Single-walled carbon nanotubes toxicity to the freshwater amphipod *Hyalella azteca*: influence of sediment and exposure duration

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Abstract

Carbon nanomaterials are present in various industrial applications and therefore their release into the environment including freshwater ecosystem is expected to increase. The aim of the present study was to investigate the influence of several parameters on the toxicity of single-walled carbon nanotubes (SWCNT) to the freshwater amphipod, *Hyalella azteca*. The effect of period of exposure, sediment presence and possible impurities released during SWCNT preparation on survival and/or growth of such organism was evaluated. We measured a reduction of survival at concentrations ranging from 10 to 40 mg/L after 96-h exposure, while no mortality was observed with the same concentrations and in the presence of artificial sediment after 14 days of exposure. It is possible that SWCNT are adsorbed on the organic matter from the artificial sediment leading to a decrease of SWCNT bioavailability. The survival and growth toxicity tests revealed a stronger effect at 28 days compared to the 14 days of exposure, and full mortality of organisms at 1000 mg/L for both exposure times. The presence of SWCNT in the gut of survived organisms was observed. The present study demonstrates that the interaction with sediment should be considered when carbon nanotubes toxicity through water exposure is investigated.

Introduction

Single-walled carbon nanotubes (SWCNT) are hollow graphene cylinders with size of 1000 nm to >10^6 nm in length and 1-2 nm in diameter. They have many applications because of their mechanical properties (toughness and strength), high electrical and thermal conductivity, and unique electronic and optical characteristics.[1] Nowadays, they are used in several types of products such as textiles, automobiles or sports equipment.[2] Carbon nanotubes are expected to be extensively used for water treatment and medical applications.[3,4] Therefore, investments in research and development of carbon nanotubes by governments and industries are increasing all over the world. For instance, the National Nanotechnology Initiative from the U.S. Government research and development had a Federal Budget of more than $1.7 billion for 2014.[5] Consequently, the release of manufactured nanoparticles, including carbon nanomaterials, into the environment is likely to increase and concern over the potential impacts for the aquatic ecosystem is growing.

Toxicity of SWCNT has been studied with several model organisms such as algae, invertebrates, and fish.[6-9] There have been few investigations conducted on benthic invertebrates and very limited toxicity data are available on amphipods.[10] In recent studies, toxicity of carbon nanomaterials was examined in selective matrices with the organisms being exposed through water or sediment.[11,12] Other routes of exposure for the crustacean *Daphnia magna*, such as dietary, have been studied elsewhere.[13,14] Previous studies have shown that size, surface of functional groups and surface charge of carbon nanotubes could affect toxicity.[15] Others have reported that as-produced SWCNT could be more toxic than functionalized carbon nanotubes,[16] where toxicity is due to metal impurities rather than the nanotube itself.[17-19] Therefore, it is recommended to measure impurities possibly released in the medium or to conduct a filtrate-only control experiment.[20] Nevertheless, the mechanism of SWCNT toxicity is not well understood. In addition, some studies did not detect any SWCNT toxicity on fish or benthic organisms.[21,22]

The hydrophobic nature of SWCNT relates to their tendency to sink in water. But in the aquatic environment, natural coatings such as organic matter can increase the carbon nanotubes dissolution by covering their surface.[14] Therefore, the use of organisms living at the interface of water and sediment is highly relevant to estimate their impacts on aquatic ecosystems. *Hyalella azteca* is a freshwater amphipod living on the surface of sediment and is frequently used for toxicity studies because of its ubiquitous presence in the freshwater environment, contact with sediment, ecological importance and ease of culture in the laboratory.[23] A previous study demonstrated that purified or as-produced carbon nanomaterials could diminish the survival and growth of *H. azteca* when exposed through water only.[17] It has been shown that carbon nanomaterials can be toxic for organisms when exposed through the water column whereas toxicity can be either reduced or is not detected when carbon nanomaterials are mixed with sediments.[21,24] Several studies have reported that carbon nanomaterials can reduce the bioavailability of organic contaminants to various terrestrial and aquatic organisms including benthic invertebrates.[24-26] To the best of our knowledge, thus far no study describes the toxicity of SWCNT on *H. azteca* through water exposure and on artificial sediments.

