

Toxic effect of single walled carbon nanotubes combined with cadmium to the crustacean *Daphnia magna*

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ABSTRACT

The aim of this study was to assess the impact of single-walled carbon nanotubes (SWCNT) on the toxicity of cadmium (Cd) using the crustacean *Daphnia magna*. LC50 of Cd and SWCNT alone and combined were calculated and compared. Sorption of Cd on SWCNT was also quantified in separate batch experiments. Results showed that the maximum adsorption of Cd onto SWCNT calculated by the Langmuir equation was 24.4 mg kg⁻¹. LC50s for Cd and SWCNT alone were 252.3 µg L⁻¹ and 1400 µg L⁻¹, respectively. In the presence of 500 and 1000 µg L⁻¹ of SWCNT, Cd LC50s were 127.2 and 120.1 µg L⁻¹ respectively. Therefore, Cd toxicity increased when organisms were exposed to both contaminants which indicated that SWCNT induces a synergistic toxic effect on the survival of *D. magna*. It appears that even if SWCNT had a low adsorption capacity for Cd, toxicity of the metal can be increased. Our study shows the complexity of SWCNT toxicity and how the understanding of their interactions with other contaminants is crucial to determine the consequences of their release into the environment.

1. INTRODUCTION

Carbon based nanomaterials are used in a number of products such as sports equipment, textiles or automobiles (Wijnhoven et al. 2011). Among these nanomaterials, single-walled carbon nanotubes (SWCNT) offer many benefits such as toughness, strength and high conductivity making them of interest for many applications in material science (Dresselhaus et al. 2004). Carbon nanotubes are also widely applied for environmental monitoring and wastewater treatment due to their large surface area (Tan et al. 2012). If the large surface area of SWCNT enhance their ability to adsorb hydrocarbons or metals present in water or soil and hence be of use for decontamination purpose (Baun et al. 2008, Qu et al. 2014), it can also modify the availability of these contaminants to organisms. The interactions of SWCNT with other contaminants needs to be closely addressed in order to fully understand the environmental impact of these nanomaterials upon release in the environment (Moore 2006).

The toxicity carbon nanotubes has been previously studied on algae (Youn et al. 2012), copepods (Templeton et al. 2006), fish (Cheng et al. 2007, Martinez et al. 2013) and the crustacean *Daphnia magna* (Petersen et al. 2009, Alloy and Roberts 2011, Li and Huang 2011). The effects measured have been mainly attributed to the action of the carbon nanotube itself, either by blocking the digestive tract and by “sticking” to the body’s surface of organisms (Kennedy et al. 2008, Mwangi et al. 2012), or by releasing impurities - most commonly iron, cobalt and nickel - embedded in the nanoparticles. A large number of studies reported the ability of carbon nanotubes to adsorb heavy metal ions but few investigated the effect of this property on the organisms potentially exposed (Li et al. 2004, Li et al. 2005, Lu et al. 2006). A previous study described a

potentiating toxicological effect of SWCNT, suspended with organic matter in seawater, co-exposed with divalent metals at very low concentration ($\mu\text{g/L}$ range) on DNA integrity of the mussel *Mytilus edulis* (Al-Shaeri et al. 2013). In other studies, lead toxicity to a freshwater fish increased in the presence of purified MWCNT (Martinez et al. 2013), and SWCNT in sediments significantly reduced bioaccumulation of hydrophobic carbon to the deposit/suspension-feeding of a polychaete (Ferguson et al. 2008). Despite this data, very little is known regarding the impact of carbon nanotube on the toxicity of metals to aquatic organisms.

Cadmium (Cd) is a heavy metal very toxic to health and environment at both acute and chronic exposure (Attar and Maly 1982, Bodar et al. 1988, Giusto et al. 2012, Souid et al. 2013). It is one of the most common pollutants found in both industrial and urban effluents due to its presence in batteries, pigments paint, pulp and paper production and wastewater treatment plants (Hare 1992, USGS 2013). Cd is relatively soluble in water and therefore is bioavailable and tends to bioaccumulate in aquatic organisms (Wang and Rainbow 2006, Vellinger et al. 2012). Many studies have shown negative effects, such as decrease in survival (Qu et al. 2013) or growth (Giusto et al. 2012), and oxidative stress on several organisms including *D. magna* (Wen-Hong et al. 2009, Souid et al. 2013). The use of *D. magna* as a standard test organism is recommended by many international organizations to test the toxicity of chemicals, effluents, and contaminated water (OECD, Biesinger et al. 2002, Lazorchak et al. 2009).

