International Amphibian Micronucleus Standardized Procedure (ISO 21427-1) for *In Vivo* Evaluation of Double-Walled Carbon Nanotubes Toxicity and Genotoxicity in Water

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ABSTRACT: Considering the important production of carbon nanotubes (CNTs), it is likely that some of them will contaminate the environment during each step of their life cycle. Nevertheless, there is little known about their potential ecotoxicity. Consequently, the impact of CNTs on the environment must be taken into consideration. This work evaluates the potential impact of well characterized double-walled carbon nanotubes (DWNTs) in the amphibian larvae *Xenopus laevis* under normalized laboratory conditions according to the International Standard micronucleus assay ISO 21427-1:2006 for 12 days of half-static exposure to 0.1–1–10 and 50 mg L^{-1} of DWNTs in water. Two different endpoints were carried out: (i) toxicity (mortality and growth of larvae) and (ii) genotoxicity (induction of micronucleated erythrocytes). Moreover, intestine of larvae were analyzed using Raman spectroscopy. The DWNTs synthetized by catalytic chemical vapor deposition (CCVD) were used as produce (experiment I) and the addition of Gum Arabic (GA) was investigated to improve the stability of the aqueous suspensions (experiment II). The results show growth inhibition in larvae exposed to 10 and 50 mg L^{-1} of DWNTs with or without GA. No genotoxicity was evidenced in erythrocytes of larvae exposed to DWNTs, except to 1 mg L^{-1} of DWNTs with GA suggesting its potential effect in association with DWNTs at the first nonacutely toxic concentration. The Raman analysis confirmed the presence of DWNTs into the lumen of intestine but not in intestinal tissues and cells, nor in the circulating blood of exposed larvae. Environ Toxicol 26: 136–145, 2011.

Keywords: double-walled carbon nanotubes; toxicity; genotoxicity; amphibian larvae; *Xenopus laevis*; Raman analysis

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INTRODUCTION

Carbon nanotubes (CNTs), a man-made form of carbon, are one dimensional nanoscale objects, characterized by exceptional properties, in relation with their nanosize. They are allotropes of carbon and their structure can be described as a graphene sheet rolled up to form a cylinder. There are two main types of CNTs (Bethune et al., 1993; Iijima and Ichihashi, 1993): single-walled CNTs (SWNTs) and multi-walled CNTs (MWNTs) depending on the number of concentric walls. Among the MWNTs, double-walled carbon nanotubes (DWNTs) are at the frontier between SWNTs and MWNTs. Their morphology is very close to SWNTs. CNTs have a diameter from 1 nm and a length up to tens of μm or more, giving them a very high aspect ratio. Their specific surface area is generally important and can theoretically reach to 1310 $m^2\ g^{-1}$ in the case of closed SWNTs.

Since their discovery in 1991 by Iijima (Iijima, 1991; Hata et al., 2004), interest in CNTs has grown rapidly due to their unique physical (mechanic, electronic, thermal) and chemical properties. CNTs represent one of the fastest developing nanoparticle materials. Applications of CNTs are numerous including TV screens (flat-screens), sport equipments (bike frame, baseball bat, tennis rackets), and tires (Baughman et al., 2002). Some others are in preparations such as in paints and composite materials in general, special technical clothes, medical, and pharmaceutical products.

Because of their increasing production, use, and applications, it is likely that some of them will get into the environment during each step of their life cycle (production, use, and disposal), especially in the aquatic compartment which concentrates all kinds of pollution. The presence of CNT-contaminated waste could lead in the near future to ecotoxicity problems. CNTs releases may come from (i) different point sources in relation with their production (manufacturing, wastewater effluents), landfills, and (ii) nonpoint sources corresponding to their use and application until their end of life, such as wet deposition from the atmosphere, storm-water runoff, groundwater, surface water leakage, and attrition of products containing CNTs. Consequently, CNTs must receive considerable attention as new, unknown, and potentially hazardous materials. Nevertheless, to our knowledge, there is little known about their potential ecotoxicity, especially on aquatic organisms, which are likely to enter the human food chain (Farré et al., 2009). Only few studies on different aquatic organisms exposed to CNTs are available. All of them indicated that exposure to CNTs generally lead to biological disorders at different levels, usually above 10 mg L^{-1}.

Until now, amphibians have not yet been really used to characterize the potential toxic effects of CNTs in the aquatic medium. Nevertheless, amphibians are well-known environmental health warning organisms due to their biphasic life cycle, permeable eggs, skin, and gills (Gauthier, 1996). Their specific physiology makes them particularly sensitive to the presence of contaminants in the water, influencing their behavior, so that they are more and more used as monitoring systems for water quality assessment (Bridges et al., 2002; Gauthier et al., 2004).

To our knowledge, only two studies (Mouchet et al., 2007a; Mouchet et al., 2007b; Mouchet et al., 2008) are devoted to the assessment of the potential genotoxic effects of CNTs on amphibian larvae *in vivo*.

Among toxic actions, genotoxic effects may durably affect the aquatic ecosystems and the presence of genotoxic compounds in water can also have repercussions on nonaquatic species, via food chains, or simply as a result of drinking water. The interaction of genotoxic compounds with DNA initially may cause structural changes in the DNA molecule. Unrepaired damage can generate other cell lesions and thus lead to tumor formation (Vuillaume, 1987; Malins et al., 1990). A number of tests have been developed to assess the genotoxic potency of water samples, using either plants or aquatic animals (see for review Jaylet et al., 1990).

In amphibian larvae, as in most eukaryotes, genome mutations may result in the formation of micronuclei, which are a consequence of chromosome fragmentation or malfunction of the mitotic apparatus. The micronucleus test (MNT) has been widely used with many amphibian species (*Pleurodeles waltl*, *Ambystoma mexicanum*, and *Xenopus laevis*) in the laboratory (Gauthier, 1996; Ferrier et al., 1998; Gauthier et al., 2004; Mouchet et al., 2005, 2006a,b, 2007a,b). The sensitivity and reliability of the MNT to detect chromosomal and/or genomic mutations makes it a good method to analyze the potential cytogenetic damage caused by pure substances for instance (Jaylet et al., 1990; Gauthier, 1996; Mouchet et al., 2005, 2006a,b, 2007a). This method has been standardized on *Xenopus laevis* in French (AFNOR, 2000) and International (ISO, 2006) recommendations. One of the key functions of such biomarkers (micronucleus) is to provide an "early warning" signal of significant biological effects (changes at the genetic/molecular level) with suborganism (molecular, biochemical, and physiological) responses preceding those occurring at higher levels of biological organization such as cellular, tissue, organ, whole-body levels, and *in fine* at population level. In this way, the use of the MNT may provide an important tool for the prediction of the potential long-term effects on amphibians in the environment.

