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Interactions between multi-walled carbon nanotubes and plankton as detected by Raman spectroscopy

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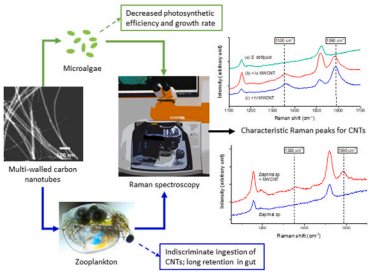
HIGHLIGHTS

- Raman spectroscopy was able to detect MWCNTs in biological specimens *in vivo*.
- Exposure to MWCNTs decreased phytoplankton photosynthetic efficiency and/or specific growth rate.
- Zooplankton could dislodge some of the externally attached MWCNTs by grooming.
- Zooplankton ingested and retained MWCNTs in their guts for an extended time.

GRAPHICAL ABSTRACT



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ABSTRACT

Raman spectroscopy has been commonly used in materials science to detect chemicals. Based on inelastic scattering of light after incident photons interact with a molecule, it has high potential for non-destructive detection of specific contaminants in living biological specimens. The increasing use of carbon nanotubes (CNTs) increases its chance to enter the aquatic habitats through direct discharge, surface runoff and atmospheric deposition, but their potential environmental impacts remain poorly known. We tested the use of Raman spectroscopy to investigate the interactions between multi-walled CNTs (MWCNTs) and aquatic plankton *in vivo*. For phytoplankton cells (*Scenedesmus obliquus*) that were exposed to MWCNTs, Raman spectroscopy was able to distinguish between background biological material and MWCNTs that adhere to the cells (G-band peak at 1590 cm^{-1} and D-band peak at 1350 cm^{-1} in the Raman spectra that were unique to MWCNTs). Harmful effects of MWCNT exposure manifested as lower photosynthetic efficiency and/or lower specific growth rate in the phytoplankton. MWCNT particles also adhered to the body surface of zooplankton, especially the carapace. Both *Ceriodaphnia* sp. and *Daphnia* sp. ingested MWCNTs directly, which was verified by the signature G-band and D-band Raman peaks in the zooplankton gut region. MWCNTs remained in the gut overnight after the zooplankton had been returned to clean water, showing that the zooplankton retained MWCNTs inside their body for an extended time, thereby increasing the chance to disperse and transfer the contaminants throughout the aquatic

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food web. Our results demonstrate that Raman spectroscopy is a promising method for non-destructive investigation of the uptake and dynamic fate of CNTs and other contaminants in aquatic organisms.

1. Introduction

Carbon nanotubes (CNTs) are hollow cylindrical structures made of graphene rings (Roco, 2003) and are available in multi-walled (MWCNTs) and single-walled (SWCNTs) forms (Liu and Fan, 2005; Ebbesen and Ajayan, 1992). Thanks to their declining production cost, high mechanical strength and versatility (Mauter and Elimelech, 2008; De Volder et al., 2013), industrial production and usage of CNTs is rapidly expanding, which could increase pollution risks with unknown consequences (Du et al., 2013). Most CNTs are expected to enter landfills (Gottschalk et al., 2009) and the waterways (Köhler et al., 2008). Exposed of some aquatic organisms to high CNT concentrations in laboratory experiments have resulted in compromised growth and development, even death (e.g. Templeton et al., 2006; Cheng et al., 2007; Jang and Hwang, 2018). For prevention and monitoring purposes, it is desirable to be able to rapidly detect CNTs within environmental samples. Phytoplankton and zooplankton are microscopic organisms that form the base of the aquatic food web. Interactions between CNTs and plankton are of particular interest because they can be a pathway for bioaccumulation of material to the higher trophic levels.

Raman spectroscopy is based on the inelastic scattering of light when incident photons interact with a molecule (Kneipp et al., 2007). For CNTs, this shifts the frequency of light in a way that corresponds to the vibrational and rotational modes of the carbon atoms, thereby providing a chemical fingerprint of the CNTs (Dresselhaus et al., 2005). Raman spectroscopy is commonly used in materials science (Roy et al., 2004; Carvalho et al., 2017) but its applications for *in vivo* imaging of biological samples are rare (Rae et al., 2014). The method offers many advantages; e.g. it does not require wet chemistry and is non-destructive to the samples, and the laser can be targeted at specific areas to map the distributions of CNTs in the sample. MWCNTs give specific Raman signals (G-band and D-band peaks at Raman shift ~1590 cm^{-1} and ~1350 cm^{-1}, respectively) that distinguish them from biological constituents (Malard et al., 2009).

