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A trophic transfer study: accumulation of multi-walled carbon nanotubes associated to green algae in water flea *Daphnia magna*

Irina Politowski [a,*], Fabian Wittmers [a], Michael Patrick Hennig [a], Nina Siebers [b,c], Birgitta Goffart [a], Martina Roß-Nickoll [a], Richard Ottermanns [a], Andreas Schäffer [a]

[a] Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
[b] Forschungszentrum Jülich GmbH, Agrosphere (IBG-3) Institute of Bio- and Geosciences, Wilhelm-Johnen-Straße, 52425 Jülich, Germany
[c] Forschungszentrum Jülich GmbH, Ernst Ruska-Centre for Microscopy and Spectroscopy with Electrons (ER-C), Wilhelm-Johnen-Straße, 52425 Jülich, Germany

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ABSTRACT

Carbon nanotubes (CNT) are promising nanomaterials in modern nanotechnology and their use in many different applications leads to an inevitable release into the aquatic environment. In this study, we quantified trophic transfer of weathered multi-walled carbon nanotubes (wMWCNT) from green algae to primary consumer *Daphnia magna* in a concentration of 100 μg L^{-1} using radioactive labeling of the carbon backbone (^{14}C-wMWCNT). Trophic transfer of wMWCNT was compared to the uptake by daphnids exposed to nanomaterials in the water phase without algae. Due to the rather long observed CNT sedimentation times (DT) from the water phase (DT_{50}: 3.9 days (d), DT_{90}: 12.8 d) wMWCNT interact with aquatic organisms and associated to the green algae *Chlamydomonas reinhardtii* and *Raphidocelis subcapitata*. After the exposition of algae, the nanotubes accumulated to a maximum of 1.6 ± 0.4 μg ^{14}C-wMWCNT mg^{-1} dry $weight^{-1}$ (dw^{-1}) and 0.7 ± 0.3 μg ^{14}C-wMWCNT mg^{-1} dw^{-1} after 24 h and 48 h, respectively. To study trophic transfer, *R. subcapitata* was loaded with ^{14}C-wMWCNT and subsequently fed to *D. magna*. A maximum body burden of 0.07 ± 0.01 μg ^{14}C-wMWCNT mg^{-1} dw^{-1} and 7.1 ± 1.5 μg ^{14}C-wMWCNT mg^{-1} dw^{-1} for *D. magna* after trophic transfer and waterborne exposure was measured, respectively, indicating no CNT accumulation after short-term exposure via trophic transfer. Additionally, the animals eliminated nanomaterials from their guts, while feeding algae facilitated their excretion. Further, accumulation of ^{14}C-wMWCNT in a growing population of *D. magna* revealed a maximum uptake of 0.7 ± 0.2 μg mg^{-1} dw^{-1}. Therefore, the calculated bioaccumulation factor (BAF) after 28 d of 6700 ± 2900 L kg^{-1} is above the limit that indicates a chemical is bioaccumulative in the European Union Regulation REACH. Although wMWCNT did not bioaccumulate in neonate *D. magna* after trophic transfer, wMWCNT enriched in a 28 d growing *D. magna* population regardless of daily feeding, which increases the risk of CNT accumulation along the aquatic food chain.

1. Introduction

Nanotechnology and nanoscience have gained more and more attention over the past 20 years (Roco, 2011). Since their discovery in 1991 (Iijima, 1991), the production of carbon nanotubes (CNT) rose continuously and in 2020, the industrial company OCSIAL announced the commissioning of the largest plant for the production of CNT to date, which will produce 100 tons of CNT annually (KunststoffWeb, 2020; OCSIAL, 2020). Due to their novel and alterable properties CNT are used in manifold applications like nanocomposites (Barra et al., 2019), energy storage (Mauter and Elimelech, 2008; Thauer et al., 2020), drug delivery (Newland et al., 2018) and water treatment (Zaib et al., 2014).

Within the life cycle of CNT, most of the material is released during synthesis and handling (Yeganeh et al., 2008; Tsai et al., 2009). CNT are most widely used in nanocomposites, i.e., plastic materials with embedded CNT, which is why their disposal can result in additional CNT release. For nanocomposites it was reported that the plastic polymer degrades by UV irradiation leaving the nanotube network exposed on the nanocomposites surface. Further, physical transformation processes, e.g., by abrasion, or sunlight can impair the CNT network leading to the release of nanomaterial (Wohlleben et al., 2013; Petersen et al., 2014; Hou et al., 2014; Schlagenhauf et al., 2015). Eventually, either through

* Corresponding author.
E-mail address: irina.politowski@rwth-aachen.de (I. Politowski).

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direct disposal of CNT-containing products in aqueous compartments, or through their weathering on landfills and subsequent leaching into water bodies, CNT enter the aquatic environment (Sarma et al., 2015). Thereby CNT will interact with co-contaminants and natural particles (Holden et al., 2016; Naasz et al., 2018; Glomstad et al., 2018). While lingering in the environment, weathering impacts (e.g., irradiation) might alter physico-chemical properties of CNT and as a result influence their environmental behavior like bioaccumulation and toxicity to organisms (Mitrano et al., 2015).

Bioaccumulation is generally defined as the increase in internal concentration of chemicals in an organism exceeding the concentration in the surrounding medium, food or both (Gobas et al., 2009).

To date, accumulation of CNT in aquatic organisms was investigated but due to the lack of suitable quantification methods (Mortimer et al., 2016), nanomaterial concentrations, several orders of magnitude higher than found in natural environments, were used (Parks et al., 2013; Maes et al., 2014; Rhiem et al., 2015; Zhu et al., 2018), except for a few studies (Petersen et al., 2009; Petersen et al., 2011; Cano et al., 2017; Cano et al., 2018). Since the estimated environmental concentration of CNT is in the ng L^{-1} range (Gottschalk et al., 2009; Gottschalk and Nowack, 2011), there is a need for studies that approach such low concentrations. Consequently, we quantified the association of CNT to algae in the μg L^{-1} range which is below the tested concentration in other studies.

Despite of the CNT concentrations used, CNT generally show a low bioaccumulation potential (Bjorkland et al., 2017), since these nanomaterials do not pass membranes like classical compounds and are therefore mainly accumulated in the gastrointestinal tract, as well as adhering to external structures (e.g., gill, skin). In addition, it was increasingly observed that orally ingested CNT are eliminated again, especially after food intake, which leads to a low body burden in the organism (Petersen et al., 2009; Guo et al., 2013; Maes et al., 2014). As under environmental conditions mostly food is available, trophic transfer thus needs to be the focus of further research.

Since green algae represent primary producers in the aquatic food chain the interaction with and the uptake of radioactively labeled MWCNT (1 mg L^{-1}) in *Desmodesmus subspicatus* was reported by Rhiem et al. (2015). Using radioactive labeling, the uptake of multi-walled CNT (MWCNT) was also quantified in five to seven days old *Daphnia magna* where a maximum body burden of 63 μg mg^{-1} after exposure to 400 μg L^{-1} over 24 h was obtained (Petersen et al., 2009). The exposure of the same stock of *D. magna* with 250 μg L^{-1} of polyethyleneimine grafted MWCNT resulted in a maximum internal concentration of 12 μg mg^{-1} (Petersen et al., 2011). In both studies, elimination into clean medium was low, however, in the presence of algae, nearly complete excretion of nanomaterial was observed, leading to low bioaccumulation. Guo et al. (2013) worked with *D. magna* neonates to investigate the uptake of ^{14}C-labeled graphene. At 250 μg graphene L^{-1} the maximum body burden was 7.8 μg mg^{-1}, whereas depuration in clean water led to nearly no change in body burden after 24 h, but as in case of CNT, excretion was facilitated in the presence of algae. Since neonate daphnids show a higher sensitivity toward chemical exposure than adult animals (Gerritsen et al., 1998; Preuss et al., 2010), the data obtained from the studies performed with adult *Daphnia* are of limited value. Therefore, the accumulation of CNT in neonate *D. magna* is still unknown but necessary and consequently in this study, we focussed on the uptake of CNT in neonate animals.

In more recent studies the accumulation of pristine MWCNT (0.1 mg L^{-1}), applied together with algae, in *D. magna* (five to 15 days old) was quantified using a microwave induced heating technique. An enrichment of above 0.2 μg MWCNT g^{-1} after 24 h in a *D. magna* population of 60 individuals and of 0.02 to 0.05 μg MWCNT g^{-1} (BCF < 1) after three days (ten animals in 50 mL) indicated a low bioaccumulation of CNT in *D. magna* under environmentally relevant feeding conditions (Cano et al., 2017; Cano et al., 2018). Further, trophic transfer of MWCNT from bacteria *Pseudomonas aeruginosa* to protozoan *Tetrahymena thermophila* (Mortimer et al., 2016) and from water flea *D. magna* to fathead minnow (Cano et al., 2018) was recently observed. These more complex studies demonstrated the food web transfer of CNT over a short period of time, using animals that were several days to weeks old but did neither look at population dynamics, nor long-term exposures. Consequently, the influence of complexity of test systems and prolonged investigations on enrichment of CNT in *D. magna* remains to be unclear. To close this knowledge gap, we investigated the accumulation of weathered CNT in a growing population of *D. magna* in long-term exposure, using radiolabeled nanomaterials.

So far, only few studies on carbonaceous nanomaterials have been performed at concentrations that are expected in the environment caused by the absence of quantification methods for CNT in complex environmental matrices. We aimed to investigate trophic transfer of ^{14}C-MWCNT from green algae to *D. magna* under low nanomaterial concentrations. Herein, the presence of nutrition on uptake and depuration was assessed. Additionally, we performed a long-term microcosm experiment to gather information on uptake of ^{14}C-MWCNT in a growing population of *D. magna*, which, to our knowledge, has not been investigated until now.

