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Characterisation and *in vivo* ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*

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Abstract

Because of their outstanding properties, carbon nanotubes (CNTs) are being assessed for inclusion in many manufactured products. Due to their massive production and growing number of potential applications, the impact of CNTs on the environment must be taken into consideration.

The present investigation evaluates the ecotoxicological potential of double-walled carbon nanotubes (DWNTs) in the amphibian larvae *Xenopus laevis* at a large range of concentrations in water (from 10 to 500 mg L^{-1}). Acute toxicity and genotoxicity were analysed after 12 days of static exposure in laboratory conditions. Acute toxicity was evaluated according to the mortality and the growth of larvae. The genotoxic effects were analysed by scoring the micronucleated erythrocytes of the circulating blood of larvae according to the International Standard micronucleus assay. Moreover, histological preparations of larval intestine were prepared after 12 days of exposure for observation using optical and transmission electron microscopy (TEM). Finally, the intestine of an exposed larva was prepared on a slide for analyse by Raman imaging.

The results showed no genotoxicity in erythrocytes of larvae exposed to DWNTs in water, but acute toxicity at every concentration of DWNTs studied which was related to physical blockage of the gills and/or digestive tract. Indeed, black masses suggesting the presence of CNTs were observed inside the intestine using optical microscopy and TEM, and confirmed by Raman spectroscopy analysis. Assessing the risks of CNTs requires better understanding, especially including mechanistic and environmental investigations.

Keyword: Carbon nanotubes, Eco(geno)toxicity, Amphibian larvae, *Xenopus laevis*, Microscopy, Raman imaging

1. Introduction

Due to their unique physical (mechanic, electronic, thermal) and chemical properties, CNTs are intensively studied since 15 years by the scientific community. They have a diameter around 1 nm and a length up to 10 μm or more, giving them a very high aspect ratio. Their specific surface area is generally important and can theoretically reach 1310 $m^2\,g^{-1}$ in the case of single-wall carbon nanotubes (SWNTs), closed and isolated. Practically, the formation of bundles, the contamination by by-products and the increase in the number of walls (multi-walled carbon nanotubes, MWNTs) lead to much more modest values.

CNTs are envisioned to be included in many manufactured products such as flat screens, tyres, composite materials in general, special technical clothes, medical and pharmaceutical products. The industrial production of CNTs, currently on the ton scale, is expected to increase steadily in the next decade. For instance, in 2003, Single-walled and multi-walled nanotubes had a worldwide production over 2900 kg (Borm et al., 2006). Double-walled carbon nanotubes (DWNTs) are at the frontier between SWNTs and MWNTs. The possibility to functionalise the outer wall to ensure the interaction with the external environment, while retaining the remarkable mechanical and electronic properties of the inner nanotube, makes them very attractive for their integration into systems and composites (Flahaut et al., 2003). The general properties of DWNTs are quite similar to those of SWNTs as the physical properties of CNTs tend to be more and more similar to those of graphite with the increase in

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the number of walls. DWNTs thus seem to be the best compromise for many applications.

Due to their massive production and utilization, CNTs must receive considerable attention as new, unknown and potentially hazardous materials at each step of their life cycle. Moreover, potential extensive contamination of the environment must be examined. Indeed, the presence of these products in the environment at the end of their use and the presence of CNT-contaminated waste could lead in the near-future to ecotoxicity problems, which have hardly been evaluated to any extent. In spite of this economical and technological context, it is surprising that only very few studies have been published about their potential toxicity. The vast majority of the studies focusing on the impact of CNTs has been devoted to the investigation of the toxicity and immunotoxicity in *in vitro* cellular models on human or rat cells (Shvedova et al., 2003; Muller et al., 2005; Monteiro-Rivière et al., 2005; Jia et al., 2005; Cui et al., 2005; Salvador-Morales et al., 2006; Pulskamp et al., 2007; see for review Smart et al., 2006; Oberdörster et al., 2007; Helland et al., 2007). Smart et al. (2006) indicated that in the last 5 years, disturbing and often conflicting data have emerged concerning their safety. To our knowledge, very little information is currently available about the toxic effects of such materials in *in vivo* studies, especially in aquatic organisms, except the following studies on (i) freshwater crustaceans (*Daphnia magna*) exposed to SWNTs (Roberts et al., 2007), (ii) freshwater crustaceans (*D. magna* and *Hyalella azteca*), a marine copepod and fish (*Pimephales promelas* and *Oryzias latipes*) exposed to C60 (fullerenes) (Oberdörster, 2004a,b; Oberdörster et al., 2006), (iii) zebrafish embryos (*Danio rerio*) exposed to SWNTs and DWNTs (Cheng et al., 2007), (iv) estuarine copepod (*Amphiascus tenuiremis*) exposed to SWNTs (Templeton et al., 2006) and (v) juvenile trout (*Oncorhynchus mykiss*) exposed to SWNTs (Smith et al., 2007). None of these *in vivo* studies on aquatic organisms are devoted to genotoxic effects of CNTs. Indeed, among toxic actions, genotoxic effects may durably affect the aquatic ecosystems and the presence of genotoxic compounds in water can also have repercussions on non-aquatic species, via food chains, or simply as a result of drinking the water. One should therefore be aware of the hidden risks of potentially genotoxic substances in the aquatic environment. 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 tumour 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 (for a review see Jaylet et al., 1990). The assays can be carried out with intact animals, taking into account uptake and elimination, internal transport and metabolism. An example is the use of amphibians, which have proved to be valuable bio indicators and sensitive models for environmental studies (Jaylet et al., 1986; Mann, 2006). 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. Thus, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. The micronucleus test (MNT) has been widely used in many amphibian species (*Pleurodeles waltl*, *Ambystoma mexicanum* and *Xenopus laevis*) in the laboratory (Gauthier, 1996; Ferrier et al., 1998; Jaylet et al., 1990; Gauthier et al., 2004; Mouchet et al., 2005, 2006a,b, 2007a,b. The sensitivity and reliability of the MNT to detect chromosomic and/or genomic mutations makes it a good method to analyse the potential cytogenetic damage caused by pure substances (Gauthier, 1996; Jaylet et al., 1990; Mouchet et al., 2005, 2006a,b, 2007a). This method has been standardised on *X. 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 sub-organism (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 in amphibians in the environment.

