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

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Abstract

Consumer goods contain multiwall carbon nanotubes (MWCNTs) that could be released during product life cycles into the environment, where their effects are uncertain. Here, we assessed MWCNT bioaccumulation in the protozoan *Tetrahymena thermophila* via trophic transfer from bacterial prey (*Pseudomonas aeruginosa*) versus direct uptake from growth media. The experiments were conducted using ^{14}C-labeled MWCNT (^{14}C-MWCNT) doses at or below 1 mg/L, which proved subtoxic since there were no adverse effects on the growth of the test organisms. A novel contribution of this study was the demonstration of the ability to quantify MWCNT bioaccumulation at low (sub μg/kg) concentrations accomplished by employing accelerator mass spectrometry (AMS). After the treatments with MWCNTs at nominal concentrations of 0.01 mg/L and 1 mg/L, *P. aeruginosa* adsorbed considerable amounts of MWCNTs: (0.18 ± 0.04) μg/mg and (21.9 ± 4.2) μg/mg bacterial dry mass, respectively. At the administered MWCNT dose of 0.3 mg/L, *T. thermophila* accumulated up to (0.86 ± 0.3) μg/mg and (3.4 ± 1.1) μg/mg dry mass by trophic transfer and direct uptake, respectively. Although MWCNTs did not biomagnify in the microbial food chain, MWCNTs bioaccumulated in the

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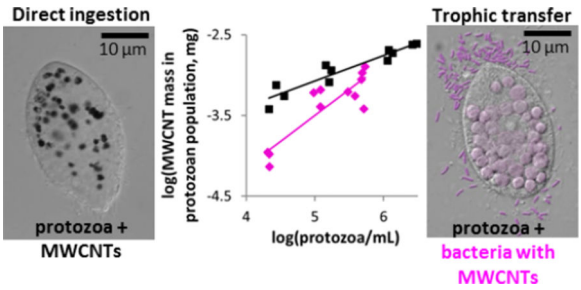
Supporting Information. Additional materials and methods of MWCNT characterization, test organism growth and media, acute toxicity assays, cell number determination, density gradient centrifugation, calculations of VCFs, BCFs and TTFs, microscopy and image analysis; figures and tables as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

protozoan populations regardless of the feeding regime, which could make MWCNTs bioavailable for organisms at higher trophic levels.

Graphical abstract



Introduction

Worldwide production capacity of carbon nanotubes (CNTs) has been reported to exceed several thousand tons per year, and CNT powders have already been incorporated into many commercial applications such as catalysts, water purification systems, coatings, and composites.[1] It has been proposed that CNT release during product lifecycles occurs by abrasion from nanocomposites and matrix degradation.[2, 3] These processes could introduce the largely biodegradation-resistant CNTs into soils, sediments and sewage sludge[4] where they could sorb and modulate the toxicity of other contaminants or vice versa.[5] In addition, weathering factors such as UV irradiation and precipitation could alter physico-chemical properties of CNTs and thereby change their bioavailability and toxicity.[6]

Studies regarding CNT environmental hazards indicate that the bioaccumulation potential of CNTs varies with exposure conditions, test organisms and physico-chemical properties of the CNTs.[7] At various exposure concentrations, single-wall carbon nanotubes (SWCNTs) were neither toxic nor bioaccumulative in marine benthic organisms (at up to 100 mg SWCNTs/kg sediment for 14 days),[8] marine bivalves (100 mg and 1000 mg SWCNTs/kg dry algae for 28 days),[9] earthworms (up to 100 mg SWCNTs/kg soil for 28 days),[10] or in aquatic plants and vertebrates in a wetland mesocosm over the 10 month incubation (2.5 mg/L SWCNTs).[11] Similarly, MWCNTs did not bioaccumulate in oligochaetes when ingested from MWCNT-spiked soils (30 mg/kg and 300 mg/kg dry soil) or sediments (37 mg/kg and 370 mg/kg dry sediment) into the organism guts, as there was no apparent absorption into tissues after the 28 day exposure and 6 h depuration phases.[10, 12, 13] Still, *Daphnia magna*, exposed to a non-toxic concentration of MWCNTs (up to 0.4 mg/L) for 24 h, retained nanotubes in the gut when placed in clean water for up to 48 h, and excreted most nanotubes only after feeding on algae.[14, 15] Recently, MWCNTs were shown to adsorb to algal cells grown for 48 h with MWCNTs, with some nanotubes also entering in the cytoplasm.[16] Also, a 2 week exposure of zebrafish to a non-toxic MWCNT concentration of 1 mg/L resulted in uptake and retention of approximately 5 mg MWCNTs/kg dry fish.[17] In the latter study, small fractions of MWCNTs accumulated in the fish blood and muscles, indicating the potential for CNT transfer in the food chain. While such studies suggest the potential for trophic transfer and bioaccumulation, most have used relatively high exposure

concentrations. As such, understanding the fate of released CNTs is still limited for low (μg/L) concentrations that are estimated to be present in aqueous environments.[18]

The assessment of trophic transfer and bioaccumulation at the low CNT concentrations predicted to occur in the environment has generally been hindered by the lack of suitable quantification methods of CNTs in complex environmental matrixes.[19] To overcome this challenge, we used ^{14}C-labeled MWCNTs (^{14}C-MWCNTs) to study their accumulation and trophic transfer in a microbial food chain of prey, the bacterium *Pseudomonas aeruginosa,* and predator, the protozoan *Tetrahymena thermophila*. The use of a sensitive detection method – accelerator mass spectrometry (AMS) - allowed for tracing ^{14}C-MWCNTs in the biological matrices at low (sub μg/kg) levels; this is the lowest detection level obtained to date for CNT quantification in tissues to our knowledge.[19, 20] Since MWCNTs were not expected to biodegrade under the experimental laboratory conditions of this study, quantification of ^{14}C could be used to trace MWCNTs in biota. Two environmentally relevant scenarios of CNT transfer to ciliates were compared at the same MWCNT doses: (i) MWCNT uptake via bactivory of MWCNT-encrusted bacteria, and (ii) grazing on medium-dispersed MWCNTs. The potential for MWCNT bioaccumulation and biomagnification in protozoa was assessed.

Materials and Methods

MWCNT Synthesis and Characterization

MWCNTs and ^{14}C-MWCNTs were synthesized using a modified chemical vapor deposition technique, purified by bath sonication with concentrated hydrochloric acid, and surface-modified with a 3:1 v:v ratio of concentrated nitric and sulfuric acid as described previously.[10, 13] The specific activity of the ^{14}C-MWCNTs was 0.015 mCi/g (555 kBq/g) as measured by liquid scintillation counting (LSC). For safety reasons, the physico-chemical characterization was performed with unlabeled MWCNTs, synthesized by the same method as the ^{14}C-MWCNTs. More than 90 % of the nanotubes were under 500 nm long, and the average diameter was 36.5 nm ± 12.7 nm as reported previously.[21] The Supporting Information (SI, Figure S1) provides additional characterization information.