2. Materials and methods

2.1. Synthesis and purification of ^{14}C-labeled MWCNT (^{14}C-MWCNT)

Synthesis of ^{14}C-MWCNT has already been described before (Maes et al., 2014; Rhiem et al., 2015). For a brief overview, ^{14}C-MWCNT were synthesized by catalytic chemical vapour deposition. To remove remains of catalyst, the ^{14}C-MWCNT were washed using 12.5% hydrochloric acid solution resulting in a C-purity for the product of 95%. Obtained ^{14}C-MWCNT consisted of 3–15 walls (4 nm for inner and 5–20 nm for outer diameter) and a length of ≥1 μm (Rhiem et al., 2015). Unlabeled MWCNT (Baytubes® C150P) were provided by Bayer Technology Services GmbH (BTS, Leverkusen, Germany) and produced under the same conditions as ^{14}C-MWCNT. The structural similarity of the nanomaterials was shown by means of transmission electron microscopy (TEM) (Rhiem et al., 2016).

2.2. Weathering and dispersion of nanomaterials

Unlabeled MWCNT and ^{14}C-MWCNT were weathered by simulated sunlight radiation for three months (2160 h) according to ISO 3892-2:2006 with minor changes, using a weathering testing apparatus (Suntest™ CPS+, Altas Material Testing Technology, Germany, standard black temperature 65 °C, dry conditions). The device provided light with a wavelength range from 300 to 400 nm by means of an air-cooled xenon lamp (1500 W) with a daylight UV filter. Irradiation intensity was set to 65 W m^{-2} and the total applied energy was 505,441 kJ m^{-2}. During exposure period, samples ((^{14}C)-MWCNT) of 0.8 g CNT agglomerates were placed into each of four petri dishes with glued-on lids made of quartz glass (transmissibility for UV light). Irradiation was performed without simulated rain or air humidity. Meanwhile, the internal sample table was cooled with a constant flow of cold water. Samples were shaken daily and once a week the position of sample bins was changed in order to achieve a uniform irradiation. After weathering process, the specific radioactivity of ^{14}C-labeled weathered MWCNT (^{14}C-wMWCNT) was determined to 1.66 MBq mg^{-1}. In order to characterize wMWCNT, thermogravimetric analysis (TGA) and Fourier-transform infrared spectroscopy (FTIR) methods were used. Neither differences nor heterogeneous functionalities on surface structures compared to pristine material were detected (see SI).

For the dispersion of CNT, the needed amount of (^{14}C)-wMWCNT was weighted on a microbalance (MYA 5.3Y, Radwag) and filled in a flask containing 50 mL (volume was adjusted according to CNT quantity) of the required test medium. Subsequently, the flask was put into an ice bath and nanomaterials were dispersed by means of ultrasonication with a micro tip for 10 min (Sonopuls HD 2070, 70 W, pulse: 0.2 s,

pause: 0.8 s). Subsequently, the stock dispersion was diluted by test medium and dispersed a second time as described above to obtain the target test dispersion. The used dispersion method has already been described (Rhiem et al., 2015; Hennig et al., 2019). Herein, the two-stage dispersion process as described is a deviation from this method. An investigation using TEM revealed that wMWCNT test dispersion contained small agglomerates as well as single tubes (length: 0.2–1 μm) and was therefore appropriate for dispersion of wMWCNT in aqueous solution.

2.3. Deposition of ^{14}C-wMWCNT

The deposition behavior of ^{14}C-wMWCNT (100 μg L^{-1}) in a static test system was assessed. A total amount of 219 μg ^{14}C-wMWCNT was weighted and filled in a flask containing M4 test medium (main constituents: 294 mg $CaCl_2 \times 2\ H_2O\ L^{-1}$, 123.3 mg $MgSO_4 \times 7\ H_2O\ L^{-1}$, 5.8 mg KCl L^{-1}, 64.8 mg $NaHCO_3\ L^{-1}$, see SI) with a pH value between 7 and 8 (OECD, 2004) and dispersed as described above. Subsequently, the stock dispersion was diluted by test medium and dispersed a second time to obtain 2 × 550 mL (100 μg ^{14}C-wMWCNT L^{-1}) test dispersions. Two subsamples of 1 mL were drawn, 2 mL of Ultima Gold™ XR scintillation cocktail (Perkin Elmer, Germany) was added and samples were submitted to LSC (liquid scintillation counter, Hidex SL 600, Hidex, Germany) to verify the achieved ^{14}C-wMWCNT concentration, respectively. Out of test dispersions four replicates with each 250 mL were prepared in glass flasks. Samples were incubated at a temperature of 20 ± 2 °C and a photoperiod of 16 h light/8 h dark. At test start (0 h), radioactivity of each replicate was determined by means of LSC (100% value: 107 ± 2 μg L^{-1}). For sampling, an aliquot of 1 mL was drawn from the water surface (layer depth of 0.5 cm) of one replicate. Initially, a sample was taken every 15 min. During the experiment, the interval between the individual measurements was increased continuously. The last sample was taken on day 21.

2.4. Test organisms

Green algae *Chlamydomonas reinhardtii* (strain no. 23.90) and *Raphidocelis subcapitata* (strain no. 61.81) were obtained from SAG Göttingen and cultured in BG 11 culture medium (SAG, 2013) and culture medium according to Kuhl and Lorenzen (1964), respectively. Cultures of algae were kept at 20 ± 2 °C, permanent aeration and constant illumination. Renewal of medium was performed every two weeks. *Daphnia magna* (Straus) were cultured in M4 medium (OECD, 2004) (20 ± 2 °C, 16 h dark/8 h light photoperiod). Daphnids were fed daily, except on weekends from a culture of the green alga *Desmodesmus subspicatus*. In addition, *Daphnia* was fed with yeast (*Saccharomyces cerevisiae*) once a week during medium renewal.

2.5. Characterization of wMWCNT in micro batch experiments

The following approaches were prepared in small-scale:

a) wMWCNT dispersion (0.1 mg L^{-1}, 20 mL)
b) wMWCNT (1 mg L^{-1}) and green algae *R. subcapitata* (2 × 20 mL, 24 h)
c) wMWCNT (1 mg L^{-1}) and five adult *D. magna* (100 mL) – uptake for 24 h (water exposure) and excretion for 24 h in presence of algae
d) wMWCNT (0.1 mg L^{-1}) and three adult daphnids (100 mL) for 72 h

M4 medium was used for all test approaches. A wMWCNT concentration of 1 mg L^{-1} was chosen in b) and c) as former tests using a wMWCNT concentration of 0.1 mg L^{-1} have not led to qualitative results (data not shown). Dispersion of nanomaterial was performed as described above. Scenario a) – c) have been characterized by means of TEM. To characterize wMWCNT in dispersion, copper grids (Plano GmbH) for TEM analysis were submerged in nanomaterial dispersion directly after assembly and placed on a filter heated up to 50 °C. In presence of organisms, subsamples of maximum 40 μL (2 × 20 μL) were taken from the bottom of test vessels using a pipette and placed onto copper grids. During the process, copper grids were placed on a paper filter. Samples were dried as described above. After desiccation, copper grids were subjected to TEM analysis using a Philips CM 20 FEG operating at 200 keV. Test organisms of scenario d) were analyzed using a light microscope to visualize internalized nanomaterials.

2.6. Interaction of ^{14}C-wMWCNT with green algae

A suspension of green alga *Chlamydomonas reinhardtii* was separated from culture medium by centrifugation (10 min, 943 ×*g*) and resuspended in BG 11 medium (main constituents: 150 mg $NaNO_3\ L^{-1}$, 4 mg $K_2HPO_4 \times H_2O\ L^{-1}$, 7.5 mg $MgSO_4 \times 7\ H_2O\ L^{-1}$, 3.6 mg $CaCl_2 \times 2\ H_2O\ L^{-1}$, pH value between 7 and 8, see SI). Cell density was determined using a fluorescence reader (Tecan M200, Männedorf, Schweiz, Software: Magellan). A quantity of 703 μg ^{14}C-wMWCNT was weighted out on a microbalance (MYA 5.3Y, Radwag) and dispersed in 100 mL BG 11 medium for 10 min as described above. Three times, 12.7 mL of this dispersion were then diluted each with 737.3 mL BG 11 medium in a flask and dispersed for further 10 min in order to obtain an application volume of 2250 mL (3 × 750 mL suspension). Four aliquots of 1 mL were drawn from each bottle and analyzed using LSC. Furthermore, 84 mL of each ^{14}C-wMWCNT dispersion were mixed with 16 mL algal suspension in a 250 mL Erlenmeyer flask. In this way, a ^{14}C-wMWCNT concentration of 122 ± 2 μg L^{-1} and an algal cell density of 1×10^{6} cells mL^{-1} was achieved. The prepared samples were weighted and incubated at 22 ± 2 °C at a diurnal rhythm of 16 h light to 8 h dark on a laboratory shaker (80 rpm). Immediately after completion ($t = 0$ h) and after 24, 48, 72 and 96 h four replicates were sampled. Three aliquots of 0.5 g were taken and measured by means of LSC to determine the 100% value. Algal density was determined as well. In case of t = 0 h this corresponded to an initial concentration of 1×10^{6} cells mL^{-1}. Subsequently, free CNT were separated from CNT associated to algae by means of density gradient centrifugation (see SI). The suitability of this method was already described before (Rhiem et al., 2015). After the last step of separation and washing algae cells with BG 11 medium, one to three drops of remaining algal pellet (five times per sample) were placed in a single well on a 24-well plate and filled up with BG 11 medium to a weight of 2 g (corresponding to 2 mL) and cell count was determined. The contents of each well were subsequently transferred completely (rinsed using methanol) to an LSC vial. The following measurement of the sample using LSC revealed the amount of ^{14}C-wMWCNT associated with the algal cells. A complete recovery was performed by quantifying radioactivity in all compartments accruing during reprocessing (see SI). The same was performed using the green algae *Raphidocelis subcapitata*. An initial ^{14}C-wMWCNT concentration of 123 ± 3 μg L^{-1} and an algal density of 2×10^{6} cells mL^{-1} was applied in culture medium (main constituents: 1011 mg $KNO_3\ L^{-1}$, 621 mg $NaH_2PO_4 \times H_2O\ L^{-1}$, 71 mg $Na_2HPO_4\ L^{-1}$, 246.5 mg $MgSO_4 \times 7\ H_2O\ L^{-1}$, 14.7 mg $CaCl_2 \times 2\ H_2O\ L^{-1}$, pH between 7 and 8, see SI). A higher starting cell count in the case of *R. subcapitata* was necessary to ensure the success of density gradient centrifugation at the time of the first sampling (t = 0 h).

