Short term exposure to multi-walled carbon nanotubes induce oxidative stress and DNA damage in *Xenopus laevis* tadpoles

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ABSTRACT

The potential impact of Multiwalled Carbon NanoTubes (MWCNTs) was investigated on *Xenopus laevis* tadpoles exposed to 0.1, 1 and 10 mg/L. Oxidative stress was measured in entire larvae exposed and DNA damage (Comet assay) was carried out in erythrocytes of circulating blood from 2 h to 24 h according to standardized recommendations. Results showed significant H_2O_2 production when larvae were exposed to 1 mg/L and 10 mg/L of MWCNTs after 4 h and 2 h of exposure, respectively. Antioxidant enzyme activities showed significant induction of catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) from only 2 h of exposure to 10 mg/L of MWCNTs. In presence of 1 mg/L of MWCNTs, only GR and CAT activities were significantly induced at 4 h. Enzyme activities do not follow a simple dose-effect relation, but the time of induction is shortened in relation with the tested concentration. The Comet assay results showed significant DNA damages with a dose dependent response. The profiles of DNA damages show fluctuations, in course of time, which are characteristics of oxidative stress response in relation with the continuous balance between damage and compensation process.

1. Introduction

Due to their massive production in relation with their number of potential applications, the impact of carbon nanotubes (CNTs) on the environment must be taken into consideration, especially in aquatic ecosystems which are one of the major potential receptacle compartments of pollutants. Previous work indicates that CNTs may lead to hazardous effects on cells, tissues, and organisms (van der Zande et al., 2011; Zhao and Liu, 2012), including plants (Patlolla, 2013), human (Bottini et al., 2006; Monteiro-Riviere et al., 2005), mammals (Mitchell et al., 2007; Poland et al., 2008) but also aquatic organisms (Krysanov et al., 2010). It has been reported a growth inhibition of the green alga *Chlorella* sp. after 96 h of exposure to 100 mg/L of MWCNTs (Long et al., 2012). A reduced survival and growth were observed on chironomid larvae (*Chironomus dilutus*) after 12 h of exposure to MWCNTs (Mwangi et al., 2012). MWCNTs can also cause developmental toxicity, gill, liver, brain, intestine pathologies (edema, altered mucocytes, hyperplasia), respiratory toxicity and oxidative stress in Rainbow trout (*Oncorhynchus mykiss*) and Zebrafish (*Danio rerio*) according to different exposure conditions (Du et al., 2013).

Moreover, there is evidence suggesting that oxidative stress may occur in presence of CNTs, at the origin of reactive oxygen species (ROS) production (Chen and Jafvert, 2010; Petersen and Nelson, 2010; Thurnherr et al., 2011). Oxidative stress corresponds to a disturbance of the redox status of the cells and is related to an increase of ROS such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2). To prevent ROS injuries, organisms have developed various defense mechanisms in order to transform ROS into less-toxic products. The majority of these mechanisms depend on metabolic mediation of natural compounds and enzymatic antioxidant systems, among them catalase

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(CAT and EC 1.11.1.6), superoxide dismutase (SOD and EC 1.15.1.1), glutathione peroxidase (GPX and EC 1.11.1.19) and glutathione reductase (GR, EC 1.6.4.2). The increased levels of these metabolic intermediary compounds, and of antioxidant enzymes, lead to increased stress tolerance against ROS (Matés, 2000).

Over-production of ROS can lead to membrane alteration, biological macromolecule deterioration, ion leakage, lipid peroxidation and DNA-strand cleavages (Halliwell and Aruoma, 1991; Hensley et al., 2000; Nel, 2006). The DNA breaks are often measured by the Comet assay. The single cell gel electrophoresis (Comet assay) in alkaline conditions is a highly sensitive biomarker. It detects and quantifies DNA damage, such as single- and double-strand breakage and alkali-labile sites (Tice et al., 2000). These types of damage can be induced directly by the contaminant, or indirectly via oxidative stress and/or the repair processes (Tice et al., 2000).

In this study, we have focused our attention on the impact of MWCNTs using the amphibian model *Xenopus leavis*, well-known to be an environmental health warning organism due to their biphasic life cycle, their permeable eggs, skin and gills (Mouchet and Gauthier, 2013).

Preliminary studies with MWCNTs helped us to highlight the effects of chronic toxicity (growth inhibition) at low concentrations under certain experimental conditions (Mouchet et al., 2010). To better understand the mechanism of toxicity of these tiny particles and whether they are able to induce an early response, amphibian larvae were exposed for short times (2, 4, 8, 12 and 24 h) to three different concentrations of MWCNTs (0.1, 1 and 10 mg/L) to evaluate their potential toxicity in term of oxidative stress and DNA damages induction.

2. Material and methods

2.1. MWCNTs, production and characterization

MWCNTs (Graphistrength C100, Arkema France) were produced by Catalytic Chemical Vapor Deposition (CCVD) by Arkema facility (Lacq, France) on a Fe–Al_2O_3-based supported catalyst using a fluidized bed process. Composition is graphite (>90 percent, [7782-42-5]), Aluminum oxide (Al_2O_3, ≤ 7 percent, [1344-28-1]) and Iron oxide (Fe_2O_3, ≤ 5 percent, [1309-37-1]). The carbon content of the MWCNTs sample was ca. 90 wt%, as obtained by elemental analysis. The physical characteristics and Transmission Electron Microscopy (TEM) observation of the MWCNTs used in this study were previously described by Mouchet et al. (2010). According to the theory of Brunauer, Emmett and Teller (BET), the specific surface area was measured after degassing the sample in N_2 for 4 h at 120 °C and the adsorption of nitrogen gas at the temperature of liquid nitrogen (Micrometrics Flow Sorb II 2300; measurement accuracy $\approx \pm 3\%$). MWCNTs had 5 to 15 walls, their length ranged from 0.1 μm to 10 μm, and their mean agglomerate size is between 200 μm and 500 μm (laser scattering granulometer, d (v: 0.5)). Each suspension of 0.1, 1 and 10 mg/L of MWCNTs were purchased in 20 ml of deionized water.