Preparation and Characterization of MWCNT Stock Suspensions

Stock suspensions of MWCNTs and ^{14}C-MWCNTs were prepared at 200 mg/L in Nanopure water. To prepare the stocks, both MWCNTs and ^{14}C-MWCNTs were weighed into acid-washed and autoclaved 118-mL flasks to which water (70 mL) was added. The flasks were placed in an ice bath and the suspensions sonicated to disperse (40 % amplitude for 1 h, pulsing for 30 s on and 10 s off), using a Cole-Parmer 750-Watt Ultrasonic Homogenizer with a 13-mm diameter probe and replaceable tip, fabricated from titanium alloy Ti-6Al-4V. The output power, measured as described previously,[22] was 27 W. Probe sonication was not expected to shorten the MWCNTs, since similar sonication procedures were used previously for similarly-synthesized MWCNTs, and no change in the length distribution was observed.[21, 23] The stock suspensions were maintained at room temperature in the dark until addition to the experimental test media. Most (88 % ± 1.4 %; n = 3, uncertainty indicates standard error of the mean) of the MWCNTs were stably dispersed in Nanopure water four

days after sonication and remained dispersed over six months, as confirmed by the ^{14}C-MWCNT specific activity measurements. Hydrodynamic diameters and zeta-potential of MWCNTs were measured as described in the SI.

Assessment of MWCNT Effects on *P. aeruginosa* and *T. thermophila*

MWCNT toxicity to *P. aeruginosa* was assessed by measuring membrane integrity using the LIVE/DEAD Bac Light Bacterial Viability Kit L7012, reductase activity using the BacLight™ RedoxSensor™ Green Vitality Kit (both from Molecular Probes, Invitrogen, CA, USA) and growth by measuring the time course optical density (600 nm). Viability of *T. thermophila* upon direct exposure to MWCNTs in acute conditions (non-growing culture) was assessed by cell counting and membrane integrity as in *P. aeruginosa* above. Experimental details are in the SI.

Preparation of *P. aeruginosa* for Trophic Transfer Experiments

A Gram-negative bacterial strain, *P. aeruginosa* PG201,[24–27] was used for ^{14}C-MWCNT sorption studies and for *T. thermophila* feeding (trophic transfer) experiments. As detailed in the SI, *P. aeruginosa* was cultured (18 h, 30 °C) with shaking at 26 rad/s (250 rpm) in Erlenmeyer flasks containing half-strength 21C growth medium (50 mL) until late exponential growth phase (optical density at 600 nm [OD_{600}] 0.7, Figure S2A). The ^{14}C-MWCNT stock dispersion (mixed with 2× concentrated bacterial growth medium at a ratio of 1:1, v:v) was added to bacterial culture in the medium with undefined chemistry, due to bacterial growth and excretion of metabolites, yielding a final nominal ^{14}C-MWCNT concentration of either 0.01 mg/L or 1 mg/L (Table S1). Replicates with unlabeled MWCNTs were included for cell counting. Bacteria were incubated at 30 °C, while shaking at 26 rad/s (250 rpm), for 1 h with or without MWCNTs, then harvested by differential centrifugation (9, 715g, 10 min). Bacteria were separated from unassociated MWCNTs by density gradient centrifugation (SI) using sucrose which was biocompatible for *T. thermophila* trophic transfer experiments. ^{14}C-MWCNT concentrations associated with bacteria were quantified as described below. Bacterial cell numbers were determined by direct counting using epifluorescence microscopy (SI). The mass of an individual dry bacterial cell was determined in a prior study.[26]

Exposure of *T. thermophila* to MWCNTs with *P. aeruginosa* Prey and in Axenic Cultures

T. thermophila strain SB210E[26] was cultured in Dryl's medium (SI) with *P. aeruginosa* to determine protozoan growth rates and yields, and to quantify the uptake of ^{14}C-MWCNTs when bacterial prey was the only food source. *P. aeruginosa*, with or without MWCNTs, recovered from sucrose density gradients and resuspended in Dryl's medium (10 mL), were pipetted into sterile polystyrene Petri plates (10 cm by 15 mm). MWCNT doses supplied to protozoa via MWCNT-encrusted bacteria were 0.004 mg/L and 0.3 mg/L, following nominal exposure concentrations to bacteria of 0.01 mg/L and 1 mg/L, respectively. For exposures in axenic cultures, the MWCNT stock was diluted to a final concentration of either 0.3 mg/L (to equal one of the two MWCNT doses in the trophic transfer experiment) or 1 mg/L in a proteose peptone-based growth (SSP) medium (10 mL in Petri plates; SI). Starved *T. thermophila* cells were added to achieve an initial cell density of ca. 10^4 cells/mL. Replicate Petri plates were prepared for each treatment and time point of culture harvest (Table S2).

More Petri plates were prepared for sampling at earlier time points when the cell concentrations were low because larger volumes were needed to harvest sufficient biomass for analysis (Table S2). *T. thermophila* was cultured in the dark in a humidity chamber (30 °C) without agitation. At 2 h, 8 h, 16 h, and 22 h, the cultures were subsampled for microscopy, cell counting, and for total ^{14}C-MWCNT quantification; for the remaining volume of the culture, protozoa were separated from bacteria, fecal pellets, and unassociated MWCNTs by density gradient centrifugation in OptiPrep™ (Axis-Shield, Oslo, Norway) as described in the SI.

Quantification of ^{14}C-MWCNTs

Either LSC or AMS was used to quantify high or low ^{14}C-MWCNT concentrations, respectively, associated with bacteria and protozoa (Table S1).

LSC—Bacterial or protozoan pellets, recovered using density gradient centrifugation (as per the SI), were digested in 2.5 mL of 0.1 % sodium dodecyl sulfate (SDS) in 0.1 mol/L NaOH by vortexing[28] and incubating the samples (55 °C, 45 min).[29] Two and one half mL of UltimaGold XR (Perkin Elmer, Groningen, The Netherlands) liquid scintillation cocktail were added to the digested samples and the mixtures were kept in the dark for 1 h before LSC (LS 6500, Beckman Coulter Inc., Fullerton, CA) with the counting time set to 10 min. For quantification of ^{14}C-MWCNTs in the total bacterial or protozoan cultures, 1 mL of 0.1 % SDS in 0.1 mol/L NaOH was added to 1.5 mL of the culture, vortexed, then heated and mixed with the cocktail, similarly to how cell pellets were treated. Measured counts per minute (CPM) were converted to disintegrations per minute (DPM) by subtracting the background CPM from the sample CPM and dividing this net CPM by the fractional efficiency (0.95). Quenching of ^{14}C by bacterial and protozoan samples was between 5 and 10 % which was accounted for by spiking the unamended samples (cell pellets or suspensions) with a known mass of ^{14}C-MWCNTs. MWCNT mass in the MWCNT-exposed bacterial and protozoan samples was then calculated as follows:

$$m_{(MWCNTs,\ \text{sample})} = \frac{DPM_{(\text{sample})} \times m_{(MWCNTs,\ \text{spiked})}}{DPM_{(\text{spiked sample})}} \quad (1)$$

where $DPM_{(sample)}$ is the activity of the sample in DPM, $m_{(MWCNTs,\ spiked)}$ is the mass of MWCNTs added to the unamended samples, and $DPM_{(spiked\ sample)}$ is the activity of the MWCNT-spiked sample in DPM.

