Carbon nanotube ecotoxicity in amphibians: assessment of ultiwalled carbon nanotubes and comparison with double-walled carbon nanotubes

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The potential impact of industrial multiwalled carbon nanotubes (MWNTs) was investigated under normalized laboratory conditions according to the International Standard micronucleus assay ISO 21427–1 for 12 days of half-static exposure to 0.1, 1, 10 and 50 mg/l of MWNTs in water. Three different end points were carried out for 12 days of exposure: mortality, growth inhibition and micronuclei induction in erythrocytes of the circulating blood of larvae. Raman spectroscopy analysis was used to study the presence of carbon nanotubes in the biological samples. Considering the high diversity of carbon nanotubes according to their different characteristics, MWNTs were analyzed in *Xenopus* larvae, comparatively to double-walled carbon nanotubes used in a previous study in similar conditions. Growth inhibition in larvae exposed to 50 mg/l of MWNTs was evidenced; however, no genetoxicity (micronucleus assay) was noticed, at any concentration. Carbon nanotube localization in the larvae leads to different possible hypothesis of mechanisms explaining toxicity in *Xenopus*.

KEYWORDS: amphibian larvae double-walled carbon nanotube genotoxicity multiwalled carbon nanotube Raman analysis toxicity *Xenopus laevis*

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Carbon nanotubes (CNTs), one allotrope of carbon, are one dimension nanoscale objects. Their structure can be described as a graphene sheet rolled up to form a cylinder. There are two main types of CNT: single-walled CNTs (SWNTs) and multiwalled CNTs (MWNTs), depending on the number of walls. Among the MWNTs, double-walled carbon nanotubes (DWNTs) are at the frontier between SWNTs and MWNTs, with very close morphology and properties to SWNTs. CNTs have a diameter between 1 and 100 nm and a length from less than 1 µm up to tens of µm or more [1], characterized by exceptional physical (i.e., mechanic, electronic and thermal) and chemical properties. However, CNT properties look more and more like those of graphite as the number of walls increases. Consequently, interest in CNTs has grown rapidly and their current applications are numerous (e.g., flat-screens, sport equipments and tyres). Some others are in preparations (e.g., paints, technical clothes and pharmaceutical products). In 2002, global CNT production capacity was estimated over 2.5 metric tons per day [2]. The global market for CNTs was estimated at US$12 million for 2002 and was expected to grow up to $700 million by 2005 [2]. Therefore, it is likely that some of them will get into the environment during each step of their lifecycle (i.e., at production, use and disposal), especially in the aquatic compartment which concentrates all kinds of pollutions. Thus, CNTs must receive considerable attention as new, unknown and potentially hazardous materials. Nevertheless, there is little known about their potential ecotoxicity, especially on aquatic organisms, with only few available studies [3]. Most published results indicate that exposure to CNTs generally leads to biological disorders at different levels, usually above 10 mg/l [3].

Among these studies, some are devoted to the assessment of the *in vivo* potential effects of CNTs in amphibian larvae [4–6]. Amphibians are well-known environmental health warning organisms due to their biphasic lifecycle, permeable eggs, skin and gills [7]. Their specific physiology makes them particularly sensitive to the presence of contaminants in the water, influencing their behavior [8,9], so that they are used more and more as monitoring systems for water quality assessment [10].

Among toxic effects, genotoxicity may durably affect the aquatic ecosystems. The interaction of genotoxic compounds with DNA may initially cause structural changes in the DNA molecule. Unrepaired damage can generate other cell lesions and, thus, lead to tumor formation [11]. In amphibian larvae, 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* [7,8,12–14]. 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 [13,15–17]. This method has been standardized on *X. laevis* in French [18] and international [19] recommendations. The use of 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 into the environment, using the standardized method ISO 21427–1 in *Xenopus* larvae [18]. A previous study showed that acute toxicity was observed in *Xenopus* exposed to 10 and 50 mg/l of raw DWNTs, whereas no toxicity was observed to lower concentrations (0.1 and 1 mg/l) [6]. No genotoxicity was evidenced in these conditions. Due to the important different nature existing among kinds of CNTs, their potential toxic effect would be expected to be different and, therefore, must be investigated. The present work presents the evaluation of the potential toxicity in *Xenopus* larvae exposed in the presence of industrial MWNTs. It then proposes to compare the results of the biological effects observed in larvae exposed in the presence of DWNTs in earlier work [6]. *Xenopus* larvae were therefore exposed to MWNTs in order to evaluate three different end points on larvae after 12 days of exposure: mortality, growth inhibition and micronucleus induction in erythrocytes in the running blood as the expression of the clastogenic and/or aneugenic effect. Following this, the presence of CNTs was investigated in the larvae using traditional microscopy methods, but also by Raman spectroscopy to study their presence.

Materials & methods

Preparation of MWNT samples

Multiwalled carbon nanotubes (Graphistrength C100, France) were produced by catalytic chemical vapour deposition (CCVD) in a French facility (Arkema®) on a Fe-Al_2O_3-based supported catalyst and using a fluidized bed process. Composition is graphite (>90%, [7782–42–5]), aluminium oxide (Al_2O_3, ≤7%, [1344–28–1]) and iron oxide (Fe_2O_3, ≤5%, [1309–37–1]. They are produced available as a dry powder. Initial suspensions of MWNTs in 50 ml of water, at the final concentrations (0.1, 1, 10 and 50 mg/l), were supplied by Arkema.

Xenopus rearing & breeding

The *Xenopus* males were injected with 50 IU of Pregnant Mare's Serum Gonadotrophin (PMSG 500; Intervet, France, [9002–70–4]) and the females with 750 IU of human chorionic gonadotropin (HCG; Organon, France, [9002–61–3]) to induce spawning. 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.