2.2. Animals, breeding and housing

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. Each pair was then placed together in normal tap water filtered through active charcoal at 22 ± 2 °C. After 48 h, the pairs were separated and viable eggs were maintained in an aquarium containing normal tap water filtered through active charcoal (Veolia Water, France [E300CA-7003]) at 21 ± 1 °C supplemented with nutritive salt (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) (ISO, 2006), until they reached a development stage appropriate for experimentation, i.e. stage 50, (Nieuwkoop and Faber, 1956). The larvae were fed every day on dehydrated aquarium fish food (TetraPhyll®).

2.3. Exposure conditions

Exposure began on larvae at stage 50 of the Xenopus development table (Nieuwkoop and Faber, 1956) characterized by the hind lim bud, constricted at the base. For each exposure time, larvae were chosen from the same hatch to reduce inter-animal variability and were placed in Pyrex crystallizing dishes containing 2 L of either reconstituted water (RW: normal tap water filtered through active charcoal, to which were added nutritive salts (ISO, 2006) corresponding to negative control, or RW supplemented with carbon nanotubes (0.1, 1 and 10 mg/L). Before tadpoles' exposure, each CNT vial was sonicated (Bioblock 89863, typ 570 HF Freq 35 kHz) for 5 min. The larvae were submitted to a natural light–dark cycle at 22.0 ± 0.5 °C during exposure.

For enzymatic assays and biochemical assays, 50 tadpoles were exposed in the same dish for each exposure condition and time: 0.1, 1 and 10 mg/L of MWCNTs at 2, 4, 8, 12 and 24 h of exposure (15 dishes). For each exposure time, a common control was carried out (5 dishes). All along the experiments, dishes were dark covered to prevent external disturbance. At the end of each exposure time, the larvae were frozen into liquid nitrogen and stored at −80 °C. Finally, five groups of ten tadpoles were taken from each dish and analyzed separately.

For Comet assays, larvae were exposed in groups of 25 animals in 5 L glass flasks containing 2 min L of RW (negative control), methyl methanesulfonate (MMS, [66-27-3], purity ~99%, Sigma France) at 6.24 mg/L (positive control), or different concentrations of MWCNTs (0.1, 1, 10 mg/L). To avoid possible artefact due to separate migration (eight different run), a negative control was realized for each MWCNTs exposure conditions. A positive control (PC) has been performed in order to check the sensitivity of the amphibian larvae and to validate the experiments. For the Comet assays, the concentrations of MWCNTs tested were 0.1, 1 and 10 mg/L during 2, 4, 8 and 24 h. At the end of each exposure time, 5 tadpoles were randomly sampled (including controls).

2.4. Acute toxicity

Acute toxicity to the larvae exposed to 0.1, 1 and 10 mg/L of MWCNTs was examined for 2, 4, 8, 12 and 24 h by visual inspection: death, no growth (severe toxicity) and abnormal behavior/reduced size/diminished food intake (weak toxicity), according the ISO recommendations (ISO, 2006). Recording the toxicity at each experimental time in treated larvae ensured that both biochemical and Comet assays were performed under conditions that were not acutely toxic.

2.5. Biochemical assays

2.5.1. H_2O_2 evaluation

Hydrogen peroxide was measured according to Islam et al. (2008). Tadpoles were homogenized in trichloroacetic acid (TCA) 0.1% (W/V) and centrifuged at 13,500*g* for 20 min. The mixture assay contained 25% of the supernatant added to 25% of 10 mM potassium phosphate buffer (pH 7.0, Sigma) and 50% of 1 M KI (Sigma, France). Absorbance was determined at 390 nm and the content of H_2O_2 was evaluated using a standard curve under the same conditions.

2.5.2. Enzyme activities assay

Tadpoles were homogenized in ice-cold 50 mM phosphate buffer Na/K (pH 7) containing 1.15% KCl, 0.25% protease inhibitor cocktail, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.1 mM aprotinin. The homogenate was centrifuged at 15,800*g* for 20 min at 4 °C. The protein contents were determined spectrophotometrically (Helios Alfa, Thermo Electron Corporation, USA) according to Bradford (1976), using bovine serum albumin (BSA, Sigma, France) as standard. The supernatant was also used for antioxidant enzyme activity. GR activity was assayed according to Dringen and Gutterer (2002), using Glutathione Reductase Assay Kit (GRSA-1KT, Sigma, France). The reaction was monitored by following the change in at 340 nm as oxidized glutathione-dependent oxidation of NADPH for 1 min. CAT activity was estimated spectrophotometrically using Aebi method (Aebi, 1984). The decomposition of H_2O_2 was followed at 240 nm (extinction coefficient of 39.4 M^{-1} cm^{-1}) for 1 min at 25 °C. SOD activity was measured using a SOD assay kit (19160-1KT-F, Sigma, France) according to Mockett et al. (2002), based on the production of $O_2^{\cdot-}$ by xanthine–xanthine oxidase, which reduces nitroblue tetrazolium (NBT) to blue formazan. Inhibition of the reaction by SOD was monitored spectrophotometrically at 560 nm for 6 min.

