Environmental and Experimental Biology (2011) 9: 99–103 *Original Paper*

Toxicity assessment of carbon nanomaterials using *Brachionus calyciflorus* test

Anda Baumerte*

Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Rīga LV–1586, Latvia

*Corresponding author, E-mail: anda.baumerte@gmail.com

Abstract

Nanomaterials are being intensively studied due to their potential applications, and in recent years attention is being paid also to toxicity of nanomaterials that may be released into the environment as pollution and cause health risks. In the current study, the Rotoxkit F (*Brachionus calyciflorus*) toxicity test was applied to assess fullerene and carbon nanotube toxicity in an aquatic environment with dimethylsulfoxide and humic substances as solvents. The results showed that carbon nanomaterials are potential toxic pollutants in aquatic solutions, even though nanomaterials did not fully dissolve.

Key words: biotests, carbon nanomaterials, humic substances, toxicity assessment.
Abbreviations: DMSO, dimethylsulfoxide; FA, fulvic acid; HA, humic acid; HS, humic substances; MWCNT, multi-walled carbon nanotubes; SWCNT, single walled carbon nanotubes.

Introduction

The term „nanotechnology" arouse in 1980s with the development of atomic force and scanning tunnelling microscopy and lithography techniques. Today, many definitions for "nanomaterials" and "nanotechnology" exist (gr. „nannos" – *dwarf*) (Ten Have 2007). The International Organization for Standardization defines manufactured nanomaterials as: „materials with any external dimension in the nanoscale – size range from approximately 1 nm to 100 nm – or having internal structure or surface structure in the nanoscale" (1 nm= 1 10^{-9} m) (https://cdb.iso.org/).

The physical and chemical properties of nanomaterials differ from those of bulk substances, and this fact has boosted research on new nanomaterial applications and their production. Due to their specific properties, carbon nanomaterials (fullerenes, single and multi-walled carbon nanotubes, and also graphene) deserve special attention. They can be used in production of strong material (for sports equipment, prostheses etc.) and other areas, e.g. combating HIV virus (Wiesner, Bottero 2007) and controlling environmental pollution. About 1000 t of these nanomaterials are produced annually, and their global market is projected to grow at a compound annual growth rate of over 18%, while the global market for nanotechnology incorporated in manufactured goods amounted to 1.6 trillion USA dollars in 2010, with a 50% compound annual growth rate over the period 2010 to 2013 (Farré et al. 2011).

Along with the wide introduction of new materials and technologies, undefined risks and toxicity for environment and human health exist. According to the SEC (2008) 2036, Accompanying document to the Communication from the Commission to the European Parliament, the Council, and the Europaen Economic and Social Committee (17.06.2008.) "Regulatory aspects of nanomaterials. Summary of legislation in relation to health, safety and environment aspects of nanomaterials, regulatory research needs and related measures", these materials are not directly addressed under EU law.

Nanomaterial toxicity has been studied *in vitro* and *in vivo*, using organisms such as bacteria (e.g. *Escherichia coli*), algae, crustaceans (*Daphnia* sp., *Thamnocephalus platyurus*), fish, rodents and plants. No tests have been carried out using *Brachionus calyciflorus*.

The results of previous studies are contradictory, e.g. fullerene is known to produce reactive oxygen species that act as free radicals and damaging agents in organism (Takenaka 1999; Wiesner, Bottero 2007; Pérez et al. 2009) and also having no or positive effects in cells (Wiesner, Bottero 2007; Klaine et al. 2008). In most of the conducted studies toxic effects have been observed, such as acute lung toxicity and mortality in rodents (Warheit et al. 2004; Smart et al. 2005; Wiesner, Bottero 2007), dermal toxicity *in vitro*, and increased or decreased solvent/pollutant toxicity by adsorption on surface of the nanomaterial (Baun et al. 2007; Wild, Jones 2009; Kim et al. 2010).

Nanomaterials can be easily incorporated into cells via endocytosis, adhesion or transport through the cellular membrane (Geiser et al. 2005; Kim et al. 2006; Lin et al. 2007) and can reach different organs and even cross the blood-brain barrier. As these properties bestow various

medical applications for carbon nanomaterials, they are stressed frequently. It is important to determine side effects and to predict possible negative effects, if they exist, in cases when nanoparticles or products are ingested accidentally.