It is well known that in natural waters, organisms are exposed to a variety of contaminants. In this study, our goal is to identify the potential impact of SWCNT on the toxicity of Cd to the crustacean *D. magna*. For this purpose, we chose to conduct 48-h lethality tests with Cd and SWCNT alone or in combination. A study of the adsorption capacity of SWCNT for Cd was also conducted to evaluate the physical interaction between the two toxicants.

2. MATERIALS AND METHODS

Preparation and characterization of SWCNT

SWCNT provided by Dr. Benoit Simard's research team (NRC Canada) were synthesized using a plasma system modified from a previous method (Kim et al. 2007). The chemical properties of dry SWCNT were measured using thermo-gravimetric analysis (TGA) and Raman spectrometry (WiTec confocal microscope fiber coupled to an Acton 2500i spectrograph fitted with a Roper Scientific CCD array detector). The diameter of SWCNT (D) was calculated from the characteristic radial breathing (RBM) wave number (ω) using the following relation: $D(\text{nm}) = 248/\omega$. Dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments) 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 (Eq. (1)) with the Smolchenski approximation:

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

Where U_E is the electrophoretic mobility ($\text{m}^2 \text{s}^{-1} \text{V}^{-1}$), ϵ is the dielectric constant of the medium, z is the zeta potential (mV), $f(ka)$ is Henry's function and correspond to the ratio of particle radius to double layer thickness (1.5) and η is the viscosity ($\text{kg m}^{-1} \text{s}^{-1}$).

Morphology was determined by field emission gun scanning electron microscopy (FEG-SEM, Hitachi 4700) operated at 2 kV and transmission electron microscopy (TEM, Hitachi H-7100).

Artificial freshwater called ASTM medium, consisting of NaHCO_3 (192 mg L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (120 mg L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (120 mg L^{-1}) and KCl (8 mg L^{-1}) was prepared with Milli-Q water ($>18 \text{ mV}$) according to ASTM E729-96 (ASTM 2014). SWCNT stock solution (0.1 g L^{-1}) was prepared by transferring 100 mg of SWCNT in 1 L of the ASTM medium in a Pyrex glass

beaker and dispersing using probe sonication for 10 min at 80 W (Ultrasonic processor, frequency 25 kHz, Misonix S4000).

Culture of *D. magna*

D. magna were cultured according to Environment Canada procedures, in reconstituted M4 medium: NaHCO₃ (64.8 g L⁻¹); CaCl₂ 2H₂O (293.8 g L⁻¹); MgSO₄ 7H₂O (123.3 g L⁻¹) and KCl (5.8 g L⁻¹); pH 7-8; hardness 230-260 mg CaCO₃ L⁻¹ (Environment-Canada 1990). Organisms were placed into an experimental chamber with programmed temperature and photoperiods (20°C ± 2, 16/8 h Light/Dark). Animals were fed daily with algae *Pseudokirchneriella subcapitata* (30 mL; 1.8 × 10⁶ cells mL⁻¹) and once a week with beef extract. The medium was renewed every week. All assays were conducted in a chamber under the same temperature and photoperiod regime using ASTM medium.

Adsorption of Cd on SWCNT

The interaction of Cd with SWCNT was measured by performing traditional adsorption batch experiment and measuring Cd concentration in the aqueous phase by ICP-MS. Various volumes of SWCNT stock solution (0.1 g L⁻¹) were mixed with various volumes of Cd stock solution (CdCl₂; 25 to 450 µg Cd L⁻¹) and water. Cd adsorption at a specific concentration of SWCNT (1 mg L⁻¹) was investigated at various metal concentrations (25, 50, 100, 200, and 450 µg Cd L⁻¹). The pH of the suspensions was measured and found to be between 8 and 8.5. Cd-SWCNT suspensions were prepared in duplicate and shaken for 48 h to achieve the adsorption equilibrium at 23 ± 1°C. In addition, a control with cadmium alone (415 µg L⁻¹) was used to examine the potential occurrence of Cd precipitation under the current conditions. Small sample aliquots were withdrawn at 24 h and 48 h for further analysis by ICP-MS. The amount of Cd on SWCNT was calculated by Eq. (2) (Moradi et al. 2011):