The present work constitutes a preliminary study on the impact of CNTs, in particular DWNTs in *X. laevis*. To date, it is very difficult to evaluate any environmentally realistic CNTs concentrations. Working on water-soluble contaminants, the substance concentrations in water are quite easy to determine using classical analytical methods. In contrast, no method for the quantification of CNTs in natural media is available so far. In the present work, the strategy was based on a classical risk assessment method, to evaluate the potential risk of this new contaminant into the environment. In this way, different conditions of exposure have been investigated. A first step consists in determining the potential acute toxic effects of this new material towards the organism using *Xenopus* larvae. Because of the absence of biological effects of DWNTs on *Ambystoma* larvae exposed to a wide range of DWNTs concentrations in a previous study (Mouchet et al., 2007b), *Xenopus* larvae were exposed to a wide range of DWNTs doses, including very high concentrations (from 10 to 500 $mg\,L^{-1}$), in order to observe potential toxic effects on this test-organism.

In the present work, *Xenopus* larvae were exposed to DWNTs in controlled laboratory conditions in order to evaluate two different endpoints after 12 days of exposure according to the French and the International standard (AFNOR, 2000; ISO, 2006): (i) acute 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 light and electron microscopy methods. The Raman spectrometry analysis was used to confirm the presence of DWNTs.

2. Materials and methods

2.1. Preparation of CNTs samples

DWNTs were prepared by catalytic chemical vapour deposition (CCVD) by decomposition of a H_2–CH_4 mixture over an MgO-based catalyst (Flahaut et al., 2003). The carbon content

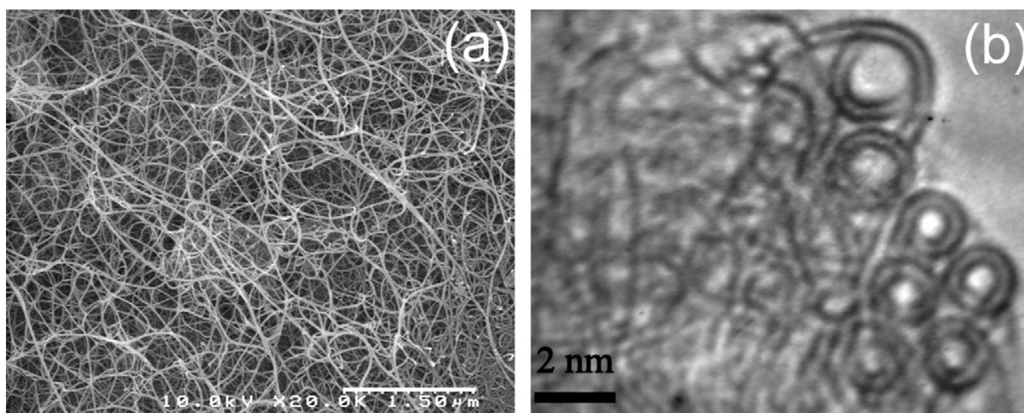

Fig. 1. (a) Field emission gun-scanning electron microscopy (FEG-SEM) and (b) transmission electron microscopy (TEM) images of raw catalytic chemical vapour decomposition (CCVD) sample.

of the as-produced CCVD product was *ca.* 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 are then obtained by treating the required amount of CCVD product with a concentrated aqueous hydrochloric acid (HCl) solution. After washing with deionised water until neutrality, the CNTs are maintained in wet conditions in order to limit agglomeration. A sample was taken and dried for further characterisation (elemental analysis, BET, Raman spectroscopy, SEM, TEM, XRD). The carbon content of the CNTs sample was *ca.* 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 Brunauer–Emmett Teller (BET)-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 sample. Analysis of the radial breathing modes (the frequency of which 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. Fig. 1(a) shows a representative field emission gun-scanning electron microscopy (FEG-SEM) image of the raw CNT sample (as-produced CCVD product), showing a very high density of CNT bundles, with extensive branching. Their diameter typically ranged between 10 and 20 nm but numerous individual CNTs were also present. No carbon nanofibre (a typical by-product of CVVD methods) was observed in the sample. high resolution transmission electron microscopy (HRTEM) observation was performed on the CNTs after elimination of the catalyst (Fig. 1(b)) and revealed clean CNT surfaces; as suggested by SEM, the CNTs are mainly isolated, or gathered into small bundles. The CNTs obtained in those conditions contain *ca.* 80% DWNTs, together with *ca.* 15% single-wall carbon nanotubes and *ca.* 5% triple-walled carbon nanotubes. The outer diameter of DWNTs is typically ranging between 1 and 3 nm (Figs. 1(b), 2–4).

