Supporting Information

# Bioaccumulation of Multiwall Carbon Nanotubes in *Tetrahymena thermophila* by Direct Feeding or Trophic Transfer

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## Additional Materials and Methods

### Characterization of MWCNTs

Environmental Scanning Electron Microscopy (ESEM). The MWCNT powder was imaged on a double-sided adhesive copper conductive tape mounted on an aluminum stub (Ted Pella, Redding, CA, USA) using an FEI XL30 field emission gun environmental scanning electron microscope (Philips Electron Optics, Eindhoven, the Netherlands) at a 15-kV accelerating voltage with a gaseous secondary electron detector in the wet mode (3 torr; Figure S1A).

Thermogravimetric Analysis (TGA). TGA of MWCNTs was performed as recommended in the ISO/TS 11308:2011 standard method.[1] Approximately 10 mg of MWCNTs was weighed on a microbalance into an alumina sample pan with an outer diameter of 6 mm and height 4.5 mm (AdValue Technology, AZ, USA). Samples were heated in the Thermogravimetric Analyzer/sDTA 851e (Mettler Toledo, OH, USA) from 25 °C to 900 °C at a rate of 5 °C/min in 50 mL/min air flow. TGA scans were conducted for three separate samples. An empty sample pan was run as a blank and subtracted from sample results to correct for buoyancy effects. Non-carbon content of MWCNTs was recorded as % residual weight ($W_{res}$) of the sample at 900 °C, and was 5.8 % ± 2 % (average of 3 replicate samples ± standard deviation). This value is higher than reported previously for MWCNTs synthesized by the same method[2, 3] and likely reflects batch-to-batch variation in CNT synthesis. The thermal stability of MWCNTs, defined as the temperature at which the highest fraction of carbon content oxidizes, was assessed by the primary oxidation temperature ($T_{ox}$), which is the temperature at which the most intense peak occurs in the derivative thermogravimetric curve (Figure S1B). The $T_{ox}$ of the MWCNTs herein was 594 °C± 0.9 °C.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The metallic composition of the non-carbon content of the MWCNTs was determined by recovering the residue from TGA and digesting this in a mixture of concentrated plasma-pure $HNO_3$ and HCl (1:3; i.e., *aqua regia*). For that, the residues from the TGA were transferred from the crucibles to 15-mL clear polypropylene centrifuge tubes (Corning, Corning, NY, USA) by pipetting 50 µl aliquots of *aqua regia* to the crucible and pipetting the suspended residue into the 15-mL tube. This step was repeated 4 times. The tubes containing TGA residues in the final volume of 200 µl *aqua regia* were heated at 100 °C for 1 h. Acid-dissolved samples were diluted with Nanopure $H_2O$ to a final acid concentration of 5 %. The samples were analyzed on an iCAP™ 6300 ICP-OES Analyzer (Thermo Scientific). The analysis showed that the MWCNTs contained nickel as a catalyst residue at $2.6 \pm 0.01$ mg/g (average of 3 measurements ± standard deviation) MWCNTs.

Dynamic Light Scattering and Zeta-Potential Measurements. The average hydrodynamic diameter, electrophoretic mobility and ζ potential of MWCNT agglomerates in aqueous suspensions at 10 mg/L were measured using a Zetasizer Nano ZS-90 (Malvern Instruments; Table S3).

**Growth Media and Microbial Cultivation**

*P. aeruginosa* PG201 was maintained at -80 °C and cultured as described previously.[4-7] The bacterial inoculum was streaked from frozen stock (-80 °C, preserved in 70 % Luria Bertani, or LB, broth plus 30 % [v/v] glycerol) onto LB agar and cultivated in the dark (12 h, 30 °C). Several colonies were transferred from the solid media into 5 mL of liquid media, grown aerobically overnight, transferred to a larger volume of fresh liquid medium and grown to late

exponential phase (approximately 18 h at 30 °C). Bacteria were cultivated in half-strength 21C[8] media which contained 0.5 g $NH_4Cl$, 1.725 g $Na_2HPO_4 \cdot 7H_20$, 1.38 g $KH_2PO_4$ per L of Nanopure water and 1 % v/v Hutner's mineral solution.[9] Glucose at 3.4 g/L was added as a carbon source from a separate filter-sterilized (0.2 μm) stock solution.

*T. thermophila* SB210E cells, which were maintained axenically by passaging every 3 weeks in 2 % proteose peptone broth at room temperature, were inoculated into standard (10 cm by 15 mm) sterile polystyrene Petri dishes containing 10 mL of a proteose peptone-based growth media (SSP, 1 % proteose peptone, 0.1 % yeast extract, 0.2 % dextrose, 0.003 % Fe-EDTA, all percentages are by volume) and grown in a humidity chamber without shaking (17 h, 30 °C), as described previously.[6, 7] After reaching mid to late exponential phase, the culture was centrifuged at 1000g for 10 min. The cell pellet was washed once with starvation medium (Dryl's medium [2 mmol/L sodium citrate, 2 mmol/L $NaH_2PO_4 \cdot H_2O$, 1 mmol/L $Na_2HPO_4$, 1.5 mmol/L $CaCl_2$, pH 7.4]), and cells were resuspended to a concentration of ca. 650 000 cells/mL. A 10 mL aliquot of cell suspension in Dryl's medium was pipetted into Petri dishes and starved in humidity chambers (17 h, 30 °C). The starved *T. thermophila* cells were centrifuged, washed in Dryl's medium, and resuspended in either SSP medium for growth experiments involving direct CNT exposure or Dryl's medium for trophic-transfer studies.

**Growth Assays with *P. aeruginosa***

The cultivation of *P. aeruginosa* for determining the effects of MWCNT exposure on growth was performed essentially as in Horst et al., 2010.[5] In brief, 200 μL of half-strength 21C medium (with or without 0.1 mg/L or 1 mg/L MWCNTs) was pipetted in the wells of a 96-well plate (flat-bottom polystyrene with clear bottom and sides; Corning Incorporated) and inoculated with

exponential-phase *P. aeruginosa* grown as described previously.[5] For the MWCNT treatments, the MWCNT stock in Nanopure water was diluted with 2× concentrated growth medium to obtain the final MWCNT concentrations, pipetted into wells, and inoculated with *P. aeruginosa*. Each treatment, including uninoculated controls, was prepared in triplicate. The multiwell plates were incubated at 30 °C, 200 rpm (21 rad/s) in a Synergy 2 Multi-Mode microplate reader (Biotek Instruments, Winooski, VT) equipped with a xenon lamp set to measure optical density at 600 nm ($OD_{600}$) regularly over time.

**Acute Toxicity Assays**

For the acute toxicity assays, bacteria and protozoa were grown to late exponential growth phase as described above, harvested by centrifugation at 10 000g or 1000g, respectively, for 10 min, and washed once with Dryl's medium. The pellet was resuspended in Dryl's medium to yield an $OD_{600} = 0.3$ (corresponding to $6\times10^8$ cells/mL) for bacteria and a cell density of $10^5$ cells/mL for protozoa. An aliquot of 100 µL of bacterial or protozoan suspension was pipetted into the wells of opaque black polystyrene 96-well plates (Corning Incorporated, MA, USA) containing 100 µL of MWCNT dispersions in Dryl's medium or Dryl's medium without MWCNTs (unamended controls). Each concentration was tested in three replicates. MWCNT dispersions in Dryl's medium were used as abiotic controls. 70 % isopropanol and 20 µmol/L carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used as positive controls in membrane integrity and reductase activity assays, respectively. The plates were covered with lids and incubated at 30 °C. After 3 h, membrane integrity was measured in protozoa. After 5 h and 24 h, membrane integrity and reductase activity were each measured in bacteria using fluorescent stains as described below. Measurements taken to ensure that artefactual results from the

presence of MWCNTs in the toxicity assays did not impact the results are described in a later section.

Membrane Integrity Assay. The stocks of SYTO9, a green fluorescent nuclear stain, (3.34 mmol/L) and propidium iodide (PI, 20 mmol/L) in DMSO included in the LIVE/DEAD Bac Light Bacterial Viability Kit L7012 (Molecular Probes, Invitrogen, CA, USA) were diluted in Nanopure $H_2O$ to yield a 20 × working solution (final concentrations in the assay: 4.73 µmol/L SYTO9 and 28.3 µmol/L PI). 10 µl of working solution was pipetted into the wells, and the plates were incubated for 15 min in the dark at room temperature. Fluorescence was measured first at Ex/Em 485 nm/530 nm and then at 485 nm/630 nm using a Synergy 2 Multi-Mode microplate reader (Biotek Instruments, Winooski, VT). Data were expressed as a ratio of green/red fluorescence.