AMS—Each liquid sample (supernatant or suspended pellet) containing at least 30 μg carbon was transferred by pipet to a prebaked (900 °C for 3.5 h) quartz tube (≈ 6 mm × 30 mm, 4 mm i.d.) located inside two borosilicate glass culture tubes (10 mm × 75 mm in 12 mm × 100 mm) and dried overnight in a vacuum centrifuge. An excess of CuO (≈ 40 mg) was added and the inner quartz vials were transferred to quartz combustion tubes, evacuated and sealed with a torch. The samples were combusted at 900 °C for 3.5 h to oxidize all organic carbon to CO_2 and then reduced to filamentous carbon as previously described.[30] Carbon samples were packed into sample holders and carbon isotope ratios were measured on a National Electrostatics Corporation (Middleton, WI) compact 250 kV AMS

spectrometer at the Lawrence Livermore National Laboratory. Typical AMS measurement times were 5 min/sample to 10 min/sample, with a counting precision (relative standard deviation, RSD) of 0.5 % to 3 % and a standard deviation among 3 to 10 measurements of 1 % to 3 %. The $^{14}C/^{13}C$ ratios of the samples were normalized to measurements of four standard samples prepared using the same method of known isotope concentration (IAEA C-6 also known as ANU sucrose) and converted to units of g MWCNTs/g sample.[31] The limit of quantitation (LOQ) of ^{14}C-MWCNT in bacteria and protozoa was typically 0.05 μg/kg to 0.07 μg/kg based on the average of 3–9 undosed controls (samples without ^{14}C-MWCNTs) plus 3 times their standard deviation. Undosed controls were analyzed with each batch of samples to establish the LOQ for each set of exposures. The carbon content of each sample type was determined with 3 to 5 replicates using a CE-440 elemental analyzer (Exeter Analytical, Inc. North Chelmsford, MA).

MWCNT concentrations in bacteria and protozoa, were calculated as described in SI. Both volumetric bioconcentration factors (VCF, unitless) and bioconcentration factors (BCF, L/kg) were calculated for all the treatments: for the direct (via the media) bacterial and protozoan exposures to MWCNTs and for protozoan exposures to MWCNTs via bacteria (dietary exposure, SI). Trophic transfer factors (TTF) were also calculated for protozoan exposures to MWCNTs via bacteria (SI). MWCNT mass in protozoa was also estimated by analyzing optical microscopy images (SI) and the results were compared to ^{14}C-MWCNT concentrations quantified by LSC.

Statistical Analysis

After testing the normality using quantile-quantile plot statistical significances of means differences were determined using one-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparisons test (R, http://www.r-project.org/) or regression analysis (Microsoft Excel, Microsoft Corporation) with a p-value < 0.05 considered statistically significant. The values reported throughout the text are the mean values of at least 3 replicate samples ± standard deviation.

Results and Discussion

MWCNT Characteristics in Media and Effects on Bacterial Growth

The MWCNTs were relatively short (under 500 nm)[21] and well dispersed both in Nanopure water and bacterial growth medium (half-strength 21C; Table S3). The acid treatment during the MWCNT purification and surface-modification process added O-containing groups as indicated by the X-ray photoelectron spectroscopy (XPS) performed previously[21] and the negative ζ-potential values at neutral pH (Table S3). This contributed to the MWCNTs' high aqueous dispersibility and stability. Previously, short functionalized MWCNTs have exhibited strong antibacterial effects when deposited on filters,[32] although acid-treated MWCNTs in suspensions had no antimicrobial activity up to concentrations of 500 mg/L to 875 mg/L.[33] Here, MWCNTs suspended in bacterial growth medium at 0.1 mg/L to 1 mg/L did not affect the specific growth rate and maximum yield of *P. aeruginosa* (Figure S2B). Similar results showing a lack of a toxic effect on specific algal growth rate at a comparable dose of MWCNTs (1 mg/L) were recently observed.[16]

Quantification of MWCNTs Associated with *P. aeruginosa*

At the nominal ^{14}C-MWCNT concentrations of 0.01 mg/L and 1 mg/L, the measured total ^{14}C-MWCNT concentrations in the bacterial suspensions were (0.0058 ± 0.0005) mg/L and (0.64 ± 0.12) mg/L, respectively, indicating that approximately 40 % of added MWCNTs had adsorbed to the flask walls during the incubation and vigorous shaking (250 rpm [26 rad/s]) of the cultures. Thus, in the *P. aeruginosa* cultures prepared for trophic transfer, the recovery of ^{14}C label after 1-h incubation with ^{14}C-MWCNTs was approximately 60 %.

After separating unbound MWCNTs from bacteria by sucrose density gradient centrifugation, the ^{14}C-MWCNT mass associated with the bacterial cells was measured and normalized to the bacterial cell count in the harvested culture ([1.9×10^8 ± 2×10^7] cells/mL and [1.7×10^8 ± 3×10^7] cells/mL, in the 0.01 mg/L and 1 mg/L of MWCNTs treatments, respectively). At nominal concentrations of 0.01 mg/L and 1 mg/L, (76 ± 17) % and (70 ± 15) % of the recovered total MWCNT mass in the cultures was adsorbed to the bacterial cells. The calculated MWCNT masses per *P. aeruginosa* cell were (0.022 ± 0.005) fg and (2.7 ± 0.5) fg, respectively. Assuming a bacterial cell mass of 0.12 pg as determined previously[26] (SI, p. S10), the respective MWCNT masses per dry mass of bacteria were (0.18 ± 0.04) μg/mg and (21.9 ± 4.2) μg/mg. In comparison, when the alga *Desmodesmus subspicatus* was grown with 1 mg/L of ^{14}C-MWCNTs, the mean MWCNT concentration associated with algae increased over time, and reached 4.98 μg/mg dry mass of algae by 72 h.[16] This value is approximately 20 % of that measured for bacteria in this study at the dose of 1 mg/L of MWCNT and can likely be explained by the lower surface area per unit dry mass of algae available for MWCNT association. Although some MWCNTs were shown to enter the algal cytoplasm, most were agglomerated around the cell,[16] which was also the likely association between bacteria and MWCNTs in this study. The retention of the ^{14}C label, as a tracer for MWCNTs, in the bacterial pellet after density gradient centrifugation indicates that MWCNTs and bacteria were strongly associated, possibly facilitated by interactions with extracellular polymeric substances (EPS).[34–36] MWCNT association with cell envelopes of bacteria without internalized MWCNTs has been demonstrated by other researchers using transmission electron microscopy.[37, 38] Since MWCNTs did not damage the bacterial membranes (Figure S3), the MWCNTs were assumed not to enter bacterial cells. Thus, MWCNT adsorption to the cell surface rather than accumulation inside bacteria is a plausible scenario for the trophic transfer of MWCNTs.