2.6. Comet assay

The Comet assay, also known as the single-cell gel test (SCGE), was performed according to alkaline procedure described by Singh et al. (1988), with adaptation to *Xenopus laevis* larvae (Mouchet et al., 2005). After cardiac puncture on larvae exposed to MWCNTs (0.1, 1 and 10 mg/L) at 2, 4, 8 and 24 h, an aliquot of heparinised blood cell suspension was immediately diluted 50-fold in Phosphate buffered saline (PBS). The slides were then treated according to Mouchet et al. (2005). The electrophoresis was carried out in alkaline buffer [4 °C; 0.3 M NaOH, 1 mM Na_2EDTA (pH > 13)] for 20 min by applying and electric field of 20 V and adjusting the current to 30 mA. Five larvae were used for NC (negative control), PC (positive control) and MWCNTs conditions. Slide analysis was performed using an epi-fluorescence microscope (LSM 410 ZEISS microscope) at 400× magnification, after staining the slides with a 0.05 mM ethidium bromide solution. DNA

strand breakage was quantified as the tail DNA percentage and tail length (TL: distance between the head and the last DNA fragment) using an image-analysis system (Komet 5.5; Andor Technology®). For each individual, two slides were coded and 50 cells were randomly analyzed in each slide. The data obtained from two slides per animal were pooled for the final processing. Comets with completely fragmented DNA (hedgehog-like figures with no apparent head) that could not be measured by the image analysis system were not taken into account (Hartmann et al., 2003).

For this experiment, erythrocyte viability was determined using the Trypan blue exclusion test, with samples showing $<90\%$ viability discarded (Collins, 2002). In this way, Comet assay has not been carried out in erythrocytes of larvae exposed to 10 mg/L of MWCNTs.

2.7. ICP-MS analysis

MWCNTs (0.1, 1 and or 10 mg/L) were placed in pyrex crystallizing dishes containing 2 L of RW. Before contamination, each CNT vial was sonicated (Bioblock 89863, typ 570 HF Freq 35 KHz) for 5 min. No CNT was added for the negative control.

After 24 h, water was recovered, filtered through 0.2 μm filters (514-0061, VWR, France), acidified with 3 drops of 69% HNO_3 (309079, Sigma, France) and stored at 4 °C. Before analysis 0.05% of In/Re and 0.3% of HNO_3 at 69% were added to 10 ml of samples. Analysis of metallic trace elements (Al, Fe and MO) was performed by ICP-MS Agilent 7500ce quadruple equipped with a collision cell He. The ionization source device consists of an inductively coupled plasma generator coil (auto frequency generator adjusted 27 MHz 1550 W). The analyzer comprises a quadruple mass spectrometer for sorting ions by mass / electrical load (about 1 amu resolution). The detector is comprised of a dual mode analog and pulse counting (electron multiplier) with counting time of 0.1 ms for major elements, 0.3 ms for trace elements and 1.5 s for the ultra-trace elements. The collision cell gas (He) eliminates some of the polyatomic interference by kinetic energy discrimination (KED). To minimize interference related to oxide ions and charged doubling the reports CeO+/Ce+ and Ce++/Ce+is controlled and was, respectively, <1percent and <3percent. Typical detection limits were between 0.1 ng/kg and 100 ng/kg. Indium and rhenium were used as internal standards to correct for instrumental drift and matrix effects.

2.8. Larvae macro-observations

Macro observations of larvae have been realized on larvae used for the Comet assay (after blood sampling preventing stress on animals for comet analysis). The general aspect of the larvae exposed to MWCNTs was visually compared to that of negative control group under microscope (Olympus CX41) at X4 magnification.

2.9. Statistical analysis

Results (enzymatic, biochemical, ICP-MS, and COMET) are all presented as mean ± standard error of the mean (SEM). Comparison between group pairs is achieved with Student *t*-test. Normality and homogeneity of variances are checked by using Shapiro–Wilk and *F* tests, respectively. Data transformation is used to correct (when required) deviation from normality and to stabilize the variance. We considered power transforms which best candidates were chosen as guided by the Box–Cox transformation procedure. The significance level is 5percent ($p<0.05$). Statistical computations were achieved using R Development Core Team (2012).

3. Results

3.1. Acute toxicity

No signs of acute toxicity were observed for *Xenopus* tadpoles exposed to MWCNTs to 0.1–1 and 10 mg/L, whatever the exposure time (2, 4, 8, 12 and 24 h).

3.2. H_2O_2 production

Results obtained are represented in Table 1. Compared to the control, tadpoles exposed to 0.1 mg/L of MWCNTs showed no significant H_2O_2 production. At 1 mg/L of MWCNTs, high H_2O_2 production was observed from 4 h of exposure ($p<0.05$). This treatment showed H_2O_2 production of 3 ± 0.3 mmole/mg of fresh weight (FW), while control was 1.1 ± 0.1 mmole/mg of FW. This production remained constant until 12 h, then decreased to 1.7 ± 0.1 mmole/mg of FW at 24 h ($p<0.05$). With 10 mg/L of MWCNTs, H_2O_2 production was 2.6 ± 0.5 mmole/mg of FW after only 2 h of exposure whit control concentrations at 1.2 ± 0.3 mmole/mg of FW. The H_2O_2 production decreased until 8 h, and then rose 2.2 ± 0.1 mmole/mg of FW at 12 h to ($p<0.05$). After 24 h, H_2O_2 production diminished significantly to production very near the negative control (1.3 ± 0.1 mmole/mg of fresh weight vs. 1.3 ± 0.1 mmole/mg of fresh weight).