Exposure conditions

The exposure was performed according to the French Standard AFNOR NF T90–325 [18] and the International Standard 21427–1 [19], in semistatic exposure conditions. *Xenopus* larvae were exposed for 12 days to 0.1, 1, 10 and 50 mg/l of MWNTs in reconstituted water (RW, distilled tap water to which nutritive salts were added [294 mg/l $CaCl_2 \cdot 2H_2O$; 123.25 mg/l $MgSO_4 \cdot 7H_2O$; 64.75 mg/l $NaHCO_3$; 5.75 mg/l KCl]). *Xenopus* exposure began on larvae at stage 50 [20]. Larvae were exposed in groups of 20 animals in crystallizing dishes containing either control media (negative controls [NCs] and positive controls [PCs]) or test media (0.1, 1, 10 and 50 mg/l of MWNTs in RW). The final suspensions of CNTs were prepared by adding 1550 ml of RW to lead to 2 l as final volume. The NC was the RW alone. The PC was cyclophosphamide monohydrate (CP, [6055–19–2], Sigma, France) in RW at 20 mg/l, which allows checking the responsiveness of the amphibian larvae. The larvae were submitted to a natural light–dark cycle at 22.0 ± 0.5°C during the 12 days of exposure. They were fed every day on dehydrated aquarium fish food.

Toxicity

Mortality of larvae exposed to CNTs was examined for 12 days according to the standardized recommendations [18,19] by visual inspection. Growth inhibition was evaluated by measuring the size of each larva (n = 20) at the beginning of the exposure (t_0) and at the end of the exposure (t_{12}) using the Mesurim image analysis software [101]. Statistical analyses were performed using SimagStat 3.1 according to nonparametric tests (Kruskal-Wallis followed by Dunn's or Dunnet's test and Mann-Whitney test) described in previous studies [5]. Graphic representations are proposed, based on the growth rate calculated, as described in these previous studies.

Genotoxicity assay

At the end of exposure, a blood sample was obtained from each anesthetized larva (MS222, Sandoz, France). Technical procedures are well described on the standardized recommendations fascicles [18,19]. The number of erythrocytes that contained one micronucleus or more (micronucleated erythrocytes [MNE]) was determined in a total sample of 1000 erythrocytes per larva. Based on median values and quartiles [21], the number of micronucleated erythrocytes per thousand, MNE ‰ is presented with their 95% confidence limits expressed by the median ± 1.57 × interquartile range (IQR; upper quartile – lower quartile)/√n. The difference between the theoretical medians of the test groups and the theoretical median of the NC group is significant to within 95% certainty if there is no overlap.

Larvae macro-observations, histological preparations for transmission electron microscopy observations

After puncturing, the general aspect of the larvae exposed to CNTs was visually compared with that of the NC group under the binocular. Larvae exposed in the presence of MWNTs were also compared with larvae exposed in the presence of DWNTs in a previous study [6]. After dissection of some larvae of each group, their guts were then observed under the binocular (magnification ×15) to observe the presence or absence of CNTs. Histological preparations from intestine and liver were realized for transmission electron microscopy (TEM) observation at the Centre de Microscopie Electronique Appliquée à la Biologie of the Medical University of Rangueil (Toulouse, France) according to the technical procedure described in the previous study [6].

Raman spectroscopy analysis

Raman spectrometry analysis (Renishaw spectrometer, green laser excitation 514.5 nm-25 mW/µm^{-2}, objective magnification of ×50, spot size ca. 3 µm diameter) was performed on contrasted ultra-thin sections. CNTs have several characteristic spectral bands. The G band is characteristic to sp2 carbon, located for graphite at 1581 cm^{-1}, a little bit higher for SWNTs, broadened by a D′ band approximately 1615 cm^{-1} for defective MWNTs. The D band is due to defects. Its spectral position, located near 1350 cm^{-1} for green excitation, is wavelength dependent. The G′2D band is always present for carbon in sp2 form and is also wavelength dependent. The G band is located at 1590 cm^{-1}, while the matrix is located closely at 1630 cm^{-1}. With MWNTs, the G and D band have approximately the same relative intensity and as the surface to volume ratio is very small, we can use G, D or G′2D band for identification of MWNTs by Raman spectroscopy. In this study, we have used both D and G bands (fitting required) to determine where the CNTs are localized. In the D-band region, no amino acid Raman signal is present, giving a very good sensitivity. As the G band is not sensitive to its surrounding, its intensity can be easily extracted and used without the cautions needed in the case of SWNTs.

Results

Physical characteristics of MWNT samples

Transmission electron microscopy observation of the raw MWNTs (Figure 1A) indicates the presence of Fe-nanoparticles (catalyst) and several

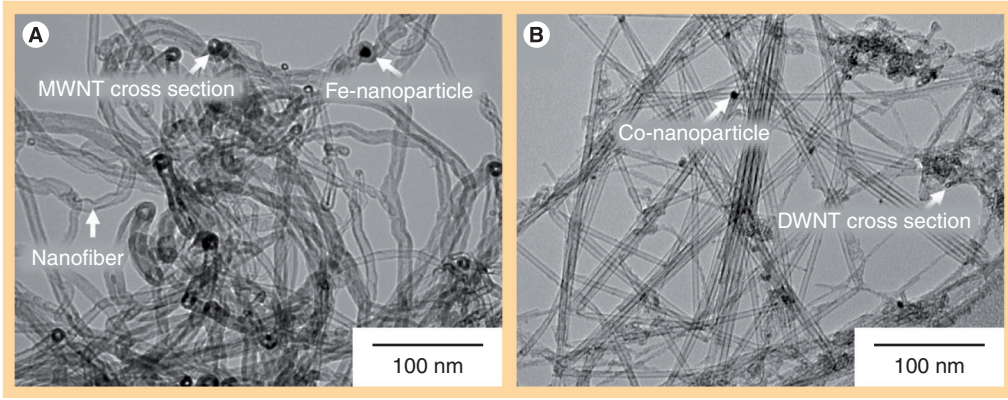

Figure 1. Representative transmission electron microscopy images of (A) raw multiwalled carbon nanotube sample (as-produced catalytic chemical vapor deposition product), and (B) double-walled carbon nanotube sample containing a very high density of carbon nanotube bundles, with extensive branching. Images are at the same magnification.
DWNT: Double-walled carbon nanotubes; MWNT: Multiwalled carbon nanotubes.