Influence of Feeding Regime on *T. thermophila* Growth and MWCNT Effects on the Protozoa

Trophic transfer of MWCNTs by bacteria to protozoa was studied in comparison to direct uptake of MWCNTs from the medium. At the MWCNT concentrations tested (0.004 mg/L to 1 mg/L), *T. thermophila* population growth was unaffected either during axenic growth in rich medium or in Dryl's medium with *P. aeruginosa*, indicated by the fact that the specific growth rates and maximum yields were not significantly different from control cultures (Table S4 and Figure S4). The growth of *T. thermophila* was exponential between 2 h and 16 h both in rich medium and in Dryl's medium containing *P. aeruginosa* (Figure S4). However, *T. thermophila* grew significantly (two-sample t-test, $p \leq 0.05$) faster and yielded higher cell

numbers in rich growth medium than when feeding on *P. aeruginosa*, despite the longer lag phase in rich medium (Table S4 and Figure 1). The latter was likely caused by the adaptation phase after transferring protozoan cultures, which had been previously starved overnight in Dryl's medium, to the rich medium. In other studies that used different media, SWCNTs at concentrations above 6.8 mg/L induced cell death in *T. thermophila* incubated in non-nutrient medium,[39] and MWCNTs administered at 100 mg/L were growth inhibitory to *T. pyriformis* in filtered pond water.[40] In the current study, besides not affecting *T. thermophila* population growth in either feeding regime (i.e. in either rich medium, or in starvation medium with bactivory), MWCNT exposure also did not impair membrane integrity and was not lethal in Dryl's medium at concentrations up to 1 mg/L and 5 mg/L, respectively (Figure S5).

MWCNT Uptake by *T. thermophila* Administered Directly in the Medium

MWCNT mass per cell was measured for *T. thermophila* exposed to 0.3 mg/L or 1 mg/L of MWCNTs over the course of a 22-h growth period in the rich medium (Figure 1A). The MWCNT mass per cell clearly depended on MWCNT dose during the first 16 h of exposure. For both MWCNT doses, the MWCNT mass per protozoan cell was the highest at 2 h and then decreased as the cell concentration increased over time (Figure 1A). The trend is clearly shown in the scatter plot of logarithm-transformed MWCNT masses and protozoan cell densities (Figure S6A). The decreasing cellular content of MWCNTs, as the biomass increased while the mass of MWCNTs in the system remained the same, was also apparent in Nomarski microscopy images of *T. thermophila* acquired over the time course of direct feeding of MWCNTs in rich media (Figure 2).

However, at the population level, the MWCNT mass retained in the protozoa correlated positively with the cell number (Figure S6B). The fraction of total administered MWCNTs in protozoan populations increased over the first 8 h independently of administered MWCNT dose (Figure 3). The maximum percentage of MWCNTs in the population was reached twice as quickly for the 1 mg/L (8 h) compared to for the 0.3 mg/L concentration (16 h). The final MWCNT masses within the entire population were (0.003 ± 0.0004) mg and (0.007 ± 0.002) mg for the 0.3 mg/L and 1 mg/L doses, respectively. These statistically similar masses constituted between 70 % to 80 % of the initially added MWCNTs and did not statistically change between 8 h and 16 h (Figure 3), indicating a maximum uptake level of the administered MWCNTs by the growing protozoan populations. That the MWCNT mass in the total population remained below 100 % is likely a result of the dynamics of ingestion, egestion and reuptake of particulate matter by protozoa as discussed in more depth in subsequent sections. This was also evident in a TiO_2 nanoparticle (NP) direct uptake study, where, at a comparable cell density to this study, 35 % of the total administered TiO_2 at a dose of 100 mg/L was within the total population by 22 h.[27] However, in the prior study where the supply of NPs was not limited (at 100 mg/L of TiO_2 NPs), protozoa were capable of ingesting a 60-fold higher mass of NPs (0.42 mg TiO_2 NPs *versus* 0.007 mg MWCNTs). Thus, even when taking into account the difference in densities of TiO_2 (3.97 g/cm^3) and MWCNTs (1.5 g/cm^3), we conclude that the dose of MWCNTs was a limiting factor to the uptake, and most of the MWCNTs were ingested by the protozoa by 8 h.

Uptake of MWCNTs by *T. thermophila* Trophically Transferred via MWCNT-Encrusted *P. aeruginosa*

In the trophic transfer experiments, *P. aeruginosa* that had been pre-exposed to 0.01 mg/L or 1 mg/L of MWCNTs and suspended in Dryl's medium at respective concentrations of $(1.8\times10^8 \pm 1.8\times10^7)$ cells/mL and $(1.2\times10^8 \pm 2\times10^7)$ cells/mL, resulted in doses to *T. thermophila* of 0.004 mg/L and 0.3 mg/L of MWCNTs, respectively (Table S1). As in the direct exposures, the MWCNT mass per *T. thermophila* cell was dose-dependent at each time point measured (Figure 1B). The MWCNT uptake trends over the 22-h growth period differed from those of direct uptake, but also differed at lower and higher MWCNT concentrations within the feeding regime: *T. thermophila* grazing on bacteria with 0.3 mg/L MWCNTs contained significantly higher levels of MWCNTs per cell at 2 h and 8 h of growth than at 16 h and 22 h, while there was no significant difference in the mass of MWCNTs per cell during growth when protozoa were fed bacteria with 0.004 mg/L of MWCNTs. Similarly to direct uptake, a decrease in MWCNT mass per *T. thermophila* cell occurred over time. The trend was statistically significant during trophic transfer of 0.3 mg/L of MWCNTs, but not for the lower MWCNT dose (0.004 mg/L, Figure S6A and Figure 1B).

Across the whole population, the retained MWCNT mass increased with higher protozoan cell numbers (Figure S6B). The fraction of total administered MWCNTs in protozoan populations increased over the first 8 h during the trophic transfer experiments for both MWCNT doses, and the maximum was reached at 16 h (Figure 3). Differently from the direct uptake of MWCNTs, the fraction of MWCNTs in the protozoan populations decreased to approximately 15 % by 22 h. Although the total cell number of *T. thermophila* grown with *P. aeruginosa* was approximately 1/6 of that in rich medium at 22 h, all cultures had reached stationary growth phase by the end of the experiment (Figure S4). Thus, the difference in MWCNT accumulation in protozoan populations during the two feeding regimes can be explained by the feeding patterns of *T. thermophila* and the availability of MWCNTs for reuptake after cellular excretion. In the trophic transfer experiments, the protozoan food vacuoles were packed with bacteria which limited the amount of MWCNTs internalized by protozoa, while there was no such physical restriction in the direct uptake exposure conditions. Accumulation of fecal pellets and agglomerated bacteria was evident in the Nomarski images at later trophic transfer time points (16 h and 22 h; Figure 4), suggesting that excreted MWCNTs were incorporated into fecal pellets that were not reingested by protozoa. This explains the decrease in the relative MWCNT mass in the protozoa at 22 h (Figure 3). Accumulation of fecal pellets in the medium was not evident in the images of *T. thermophila* grown in rich medium (Figure 2), indicating that MWCNTs were excreted as aggregates that were small enough for reuptake, resulting in a higher percentage of administered MWCNTs in the protozoan population (Figure 3). Comparatively, Chan et al.[41] showed that initial ingestion of subtoxic amounts of SWCNTs by *T. thermophila* impaired subsequent digestion of *Escherichia coli* and increased the number of egested fecal pellets. Here, grazing on MWCNT-amended *P. aeruginosa* did not appear to alter the numbers of fecal pellets compared to control cultures (Figures 4 and S7).