The aim of the present work is to contribute to the ecotoxicological assessment of the potential impact of CNTs using the standardized method ISO 21427-1 (ISO, 2006). *Xenopus* larvae were exposed to DWNTs at concentrations ranging from 0.1 mg L^{-1} (to mimic potential environmental doses) to 50 mg L^{-1} (which may represent an accidental release, and optimize the observation of the potential toxic effects), with and without Gum Arabic (GA), a natural polysaccharide which acts as a dispersant, under controlled

laboratory conditions to evaluate two different endpoints after 12 days of exposure: (i) toxicity on larvae (mortality and growth) and (ii) genotoxicity as the expression of the clastogenic and/or aneugenic effects observed in erythrocytes in the running blood. Then, the presence of DWNTs was investigated in the larvae using traditional microscopy methods (photonic and electronic), but also by Raman spectroscopy to confirm the presence of DWNTs.

MATERIALS AND METHODS

Preparation of CNTs Samples

DWNTs were prepared by catalytic chemical vapor deposition (CCVD) by decomposition of a H_2—CH_4 mixture over an MgO-based catalyst (Flahaut et al., 2003). The carbon content of the as-produced CCVD product was about 7.8 wt %, as determined by elemental analysis (flash combustion). Assuming that all the carbon is present in the form of CNTs (Flahaut et al., 2000), it is thus possible to calculate the amount of as-produced CCVD product corresponding to a given amount of CNTs. CNTs were then obtained by treating the required amount of CCVD product with a concentrated aqueous hydrochloric acid (HCl) solution. After washing with deionized water until neutrality, the CNTs were maintained in wet conditions to limit aggregation. A sample was taken and dried for further characterizations (elemental analysis, BET, Raman spectroscopy, SEM, TEM, XRD). The carbon content of the CNTs sample was about 90 wt %, as obtained by elemental analysis. This corresponds to more than 97.7 mol % of carbon, assuming that the sample contains mainly Co and C. The remaining Co was assumed to be present only as carbon-encapsulated nanoparticles (Flahaut et al., 2000, 2002). The BET (Brunauer Emmett Teller) specific surface area measured was between 800 and 900 $m^2\ g^{-1}$. Raman analysis ($\lambda = 488$ nm, not shown) revealed that the ratio between the intensity of the D and G bands was close to 10%, corresponding to a good structural quality of the CNTs. Analysis of the radial breathing modes (which frequency can be easily associated to the diameter of the CNTs) indicated the presence of CNTs with diameters ranging from 0.7 to 2.2 nm. Figure 1(a) shows a representative FEG-SEM (Field Emission Gun-Scanning Electron Microscopy) image of the raw CNTs sample (as-produced CCVD product), showing a very high density of CNTs bundles, with extensive branching. Their diameter (bundles) typically ranged between 10 and 20 nm but numerous individual CNTs were also present. No carbon nanofibre (a typical by-product of CCVD methods) was observed in the sample. HRTEM (High Resolution Transmission Electron Microscopy) observation was performed on the CNTs after elimination of the catalyst by HCl washing [Fig. 1(b)] and revealed clean CNTs surfaces; as suggested by SEM, the CNTs are mainly isolated, or gathered into small bundles. The CNTs obtained in those conditions contain about 80% DWNTs, together with about 15% single-wall carbon nanotubes (SWNTs), and about 5% triple-walled carbon nanotubes. The outer diameter of DWNTs is typically ranging between 1 and 3 nm. The concentration of CNT in suspension in water after dispersion was monitored and published in a previous work (Datsyuk et al., 2009).

Xenopus Rearing and Breeding

The *Xenopus* males were injected with 50 IU of PMSG 500 (Pregnant Mare's Serum Gonadotropin, Intervet, France) and the females with 750 IU of HCG (Human Chorionic Gonadotropin, Organon, France) to induce spawning. Each pair was then placed together in normal tap water filtered through active charcoal at (22 ± 2)°C. Twenty four hours later, the pair was separated and viable eggs were maintained in an aquarium also containing normal tap water filtered through active charcoal at 20–22°C, until they reached a development stage appropriate for experimentation. The larvae were fed every day on dehydrated aquarium fish food. Two different hatches were used in the present work (one for experiment I and the other for the experiment II).

Exposure Conditions

The exposure was performed according to the French Standard AFNOR NF T90-325 (AFNOR, 2000) and the International Standard 21427-1 (ISO, 2006). Two independent experiments (I and II) were conducted, in semi-static exposure conditions consisting in a daily renewal of the medium during the 12 days of exposure. In both experiments, *Xenopus* larvae were exposed for 12 days to the same DWNTs concentrations i.e., 0.1, 1, 10, and 50 mg L^{-1} of DWNTs without GA (experiment I) or with GA (experiment II). The choice of adding a surfactant is justified by both the stabilization of the exposure media and the limitation of the size of the aggregates of CNTs. Moreover, CNTs which could end up in the environment would be likely to have adsorbed natural compounds such as natural sugars present into the aquatic compartment. Primarily used in the food industry as a stabilizer, GA was used because it is composed of polysaccharides which are secreted by most of photosynthetic organisms into the environment.

