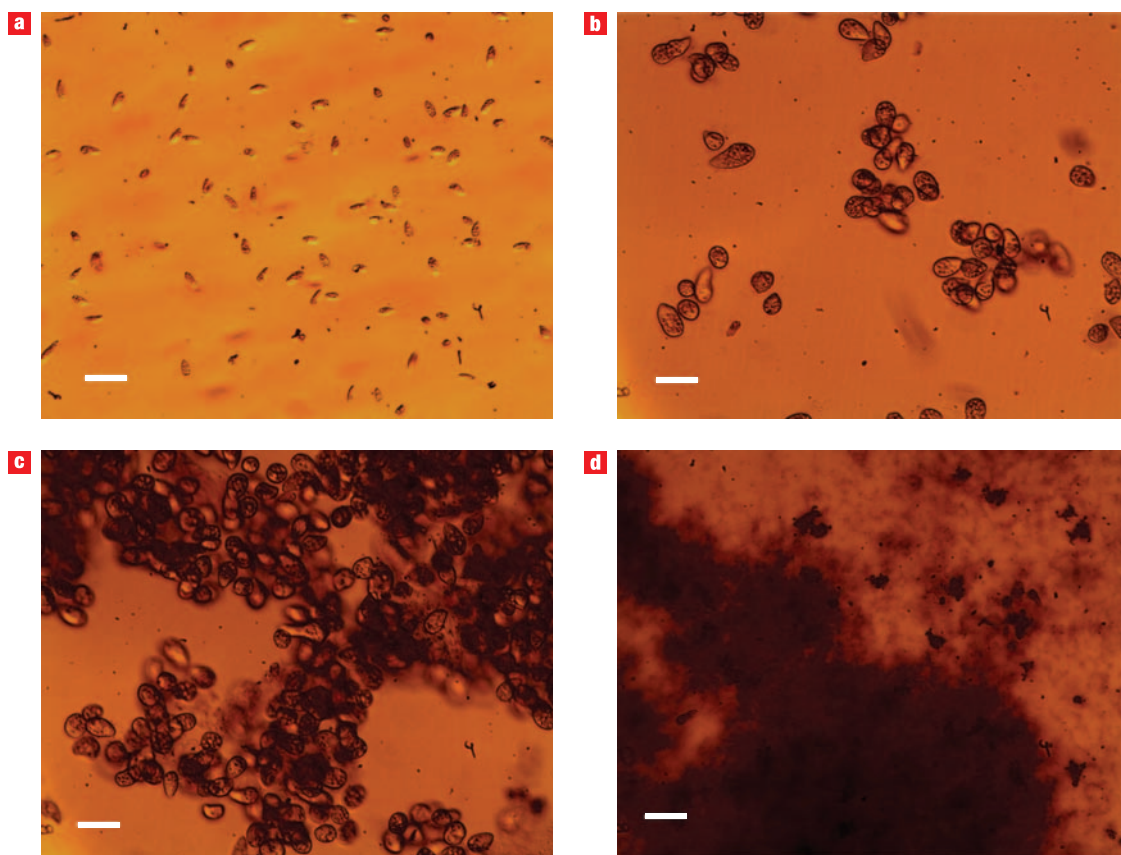


Figure 2 Phase contrast micrographs of *T. thermophila* after addition of SWNTs. **a–d**, Still images of control cultures (**a**) and 0.5 h (**b**), 1 h (**c**), 24 h (**d**) after addition of $11.9 \mu\text{g ml}^{-1}$ SWNTs. Owing to the high mobility of the control cells, image **a** was taken at a lower magnification. Scale bar for **a**, 100 μm ; scale bars for **b–d**, 50 μm .