Toxic effect of nanomaterials can be determined by their biological persistence, reactivity, chemical structure (functional groups, impurities) and material dimensions – long particles can be inhaled, if their diameter is less than 5 μm and, as particles longer than 17 μm cannot be ingested by macrophages, they may initiate inflammation and fibrosis (Smart et al. 2005).

Nanomaterials can aggregate forming agglomerates in water solutions, lowering total solubility. To completely dissolve nanomaterials, solvents (surfactants) and chemical modifications are used. In solutions, humic substances (heterogenic compounds which comprise the bulk of natural organic matter) can also act as surfactants (Chapell et al. 2008). Salinity increases solution stability and therefore toxicity. In seawater (3.3%), nanomaterials aggregate (Klaine et al. 2008), but nevertheless lethal and toxic effects have been observed. It is possible that greater toxicity can occur at lower concentrations, where the tendency to aggregate is less likely (Tiede et al. 2008). This has been shown in tests with fullerenes and *Daphnia magna* (Pérez et al. 2009).

The aim of this study was to assess the toxicity of carbon nanomaterials on *B. calyciflorus* in humic substance (HS) and dimethylsulfoxide (DMSO) solutions.

Materials and methods

The preparation of stock solutions

The study was conducted in the Laboratory of Environmental Quality Monitoring, Faculty of Geography and Earth Sciences, University of Latvia.

Stock solutions contained common carbon nanomaterials: fullerene C_{60} (TCI Europe, Belgium, purity > 99.0 %), or single-walled or multi-walled carbon nanotubes (SWCNT and MWCNT; Tokyo Chemical Industry Corporation Ltd., Japan; length 5 to 15 μm, diameter 2 nm). Humic substances used as solvents were Aldrich humic acid, Nr. S15539-104, Sigma-Aldrich (Aldrich HA), or standard fulvic acids (FA; IHSS Pahokee Peat FA Standard, Nr. 2S103F or IHSS Suwannee River FA Standard II, Nr. 2S101F). Dimethylsulfoxide (DMSO) C_2H_6OS (Penta, 99%, analytically pure; Enola Ltd., analytically pure) was also used as a co-solvent. The choice in favour of DMSO was done made to its low toxicity in comparison to available alternatives – as previous studies suggest, various co-solvents can induce toxic effects in test organisms (Kim et al. 2010).

The stock solutions were prepared by weighing (analytic scales KERN ALJ 220-4, ± 0.1 mg) carbon nanomaterials and HS, which then were diluted with DMSO and a small amount of standard freshwater (distilled water with $NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl), as follows: 2 mg SWCNT or MWCNT and 20 mg Aldrich HA (5.4% DMSO), 10 mg C_{60} or SWCNT or MWCNT, 10 mg Aldrich HA (6% DMSO), 12.5 mg C_{60} and 12.5 mg Pahokee Peat FA or Suwannee River FA (4.8% DMSO), and five different concentrations of C_{60} in 50% DMSO.

The solutions were subjected to sonication (Cole-Parmer, 70 W, 42 Hz ± 6%) for 6 to10 h. When little or no residue was visible in the flask, more freshwater was added to the solution and sonication was repeated (30 min to 2 h). Solutions were then filtered with filter paper, achieving different maximum concentrations (20, 50 and 100 mg L^{-1}) of nanomaterials and HS. Because part of the solutions still contained insoluble residue, the actual concentration was likely lower and the derived concentrations are referred to as "dilutions".

Brachionus calyciflorus *test (Rotoxkit F)*

The acute toxicity test (24 h) Rotoxkit F with *Brachionus calyciflorus* is designed for chemical substance toxicity assessment in freshwater, estuaries and sea. It contains six tubes with dormant *B. calyciflorus* cysts, six microplates (13.5 × 9.5 cm) with a hatching trough, rinsing troughs and 36 test wells, five (15 mL) glass vials with concentrated hatching and toxicant dilution medium ($NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl) for preparing 1 L standard freshwater, six polyethylene micropipettes, 10 × 15 cm Parafilm, a Standard Operational Procedure brochure, abbreviated Bench Protocol, data scoring sheets and graphical L(E)C_{50} calculation sheets.