Quantification of MWCNT Bioaccumulation and Biomagnification

Classical risk assessment of dissolved chemicals defines bioconcentration as increase in the concentration of a chemical substance in or on an organism relative to the concentration of the chemical in the surrounding medium, and bioaccumulation as a process in which the chemical concentration in an organism exceeds that in the medium and the diet.[42] However, it has been acknowledged that quantification and interpretation of NP bioaccumulation requires a different approach because of properties of NPs that are distinct from those of hydrophobic organic contaminants (HOC) or metals.[43, 44] Translocation of NPs, particularly carbonaceous ones, across epithelial cells (e.g., microvilli) and into organisms' tissues is generally limited, but NPs may become trapped in the digestive tract and not eliminated even after organismal feeding;[14, 45, 46] in these cases, NPs could still be considered as being accumulated.[47]

In the current study, MWCNTs became adsorbed to the surface of *P. aeruginosa*. MWCNTs were accumulated in the food vacuoles of *T. thermophila* when they were directly exposed to MWCNTs in the medium or fed MWCNT-encrusted bacteria. To demonstrate the magnitude of association between MWCNTs and test organisms, and to compare with the published literature, bioconcentration factors (BCF) were calculated in two ways (SI). The first followed the definition conventionally used in risk assessment of chemicals (BCF expressed in L/kg dry mass)[42] and the second was the unitless volumetric concentration factor (VCF).[26, 27]

The BCFs of MWCNTs for *P. aeruginosa* were (230,000 ± 180,000) L/kg dry mass and (130,000 ± 50,000) L/kg dry mass of bacteria after exposure to 0.01 mg/L and 1 mg/L MWCNTs, respectively. These two BCFs, which are not statistically different, indicate a high propensity of MWCNTs to associate with bacterial cells. The corresponding VCFs were 40,000 ± 30,000 and 35,000 ± 10,000 after exposure to 0.01 mg/L and 1 mg/L MWCNTs, respectively. In comparison, CdSe quantum dots that damaged bacterial membranes and bioaccumulated in cells resulted in much lower VCF of 70.[26] However, 100 mg/L TiO_2 NPs that, similarly to this study, did not enter cells, fully adsorbed to bacterial membranes.[27] In the latter case, the putative BCF is infinity and thus not meaningful, but — despite the difference in NP morphologies — the comparison may indicate that BCFs could have been greater at higher MWCNT exposure concentrations. A direct comparison for MWCNTs was only available for unicellular algae, with a BCF of 5000 L/kg dry mass.[16] This value is two orders of magnitude lower than in this study, likely because of the lower available surface area per unit dry mass of algae compared to bacteria.

In prior studies, NP-amended *P. aeruginosa* were fed to *T. thermophila,* and NPs accumulated in protozoa through dietary intake, with biomagnification of QDs[26] and without biomagnification of TiO_2 NPs.[27] Herein, MWCNTs in the same microbial food chain were trophically transferred similarly to TiO_2 NPs in that MWCNTs accumulated in *T. thermophila* but did not biomagnify, as indicated by trophic transfer factors (TTF) below 1 (ranging from 0.01–0.04) for both MWCNT doses and all time points (Table S6). MWCNTs, like TiO_2 NPs, accumulated in the cells but were confined to the food vacuoles and were continuously excreted into the surrounding medium. The fact that localization of MWCNTs was likely limited to protozoan food vacuoles was supported by significant linear

correlations between MWCNT mass versus MWCNT area per cell as measured in the Nomarski images after direct MWCNT uptake (Figure S8), and MWCNT mass versus the total number of food vacuoles in *T. thermophila* population in trophic transfer experiments (Figure S9). Among other test systems where NPs have been shown to be trophically transferred,[48–50] only a few have indicated biomagnification.[51, 52]

The BCFs calculated herein for *T. thermophila* grown in MWCNT-amended medium or when grazing on MWCNT-encrusted bacteria, and when sampled at different times, ranged from 35,000 L/kg [log BCF = 4.5] to 800 L/kg [log BCF = 2.9] (Tables S5 and S6, Figure 5). These values are within the same order of magnitude as the logarithm-transformed BCF values of 3.74 to 5.64, calculated for CNTs in daphnids after exposure to between 0.04 mg/L and 0.4 mg/L of ^{14}C-labeled CNTs.[53] Considering that "very bioaccumulative" substances, as defined by regulatory agencies in the Unites States, the European Union and Canada, have log BCF values ≥ 3.7,[54] the values calculated herein and also those reported in the literature for daphnids[53] suggest that NPs have a high propensity for bioaccumulation both in protozoa and daphnids. However, considering that MWCNTs have a low potential for crossing the cell membranes or for absorption into tissues,[55, 56] the accumulated MWCNTs are likely retained in the digestive system. Thus, the BCFs are not directly comparable to those calculated for HOCs or metals.

Comparison of the BCFs calculated for *T. thermophila* at different time points during direct exposure and trophic transfer of MWCNTs indicated higher bioaccumulation of MWCNTs when taken up directly from the medium than by bactivory at 2 h and 8 h (Figure 5). However there appeared to be no BCF dependence on dose or feeding regime at 16 h and 22 h. Higher accumulation of NPs in the case of direct aqueous exposure compared to trophic transfer has been reported previously for gold NP transfer from algae to mussels,[57] and for TiO_2 NPs from daphnids to zebrafish.[58] However, marine mussels accumulated CeO_2 NPs in equal amounts, regardless of whether the NPs were associated with phytoplankton or as free particles in the water column[59] and freshwater snails accumulated higher amounts of CuO NPs via dietary intake compared to waterborne exposure.[60] *T. thermophila* accumulated similar masses of TiO_2 NPs by direct exposure in the medium and via feeding TiO_2 NP-encrusted bacteria.[27] For a fast growing unicellular organism, like *T. thermophila*, and in the limiting MWCNT exposure concentrations used here, the decrease of calculated BCF values observed as a function of time during population growth in direct feeding on MWCNTs (Figure 5) likely reflects the changing ratio between the biomass and MWCNT mass in the system: as the biomass increased over time (from 2 h to 22 h, Figure 1 and S6), the BCF values generally decreased at each administered MWCNT dose (Figure 5). Still, both direct exposure and trophic transfer of MWCNTs resulted in similar BCFs by the end of exposure (22 h), indicating that regardless of MWCNT dose and feeding regime, MWCNTs bioaccumulated in protozoa.