2.7. Experiment for uptake of ^{14}C-wMWCNT by D. magna

Uptake of ^{14}C-wMWCNT in *D. magna* was investigated via water exposure and trophic transfer. Body burden (application: 100 μg ^{14}C-wMWCNT L^{-1}) was determined after 2, 18, 24, 48 and 72 h in four replicates each (water exposure: 72 h, $n = 5$; trophic transfer: 2 and 24 h, n = 5). As test medium, oxygen saturated M4 medium was used. For water exposure a total mass of 107 μg ^{14}C-wMWCNT was weighted on a microbalance (MYA 5.3Y, Radwag), transferred to a flask containing 100 mL of test medium and dispersed as described above. Afterwards, a certain volume (32.71 mL) was taken from this stock dispersion and

diluted by test medium (317.29 mL) to obtain the test dispersion with 100 μg ^{14}C-wMWCNT L^{-1} (2 × 350 mL). The test solution was dispersed for another 10 min (see above). Homogeneity and ^{14}C-wMWCNT concentration in test dispersion were monitored directly after sonication. Therefore, three aliquots of 1 mL were mixed with 2 mL of Ultima Gold™ XR scintillation cocktail (Perkin Elmer, Germany) and measured by means of LSC.

Immediately after LSC measurements, 30 mL of test dispersion were filled in small glass beakers. Radioactivity in every test beaker (2 × 0.5 mL) was determined using LSC. Achieved ^{14}C-wMWCNT concentration was 116 ± 5 μg L^{-1} instead of 100 μg L^{-1}. 10 daphnids ≤24 h were placed into each beaker. Test organisms were kept at a temperature of 20 ± 2 °C and a photoperiod of 16 h light/8 h dark. The animals were not fed during this experiment and were last fed 24 h before test start.

For sampling, daphnids were taken out of test beakers, placed into a plain glass dish and washed two times extensively with test medium and distilled water. Washed animals were then transferred to aluminium boats and dried at 65 °C for 24 h. The amount of radioactivity in test beakers and in water phases from cleaning steps was determined by LSC (obviously immobile or dead organisms were captured in this fraction). Glass ware and used pipettes were cleaned using methanol moistened tissues. To quantify the radioactivity of ^{14}C-wMWCNT attached to the working utensils, tissues were submitted to LSC as well. After determination of dry weight, test animals were transferred to a micro glass mortar and homogenized using methanol. This way, radioactivity trapped in the animals was released and detected by LSC to avoid a defective quantification due to quenching processes by animal's carapace or compact agglomerated CNT. Afterwards, the crushed animal/methanol mass was transferred to an LSC Vial. The micro mortar was cleaned carefully, and water used for rinsing was placed in the same scintillation vial. Quantification of accumulated radioactivity in *D. magna* was performed by means of LSC.

For the trophic transfer experiment, the green alga *Raphidocelis subcapitata* was pre-loaded by ^{14}C-wMWCNT and subsequently fed to *D. magna*. A two weeks old culture of *R. subcapitata* was centrifuged (10 min, 943 ×*g*) and the pellet was resuspended in 100 mL Kuhl medium. A total of 790 μg ^{14}C-wMWCNT was weighted on a micro balance and dispersed in 100 mL culture medium as described above. This dispersion was further diluted by 550 mL culture medium and dispersed again for 10 min. Algae suspension was added to ^{14}C-wMWCNT dispersion and incubated for three days (aerated in a round-bottomed flask) as described for *D. magna* previously. Determined ^{14}C wMWCNT concentration was 1.3 mg L^{-1}. After incubation algae were centrifuged (10 min, 943 ×*g*), supernatant discarded and the pellet washed (centrifugation, 10 min, 943 ×*g*) two times using 50 mL of M4 medium.

The remaining algal pellet was resuspended in culture medium. ^{14}C-wMWCNT concentration was determined using LSC up to 1.5 mg L^{-1}. For the volume of test medium required (750 mL) and a ^{14}C wMWCNT concentration of 120 μg L^{-1} (see water exposure) a volume of 58.8 mL of suspension with ^{14}C-wMWCNT-loaded algae was diluted with M4 medium to 750 mL test medium. Concentration of algae was 1.6 × 10^{6} cells mL^{-1}. A test concentration of 120.3 μg ^{14}C-wMWCNT L^{-1} was verified by means of LSC (three aliquots each 1 mL). Setup of test, incubation and determination of body burden was performed as described in case of water exposure.

2.8. Experiment for elimination of ^{14}C-wMWCNT by D. magna

Uptake of 120 μg ^{14}C-wMWCNT L^{-1} in *D. magna* was conducted for 24 h. 120 μg ^{14}C-wMWCNT were weighted on a microbalance and transferred to a flask containing 100 mL M4 medium. After dispersion, twice 45 mL of stock dispersion were diluted by 405 mL M4 medium and dispersed for another 10 min (for dispersion details see above) to obtain a test dispersion of 120 μg ^{14}C-wMWCNT L^{-1}. Aliquots were taken and measured by means of LSC. Aberration from one test dispersion to another was only about 3% and the effect on uptake and elimination of CNT in *D. magna* is considered as admissible. The test setup was the same as described for uptake experiments, 27 samples were prepared. After uptake phase, 7 replicates were sampled to calculate 100% value (0 h) for excretion experiment. The test organisms from the remaining 20 samples were transferred to clean M4 medium. For every replicate, daphnids were taken out and washed vigorously using pipettes to subduct nanomaterial attached to the animal's surface. With a small amount of fresh M4 medium the daphnids were then transferred into the elimination beakers. Time was noted for every beaker to verify the start of excretion process. The described test approach was prepared twice. One test was set up for excretion in water phase (without food, −algae) and one for excretion in presence of food (+algae). Due to poor recovery, only 5 replicates were included in the calculation of the 100% value at 0 h for the scenario without algae. After animal transfer to clean medium, 0.1 mg carbon through green alga *D. subspicatus* was added to the samples. For incubation conditions see the uptake experiment. Four replicates (3 replicates after 48 h in the scenario without algae) were sampled after 2.5, 5, 19, 24 and 48 h, respectively. Sampling of test animals and quantification of accumulated nanomaterial was performed as described above.

2.9. D. magna population experiment

Uptake of 100 μg ^{14}C-wMWCNT L^{-1} was assessed over 4 weeks in an evolving population of *D. magna*. A total of 1.8 mg ^{14}C-wMWCNT was weighted out on a microbalance and dispersed for 10 min (Sonopuls HD 2070, 70 W, pulse: 0.2 s, pause: 0.8 s) in 200 mL ice bath cooled M4 medium. Volume for stock dispersion was doubled in this case due to the high amount of ^{14}C-wMWCNT required. Afterwards a volume of 22.75 mL was diluted by 1977.25 mL M4 medium and dispersed for another 10 min (see above). Two aliquots of 1 mL were taken from each test dispersion and radioactivity was measured using LSC. 800 mL of ^{14}C-wMWCNT test dispersion were filled in 1 L glass beakers. Subsequently 3 adult (3 to 4 weeks old) and 5 neonate (≤ 24 h) daphnids were placed into each sample and gentle aeration was fixed to beakers. Additionally, four controls without ^{14}C-wMWCNT were prepared for supervision of *D. magna* population growth at every sampling time. Samples were kept at 20 ± 2 °C and a photoperiod of 16 h light/8 h dark. Animals in control and treatment group were fed daily 0.5 mg carbon L^{-1} via a culture of *D. subspicatus* (Hammers-Wirtz and Ratte, 2003).

In treatments, once a week renewal of test medium and reapplication of ^{14}C-wMWCNT (100 μg L^{-1}) was performed (semi-batch approach). Therefore, test animals were sieved (mesh for brine shrimp, 180 μm), washed with 100 mL clean M4 medium and transferred (with 100 mL clean M4 medium, dilution was considered while ^{14}C-wMWCNT preparation of test dispersion) to beakers containing a newly prepared (100 μg ^{14}C-wMWCNT L^{-1}) nanomaterial dispersion. Just as in the treatments, the medium of the controls was renewed every week. Animals from controls were sieved (mesh for brine shrimp, 180, 300, 560 and 900 μm) and distributions of individuals per size were noted. Counted daphnids were washed into fresh M4 medium and incubated as described before.