$$q_e = (C_0 - C_e)V/w \quad (2)$$

Where C₀ (mg L⁻¹) is the initial Cd concentration, C_e (mg L⁻¹) is the final Cd concentration at equilibrium (mg g⁻¹), V (L) and w (g)

3. TOXICITY TESTS

Exposure to Cd or SWCNT

Before and during the study, K₂Cr₂O₇ (Sigma-Aldrich) was used as a reference toxicant (positive control) to evaluate the quality of cultured organisms (concentration range 0.175 - 2.8 mg L⁻¹). Other test validation criteria (e.g. response in the negative control, temperature) were verified against guidelines criteria and laboratory in-house control data.

Stock solutions of Cd (CdCl₂, 1 g L⁻¹) and SWCNT (0.1 g L⁻¹) were prepared directly in the ASTM culture medium. Organisms were exposed according to standardised tests (Environment-Canada 1990). Five test concentrations and one control group were used, each with four replicates. Each replicate consisted of five *D. magna* neonates (< 24 h) in a 150 mL glass beaker containing 100 mL test solution. CdCl₂ and SWCNT concentrations used were respectively between 25 - 525 µg L⁻¹ (measured concentrations of Cd) and 0.5 - 10 mg L⁻¹ (nominal concentrations of SWCNT). The glass beakers were covered with a glass lid and beakers were incubated for 48 h at 20 ± 2 °C and 16/8 h Light/Dark condition. After 48 h of exposure, immobile organisms were placed under a binocular microscope for heart beat observation and the percentage of surviving organisms was calculated for each condition. The residual suspensions of test solutions were used for the measurement of Cd concentration.

To test if the toxicity observed could be related to impurities released from SWCNT, *D. magna* were exposed to filtered solutions of SWCNT. The nominal test concentrations assayed were prepared by sonication of SWCNT in ASTM 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 their absorption at 800 nm using spectrophotometry and TEM analysis.

Toxicity test of Cd and SWCNT combined

The potential impact of SWCNT on Cd toxicity was investigated under the same conditions as the Cd toxicity evaluation described above. Briefly, two series of different concentrations of Cd (25 to 525 $\mu\text{g L}^{-1}$) were prepared as described previously. Then, each series received a non toxic concentration of SWCNT (500 or 1000 $\mu\text{g L}^{-1}$). The suspensions were mixed prior to the addition of *Daphnia* neonates.

Chemical analysis

Cd concentrations were measured at the end of each lethality test and sorption experiment. Briefly, the solutions were transferred into polypropylene tubes with 5 % nitric acid. Samples with SWCNT and/or Cd were filtered with a 0.22 μm filter (EMD, PES). After dilution, residual Cd concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS, X series 2, Thermo scientific).

During analysis, blanks and two certified reference materials (ERA from water company and SLRS-5: River water reference material for trace metals from National research Canada) were also analyzed to verify contamination and analytical accuracy. 95% recovery values were obtained.

Statistical analysis

The lethal concentration (LC50) was calculated using probit analysis or Trimmed Spearman-Kaber with the TOXCAL program (1996, software package version 5).

Statistical differences between treatments were assessed by Shapiro–Wilk test; the Bartlett test and the Levene test to assess normality and homoscedasticity, respectively. Data that fulfilled the normality and homoscedasticity assumptions were analyzed through one-way ANOVA followed by Tukey’s test. Remaining data were analyzed with the Kruskal–Wallis test followed by multiple comparisons like Steel Dwass. Analysis was performed with JMP IN 4 software.