Environmental Implications

T. thermophila was exposed to MWCNTs via direct feeding in rich media or via trophic transfer by bactivory of MWCNT-encrusted *P. aeruginosa*. Nominal exposure concentrations of MWCNTs in media were on the same order of magnitude as those predicted in aquatic

environments by modeling, i.e. down to the μg/L level.[18] Working with such low concentrations was enabled by the novel application of AMS to quantify very low levels of ^{14}C from ^{14}C-MWCNTs sorbed to bacteria or bioaccumulated in protozoa. At low exposure concentrations of MWCNTs, *T. thermophila* indiscriminately ingested and bioaccumulated MWCNTs in a closed system, regardless of whether MWCNTs were made available as free agglomerates or as coatings on bacterial prey. Since for either feeding regime there was bioaccumulation of MWCNTs during population growth, protozoa would be reliable vectors for transferring MWCNTs to the next trophic level. This research also showed that, depending on the objective, future studies can be simplified by focusing on quantitative image analysis to assess *T. thermophila* bioaccumulation of carbonaceous nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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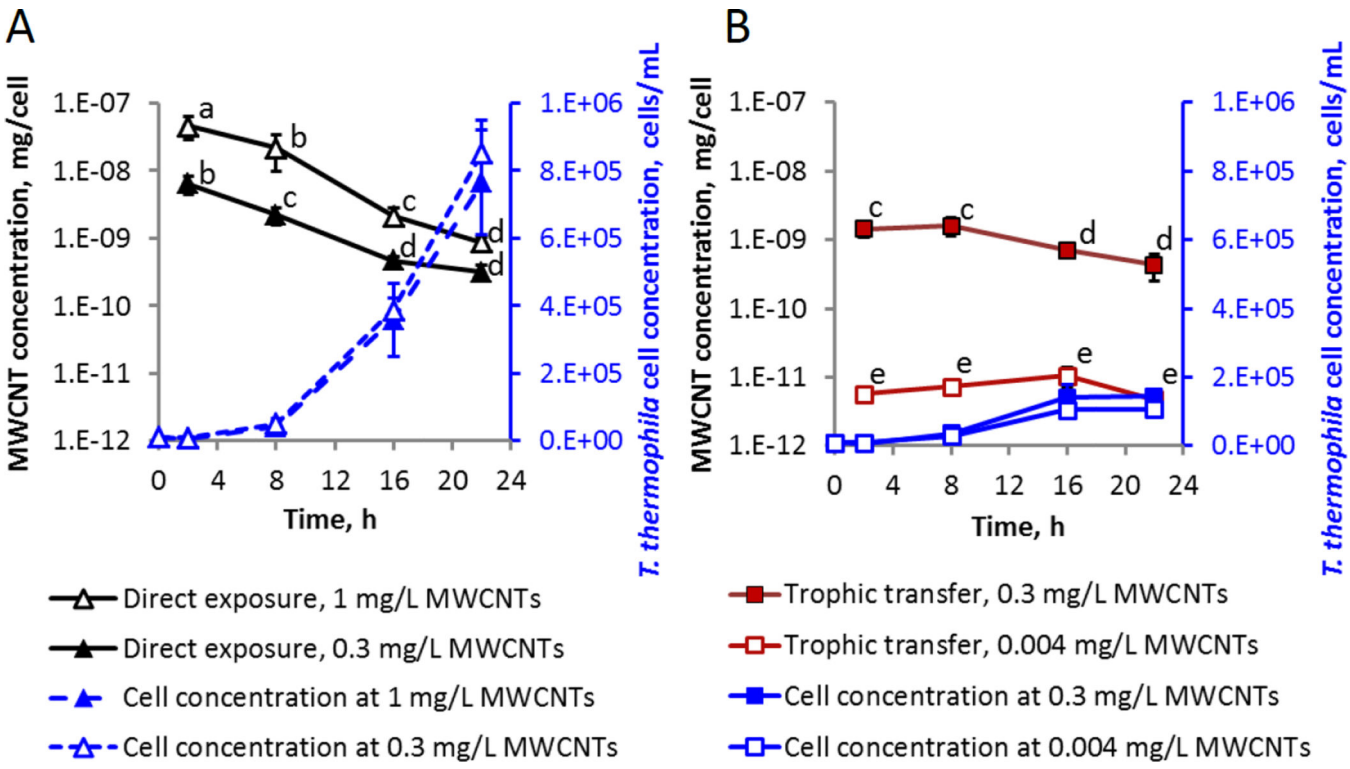

Figure 1.

MWCNT masses per *T. thermophila* cell and the cell densities of *T. thermophila* during the direct exposure to (A), and trophic transfer of (B), MWCNTs. Data points are average values of at least 3 replicates; error bars indicate standard deviation. In cases of very small standard deviations, error bars are not visible beyond the symbol. Data points with the same letter are not significantly different from one another; Tukey's multiple comparisons test, $p \leq 0.05$. Note the logarithmic scale of the left vertical axis. MWCNT doses listed in the legend are the nominal doses in the case of the direct exposures, and bacterial cell-associated doses in the trophic transfer experiments (Table S1). Note that the *T. thermophila* growth curves corresponding to the control (no MWCNTs) treatments in each media (SSP for direct exposure, or Dryl's medium with *P. aeruginosa* for trophic transfer) are not shown for simplicity, since the exposure to MWCNT within each feeding regime did not affect the *T. thermophila* specific growth rate (Figure S4 and Table S4).

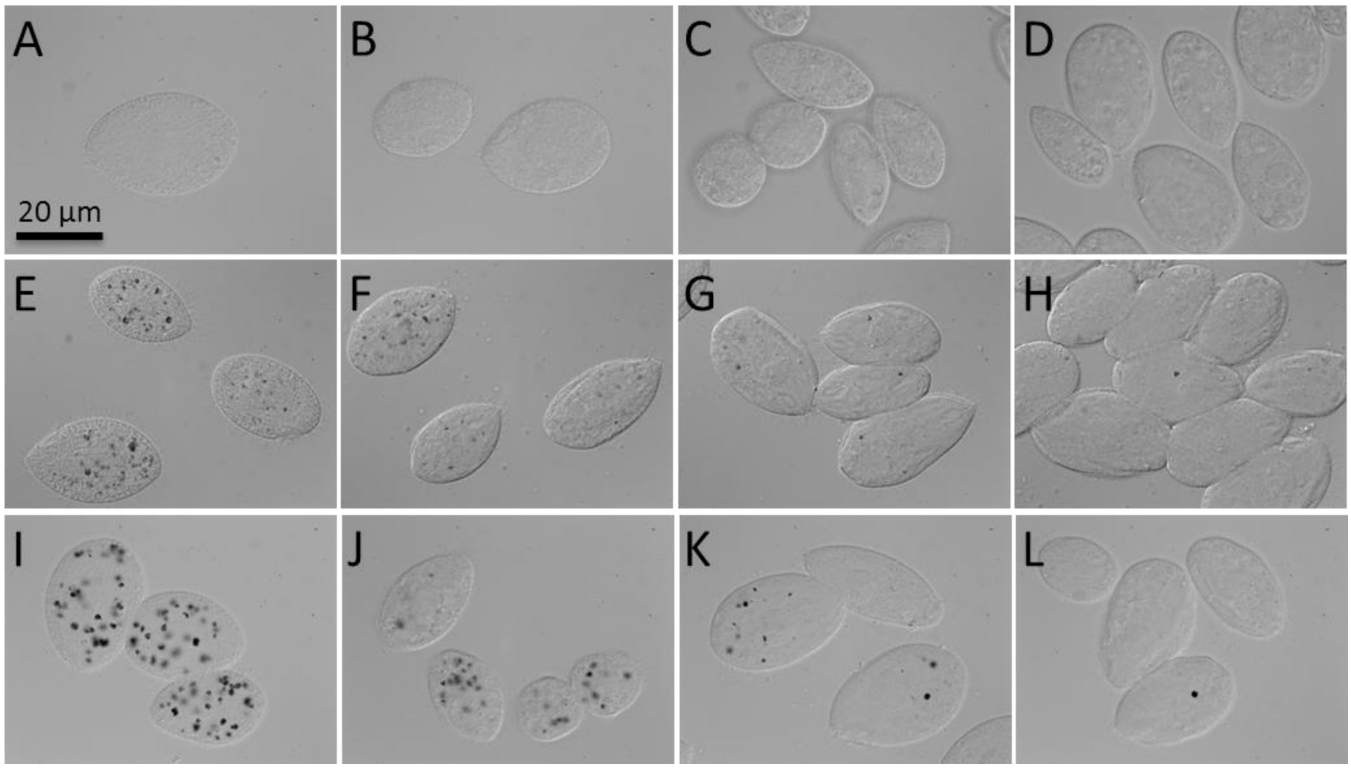

Figure 2.