Table 1. Comparative of physical and chemical characteristics of raw multiwalled carbon nanotube and double-walled carbon nanotube samples.

Characteristic	MWNT	DWNT
Synthesis	CCVD	CCVD
Catalyst	Fe-Al_2O_3	Co/Mo-MgO
Carbon content (wt.%)	90	90
Aspect	Solid (powder)	Solid (powder)
Solubility	Indissoluble in water and others organic solvents	Indissoluble in water and others organic solvents
Number of walls	5–15 (100% MWNT)	80% of DWNT, 15% of SWNT and 5% of TWNT
Diameter	10–15 nm	1–3 nm
Length	0.1–10 µm	1 to > 100 µm (in bundles)
Specific surface area	210–260 m^2/g	980 m^2/g

DWNT: Double-walled carbon nanotube; MWNT: Multiwalled carbon nanotube; SWNT: Single-walled carbon nanotube; TWNT: Triple-walled carbon nanotube.

carbon nanofibers (CNTs with a lot of structural defects). MWNTs were prepared by CCVD in a fluidized bed with a Fe-Al_2O_3-based catalyst (Table 1). The carbon content of the MWNT sample was ca. 90 wt%, as obtained by elemental analysis. The Brunauer–Emmett Teller (BET)-specific surface area was measured between 210 and 260 m^2/g. Their diameter typically ranged between 10 and 15 nm and their length ranged from 0.1 to 10 µm. The grading for 50% of them is between 400 and 500 µm and for 0.5% less than 10 µm. The CNTs obtained have 5 to 15 walls.

Acute & chronic toxicity

The results show no mortality in larvae exposed in the presence of MWNTs (Table 2), whatever the concentration. The measurements of the larval size show that larvae exposed in the presence of 50 mg/l of MWNTs have significantly reduced size compared with the NC in a dose-dependent manner (Figure 2). Larvae exposed to 10 mg/l of MWNTs showed nonsignificant growth inhibition. Larvae exposed to 0.1 and 1 mg/l of MWNTs did not show any sign of toxicity compared with the NC.

Table 2. Results of acute toxicity (mortality) in larvae exposed to 0.1, 1, 10 and 50 mg/l of multiwalled carbon nanotubes and comparison with results of acute toxicity in larvae exposed to the same concentrations of double-walled carbon nanotubes.

CNT exposure (mg/l)	Acute toxicity (motality) (%)
MWNT	
NC	0
0.1	0
1	0
10	0
50	0
DWNT	
NC	0
GAC	0
0.1	0
1	0
10	5
50	15

CNT: Carbon nanotube; DWNT: Double-walled carbon nanotube; GAC: Gum arabic control; MWNT: Multiwalled carbon nanotube; NC: Negative control.
Data from [6].

Genotoxicity

The median value of MNE ‰ for the NC was 4 ± 1.6 (Figure 3). The PC (9.5 ± 1.49) showed significantly higher MNE ‰ as compared with the NC group. No genotoxicity via micronucleus induction in erythrocytes of *Xenopus* larvae was observed, whatever CNT concentration. The levels of MNE induced were 4, 3, 4 and 1%, respectively for 0.1, 1, 10 and 50 mg/l of MWNTs. In each group, the level of MNE induced was in the same range as the NC, whatever the concentration.

Macro-observations of larvae & dissection

Xenopus larvae exposed over 12 days to CNTs in water displayed a particular visual aspect compared with NC larvae (Figure 4). Gills showed no difference whatever the MWNT concentration, whereas intestinal tract from exposed larvae had black masses that were particularly visible through the thin peritoneal membrane of the larvae owing to their deep black color. Figure 5 shows that the proportion of black masses seemed to increase with the MWNT concentration.

TEM observations

Transmission electron microscopy observations of intestines of larvae exposed in presence of MWNTs confirmed the visual observations (Figure 6). MWNTs were observed both in the lumen and in the villi. TEM observations were

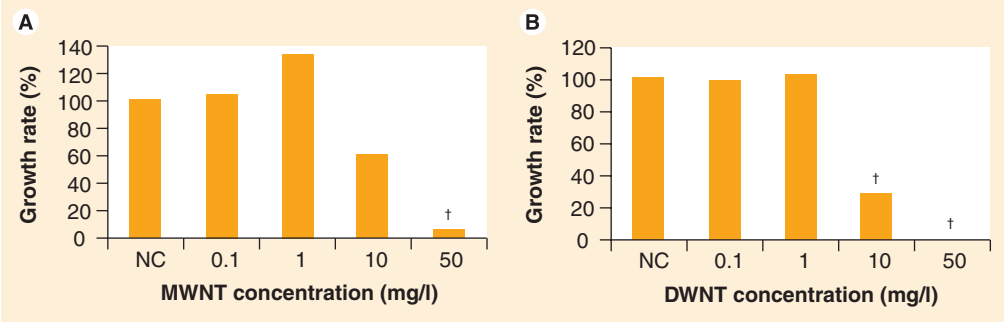

Figure 2. Chronic toxicity results in terms of growth inhibition of larvae exposed during 12 days to (A) multiwalled carbon nanotubes or (B) double-walled carbon nanotubes.
†Corresponds to a significant different size of larvae compared with the NC group (mean value).
DWNT: Double-walled carbon nanotubes; MWNT: Multiwalled carbon nanotubes; NC: Negative control.

easier than in the DWNTs owing to their higher contrast and diameter.