2.2. *Xenopus* rearing and breeding

The *Xenopus* males were injected with 50 IU of PMSG 500 (Pregnant Male Serum Gonadotrophin, 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 experimenta-

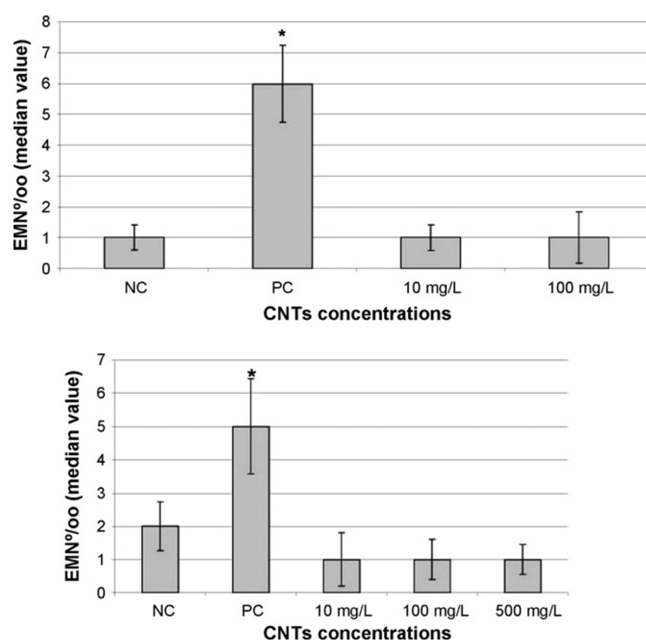

Fig. 2. Results of the micronucleus assay in larvae exposed in static exposure to 10, 100 and 500 $mg\ L^{-1}$ of CNTs in experiment I (without added air in media) and II (with air bubbling). NC, negative control; PC, positive control (cyclophosphamide, 20 $mg\ L^{-1}$); CNTs, carbon nanotubes. Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE ‰) and their 95% confidence limits. Five hundred milligrams per litre of CNTs has not been evaluated in the experiment I because of the high mortality rate.