Here we test the use of Raman spectroscopy to detect the interactions between MWCNTs and plankton in terms of adhesion and internalisation. We also made ancillary observations to explore potential sublethal effects of the interactions.

2. Materials and methods

2.1. Preparation of MWCNT suspensions

Raw MWCNTs (0.991 g) were created by chemical vapour deposition of toluene/polystyrene feedstock at 750 °C using ferrocene catalyst as described previously (Hedayati et al., 2019; Orbaek White et al., 2021). All MWCNTs used in the experiments were taken from the master batch. The CNTs were characterised with transmission electron microscopy (TEM) and Raman spectroscopy. The TEM was conducted with a Thermo Fisher Scientific Talos Transmission Electron Microscope with high-resolution TEM mode operating at 200 kV. The TEM samples were prepared by dipping graphene coated holey carbon TEM grids into CNT powders. Raman spectroscopy was conducted with a Renishaw inVia Raman microscope with 633 nm laser wavelength that is typically operated at < 5% beam power between 100 cm^{-1} and 3200 cm^{-1} Raman shift. The laser beam is usually focused by maximizing the intensity of the G-peak to confirm beam length alignment between detectors and the sample. Images were analysed using ImageJ software for diameter, tube length and topological morphology.

Deionised water and all glassware were autoclaved before use. MWCNTs were weighed out, mixed into water in a flask and stirred with a glass rod to separate large agglomerates. The flask was then placed in a water bath and sonicated with a titanium probe (Cole-Parmer CP505; frequency 20 kHz; actualised power output 205.46 J s^{-1}) for 20 min to create a homogeneous MWCNT suspension (Taurozzi et al., 2011). Water without MWCNTs was treated in the same way and used for the control.

2.2. Phytoplankton experiments

The chlorophyte *Scenedesmus obliquus* was cultured in BG-11 medium (1:100 v/v; Sigma-Aldrich). To begin the experiments, aliquots of the culture were mixed into 200 mL of MWCNT suspensions (nominal concentrations: 0, 5 and 50 mg L^{-1}) in flasks to achieve a starting cell concentration of 1.20×10^7 cells mL^{-1} (n = 4). The flasks were capped and placed in an incubator (16 h light: 8 h dark; 20 °C).

Daily, the flask content was gently mixed; a 2-mL aliquot was removed to measure photosynthetic efficiency in terms of quantum yield (QY) using an Aquapen (AP 110-C; Photon Systems Instruments). QY was measured without dark adaptation to obtain readings that were indicative of the real-time photosynthetic activity of the cells (Murchie and Lawson, 2013). A QY value around 0.6 indicates healthy cells, whereas a precipitous drop in QY would indicate stress (Rascher et al., 2000; Shelly et al., 2010). Another 1-mL aliquot was transferred to a Sedgwick-rafter counting chamber for cell counts. The procedures were repeated for 5 days. At the end of the experiments, aliquots were removed for Raman spectroscopy, using silicon wafer for calibration. Sample slides were prepared without coverslip to minimise interference. The laser was focussed at the cells to check for MWCNT adhesions while avoiding any free MWCNTs in the surrounding water. Unexposed *S. obliquus* cells were used as the control.

2.3. Zooplankton experiments

The cladocerans *Daphnia* sp. and *Ceriodaphnia* sp. collected from a local pond were maintained in stock cultures in an incubator (20 °C, 16 h:8 h light: dark cycle), and were fed uncontaminated *S. obliquus* to maintain healthy animals before doing subsequent experiments. To test for MWCNT adhesion, the animals were placed individually into beakers (n = 8) with 50 mL of MWCNT suspension (nominal concentration 25 mg L^{-1}). Each individual was observed for external adhesions under a light microscope (Olympus model: BX43) every 2 h for 10 h. Raman spectroscopy was also used to detect adhesions by focusing the laser on the exterior surfaces where adhesions were observed. Animals were individually placed on a glass slide and the surrounding water was removed with absorbent paper to minimise background fluorescence (Auer and Skinner, 2008).