Reductase Activity. RedoxSensor™ Green reagent (1 mmol/L in DMSO) included in the BacLight™ RedoxSensor™ Green Vitality Kit (Molecular Probes, Invitrogen, CA, USA) was diluted in phosphate buffered saline (PBS, pH 7.4) to yield a 20× working solution. The final concentration of the stain in the assay was 1 µmol/L. After pipetting 10 µL of the working solution into the wells, the plates were incubated in the dark for 10 min and the fluorescence was measured at Ex/Em 485/530nm. The data were expressed as intensity of green fluorescence.

Data Analysis of the Fluorescence Assays. Each plate included the following combinations of treatments and fluorescent stain: bacterial or protozoan cells, with or without MWCNTs + fluorescent stain; bacterial or protozoan cells, with or without MWCNTs, no stain added; medium with or without MWCNTs + fluorescent stain. To account for autofluorescence of the MWCNTs and quenching of the stain fluorescence by MWCNTs, the following formula was used to calculate the fluorescence of the stain at each concentration of MWCNTs:

$$F = \left(F_{exposure} - F_{background}\right) \times \frac{F_{abiotic}}{F_{abiotic\ with\ MWCNTs}}$$

where $F_{exposure}$ is the fluorescence of combined stain, cells and MWCNTs; $F_{background}$ is the fluorescence of the cells with MWCNTs, but with no stain added; $F_{abiotic}$ is the fluorescence of the stain in the test medium; and $F_{abiotic\ with\ MWCNTs}$ is the fluorescence of the medium containing stain and MWCNTs.

Viability. The viability of *T. thermophila* upon direct exposure to MWCNTs in acute conditions (non-growing culture) was assessed by cell counting. Protozoans were incubated in Petri plates containing 10 mL of Dryl's medium, either with or without added MWCNTs, at 30 °C in the humidity chamber. After 3 h and 24 h, replicate 100-µL samples were pipetted from the Petri plates and fixed with 2.5 % glutaraldehyde (by volume) for counting in a hemocytometer.

**Cell Counting and Determination of Cell Numbers in $^{14}$C-MWCNT-exposed Samples in Growth Experiments**

*P. aeruginosa* and *T. thermophila* in the control cultures (either without MWCNTs or treated with unlabeled MWCNTs) were counted directly using optical microscopy. Cell samples were fixed with glutaraldehyde (2.5 % by volume) and stored at 4 °C until counting (less than 48 h). Fixed *P. aeruginosa* cells were stained with SYBR Gold (Life Technologies, Carlsbad, CA) and counted by epifluorescence microscopy (Nikon E800, 1 000× magnification) as described previously.[4] *T. thermophila* population growth was assessed by counting the cells in a 100-µL sample (fixed in 2.5 % glutaraldehyde) periodically (2, 8, 16, and 22 h) using a hemocytometer. Duplicate counts were averaged for each time point. To determine the cell numbers in the $^{14}$C-

MWCNT treated samples, 100 µL of the fixed *P. aeruginosa* and *T. thermophila* cells were pipetted into the wells of opaque black polystyrene 96-well plates, stained with SYBR Gold for 35 min, and the fluorescence quantified in the plate reader at Ex/Em 485 nm/530 nm. At each measurement, control cell samples with several different known cell concentrations, as determined by direct counts, were used to establish a calibration curve for SYBR Gold fluorescence and cell numbers.

**Sucrose Density Gradient Centrifugation**

As per a detailed proven methodology,[10] bacteria were separated from unbound MWCNTs and MWCNT agglomerates by sucrose-based density gradient centrifugation. Ten mL of bacterial culture with or without MWCNTs was pelleted by centrifugation at 9715g for 10 min, resuspended in 0.5 mL of Dryl's medium and pipetted on top of the discontinuous sucrose gradient consisting of 50 % and 60 % sucrose (w/v) layers, both in Dryl's medium. The tubes were centrifuged at 4194g for 10 min at 10 °C in a swinging-bucket rotor (Sorwall SH-3000). The top and sucrose fractions (4.5 mL total) were removed and discarded. The bacterial pellet was washed by vortexing (1 min) in 10 mL of Dryl's medium, followed by centrifugation at 9715g for 10 min at 10 °C. The washing step was repeated and the pellet was suspended either in 1 mL of 10 mM potassium phosphate buffer, pH 7.4, 2.5 mL of 0.1 % sodium dodecyl sulfate (SDS)/0.1 mol/L NaOH or 10 mL of Dryl's medium for AMS, LSC or trophic transfer, respectively. LSC samples were processed and measured immediately after harvesting, AMS samples were pipetted into cryovials (Thermo Scientific Nalgene 1.2-mL Cryogenic Vials) and stored at -80 °C until shipment on dry ice to the Center for Accelerator Mass Spectrometry (CAMS; Lawrence Livermore National Laboratory or LLNL, Livermore, CA) for analysis.

**Density Gradient Centrifugation in OptiPrep™**

As per a methodology proven in detail,[10] *T. thermophila* was purified from bacteria, fecal pellets and unassociated MWCNTs by centrifugation in OptiPrep™ density gradient medium. After either direct uptake of MWCNTs in the rich medium or growth with *P. aeruginosa* as prey, *T. thermophila* was harvested (see Table S2 for harvested volumes at each time point) by low-speed centrifugation (607g, 5 min, 10 °C). The concentrated cell suspension was gently pipetted over 10 % OptiPrep™ and centrifuged (1864g, 5 min, 10 °C). The pellet was resuspended in 500 µL of Dryl's medium and pipetted over 20 % OptiPrep™ solution. The centrifugation was repeated, the top 2 mL containing the protozoan cells were pipetted into a clean 15-mL tube, and the cells were washed with Dryl's medium and pelleted by centrifugation (607g, 5 min, 10 °C). The pellet was suspended either in 1 mL of Dryl's medium or 2.5 mL of 0.1 % SDS/0.1 mol/L NaOH for AMS or LSC, respectively.

**Calculation of MWCNT Doses in the Trophic Transfer Experiments, MWCNT Volume- and Mass-Based Concentrations in *P. aeruginosa* and *T. thermophila,* and Bioconcentration and Trophic Transfer Factors**

The mass of MWCNT adsorbed to *P. aeruginosa* or accumulated in *T. thermophila* (expressed as MWCNT mass per cell, $m_{(MWCNTs,\ bacterial\ cell)}$ and $m_{(MWCNTs,\ protozoan\ cell)}$, respectively), was calculated by dividing the MWCNT mass measured in the samples of density gradient-separated cells by the number of cells in the sample ($n_{(bacteria)}$ or $n_{(protozoa)}$, respectively).

Cell volume- and mass-based concentrations of MWCNTs ($C_{volume\ (MWCNTs)}$, g MWCNTs/L of cells, and $C_{mass\ (MWCNTs)}$, g MWCNTs/kg of cellular dry mass, respectively) in bacteria and protozoa were calculated as follows:

$$C_{volume\ (MWCNTs)} = \frac{m_{(MWCNTs,\ cell)}}{V_{cell}}\ (1);$$

$$C_{mass\ (MWCNTs)} = \frac{m_{(MWCNTs,\ cell)}}{m_{cell}+m_{(MWCNTs,\ cell)}}\ (2),$$

where $m_{(MWCNTs,\ cell)}$ is the mass of MWCNTs per cell, $V_{cell}$ is the cell volume and $m_{cell}$ is the dry mass of the cell. As per Werlin et al., 2011,[6] 0.37 µm$^3$ and 7 042 µm$^3$ were respectively used for *P. aeruginosa* and *T. thermophila* volumes, and 0.12 pg and 1 860 pg as respective cellular dry weights. The cell dimensions of *T. thermophila* and *P. aeruginosa* were measured from Nomarski images and were confirmed to be within the same range as those reported in Werlin et al., 2011.[6]

The concentration of free MWCNTs in the bacterial culture medium ($C_{(MWCNTs,\ bacterial\ medium)}$, g/L) was calculated by subtracting the concentration of MWCNTs associated with bacteria from the measured concentration of MWCNTs in the total bacterial culture ($C_{(MWCNTs,\ culture,\ measured)}$, g/L):

$$C_{(MWCNTs,\ bacterial\ medium)} = C_{(MWCNTs,\ culture,\ measured)} - \frac{m_{(MWCNTs,\ bacterial\ cell)} \cdot n_{(bacteria)}}{V_{culture}}$$

(3)

where $m_{(MWCNTs,\ bacterial\ cell)}$ is cell-associated MWCNT mass per bacterial cell (in grams), $n_{(bacteria)}$ is number of bacteria in 1 liter of culture ($V_{culture}$).