In case of treatments four replicates were sampled after 7, 14, 21 and 28 d. The water body of every sample was sieved completely using a 4-part brine shrimp sieve set (see above). After that, 100 mL tap water were filled in test beaker and remaining daphnids were flushed out and sieved. The complete volume of water phase was collected in a 1 L bottle. Using tap water, size sorted animals were transferred in 4 single petri dishes and sieves were washed thoroughly. Subsequently, water was removed in order to immobilize test organisms and individuals per size distribution were acquired. After counting, the animals sorted by size were conveyed to smaller petri dishes and washed extensively using tap water to remove radioactive material from the animals' surface. The water used for rinsing was withdrawn and the animals were transferred to pre-weighted aluminium boats using distilled water. Boxed animals were dried for 24 h at 65 °C. If the number of individuals in a size class

was < 10 and an exact weight determination therefore impossible, the animals were added to the aluminium boat of the next larger class. For a complete recovery, working utensils were wiped clean using methanol moistened tissues, which were submitted to LSC. The rinsing water was pooled with the collected water phase and the volume was dispersed for 10 min to obtain a homogeneous distribution of radioactivity. For quantification of radioactivity in water phase, two subsamples of 1 mL per flask were mixed with 2 mL Ultima Gold™ XR scintillation cocktail (Perkin Elmer, Germany) and measured by means of LSC.

After determination of dry weight, animals were transferred to 20 mL LSC Vials using methanol. Vials were kept for 24 h under a fume hood (evaporation of methanol), subsequently 1 mL of solubilizer Soluene-350® (PerkinElmer) was added to the samples. Sample vials were closed and incubated at 60 °C. In the first hours, the liquid was swivelled every 30 min and incubation was finished after complete dissolving of biological matrix. After cooling the samples to room temperature, 19 mL LSC cocktail was added to each vial and the amount of radioactivity was determined.

2.10. Data evaluation and statistical analysis

Collected data was processed using Microsoft Excel® (Microsoft Office 365 ProPlus), GraphPad Prism (GraphPad Prism 5, USA), and SigmaPlot (SigmaPlot Version 12.0, USA). Outliers were identified by Dixon's Q test ($\alpha = 0.05$). To identify differences between treatments either a two-sample *t*-test by comparing the t-based 95% confidence intervals for mean values or a common t-test (*p* value to reject = 0.05) was performed. Raw data were tested for normal distribution with Shapiro-Wilk test (p value = 0.05) and variance homogeneity with Levene's test (p value = 0.05). If any of the above test criteria could not be met, a Mann-Whitney Rank Sum test was performed.

For data sets from the deposition experiment, in addition to evaluation of experimental data, the deposition kinetics were evaluated using Computer assisted kinetic evaluation (CAKE, Version 3.3 (Release), Tesella). A Hockey Stick (HS) model, i.e., an exponential decline function with a breaking point separating two kinetics (k_1 and k_2), was selected for the description of the data set.

Since the usual definition of bioaccumulation is inappropriate regarding nanomaterial uptake, an approximation of bioaccumulated wMWCNT in different aquatic organisms was performed based on a one-compartment model according to Connel (1998) (Eq. 1):

$$C_{b(t)} = C_w \frac{k_1}{k_2}\left(1 - e^{-k_2 t}\right) + C_{b(t0)} e^{-k_2 t} \quad (1)$$

C_b and C_w are the wMWCNT concentration in biota (mg kg^{-1}) and water phase (mg L^{-1}), respectively, k_1 (L kg^{-1} h^{-1}) and k_2 (h^{-1}) represent the uptake and elimination rate constants and t indicates time (h). Assuming that C_w as well as k_1 and k_2 are constant over the test period, the bioaccumulation factor can be calculated as follows: BAF = $k_1/k_2 = C_b/C_w$. For green algae, a bioconcentration factor (BCF) was calculated using C_b/C_w (Bjorkland et al., 2017), where C_w was the applied wMWCNT concentration (0.1 mg L^{-1}). In case of *D. magna*, BCF in water exposure scenario was calculated using C_b/C_w and k_1/k_2, where C_w was the wMWCNT concentration in water measured at sampling date. Using k_1/k_2, uptake rate k_1 varied over time, so k_1 was calculated from time points where interaction of test organism and wMWCNT were at maximum (pseudo-steady-state). According to Bjorkland et al. (2017), BAF was calculated using C_b/C_w, where C_w was the wMWCNT concentration in water and diet.

3. Results

3.1. Deposition of ^{14}C-wMWCNT

Concentration of ^{14}C-wMWCNT in top layer of static water phase (M4 medium) decreased over time (Fig. 1). After one day and three days the radioactivity level in the top layer of water dropped to a value of 87.7 ± 1.6% and 69.5 ± 0.9%, respectively. After 21 days only <0.35% (0.2 ± 0.1%) of the initially applied radioactivity remained at the top layer of the water phase. An exponential regression fit to the decreasing concentrations resulted in a DT_{50} and DT_{90} of 3.91 d and 12.82 d, respectively. The application of a HS model to the data set produced very similar deposition times (DT_{50} = 4.38 d and DT_{90} = 10.50 d). Both, the exponential fit and the modelling (Fig. S1, Table S1) revealed a coefficient of determination of $R^2 = 0.99$ and showed a good description of the experimental data.

3.2. Characterization of wMWCNT

Fig. 2 - B shows that the optimized method to disperse the used nanomaterials (wMWCNT concentration: 0.1 mg L^{-1}) resulted in small agglomerates (bundles) and single strand exfoliated CNT. Weathered MWCNT showed a length of 200 nm to 3 µm, visualized by means of TEM. The observed small agglomerates contained loosely associated individual CNT (Fig. 2 – B). The sickle-shaped structure in Fig. 2 - A shows a single cell of the green alga *R. subcapitata* to which long wMWCNT fibers are attached (Fig. 2 - A; Fig. S2). We also identified

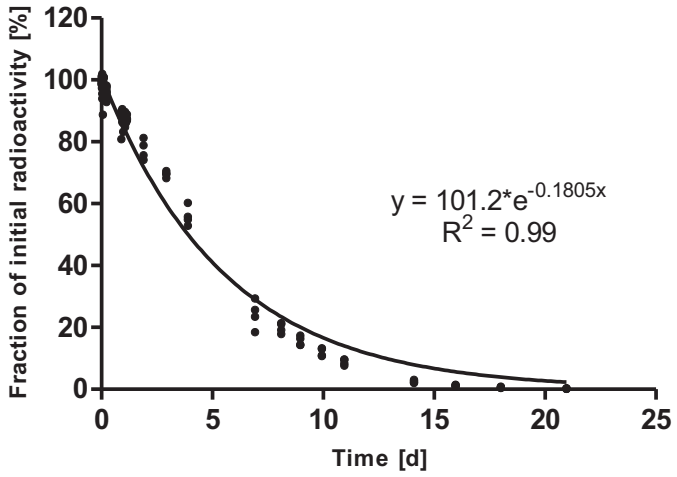

Fig. 1. Deposition of ^{14}C-wMWCNT in a static water phase (M4 medium) over 21 days (d). The measured amount of radioactivity at $t = 0$ d was set to 100%. The proportion of decrease (%) of the initially applied radioactivity over the test period is shown. An exponential decay equation model was fitted to the experimental data. Data points represent single replicates ($n = 4$). (R^2 = coefficient of determination).

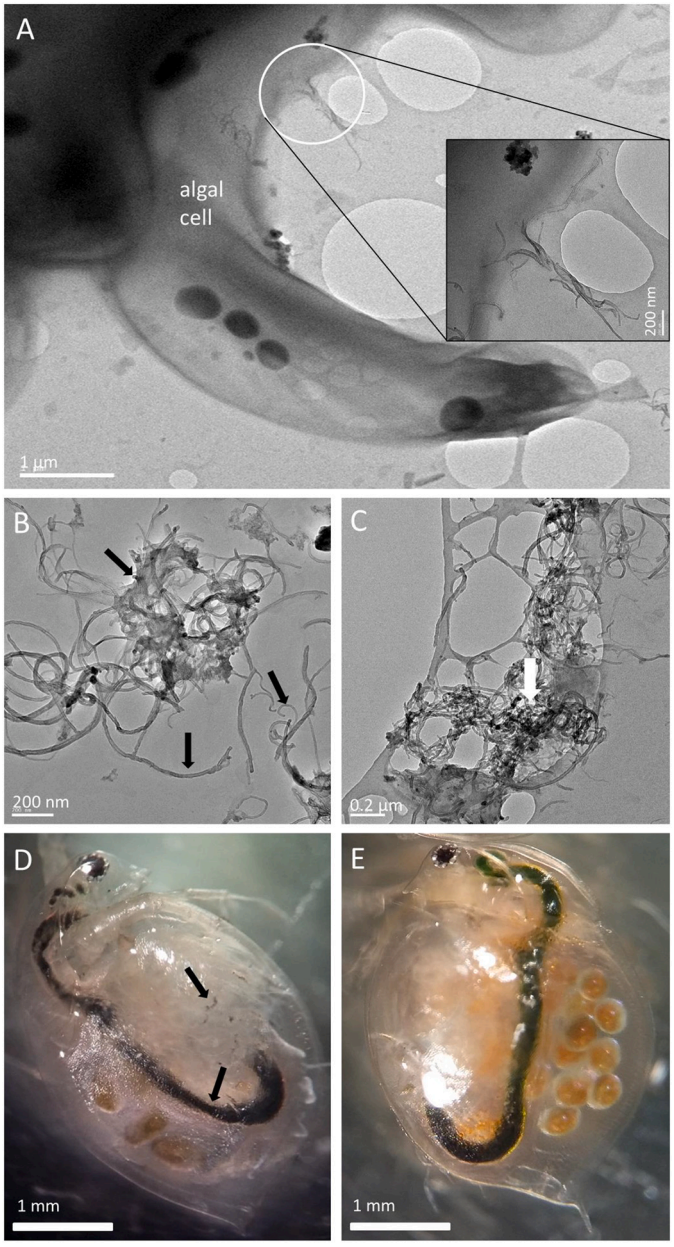

Fig. 2. (A – E): Transmission electron microscopy (TEM) images of wMWCNT in association with green alga *R. subcapitata* (A), wMWCNT dispersion (0.1 mg L^{-1}) (B) and excreted wMWCNT by *D. magna* after uptake period of 24 h (C). D and E show the light microscopy image of an adult *D. magna* exposed to 100 µg wMWCNT L^{-1} and without exposure over a period of 72 h, respectively.