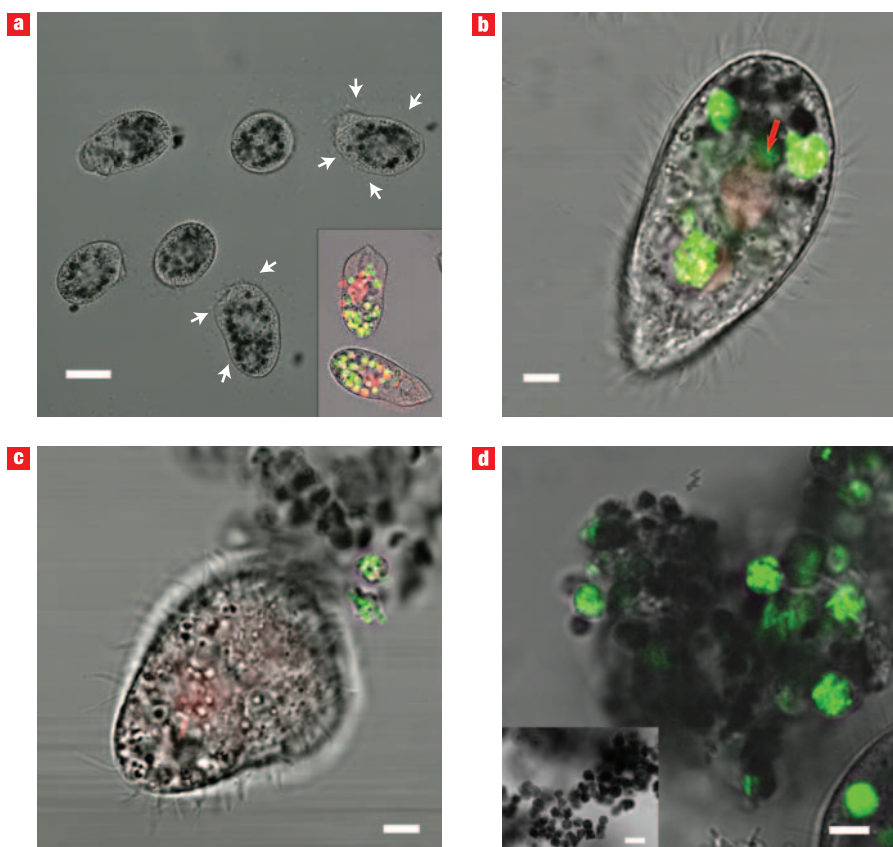


Figure 3 Confocal and white light images of *T. thermophila*. **a**, Ingestion of SWNTs (black granules) stimulated the release of mucus-like substance (white arrow). Internalized SWNTs are then egested as aggregates. Inset shows ingestion of *E. coli*-gfp alone (green-yellow). **b**, Ingestion of SWNTs and *E. coli*-gfp inside the same (red arrow) or different food vacuoles. **c,d**, *T. thermophila* egesting SWNT aggregates and remnant or viable *E. coli*-gfp after 4 h (**c**) and 24 h (**d**). Scale bar for **a**, 20 μm ; scale bars for **b–d**, 5 μm .

Information). The lowest level of recovery occurred at the highest SWNT concentrations.

When SWNT-treated cultures were examined more closely by confocal microscopy, five interactions between SWNT and *Tetrahymena* were seen. First, a faint matrix was seen to surround some individual cells shortly after SWNT exposure (Fig. 3a, white arrows). These resemble the capsules shed upon exposure of *Tetrahymena* to Alcian blue¹⁵. 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^{15,16}. Therefore, the induction of capsule formation could be responsible for the decreased motility and early aggregation of *Tetrahymena* with SWNTs. How similar the phenomena induced by SWNT and Alcian blue are will require further investigation, but structurally the inducers are very different. For long-term exposures to high SWNT concentrations, continued stimulation of mucocyst discharge might have led to excessive loss of membrane and cell death.

Second, *Tetrahymena* internalized the SWNTs. This is the interpretation given to the dark structures appearing within ciliates after incubation in SWNT solutions for roughly 2 h (Fig. 3a). When *Tetrahymena* were examined after being fed *E. coli*-gfp (green fluorescent protein), but no SWNTs, fluorescent structures of a similar size to the dark structures were seen (Fig. 3a, inset), suggesting that, like bacteria, SWNTs were engulfed or phagocytized into food vacuoles. Exposure of

Tetrahymena to *E. coli*-gfp and SWNTs led to vacuoles that contained both SWNTs and the bacteria (Fig. 3b).

Third, *Tetrahymena* egested SWNT. Under continuous microscopic observation, some ciliates (after a few hours in SWNT solution) excreted SWNT globules through their posterior ends while swimming around with no obvious signs of toxicity. SWNT egestion was observed with (Fig. 3c) or without (Fig. 3a) the presence of *E. coli*, suggesting that this behaviour is triggered solely by SWNTs. Others have previously observed the food vacuole contents of *Tetrahymena* being egested from the cytoproct in defecation balls^{11,17,18}. For example, India ink was egested as carbon-containing faecal pellets without membranes¹¹. 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 2d,3d). An SEM image of a sample taken from the dark matrix shows heavily coated SWNT amorphous tangles (Fig. 1c). 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. 3a). 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.