Raman spectroscopy analysis

Carbon nanotubes were tracked using their Raman signal. To see more clearly the variation at the frontier, scan line analysis has been performed. For each line, the G-band intensity has been plotted (which is approximately the same as D band for MWNTs, and which is more intense than D band for DWNTs) versus the position and the resulting graph has been superimposed to the optical image. Typical lines were reported on intestinal section of larvae exposed to CNTs (Figures 7 & 8). In the lumen, a high intensity of the G band for all concentrations ranging from 0.1 to 50 mg/l has been found, corroborating the visual and TEM observations, evidencing the presence of CNTs. In the intestinal barrier, the intensity of the G band was zero, thus no CNTs were localized in the barrier and beyond it. We observed no gradient of G-band intensity between the lumen and the intestinal cells, suggesting that CNTs do not cross the intestinal wall.

Discussion

The comparative TEM images of CNTs (Figure 1) show the concomitant presence of carbon-coated cobalt nanoparticles in the case of DWNT sample and of carbon-coated iron nanoparticles as catalyst by-products in the case of MWNTs. DWNTs are observed more in bundles than MWNTs. In the case of MWNTs, some carbon nanofibers are observed. DWNTs seem to be more linear than MWNTs, with less structural defects.

Both kinds of CNTs were prepared by CCVD with a Fe-Al_2O_3-based catalyst in the case of MWNTs and a Co/Mo-MgO-based one in the case of DWNTs (Table 1). The carbon content

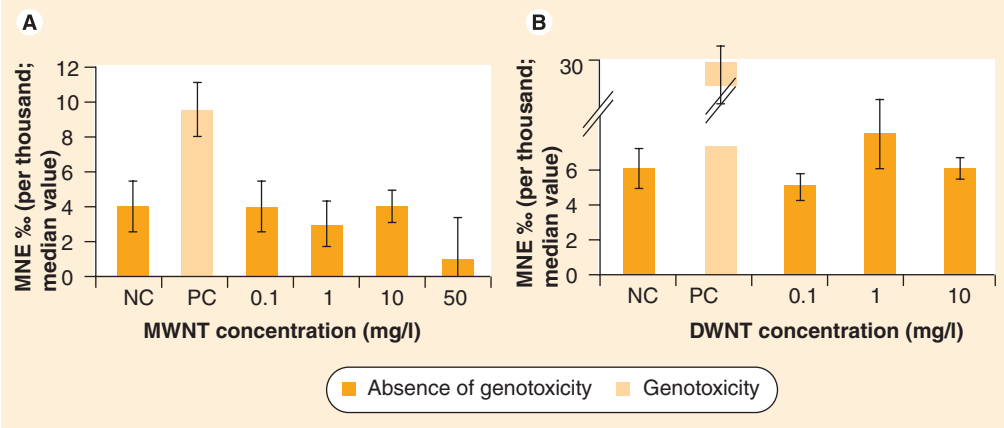

Figure 3. Results of micronucleus assay in erythrocytes of *Xenopus* larvae exposed during 12 days according to standardized conditions to (A) multiwalled carbon nanotubes or (B) double-walled carbon nanotubes.
50 mg/l of DWNT has been not explored owing to the high toxicity.
DWNT: Double-walled carbon nanotubes; MNE: Micronucleated erythrocyte; MWNT: Multiwalled carbon nanotubes; NC: Negative control; PC: Positive control.

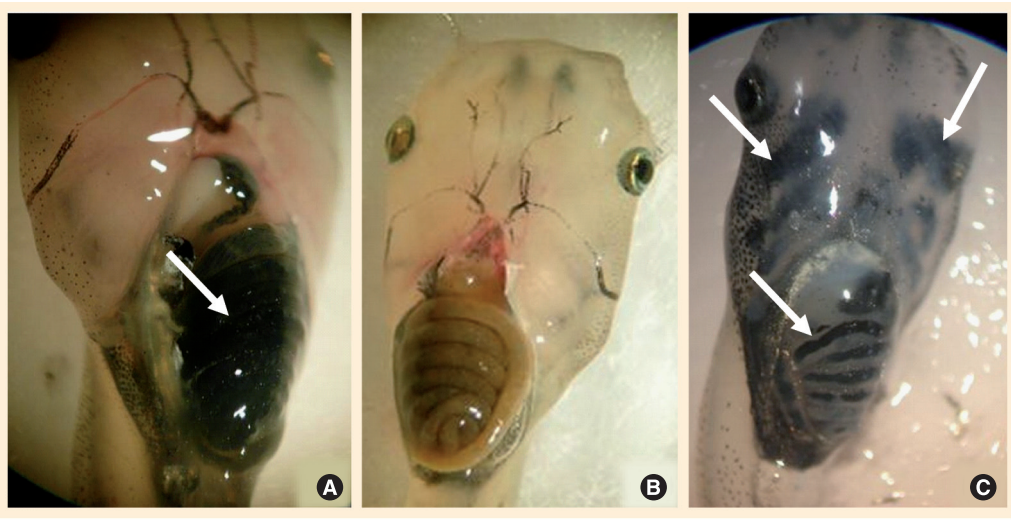

Figure 4. Localization of carbon nanotubes (CNT) in larvae exposed to 10 mg/l of multiwalled CNTs (A) or double-walled CNTs (C), in comparison to a control (exposure without CNT) larva (B). White arrows indicate the presence of CNT in gills in the case of double-walled CNT exposure **(C)** and in the intestine in the case of both CNT **(A & C)**.

of both kinds of samples was approximately 90 wt%, as obtained by elemental analysis. This corresponds to more than 97.7 mol.% of carbon, assuming that the sample of DWNTs contains mainly Co and C; the remaining Co was assumed to be present only as carbon-encapsulated nanoparticles [22–23]. The BET-specific surface area was higher for DWNTs than for MWNTs. The diameters of MWNTs are higher than those of DWNTs. The DWNTs obtained in those conditions contain approximately 80% DWNTs, together with ca. 15% single-wall carbon nanotubes and approximately 5% triple-walled carbon nanotubes, whereas the MWNTs obtained have a range of walls between 5 and 15.