wMWCNT excreted by *D. magna*. Excreted wMWCNT in faeces of *Daphnia* (Fig. 2 - C) occur in agglomerates, which appear much more condensed than, e.g., agglomerates in dispersion of 0.1 mg wMWCNT L^{-1} (Fig. 2- B). Due to the compression of the nanomaterials, an exact estimation of wMWCNT length of the few recognizable single strands is difficult (about 0.8 µm to 1 µm). Fig. 2 - D shows an adult daphnid with internalized wMWCNT after an incubation period of 72 h. The black colored intestine, which appears green under normal conditions (Fig. 2 – E), is particularly well visible. Furthermore, associated small black particles can be seen on the filter apparatus (black arrow). The rest of the animal's body is free of foreign particles. Embryos are visible in the brood chamber. The animal appears pale and colourless.

3.3. Interaction of ^{14}C-wMWCNT with green algae

Recovery of radioactivity in tests with green algae was 95% to 112%. In a pretest, a wMWCNT concentration of 100 µg L^{-1} had no effect on the growth of green algae (data not shown). For *C. reinhardtii*, interaction with ^{14}C-wMWCNT increased in the first 24 h (Fig. 3 - A), but between 24 h (maximum uptake: 1.6 ± 0.4 µg wMWCNT mg^{-1} dw^{-1}) and 96 h (0.9 ± 0.1 µg wMWCNT mg^{-1} dw^{-1}) a decrease in wMWCNT associated to the algae was recorded. Further, wMWCNT association with *R. subcapitata* was considerably lower and resulted in a maximum uptake after 48 h (0.7 ± 0.3 µg wMWCNT mg^{-1} dw^{-1}). Subsequently, the associated amount of wMWCNT decreased to 0.1 ± 0.03 µg wMWCNT mg^{-1} dw^{-1} (96 h) (Fig. 3 - A). During the uptake experiments an exponential growth for both algae species was observed. Initial cell counts increased 5 times and 2.5 times within 96 h in case of *C. reinhardtii* and *R. subcapitata*, respectively (Fig. 3 - B), indicating a significantly higher growth rate for *C. reinhardtii*. Considering the amounts of radioactivity associated with the algae a 24 h BCF (C_b/C_w) of 13,700 L kg^{-1} and a 48 h BCF (C_b/C_w) of 6800 L kg^{-1} was calculated for *C. reinhardtii* and *R. subcapitata*, respectively.

In test systems with both algal species a total of 12–13 µg ^{14}C-wMWCNT was applied. The total associated amount of wMWCNT to green algae was calculated by extrapolation over time. After 48 h 14 ± 3 µg wMWCNT (per 9 ± 0.1 mg dry weight of algae) were already associated with *C. reinhardtii*, leaving no bioavailable fraction of wMWCNT. The total amount of wMWCNT associated to *R. subcapitata* fluctuated over the test period and after 48 h a maximum of 5 ± 2 µg wMWCNT (per 7 ± 0.2 mg dry weight of algae) was associated (Table S2).

3.4. Uptake and excretion of ^{14}C-wMWCNT by D. magna

Daphnid mortality was below 10% in every test. In the uptake experiments the recovery of ^{14}C-wMWCNT was 83 ± 3% to 96 ± 6% and 87 ± 8% to 97 ± 8% for water exposure and trophic transfer, respectively. In the water exposure scenario, an increase in body burden was observed up to 18 h (7.1 ± 1.5 µg wMWCNT mg^{-1} dw^{-1}). Subsequently, elimination process exceeded the ingestion resulting in decreasing internal wMWCNT concentrations. For trophic transfer, the highest body burden was already observed after 2.5 h (0.07 ± 0.01 µg wMWCNT mg^{-1} dw^{-1}), which corresponds to an uptake of 2 orders of magnitude smaller compared to water exposure. It has been shown that the addition of food significantly influences wMWCNT uptake in *D. magna*. Afterwards, elimination processes predominated ingestion. After 72 h only 0.014 ± 0.004 µg wMWCNT mg^{-1} dw^{-1} remained in test organisms. Without food addition, the elimination rate constant (k_2 = 0.015 h^{-1}, exponential fit, Fig. 4) was significantly lower than in presence of food (k_2 = 0.038 h^{-1}).

Even though the application of bioaccumulation factors to nanomaterials is not suitable, in the following accumulation factors to compare with the existing literature are given. For maximum uptake (18 h) in water exposure scenario a BCF (C_b/C_w) of 98,000 ± 30,000 L kg^{-1} was determined. Since no steady state was reached, the BCF changed over time and after 72 h the value was 40,000 ± 17,000 L kg^{-1}. Bioaccumulation changing over time were also observed in the trophic transfer scenario. After 2.5 h (maximum uptake) and 72 h the BAF's (C_b/C_w) amounted to 600 ± 70 L kg^{-1} and 120 ± 40 L kg^{-1}, respectively.

Elimination of ingested wMWCNT by *D. magna* was significantly faster in the setup with food supply, indicated by k_2 of 1.079 h^{-1} compared to that in absence of algae (0.032 h^{-1}; Fig. 5). In presence of algae a portion of 6, 1.6, 0.97, 0.71 and 0.84% of the initial body burden remained after 2.5, 5, 19, 24 and 48 h in the test organisms, i.e., after approximately 5 h the gut was almost completely cleared. At the same time, elimination in clean M4 medium was slower, i.e., 52, 45, 42, 34 and 15% of the initial body burden remained in *Daphnia*. Thus, the presence of food has a significant influence on the uptake of wMWCNT as well as on its depuration.

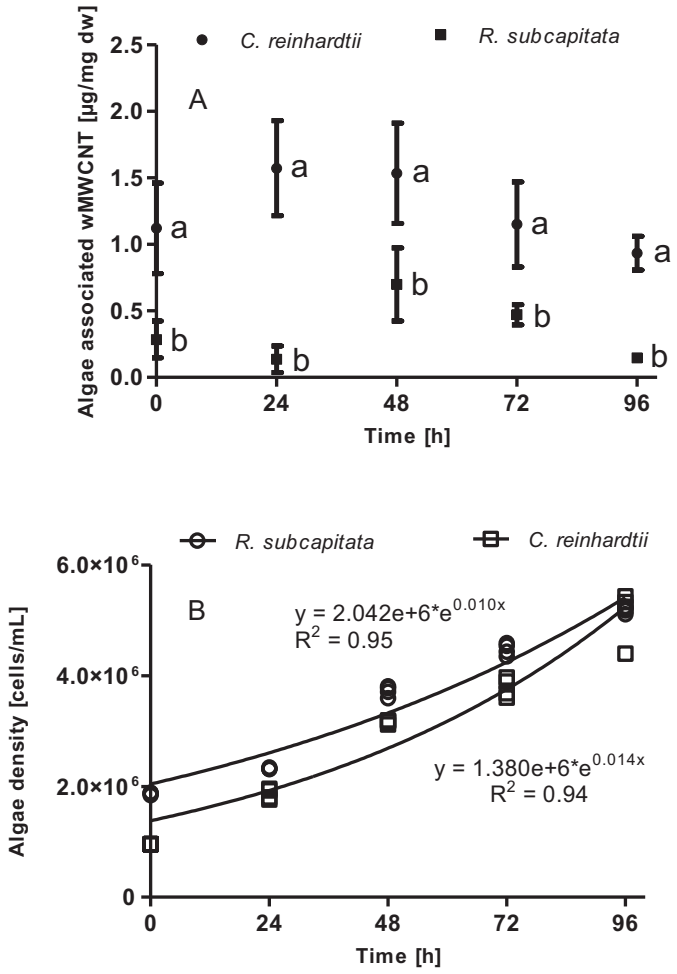

Fig. 3. Weathered multi-walled carbon nanotube (wMWCNT) concentration linked to green algae over time. A ^{14}C-wMWCNT concentration of 100 µg L^{-1} was applied to suspensions of *R. subcapitata* (circles) and *C. reinhardtii* (squares) (A). The mean values and standard deviations are given ($n = 4$). For all timepoints, mean values for uptake of wMWCNT in *R. subcapitata* and *C. reinhardtii* proved to be significantly different (visualized using the letters a and b, *t*-test, $\alpha = 0.05$). An exponential fit was plotted for the corresponding growth curves, algae density (cells mL^{-1}) was measured over time in uptake experiment (B). Growth rates for *C. reinhardtii* and *R. subcapitata* are $\mu = 0.014\ h^{-1}$ and $\mu = 0.010\ h^{-1}$, respectively. A significant difference over time was identified (two-sample *t*-test). Data points represent single replicates ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

With the use of a one compartment model, fitted to the data from 0 h to 18 h a kinetic BCF (k_1/k_2) for the water exposure scenario was determined. The uptake rate constant k_1 was calculated from the experimental data and k_2 was taken from the elimination experiment without food supply ($k_2 = 0.032\ h^{-1}$, Fig. 5). Nevertheless, that only two time points could be applied, the coefficient of determination $R^2 = 0.96$ showed a good fit (Fig. 4). A kinetic BCF of 140,000 L kg^{-1} was calculated, three orders of magnitude greater compared to BAF's from trophic transfer. Due to the missing uptake data in trophic transfer scenario (0 h to 2.5 h), no k_1 could be calculated and therefore no kinetic BAF is given.