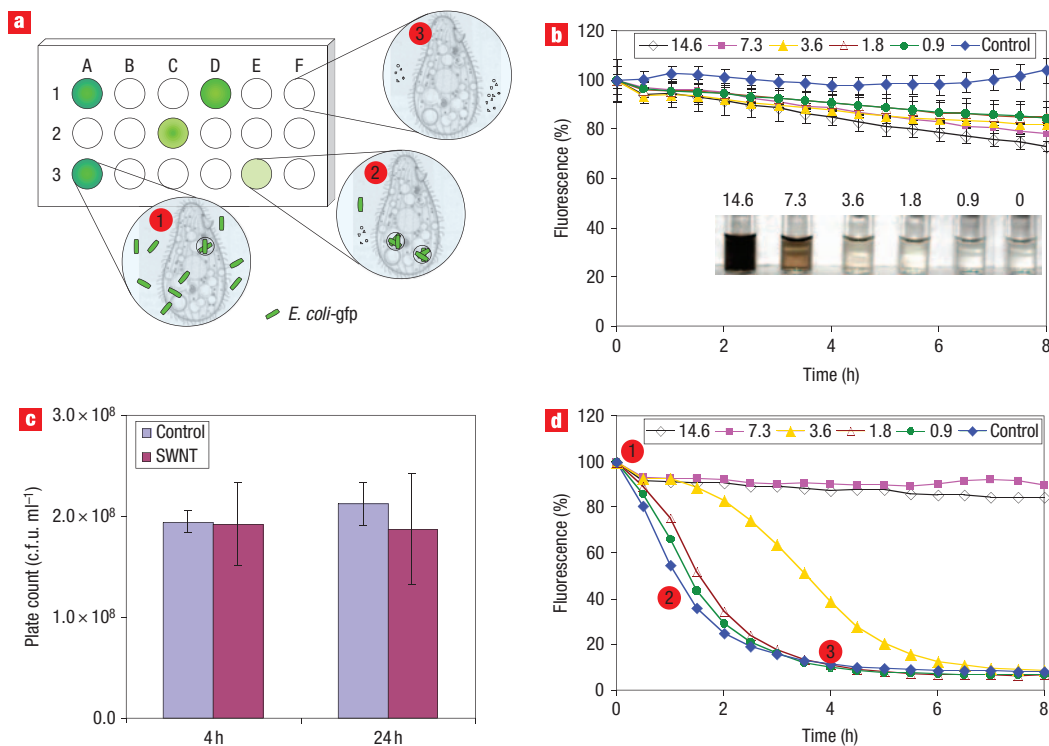


Figure 4 Effect of SWNTs on bacterivory of *T. thermophila*. **a**, CB assay detects the gradual disappearance of fluorescence upon exposure (1), ingestion (2) and eventual destruction (3) of ingested *E. coli-gfp* by *T. thermophila*. **b**, CB assay on *E. coli-gfp* alone (no *T. thermophila*) shows 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. **c**, Plate counts from a colony forming assay of *E. coli-gfp* with and without SWNTs show no difference. **d**, Various concentrations of SWNTs (final concentrations in $\mu\text{g ml}^{-1}$) were added concurrently with *E. coli-gfp* to microwells containing *T. thermophila*. High concentrations of SWNTs (7.3 and $14.6 \mu\text{g ml}^{-1}$) blocked bacterivory.

Finally, two observations suggest that SWNTs could be inhibiting bacterivory. When *E. coli-gfp* was presented together with high SWNT concentrations ($>7.3 \mu\text{g ml}^{-1}$), fewer ciliates were seen to have incorporated *E. coli-gfp* (see Supplementary Information), suggesting that phagocytosis was impaired. Also, at a concentration of $11.9 \mu\text{g ml}^{-1}$, more round fluorescent aggregates were seen outside the ciliates than in control cultures (Fig. 3c,d). These might be egested digestion remnants or viable bacteria, which would suggest that SWNTs interfered with the digestion process. *Tetrahymena* have been shown to release viable bacteria within vesicles¹⁹. Alternatively, the fluorescent aggregates might have been individual bacteria that aggregated as a result of matrix buildup.

The effect of SWNTs on the consumption of *E. coli-gfp* by *Tetrahymena* was quantified over time with a fluorescence plate reader in a bacterivory assay (Fig. 4a, see Methods). SWNTs alone had little effect on *E. coli-gfp* viability, as they continued to fluoresce over time, despite initial quenching, and form colonies (Fig. 4b,c). With the control (no SWNT), fluorescence declined rapidly over 2 h due to the engulfment and digestion of bacteria by *Tetrahymena* (Fig. 4d)¹⁰. With 0.9 and $1.8 \mu\text{g ml}^{-1}$ SWNTs, bacterivory proceeded only slightly slower than the control. At high SWNT concentrations (7.3 and $14.6 \mu\text{g ml}^{-1}$), bacterivory was completely blocked, whereas at $3.6 \mu\text{g ml}^{-1}$ bacterivory was delayed but eventually proceeded to the same extent as the control. For high concentrations this is attributed to the diminished motility and increased aggregation of SWNT-treated ciliates blocking their bacterivorous capacity, whereas for $3.6 \mu\text{g ml}^{-1}$, many ciliates eventually recovered their motility and

were capable of bacterivory. However, an interaction between SWNTs and the bacteria, such as SWNTs inducing change in their palatability, could also have contributed to the diminished bacterivory.