Standard freshwater was prepared by pouring concentrated salts into distilled water and aerating solutions for 15 min. These were then maintained at room temperature before use. A day before the test, one vial of *B. calyciflorus* cysts was added to 2 mL of standard freshwater in a hatching trough, the plate was covered with Parafilm and the lid, and then left to incubate at 25 °C temperature for 16 to 18 h (3000 to 4000 lux illumination).

On the test day, microplates were filled with standard-water-diluted DMSO control in the first row and increasing concentrations (dilutions) of carbon nanomaterial and HS-carbon nanomaterial solutions (0.7 mL in rinsing troughs and 0.3 mL in test wells). Approximately five hatched rotifers were transferred with a micropipette to the rinsing trough and from rinsing to test wells. Microplates were covered with Parafilm and lid, and incubated at 25 °C temperature for 24 h in the dark (Rotoxkit 1992).

After incubation, the number of living and dead rotifers in each well was recorded in the test protocol. The lethal concentration for 50% test organisms (LC_{50}) after 24 h exposure was obtained graphically from the ordinate axis of a dose-response curve (Rotoxkit 1992). In addition, probit regression analysis was used to derive regression lines for binomial response of mortality to concentration. The calculations were conducted in MS Excel, using the function NORMINV.

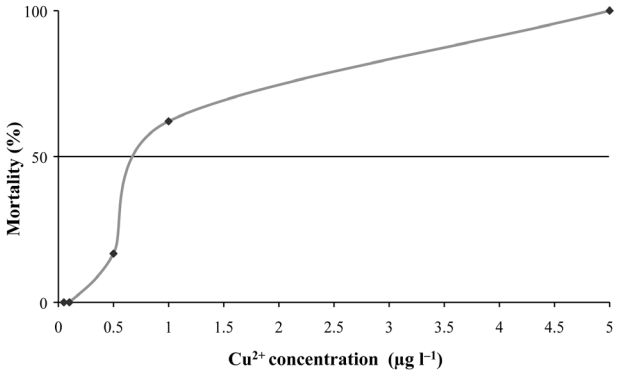

Fig. 1. *Brachionus calyciflorus* dose-response in Cu^{2+} solution after 24 h exposure.

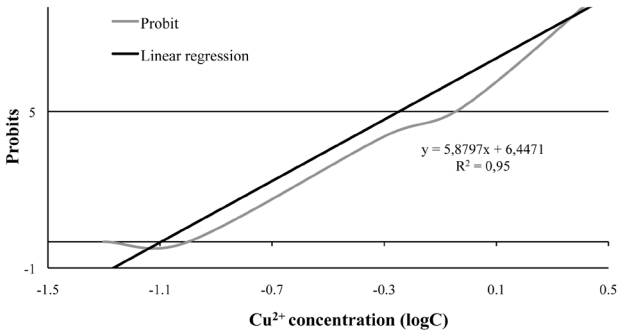

Fig. 2. Probit analysis for *Brachionus calyciflorus* mortality in relation to concentration of Cu^{2+} solution. logC – cocncentration logarithm; probits – probability units. Estimated LC_{50} = 0.57 µg L^{-1}.

Results

A control experiment in solution with Cu^{2+} was carried out to assess *B. calyciflorus* sensitivity. Probit analysis indicates LC_{50} = 0.57 µg L^{-1} (R^2 = 0.98) for Cu^{2+} after 24 h (Fig. 1 and 2).

After exposure to an Aldrich HA-SWCNT (10 mg HA, 10 mg SWCNT, 11 mL DMSO, 200 mL) solution, LC_{50} at 66.9% dilution (R^2 = 0.73) was found. In 90 to 100% dilutions particles aggregated around rotifers (Fig. 3 and 4).