4. RESULTS AND DISCUSSION

Characterization of SWCNT

Characterization of dry SWCNT by TGA analysis confirmed raw nature of the sample with 60% of unpurified SWCNT oxidized, 20% of highly pure and defect-free SWCNT and residual mass of 20 % corresponding to the fully oxidized catalysts metals, as previously reported (Dillon et al. 2005, Kim et al. 2007). Raman spectrum analysis revealed the presence of aldehyde groups (CHO) at the end of nanotubes and inclusions other than pure carbon in the sample like amorphous carbon (data not shown). The SWCNT diameter was estimated at 1.53 nm. The FEG-SEM and TEM analysis revealed the presence of impurities on nanotubes (Figure 1). In solution, SWCNT formed large agglomerates with an average size ranging from 582.6 to 1614 nm, and an electrophoretic mobility with a minimum value of $-1.6 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ and a maximum of $-1.4 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ (mean = $-1.1 \pm 0.1 \text{ SD}$).

Adsorption characteristics of Cd on SWCNT

Adsorption experiments were carried out by mixing known amounts of carbon nanotubes with Cd solutions at different concentrations. The interaction between SWCNT and Cd was evaluated by measuring the sorption equilibrium concentration (Figure 2) after 48 h.

The Langmuir isotherm was used to fit the experimental data with Eq. (3) (Jaroniec 1983):

$$q_e = \frac{K_a q_m C_e}{1 + K_a C_e} \quad (3)$$

Where C_e (mg L^{-1}) is the equilibrium Cd concentration (mg kg^{-1}), q_m (mg kg^{-1}) is the maximum adsorption capacity which considers a complete monolayer coverage and K_a (L mg^{-1}) is the Langmuir constant indirectly related to the energy of adsorption.

The reasonable fit of the Langmuir isotherm model was confirmed by the correlation coefficient tested ($r^2 = 0.88$) which is close to 1. The q_m value calculated by the Langmuir equation is 24.4 mg kg^{-1} . Therefore, the toxic effect of a Cd-SWCNT combination should be similar to effect of Cd alone. The Langmuir equation has been widely used in metal adsorption studies. This model assumes a monolayer coverage of adsorbate over a homogeneous adsorbent surface with an equal adsorption activation energy for each molecule adsorbed onto the surface.

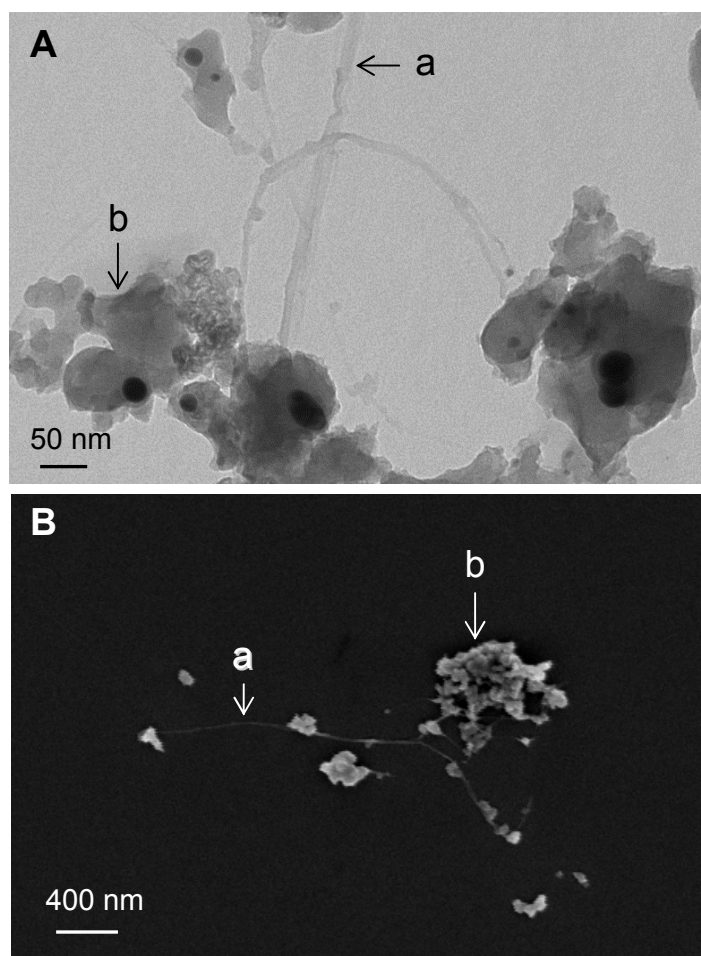


Figure 1. SWCNT observed with (A) field emission gun scanning electron microscopy and (B) transmission electron microscopy. Both techniques allow visualisation of (a) carbon nanotubes and (b) impurities forming rounded aggregates.