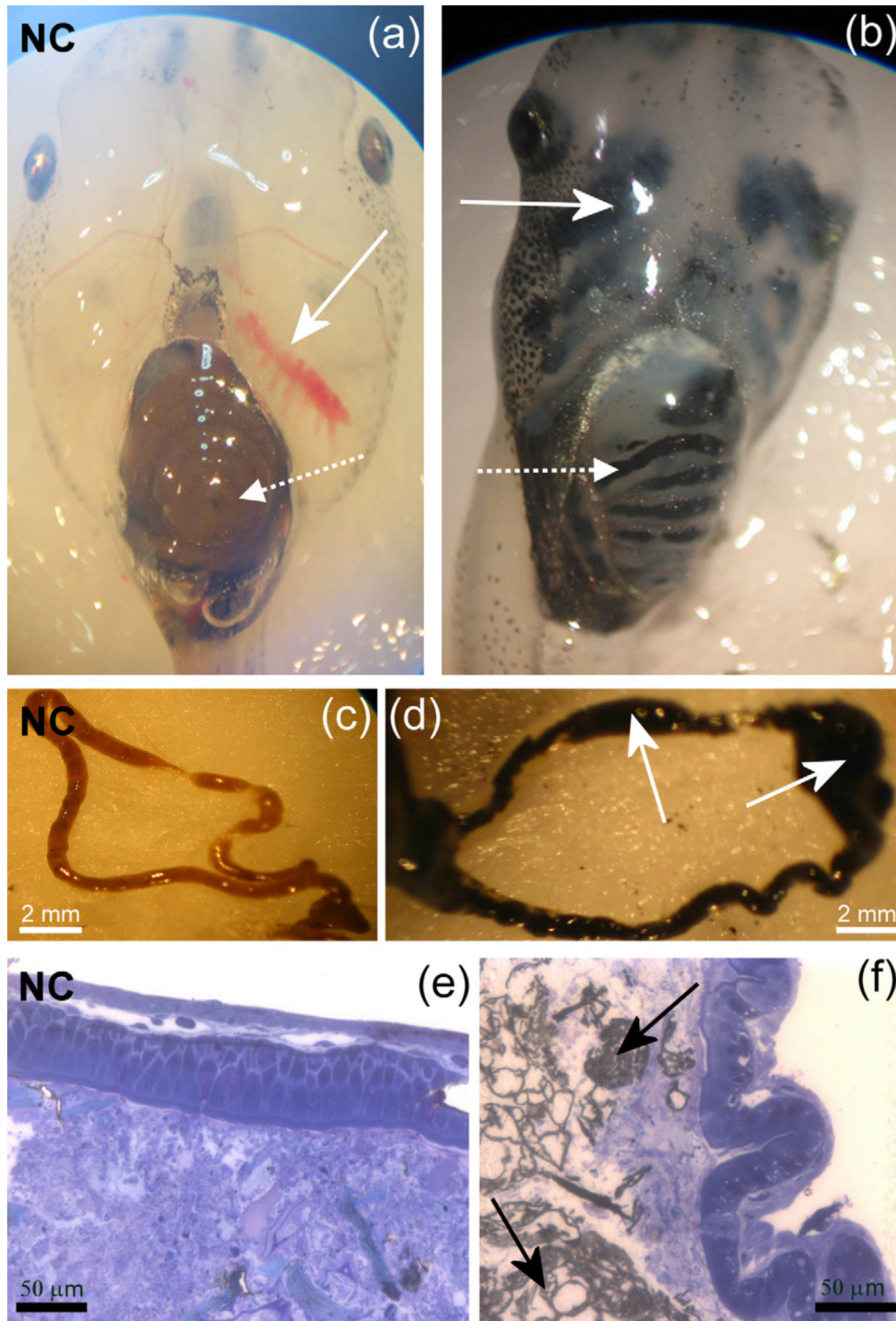

Fig. 3. Light photomicrographs of *Xenopus* larvae exposed 12 days to 10 mg L^{-1} of CNTs compared to the control group: general observation of entire larvae, binocular observation and histology of intestine. NC: negative control. On Fig. 3(a and b), plain white arrows indicate traps and dotted white arrows show the intestine. Plain white arrows on Fig. 3(d) show the distended aspect of the intestine in larvae exposed to CNTs, as compared to the intestine of non-exposed larvae (Fig. 3(c)). Black arrows in Fig. 3(f) indicate the presence of black fibrous structures in the intestine lumen of CNT-exposed larvae. Sections shown in Fig. 3(e) and (f) were stained with methylene blue.

tion. The larvae were fed every day on dehydrated aquarium fish food.

2.3. 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). However, in order to adapt the exposure conditions to more realistic environmental conditions, we modified the standardised procedure. Two independent experiments (I and II) were conducted in static exposure conditions (without any renewal of the medium during test exposure) instead of the semi-static exposure condition (daily renewal of the medium) of the standardised procedure. In experiment I, *Xenopus* larvae were exposed for 12 days in static conditions to 10, 100 and 500 mg L^{-1} of DWNTs. In experiment II, the

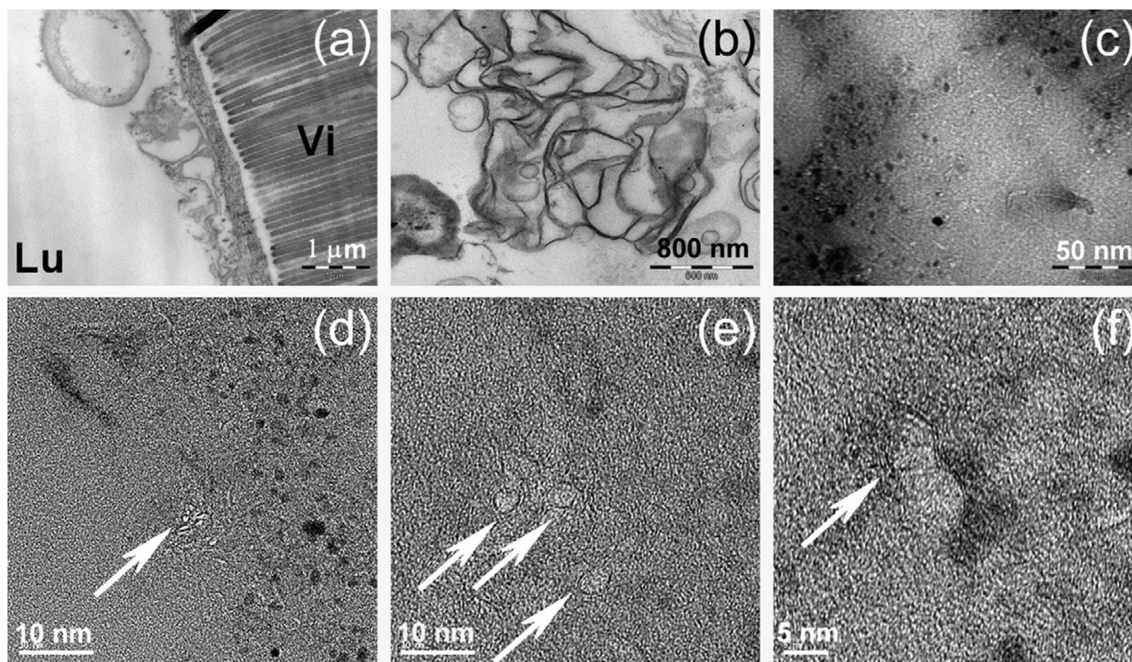

Fig. 4. Observation of an area containing black fibrous structures in the intestine lumen of CNTs exposed larvae using transmission electron microscopy (TEM) and high resolution transmission electron microscopy (HRTEM) micrographs of the intestine of *Xenopus* larvae exposed to 100 mg L^{-1} of CNTs. The Fig. 4(a) shows the lumen (on the left) where digested material passes through and from where nutrients are absorbed and the villi (on the right). Fig. 4(b and c) correspond to successive magnifications of Fig. 4(a). White arrows in HRTEM images 4(d–f) indicate structures corresponding to CNTs. Lu, lumen; Vi, villi.

larvae were exposed to the similar conditions as in experiment I, but an air diffuser was placed in the water of each group of larvae in order to promote DWNTs dispersion in the media and to compensate the potential oxygen deficit in water, due to static exposure conditions.

The amphibian larvae were exposed in reconstituted water (distilled tap water to which nutritive salts were added [294 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 123.25 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 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) characterized by the hind limb bud longer than broad, constricted at the base. For a given experiment, the larvae were taken from the same hatch to reduce inter-animal 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 (10, 100 and 500 mg L^{-1} of DWNTs). The suspensions of DWNTs were prepared by adding reconstituted water to the corresponding wet amount of DWNTs, up to 2 L.

The negative control (NC) was the reconstituted water alone. The positive control (PC) was cyclophosphamide (CP, [6055-19-2], Sigma France) in reconstituted water 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 included in each experiment to check the responsiveness of the amphibian larvae. The larvae were submitted to a natural light–dark cycle at $22.0 \pm 0.5\,^{\circ}$C during the 12 days of exposure. They were fed every day on dehydrated aquarium fish food.