The aim of this study was to investigate the influence of period of exposure, sediment presence and possible impurities released during SWCNT preparation on the toxicity of SWCNT to the freshwater amphipod *H. azteca.* The objectives were: i) to compare the effects of acute and chronic exposure; ii) to investigate the toxic effect of impurities released by using a filtered solution of SWCNT; and iii) to evaluate the effect of the presence of sediment on toxicity.

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Materials and Methods

Characterization and preparation of single-walled carbon nanotubes

SWCNT were provided by Dr. Benoit Simard's (NRCC, Ottawa) research team. The chemical properties of dry SWCNT were measured using thermo-gravimetric analysis (TGA) and the ratio of the G-band (~1580 cm^{-1}) to the D-band (~1350 cm^{-1}) was established using Raman spectrometry (WiTec confocal microscope fiber coupled to an Acton 2500i spectrograph fitted with a Roper Scientific CCD array detector). The diameter of SWCNT was calculated from the RBM wave number (ω) using the following relation: D (nm) = 248/ω. Dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK) was used to determine the particle size and electrophoretic mobility of SWCNT suspensions. The latter was calculated from the zeta potential values using the Henry equation with the Smolchenski approximation:

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \quad (1)$$

where *UE* is the electrophoretic mobility, ε is the dielectric constant of the medium, z is the zeta potential, f (ka) is Henry's function (1.5) and η is the viscosity.

Morphology and diameter were determined by field emission gun scanning electron microscopy (FEG-SEM) (Hitachi 4700; Hitachi Ltd., Tokyo, Japan) operated at 2 kV and transmission electron microscopy (TEM) (Hitachi H-7100; Hitachi Ltd.).

Artificial freshwater medium (M4) was prepared with $NaHCO_3$, 64.8 g/L; $CaCl_2$ $2H_2O$, 293.8 g/L; $MgSO_4$ 7 H_2O, 123.3 g/L and KCl, 5.8 g/L diluted in Milli-Q water (>18 mV). For each test, the appropriate quantity of SWCNT (10-1000 mg) was added to 1 L of the medium in a Pyrex glass beaker and dispersed by probe sonication for 15 min at 80 W (Ultrasonic processor, frequency 25 kHz, Misonix S4000; Misonix, Inc., Farmingdale, NY, USA).

Test organisms and artificial sediment

H. azteca were maintained in lab cultures with reconstituted M4 medium (pH, 7-8; hardness, 230-260 mg $CaCO_3$ mg/L).[27] Organisms were placed into an experimental chamber with programmed temperature and photoperiod (23°C±3, 16/8 h light/dark). Organisms were fed twice a week with a mixture of yeast, tetramin and rabbit pellets (YCT) and algae *Pseudokirchneriella subcapitata* (50 mL; 1.5×10^6 cells/mL). All assays were conducted in a chamber under the same temperature and photoperiod regime using M4 as dilution medium. Constant aeration was applied.

Artificial sediment was prepared according to the Organization for Economic Cooperation and Development (OECD) protocol 218 by mixing 5% peat powder on a dry-mass basis, 75% quartz sand (50% of the particles in the range of 50-200 mm), 20% kaolinite clay, and 0.1% of $CaCO_3$ to adjust sediment pH.[28] Experiments were conducted in 300 mL polypropylene beakers containing 100 mL sediment and 160 mL of the test solution as overlying water.

Toxicity test and quality control

Before and during the study, $CdCl_2$ (Sigma-Aldrich, Buchs, Switzerland) was used as a reference toxicant (positive control, concentration range 80-1280 μg/L) using the 96-h water-only assay to evaluate the quality of cultured organisms. Other test validation criteria were verified against guidelines criteria and laboratory in-house control data. At the beginning of each test, the following variables were measured in SWCNT solutions: temperature, dissolved oxygen, pH and conductivity, water hardness, ammonia and nitrite concentration.

Effects of acute and chronic exposure

Here, we chose to use a very wide range of concentrations, which are not representative of what can be estimated in the environment, because we found an important variability in values of toxic concentrations in the literature.[13,17] In addition, we wanted to identify mechanisms of toxicity at the species level and establish a dose-response relationship which could be used for hazard assessment.