Raman analysis was performed at $\lambda = 488$ nm (not shown) on DWNT and MWNT samples. Five Raman spectra were averaged for each sample. They revealed that the ratio between the intensity of the D and G bands was close to 0.1 for DWNTs, corresponding to a good structural quality of the sample. In the case of MWNTs this ratio was close to 1.4, corresponding to the presence of numerous CNT structural defects. This observation confirms our previous TEM observations where DWNTs appear to be more linear than MWNTs (Figure 1).

In the presence of MWNTs, we observed no mortality of the exposed larvae whatever the concentration; whereas low mortality was obtained in previous work for larvae exposed to 10 and 50 mg/l of DWNTs [6].

Growth inhibition was only observed in larvae to 50 mg/l of MWNTs. In the previous work [6], growth inhibition was observed from 10 mg/l of DWNTs. Therefore, acute and chronic toxicity

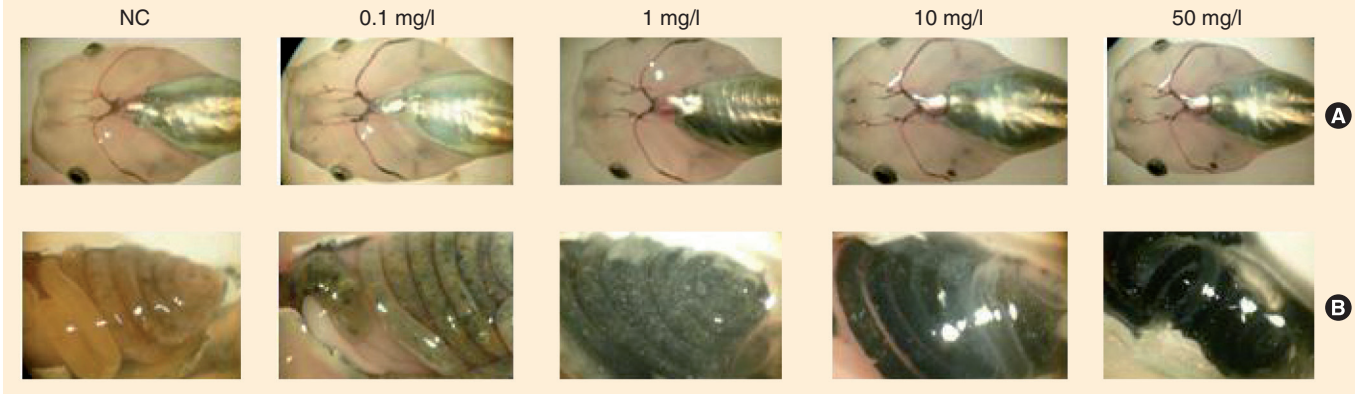

Figure 5. Comparative aspect of larvae from negative control to 50 mg/l of multiwalled carbon nanotubes, observed under binocular. Black masses increase in the gut with increasing multiwalled carbon nanotube concentration. **(A)** Body of *Xenopus* larvae showing cardiac and intestinal cavities covered by the silvery tissue, ventral view and **(B)** discovered intestinal cavity showing intestine in place under its natural curling position.
NC: Negative control.

in larvae seem to be lower in the presence of MWNTs than DWNTs in terms of concentration (mg/l), which may be due to the larger size of MWNTs or larger aggregate size, leading to more difficulties when entering into organisms. Moreover, raw (pristine, and nonfunctionalized) CNTs are inherently hydrophobic and aggregation occurs quickly in water due to strong van der Waals interactions; therefore, expected aggregation of CNTs, which was observed in water media, leads to larger particle size of CNTs, especially for MWNTs compared with DWNTs. The presence of salts in the exposure media (RW) may have contributed to aggregate CNTs. The size of aggregates of CNTs is thought to be a primary concern for toxicity.

It was difficult to compare CNT effects in terms of concentration owing to their very different morphologies. DWNTs have only two walls compared with MWNTs, which have up to 15 walls. It was possible to evaluate the mass ratio between DWNTs and MWNTs. We consider that graphene has a weight surface of 7.68 g/m^2 [24]. Specific surface area is the ratio between external surface and CNT weight, so this parameter depends only on diameter and number of walls. We have calculated that one MWNT weighs 25-times more than a DWNT. Weight comparison between DWNTs and MWNTs seems to be more relevant than a comparison in terms of concentration. Concentration could be used only in the case of comparison of a same kind of CNTs (e.g., raw DWNTs, purified DWNTs and functionalized DWNTs).

Photonic observations of larvae exposed to MWNTs indicate presence of CNTs only in the lumen and not in the gills (Figure 4), even at the highest concentration. By contrast, in the case of larvae exposed to DWNTs, black masses were observed in gills whatever the concentration. In the latter, toxicity may be mediated by branchial obstruction, potentially generating gaseous exchanges perturbations and/or anoxia. Recently, other authors demonstrated the link existing between the presence of CNTs in water and the appearance of respiratory pathologies in aquatic organisms. For instance, Smith *et al.* have shown that exposure of juvenile trout to dispersed SWNTs (prepared in presence of sodium dodecyl sulphate and assisted by a sonication step) for up to 10 days caused respiratory toxicity and gill pathologies [25]. In the case of SWNTs in the mice after intravenous administration, long-term accumulation