Previously, SWCNT were reported to have various adsorption capacities towards metals. For Cd, adsorption capacities of 18.58 and $52.35 \text{ mg Cd g}^{-1}$ of as-produced or carboxylate group functionalized SWCNT were calculated respectively (Moradi et al. 2011). It appears that the variability of Cd adsorption capacity on SWCNT is related to the different production and purification methods (Li et al. 2004, Li et al. 2005). In particular, the presence of carboxylate groups on the surface of SWCNT enables a more effective metal adsorption.

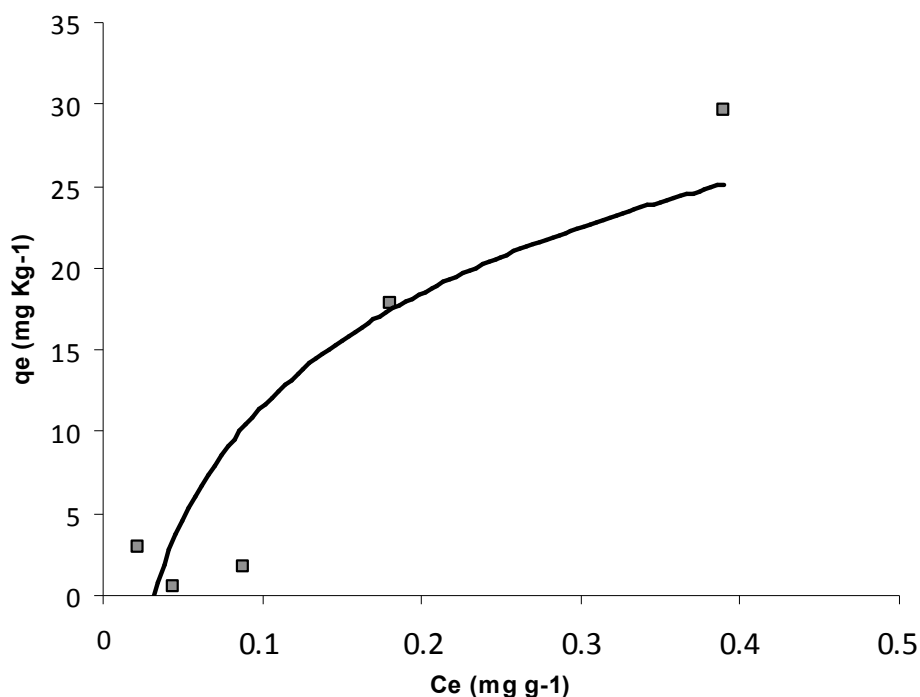


Figure 2. Adsorption isotherm for Cd ions at a concentration of 1 mg/L SWCNT.

Toxicity of Cd or SWCNT

The water quality parameters for the toxicity tests were all within the acceptable ranges: 19.5–21°C for temperature, 95–100 % for dissolved oxygen, 8.0 – 8.1 for pH and 643–750 $\mu\text{S}/\text{cm}$ for conductivity. Ammonia and nitrite concentration remained very low and water hardness was measured to be between 240 and 290 mg L^{-1} . All toxicity tests performed were valid according to the criteria mentioned in the respective guidelines, i.e. for reproducible tests mortality is inferior to 10%.

Figure 3 presents the results of three tests where *D. magna* neonates were exposed to Cd alone. Reproducible dose-response curves were obtained and the LC50 determined was 252.3 $\mu\text{g L}^{-1}$. Previous studies have reported the toxicity of Cd to *D. magna* under different medium and culturing conditions than those mentioned in the current publication (Bodar et al. 1988, Ferreira et al. 2008, Qu et al. 2013). These studies showed a decrease in survival of *D. magna* at concentrations up to 300 $\mu\text{g L}^{-1}$ in good agreement with the values measured herein.