3.5. Uptake of ^{14}C-wMWCNT by D. magna population

No significant differences in population size between control and wMWCNT treatment groups were observed except for the last sampling date (Fig. 6). Within 14 days *D. magna* populations grew up rapidly from the start with 8 daphnids per sample to a maximum of approximately 200 individuals (control: 209 ± 40 individuals; wMWCNT treatment: 159 ± 85 individuals) and afterwards dropped to half of the population density. As shown in Fig. 7, body burden for ^{14}C-wMWCNT uptake in *D. magna* increased over time and a maximal value of 0.7 ± 0.2 µg wMWCNT mg^{-1} dw^{-1} was achieved after 28 days. A steady state was reached after weekly applications of 100 µg ^{14}C-wMWCNT L^{-1}. The 28 d BAF (C_b/C_w) was 6700 ± 2900 L kg^{-1}.

4. Discussion

4.1. Dispersion and deposition of wMWCNT

Dispersion of 100 µg wMWCNT L^{-1} in the test media resulted in small agglomerates, formed by van der Waals forces and electrostatic interactions (Kennedy et al., 2008; Zhou et al., 2015), and some single strands visualized by means of TEM. Similar behavior of CNT after a dispersion period of 4 h has been reported (Zhou et al., 2015), indicating that an increase of dispersion interval does not lead to a more homogeneous dispersion. The use of intensive sonication can further result in damage or shortening of CNT strands (Zhu et al., 2006; Saleh et al., 2008).

The M4 medium used contains among other ingredients divalent cations like Mg^{2+} and Ca^{2+} which will further influence the dispersion stability of CNT (Hyung et al., 2007; Chen et al., 2010; Schierz et al., 2014; Glomstad et al., 2018). Additionally, surface functionalization of CNT increases the stability of CNT dispersion in aqueous media (Jackson et al., 2013). Our weathering procedure of CNT using simulated sunlight irradiation was expected to lead to surface functionalization like hydroxyl groups (Klaine et al., 2008), however, we were not able to detect

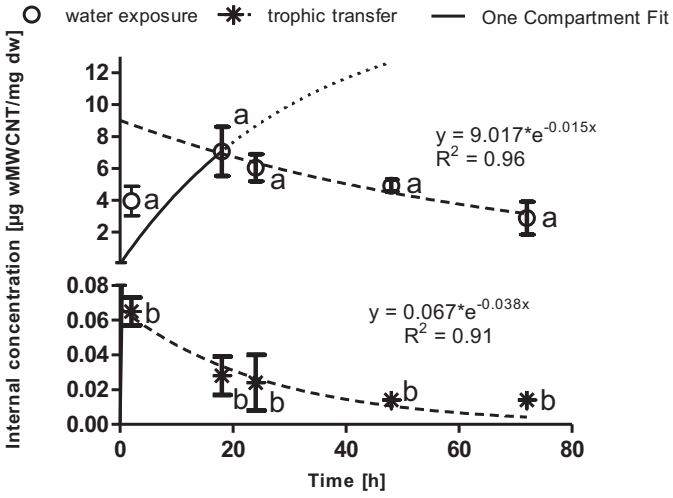

Fig. 4. Uptake of ^{14}C-wMWCNT in *D. magna* after waterborne exposure (circles) and trophic transfer (asterisks). For trophic transfer green algae *R. subcapitata* was pre-loaded by radioactively labeled wMWCNT over 72 h. A one compartment model ($R^2 = 0.96$) was fitted to experimental data of uptake via water exposure (solid line, 0 h – 18 h). Additionally, the further course of the modelling is shown (dotted line). An exponential fit was adapted to areas with uptake decline for water exposure (dashed line, 18 h – 72 h) and trophic transfer (dashed line) scenario. Error bars indicate standard deviation ($n = 4$). For all timepoints, mean values of uptake via water exposure and trophic transfer proved to be significantly different (visualized using the letters a and b, *t*-test, $\alpha = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

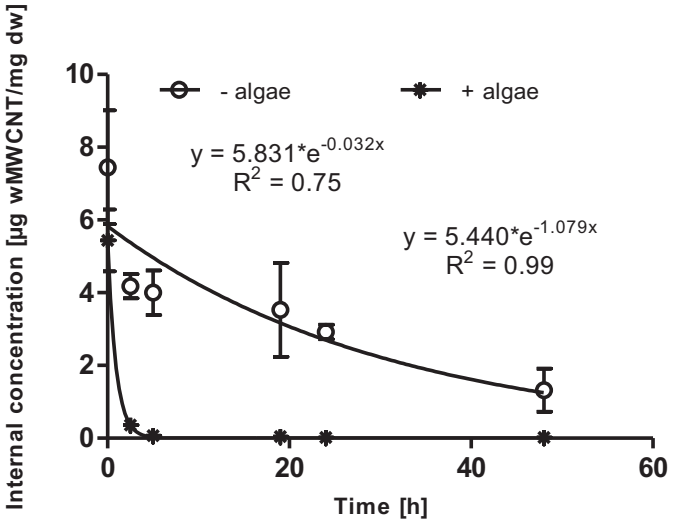

Fig. 5. Internal ^{14}C-wMWCNT concentration in *D. magna* after depuration over a period of 48 h. Start wMWCNT-concentration in -algae (circles) and + algae (asterisks) scenario was calculated from 5 and 7 replicates, respectively. *Daphnia* were fed with green algae (0.1 mg carbon) after transfer to clean medium. Elimination rates (k_2) were determined (exponential fit) to 0.032 h^{-1} and 1.079 h^{-1} in scenario -algae and + algae, respectively. Elimination in presence and absence of algae was significantly different over time (two-sample *t*-test). Error bars indicate standard deviation ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

changes of the surface structure compared to the pristine material by using TGA and FTIR. A possible explanation is the autoxidation of the pristine MWCNT, which can occur due to the prolonged storage of this material. This is a change in surface functionalization, especially also as a function of surface occupancy. Other reasons could be that, e.g., COOH functionalities decarboxylate over time and thus the oxidative functionality cannot be detected, or that the sensitivity of the used method was not adequate to detect the surface modifications. Due to the use of radioactively labeled (^{14}C) wMWCNT, a homogeneous distribution in M4 medium was demonstrated by means of LSC.

In the present study a DT_{50} of 3.8 to 3.9 days was calculated for deposition of 100 µg wMWCNT L^{-1} under non-stirred (static) conditions. Kennedy et al. (2008) found a sedimentation half-life for MWCNT (100 mg L^{-1}) in reconstituted freshwater containing 100 mg L^{-1} natural organic matter (NOM) of 9 min. Schierz et al. (2014) calculated a half-life for single wall carbon nanotubes (SWCNT, 2.5 mg L^{-1}) of 7.4 h in a mesocosm ecosystem. Since the addition of NOM increases the stability of CNT dispersions and thus reduces aggregation and deposition (Schierz et al., 2014; Glomstad et al., 2018), we conclude that the considerably higher DT_{50} values are caused by the lower initial concentration of introduced CNT. In addition, low CNT concentrations, as in our study, will reduce the probability that individual particles collide and interact. Thus, homo- and heteroaggregation, which is also influenced by the physical and chemical properties of the particles and the aqueous medium (Chen et al., 2010), in our experiment is rather low and consequently the sedimentation rate is reduced in comparison to other studies. We expect that sedimentation will be similarly slow or even slower at the much lower environmental concentration in the ng L^{-1}

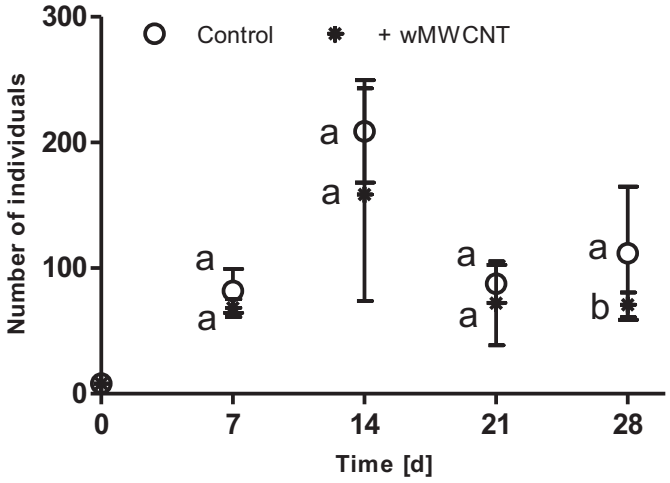

Fig. 6. *D. magna* population dynamics. In 800 mL M4 medium, a population of *D. magna* developed from initially 5 neonate and 3 adult animals over 28 days. *D. magna* were fed daily 0.5 mg carbon (green algae). Two wMWCNT concentrations were tested: 0 mg L^{-1} (Control, circles) and 0.1 mg L^{-1} (asterisks). Total number of individuals over time is given. Error bars indicate the standard deviation on the mean of four replicates ($n = 4$). The combination of the letters a and b visualizes the identified significant difference (*t*-test, $\alpha = 0.05$).