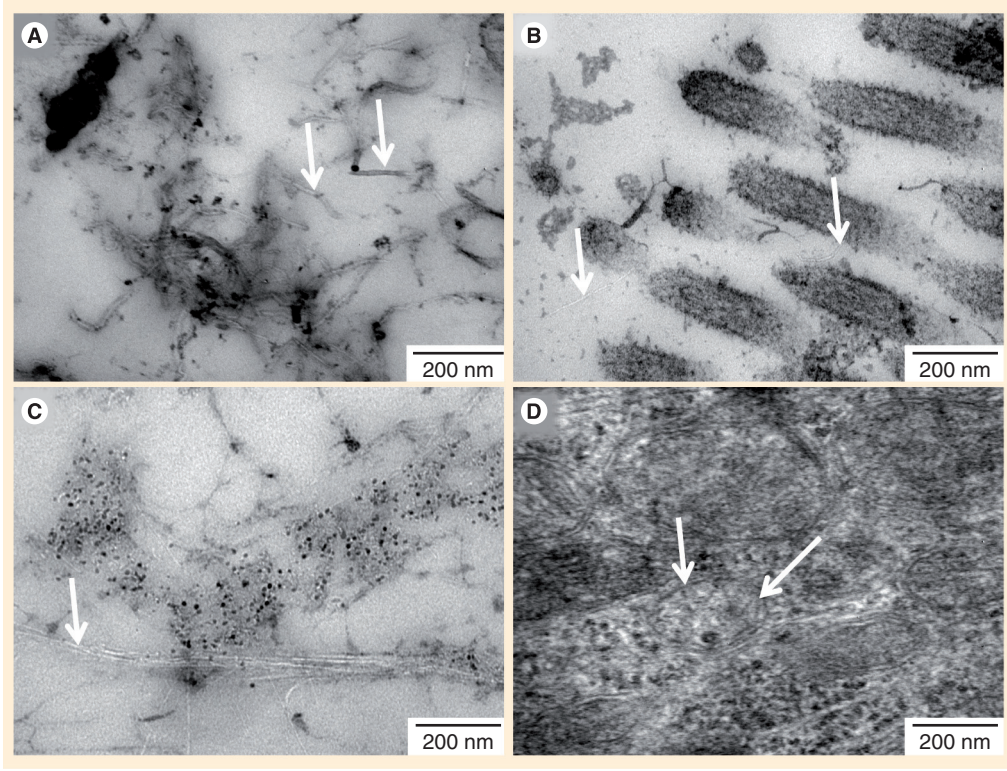

Figure 6. Transmission electron microscope images at 10 mg/l of carbon nanotubes visualized by white arrows. Multiwalled carbon nanotubes **(A)** in the lumen and **(B)** in the villi, **(C)** double-walled carbon nanotubes in the lumen and **(D)** structures in intestinal cells looking like multiwalled carbon nanotubes (white arrows).

in the main organs was evidenced by Yang *et al.* inducing slight inflammation and inflammatory cell infiltration in the lung [26]. In the case of Graphistrength C100, Muller *et al.*, showed in rat, after intratracheal administration, that CNTs persist in the lung after 60 days and induced fibrotic reactions [27]. We have found MWNTs and DWNTs in the intestines of larvae in our experiment (Figure 4). Toxicity may also be mediated by intestinal obstruction due to both kinds of CNTs ingested from the water exposure medium. In the same way, some other authors also observed absorption of CNTs in the intestine of organisms, such as trout exposed to SWNTs [25], *Daphnia* exposed to coated SWNTs [28], *Oligochaetes* exposed to ^{14}C-labelled SW and MWNTs [29], and crustaceans exposed to raw and oxidized MWNTs [30], inducing different kinds of toxicity, via inflammatory processes, for example, in trout [25] or mortality and immobilization in crustacean [29]. A competition between CNTs and nutrients could also in this case explain growth inhibition of larvae in the presence of MWNTs.