Doses of MWCNTs delivered to protozoa by bacteria in the trophic transfer experiments ($C_{i\ (MWCNTs,\ medium,\ T)}$, g/L) were calculated by multiplying the MWCNT mass per *P. aeruginosa* ($m_{(MWCNTs,\ bacterial\ cell)}$) by the bacterial cell concentration at the beginning of the trophic transfer experiment ($n_{i\ (bacteria)}/V_{medium}$):

$$C_{i\,(MWCNTs,\,medium,\,T)} = \frac{m_{(MWCNTs,\,bacterial\,cell)} \cdot n_{i\,(bacteria)}}{V_{medium}} \quad (4)$$

where $n_{i\,(bacteria)}$ is the number of bacteria in 1 liter of exposure medium ($V_{medium}$).

The MWCNT concentration in the trophic transfer exposure medium (i.e., MWCNTs not accumulated in protozoa, including MWCNTs associated with bacteria, fecal pellets and possibly free MWCNTs disassociated from bacteria) at time t ($C_{t\,(MWCNTs,\,medium,\,T)}$, g/L) was calculated by subtracting the measured MWCNT mass in *T. thermophila* at time t from the measured MWCNT mass in bacteria at the beginning of the experiment ($C_{i\,(MWCNTs,\,medium,\,T)}$):

$$C_{t\,(MWCNTs,\,medium,\,T)} = C_{i\,(MWCNTs,\,medium,\,T)} - \frac{m_{t\,(MWCNTs,\,protozoan\,cell,\,T)} \cdot n_{t\,(protozoa,\,T)}}{V_{medium,\,T}} \quad (5)$$

where $m_{t\,(MWCNTs,\,protozoan\,cell,\,T)}$ is the mass of MWCNTs per protozoan cell at time t, $n_{t\,(protozoa,\,T)}$ is the number of protozoa in 1 liter of the exposure medium ($V_{medium,\,T}$) at time t in the trophic transfer experiment.

The concentration of free MWCNTs in the medium at time t during direct MWCNT exposure to protozoa ($C_{t,\,(MWCNTs,\,medium,\,D)}$, g/L) was calculated by subtracting the measured MWCNT mass in *T. thermophila* from the nominal administered MWCNT mass at the beginning of the experiment ($C_{i\,(MWCNTs,\,nominal)}$):

$$C_{t\,(MWCNTs,\,medium,\,D)} = C_{i\,(MWCNTs,\,nominal)} - \frac{m_{t\,(MWCNTs,\,protozoan\,cell,\,D)} \cdot n_{t\,(protozoa,\,D)}}{V_{medium,\,D}} \quad (6)$$

where $m_{t\,(MWCNTs,\,protozoan\,cell,\,D)}$ is the mass of MWCNTs per protozoan cell at time t, $n_{t\,(protozoa,\,D)}$ is the number of protozoa in 1 liter of the exposure medium ($V_{medium,\,D}$) at time t in the direct

exposure experiment.

Volumetric concentration factors for MWCNT encrusted bacteria ($VCF_{bacteria}$) and protozoa at time t during direct exposure to MWCNTs ($VCF_{t,\ protozoa,\ D}$) and during trophic transfer experiments ($VCF_{t,\ protozoa,\ T}$), respectively, were calculated by dividing the cellular volume-based MWCNT concentration by the free MWCNT concentration in the medium:

$$VCF_{bacteria} = \frac{C_{volume\ (MWCNTs,\ bacteria)}}{C_{(MWCNTs,\ bacterial\ medium)}} \qquad (7);$$

$$VCF_{t,\ protozoa,\ D} = \frac{C_{t,\ volume\ (MWCNTs,\ protozoa,\ D)}}{C_{t\ (MWCNTs,\ medium,\ D)}} \qquad (8)$$

$$VCF_{t,\ protozoa,\ T} = \frac{C_{t,\ volume\ (MWCNTs,\ protozoa,\ T)}}{C_{t\ (MWCNTs,\ medium,\ T)}} \qquad (9)$$

where $C_{volume\ (MWCNTs,\ bacteria)}$ is the bacterial cell volume-based MWCNT concentration (g MWCNTs/L of bacterial cells) calculated using equation (1), and $C_{t,\ volume\ (MWCNTs,\ protozoa,\ D)}$ and $C_{t,\ volume\ (MWCNTs,\ protozoa,\ T)}$ are protozoan cell volume-based MWCNT concentrations at time t during direct exposure and trophic transfer, respectively (g MWCNTs/L of protozoan cells), calculated using equation (1).

Bioconcentration factors for MWCNT encrusted bacteria ($BCF_{bacteria}$, L/kg) and protozoa at time t during direct exposure to MWCNTs ($BCF_{t,\ protozoa,\ D}$ L/kg) and during trophic transfer experiments ($BCF_{t,\ protozoa,\ T}$, L/kg), respectively were calculated by dividing the cellular mass-based MWCNT concentration by the free MWCNT concentration in the medium:

$$BCF_{bacteria} = \frac{C_{mass\ (MWCNTs,\ bacteria)}}{C_{(MWCNTs,\ bacterial\ medium)}} \qquad (10);$$

$$BCF_{t,\,protozoa,\,D} = \frac{C_{t,\,mass\,(MWCNTs,\,protozoa,\,D)}}{C_{t\,(MWCNTs,\,medium,\,D)}} \qquad (11)$$

$$BCF_{t,\,protozoa,\,T} = \frac{C_{t,\,mass\,(MWCNTs,\,protozoa,\,T)}}{C_{t\,(MWCNTs,\,medium,\,T)}} \qquad (12)$$

where $C_{mass\ (MWCNTs,\ bacteria)}$ is bacterial cell mass-based MWCNT concentration (g MWCNTs/kg bacterial dry mass) calculated using equation (2), and $C_{t,\ mass\ (MWCNTs,\ protozoa,\ D)}$ and $C_{t,\ mass\ (MWCNTs,\ protozoa,\ T)}$ are protozoan cell mass-based MWCNT concentrations at time t in direct exposure and trophic transfer, respectively, (g MWCNTs/kg protozoan dry mass) calculated using equation (2). It should be noted that the equations 10-12 are typically used under steady-state assumptions and are used here although steady state conditions between the MWCNT concentration in the medium and in/on the organisms were not reached in the batch experimental setup used herein.

The trophic transfer factor ($TTF_t$) at time t was calculated by dividing the MWCNT mass per dry mass of *T. thermophila* at time t during the trophic transfer experiment ($C_{t,\ mass\ (MWCNTs,\ protozoa,\ T)}$, g MWCNTs/kg protozoan dry mass) by the MWCNT mass per dry mass of *P. aeruginosa* ($C_{mass\ (MWCNTs,\ bacteria)}$, g MWCNTs/kg bacterial dry mass)*:*

$$TTF_t = \frac{C_{t,\,mass\,(MWCNTs,\,protozoa)}}{C_{mass\,(MWCNTs,\,bacteria)}} \qquad (13)$$

The results of the calculations for protozoan exposures to MWCNTs directly via medium and by grazing on MWCNT-encrusted bacteria are shown in Tables S5 and S6, respectively.