In Aldrich HA-C_{60} solution (10 mg HV, 10 mg C_{60}, 11 mL DMSO, 200 mL), LC_{50} was estimated to be at 10.2% (maximum HA and C60 concentration 5.1 mg L^{-1}; R^2 = 0.898) (Fig. 5 and 6). In 16.7% dilution, 100% mortality of test organisms was found, and a dark substance was visible in organisms in 50 to 100% dilutions. No toxicity was found in fullerene and Pahokee Peat FA, Suwannee River FA solutions with equal concentration in 16.7 % dilution.

Toxicity was not found also in 100% MWCNT solution (10 mg MWCNT, HA, 11 mL DMSO, 200 mL) and 50% solutions with lower nanomaterial and higher HA maximum concentration (2 mg SWCNT or MWCNT, 20 mg HA, 5 mL DMSO, 100 mL).

In a fullerene-DMSO solution mortality of 78.3% test organisms was observed at a concentration of 0.001 µg L^{-1}, but no LC_{50} value was established.

To compare the toxic effect of a co-solvent, a control experiment with DMSO dilutions (1 to 5%) was performed. No mortality of *B. calyciflorus* was observed up to 5% dilution, which corresponds to the dilutions used in nanomaterial testing.

Discussion

We observed toxicity of unmodified carbon nanomaterials are toxic to *B. calyciflorus* species in a dose-dependent manner. Aldrich HA-nanomaterial solutions can act as pollutants at environmentally relevant concentrations (Boxall et al. 2007) and, as the production of nanomaterials continues to grow, regulations will be necessary to determine acceptable uses or thresholds. SimpleBox models imply that carbon nanomaterials could be found at concentrations of 1 to 100 µm L^{-1} in the environment, at 1 to 10 mg L^{-1} concentrations in dissoluted and colloid form in freshwaters (Boxall et al. 2007). The most plausible compartment for accumulation of nanomaterials is sediments (Cheng et al. 2008; Klaine et al. 2008). Nanomaterials can also be bioaccumulated or recycled in biota, depending on their properties.

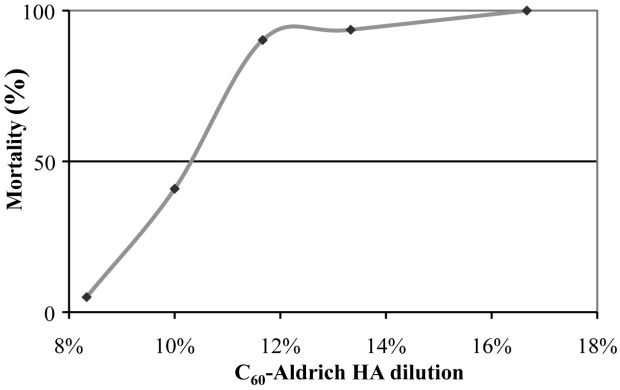

Fig. 3. *Brachionus calyciflorus* dose-response curve for C_{60}-Aldrich HA solution after 24 h exposure.

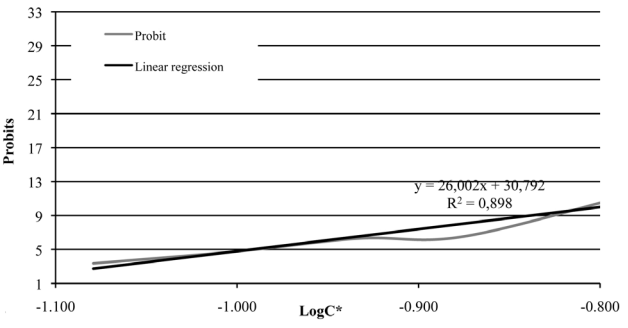

Fig. 4. Probit analysis for *Brachionus calyciflorus* mortality assesment in C_{60}-Aldrich HA solution. logC* – dilution (%) logarithm; probits – probability units. Estimated LC_{50} = 10.3%.