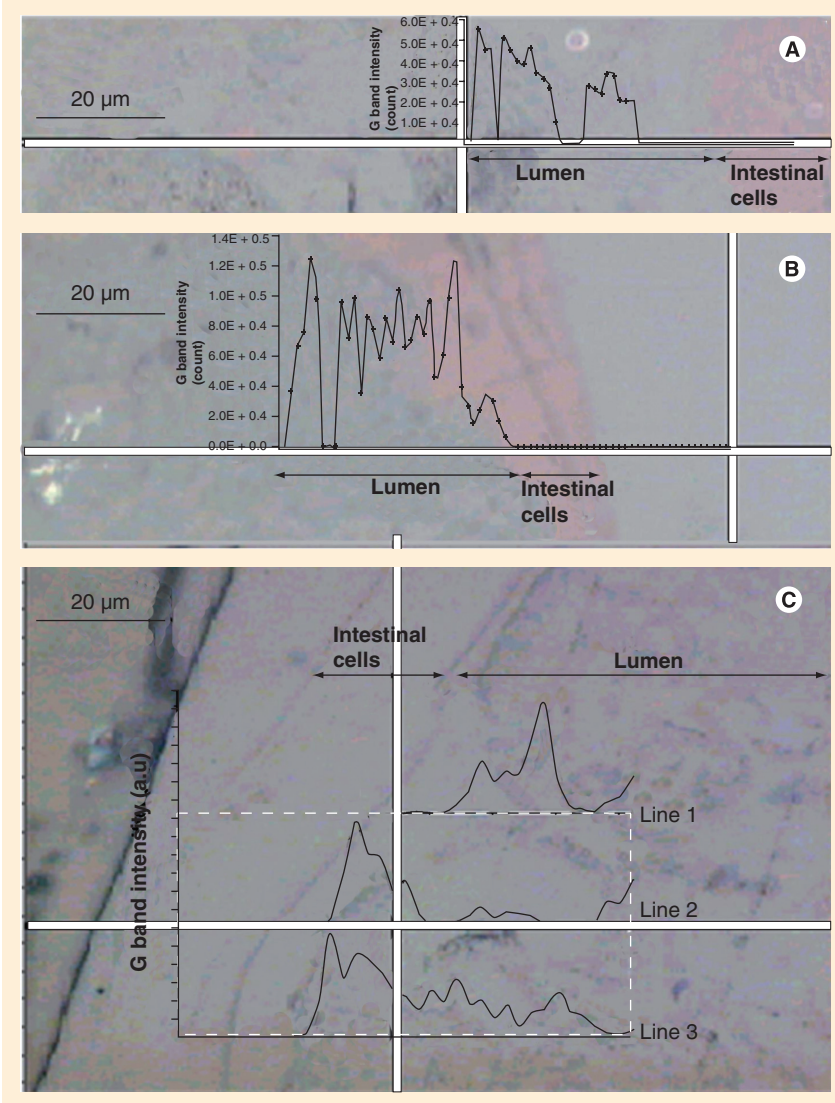

Figure 7. Superposition of Raman spectra and photonic microscopy images of the biological matrix. Raman spectra (scans line) on intestine cross section (lumen and intestinal cells) of *Xenopus* larvae exposed to **(A)** 10 mg/l of multiwalled carbon nanotubes and **(B)** 50 mg/l of multiwalled carbon nanotubes, and **(C)** 10 mg/l of double-walled carbon nanotubes.

Transmission electron microscopy observations of CNTs in the biological matrix are very different. DWNTs have a small diameter and only two walls, so they have a lower contrast than MWNTs when observed by TEM in the biological matrix. Thus, MWNTs are more easily observed than DWNTs due to their larger diameter and number of walls (Figure 6). CNTs have similar size and morphology to a lot of cellular structures, such as ribosomal structures (i.e., ~25 nm; Figure 9) [31]. Therefore, it was not possible to clearly identify CNTs in cells, if present. Thus, TEM is a restrictive technique (local and confusing) to localize CNTs in biological matrix (Figure 7), although the presence of companion carbon-encapsulated metal nanoparticles can be considered as convincing evidence, but necessitates a supplementary x-ray analysis.

Therefore, Raman spectroscopy analysis was used to identify and characterize CNTs in biological matrix. In the lumen, Raman analysis evidences the presence of both kinds of CNTs, whereas no CNTs were localized in the intestinal cells, suggesting that CNTs do not cross the intestinal barrier. Raman spectroscopy is a more realistic technique than TEM, especially owing to its sensitivity. With line scans, CNTs were also localized only in the lumen. A large mapping of the liver (well known for its accumulation capacity) has been performed with 40 × 40 spectra (Figure 8). In intestine, the MWNT signal is strong. A total of 1600 spectra were treated in liver samples to find no signal associated to CNTs. TEM observations of different tissues of the larvae (especially liver) may lead to confusion of identification. In this study, the presence of CNTs was evidenced neither in blood (results not shown), nor in the liver of amphibian using Raman spectroscopy analysis. In the same way, Tabet *et al.* demonstrate that MWNTs exert adverse effects without being internalized by human epithelial and mesothelial pulmonary cell lines [32].

No genotoxic effects via micronucleus induction were observed. This result is in agreement with those obtained previously with DWNTs

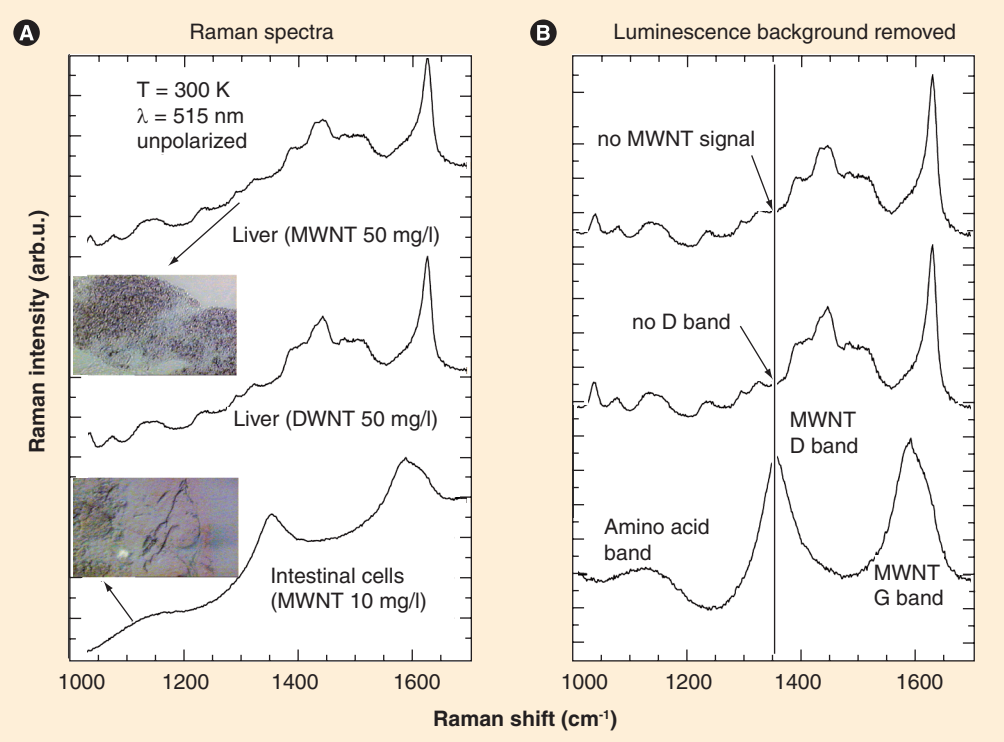

Figure 8. Average Raman spectra for three samples (two livers at the top and one intestine at the bottom) (A) without and (B) with background correction. In intestine, the MWNT signal is intense. In liver, the Raman signal is due to amino acids. No D band was observed. The signal was the same for liver with *Xenopus* in presence of DWNT or MWNT.
DWNT: Double-walled carbon nanotube; MWNT: Multiwalled carbon nanotube.

on amphibians *Ambystoma mexicanum* [4] and *X. laevis* [5]. Raw CNTs probably can not enter into the cells owing to their size due to their strong aggregation in water. Further investigations on stabilized suspensions of individual CNTs will allow evaluation of the potential biological effects of CNTs at the real nanolevel. Another possibility would be that erythrocytes are not adequate or sensitive targets to evaluate the potential genetic toxicity of CNTs and that finally, micronucleus induction is not a relevant biomarker for CNTs. Further investigations must be carried out before concluding on the absence of genetic diseases in amphibian larvae after exposure to CNTs, since genetic damage, 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 of intracellular reactive oxygen species was explained by the metal traces associated with the commercial nanotubes [33]. In our experiments, the metal particles (Co) associated to DWNTs used are supposed to be biologically inert [23], but today no data are available about the potential toxicity of Fe-particles contained in the samples.