The amphibian larvae were exposed in reconstituted water (RW) (distilled tap water to which nutritive salts were added [294 mg L^{-1} $CaCl_2.2H_2O$, 123.25 mg L^{-1} $MgSO_4.7H_2O$, 64.75 mg L^{-1} $NaHCO_3$, 5.75 mg L^{-1} KCl]). *Xenopus* exposure began on larvae at stage 50 of the *Xenopus* development table (Nieuwkoop and Faber, 1956). For a given experiment, the larvae were taken from the

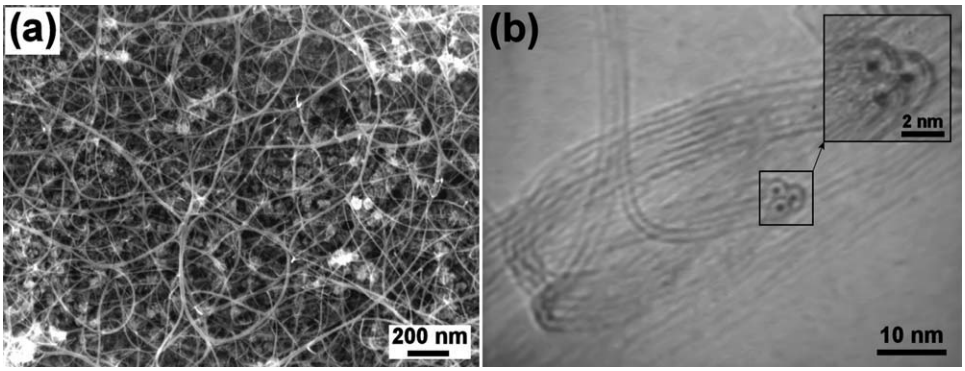

Fig. 1. (a) FEG-SEM (field emission gun-scanning electron microscopy) and (b) TEM (transmission electron microscopy) images of raw CCVD (catalytic chemical vapor decomposition) sample. Both the inner and outer wall of the DWNTs are clearly visible on (b).

same hatch to reduce interanimal genetic variability within each experiment. Larvae were exposed in groups of 20 animals (100 mL/larva) in 2 L pyrex crystallising dishes containing either the control medium (negative and positive controls) or the test medium (0.1–1–10 and 50 mg L^{-1} of DWNTs with or without GA), or the GA control (50 mg L^{-1}). An initial stock suspension of CNTs with or without GA containing the total amount of CNTs required for the 12 days of exposure was prepared by adding RW to the corresponding amount of wet DWNTs. Each day, DWNTs stock suspensions (with or without GA) were homogenized by bath sonication for 20 min before taking the required volume to obtain the target concentration by dilution in RW (final volume: 2L). The final suspensions of CNTs were then homogenized by mechanical stirring (Ultraturax) for 5 min at 9500 rpm. In the case of the addition of GA, the concentration was 50 mg L^{-1}. The negative control (NC) was the RW alone. The positive control (PC) was monohydrated cyclophosphamide (CP, [6055-19-2], Sigma France) in RW at 20 mg L^{-1} (ISO, 2006). CP is a standard indirect mutagen requiring metabolic activation in liver prior to becoming effective. Positive control was systematically performed in each experiment to check the responsiveness of the amphibian larvae. The larvae were submitted to a natural dark cycle at 22.0°C ± 2°C during the 12 days of exposure. They were fed every day on dehydrated aquarium fish food.

Toxicity

Acute toxicity (death or abnormal behavior) of larvae exposed to CNTs was examined for 12 days according to the standardized recommendations (AFNOR, 2000; ISO, 2006) by visual inspection compared to NC. Abnormal behavior corresponding to reduced and/or stopped growth of larvae, reduced food intake and abnormal motility. The visual inspection of the size of larvae was completed by measuring the size of each larva at the beginning of the exposure (Time 0 = t_0) and at the end of the exposure (Time 12 days = t_{12}). For this, larvae were preliminarily anesthetized (Tricaÿne methane sulfonate, MS 222, Sandoz) and photographed (Leica, France). The measure was then performed on photograph of each larva using the Mesurim image analysis software (Madre, 2006). Statistical analyses were performed using SimagStat 3.1. Nonparametric tests were preferred because of (i) nonnormality, (ii) and/or nonequivalence of variances and (iii) samples size ($n < 30$). For each time of exposure (t_0 and t_{12}), the data (size of larvae expressed in cm) were compared to (i) confirm that there is not a significant difference at t_0 between larval size from a same condition on one hand [i.e., larval size was in accordance with the stage 50 of the development stage table (Nieuwkoop and Faber, 1956)] and between larvae size from the different conditions on the other hand, (ii) conclude at t_{12} about the significant difference between the different conditions compared to NC and (iii) conclude about the significant difference for a given condition between t_0 and t_{12}. Kruskal-Wallis test (variance analyze on ranks) was performed to compare between all conditions, followed by Dunn's (same size of sample) or Dunnet's (different size of sample) test to isolate the group(s) that differ(s) from the others using a multiple comparison procedure, with unpaired date, versus the negative control group. The Mann–Whitney *U*-test was performed to compare on the basis of two and two conditions.

Graphic representations are proposed, based on the growth rate calculated as follow:

$[((\mathrm{mCd}t^{\circ}\mathrm{X}t_{12} - \mathrm{mCd}t^{\circ}\mathrm{X}t_0) - (\mathrm{mCd}t^{\circ}\mathrm{NC}t_{12} - \mathrm{mCd}t^{\circ}\mathrm{NC}t_0))/(\mathrm{mCd}t^{\circ}\mathrm{NC}t_{12} - \mathrm{mCd}t^{\circ}\mathrm{NC}t_0)] \times 100]$ where $\mathrm{mCd}t^{\circ}\mathrm{X}t_{12}$ and $\mathrm{mCd}t^{\circ}\mathrm{X}t_0$ represent the mean value of the size of larvae exposed to the condition X and measured at respectively t_{12} and t_0, $\mathrm{mCd}t^{\circ}\mathrm{NC}_{t12}$ and $\mathrm{mCd}t^{\circ}\mathrm{NC}_{t0}$ represent the mean value of the size of larvae exposed to the condition NC and measured at respectively at t_{12} and t_0.

Statistics analyses are realized on the size of larvae (mean value) measured at respectively, t_{12} and t_0. Asterisks are written on the graphic representation based on the growth rate when a significant different size of larvae compared to the negative control group (mean value) is concluded.