In conclusion, we have shown that SWNTs are capable of entering the ciliated protozoan, *T. thermophila*. *Tetrahymena* ingested SWNTs and bacteria with no apparent discrimination. Both soluble and insoluble SWNTs could contribute to the microscopically visible aggregates within the *Tetrahymena* cytoplasm. The effects of SWNTs on ciliate mobility, bacterivory and viability were observed starting at $1.6 \mu\text{g ml}^{-1}$, $3.6 \mu\text{g ml}^{-1}$ and $6.8 \mu\text{g ml}^{-1}$, respectively. The exact internalization routes^{20–22} of SWNTs and the mechanisms leading to *Tetrahymena* immobilization/aggregation require further investigation. The interactions between *T. thermophila* and SWNTs have several ecotoxicological implications. Internalization within ciliates followed by consumption of the ciliates by multicellular animals could be a route for SWNTs to move up food chains. The apparent ability of the ciliates to aid SWNT aggregation through exudates or the egestion of SWNT clumps could help incorporate SWNTs into normal ecological processes. The heavily coated SWNT tangles can be considered as another form of detritus, which is dead particulate organic matter that contributes to food webs by providing microhabitats for colonizing bacteria²³. SWNTs inhibited ciliate bacterivory, which means CNTs could potentially disrupt the role of ciliates in regulating bacterial populations. Whether other grazing protists will show similar susceptibility to SWNTs remains to be established. Finally, nanomaterials can be considered to be potential toxicants, but could also be assets in

wastewater treatment²⁴. SWNTs caused *Tetrahymena* to release excess exudates, which contribute to floc formation, so they could be used to improve the efficiency of ciliates in wastewater treatment^{8,16}, although effective measures to control and monitor SWNT release would be necessary.

METHODS

SWNT PREPARATION AND CHARACTERIZATION

The SWNTs were produced by acid oxidation, which is a widely used method to render purified, shortened and water-soluble CNTs²⁵, and characterized using several techniques. AFM and 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 6 M HNO₃ 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 1 h. Centrifugation (22,000g, 5 h) 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*, a stock solution of SWNTs was serially diluted in Osterhout's minimal salts medium (5.2 g NaCl, 0.907 g MgCl₂·6 H₂O, 0.2 g MgSO₄, 0.115 g KCl, 0.066 g CaCl₂·2 H₂O, in 100 ml of distilled water) or PPYE medium. UV-visible-NIR spectroscopy was used to characterize SWNT concentration²⁶.

OPTICAL 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, 5×10^5 cells ml⁻¹ for *T. thermophila* and 5×10^8 c.f.u. ml⁻¹ for *E. coli*. *Tetrahymena* nuclei were stained red using DRAQ5. The yellow appearance of *E. coli*-gfp is due to overlapping of green (gfp) and red (stained by DRAQ5).

BIOASSAYS

A schematic of the ciliate bacterivory (CB) assay is presented in Fig. 4a. 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* (5×10^8 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 assay was begun by the addition of *E. coli*-gfp, SWNTs and *Tetrahymena* and was monitored with a fluorescence plate reader (Victor V, PerkinElmer) that repeatedly measured over time the relative fluorescence units (RFUs) per well as described previously¹⁰. 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 (Fig. 4b,c). 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 µg ml⁻¹), 133,084 ± 11,756 (1.8 µg ml⁻¹), 130,763 ± 441 (3.6 µg ml⁻¹), 87,660 ± 2,578 (7.3 µg ml⁻¹) and 54,309 ± 4,997 (14.6 µg 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 37 °C overnight. Colonies were counted and expressed as colony forming units c.f.u. ml⁻¹.

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Author contributions

P.G. and X.S.T. conceived and designed the experiments, carried out experiments and data analysis, and co-wrote the paper. C.H.St.D. carried out the colony forming assay, replicated the CB assay, and supplied *E. coli*-gfp and *Tetrahymena* stock. M.E.P. carried out initial data acquisition and provided bioassay and instrument training. X.J. carried out AFM characterization of the SWNTs. V.T. carried out the survey and video recording under the contrast phase optical microscope. H.S.M. did EDAX characterization of SWNTs. N.C.B. conceived and designed the experiments, provided material support, co-analysed data and co-wrote the paper. All authors discussed the results and commented on the manuscript.

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