The absence of genotoxicity reported in this work could be correlated to structurally pure CNTs employed since Fenoglio and collaborators [34], and Muller and collaborators [35] demonstrate that structural defects play an important

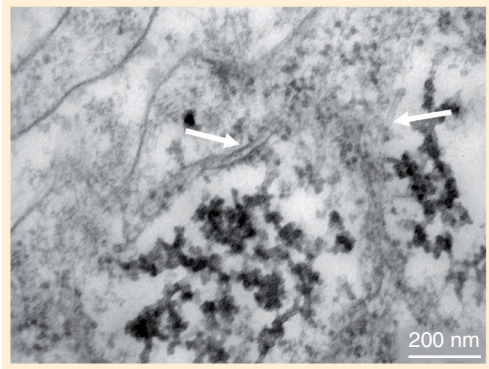

Figure 9. Observation of the liver of larvae exposed to 10 mg/l of multiwalled carbon nanotubes using transmission electron microscopy. White arrows indicate structure, which would remain multiwalled carbon nanotubes considering its size and morphology, but Raman analysis radically invalidates this observation (Raman analysis has been realized on several samples at random and not specially on this observation).

role in lung acute toxicity. However, in a previous study in amphibian larvae [6], using the same DWNTs, a genotoxic effect has been observed in the presence of addition of dispersing agent (gum arabic). These data suggest an important role in the state of dispersion of nanotubes.

Conclusion

The potential and the growing use of CNTs and their mass production have raised several questions regarding their safety and environmental impact [36]. The present work constitutes a contribution to the ecotoxicological assessment of the potential toxicity of CNTs. As CNTs are all different, this work proposes a comparative evaluation between two kinds of CNTs (DWNTs, laboratory production; and MWNTs, industrial production) on amphibians *Xenopus* larvae in standardized exposure conditions [18]. It is difficult to compare the relative toxicity of each kind of CNTs because their purities differ, different catalysts were used for their synthesis, but methods of exposure were exactly the same. Growth inhibition was observed from 10 mg/l of DWNTs and 50 mg/l of MWNTs. This toxicity could be explained by branchial and/or intestinal obstructions in the case of DWNTs and by intestinal obstructions for MWNTs because no MWNTs were localized in the gills. By contrast, no genetoxicity was evidenced in both experiments. Since both kinds of CNTs are ingested by larvae, the possibility that CNTs may be found later in the food chain cannot be excluded, once released into the environment.

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Executive summary

Physicochemical characteristics of carbon nanotube samples

- Concomitant presence of carbon-coated cobalt-nanoparticles in the case of double-walled carbon nanotubes (DWNTs) sample and of carbon-coated iron-nanoparticles as catalyst by-products in the case of multiwalled carbon nanotubes (MWNTs) are observed.

Acute & chronic toxicity of carbon nanotube in amphibians

- In the presence of MWNTs, no mortality of the exposed larvae was observed.
- Growth inhibition was only observed in larvae exposed to 50 mg/l of MWNTs.
- Acute and chronic toxicity in larvae seem to be lower in the presence of MWNTs than DWNTs in terms of concentration (mg/l). This may be due to the larger size of MWNTs or larger aggregate size, leading to more difficulties to enter into organisms. The presence of salts in the exposure media may have contributed to aggregate carbon nanotube (CNT). The size of aggregates of CNTs is thought to be a primary concern for toxicity.

Localization of CNTs in biological samples – studying methods – potential toxicity mechanisms

- Photonic observations of larvae exposed to MWNTs indicate presence of CNTs in the lumen and not in the gills. By contrast, in the case of DWNT exposure, black masses were observed in gills whatever the concentration. Toxicity may be mediated by branchial obstruction, potentially generating gaseous exchanges perturbations and/or anoxia.
- MWNTs and DWNTs were found in the intestines of larvae. Toxicity may also be mediated by intestinal obstruction due to both kinds of CNTs ingested from the water exposure medium. A competition between CNTs and nutrients could also explain growth inhibition of larvae in the presence of MWNTs.
- It was not possible to clearly identify CNTs in cells, if present. Transmission electron microscope (TEM) is a restrictive technique to localize CNTs in biological matrix although the presence of companion carbon-encapsulated metal nanoparticle can be considered as convincing evidence, but necessitates a supplementary x-ray analysis.
- Raman analysis evidences the presence of CNTs in the lumen, but not localized in the intestinal cells, suggesting that CNTs do not cross the intestinal barrier. Raman spectroscopy is a more realistic technique than TEM. TEM observations of different tissues of the larvae may lead to confusion of identification.
- The presence of CNTs was evidenced neither in blood nor in liver of amphibian using Raman spectroscopy analysis.

Potential genotoxicity of CNTs in amphibians

- No genotoxic effects via micronucleus induction were observed. Raw CNTs probably can not enter into the cells owing to their size due to their strong aggregation in water. Further investigations must be carried out before concluding on the absence of genetic diseases in amphibian larvae after exposure to CNTs, since genetic damage, such as oxidative stress, was highlighted by some authors as a potential way of CNT toxicity. The absence of genotoxicity could be related to structural pure CNTs.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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