2.4. Acute toxicity

Acute toxicity (death or abnormal behaviour) of larvae exposed to CNTs was examined for 12 days according to the standardised recommendations (AFNOR, 2000; ISO, 2006) by visual inspection and comparison to NC. Abnormal behaviour corresponding to reduced and/or stopped growth of larvae, reduced food intake and abnormal motility was recorded during the 12 days of exposure.

2.5. Micronucleus test, genotoxicity assay

At the end of exposure, a blood sample was obtained from every anaesthetized 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 or more micronuclei (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 every group of animals, the results (number of micronucleated erythrocytes per thousand, MNE ‰) obtained for every larva were arranged in increasing order of magnitude. The medians and quartiles were then calculated. The statistical method used to compare the medians consists of determining the theoretical medians of samples of size n (where $n \geq 7$) and their

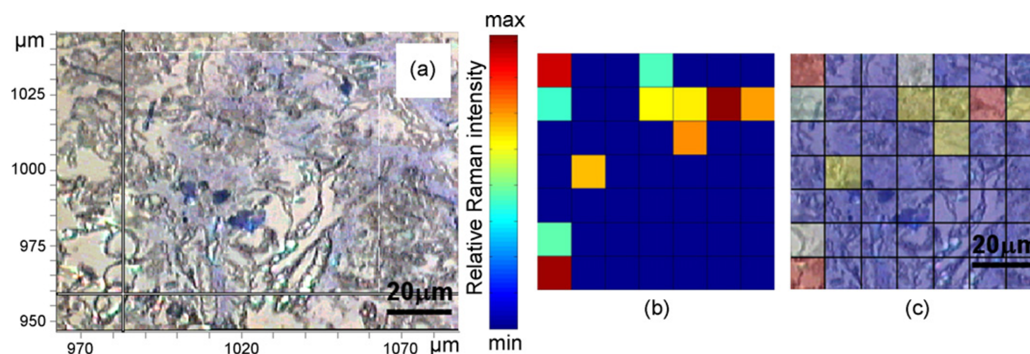

Fig. 5. (a) Light microscope image of the studied intestine area, (b) Raman intensity map corresponding to the selected observation area of (a) and (c) superposition of optical image and Raman intensity map.

95% confidence limits expressed by $M \pm 1.57 \times \text{IQR}/\sqrt{n}$, 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.

2.6. Animal observations, dissections

After puncturing, the general aspects of the larvae were compared in negative control group and in groups exposed to CNTs. Some animals of both groups were then dissected and the guts observed under the binocular at a magnification of ×15 to observe the presence or absence of CNT agglomerates. Additionally, in some cases, further investigations were conducted aiming at identifying CNTs in larval intestine under light and electron microscope. The histological sections were prepared at the Centre de Microscopie Électronique Appliquée à la Biologie of the Medical University of Rangueil in Toulouse.

2.7. Histological preparation, light and transmission electron microscopy (TEM)

Tissue were fixed at 4 °C in 2% glutaraldehyde in sodium phosphate buffer, post fixed in 1% osmium tetroxide, dehydrated through graded ethanol solutions and then embedded in Embed812-Araldite502 resin (EMS). For optical microscopy, semi-thin sections (1 µm of depth) were stained with methylene blue. These sections were then examined microscopically and digitally photographed with a SPOT digital camera connected to an Olympus BX 41 microscope.

For transmission electron microscopy, ultra-thin sections (from 50 to 90 nm of depth) were mounted on copper grids, and then stained in lead citrate and uranyl acetate solutions for examination. Two types of observations were conducted. The first one used a classical transmission electron microscope, TEM JEOL 1011, operated at 100 kV), and the second used the high resolution TEM (HR TEM, JEOL 2100F, operated at 200 kV) to ensure at higher magnification that observable fibres at the nanolevel are indeed CNTs.

2.8. Raman spectrometry analysis

The sample was prepared by simple pressing of intestine fragments between two glass slides, without further treatment and then observed by Raman analysis. The Raman spectra were recorded on a Renishaw spectrometer with a green laser excitation (514.5 nm) at a wave number around 2675 cm^{-1}, corresponding to the 2D band of CNTs. This allowed avoiding any interference with the biological matrix. 2D signal being superimposed to an intense flat signal. Ten spectra were acquired for each location on the sample (to improve the signal to noise ratio) with a total accumulation time of 270 s. The laser power was kept at 25 mW with an objective power of ×50 (spot size *ca.* 3 µm diameter), leading to *ca.* 0.25 mW μm^{-2} on the sample. The size of analysis pixel was *ca.* 10 µm × 10 µm. The CNT signal represented only *ca.* 2% of the total intensity. When the intensity was lower than 0.5%, we were not able to accurately extract it and consequently reported no coloration on the image (Fig. 5), which only corresponds to a concentration lower than 25% of the maximum concentration. No particular care was taken to avoid heating effect, as only the intensity was used to evidence the CNTs location.

3. Results

3.1. Acute toxicity

The results show no toxicity in either NC or PC in our two experiments (Table 1). A high mortality level was observed at 500 mg L^{-1} of DWNTs in experiment I (85%) whereas a lower one was observed in experiment II (5%) at the same exposure concentration. Lethality was also observed at 10 and 100 mg L^{-1} of DWNTs in experiment II (15% and 5%, respectively) whereas no mortality was recorded to these DWNTs exposure concentrations in experiment I.