To identify the influence of the period of exposure, we performed two standardized tests: the acute 14-d and the chronic 42-d toxicity tests, according to the guidelines of Environment Canada.[27] The tests were conducted using early life stage (7 to 14 days old) organisms exposed to overlying water-spiked SWCNT on artificial sediment. The nominal test concentrations assayed were 0, 10, 32, 100, 320, and 1000 mg/L (prepared as described above) and a minimum of 5 replicates were used. Because SWCNT solutions were added on the sediment and not mixed with it, no decrease in SWCNT concentration could occur. Seven days prior to the addition of organisms, aeration was applied to each test beaker to allow them to equilibrate before the beginning of the experiment. At the start of the test, SWCNT were agglomerated and a distinct layer (its thickness increased with SWCNT concentration) was visible on sediment. Organisms were randomly assigned to the test beakers until each beaker contained 10 organisms. Daily, 0.750 μL of food (YCT) was added to each test chamber.[27]

The number of living organisms (identified by their ability to swim) was counted after 14 days (for the 14 days test) or 28, 35 and 42 days (for the 42 days test). According to test guidelines, survivors from 14 days and 28 days counts were kept and frozen (−20°C) until growth measurement was performed on each organism of each replicate. A sample of organisms taken at the beginning of each test was kept and frozen under the same conditions. As recommended by the guidelines, a test was considered invalid if the survival for the control treatment was <80%.[27]

Effects of a filtered solution of single-walled carbon nanotubes

Here, we indirectly tested the hypothesis that toxicity is caused by catalyst impurities added during the preparation of SWCNT.

Amphipods were exposed to a filtered solution of SWCNT initially at 0, 100, 320 and 1000 mg/L, according to the guidelines of Environment Canada.[27] The nominal test concentrations assayed were prepared by sonication of SWCNT (previously described) in artificial medium (M4) and then filtered using a 0.22 μm filter (EMD, PES membrane) to remove carbon nanotubes. Reduction/elimination of carbon nanotubes was verified by measuring the quantity of SWCNT in filtrate using their absorption at 800 nm with optical spectrophotometry measurements and TEM analysis. Three replicates containing 10 organisms from 2 to 9 days old were exposed to 100 mL (in polypropylene beakers of 150 mL) of the corresponding test solution with a piece of mesh fabric (cheesecloth) for substrate. On the third day animals were fed with 0.750 μL of YCT.

The number of living organisms was counted after 96-h of exposure and a test was considered invalid if the survival for the control treatment was <90%.

Influence of sediment presence on toxicity

To test if the presence of sediment could influence SWCNT toxicity, two acute standardized tests were performed. A 96-h water only and a 14-d toxicity test were conducted using the following nominal test concentrations 0, 2, 5, 10, 20, and 40 mg/L. The assays were performed according to the guidelines of Environment Canada and were conducted as described above.[27]

Observation of test organisms

To confirm the interaction between SWCNT and *H. azteca*, the presence of SWCNT in the entire gut of organisms from the two 14-d toxicity test was established during growth measurement. Amphipods with more than half of their gut filled with an intense black color (interpreted as SWCNT) were counted for each replicate. The percentage of organisms with carbon nanotubes inside them was calculated as a mean for each condition.

Data analysis

Growth rate was determined by measuring survivor's length which is a more sensitive parameter than weight measurement for *H. azteca*.[29] A binocular microscope (Stemi 2000-C; ZEISS International, Oberkochen, Germany) associated with a camera (Powershot G6, Canon, Inc., Tokyo, Japan) was used to photograph organisms. The length of each amphipod was then measured using the ImageJ software. Growth data are expressed as percentage increase and average per replicate and concentration were established.

The lethal concentration (LC_{25}, LC_{50}) was calculated using probit analysis or Trimmed Spearman-Karber using the TOXCAL software (version 5).[30] Effective concentration (EC_{50}) values were calculated using nonlinear regression techniques or linear interpolation when data were not amenable to nonlinear regression.