Micronucleus Test, Genotoxicity Assay

Formation of micronuclei is the consequence of chromosome fragmentation or malfunction of the mitotic apparatus and may result in genome mutations. In both cases, entire or fragmented chromosomes can no longer migrate to the cellular poles in the anaphase of the cell cycle resulting in a little clump of chromatin, called a micronucleus, near the principal nucleus in the cytoplasm of the daughter cells. Thus, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. At the end of exposure, a blood sample was obtained from each anesthetized larva (MS222, Sandoz, France) by cardiac puncture with heparinized micropipettes (20% solution at 5000 IU mL^{-1}, Sigma, France). After fixing in methanol and staining with hematoxylin (Sigma, France), the smears were screened under the microscope (oil immersion lens, 1500×). The number of erythrocytes that contained one micronucleus or more (micronucleated erythrocytes, MNE) was determined in a total sample of 1000 erythrocytes per larva. All slides were scored blinded by only one individual. Since micronucleus frequency was not normally distributed, median values and quartiles were calculated instead of means (McGill et al., 1978). For each group of animals, the results (number of micronucleated erythrocytes per thousand, MNE ‰) obtained for each larva were arranged in increasing order of magnitude. The medians and quartiles were then calculated. The statistical method used to compare the medians consists in determining the theoretical medians of samples of size n (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times \mathrm{IQR}/\sqrt{n}$, where M is the median and IQR is the Inter-Quartile Range (upper quartile - lower quartile) (McGill et al., 1978). The difference between the theoretical medians of the test groups and the theoretical median of the negative control group is significant to within 95% certainty if there is no overlap. In this case, the induction of micronucleus in exposed larvae, compared to the control, is considered as a genotoxic response.

Raman Spectrometry Analysis

Raman spectrometry analysis was performed on an intestine sample of larvae exposed to a concentration of 100 mg L^{-1} (higher than the maximum 50 mg L^{-1} concentration investigated in this study). Exposure at this concentration was carried out only for the analysis with the Raman spectrometry to increase the chances of tissue contamination. Such a concentration is clearly toxic for *Xenopus* (Mouchet et al., 2008). Histological preparation of intestine (semithin sections of 1 μm of depth) was observed by Raman analysis. The Raman spectra were recorded on a Renishaw spectrometer with a green laser excitation (514.5 nm). The presence of CNTs was evidenced using the G'_{2D} band of CNTs at a wave number of about 2675 cm^{-1}. This allowed avoiding any interference with the biological matrix. The laser power was kept at 25 mW with an objective magnification of 50× (spot size about 3-μm diameter), leading to about 0.25 mW μm^{-2} on the sample. No particular care was taken to avoid heating effect, as only the intensity was used to evidence the CNTs presence and location.

TABLE I. Results of acute toxicity in larvae exposed to 0.1, 1, 10, and 50 mg L^{-1} 554 of DWNTs in experiment I (without GA) and II (with GA)

DWNTs Concentrations (mg L^{-1}) − GA						
Experiment I	NC		0.1	1	10	50
Mortality	0%		0%	0%	5%	15%
Visual inspection			–	–	*	***
DWNTs concentrations (mg L^{-1}) + GA (50 mg L^{-1})						
Experiment II	NC	GAC	0.1	1	10	50
Mortality	0%	0%	0%	0%	0%	0%
Visual inspection			–	–	*	***

"–": No sign of acute toxicity compared to the negative control group (visual inspection). "*": reduced or stopped size, anaemia signs compared to the negative control group (visual inspection). The number of asterisks is function of the intensity of increasing effects. NC: Negative Control; GAC: Gum Arabic Control (50 mg L^{-1} 558 of GA alone).

RESULTS

Toxicity

The results show no mortality of the larvae whatever the experimental condition and the experiment (I and II), except at 10 and 50 mg L^{-1} without GA, where 5 and 15% of mortality were respectively observed in experiment I (Table I). The visual inspection of the acute toxicity in larvae was confirmed by measurements of the size of the larvae and shows that larvae exposed in presence of 10 and 50 mg L^{-1} of DWNTs with (experiment II) or without GA (experiment I) have reduced size compared to the NC in a dose dependant manner (Table I, Fig. 2). In contrast, larvae exposed to 0.1 and 1 mg L^{-1} of DWNTs do not show any sign of toxicity compared to the NC. Furthermore, the results show that the growth rate decreases in a dose dependant manner in larvae of both experiments (Fig. 2).

Genotoxicity

The median value of MNE ‰ for the negative control was 6.0 ± 1.2 in experiment I [Fig. 3(a)] and 1.5 ± 0.4

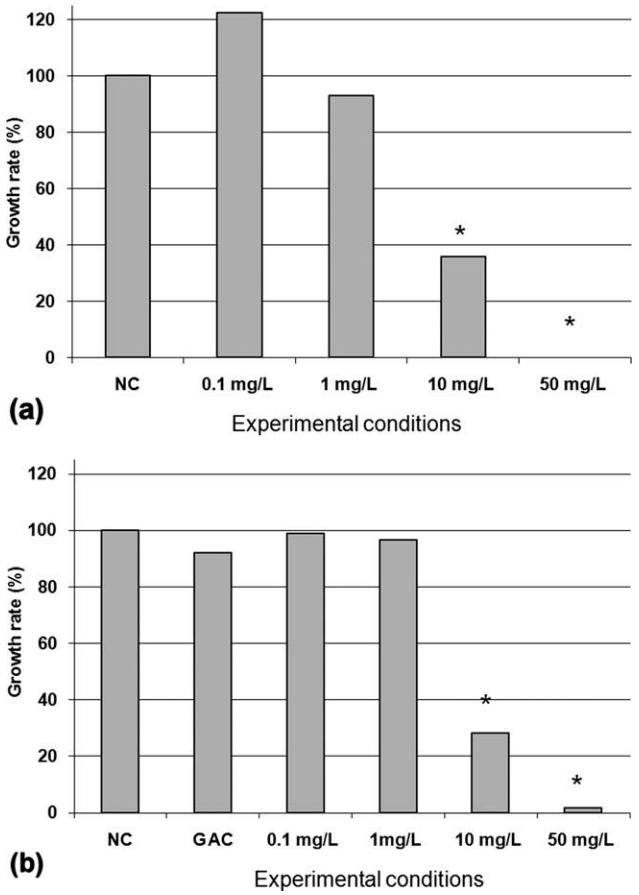

Fig. 2. Growth rate of *Xenopus* larvae exposed to 0.1, 1, 10, and 50 mg L^{-1} of DWNTs. (a) Experiment I without GA and (b) Experiment II with GA. '*' corresponds to a significant different size of larvae compared to the negative control group (mean value). NC: Negative Control; GAC: Gum Arabic Control (50 mg L^{-1})

in experiment II [Fig. 3(b)]. The positive control showed significantly higher MNE ‰ as compared to the NC group in experiments I and II (30.0 ± 4.7 and 10.5 ± 1.6, respectively). Larvae exposed to 50 mg L^{-1} of DWNTs with or without GA, were not punctured because of the toxicity (growth inhibition and lack of cell divisions) observed at this concentration. Indeed, genotoxic effects are usually expressed at subtoxic concentrations of the tested substance, following DNA damage, micronucleus induction is tributary to cellular division and hence to the mitotic index of the red blood cells. In experiment I, the results indicate no genotoxicity via micronucleus induction in erythrocytes of *Xenopus* larvae, whatever the DWNTs concentration tested without GA. No genotoxicity was also observed in larvae exposed to GA alone (2‰). In contrast, a significant micronucleus induction was observed in larvae exposed to 1 mg L^{-1} of DWNTs in presence of GA (3‰).