Separate experiments were conducted to test for ingestion of MWCNTs by the zooplankton. Individuals (n = 10) were first fed *S. obliquus* until their guts were filled with food. Then, they were transferred to clean water and allowed to empty their guts through peristalsis. Afterward, the animals were exposed to a MWCNT suspension (25 mg L^{-1}) for 2 h. Images of the animals were taken throughout to follow the changes in gut colour from green (with *S. obliquus*) to colourless (empty gut) to black (with CNTs). The sequential colour change would confirm ingestion of MWCNTs. For *Daphnia* sp., the animals were kept in the MWCNT suspensions overnight after the experiment, then imaged by Raman spectroscopy in the following day by focusing the laser at the gut region; unexposed animals were used as the control. Afterward, the remaining animals with CNT-filled gut were returned to clear water overnight and examined the next day.

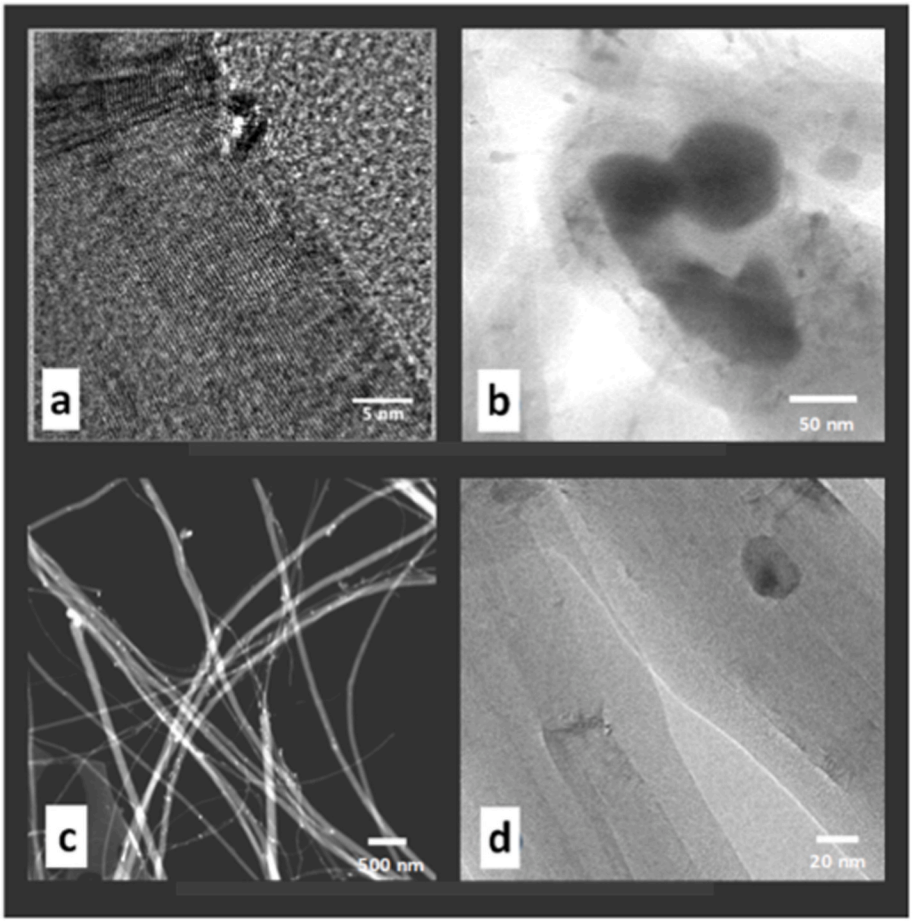

Fig. 1. TEM images of MWCNTs. (a) A single MWCNT with a kink; (b) MWCNT bundle; (c) MWCNT agglomerate structure; (d) Two MWCNTs with topological defects.