Statistical differences between treatments were assessed using one-way ANOVA followed by Tukey's test. Remaining data were analyzed with the Kruskal-Wallis test followed by multiple comparisons like Steel dwass. Calculations were performed with JMP 4 software.

Results and Discussion

Characterization of dry test product and prepared solutions

Characterization of dry SWCNT by TGA analysis confirmed that SWCNT were as-produced.[31,32] Raman spectrometry analysis revealed the presence of aldehyde groups (CH) at the end of the nanotubes and inclusions other than pure carbon in the sample like amorphous carbon (*data not shown*). The SWCNT diameters were estimated at 1.53 nm.

The FEG-SEM images confirmed the presence of impurities on nanotubes (Figure 1A and B) like amorphous carbon particles.[31] The characterization of nanotube structural parameters by TEM analysis (Figure 1C and D) revealed that SWCNT are long and without a defined inner channel.

For water-only tests, SWCNT formed large agglomerates with an average size ranging from 287 to 2063 nm, and an electrophoretic mobility with a minimum value of 1.4×10^{9} m^2/Vs and a maximum of 0.8×10^{9} m^2/Vs [mean= 1.1±0.1 standard deviation (SD)]. Large agglomerates were also measured in water from sediment-toxicity tests with an average size ranging from 766 to 983 nm and an electrophoretic mobility ranging from 1.6 to 1.3×10^{9} m^2/Vs (mean= 1.5±0.1 SD). Organisms are then expected to interact with agglomerated forms of carbon nanotubes. Indeed, agglomerates of SWCNT were visible in the amphipods gut (example shown in Figure 2). Whereas no SWCNT were visible in the organisms at 2 mg/L, agglomerates were observed for the following concentrations: 5, 10, 20, 32, 40, 100, 320 mg/L and the percentage of organisms having nanotubes in their gut increased along with the values of the concentrations tested (Figure 2C). In a previous study, carbon nanotubes were observed in the gut of test organisms using transmission electron microscopy.[17] It is likely that adverse effects of SWCNT observed on amphipods are caused by the blocking of the digestive track that could lead to a decrease in food intake (not tested in this study). Another investigation showed no dietary effect of carbon nanotubes in *Drosophila melanogaster*.[33] A study using *Ceriodaphnia dubia* demonstrated the difficulty of ridding large CNT agglomerates from the gut of organisms which eventually caused immobilization and increased mortality.[16]

The same study demonstrated that exposure of *H. azteca* to high concentrations of multi-walled carbon nanotubes (MWCNT) in the presence of sediment can cause lethality, with an LC_{50} of 50 to 264 g/kg. Nevertheless, even if carbon nanotubes were visualized into organisms, nothing indicates that SWCNT actually penetrated the cells of amphipods. This possibility is unlikely considering that carbon nanotubes tended to form large agglomerates in our medium. Earlier studies reported that purified and as-produced SWCNT were not adsorbed into tissues of an oligochaete, a copepod, a polychaete, and daphnids.[24,34,35] Similarly, TEM analysis of fullerene uptake by the oligochaete *Lumbriculus variegatus* did not indicate absorption across the gut tract. Although we did not study SWCNT bioaccumulation in *H. azteca*, the ingestion of carbon nanomaterials suggests that it could eventually be transferred in the food chain through the ingestion of amphipods by other organisms, and potentially be toxic to organisms indirectly exposed. Bioaccumulation and biomagnification were previously confirmed with the fish *Danio rerio* exposed to MWCNT and the ciliated protozoan *Tetrahymena thermophila* exposed to cadmium quantum dot, respectively.[36,37]

Toxicity of single-walled carbon nanotubes: influence of exposure time

The water quality parameters for the toxicity tests were all within the acceptable ranges: 21-24°C for temperature, 91-97% of dissolved oxygen, 7.3-7.8 for pH and 643-750 μS/cm for conductivity. Ammonia and nitrite concentrations remained very low and water hardness was estimated to be 240-290 mg/L.