Table 1
Hydrogen peroxide production in *Xenopus laevis* tadpoles exposed to three different concentrations of MWCNTs.

Time	CTRL	MW 0.1	MW 1	MW 10
2	1.2 ± 0.3^a	1.9 ± 0.2^a	1.3 ± 0.3^a	2.6 ± 0.5^b
4	1.1 ± 0.1^a	0.9 ± 0.1^a	3.0 ± 0.3^b	1.3 ± 0.1^c
8	2.2 ± 0.2^a	2.0 ± 0.1^a	2.9 ± 0.1^b	2.1 ± 0.4^a
12	1.9 ± 0.1^a	1.7 ± 0.1^a	3.2 ± 0.2^b	2.2 ± 0.1^c
24	1.3 ± 0.1^a	1.4 ± 0.1^a	1.7 ± 0.1^b	1.3 ± 0.1^a

Hydrogen peroxide production in *Xenopus leavis* tadpoles exposed during 2, 4, 8, 12 and 24 h to 0.1, 1 and 10 mg/L of MWCNTs compared to negative control condition (CTRL).
Values expressed as mean ± SEM. Concentrations are in millimole/mg of fresh weight ($n=5$).
Means of the same row followed by different letters differ significantly ($P<0.05$).

3.3. Enzyme activities

Changes in the activity levels of antioxidant enzymes in *X. laevis* tadpoles in the presence of MWCNTs are shown in Fig. 1. At 0.1 mg/L of MWCNT, induction of GR activities started significantly after 8 h ($p<0.05$) of exposure to MWCNTs to reach 84.3 ± 2.3 μU/mg protein, then gradually decreases to 38.3 ± 1.6 μU/mg protein at 24 h. With 1 mg/L of MWCNTs, both GR and CAT activities were significantly induced at 4 h of exposure to MWCNTs to reach 123.3 ± 16.8 μU/mg of protein and 257.2 ± 27.1 mU/mg of protein, respectively. After 24 h, the GR and CAT activities decreased to 39.7 ± 1.7 μU/mg of protein and 73.2 ± 5.5 mU/mg of protein, respectively. When larvae were exposed to 10 mg/L of MWCNTs, all antioxidant enzyme activities (CAT, GR and SOD) were significantly induced after only 2 h of exposure to MWCNTs. In presence of carbon nanotubes in the exposure media, GR was 94.3 ± 5.3 μU/mg of protein, CAT was 147.1 ± 3.3 mU/mg of protein and SOD was 7.1 ± 0.7 U/mg of protein. The controls were 53.6 ± 7.5 μU/mg of protein, 31.7 ± 0.9 mU/mg of protein and 3.1 ± 0.3 U/mg of protein, respectively. A slow decreased was observed for all GR activities until 24 h. For CAT and SOD activities, the same fluctuations in time was observed with a significant enhancement of values at 8 h (109.9 ± 2.6 mU/mg of protein and 5.5 ± 0.2 U/mg of protein respectively) and 24 h (only significant for SOD activity).

3.4. DNA damage

Significant DNA damages were recorded after 4–24 h in larvae exposed to 0.1 mg/L of MWCNTs (Table 2), compared to the negative control, for all parameters (Tail DNA and TL). Maximum Levels of DNA damage were observed at 24 h of exposure considering Tail DNA (14.3 ± 0.7), and after 4 h considering TL (70.7 ± 1.3). In contrast, no significant DNA damage was measured after 2 and 8 h of exposure to 0.1 mg/L of MWCNTs. At 1 mg/L of MWCNTs, significant DNA damages were recorded for all exposure times and parameters, with maximal DNA damages at 24 h according the Tail DNA (34.9 ± 1.4) and TL (55.8 ± 0.9) values. Results show no significant evolution of basal DNA damage (negative control) between each experimental time exposure, nor evolution of MMS-induced DNA damage

3.5. ICP-MS analysis

The measurement of metal concentrations (Al, Fe, Mo) released in RW containing, 0.1, 1 or 10 mg/L of MWCNTs after 24 h are presented in Table 3. Results show no significant release of Al and Fe compared to the control, for all MWCNTs concentrations in RW. In the case of Mo, a statistically significant, but very low, amount is released by MWNCTs at 1 mg/L (0.5 ± 0.02 μg/L) and 10 mg/L (2.6 ± 0.04 μg/L), compared to the control (0.2 ± 0.02 μg/L).