Nomarski images of *T. thermophila* grown without MWCNTs (A–D), with 0.3 mg/L (E–H) and 1 mg/L (I–L) MWCNTs in the rich growth medium for 2 h (A, E, I), 8 h (B, F, J), 16 h (C, G, K) and 22 h (D, H, L). MWCNT aggregates internalized by phagocytosis appear as black areas in the food vacuoles of the cells grown with MWCNTs (E –L) while no black spots were detected in the control cells (A–D).

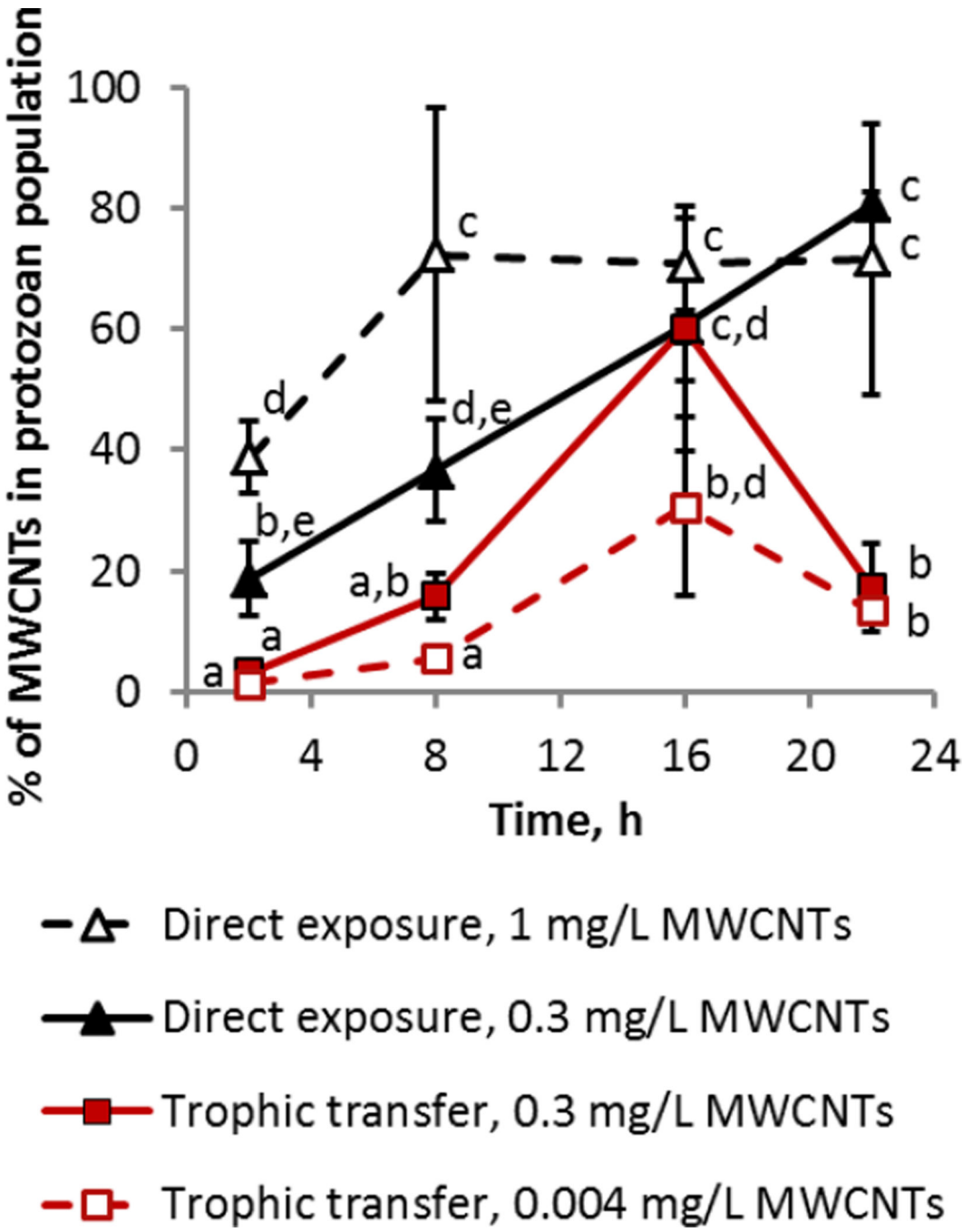

Figure 3.

Percent of administered MWCNT mass retained in the *T. thermophila* population. Average values of at least 3 replicates are graphed and the error bars indicate the standard deviation. In the case of very small standard deviations, the error bar is not visible beyond the symbol. Data points with the same letter are not significantly different from one another; Tukey's multiple comparisons test, $p \leq 0.05$.