The 14-d toxicity test showed high variabili-

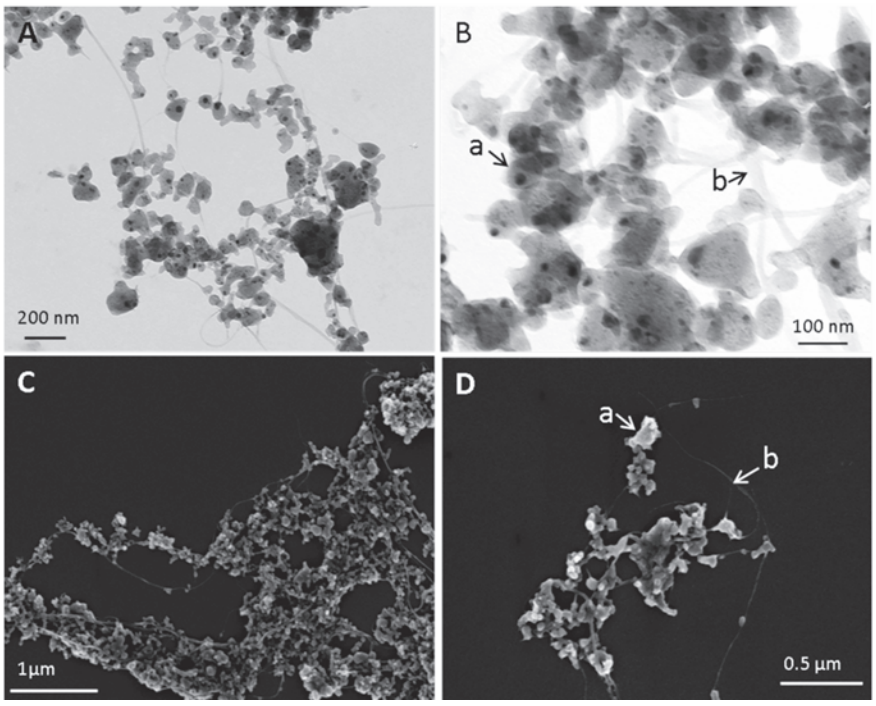

Figure 1. Single-walled carbon nanotubes (SWCNT) solubilized in M4 medium observed with field emission gun scanning electron microscopy (A and B) and transmission electron microscopy (C and D). Both techniques allow visualization of carbon nanotubes (a) and impurities (b) forming rounded aggregates.

ty of effects between replicates, which is probably due to the high variability of the agglomerates size formed in the medium. No significant differences were measured between the control and other treatments for survival or growth within the concentration range and death of all organisms occurred at 1000 mg/L (Figure 3). The 42-d toxicity test showed a statistically significant decrease in survival at ≥320 mg/L after 28 days of exposure (Figure 4) and only a trend to decrease was observed after 35 and 42 days. This is probably due to the lower number of replicates (three) used for the two endpoints. For growth, a statistically significant decrease was measured at 320 mg/L after 28 days of exposure (Figure 4). The highest concentration induced death of all organisms. For survival and growth inhibition, LC_{50} and EC_{50} were both >320 mg/L. We concluded that exposure conditions affected the toxicity measurement endpoints and that a longer period of time can lead to a stronger effect on lethality and growth toxicity endpoints for *H. azteca*.

The effects of carbon nanomaterials chronic exposure have already been reported by Alloy and Roberts (2011) who showed that MWCNT stabilized in natural organic matter decreased growth and reproduction of *D. magna*.[38] Similarly, a reduction of growth was observed for the midge *Chironomus riparius* larvae exposed to a fullerenes solution deposited on top of the sediment.[39]

Toxicity of a filtered single-walled carbon nanotubes solution

A 96-h water-only lethality test was carried out to study the effect of a filtered SWCNT solution through water only exposure. No significant differences between the control and other treatments were measured for survival with a mean of survival >80% for all treatments (*data not shown*). Therefore, filtered SWCNT solutions were not toxic even though the sonication process was used for a sufficient period of time (15 min at 80 W) to solubilize metal impurities.[40] Unfortunately this could not be confirmed by direct measurements of metal concentrations in the test medium. Previously, metal impurities in carbon nanotubes were reported to have contributed to the hatching delay of zebrafish,[8] and have stimulated oxidative stress.[41] However, here, we attributed the toxicity to accumulation of SWCNT in the amphipod guts rather than to the dissolution of impurities. Carbon nanotubes could also induce nutrient depletion by adsorbing critical components of the exposure medium.[20]