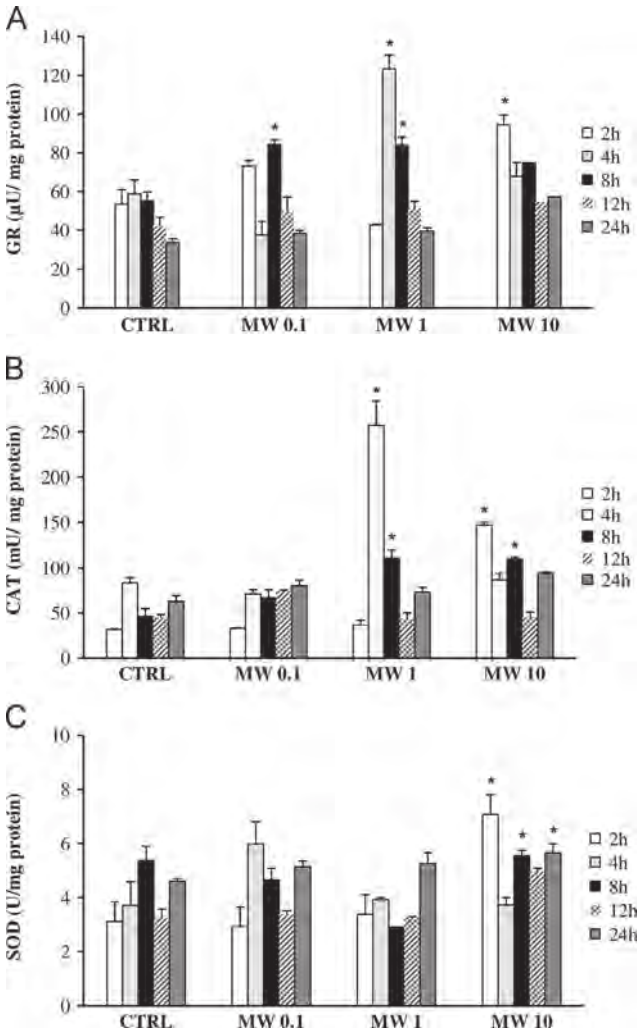

Fig. 1. Antioxidant enzymes activities in *Xenopus laevis* tadpoles exposed to different concentrations of MWCNTs. Antioxidant enzymes activities in *Xenopus laevis* tadpoles after exposure to 0.1, 1 or 10 mg/L of raw MWCNTs during 2, 4, 8, 12 and 24 h. (A) Glutathion reductase activity, (B) Catalase activity, (C) Superoxyde dismutase activity. Values expressed as mean ± SEM. * indicates a significant induction ($P < 0.05$) of antioxidant enzyme activities compared to the CTRL: Negative control, MW: MWCNTs.

3.6. Macroscopic observations

Macroscopic observations of tadpoles exposed to 0.1 and 1 mg/L during 2, 4, 8 and 24 h are shown in Figs. 2 and 3. Fig. 2 shows presence of brown masses in basket gills and intestine at 24 h of exposure in the control tadpoles. In larvae exposed to MWCNTs, black masses of carbon particles are suspected after 2 h of exposure in basket gills and intestine at 0.1 mg/L and increase with time up to 24 h. However, the agglomerated MWCNTs are not visible any more after 24 h in intestine. Larvae exposed to 1 mg/L of MWCNTs during 24 h showed the presence of agglomerated nanotubes on the surface of gills (Fig. 3D). These black spots are not to be confused with the natural pigmentation of *Xenopus laevis* tadpoles. These agglomerated MWCNTs (as black mass) are also visible under microscope on gill arches after dissection in larvae exposed for 24 h to 1 mg/L (Fig. 3E). Presence of MWNCTs can be suspected as a result of their agglomeration and their increased dimensions in agglomerates size.

4. Discussion

The result of the present study highlight an increase of the oxidative stress in *Xenopus laevis* tadpoles in presence of MWCNTs. Oxidative stress is defined as an unbalance between pro- and anti-oxidant systems. It corresponds to a disturbance of the redox status of the cells, and is related to an increase of reactive oxygen species (ROS) such as O_2^-, singlet oxygen (½O_2), hydroxyl radical. OH and hydrogen peroxide (H_2O_2). Reactive oxygen species are

Table 3
Concentrations of MWCNTs released metals in RW after 24 h.

	CTRL	MW 0.1	MW 1	MW 10
Al	30.7 ± 1.2	32.1 ± 0.3	32.4 ± 0.4	31.8 ± 0.7
Fe	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.3	0.6 ± 0.1
Mo	0.2 ± 0.02^a	0.2 ± 0.03^a	0.5 ± 0.02^b	2.6 ± 0.04^c

Concentrations of released metals after 24 h in water containing 0.1, 1 and 10 mg/L of MWCNTs, compared to negative control condition (CTRL), measured by ICP-MS. Values expressed as mean ± SEM. Concentrations are in μg/L ($n = 3$), means of the same row followed by different letters differ significantly ($P < 0.05$).
Al: aluminum, Fe: iron, Mo: molybdenum.

Table 2
Comet assay parameter variation in *Xenopus laevis* blood after exposure to MWCNTs.

Time (hours)	Measured parameter	PC	MW 0.1		MW 1	
			NC	MW	NC	MW
2	Tail DNA	22.3 ± 1.1*	7.4 ± 0.4	8.7 ± 0.5	3.1 ± 0.2	22.3 ± 1.1*
	TL	50.9 ± 0.9*	30.2 ± 0.6	35.5 ± 0.7	24.2 ± 0.4	50.9 ± 0.9*
4	Tail DNA	17.5 ± 0.8*	5.1 ± 0.2	12.4 ± 0.6*	2.5 ± 0.2	17.5 ± 0.8*
	TL	48.1 ± 0.9*	50.8 ± 1.2	70.7 ± 1.3*	30.8 ± 0.6	48.1 ± 0.9*
8	Tail DNA	14.4 ± 0.8*	5.2 ± 0.3	7.5 ± 0.4	3.1 ± 0.2	14.4 ± 0.8*
	TL	49.1 ± 0.9*	37.3 ± 0.9	35.7 ± 0.7	29.8 ± 0.6	49.1 ± 0.9*
24	Tail DNA	34.9 ± 1.4*	5.0 ± 0.2	14.3 ± 0.7*	3.2 ± 0.2	34.9 ± 1.4*
	TL	55.8 ± 1.0*	40.9 ± 0.9	44.4 ± 0.8*	31.2 ± 0.7	55.8 ± 0.9*

Comet assay results in *Xenopus laevis* blood after exposure to MWCNTs. Mean values ± SEM of Tail DNA (percentage of DNA in the tail) and TL (Tail Length) in larvae reared for 2, 4, 8, and 24 h in 0.1 mg/L (A) and 1 mg/L (B) of MWCNTs.