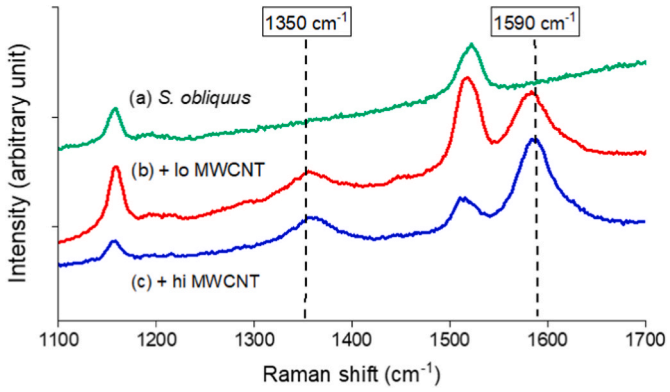

Fig. 2. Composite Raman spectra from the phytoplankton experiments. Unexposed *Scenedesmus obliquus* showed background peaks at ~1160 and ~1520 cm^{-1} (a); *S. obliquus* that had been exposed to low (5 mg L^{-1}) and high (25 mg L^{-1}) concentrations of MWCNTs showed additional peaks at 1590 cm^{-1} (G-band) and 1350 cm^{-1} (D-band) that indicate MWCNT adhesions (b and c). Note that Raman signal intensity is expressed in arbitrary unit and is not indicative of relative quantities.

3. Results and discussions

3.1. Multi-walled carbon nanotubes (MWCNTs)

The TEM images showed that the MWCNTs were intertwined networks of nanotubes with diameters in the range of 70–110 nm. Topological defects were seen as kinks (between 7- and 5- membered rings) but otherwise the nanotubes were well formed (Fig. 1).

3.2. Phytoplankton

Unexposed *S. obliquus* cells (control) showed peaks at ~1160 cm^{-1} and ~1520 cm^{-1} on the Raman spectra that likely indicated some carbon-rich constituents of the cells (Fig. 2a). Cells treated with MWCNTs (5 mg L^{-1} and 25 mg L^{-1}) showed the additional characteristic peak at 1590 cm^{-1} and 1350 cm^{-1} that signified MWCNT adhesion (Fig. 2b–c). Overall, our results showed that Raman spectroscopy can be a reliable method to detect MWCNT contamination of phytoplankton, especially in cases where MWCNTs may not be easily detected by conventional methods.

At the start of the experiments, cells in the control and treatments had similar QY values of 0.46–0.48. QY in both the control and lo-MWCNT treatment increased to ca. 0.60 towards the end of the experiment, indicating normal photosynthetic efficiency (Fig. 3a). Nevertheless, population increase in the lo-MWCNT treatment lagged behind the control, indicating that cell growth was impacted (Fig. 3b). In contrast, QY in the hi-MWCNT treatment decreased to zero after only 2 days, suggesting severe stress and highly compromised photosynthetic capacity (Fig. 3a), and cell abundance increased only slightly over the 5-day period (Fig. 3b). The equivalent specific growth rates (mean ± SE) were 0.41 ± 0.02 d^{-1} (control) 0.33 ± 0.01 d^{-1} (lo-MWCNT) and 0.12 ± 0.02 d^{-1} (hi-MWCNT), respectively (Fig. 3c), and the values were significantly different from each other (One-way ANOVA followed by Tukey test; $p < 0.05$).