**Optical Microscopy of MWCNTs and Bacteria in *T. thermophila***

*T. thermophila* cells sampled during the trophic transfer and direct uptake experiments were fixed in 2.5 % glutaraldehyde and kept at 4 °C until imaging. A total of 5 μL of cell suspension were pipetted onto a glass slide, mixed with 5 μL of Mowiol® 4-88 (Sigma-Aldrich, St. Louis, MO, USA), covered with a glass coverslip, allowed to harden at room temperature for 24 h, and kept at 4°C until imaging. An Olympus BX51 upright microscope with differential interference contrast (DIC, also known as Nomarski microscopy) optics and Retiga 2000R QImaging Camera was used to capture images using Q-Capture Pro 7 software (Surrey, BC, Canada). Several images at different focal planes were acquired from each *T. thermophila* cell to capture intracellular MWCNT aggregates in the direct MWCNT uptake samples and food vacuoles filled with bacteria in the trophic transfer samples. Phase contrast images were captured with Nikon E800 upright microscope using RS Image software (Roper Scientific).

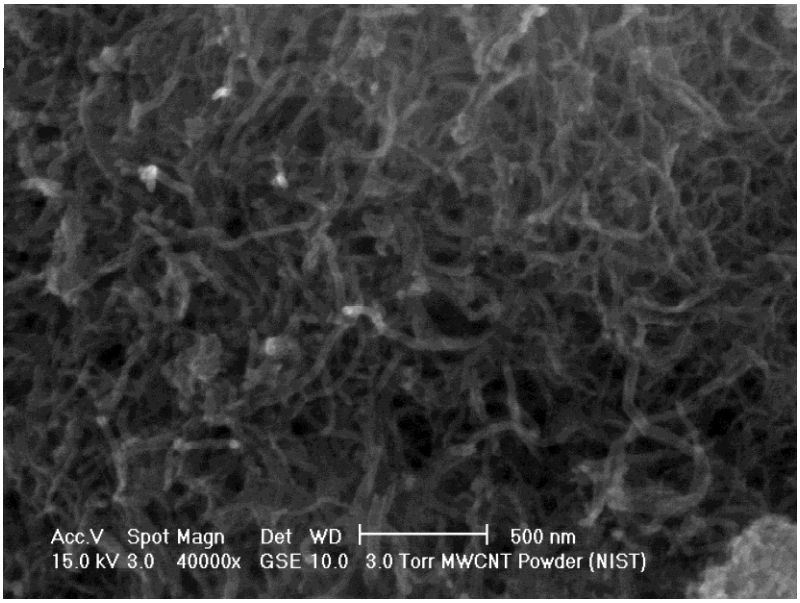
**Estimation of MWCNT Mass in *T. thermophila* Using Image Analysis**

The Nomarski images acquired from the direct MWCNT uptake samples were analyzed as follows: the images acquired from one *T. thermophila* cell at different focal panes were overlaid in Adobe Photoshop and the area of black MWCNT aggregates in the overlaid image was quantified using ImageJ [National Institutes of Health (NIH), Bethesda, MD]. The threshold was adjusted to maximize the areas of MWCNT aggregates while minimizing background noise in the binary image. Intracellular aggregates were selected with the freehand selection tool to exclude any MWCNT aggregates, which were outside the cell borders, from the area calculations, then the particle analysis tool was used for area quantification. The results were presented as agglomerate-equivalent area per cell. In the Nomarski images acquired from the

trophic transfer samples, the food vacuoles in *T. thermophila* cells were manually counted in ImageJ using the multi-point tool. The diameters of the food vacuoles, and lengths and widths of *T. thermophila* cells, were also measured manually using ImageJ. Six to twelve cells, considered to be a sufficient number of cells to capture the cell-to-cell variability, were analyzed per time point in each treatment.

Figures and Tables

**A**




**B**

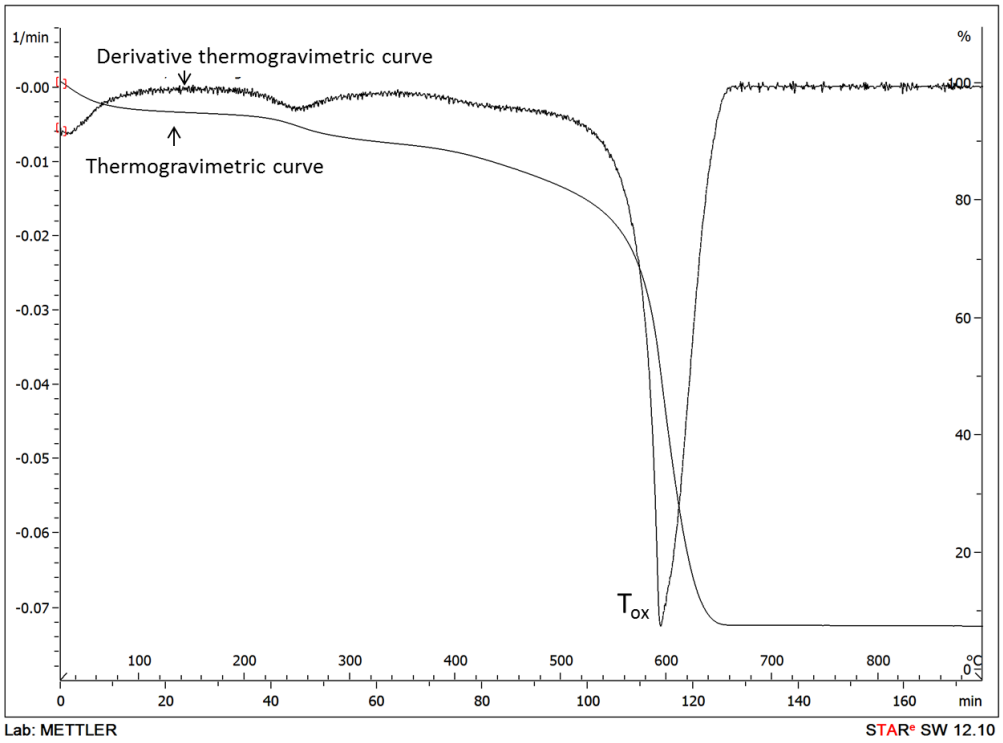
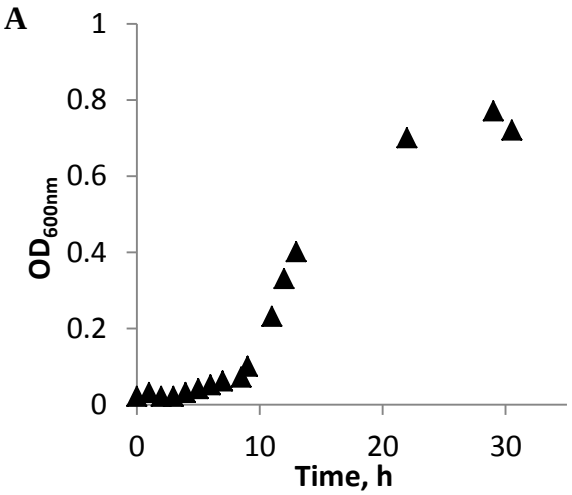
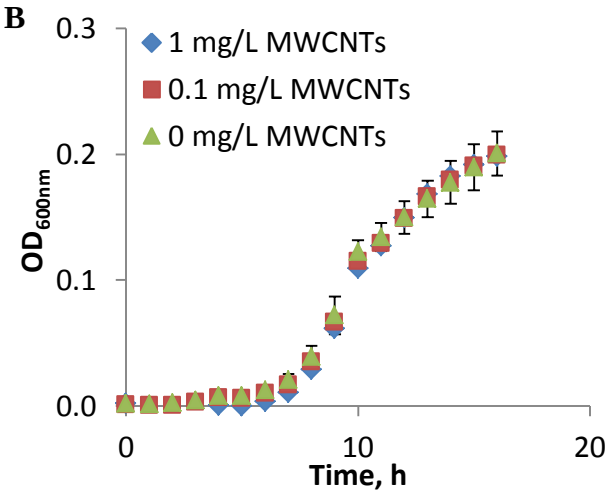



**Figure S1**. Environmental scanning electron microscopy (ESEM) image of dry MWCNTs (A), and the derivative thermogravimetric curve (primary y-axis) with the primary oxidation temperature ($T_{ox}$) peak and the thermogravimetric curve showing the % mass remaining (secondary y-axis) of MWCNTs at increasing temperatures over time (B).




| MWCNT concentration, mg/L | 0 | 0.1 | 1 |
|---|---|---|---|
| Specific growth rate (5 h to 9 h), $h^{-1}$ | 0.55 ± 0.03 | 0.56 ± 0.05 | 0.49 ± 0.03 |
| Lag phase, h | 4.9 ± 0.1 | 4.9 ± 0.06 | 4.8 ± 0.09 |
| Maximum yield, $OD_{600nm}$ | 0.2 ± 0.02 | 0.2 ± 0.01 | 0.2 ± 0.02 |

**Figure S2.** A representative growth curve of *P. aeruginosa* grown in half-strength 21C medium in 50-mL culture volume (A) and growth curves of *P. aeruginosa* cultivated with and without MWCNTs in 96-well microplate (culture volume 200 µL) (B). $OD_{600nm}$ values in panels A and B are different because of the different optical path lengths in spectrophotometer (A) and microplate reader (B). *P. aeruginosa* growth parameters listed in the table were calculated based on data presented in graph B. Values in the table and are the average of 6 replicates ± standard deviation. Data points in graph B are the average of 6 replicates and error bars indicate standard deviations.