* Indicates increased significant ($p < 0.05$) DNA damage relative to the negative control. NC/PC: negative/positive controls. Comet assay was not carried out on larvae exposed to 10 mg/L which erythrocyte viability was inferior to 90%.

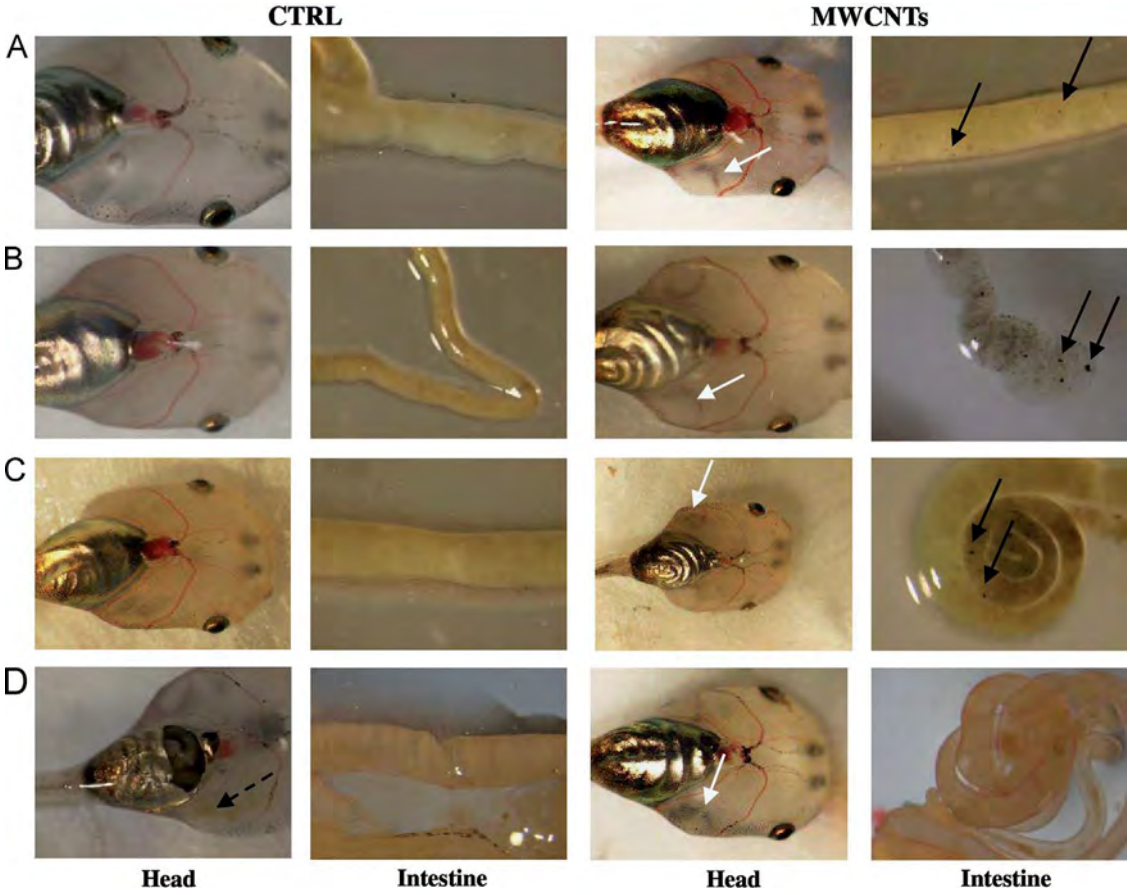

Fig. 2. Head and intestine macroscopic observations of *Xenopus laevis* larvae exposed or no to MWCNTs at 0.1 mg/L. Macroscopic observation of the Head and intestine fragment of *Xenopus* larvae after 2 (A), 4 (B), 8(C) and 24 h (D) of exposure to 0.1 mg/L of MWCNTs (MWCNT) compared to negative control larvae (CTRL). Black dotted arrow indicates the presence of brown mass of food (Tetraphyll reduced into powder) in the gill at the entire larval level in negative control condition after 24 h. White solid arrows indicate presence of dark masses of suspected agglomerated MWCNTs in gill area of MWCNT-exposed larvae. Black solid arrows show suspected agglomerated MWCNTs in the intestine after 2 h of exposure.