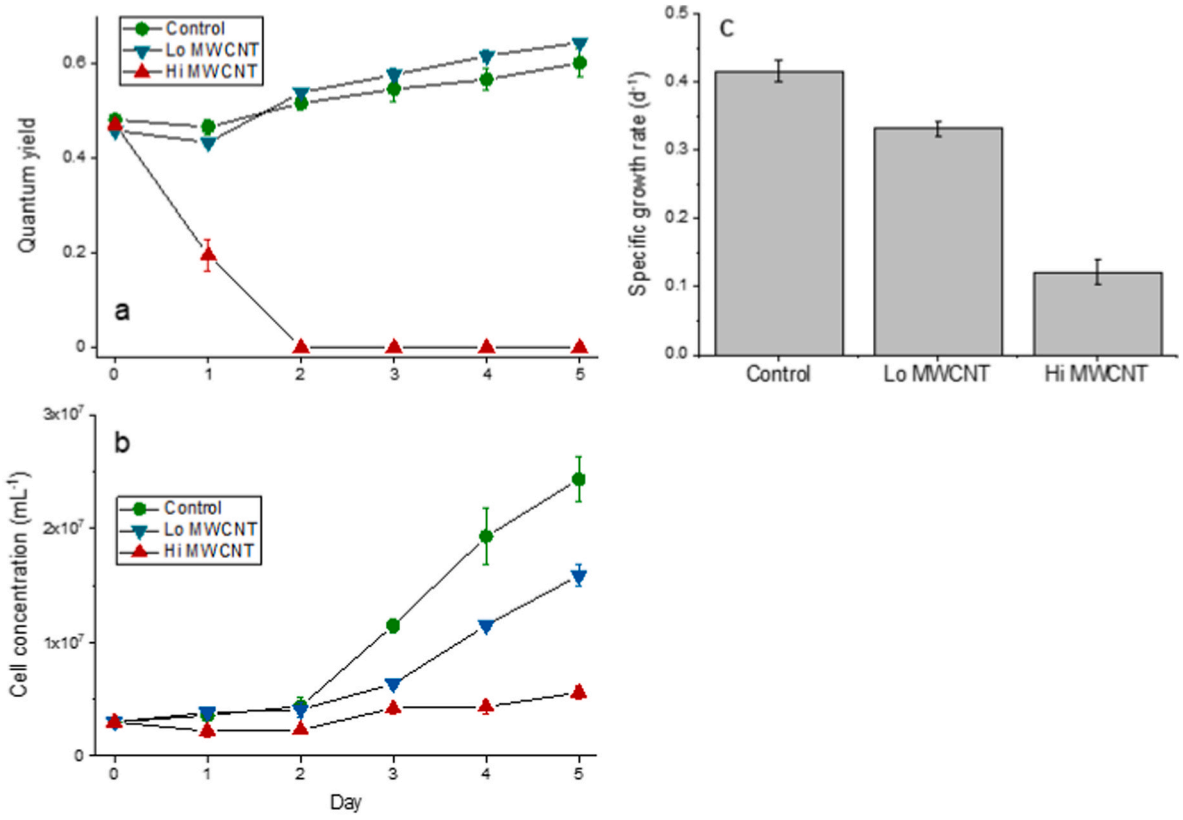

Fig. 3. Phytoplankton experiments. Response of *S. obliquus* cells to different MWCNT treatments in terms of quantum yield (a) and population growth (b). Their specific growth rates were significantly different from each other (c). Data are mean ± SE.

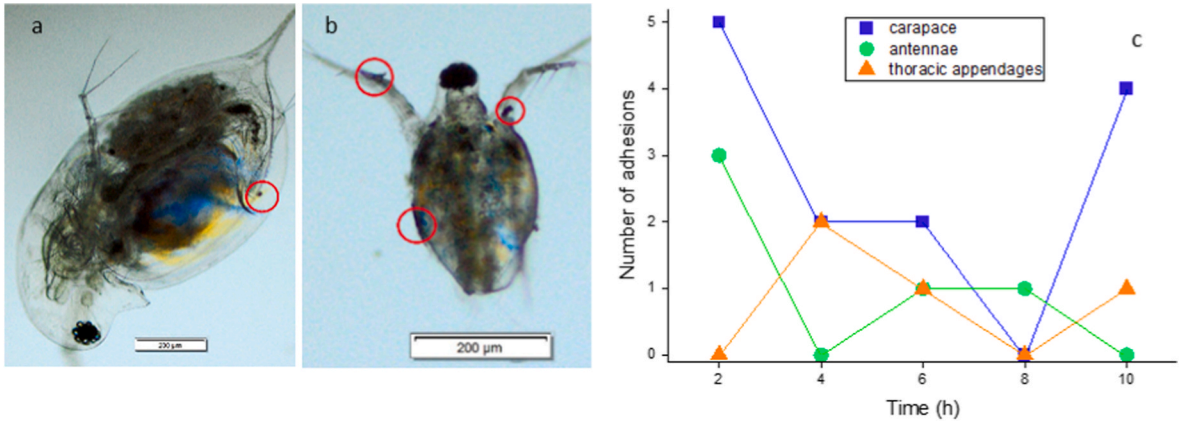

Fig. 4. Zooplankton experiments. Adhesion of MWCNTs to the exterior of *Daphnia* sp. (a) and *Ceriodaphnia* sp. (b) within 2 h of exposure (highlighted in circles; scale bar = 200 µm). Grooming behaviour in *Ceriodaphnia* sp. led to changes in number of adhesions through time (c).