Raman Spectrometry Analysis

We have performed a Raman line scan to localize the CNTs. The signal from the tissues is composed of several bands around 1450 cm^{-1} and a very intense one at 1630 cm^{-1} (Fig. 4). As CNTs present no Raman signal at this frequency, the peak at 1630 cm^{-1} was used as an indication of the presence of the tissues. In the range where D (for defect induced band) or G'_{2D} (overtone of D band) bands are present, no other band associated to organic material was visible and consequently, fitting these bands is a very good way to know where the CNTs are located. We have also fitted the G band and compared all signals coming from the tubes. To fit properly the G band, we should fix all parameters, including the signal coming from the tissues, and leave only the intensities free. We obtain the same results than with G'_{2D} band but the zero intensity is less clean (very small residual intensity sometimes). We

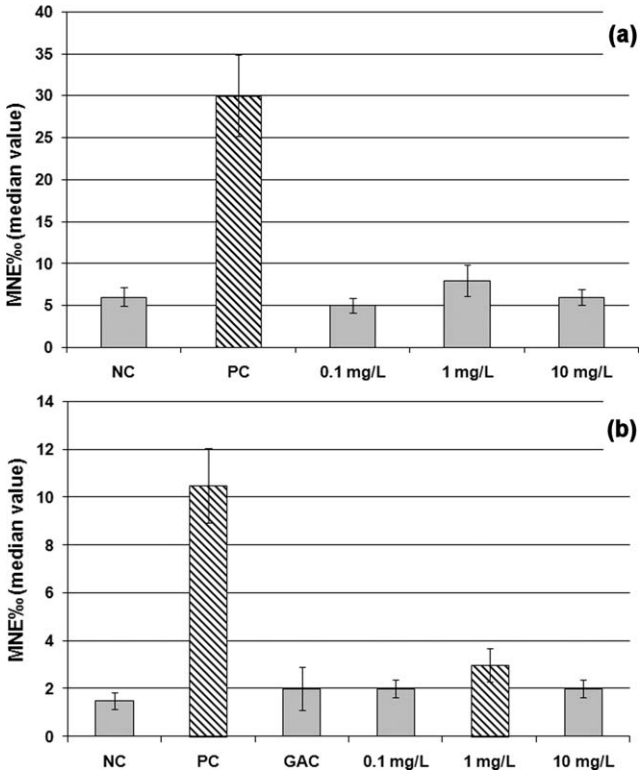

Fig. 3. Results of the micronucleus assay in larvae exposed according an half-static exposure to 0.1, 1, and 10 mg L^{-1} of DWNTs in experiment I (without GA, (a)) and II (with GA, (b)). NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg L^{-1}). Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE ‰) and their 95% confidence limits. The 50 mg L^{-1} of DWNTs has not been evaluated because of the high acute toxicity. The hatched stick indicates a genotoxic response compare to the NC. (a) Experiment I without GA and (b) Experiment II with GA.

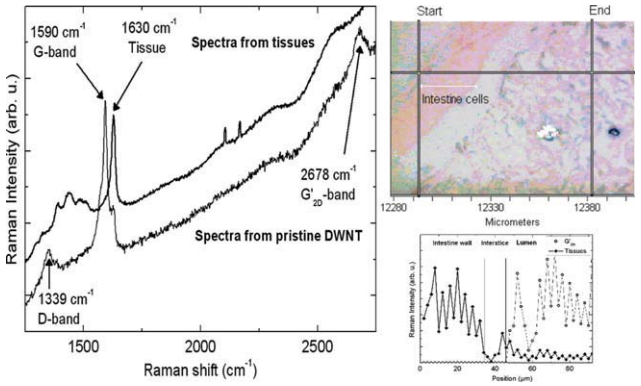

Fig. 4. (a) Compared Raman spectra of pristine DWNT and Xenipus intestine material; (b) Light microscopy image of an histological section of *Xenopus* intestine exposed to 100 mg L^{-1} of DWNTs ; (c) Horizontal line scan Raman analysis (see (a)) showing the variation of the intensity of DWNTs. (G'_{2D}) ○ and *Xenopus* tissues ◆. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

consider that both approaches are correct but we prefer to keep G'_{2D} intensity as in earlier work (Mouchet et al., 2008).

Figure 4(b) shows a histological section of the intestine of *Xenopus* exposed to 100 mg L^{-1} of DWNTs. The lumen and the intestinal wall (villi and intestinal cells) are clearly visible. On the one hand, the intensity of the G'_{2D} band strongly decreases from the lumen to the intestinal wall. On the other hand, the intensity of the signal corresponding to the *Xenopus* biological matrix increases from the lumen to the intestinal wall [Fig. 4(c)]. The presence of CNTs was clearly identified; thanks to Raman spectroscopy. However, it is unlikely that all the black material visible on the light microscopy image would correspond to DWNTs (Mouchet et al., 2008).

DISCUSSION

Considering the global planned production of CNTs on the ton scale, and their integration in everyday-life products, it is likely that some of them will enter the environment during their product's life cycle (manufacture, use, and disposal). Their widespread use thus constitutes a potential risk of exposure for all living organisms in the environment. Curiously, one of the most important areas of interest in environmental risk assessment of such new materials, i.e., the ecotoxicological field of research, remains uninvestigated. Very few data have been published on the impact of CNTs on aquatic organisms (see for review Templeton et al., 2006; Cheng et al., 2007; Helland et al., 2007; Roberts et al., 2007; Smith et al., 2007; Kennedy et al., 2008; Mouchet et al., 2007a, 2008; Petersen et al., 2008).