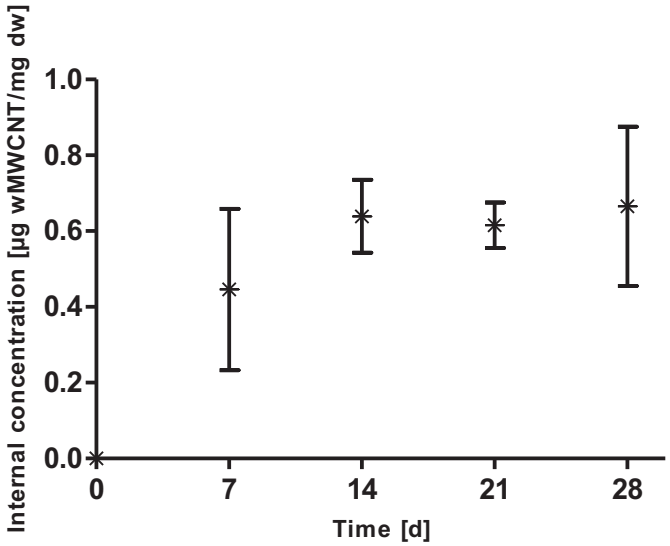

Fig. 7. Uptake of ^{14}C-wMWCNT in a developing *D. magna* population over 28 days. Renewal of medium and simultaneous reapplication of ^{14}C-wMWCNT was performed once a week. Error bars indicate standard deviations for arithmetic average of 4 replicates (n = 4).

range (Gottschalk et al., 2009; Gottschalk and Nowack, 2011).

4.2. Interaction of wMWCNT with green algae

The association of algal cells and wMWCNT was visualized and quantified. TEM analysis showed the linkage of single wMWCNT fibers to cells of *R. subcapitata*. Penetration of the algal cell wall cannot be excluded (Fig. 2 - A, Fig. S2), since this has already been observed (Wei et al., 2010; Long et al., 2012; Rhiem et al., 2015). Additionally, Rhiem et al. (2015) detected MWCNT single tubes in the cytosol of *D. subspicatus* cells but most were agglomerated at the surface of cells. An association of CNT with algal cells instead of the absorption into the cells was further suggested for MWCNT interaction with bacteria *P. aeruginosa* (Mortimer et al., 2016). Schwab et al. (2011) found strong binding of algal cells to CNT and suggested the formation of hydrogen bonds between the cell surface and oxidized gaps in the CNT structure, which could be an explanation for our findings.

During the test, the green algae *C. reinhardtii* and *R. subcapitata* initially showed an increase in the associated amount of wMWCNT which later decreased. Both algae species showed an exponential growth over the entire test period, indicating that the observed decrease in concentration is likely due to growth dilution. Furthermore, increased agglomeration with time may have reduced the bioavailability of the CNT (Klaine et al., 2008; Chen et al., 2010) and prevented further uptake of CNT. For the two algae species, a significant difference in the uptake of CNT was demonstrated: *C. reinhardtii* adsorbed 100% of applied CNT over time but R. subcapitata only ≤43%, indicating a different association behavior of CNT on the two algae species in the accumulation study.

It is known that algae produce extracellular polymeric saccharides (EPS), which interact with nanomaterials (Miao et al., 2009; Zhang et al., 2012; Adeleye and Keller, 2016; Zheng et al., 2019). Sijm et al. (1998) observed that sorption on algae exudates significantly reduced the bioavailability of hydrophobic organic chemicals as well. It is therefore likely that the two green algae produced exudates which differ in their composition (Xiao and Zheng, 2016), which might explain the

higher uptake rate for *C. reinhardtii*. Additionally, in contrast to the samples with *C. reinhardtii*, in those with *R. subcapitata* CNT aggregates, covered by an unidentified material, were observed after 72 h (Fig. S4) leading to CNT precipitation and reducing CNT bioavailability. Zhang et al. (2013) observed formations of similar agglomerates and suggested the presence of quantum dot-algae associates. We assume that these presented agglomerates are composed of algal cells, their exudates and CNT (Verdugo et al., 2004; Xiao and Zheng, 2016).

Sijm et al. (1998) observed a lower bioconcentration of hydrophobic organic chemicals in algae at higher algae densities and recognized that in addition to the physico-chemical properties of the test substance, also the physiology (e.g., species, growth stage, structure) of the algae is of particular importance. Apart from the fact that the spherical *C. reinhardtii* is larger (diameter: 14–22 μm) than the sickle-shaped *R. subcapitata* (helical diameter: 5.6–11.7 μm; width: 1.9–3.6 μm), the two green algae differ in the composition of their cell wall. While the cell wall of the algae cells from the Selenastraceae family (*Raphidocelis subcapitata*) have one homogeneous layer surrounded by a remarkable mucilage, the cell wall of *C. reinhardtii* consists of five different layers, whereas the outer layer contains frayed areas depending on the cell status (Kiefer et al., 1997; Fawley et al., 2006; Baudelet et al., 2017). Therefore, we assume that CNT less likely interact with the mucilage adhering to the cell wall of *R. subcapitata*, but more intensely adsorb onto the inhomogeneous cell wall of *C. reinhardtii*.

A 24 h BCF (C_b/C_w) of 13,700 L kg^{-1} and a 48 h BCF (C_b/C_w) of 6800 L kg^{-1} for wMWCNT accumulation was calculated for green algae *C. reinhardtii* and *R. subcapitata*, respectively. Rhiem et al. (2015) determined a bioconcentration factor of 5000 after 72 h exposure of *D. subspicatus* to MWCNT (1 mg L^{-1}). In calculating the BCF (C_b/C_w), the initial exposure concentration was used as C_w. Dispersion stability influences exposure concentration and sedimentation processes, i.e., organisms are exposed to test concentrations that change over time (Glomstad et al., 2018). In case of the test system with algae, agglomeration processes and the presence of exudates as well as the association of CNT to algae play a decisive role in the bioavailability of the nanomaterials in the water phase. Consequently, the concentration of the freely available CNT is reduced over time and C_w may have been overestimated. As a result, bioconcentration factors are underestimated and probably cannot accurately reflect the accumulation of CNT in algae. Therefore, and since the one-compartment model described has been simplified for application to the accumulation of wMWCNT in algae the given BCF's should be considered with reservation (Praetorius et al., 2014; Bjorkland et al., 2017). Regarding the uptake of wMWCNT, a differentiation between adsorption on and uptake in algal cells was not possible. Nevertheless, it has been shown that CNT associate to a large extent with green algae, which explains the observed food transfer of the nanomaterial to daphnids described below.

4.3. Uptake of wMWCNT by D. magna

Maximum uptake of wMWCNT was observed after 18 h for waterborne exposure and after 2.5 h in trophic transfer scenario. Maximum uptake within the first 24 h has been shown for MWCNT and fullerenes previously (Petersen et al., 2009; Tervonen et al., 2010; Petersen et al., 2011). In our test, maximum concentrations after water exposure and trophic transfer were 7.1 ± 1.5 μg wMWCNT mg^{-1} dw^{-1} and 0.07 ± 0.01 μg wMWCNT mg^{-1} dw^{-1}, respectively. Accumulated wMWCNT accounted for 0.71 wt% after water exposure and 0.007 wt% after trophic transfer of total *Daphnia* dry mass, similarly to a study with MWCNT by Petersen et al. (2011) and graphene by Guo et al. (2013). In a previous study, Petersen et al. (2009) measured body burdens in the range of up to 70 μg mg^{-1} dw^{-1} in a study on the uptake of MWCNT (40, 100 and 400 μg L^{-1}) from the water phase in daphnids, i.e., considerably higher than the body burdens in our experiment. The author explained that the enormous difference in body burdens can be due to the smaller weight of the *Daphnia* used (5–7 days old). The weights of the 5–7 days old *Daphnia* (0.01–0.02 mg per individual) in the Petersen study of 2009 were equivalent to the weight of the neonate animals (≤ 24 h) in our study after 18 h exposure, which on average was 0.01 mg per individual (see Table S3). In a more recent study (Petersen et al., 2011) and in the present study *Daphnia* were fed daily, which most likely resulted in fitter and healthier animals compared to the animals from the Petersen study of 2009.

As shown in Fig. 4, body burden in water exposure increased over the first 18 h, followed by a decrease from 18 h to 72 h. Guo et al. (2013) revealed very similar results for graphene uptake in 1-day old daphnids (max. uptake of 250 μg L^{-1} after 24 h: 7.8 μg mg^{-1} dw^{-1}). Decrease in body burdens after 18 h probably resulted from agglomeration and settling of CNT, and thus lower concentrations in the water column. The deposition experiment (Fig. 1) showed that after 18, 24, 48 and 72 h a ^{14}C-wMWCNT portion of 88.4, 87.7, 77.5 and 69.5% remained in the water column. In addition to this fast sedimentation behavior in an abiotic test system, in the presence of animals, it was suggested that agglomeration of CNT is enhanced by passage through the gut tract and biomodification of the CNT surface (Roberts et al., 2007; Petersen et al., 2009; Guo et al., 2013).