**Table S1.** Exposures, Doses of MWCNTs and the Respective $^{14}C$ Quantification Methods Used in the Study.

| Test organism | Exposure pathway | Nominal MWCNT concentration, mg/L | $^{14}C$ quantification method |
|---|---|---|---|
| *P. aeruginosa* | Directly in the medium | 0.01 | AMS |
| | | 1 | LSC |
| *T. thermophila* | Directly in the medium | 0.3 | LSC |
| | | 1 | LSC |
| | Grazing on MWCNT-encrusted *P. aeruginosa* | Bacteria exposed to 0.01 mg/L MWCNTs, resulting in a dose of 0.004 mg/L to protozoans | AMS |
| | | Bacteria exposed to 1 mg/L MWCNTs, resulting in a dose of 0.3 mg/L to protozoans | LSC |

AMS – accelerator mass spectrometry; LSC – liquid scintillation counting

**Table S2.** Number of Replicates and Petri Plates per Replicate, and *T. thermophila* Culture Volumes Harvested at Each Time Point per Experiment*

| Harvesting time, h | Treatment** | Number of replicates | Number of Petri plates per replicate, each containing 10 mL of culture | Volume of culture harvested per replicate, mL |
|---|---|---|---|---|
| 2 | $^{14}$C-MWCNT | 3 | 3 | 25 |
| | Control | 3 | 3 | 25 |
| | MWCNT | 1 | 3 | 25 |
| 8 | $^{14}$C-MWCNT | 3 | 2 | 16 |
| | Control | 3 | 2 | 16 |
| | MWCNT | 1 | 2 | 16 |
| 16 | $^{14}$C-MWCNT | 3 | 1 | 5 |
| | Control | 3 | 1 | 5 |
| | MWCNT | 1 | 1 | 5 |
| 22 | $^{14}$C-MWCNT | 3 | 1 | 3.5 |
| | Control | 3 | 1 | 3.5 |
| | MWCNT | 1 | 1 | 3.5 |

*Exposure experiment at 1 mg/L MWCNTs in axenic cultures was repeated twice to confirm reproducibility, the coefficients of variation (CV) were 40 % at 2 h, 50 % at 8 h, 30 % at 16 h and 20 % at 22 h.

**"Control" designates the treatments in rich medium without MWCNTs or *T. thermophila* grown with bacteria without MWCNTs; "MWCNT" denotes treatments with unlabeled MWCNTs prepared for microscopy.

**Table S3.** Hydrodynamic Diameters, Polydispersity Indices, Electrophoretic Mobilities, Zeta-Potentials, and pH Values of MWCNTs in Aqueous Dispersions

| Medium | Hydrodynamic diameter, nm | PdI* | Electrophoretic mobility, μm cm/V s | Z-potential, mV | pH |
|---|---|---|---|---|---|
| $H_2O$ | 165.6 ± 2.2 | 0.21 | -3.5 ± 0.4 | -45.0 ± 5.5 | 6.0 |
| Dryl's medium | 164.8 ± 2.1 | 0.21 | -2.6 ± 0.1 | -33.4 ± 0.75 | 7.4 |
| Half-strength 21C medium | 320.9 ± 13 | 0.24 | -2.6 ± 0.2 | -33.3 ± 2.4 | 7.0 |
| SSP medium | 1580 ± 23 | 0.71 | -0.58 ± 0.05 | -7.4 ± 0.6 | 7.0 |

*PdI – polydispersity index; the data represent average values of triplicate measurements ± standard deviation.

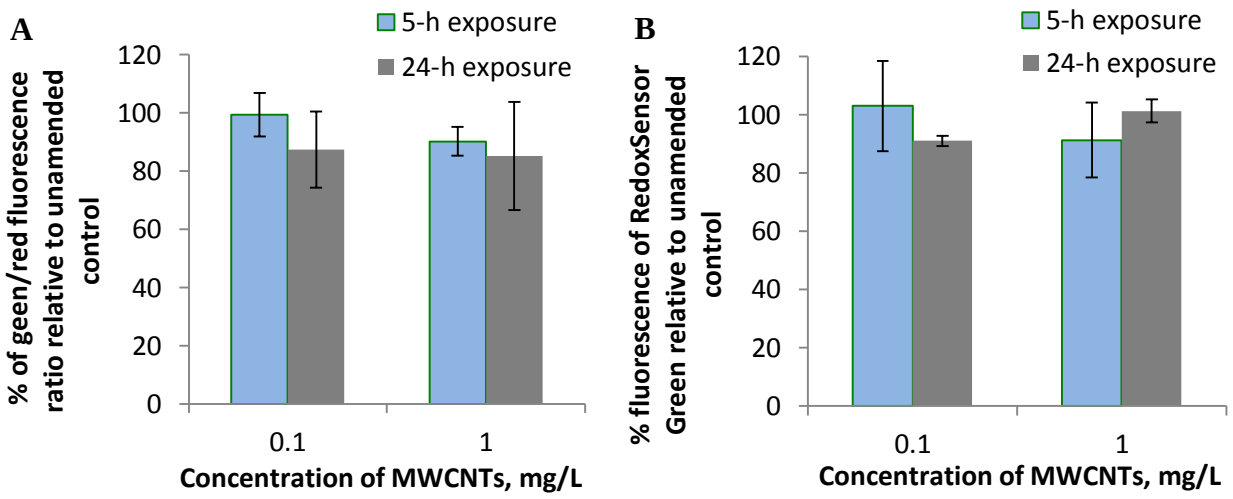



**Figure S3.** Membrane integrity (A) and reductase activity (B) of *P. aeruginosa* after incubation with MWCNTs in Dryl's medium (non-growing culture) for 5 h and 24 h, quantified by measuring fluorescence of SYTO9 (emitting green fluorescence in all cells)/propidium iodide (emitting red fluorescence in membrane damaged cells) and RedoxSensor Green, respectively. Data are the average of three replicates and error bars indicate standard deviation values. The results were not significantly different from the control (*P. aeruginosa* not exposed to MWCNTs), based on the two-sample t-test ($p > 0.05$).

**Table S4**. Growth Parameters of *T. thermophila* Grown with *P.aeruginosa* (Trophic Transfer) and in Rich Growth Medium (Direct Exposure) with and Without MWCNTs.

| Treatment | Maximum yield, cells/mL | Specific growth rate, $h^{-1*}$ | $R^{2**}$ | Lag phase, h |
|---|---|---|---|---|
| *T. thermophila* grown with *P. aeruginosa* without MWCNTs | $1.5\times10^{5} \pm 2\times10^{4}$ (a) | $0.18 \pm 0.02$ (a) | $0.945 \pm 0.01$ | $2.0 \pm 0.3$ (a) |
| Trophic transfer, MWCNT dose: 0.004 mg/L | $1.1\times10^{5} \pm 8\times10^{3}$ (a) | $0.17 \pm 0.02$ (a) | $0.996 \pm 0.005$ | $1.9 \pm 0.2$ (a) |
| Trophic transfer, MWCNT dose: 0.3 mg/L | $1.5\times10^{5} \pm 3\times10^{4}$ (a) | $0.21 \pm 0.02$ (a) | $0.979 \pm 0.005$ | $2.3 \pm 0.5$ (a) |
| *T. thermophila* grown in SSP medium without MWCNTs | $8.6\times10^{5} \pm 4\times10^{4}$ (b) | $0.30 \pm 0.04$ (b) | $0.996 \pm 0.006$ | $3.6 \pm 0.2$ (b) |
| Direct exposure to 0.3 mg/L MWCNTs | $8.5\times10^{5} \pm 1\times10^{4}$ (b) | $0.27 \pm 0.02$ (b) | $0.996 \pm 0.003$ | $3.3 \pm 0.1$ (b) |
| Direct exposure to 1 mg/L MWCNTs | $7.6\times10^{5} \pm 1.5\times10^{5}$ (b) | $0.27 \pm 0.03$ (b) | $0.996 \pm 0.005$ | $3.4 \pm 0.3$ (b) |