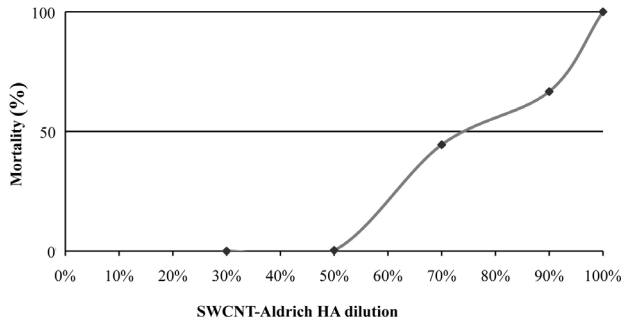

Fig. 5. *Brachionus calyciflorus* dose-response curve in SWCNT-Aldrich HA solution after 24 h exposure.

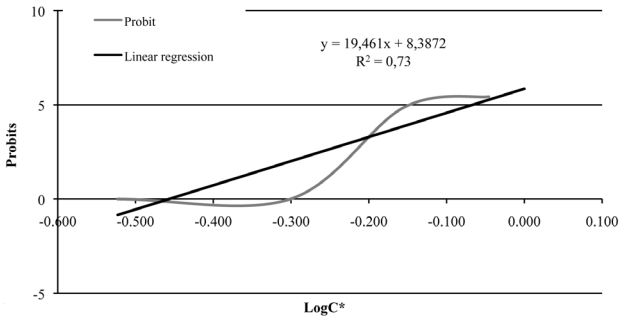

Fig. 6. Probit analysis for *Brachionus calyciflorus* mortality assesment in SWCNT-Aldrich HA solution. logC* – dilution (%) logarithm; probits – probability units. Estimated LC_{50} = 66.9%.

The stock solutions were prepared in conditions described in literature, varying periods of sonication and concentrations. The solution can be considered saturated, but aggregates and material residues were visible in solution. The most common methods for increasing solubility in toxicity testing are addition of functional groups in oxidation reactions and covalent interaction mechanisms, non-covalent interaction mechanisms (hydrophobic interactions, ionic bonds, Van der Waals forces, hydrogen bonds; organic surfactant addition) or sonication. Even considering this, Chen et al. (2007) observed that only 31% of nanomaterial amounts dissolute.

Kahru and Dubourguier (2010) reviewed available information on nanoectoxicology and identified nanotoxicology assessment methods. From the available literature, algae and crustaceans (*Daphnia*) have been identified as the most sensitive organisms to nanomaterial pollution in aquatic environment. The authors classify SWCNT and MWNCT as toxic (LC_{50} 1 to 10 mg L^{-1}). Due to unmodified nanomaterial solubility in water (~1.3 10^{-11} mg L^{-1}), rotifer toxicity is lower than *Daphnia* sp. toxicity.

High toxicity was found at a low fullerene concentration in C_{60}-DMSO solution, which may have been partly due to the amount of co-solvent DMSO. However, DMSO toxicity was low at concentrations tested. Also, with proportionally less fullerene in C_{60}-DMSO solution, less residue were observed, and generally the solubility trend in DMSO is C_{60} > SWCNT > MWCNT. Additionally, treatment in an ultrasonic bath can change nanomaterial structure and their effect on organisms (Oberdörster et al. 2006; Zhu et al. 2006; Pérez et al. 2009) and the power used in sonication can be important.

It is possible that added HS may lessen toxicity by adsorbing on nanomaterials and decreasing functional group diversity, but more comparable tests have to be made in solutions without HS to test this. Other faults in choice of method can exist, as toxicity testing with unmodified nanomaterials has been described rarely. It is possible that nonmodified nanomaterials are the least toxic.

The toxicity test Rotoxkit F with *Brachionus calyciflorus* can be a convenient method for carbon nanomaterial toxicity testing, but for toxicity interpretation and assessment in the aquatic environment, water solution preparation and quantification methods are essential. In this case, nanomaterial quantification was hindered by the presence of DMSO and HA, which excluded the most common methods: UV spectrometry and total organic carbon assessment (Farré et al. 2011).

It is important to continue toxicity testing on unmodified carbon nanomaterial solutions, improving methods for preparing aquatic solutions and for quantifying nanomaterial amounts in solutions with natural organic matter.

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Received 1 September 2011; received in revised form 10 October 2011; accepted 10 November 2011