Toxicity of single-walled carbon nanotubes: influence of sediment

After conducting a series of 96-h water-only lethality test, significant differences between

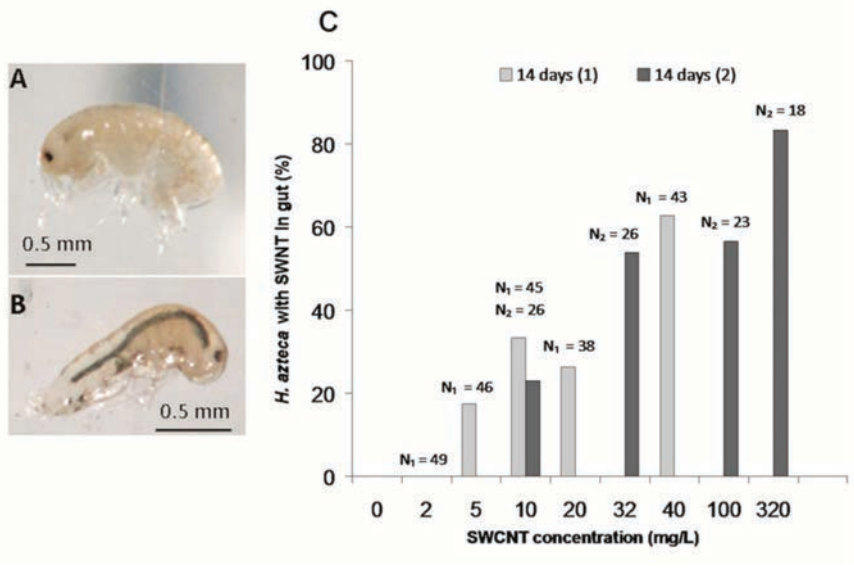

Figure 2. Accumulation of single-walled carbon nanotubes (SWCNT) in *Hyalella azteca*. Organisms exposed to medium M4 (A) or SWCNT at 320 mg/L (B) and mean proportion (%) of amphipods with SWCNT in their gut after exposure to different concentrations of SWCNT in M4 for 14 days (C). N indicates the number of organisms used for the calculation: N1 and N2 correspond to the 14 days test (1) and (2) respectively (no statistical significance tested).

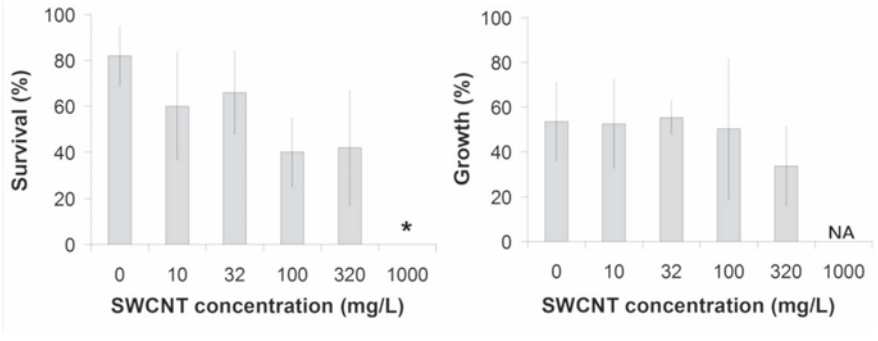

Figure 3. Mean survival and growth of *Hyalella azteca* exposed to different concentrations of single-walled carbon nanotubes (SWCNT) in M4 for 14 days. Each column is the mean of 5 replicates ± standard deviation and * indicates significant difference of group exposed compared to the negative control. NA; not applicable due to no survival.