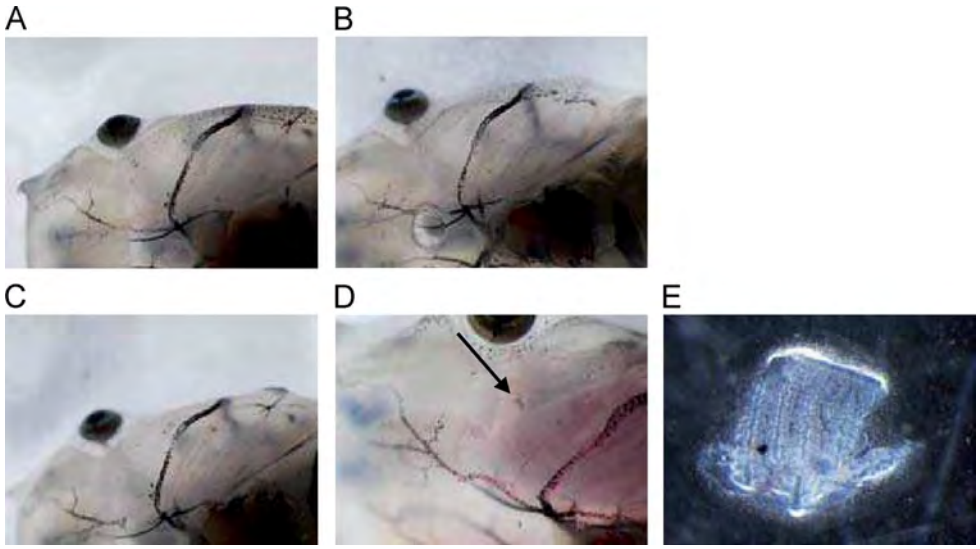

Fig. 3. Bronchial area microscopic observation of *Xenopus laevis* tadpoles exposed to 1 mg/L of MWCNTs. Macroscopic observations of branchial area of *Xenopus* larvae after 2, 4, 8 and 24 h of exposure to 1 mg/L of MWCNTs. Head macro-observation after 2, 4, 8 and 24 h (A, B, C and D) of exposure to 1 mg/L. Larvae are observed in dorsal view. Eyes are recognized by their black spherical form. Gill areas are encircled by white dots. Presence of MWCNTs on the surface of gills may be suspected at the entire larval level under binocular (black arrows) after 24 h of exposure and must be not confused with natural pigmentation of *Xenopus* larvae. (E) Gill arch macro-observation after its dissection from larvae 24 h-exposed to 1 mg/L of MWCNTs. White arrow indicates agglomerated MWCNTs (as black mass) on dissected gill tissue suggesting presence of MWCNTs in gill arcs.

short lived, unstable, and highly chemically reactive molecules, possessing unpaired valence shell electrons. Although H_2O_2 is not a radical, it is a reactive species because of its higher activity than molecular oxygen. Although all of these oxygen-based toxic species are ROS but all ROS are not oxygen radicals.

In the present experimental conditions, a significant increase of H_2O_2 was observed at 4 h, when tadpoles were exposed to 1 mg/L of MWCNTs and remained significantly different from the control until 12 h. This production decreased after 24 h. For the highest MWCNTs concentration tested (10 mg/L), H_2O_2 production was significantly increased after 2 h and decreased rapidly after 8 h. A second significant, but attenuated, production of H_2O_2 was observed after 12 h. These results indicated that (i) H_2O_2 production does not follow a simple dose-effect relation, with a lag time shortened in relation with the MWCNTs concentration and (ii) the regulation of the H_2O_2 production occurs rapidly after tadpole's exposure. ROS represent a dynamic parameter; they are continuously generated and eliminated (Lushchak, 2011). Even if ROS are toxic for organisms, they play an important role in cell signaling and regulation, particularly in cell division and apoptosis. Therefore, it is of particular importance in constant metamorphosis state of larvae as *Xenopus laevis* tadpoles (Menon and Rozman, 2007). In addition, the fast response obtained in function of the concentration is not in accordance with the intensity of H_2O_2 production; since the maximum response (3.2 ± 0.2 mmole/mg FW) of H_2O_2 was obtain in presence of 1 mg/L of MWCNTs. These results indicate that in the presence of 10 mg/L MWCNTs, the maximum was obtained before 2 h. In this case, the end of the acute oxidative stress, would be observed as defined by Lushchak (2011). In the case of in vitro studies, the increase of intracellular ROS has been explained by the metal traces associated with commercial nanotubes (Pulskamp et al., 2007). In this study the absence of significant release of Al and Fe (the metal particles associated to MWCNTs catalyze process) in exposure medium (Table 3) supposed that they are biologically inert. In contrast, very low Mo concentrations are released in water exposure with 10 mg/L of MWCNTs, but do not correspond to higher enzymatic activities in larvae. Metals from catalysers would be not responsible of oxidative stress status in the present work.

The antioxidant system includes water soluble compounds as reduced glutathione, ascorbic acid and lipid-soluble molecules as carotenoids, retinol and α-tocopherol. They generally operate as free radical scavengers but they also serve as cofactor for antioxidant enzyme such as glutathione. The most important enzymatic pathway for ROS defense are SOD that convert O_2^- into H_2O_2 and CAT which convert H_2O_2 in h_2o and O_2 (Donaldson et al., 2006). In the addition of the CAT action, GPX is involved in the mobilization of H_2O_2 by using reduced glutathione (GSH) as electron donor. This produces oxidized glutathione (GSSG) that can be reduced by the action of GR enzyme. As for H_2O_2 production, induction of antioxidant enzyme activities does not follow a simple dose-effect relation. GR activity was induced with 0.1 mg/L of MWCNTs only after 8 h. For 1 mg/L, the induction appears significant after 4 h (maximal level) and 8 h, then decreased after 24 h. For 10 mg/L, the induction was only significant after 2 h, with a rapid decrease after 4 h. In addition, SOD activity was only significant in presence of 10 mg/L of MWCNTs. Despite the high H_2O_2 production observed in tadpoles exposed to 1 mg/L of MWCNTs, the SOD activity remains low. H_2O_2 has been described to modulate the response of enzyme involved in the regulation of the oxidative stress (Lushchak, 2011). When the level of superoxide is low, the peroxidase reaction will dominate, hence regulate dismutase activity by substrate inhibition and therefore reduce the level of H_2O_2 (Gottfredsen et al., 2013). The absence of significant SOD activation in presence of low MWCNTs concentrations (0.1 and 1 mg/L) suggest different possible sources of H_2O_2 production. Several enzymes, such as glucose oxidase, uric acid or amino acid oxidase in mammalian cells, produce H_2O_2 directly (Cadenas and Davies, 2000; Szatrowski and Nathan, 1991). These enzymes are generally located in cellular organelles as peroxisomes. Monoamine oxidase present in the mitochondrial membranes is also involved in hydrogen peroxide production (Edmondson, 2014; Sandri et al., 1990).