Figure 3 presents the survival of *D. magna* neonates after exposure to SWCNT alone. The LC50 determined was 1400 $\mu\text{g L}^{-1}$. In recent years, the development of new applications for carbon nanotubes has been increasing. Several studies have investigated the toxicity of SWCNT towards aquatic invertebrates. Earlier work has shown that a 48 h exposure of carbon nanotubes at 400 $\mu\text{g L}^{-1}$ could induce lethality in *D. magna* (Petersen et al. 2009). Several authors have investigated the food particle size of *D. magna*, which appears to be between 100 and 5000 nm (Geller and Müller 1981).

Because it is in the range of agglomerates size of SWCNT measured in our study, carbon nanotubes are likely to accumulate in the digestive tract of organisms and decrease food intake (Petersen et al. 2009, Petersen et al. 2011). This hypothesis has been confirmed by our observations of survivors that showed the presence of SWCNT in the digestive tract of daphnids exposed to 500 and 1000 $\mu\text{g L}^{-1}$, while SWCNT were also observed on the body surface of daphnids exposed to 1000 $\mu\text{g L}^{-1}$ (Figure 4). A feeding rate test could complement this hypothesis. Earlier work has shown the presence of carbon nanotubes in the gastrointestinal track of *D. magna* using light,

binocular and transmission electron microscopy (Baun et al. 2008, Petersen et al. 2009, Mwangi et al. 2012).

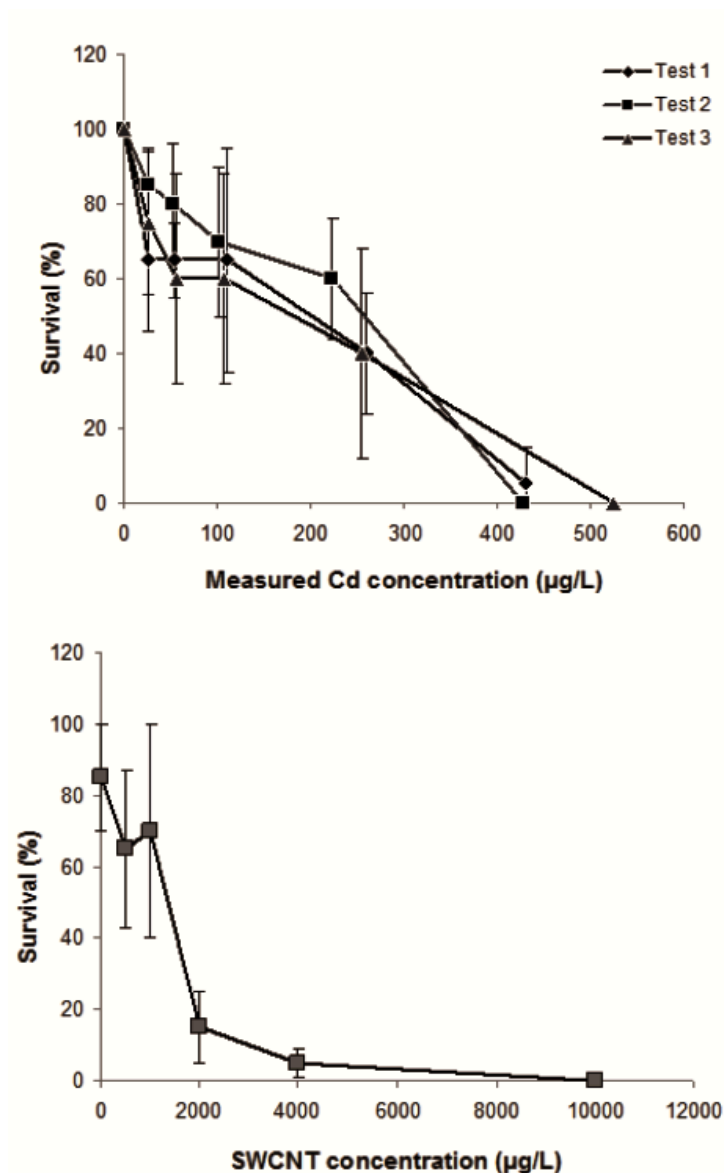


Figure 3. Toxicity of Cd and SWCNT to *D. magna* after 48 h of exposure.