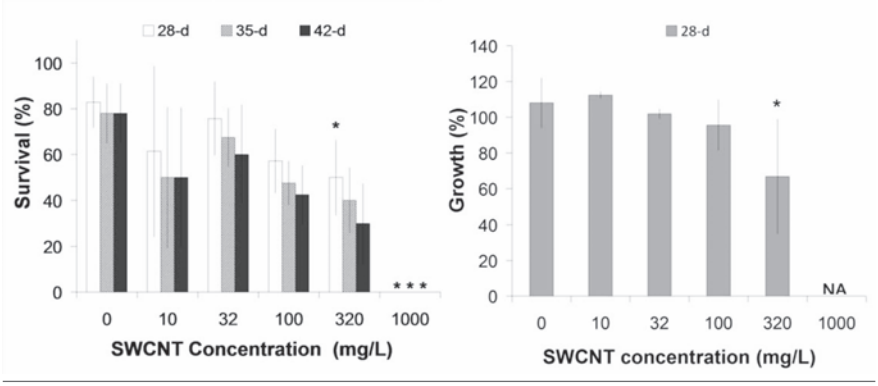

Figure 4. Mean survival and growth of *Hyalella azteca* exposed to different concentrations of single-walled carbon nanotubes (SWCNT) in M4 for 42 days. Survival of organisms after 28, 35 and 42 days of exposure and growth rate measured after 28 days of exposure. Each column is the mean of 3 to 6 replicates ± standard deviation and * indicates significant difference of group exposed compared to the control.

the control and treatments were measured at ≥5 mg/L (Figure 5). The LC_{25} was 18.87±2.04 mg/L and LC_{50}>40 mg/L, *i.e.*, greater than the higher concentration tested.

However, using the 14-d toxicity test, in the presence of sediment, and the same concentrations, no significant differences between the control and all treatments were measured for survival or growth (Figure 5). Therefore, the toxic effect of SWCNT was altered based on presence of sediment. Earlier work has shown that a 10-d exposure of SWCNT in sediment at 30 mg/kg did not induce lethality to the lugworm *Arenicola marina*.[20] The lower toxicity of SWCNT could be explained by the presence of organic matter as previously demonstrated with multi-walled carbon nanotubes, stabilized in aqueous phase by natural organic matter.[42] Even if the comparison is indirect because of different exposure durations used, differing exposure conditions (water against presence of sediment) demonstrated the potential effects of sediment presence on SWCNT bioavailability. It is important to emphasize that artificial sediment was used instead of natural sediment, in order to facilitate comparison of results among laboratories as well as with previous data. Artificial sediment has relatively high organic matter content (5% peat moss) and is consequently less representative of sediment in the natural environment. In addition, the impact of sediment on toxicity will also depend on the different concentration in fine particles in aquatic sediments. In the future, studies should be conducted using natural sediment from reference site (free of any contamination) with subsequent comparison of toxicity results for risk assessment purposes or to assess toxicity-based criteria.

Although here the presence of sediment decreases the toxicity of SWCNT, it is important to consider the possibility of a long-term accumulation in sediments, which could serve as a source of SWCNT contamination but also resuspension of carbon nanomaterials in the water column. Re-introduction of SWCNT can occur through bioturbation or dredging, which has previously been reported for metals and organic contaminants.[43]

Conclusions

Because estimating toxicity of SWCNT is a complex matter, the characterization of nanomaterials must be undertaken including details about the agglomeration state. This allows for a better forecast of the potential effects of manufactured nanomaterials. This study has shown that agglomerated forms of SWCNT can be directly toxic to the amphipod *H. azteca* by inducing lethality and growth inhibition. Our work further demonstrates that the presence of sediment mitigates SWCNT toxicity. Results also suggest that the toxicity of SWCNT may be due to digestive track blocking of organisms, which may impede nutrient uptake. Overall, effects were measured at concentrations higher than those that can be presently estimated in the aquatic environment. Modelization studies predicted carbon nanotube concentrations to be in the range of μg/g in sediments and ng/L in aquatic ecosystem, but because no reliable detection methods in complex environmental samples are yet validated no measured concentrations are available.[44,45] Hence, under low exposure conditions, SWCNT do not appear to represent an important risk for the amphipod *H. azteca*. Nevertheless, the present data offer a useful background of information for hazard assessment, criteria development and lay the ground for future toxicity studies.