Furthermore, the results show a dose-dependent reduction is size of larvae exposed in experiment I. In experiment II, a reduced size was also recorded in larvae exposed to 100 and 500 mg L^{-1} of DWNTs, in a dose-dependent manner, whereas larvae exposed to 10 mg L^{-1} of DWNTs showed no reduction in size.

Table 1
Results of acute toxicity in larvae exposed in static conditions to 10, 100 and 500 mg L^{-1} of CNTs in experiments I (without added air in media) and II (with air bubbling)

	NC	PC	DWNTs concentrations (mg L^{-1})		
			10	100	500
Experiment I					
Mortality	0%	0%	0%	0%	85%
Abnormal behaviour		−	+	++	+++
Experiment II					
Mortality	0%	0%	15%	5%	5%
Abnormal behaviour		−	−	+	+++

"−": No abnormal behaviour compared to the negative control group. "+": Abnormal behaviour compared to the negative control group (reduced and stopped size). NC, negative control group; PC, positive control group (cyclophosphamide, 20 mg L^{-1}).

3.2. Genotoxicity

The median value of MNE ‰ for the negative control was 1 ± 0.41 in experiment I (Fig. 2A) and 2 ± 0.74 in experiment II (Fig. 2B). The positive control showed significantly higher MNE ‰ as compared to the negative control group in experiments I and II (6 ± 1.25 and 5 ± 1.44, respectively). The results indicate that DWNTs did not induce any genotoxicity seen as micronucleus induction in erythrocytes of *Xenopus* larvae, whatever the DWNTs concentration tested in both experiments. In both groups, the levels of MNE induced were 1 ‰ whatever the concentration. MNE frequencies showed the same level of MN induction whatever the DWNTs concentration and the experiment.

3.3. Observations on larvae and their dissections

Xenopus larvae exposed during 12 days to 10 mg L^{-1} of DWNTs in water showed a particular visual aspect (Fig. 3(b)). Compared to negative control larvae (Fig. 3(a)), their gills and gut had black mass which was particularly visible through the thin peritoneal membrane of the larvae because of its deep black colour (Fig. 3(b)). Dissection of larvae revealed the presence of invasive black masses in the gills and in the digestive tract of larvae exposed to 10 mg L^{-1} of DWNTs, particularly in the intestine (Fig. 3(d)). Compared to control animals (Fig. 3(c)), the gut of exposed animals seemed to be morphologically dystrophic (Fig. 3(d)). The same observations, but in this case dose-dependent, were made in both experiments (I and II) in living larvae exposed to 100 and 500 mg L^{-1} of DWNTs (data not shown).

3.4. Light microscopic observations

The histological preparations from different organs showed only macroscopic levels of CNTs at the light microscopic scale. Macroscopically observable levels of CNTs were seen only in the intestine. Fig. 3 shows photomicrographs of larval intestine of *Xenopus* in control groups (Fig. 3(e)) and in animals exposed to 10 mg L^{-1} of DWNTs (Fig. 3(f)). The same tissue structures are visible in the preparations of both animal groups, except for the presence of black fibrous structures in the intestinal lumen of DWNT-exposed larvae. These black-coloured fibres were interpreted as DWNTs bundles, observable at the macroscopic (micrometric) level, present in the intestinal lumen, absorbed with the food of the animal. The same food content is observable in the gut lumen of control larvae. Similar observations were made in larvae exposed to 100 and 500 mg L^{-1} of DWNTs in both experiments (data not shown).

3.5. TEM characterisations

The thin intestine morphology of the CNT-exposed larvae was not different from that of the control. Fig. 4(a) shows fibres in the intestinal lumen of larvae exposed to DWNTs, near the microvilli but the same structures were also observed in the control group (not shown). Fibres were then observed at higher magnification (Fig. 4(b) and (c)). Discrimination of these fibres was carried under high resolution TEM (Fig. 4(d–f)). Fig. 4(d) shows isolated CNTs indicated by white arrows. Higher magnification observations were also performed (Fig. 4(e) and (f)) and also revealed the presence of CNT fibres but only in larvae exposed to CNTs.

3.6. Raman spectrometry analysis

Fig. 5 shows a superposition of the optical microscope image (slide) of an intestine sample of CNT-exposed animals with the corresponding Raman image from the same area. In the 1000–2000 cm^{-1} wave number range, many features prevent from finding the CNT signal unambiguously (presence of an additional peak at 1630 cm^{-1} close to the G band, as well as several bands in the D band wave number range). To avoid interference from Raman signals due to the biological matrix, we focussed our attention on the 2D Raman band (specific of hexagonal network of sp2 carbon and thus of CNT) located around 2675 cm^{-1} with a 514.5 nm excitation wavelength. The 2D signal was superimposed to an intense flat signal. The CNT signal represents about 2% of the total intensity. When the intensity was lower than 0.5%, we were not able to accurately extract it and consequently reported no coloration on the image, which only means concentration lower than 25% of the maximum concentration. The Raman image corresponds to the intensity level of the 2D band, which is of course absent in the negative control sample. The superposition clearly shows that on the one hand some of the black structures visible in optical microscopy do not contain any CNT (no match), but that on the other hand CNT are present in some other places although optical microscopy does not show any clear evidence (this must be related to agglomerate size). Red corresponds to maximum concentration, yellow to around half and grey to around 25%.