Toxicity

Toxicity (mainly growth inhibition) was clearly observed in *Xenopus* larvae exposed to 10 and 50 mg L^{-1} of DWNTs with or without GA according to the classical standardized test procedure of the amphibian micronucleus assay (ISO, 2006). Low mortality rate (5 and 15%) was observed at 10 mg L^{-1} and at the highest DWNTs concentration (50 mg L^{-1}), respectively in larvae exposed without GA. Nevertheless, this mortality rate is not strong enough to be considered as significant. The toxicity observed in *Xenopus* larvae from 10 mg L^{-1} in both experiments is in agreement with previous results on *Xenopus* larvae exposed to the same type of DWNTs in static conditions (Mouchet et al., 2008).

To explain the general toxicity observed in *Xenopus* larvae exposed to DWNTs, as already suggested in a previous study (Mouchet et al., 2008), different hypothesis were proposed considering the different levels of observations in larvae (binocular, photonic, or electronic microscopy). The visual inspection of the larvae under the binocular after exposure to DWNTs, whatever the concentration, shows black material inside gill suggesting first that toxicity may be mediated by branchial obstruction generating gaseous exchanges perturbation and anoxia. Recently, other authors demonstrated the link existing between the presence of CNTs in water and the apparition of respiratory pathologies in aquatic organisms. For instance, Smith et al. (2007) showed that exposure of juvenile trout (*Oncorhynchus mykiss*) for up to 10 days to dispersed SWNTs (prepared in sodium dodecyl sulfate supported by a sonication step) caused respiratory toxicity (a dose-dependant rise in ventilation rate) and gill pathologies (edema, altered mucocytes, hyperplasia).

Moreover, in amphibian larvae, "black masses" were also observed into the intestine lumen after dissection, suggesting that toxicity may be also mediated by intestinal obstruction due to the DWNTs ingested from the water exposure. In the same way, some other authors also observed absorption of CNTs in intestine of organisms such as trout exposed to SWNTs (Smith et al., 2007), daphnia exposed to coated SWNTs (Roberts et al., 2007), oligochaetes exposed to ^{14}C labeled SW and MWNTs (Petersen et al., 2008) and crustaceans exposed to raw and oxidized MWNTs (Kennedy et al., 2008) inducing different kind of toxicity, via inflammatory processes for example in trout (Smith et al., 2007) or mortality and immobilization in crustacean (Kennedy et al., 2008). In amphibian larvae, the microscopy observations of intestine cross sections in control and exposed larvae shows that black masses are clearly identifiable with the food intake in the lumen of the gut in exposed *Xenopus* larvae, whereas food is homogenously distributed into the lumen of nonexposed larvae suggesting thirdly a possible competition between DWNTs and nutritive compounds of the food. Nevertheless, neither histological preparations, nor the observation of the preparation for

TEM observations revealed black masses in epithelial or in chorionic tissues of the instestin cutting. Petersen et al. (2008) also observed that the detected ^{14}C labeled SWNTs and MWNTs were in association with sediments remaining in the gut of *L. variegatus* and were not readily adsorbed into organism tissues. In the present work, the Raman analysis carried out on the gut of exposed larvae confirms the presence of DWNTs in the lumen of the intestine, in agreement with the previous study (Mouchet et al., 2008), but the abrupt vanishing of the signal of DWNTs [G'_{2D} band, Fig. 4(c)] in tissues allows to conclude to their absence in intestinal wall (villi and cells), suggesting that DWNTs do not cross the intestinal barrier. The Raman imaging technique is very sensitive and allows assessing without doubt that the intestine of CNT-exposed animals contains CNTs, as already suggested by the simple visual inspection and/or the TEM observation. Although this analysis seems to be necessary to confirm the presence of CNTs in organisms, only one other author, to our knowledge, carried out the Raman analysis to confirm that black material was indeed CNTs in the gut of *D. magna* (Roberts et al., 2007).

In the present work, the observed toxicity in larvae was globally the same with or without GA. Gum Arabic (GA), is exempt of demonstrated toxicity in *in vivo* studies in mammals (Melnick et al., 1983; Collins et al., 1987). The presence of GA can help to improve dispersion of CNTs. In absence of larvae, suspension of DWNTs was stabilized by the addition of GA. However, suspensions were rapidly destabilized in presence of the amphibians probably due both to the ingestion of GA by larvae and consequently to decrease in GA concentration in the water, as well as the modification of exposure conditions (pH). Similarly, Roberts et al., (2007) have shown that *D. magna* exposed to SWNTs suspensions stabilized with a surfactant (lysophosphatidylcholine) were able to ingest the coated SWNTs through normal feeding behavior and to use the surfactant coating as a food source. Their study also provides some evidence of biomodification of a carbon-based nanomaterial by an aquatic organism. This is a very important point to be taken into account when dealing with ecotoxicity evaluation of CNTs in the environment, since CNTs which could end-up in the environment are likely to have been functionalized (deliberately or not) and/or to have adsorbed natural compounds such as natural sugars and organic matter etc., which are present into the aquatic compartment.

Genotoxicity

Only two studies concern genotoxicity of CNTs are available to our knowledge. Both are *in vitro* studies. Szendi and Varga (2008), using a pilot study in rat, show that oral exposure to SW and MWNTs did not increase urinary mutagenicity in rats as investigated using Ames test and that no genotoxicity effect was found using the *in vitro* micronucleus and sister chromatid exchange assays. Zhu et al. (2007) found that MWNTs can accumulate and induce apoptosis in mouse ES cells and activate the tumor suppressor protein p53 within 2 h of exposure. Among the studies available on the impact of CNTs *in vivo* none focuses on genetic effects, and especially in aquatic organisms.

The present results indicate no genotoxicity via micronucleus induction in *Xenopus* larvae exposed for 12 days in presence of pristine DWNTs (without GA), whatever their concentration in the water. This result is in good agreement with the results of the previous studies on amphibian larvae (*Xenopus* and *Axolotl*) exposed to the same type of pristine DWNTs in suspension in water (Mouchet et al., 2007a, 2008). Several hypotheses can be proposed to explain this lack of genotoxic response: (i) erythrocytes are not adequate or sensitive targets, (ii) micronuclei induction at high concentrations (>10 mg L^{-1}) would be masked by the toxicity expression, (iii) micronuclei induction is not a relevant biomarker for CNTs, since micronuclei are non repairable mutation, (iv) CNTs are present as bundles instead of individual nanoparticles, and are thus too large to penetrate into the cells, and finally (v) CNTs used in both studies do not lead to genotoxicity in amphibians.