3.3. Zooplankton

In the adhesion experiments, MWCNTs were rarely found on the body surface of *Daphnia* sp., but a few agglomerates of MWCNTs could be seen on *Ceriodaphnia* sp. under a light microscope (Fig. 4a–b). Raman spectroscopy failed to register the signature peaks, perhaps due to the small amounts of MWCNTs on the exterior. *Ceriodaphnia* sp. exhibited grooming behaviour in attempts to dislodge the particles such that the distribution of MWCNTs on its body surface changed through time (Fig. 4c). Adhesion to antennae and thoracic appendages appeared to drop off over time whereas a few adhesions remained on the carapace after 10 h.

In the ingestion experiments, both species showed the expected change in gut colour: green when being fed algae, then turned colourless after emptying in clean water, then black after ingesting MWCNTs (Fig. 5a–d). The full black gut suggests that the animals did not discriminate against MWCNTs in the feeding process. While no discernible ill effects were observed, non-discriminant ingestion of MWCNTs would inevitably diminish the nutritional intake by the organisms.

Raman spectroscopy was applied to the gut region of the *Daphnia* sp. Unexposed *Daphnia* sp. gave peaks at 1160 cm^{-1} and 1520 cm^{-1} (Fig. 5e). The peak at 1160 cm^{-1} is similar to the one for *S. obliquus* cells (Fig. 2) and may indicate some common biomolecules. The strong signal at 1520 cm^{-1} was likely caused by chitin in the carapace (Brugnerotto et al., 2001). *Daphnia* sp. that had been exposed to MWCNTs showed the