Values in the table are average of three independent measurements ± standard deviation; values with different letters within a column are significantly different, two-sample t-test, $p \leq 0.05$

*Specific growth rates were calculated using data points in the range of 2 h to 16 h

**Averaged $R^2$ for the three independent measurements of specific growth rate, using linear regression analysis of natural log-transformed data

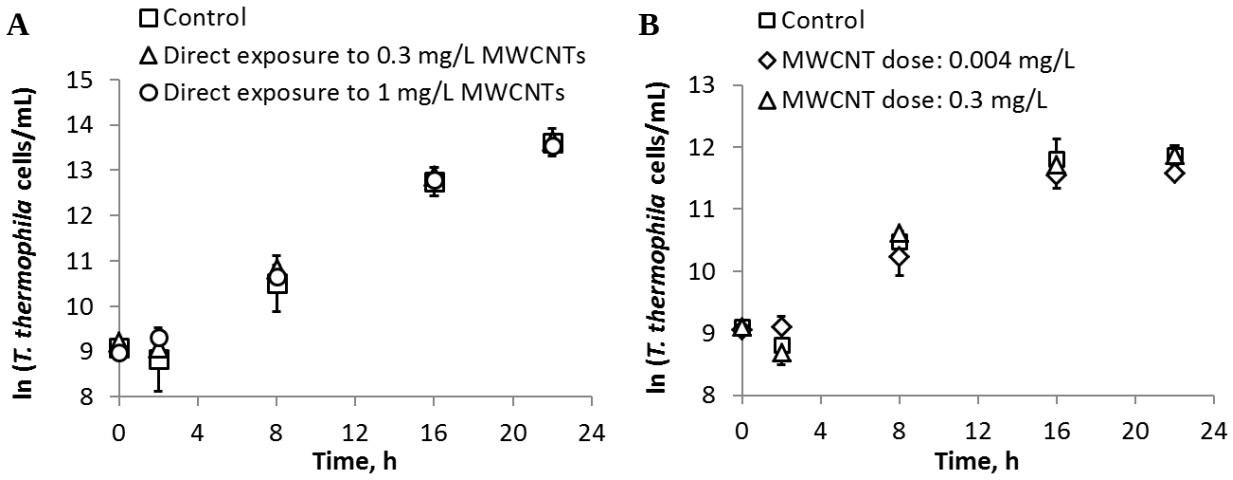



**Figure S4.** Natural log-transformed *T. thermophila* cell counts per mL over 22 h of growth in rich SSP medium with and without MWCNTs (A) and in Dryls' medium with control or MWCNT-treated *P. aeruginosa* as a food source (B). Symbols are the average of three replicates and error bars indicate standard deviation values.

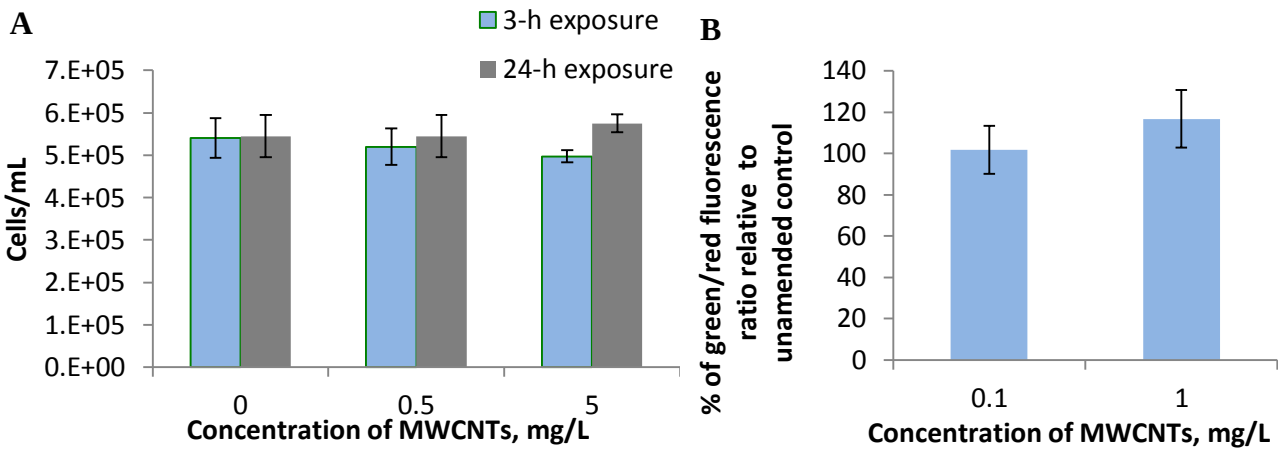



**Figure S5.** Viability (A) and membrane integrity (B) of *T. thermophila* after incubation with MWCNTs in Dryl's medium (non-growing culture) for 3 h (blue bars) and 24 h (grey bars), quantified by cell counts and measuring fluorescence of SYTO9/propidium iodide, respectively. Data are the average of three replicates and error bars indicate standard deviations. The results were not significantly different from the control (*T. thermophila* not exposed to MWCNTs), based on the two-sample t-test ($p > 0.05$).