4. Discussion

Considering the 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. The findings of toxicology studies stress the need for work to characterize the potential impact of CNTs on 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 totally uninvestigated and very little data on nanoparticles (for a review see Oberdörster et al., 2007), including CNTs (Helland et al., 2007), have been published. Concerning carbon nanoparticles, the most investigated are fullerenes (C_{60}) and some studies have demonstrated expression of toxicity to aquatic organisms such as *D. magna*, *Micropterus salmoides*, *Pimphales promelas*, mexposed to low concentrations (Oberdörster, 2004a,b; Oberdörster et al., 2006). Concerning specifically CNTs, only few studies are published (Templeton et al., 2006; Cheng et al., 2007; Smith et al., 2007).

To our knowledge, until now, amphibians have not been 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 behaviour, so that they are more and more used as monitoring systems for water quality assessment (Gauthier et al., 2004; Bridges et al., 2002). Thus, *Xenopus* larvae were exposed to a wide range of DWNTs concentrations in order to observe potentially toxic effects on this test organism. Concentrations were chosen from 10 to 500 mg L^{-1}, and may mimic accidental releases of CNTs during production or transport.

4.1. Genotoxicity

The present results demonstrated that rough DWNTs deposed in water did not lead to any induction of micronuclei in erythrocytes of *Xenopus* larvae, whatever their concentration in the water and the exposure condition. These results are in accordance with those previously obtained in our laboratory in larvae of another amphibian (*A. mexicanum*) with the same DWNTs with the same range of concentrations (Mouchet et al., 2007b). Several hypotheses can explain these results: (i) CNTs used in both studies are not genotoxic in amphibians, (ii) the size of the raw CNTs, as bundles, is too large to penetrate into the cells, (iii) erythrocytes are not adequate or sensitive targets and finally (iv) micronucleus induction is not a relevant biomarker for CNTs, since micronuclei are non repairable mutations. Further investigations must be carried out before concluding about the absence of genetic diseases in amphibian larvae, since genetic damages such as oxidative stress was highlighted by some authors as a potential way of CNT 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 metal particles (Co) associated to the purified DWNTs used are supposed to be biologically inert (Flahaut et al., 2002) and could thus explain the absence of genotoxic effects, if it is assumed that the potentially genotoxic effects observed are ROS mediated.

4.2. Acute toxicity

Acute toxicity (mortality or reduced size) was clearly observed in *Xenopus* larvae exposed to the classical standardised test procedure of the amphibian micronucleus assay (ISO, 2006), including at the lowest DWNTs concentration (10 mg L^{-1}) tested, where reduced size was mainly observed. A high mortality rate (85%) was noted at the highest DWNTs concentration (500 mg L^{-1}). Moreover, supplied air can help to physically improve dispersion of CNTs. When air was bubbled in the aquaria, high lethality rates were not recorded, even if abnormal size reduction were still observed in larvae exposed to 100 and 500 mg L^{-1} of DWNTs. The acute toxicity observed from 10 mg L^{-1} in the present work is not in accordance with those of the previous study (Mouchet et al., 2007b) since no acute toxicity was observed in axolotl larvae exposed in static conditions to the same kind of DWNTs up to 1000 mg L^{-1}. This difference of sensitivity between both amphibian species may be in relation with their biology, larval growth, behaviour, etc. A difference of sensitivity between species can be observed in response to chemical pollutants, and *Xenopus* larvae are often found to be more sensitive than urodelian species to environmental substances (Gauthier, 1996). Recently, using copepods, Templeton et al. (2006) demonstrated that these organisms ingesting purified SWNTs showed no significant effects on mortality, development and reproduction, whereas exposure to the more complex AP-SWNTs mixture (partially purified and oxidatively processed to remove metallic and carbonaceous impurities in nitric acid, i.e. dispersible in water) significantly increased life-cycle mortality, reduced fertilization rates and reduced molting success at the concentration of 10 mg L^{-1}. Such results highlight the ability of CNTs to induce acute toxicity in aquatic organisms, depending of the nature of the nanomaterial itself. Studying the effects of CNTs on aquatic vertebrates, Cheng et al. (2007) showed that exposure to raw SWCNTs induced a significant hatching delay in zebrafish embryos between 52 and 72 h postfertilization (hpf) at concentrations higher than 120 mg L^{-1}, but 99% of the exposed embryos hatched by 75 hpf. Raw DWNTs also induced a hatching delay at concentrations greater than 240 mg L^{-1}. Such developmental and physiological effects could also have been part of the effects responsible of the growth inhibition observed with *Xenopus* larvae exposed to DWNTs in our experiments.

To explain the general acute toxicity observed in *Xenopus* larvae exposed to DWNTs, different hypothesis are proposed. Acute toxicity may be mediated by several biological mechanisms: (i) branchial obstruction and/or gaseous exchanges perturbation and/or anoxia (ii) and/or intestinal obstruction (iii) and/or competition between CNTs and nutritive food. Since these hypotheses suppose that CNTs function inside the organism itself, it was necessary to show the presence of CNTs in the *Xenopus* larvae after CNTs exposure. This was first studied by visual inspection of the larvae under the binocular after exposure (Fig. 3(a) and (b)). Internal gills were black coloured

and seemed to be blocked up, so that anoxia could have been, at least in part, responsible of the toxic effects observed in *Xenopus* larvae. This could also explain the low mortality recorded in experiment II with *Xenopus* larvae exposed to 500 mg L^{-1} of DWNTs in air-bubbled medium. Recently, other authors have demonstrated that a link exists between the presence of CNTs in water and the appearance of respiratory pathologies in aquatic organisms. For instance, Smith et al. (2007) showed that exposure of juvenile trout (*O. mykiss*) for up to 10 days to dispersed SWNTs (prepared in sodium dodecyl sulphate supported by a sonication step) caused respiratory toxicity (a dose-dependent rise in ventilation rate) and gill pathologies (oedema, altered mucocytes, hyperplasia).