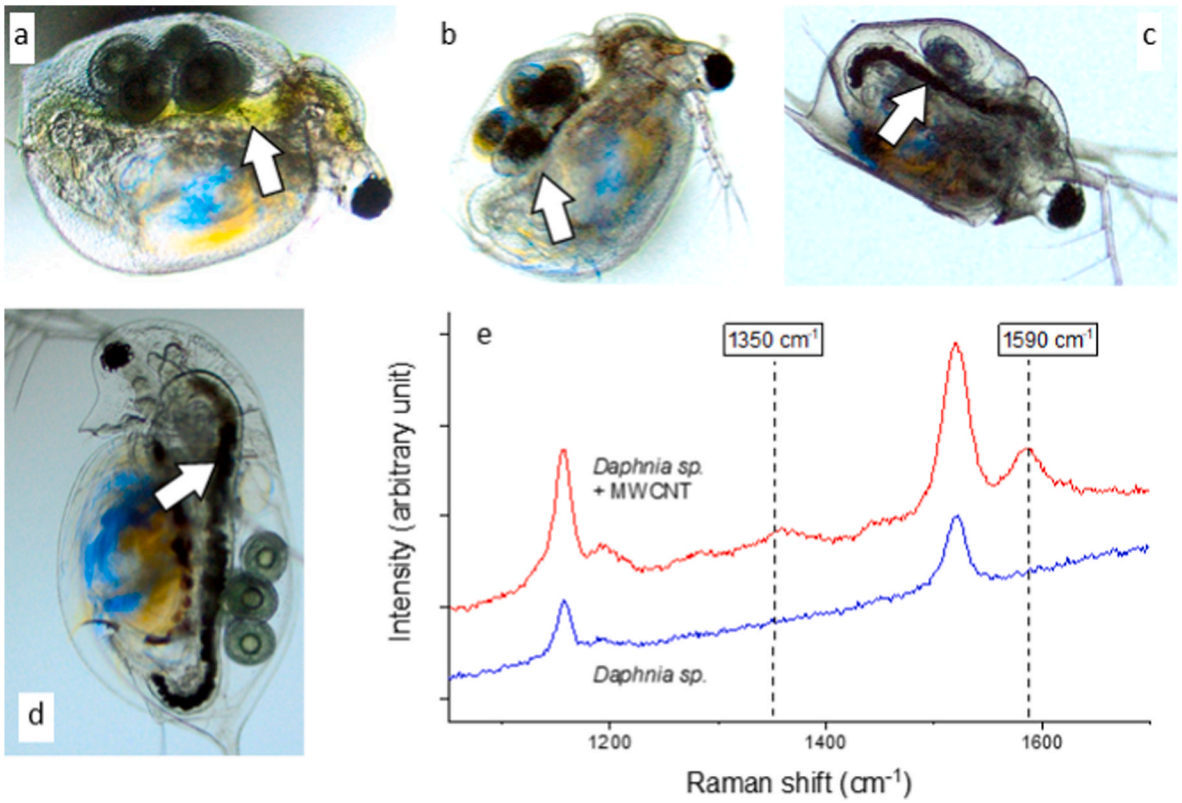

Fig. 5. Zooplankton ingestion experiments. *Ceriodaphnia* sp. showed distinct changes in gut colour from green (a; ingestion of algae), to colourless (b; empty gut), to black (c; ingestion of MWCNTs). Experiment with Daphnia sp. also showed the same changes, with a black gut after ingestion of MWCNTs (d). The gut is indicated by the arrow in each photo. Composite Raman spectra showed the D-band and G-band peaks of MWCNTs in the gut region of *Daphnia* sp. that had ingested MWCNTs (e). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

signature peaks at 1350 cm^{-1} and 1590 cm^{-1}, confirming the presence of MWCNTs in the gut (Fig. 5). The typical gut passage time of *Daphnia* sp. is between a few minutes and a couple of hours, i.e. the amount of time for ingested material to pass through the gut and be egested (Murtagh, 1985). After our experiments, remaining animals were returned to clean water; the next day (ca. 18 h later) MWCNTs were still visible, suggesting that the animals retained MWCNTs in the gut for an extended time, thereby increasing their possibility of dispersal and transfer to the higher trophic levels. Future studies may investigate whether mixing CNTs with normal food particles may change the intake and gut passage of CNTs in the zooplankton.

3.4. Raman spectroscopy for environmental monitoring

The manufacturing and use of CNTs and other nano-particles has grown exponentially in the recent decades. For example, in the United States alone, investments into nanotechnology development totalled ca. $29 billion in 2020, but less than 5% was spent on environmental, health and safety studies (Klaine et al., 2012). The durability of CNTs inevitably increases their persistence in the ecosystem (Köhler et al., 2008). Due to their microscopic sizes and inert chemical properties, conventional sampling and measurements may not be able to detect their presence or their interactions with organisms *in situ*.

In this proof-of-concept study, we showed that Raman spectroscopy can identify MWCNTs against the background biological materials. The laser was able to penetrate the zooplankton body walls and probe internalised CNTs without the need for extraction or dissection, and no special or lengthy preparation was required. For field sampling, the zooplankton can be frozen to preserve their gut contents. As such, Raman spectroscopy offers an excellent potential to easily and quickly detect and trace the flow of CNTs within the aquatic food webs.

4. Conclusions

Despite the increasing use of CNTs, information on their effects on the aquatic ecosystem is still rare. We conducted laboratory experiments to test the worst-case scenario where common plankton were exposed to high amounts of MWCNTs. The photosynthetic performance and growth rate of phytoplankton were compromised. Zooplankton ingested MWCNTs indiscriminately, and retained MWCNTs in their gut for an extended time, which may increase the chance to disperse and transfer MWCNTs through the food web. Our study showed that Raman spectroscopy is a versatile non-destructive measurement tool to verify the presence of nanomaterials in biological specimens, and would be particular useful for biological samples that are opaque to light microscopy or where CNTs are obscured by other ingested materials inside the zooplankton's gut.

Author contributions

Conceptualization: J.R.H., A.O.W. and K.W.T.; Experimentation: J.R.H.; CNT synthesis: A.H.; CNT imaging: Y.N. and R.E.P.; Data analysis: J.R.H., A.O.W. and K.W.T.; Manuscript original draft: J.R.H., A.O.W. and K.W.T. All authors contributed to final draft and approved submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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