Previously, metal impurities in carbon nanotubes were reported to disturb the hatching delay of zebrafish (Cheng et al. 2007). A 48-h lethality test was thus carried out to study the effect of a pre-sonicated filtered SWCNT solution. No significant differences between the control and other treatments were measured with a mean of survival > 85% (data not shown), and observation of organisms did not reveal any accumulation of SWCNT in the digestive tract. Therefore, we concluded that the filtered SWCNT solutions were not toxic in our model, even though the sonication process for the dispersion of SWCNT was operated for a period long enough to dissolve impurities in the medium (Toh et al. 2012). In this study, the toxicity of SWCNT to *D. magna* was thus attributed to the accumulation of SWCNT in the digestive tract rather than to the dissolution of impurities.

Toxicity of Cd in the presence of SWCNT

In natural environments, organisms are exposed to multiple contaminants. Furthermore, nanomaterials are highly reactive. To evaluate the toxicity of carbon nanomaterials it is crucial to

consider possible interactions with other toxic substances such as metals or organic contaminants. Indeed, the surface properties and adsorption capacity of carbon nanotubes facilitate binding of compounds present in the environment or in test medium such as contaminants and influence their toxicity (Bhatt and Tripathi 2011, Al-Shaeri et al. 2013).

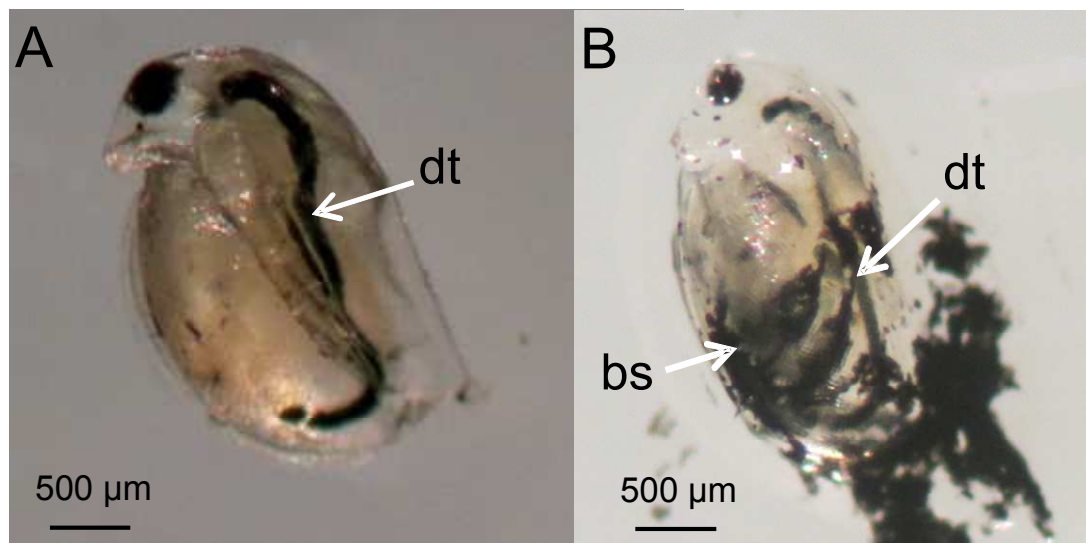


Figure 4. Binocular images of *D. magna* exposed to SWCNT after 48 h exposure to a suspension of (A) 500 µg L⁻¹ and (B) 1000 µg L⁻¹. The black colour in the digestive tract (dt) or on the body surface (bs) shows the uptake and interaction with SWCNT.