As indicated by the Raman analysis carried out on the gut of exposed *Xenopus*, and on circulating blood of *Xenopus* and *Axoltl* exposed to DWNTs (data not shown), DWNTs do not cross the intestinal barrier. These data support an indirect cytotoxic effect for DWNTs. Genotoxicity effects are usually expressed at subtoxic concentrations of the tested substance. Then, in this case, genotoxicity expression can be masked or limited by the expression of the cytotoxicity. In the present case, the mitotic index of intoxicated larvae exposed to 10 mg L^{-1} of DWNTs was reduced compared to the index in larvae exposed to lower CNTs concentrations and to the negative control (data not shown). Szendi and Varga (2008) have shown mitotic inhibition, a possible cytotoxic effect, in the human lymphocyte cultures upon treatment with SWNTs. Concerning the biomarkers, further investigations must be carried out since genetic damages such as oxidative stress was highlighted by some authors as a potential way of toxicity. For example, in the case of *in vitro* studies, the increase in intracellular reactive oxygen species (ROS) was explained by the metal traces associated with the commercial nanotubes (Pulskamp et al., 2007). In our experiments, the metals particles (Co) associated to the purified DWNTs used are supposed to be biologically inert (Flahaut et al., 2002) and could thus explain the negative genotoxic effects observed, if it is assumed that the potentially genotoxic effects observed are ROS mediated.

Nevertheless, the present results revealed genotoxicity in larvae exposed to 1 mg L^{-1} in presence of GA, whereas no genotoxicity was observed in larvae exposed to a lower (0.1 mg L^{-1}) or a higher concentration (10 mg L^{-1}). No

genotoxicity was observed in larvae exposed to GA alone which is in agreement with experiments in mammals for which GA is exempt of evidenced toxicity (Melnick et al., 1983; Collins et al., 1987). GA is a natural polysaccharide stabilizer. It acts as a dispersant agent and can promote the bioavailability of CNTs to larvae. Maybe the agglomerates of CNTs are smaller and/or fewer in presence of GA. This concentration of 1 mg L^{-1} of DWNTs is the higher non toxic concentration and the first concentration at which toxicity would not mask genotoxicty. One other hypothesis would be in relation with the association of GA and DWNTs at 1 mg L^{-1}. Indeed, the results [Fig. 3(b)] show an increased MNE ‰ (median value) in the case of larvae exposed to 1 mg L^{-1} of DWNTs in presence of GA even if there is no significant genotoxic response. Moreover, GA being a sugar, exposure to 50 mg L^{-1} (high concentration) could induce several metabolic disorders which may act in a synergetic way with DWNTs. Work is in progress to develop a dispersion protocol limiting the amount of added surfactant (Datsyuk et al., 2009).

One can note that direct comparison of the present results with the literature must be carried out with care because toxic and genotoxic results are likely to depend on the administration route (peritoneal injection for rodent and water exposure for aquatic organism), exposure conditions of aquatic species in relation with their biology and physiology. The behavior of CNTs also depends on their intrinsic structure (number of walls, diameter, etc.) and synthesis route but also on their surface chemistry (pristine or functionalized), which plays an important role on their ability to form stable suspensions.

CONCLUSION

The present work evaluates the eco(geno) toxicity of DWNTs in amphibian *Xenopus* larvae in controlled laboratory conditions (ISO, 2006) according to two different endpoints after 12 days of exposure: toxicity and genotoxicity as the expression of the clastogenic and/or aneugenic effects observed in erythrocytes of the running blood. The results highlight the potential risk of the DWNTs used in this study, since (i) toxicity was observed in larvae exposed to DWNTs to 10 and 50 mg L^{-1} with or without GA and (ii) genotoxicity was observed in larvae exposed to 1 mg L^{-1} of DWNTs with GA. Even if DWNTs were evidenced in the larvae (gills and lumen) using microscopy method as already observed in previous study (Mouchet et al., 2008), and Raman analysis confirmed their presence into the lumen of intestine, but not in intestinal cells suggesting that intestinal barrier is not crossed. Since DWNTs are ingested by the larvae, one can not exclude the possibility that DWNTs may be found later in the food chain, once released into the environment. Considering the increasing use of CNTs in commercial products, this study emphasizes further needs to study ecotoxicity of this nanomaterial and highlights that assessing the risks of the CNTs requires a better understanding of their toxicity, bioavailability and behavior into the environment.

The authors acknowledge Elodie Sarremejean and Guillaume Bernard for their technical help, Pr A. Peigney of the CIRIMAT for SEM characterization of pristine DWNTs and Georges Merlina of EcoLab for fruitful discussion.

REFERENCES

AFNOR. 2000. Association française de normalisation (the French National Organization for quality regulations). Norme NFT 90-325. Qualité de l'Eau. Evaluation de la génotoxicité au moyen de larves d'amphibien (*Xenopus laevis*, *Pleurodeles waltl*). Paris: AFNOR. pp 17.

Baughman RH, Zakhidov AA, de Heer WA. 2002. Carbon nanotubes-the route toward applications. Sciences 297:787–792.

Bethune DS, Kiang CH, Devries MS, Gorman G, Savoy R, Vazquez J. et al. 1993. Cobalt-catalyzed growth of carbon nanotubes with single-atomic-layerwalls. Nature 363:605–607.

Bridges CM, Dwyer FJ, Hardesty DK, Whites DW. 2002. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? Bull Environ Contam Toxicol 69:562–569.

Cheng J, Flahaut E, Cheng SH. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. Environ Toxicol Chem 26:708–716.

Collins TFX, Welsh JJ, Black TN, Graham SL, Brown LH. 1987. Study of the teratogenic potential of gum arabic. Food Chem Toxicol 25:815–882.

Datsyuk V, Landois P, Fitremann J, Peigney A, Galibert AM, Soula B, Flahaut E. 2009. Double-walled carbon nanotube dispersion via surfactant substitution. J Mater Chem 19:2729–2736.

Farré M, Gajda-Schrantz K, Kantiani L, Barceló D. 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment: Review. Anal Bioanal Chem 393:81–95.

Ferrier V, Gauthier L, Zoll-Moreux C, L'Haridon J. 1998. Genotoxicity tests in amphibians—A review. Microscale Test Aquat Toxicol Adv Tech Practice 35:507–519.