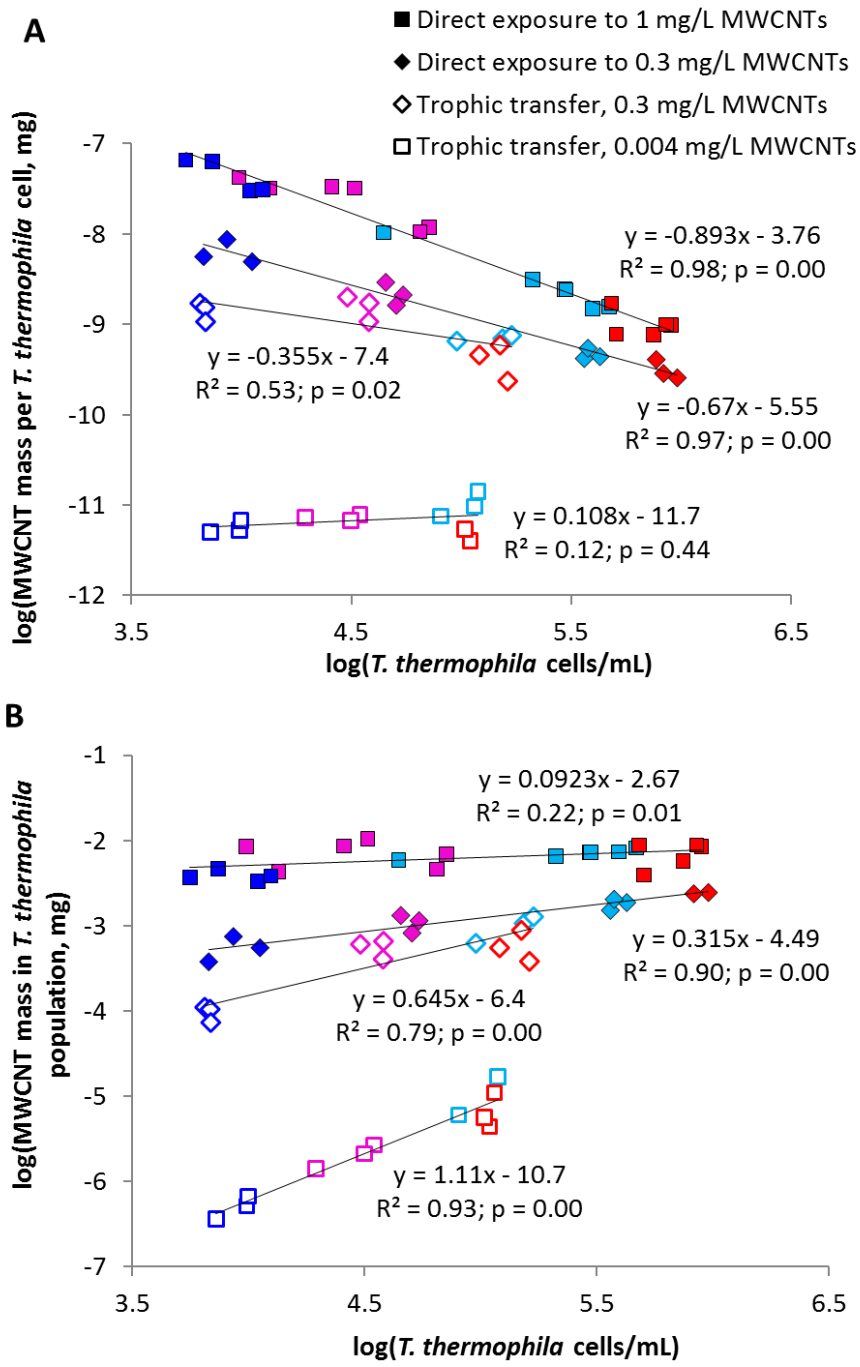



**Figure S6.** Scatter plots of log-transformed MWCNT mass per *T. thermophila* cell (A) and in *T. thermophila* population (B) versus log-transformed *T. thermophila* cell counts per mL in trophic transfer and direct exposure experiments. Data points indicate individual measurements made in triplicate (in sextuplicate in case of direct exposure to 1 mg/L MWCNTs) at 2 h (blue), 8 h (purple), 16 h (light blue) and 22 h (red). $p < 0.05$ indicates statistically significant dependence between the two variables based on regression analysis.

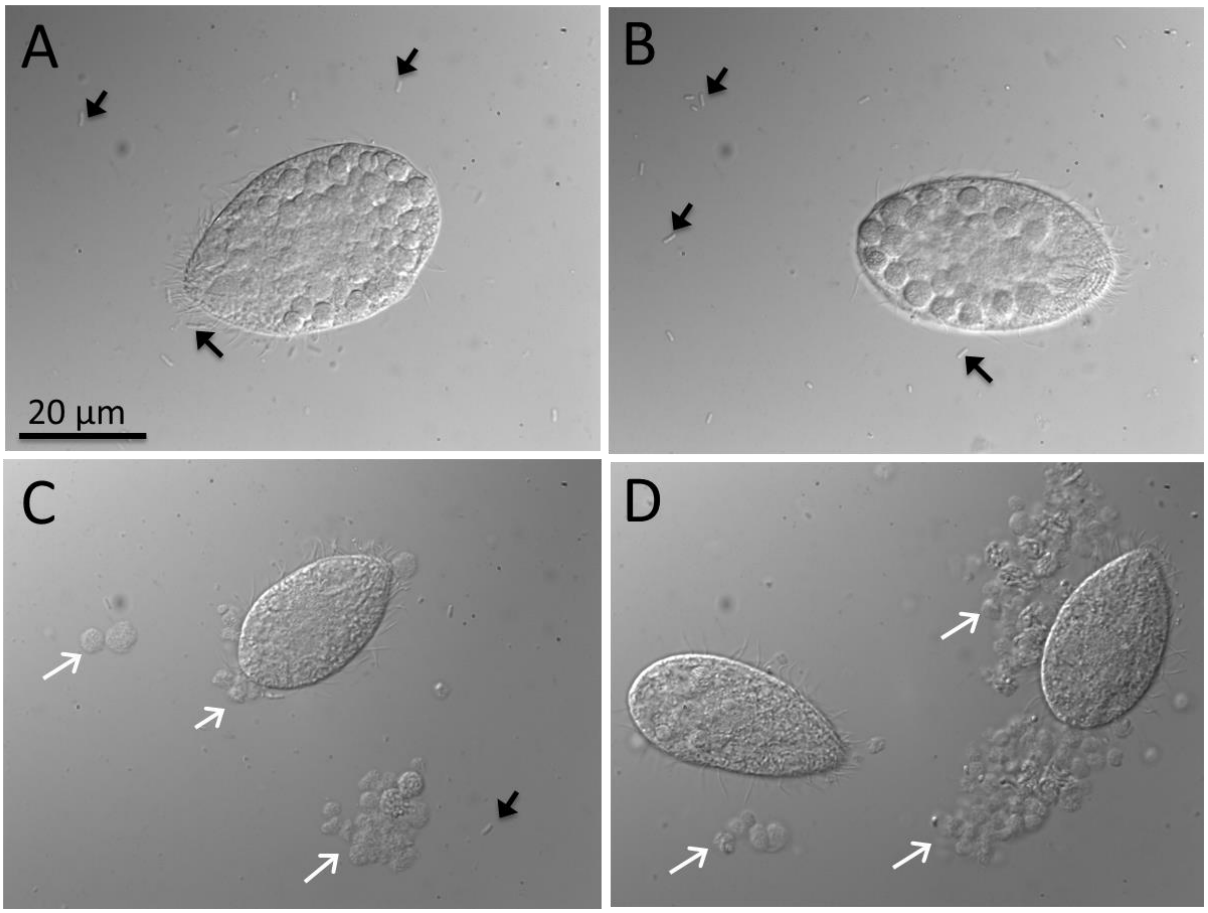



**Figure S7.** Nomarski images of *T. thermophila* grown with control *P. aeruginosa* as prey for 2 h (A), 8 h (B), 16 h (C) and 22 h (D). Black arrows indicate bacteria which are abundant at 2 h and 8 h and white arrows show fecal pellets evident at 16 h and 22 h.

**Table S5.** Calculated Values of MWCNT Volume- and Mass-Based Concentrations in *T. thermophila*, and Calculated Values of Bioconcentration Factors of MWCNTs in *T. thermophila* Grown in the Rich Growth Medium with 0.3 mg/L and 1 mg/L MWCNTs.

| 1. Time, h | 2. MWCNT concentration, mg/L | 3. *T. thermophila* cell concentration, cells/mL | 4. MWCNT mass per *T. thermophila* cell, pg | 5. MWCNT mass per *T. thermophila* cell volume, mg/L | 6. MWCNT mass per dry mass of *T. thermophila*, mg/kg | 7. MWCNT concentration in the medium, mg/L | 8. BCF, L/kg | 9. VCF |
|---|---|---|---|---|---|---|---|---|
| 2 | 0.3 | $8.8\times10^{3} \pm 2\times10^{3}$ | 6.4 ± 2 | 900 ± 300 | 3400 ± 1100 | 0.24 ± 0.02 | 14 000 ± 5000 | 3700 ± 1200 |
| | 1 | $8.0\times10^{3} \pm 4\times10^{3}$ | 46 ± 20 | 6600 ± 2000 | 24 000 ± 9000 | 0.69 ± 0.18 | 35 000 ± 16 000 | 9600 ± 4400 |
| 8 | 0.3 | $5.0\times10^{4} \pm 5\times10^{3}$ | 2.2 ± 0.6 | 320 ± 90 | 1200 ± 400 | 0.19 ± 0.03 | 6000 ± 2000 | 1700 ± 500 |
| | 1 | $4.2\times10^{4} \pm 2\times10^{4}$ | 22 ± 10 | 3100 ± 2000 | 12 000 ± 6000 | 0.34 ± 0.21 | 34 000 ± 27 000 | 9000 ± 7300 |
| 16 | 0.3 | $3.9\times10^{5} \pm 3\times10^{4}$ | 0.47 ± 0.07 | 66 ± 9 | 250 ± 40 | 0.12 ± 0.03 | 2100 ± 600 | 560 ± 150 |
| | 1 | $3.6\times10^{5} \pm 1\times10^{5}$ | 2.1 ± 0.6 | 300 ± 90 | 1100 ± 300 | 0.29 ± 0.08 | 3900 ± 1600 | 1000 ± 400 |
| 22 | 0.3 | $8.5\times10^{5} \pm 9\times10^{4}$ | 0.32 ± 0.08 | 45 ± 10 | 170 ± 40 | 0.06 ± 0.01 | 2900 ± 800 | 760 ± 210 |
| | 1 | $7.6\times10^{5} \pm 2\times10^{5}$ | 0.87 ± 0.2 | 120 ± 30 | 470 ± 100 | 0.39 ± 0.25 | 1200 ± 800 | 320 ± 220 |