The toxic effects of SWCNT are usually observed at concentrations higher than estimated concentrations in water and sediment.[13,15,21,34] But in order to determine the toxic potential of SWCNT, the possible interaction between carbon nanotubes and environmental compounds should be closely investigated. Indeed, because of SWCNT surface and adsorption properties, carbon nanotubes can interact with organic matter or contaminants, which can influence their fate, behavior and eventually their toxicity.[14]

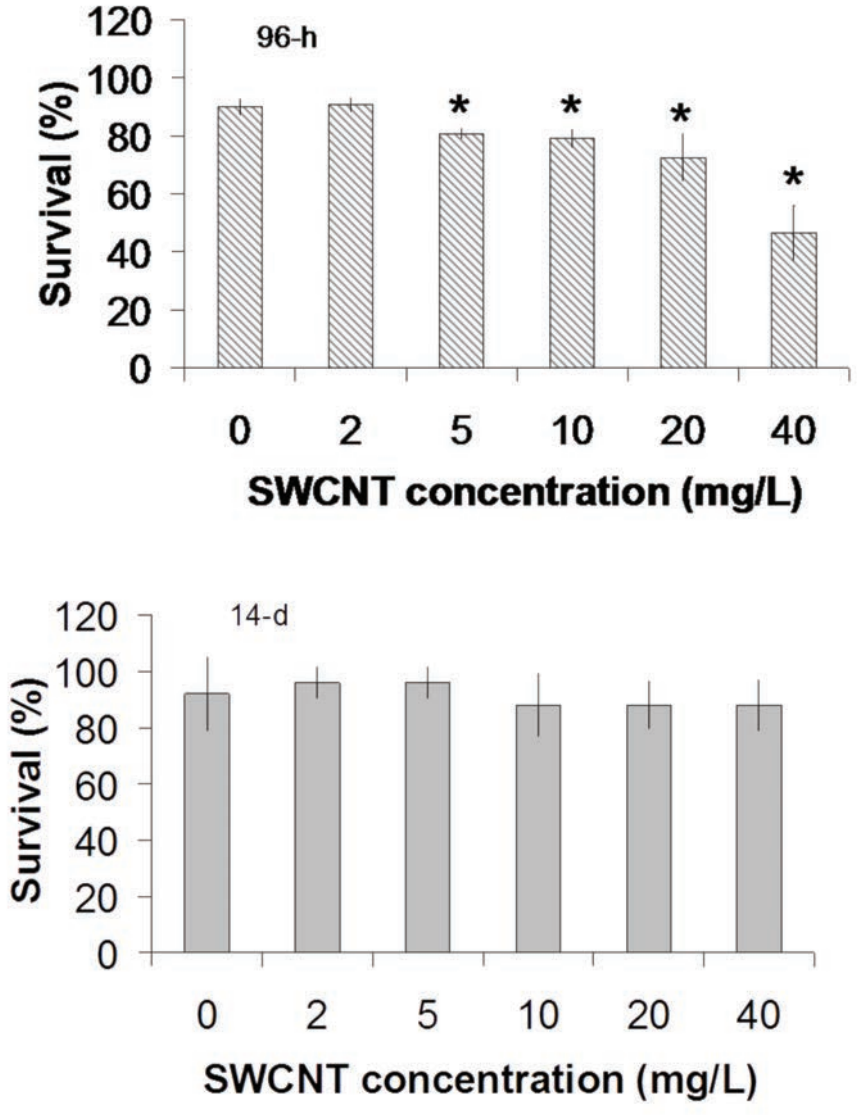

Figure 5. Mean survival of *Hyalella azteca* exposed to different concentrations of single-walled carbon nanotubes (SWCNT) in M4 for 96 h in water-only, where each column is the mean of 3 experiments (4 replicates per experiment) ± standard error, and 14 days in the presence of sediment, where each block is the mean of 6 replicates ± standard deviation. * Indicates significant difference of group exposed compared to the negative control.

Research highlights

The presence of sediment mitigates SWCNT toxicity to the amphipod *H. azteca.*

In the presence of sediment, SWCNT can induce lethality and a decrease of growth for concentrations >100 mg/L.

In the context of water only exposure, SWCNT can induce lethality for concentrations between 5 and 40 mg/L.

SWCNT are accumulated in the gut of organisms.

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