Flahaut E, Peigney A, Laurent C, Rousset A. 2000. Synthesis of single-walled carbon nanotube-Co-MgO composite powders and extraction of the nanotubes. J Mater Chem 10:249–252.

Flahaut E, Agnoli F, Sloan J, O' Connor C, Green MLH. 2002. CCVD Synthesis and Characterization of cobalt-encapsulated nanoparticles. Chem Mater 14:2553–2558.

Flahaut E, Bacsa R, Peigney A, Laurent C. 2003. Gram-scale CCVD synthesis of double-walled carbon nanotubes. Chem Commun 1442–1443.

Gauthier L. 1996. The amphibian micronucleus test, a model for *in vivo* monitoring of genotoxic aquatic pollution. Review paper. Int J Batrachol 14:53–84.

Gauthier L, Tardy E, Mouchet F, Marty J. 2004. Biomonitoring of the genotoxic potential (micronucleus assay) and detoxifying

activity (EROD induction) in the river Dadou (France), using the amphibian *Xenopus laevis*. Sci Total Environ 323:47–61.

Hata K, Futaba DN, Mizuno K, Namai T, Yumura M, Iijima S. 2004. Water-assisted highly efficient synthesis of impurity-free single-walled carbon nanotubes, Science 306:1362–1364.

Helland A, Wick P, Koehler A, Schmid K, Som C. 2007. Reviewing of the environmental and human health knowledge base of carbon nanotubes. Environ Health Perspect 115:1125–1131.

Iijima S. 1991. Helical microtubules of graphitic carbon. Nature 354:5658.

Iijima S, Ichihashi T. 1993. Single-shell carbon nanotubes of 1-nm diameter. Nature 363:603–605.

ISO. 2006. ISO International Standard. Water quality—Evaluation of genotoxicity by measurement of the induction of micronuclei—Part 1: Evaluation of genotoxicity using amphibian larvae. ISO 21427-1, ICS: 13.060.70, Genova—CH.

Jaylet A, Gauthier L, Zoll C. 1990. Micronucleus test using peripheral red blood cells of amphibian larvae for detection of genotoxic agents in freshwater pollution. In: Sandhu SS, et al., editos. *In Situ* Evaluations of Biological Hazards of Environmental Pollutants. New York: Plenum. pp 71–80.

Kennedy AJ, Hull MS, Steevens JA, Dontsova KM, Chappell MA, Gunter JC, Weiss CA. 2008. Factors influencing the partitioning and toxicity of nanaotubes in the aquatic environment. Environ Toxicol Chem 27:1932–1941.

Madre JF. 2006. Logiciel Mesurim. Académie d'Amiens. Available at: http://www.ac-amiens.fr/pedagogie/svt/info/logiciels/Mesurim2/Index.htm.

Malins DC, Ostrander GK, Haimanot R, Williams P. 1990. A novel DNA lesion in neoplastic livers of feral fish: 2, 6-diamino-4-hydroxy-5-formamidopyrimidine. Carcinogen 11:1045–1047.

McGill R, Tuckey J, Larsen W. 1978. Variations of box plots. Am Stat 32:12–16.

Melnick RLJ, Huff JK, Haseman MP, Dieter CK, Grieshaber DS, Wyand AB, Russfield ASK, Murthy Fleischman RW, Lilja HS. 1983. Chronic effects of agar, guar gum, gum arabic, locust-bean gum, or tara gum in F344 rats and B6C3F_1 mice. Food Chem Toxicol 21:305–331.

Mouchet F, Gauthier L, Mailhes C, Ferrier V, Devaux A. 2005. Comparative study of the comet assay and the micronucleus test in amphibian larvae (*Xenopus laevis*) using benzo(*a*)pyrene. ethyl methanesulfonate, and methyl methanesulfonate: Establishment of a positive control in the amphibian comet assay. Environ Toxicol 20:74–84.

Mouchet F, Gauthier L, Mailhes C, Ferrier V, Devaux A. 2006a. Comparative evaluation of the genotoxicity of captan in amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*) using the comet assay and the micronucleus test. Environ Toxicol 21: 264–277.

Mouchet F, Baudrimont M, Gonzalez P, Cuenot Y, Bourdineaud JP, Boudou A, Gauthier L. 2006b. Genotoxic and stress inductive potential of cadmium in *Xenopus laevis* larvae. Aquat Toxicol 78:157–166.

Mouchet F, Landois P, Flahaut E, Pinelli E, Gauthier L. 2007a. Assessment of the potential *in vivo* ecotoxicity of double-walled carbon nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum*. Nanotoxicology 1:149–156.

Mouchet F, Cren S, Cunienq C, Deydier E, Guilet R, Gauthier L. 2007b. Assessment of lead ecotoxicity in water using the amphibian larvae (*Xenopus laevis*) and preliminary study of its immobilization in meat and bone meal combustion residues. Biometals 20:113–127.

Mouchet F, Landois P, Sarreméjean E, Bernard G, Puech P, Pinelli E, Flahaut E, Gauthier L. 2008. Characterisation and *in vivo* ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. Aquatic Toxicol 87:127–137.

Nieuwkoop PD, Faber J. 1956. Normal Tables of *Xenopus laevis* (Daudin). Amsterdam: North-Holland.

Petersen EJ, Huang Q, Weber WJ. 2008. Ecological uptake and depuration of carbon nanotubes by L*umbriculus variegates*. Environ Health Perspect 116:496–500.

Pulskamp K, Diabaté S, Krug H. 2007. Carbon nanotubes show no sign of actute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol Lett 168:58–74.

Roberts AP, Mount AS, Seda B, Souther J, Qiao R, Lin S, Ke PC, Rao AM, Klaine SJ. 2007. *In vivo* biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. Environ Sci Technol 41:3025–3029.

Smith CJ, Shaw BJ, Handy RD. 2007. Toxicity of single walled carbon nanotubes on rainbow trout (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. Aquat Toxicol 82:94–109.

Szendi K, Varga C. 2008. Lack of genotoxicity of carbon nanotubes in a pilot study. Anticancer Res 28:349–352.

Templeton RC, Lee Ferguson P, Washburn KM, Scrivens WA, Chandler GT. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. Environ Sci Technol 40:7387–7393.

Vuillaume M. 1987. Reduced oxygen species, mutation, induction and cancer initiation. Mutat Res 186:43–72.

Zhu L, Chang DW, Dai L, Hong Y. 2007. DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. NanoLetter 7:3592–3597.