As for trophic transfer, uptake of wMWCNT was two orders of magnitude lower compared to water exposure. A study of MWCNT uptake in *D. magna* showed that the presence of food during exposure revealed body burdens of up to 0.8 μg g^{-1} and 0.02–0.06 μg g^{-1}on dry mass basis for 5 to 15 days old daphnids after 24 h and 3 d, respectively (Cano et al., 2017; Cano et al., 2018). The fact that uptake in the presence of algae results in decreasing body burdens over time (Fig. 4) and k_2 is more than twice as large in the presence of food indicates that besides agglomeration and sedimentation processes, excretion determines the body burden, as described in the model of Connel (1998). Roberts et al. (2007) showed that solubilized lipid coated SWCNT were ingested by *D. magna* and egested as precipitated SWCNT without coating. Because survival of test organisms was dose-dependent, the authors suggested that *Daphnia* were able to accept lipid coating as food source. In our trophic transfer study, wMWCNT were loaded on algae, therefore algae from ingested algae-wMWCNT complexes might be absorbed in the digestive system leading to the significant increase in weight of the exposed daphnids compared to the animals from the water exposure study (Fig. S5). Animals after 72 h water phase exposure looked pale and their gut was filled with nanomaterial (Fig. 2 - D). Well-fed daphnids are more intensely colored than starving organisms and coloring corresponds to the food intake (Ebert, 2005). Nanomaterials were mainly observed in the gastrointestinal tract of the test organisms and adhered to thoracopods. Previous studies already established that CNT are unlikely to be absorbed by the organism beyond the intestine (Petersen et al., 2009; Edgington et al., 2010; Parks et al., 2013; Edgington et al., 2014), which is one reason why they are not bioaccumulated in the classical sense like soluble chemicals (Praetorius et al., 2014; Bjorkland et al., 2017). Due to the lack of food, animals of waterborne exposure were obviously in poor physical constitution.

The calculated bioconcentration factors were 140,000 (kinetic BCF, L kg^{-1}) and 120 (BAF, L kg^{-1}) on dry mass basis for waterborne exposure and trophic transfer, respectively. For trophic transfer, bioconcentration factor was three orders of magnitude smaller than after water exposure. Mortimer et al. (2016) revealed similar differences in BCF values by two orders of magnitude of 35,000 L kg^{-1} and 800 L kg^{-1} when *T. thermophila* was exposed to MWCNT-amended medium and MWCNT encrusted bacteria, respectively. Petersen et al. (2009) described BCF values of 360,000 L kg^{-1}, 440,000 L kg^{-1} and 350,000 L kg^{-1} for exposure of *D. magna* to 40, 100 and 400 μg L^{-1} over 48 h. All above mentioned BCF values indicate high bioaccumulation of CNT which originate predominantly from accumulation of nanomaterial in the gut. CNT amounts attached to animals' outer surfaces can be considered negligible.

4.4. Elimination of wMWCNT by D. magna

As shown in Fig. 5, daphnids were able to fully purge CNT from their intestine in presence of algae. Elimination of CNT in pure M4 medium was significantly slower and incomplete. The positive influence of food on elimination processes is also shown by the different weights of the animals in the experiment. The increasing weight of *Daphnia* in the scenario with algae from 0.127 to 0.267 mg per 10 neonates (Fig. S6, Table S4) suggests that these animals were considerably fitter than their conspecifics (0.136 to 0.118 mg). Petersen et al. (2009) showed no excretion of MWCNT by *Daphnia* in clean or filtered lake water and after addition of algae during depuration, body burdens decreased only by 50–85% in the first hours. As already mentioned, *Daphnia* from this study were 5–7 days old and as heavy as the neonate daphnids in our study, which probably were not able to efficiently purge their guts due to health issues. The ability of daphnids to empty their intestines even without food source was also shown after gold nanoparticle intake (Lovern et al., 2008). It is known that daphnids can defecate by peristaltic movements, but still need the pressure of further ingested food (Ebert, 2005). Depuration of graphene and coated MWCNT in presence of algae revealed clearance of more than 90% and 89–99%, respectively (Petersen et al., 2011; Guo et al., 2013). The addition of algae as food source therefore facilitates excretion (Gillis et al., 2005; Kennedy et al., 2008; Petersen et al., 2011; Guo et al., 2013). Nevertheless, it was shown that depuration of CNT by neonate daphnids increased over time even without food intake since *Daphnia* take up water from their surroundings to stimulate their digestion (Fox, 1952).

4.5. Uptake of wMWCNT by D. magna population

As shown in Fig. 6, population of *D. magna* grew rapidly in the first two weeks to a maximum of approximately 200 animals and then population density leveled off to an equilibrium size. Similar population dynamics were observed in laboratory (Hammers-Wirtz and Ratte, 2003) and model systems (Preuss et al., 2009). In our study, no significant differences on growth of population between control and treatment groups were observed except for the last sampling date. The significantly smaller population size after 28 d in the treatment group may be due to chance, but a long-term effect on the growth of a *D. magna* population caused by a wMWCNT concentration of 100 μg L^{-1} cannot be completely excluded, especially since the population size of the treatment over the whole period shows lower numbers than the controls. Biologically evaluated, the exposure to wMWCNT under normal feeding conditions for 28 days had no effect on population dynamics. Uptake of ^{14}C-wMWCNT increased from 0 d to 14 d before steady state was reached.

Maximal body burden was 0.7 ± 0.2 μg mg^{-1} dw^{-1} after 28 d. The value for the received body burden from population experiment (Fig. 7) is smaller than the maximum body burden from experiment without food apply (water exposure) and one order of magnitude higher than maximum body burden from trophic transfer scenario, where CNT were loaded to algae prior to exposition (Fig. 4). In population experiment steady state was reached unlike water exposure and trophic transfer scenario. The significant smaller body burden in population experiment compared to the water exposure group can be explained by the facilitated excretion due to feeding and because of *D. magna* in different growth stages - from neonates to adults - existed. The ratio of accumulated nanomaterial mass to total organism mass is smaller when larger organisms are applied (Petersen et al., 2011). As for the daily feeding in population experiment, *Daphnia* do not have a pronounced food preference and consumes particles ranging in size from 0.1 to 30 μm (Lynch, 1978), which is why algae and dispersed CNT were competing for uptake. The maximum body burden of wMWCNT in *D. magna* population experiment was, despite of weekly applications over 28 days, significantly smaller compared to exposure of neonates in the test medium without food. This can serve as further indication that CNT are not enriched and stored in tissues other than the intestine (Edgington et al., 2014).

The significant higher body burden in the population experiment can be explained by the fact that daphnids were fed daily with weekly ^{14}C-wMWCNT applications, compared to the trophic transfer study where a single application of CNT-loaded algae was performed. Additionally, the reduced settlement of CNT by aeration and turbulence of water phase led to a higher bioavailability of CNT. Furthermore, it has already been shown that increasing volumes of water at the same concentration leads to higher body burdens because of the larger amount of available CNT (Petersen et al., 2009). Our results suggest that the constant discharge of CNT into a water body under normal food conditions causes the accumulation of these by *D. magna* until a steady state is reached. A further transport of CNT along the food chain is thus possible.

In the present study, a BAF of 6700 ± 2900 L kg^{-1} was calculated. Cano et al. (2017) investigated the uptake of MWCNT at a concentration of 0.1 mg L^{-1} in *D. magna* (60 individuals) at 600 mL water for 24 h and obtained body burdens up to 0.0008 μg MWCNT mg^{-1} dw^{-1} under the application of food. The body burden is two and three orders of magnitude smaller compared to our trophic transfer and population experiments, respectively, probably because of the short exposure duration of 24 h compared to 7–28 days of exposure in our study. Another reason for the lower accumulation in the study of Cano et al. (2017) may be the use of a surfactant (sodium dodecyl benzene sulfonate) that has been shown to have toxic effects on *D. magna* in high concentrations and therefore might have influenced the uptake of CNT by test animals. The influence of different food and starving conditions on uptake of CNT in an evolving *D. magna* population should be further investigated.

5. Conclusion

In the present study it was shown that weathered CNT at μg L^{-1} concentrations persist for several days in the water phase of a static aquatic system and interact with living organisms. Depending on the shape and metabolic activity of green algae, CNT associate to algal cells to a large extent and, as nanomaterial carriers, lead to food web transfer. In case of *D. magna*, trophic transfer resulted in orders of magnitude lower body burdens compared to waterborne exposure indicating no bioaccumulation. Daphnids excrete CNT in absence and presence of food.

To our knowledge we are the first to investigate uptake of CNT in a growing *D. magna* population. Multiple CNT (100 μg L^{-1}) applications lead to steady state body burdens in daphnids after 14 days but no toxic effects compared to control groups. Results indicate that uptake of nanomaterials by *D. magna* depends on CNT dispersion stability, food supply and therefore on the physiological constitution of test organisms. Finally, long residence times of CNT at low concentrations in the water phase increase the interaction and association probability with algae and subsequently lead to the food-chain transport of CNT to higher organisms.

Compared to other published investigations, our population experiment reflects natural conditions and resulted in a bioaccumulation factor of >2000 for the weathered MWCNT which would be classified as a bioaccumulative chemical and therefore hazardous under the EU REACH regulation. However, it must be considered that bioaccumulation of nanomaterials differs from that of dissolved organic chemicals, because accumulation is located mainly in the intestine of organisms. Considering that the estimated environmental concentration of CNT (ng L^{-1}) is lower than our tested concentrations by five orders of magnitude we can assume, that no risk is to be expected for aquatic organisms by exposure to CNT.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2021.100303.

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