The second hypothesis was first explored by direct visual inspection of the digestive tract under the binocular in larvae exposed to DWNTs (Fig. 3(c) and (d)). Distended aspects were observed in intestine walls of exposed animals and "black masses" were observed in the intestine lumen after dissection (Fig. 3(d)), suggesting that the larvae may have absorbed the CNTs present in the exposure medium. In amphibian larvae, apart from gills, xenobiotics enter the body from the exposure medium in two principal ways: the dermal route and the digestive route leading to potentially high ingestion rates of particles, especially particles of food susceptible to vehicle-agglutinated CNTs and water with suspended particles. Absorption of CNTs was also observed in intestine of trout exposed to SWNTs contaminated water (Smith et al., 2007). In this case, CNTs induced toxicity via, e.g., inflammatory processes.

It was then necessary to characterize the presence of CNTs in exposed *Xenopus* intestines to suggest the possible toxic effects of CNTs ingestion by the larvae. The first investigations were conducted using microscopic observations of intestine cross sections in control and exposed larvae (Fig. 3(e and f)). Black masses were clearly identifiable with the food intake in the lumen of the gut in exposed *Xenopus* larvae, but black masses were not observed in epithelial or in chorionic tissues. It was not really possible to identify CNTs in the black masses observed in the gut lumen on the light microscopy histological preparations. Sections for TEM of the intestines were prepared from control and exposed animals with the following double objective: (i) to look at CNTs inside the digestive tissue and (ii) to identify CNTs in the lumen of the gut. Normal TEM did not allow the confirmation of the presence of CNTs in the digestive tissue or intestinal lumen. Rather, it was impossible to distinguish between the lumen content of exposed and non-exposed animals, including at the level of the "black masses".

Further investigations were then designed to try to identify DWNTs in the preparations: The HRTEM observation (Fig. 4(d–f)) and the Raman analysis (Fig. 5), allow confirming the presence of CNTs in black masses. HRTM observations revealed the presence of CNTs inside the intestine lumen of exposed larvae (Fig. 4(d–f)). The dark spots correspond to nanoparticles encapsulated in graphitic shells, a by-product of the CCVD synthesis. These Co nanoparticles do not interact with the environment because the encapsulation protects them in a very efficient way (Flahaut et al., 2002). They are easily evidenced by TEM because they exhibit much higher contrast than carbon. At higher magnification, some circular features become visible (Fig. 4(d and e)), indicated by white arrows, which could correspond to cross-sections of CNTs. This is even more obvious in Fig. 4(e), where SWNTs and DWNTs become visible (again in cross-section). Fig. 4(f) shows one of these features at higher magnification and a three-walled structure is clearly visible. The Raman imaging technique is very sensitive and thus allows assessing without doubt that the intestine of CNT-exposed animals contains CNTs, as already suggested by the simple visual inspection and the TEM observation. But the image superposition analysis (Fig. 5) clearly sharpened the first interpretation obtained with the microscopic technique. Indeed, some of the "black masses" observed in light microscopy do not contain any CNT, whereas CNTs are clearly identified in some other places although light microscopy does not show any clear evidence for this.

Direct comparison of the present results with the literature must be carried out with caution, because toxic and genotoxic results are likely to depend on the route of administration (peritoneal injection for a rodent and water exposure for an aquatic organism), exposure conditions of aquatic species in relation with their biology and physiology. The behaviour of CNTs also depends on their intrinsic structure (number of walls, diameter, etc.) and synthesis route as well as on their surface chemistry (raw or functionalised), which plays an important role on their ability to form stable suspensions.

The difficulty to have and work with stable suspensions of CNTs in water is an important issue. The size of the agglomerates will probably play an important role in terms of the bioavailability and thus of toxicity of CNTs. In our static conditions, the daily addition of food to feed the animals and the progressive release of natural organic matter improved little by little the ability of the medium to keep the CNTs in suspension (Hyung et al., 2007). This was more obvious at the lowest concentrations. Although the swimming of the animals was rather efficient to re-suspend the agglomerates of CNTs, the addition of air diffusers in experiment II also helped in this purpose. Experiments are in progress with daily renewal of the medium (including the CNTs) and will allow working in more controlled conditions. However, even if more difficult to analyse, the static conditions used in this study are probably more realistic from a biological point of view.

5. Conclusion

The present work evaluates the eco(geno)toxicity of DWNTs in amphibian *Xenopus* larvae in controlled laboratory conditions, according to two different endpoints after 12 days of exposure: (i) acute toxicity and (ii) 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 acute toxicity was observed in larvae exposed to DWNTs whatever the concentration, possibly due to physical blockage of the gills and/or digestive tract. Indeed, the presence of DWNTs was first found in gills. DWNTs were also observed in the gut of lar-

vae using both electron microscopy and Raman analysis. Our results also demonstrate that light microscopy alone is not sufficient to assess the presence of CNTs in biological samples. Since DWNTs are ingested by larvae, one cannot exclude the possibility that DWNTs may be found later in the food chain, once released into the environment. In contrast, no genotoxicity was observed in larvae exposed to DWNTs in these conditions. Considering the increasing use of CNTs in commercial products, this study emphasizes further needs to study ecotoxicity and highlights that assessing the risks of the CNTs requires a better understanding of their toxicity, bioavailability and behaviour into the environment.

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