The presence of SWCNT at 500 and 1000 µg/L enhanced the toxicity of Cd by a factor 2 with a LC50 of 127.2 and 120.1 µg L⁻¹, respectively (Figure 5). A stronger effect was measured for SWCNT at 1000 µg/L with lower variability. To our knowledge this is the first study to report a decrease in *D. magna* survival by a co-exposition to SWCNT and Cd. A previous study showed an increase of Cd accumulation in the liver of the goldfish (*Carassius auratus*) after 12 d exposure in the presence of oxidized multi-walled carbon nanotubes (Qu et al. 2014). Martinez et al. (2013) reported the increase of lead toxicity to the Nile tilapia (*Oreochromis niloticus*) in the presence of purified MWCNT (Martinez et al. 2013). Another study measured a potentiating effect of SWCNT exposed with Cd or Zn on the DNA damage measured in the hemocytes of the blue mussel (*Mytilus edulis*) (Al-Shaeri et al. 2013).

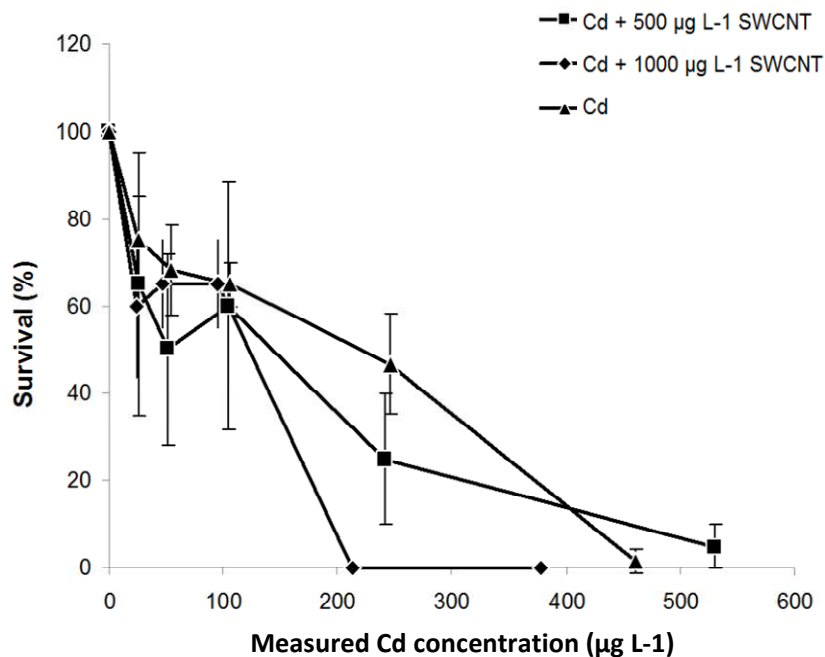


Figure 5. Toxicity of Cd with or without SWCNT after 48 h of exposure.

Although in our study SWCNT have a low adsorption capacity for Cd, the results suggest that they can still interact with Cd species and influence the toxicity to the organisms exposed. To our knowledge this is the first study highlighting such an effect in the presence of SWCNT with a low adsorption capacity. The accumulation of carbon nanotubes on the outer surface of daphnia and inside the digestive tract may induce a higher intake of Cd. The physicochemical conditions of the digestive tract of *D. magna* found in the literature (Hasler 1935, Weltens et al. 2000) support the hypothesis that Cd would then be desorbed and release into the intestine. Indeed, even though the pH is neutral in the *Daphnia* gastro-intestinal tract (Pennak 1978), enzymatic conditions can be in favour of desorption processes (Lampert 1987, Weltens et al. 2000). To confirm the mechanism implicated in toxicity, measurement of SWCNT and Cd intake by *D. magna* should be further investigated with quantification of contaminants into the organisms.

5. CONCLUSION

SWCNT possess a large surface area that enhances their ability to adsorb contaminants. This property is appealing for wastewater treatment (Tan et al. 2012), but also threatening for organisms that could interact with contaminants adsorbed on the surface of carbon nanotubes. Our goal in this study was to evaluate the effect of the presence of SWCNT on the toxicity of Cd to the crustacean *D. magna*. The results showed that SWCNT had a low adsorption capacity for Cd but increased the toxicity of the metal to *D. magna*. We assume that the observed effects are caused by the uptake of free cadmium species and Cd adsorbed on SWCNT by the organisms. Our study shows how the understanding of SWCNT interactions with other contaminants is important to evaluate the consequences of their release in the environment.

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