BCF – bioconcentration factor; VCF – volumetric concentration factor; see the section "Calculation of MWCNT Doses in the Trophic Transfer Experiments, MWCNT Volume- and Mass-Based Concentrations in *P. aeruginosa* and *T. thermophila*, and Bioconcentration and Trophic Transfer Factors" for calculation details. Data in the columns 3 to 7 are the average values of three replicates ± standard deviation, and in the columns 8 and 9, the average values of three replicates ± standard deviation derived by error propagation.[11]

**Table S6.** Calculated Values of MWCNT Volume- and Mass-Based Concentrations in *T. thermophila*, and Bioconcentration and Trophic Transfer Factors of MWCNTs in *T. thermophila* Grown with *P. aeruginosa* Exposed to 0.01 mg/L and 1 mg/L MWCNTs and Resulting in MWCNT Doses of 0.004 mg/L and 0.3 mg/L, respectively.

| 1. Time, h | 2. MWCNT dose, mg/L | 3. *T. thermophila* cell concentration, cells/mL | 4. MWCNT mass per *T. thermophila* cell, pg | 5. MWCNT mass per *T. thermophila* cell volume, mg/L | 6. MWCNT mass per dry mass of *T. thermophila*, mg/kg | 7. MWCNT concentration in the medium, mg/L[a] | 8. BCF, L/kg | 9. VCF | 10. TTF |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.004 | $9.0\times10^{3} \pm 2\times10^{3}$ | 0.0057 ± 0.001 | 0.81 ± 0.1 | 3.1 ± 0.5 | 0.0036 ± 0.00002 | 840 ± 100 | 170 ± 30 | 0.011 ± 0.005 |
| | 0.3 | $6.7\times10^{3} \pm 2\times10^{2}$ | 1.4 ± 0.3 | 200 ± 50 | 770 ± 200 | 0.28 ± 0.002 | 2700 ± 600 | 730 ± 200 | 0.04 ± 0.009 |
| 8 | 0.004 | $2.9\times10^{4} \pm 8\times10^{3}$ | 0.0073 ± 0.0007 | 1 ± 0.07 | 3.9 ± 0.3 | 0.0035 ± 0.00006 | 1100 ± 80 | 220 ± 2 | 0.015 ± 0.006 |
| | 0.3 | $3.6\times10^{4} \pm 5\times10^{3}$ | 1.6 ± 0.5 | 230 ± 70 | 860 ± 300 | 0.29 ± 0.01 | 2900 ± 900 | 770 ± 200 | 0.03 ± 0.01 |
| 16 | 0.004 | $1.0\times10^{5} \pm 2\times10^{4}$ | 0.01 ± 0.003 | 1.5 ± 0.5 | 5.6 ± 1.8 | 0.0026 ± 0.0005 | 2200 ± 900 | 400 ± 100 | 0.021 ± 0.01 |
| | 0.3 | $1.4\times10^{5} \pm 4\times10^{4}$ | 0.7 ± 0.05 | 99 ± 7 | 380 ± 30 | 0.07 ± 0.03 | 5700 ± 3000 | 1500 ± 800 | 0.02 ± 0.004 |
| 22 | 0.004 | $1.1\times10^{5} \pm 4\times10^{3}$ | 0.0047 ± 0.001 | 0.67 ± 0.1 | 2.5 ± 0.5 | 0.0032 ± 0.00009 | 790 ± 200 | 150 ± 30 | 0.009 ± 0.004 |
| | 0.3 | $1.4\times10^{5} \pm 2\times10^{4}$ | 0.43 ± 0.2 | 61 ± 30 | 230 ± 100 | 0.29 ± 0.03 | 790 ± 300 | 210 ± 90 | 0.009 ± 0.003 |

BCF – bioconcentration factor; VCF – volumetric concentration factor; TTF – trophic transfer factor; see the section "Calculation of MWCNT Doses in the Trophic Transfer Experiments, MWCNT Volume- and Mass-Based Concentrations in *P. aeruginosa* and *T. thermophila*, and Bioconcentration and Trophic Transfer Factors" for calculation details. [a]MWCNTs associated with *P. aeruginosa* administered as a food source to *T. thermophila* and MWCNTs excreted by *T. thermophila*. Data in the columns 3 to 7 are the average values of three replicates ± standard deviation, and in the columns 8 to 10, the average values of three replicates ± standard deviation derived by error propagation.[11]

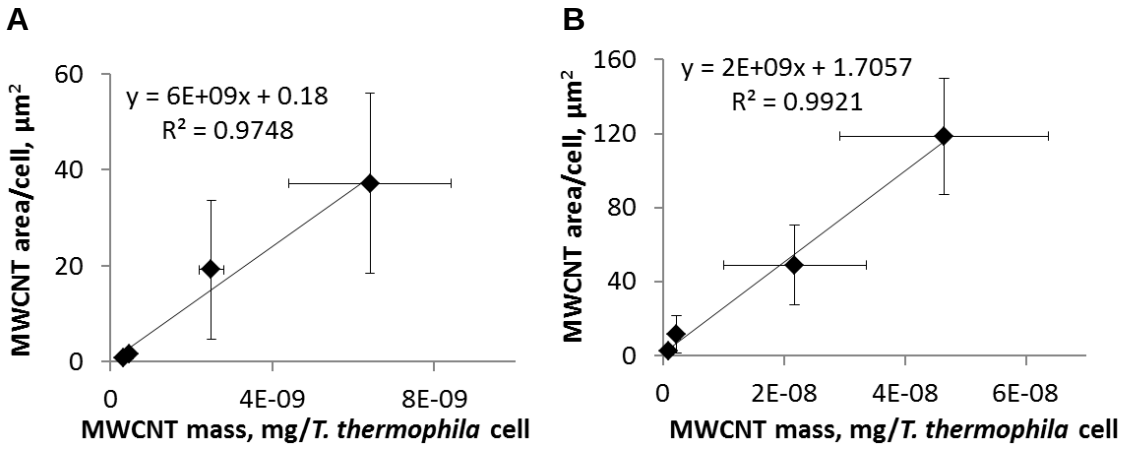



**Figure S8.** Scatter plots illustrating linear regression between the MWCNT area and MWCNT mass per *T. thermophila* cell in rich medium with 0.3 mg/L (A) and 1 mg/L MWCNT (B). MWCNT area per *T. thermophila* cell was measured in the Nomarski images using ImageJ and MWCNT mass was quantified by LSC. Data points are the average values of three replicates in mass quantification and 12 replicates in image analysis and error bars indicate standard deviation.

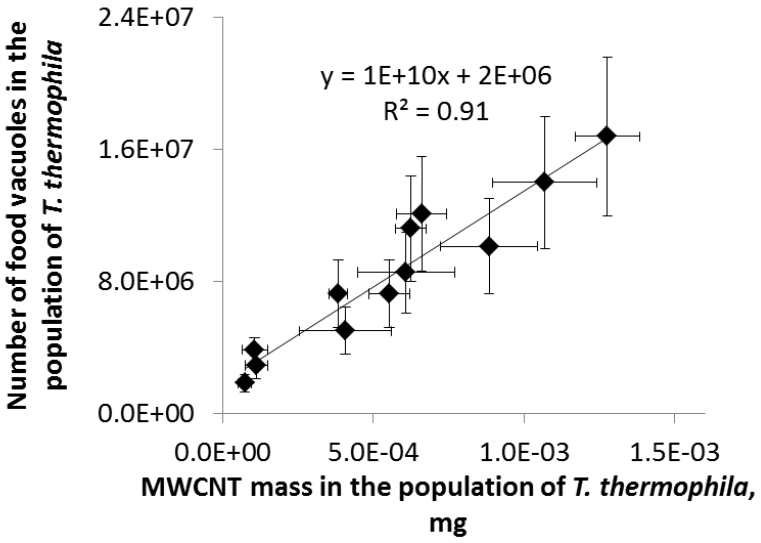



**Figure S9.** Scatter plot illustrating linear regression between the number of food vacuoles and MWCNT mass in *T. thermophila* grown with MWCNT-encrusted *P. aeruginosa* as prey at the MWCNT dose of 0.3 mg/L. Food vacuoles were counted in the Nomarski images of *T. thermophila* and MWCNT mass measured by LSC. Data points indicate individual measurements made in triplicate at 2, 8, 16, and 22 h, error bars indicate errors propagated according to standard methods.[11]

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