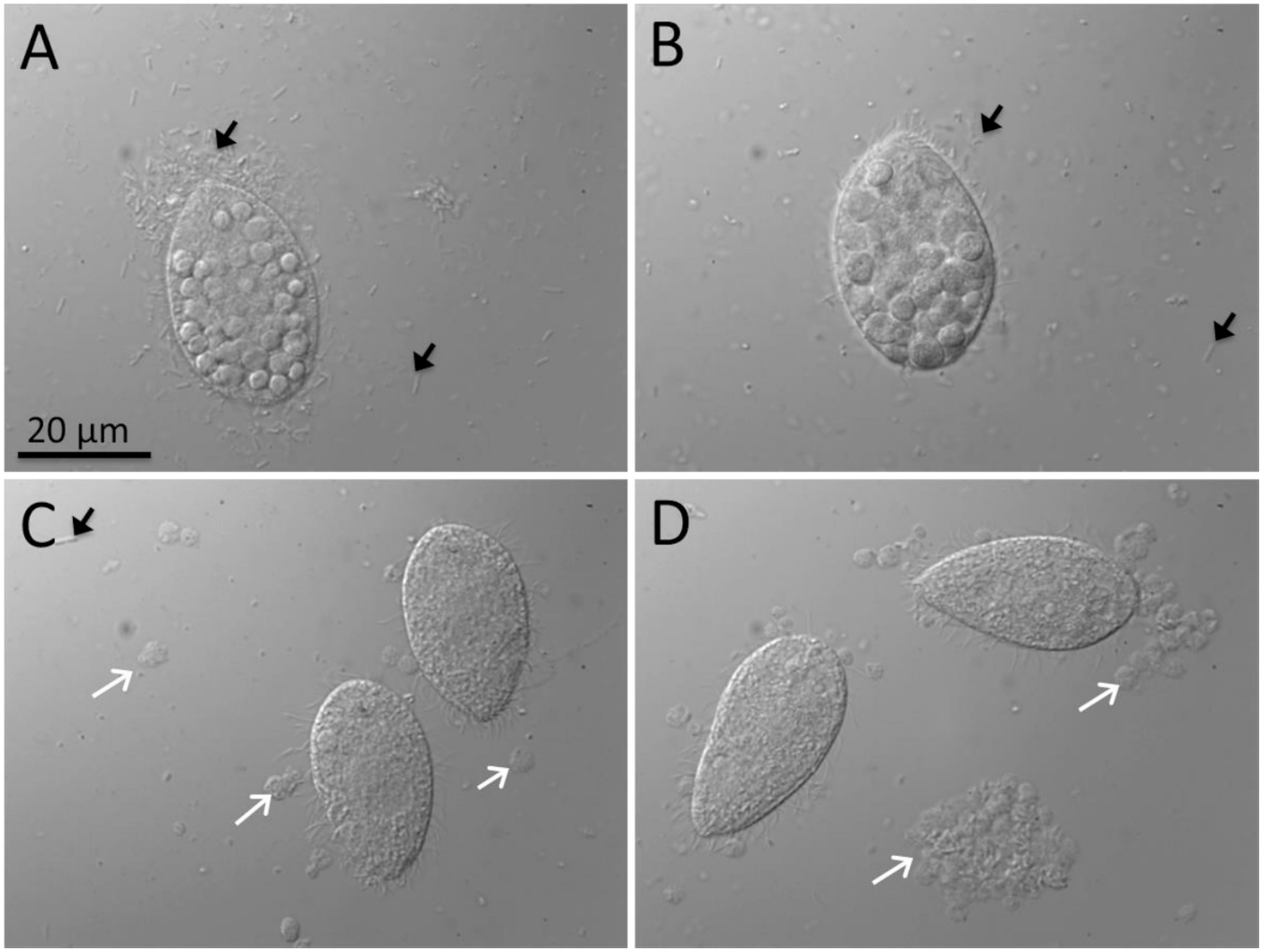

Figure 4.

Nomarski images of *T. thermophila* grown with MWCNT-encrusted *P. aeruginosa* as prey (MWCNT dose: 0.3 mg/L) for 2h (A), 8h (B), 16h (C) and 22h (D). Black arrows indicate bacteria which are abundant at 2 and 8 h and white arrows show fecal pellets evident at 16 and 22 h. The round shapes inside *T. thermophila*, well visible in A and B, are food vacuoles filled with *P. aeruginosa*.

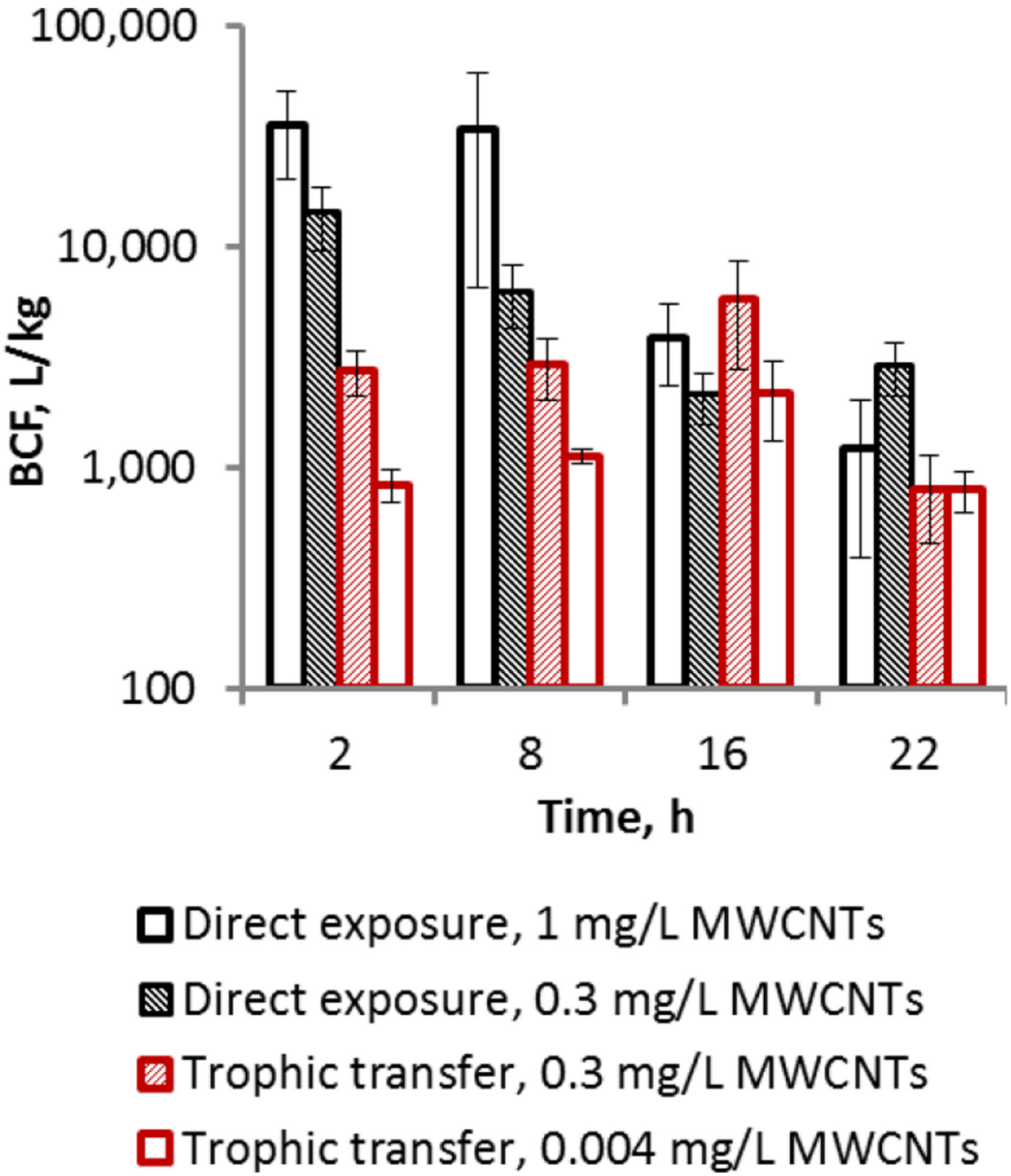

Figure 5.

Bioconcentration factors (BCFs) of MWCNTs at different time points during *T. thermophila* growth in the presence of MWCNTs, administered either directly in the medium (direct exposure) or with MWCNT-encrusted *P. aeruginosa* (trophic transfer). The bars indicate BCFs calculated using the mean MWCNT concentration values of three replicates (equations 11 and 12 in SI; Tables S5 and S6) and error bars indicate errors propagated using standard methods.