Nanomaterials, and particularly carbon nanotubes, have been described to induce oxidative stress in different organisms and cell types (Shvedova et al., 2012). They can activate the inflammosome by interaction with phagocytes leading to ROS production. CNTs could also interfere directly with cell membranes, producing cell damages at the origin of oxidative stress. In the same way, an alteration of the mitochondrial respiratory chain could promote a rise of ROS production. In addition, Fenoglio et al. (2006) reported that MWCNTs were effective scavengers of both O_2^- and OH free radicals, regardless of how the species were generated. All these data suggest a complex interaction between MWCNTs and tadpoles disturbing the redox statute by both inducing and/or scavenging ROS.

Although it does not constitute the only genotoxic pathway, oxidative stress was described as being mainly implied in DNA-damage formation (Halliwell, 1990; Petersen and Nelson, 2010). This can induce primary damages such as oxidized base, cross-links, double- and single-strand breaks, alkali-labile sites, and excision repair sites. In this work, alkaline comet assay has been used to detect primary lesions in blood erythrocytes tadpoles. Study of erythrocytes viability (blue trypan exclusion) in larvae exposed to MWCNTs, revealed a decreased of cellular viability at 10 mg/L from 2 h of exposure, preventing the realization of the Comet assay as recommended by Collins (2002). Significant DNA damages, compared to the negative control, appear after 4 h to 24 h of larvae exposed to 0.1 mg/L of MWCNTs, whereas no DNA damages are measured after 2 h and 8 h. Moreover, in larvae exposed to 1 mg/L of MWCNTs, DNA damages are significant at all experimental times. In Table 2 comparison between mean values for both MWCNTs concentrations (0.1 and 1 mg/L) are shown with exposure time. DNA damage induction at 1 mg/L of MWCNTs is higher than that of 0.1 mg/L, suggesting an increasing dose-response, regardless parameter and exposure time. This maximal response of DNA damages to 1 mg/L would correspond to the higher registered enzymatic activity and H_2O_2 production. Significant enzymatic activities and DNA damages are not induced at the same times. This observed shift could be due to the fact that DNA damages are evaluated in erythrocytes circulating blood of larvae, whereas enzymatic activities and H_2O_2 production are measured at the entire larval level. Nevertheless, profiles of DNA damages, H_2O_2 production and enzymatic activities in course of time show fluctuations which are characteristics of oxidative stress response in relation with the continuous balance between damage and compensation process. This includes the DNA repair systems based on excision repair enzymes at the origin of DNA breaks also measured with Comet assay (Petersen and Nelson, 2010). In blood erythrocytes, DNA damages are detected earlier than enzymatic induction in entire tadpoles. In this way, Comet assay could be considered as a more sensitive tool than enzyme induction measurement to evaluate the potential effect of MWCNTs on oxidative stress. The in vivo micronucleus assay in amphibians detects fixed DNA damages persisting after at least one mitotic cycle, i.e. chromosomal and/or genomic mutations (Mouchet et al., 2005). Alkaline Comet assay detects primary DNA damages which represent reversible damages (Moretti et al., 2002; Van Goethem et al., 1997). Unlike the micronucleus assay, which highlights the cumulative effect of chronic exposure, the Comet assay provides instantaneous information about the current exposure (Maluf and Erdtmann, 2000). This method, however, does not

allow for predictive information on the possible DNA damage evolution in transmissible mutations from cell generation to another.

Macroscopic observation under binocular of tadpoles exposed to MWCNTs suggests a presence of agglomerated MWCNTs with food particles on the gills surface from 1 mg/L (Fig. 3) and into intestine (data not shown). Agglomerated MWCNTs were also visible from 0.1 mg/L into intestine after 2, 4 and 8 h in a time-dependent manner (Fig. 2) and were rapidly excreted in mass not to be more visible after 24 h. Resolution of binocular is limited and CNTs must be enough agglomerated in mass to be visible under microscope. Nevertheless, gills and intestine have already been described as entry pathway in *Xenopus laevis* larvae when exposed 12 days to MWCNTs exposure (Bourdiol et al., 2013; Mouchet et al., 2010). However, it has not been demonstrated to date, the presence of CNTs in enterocytes, hepatocytes, branchial and blood cells using specific microscopy tools such as Transmission Electronic Microscopy and Raman spectroscopy. Anyway, In *Xenopus* larvae, the pathway of contaminant entry from exposure media is double: dermal (integument and gills) and breeding exposure. Indeed, *Xenopus laevis* larvae are detritivorous (Bury and Whelan, 1984), gill-breathing, microphageous feeders, thus leading to high ingestion rates of suspended particles (Wassersug, 1975), especially particles of food susceptible to bind CNTs.

It could be hypothesized that CNTs can be adsorbed on the Xenopus larval surface and especially on gill surfaces, inducing gill clogging, at the origin of gas exchanges disturbance. Gas alterations could in turn promote larval hypoxia and oxidative stress (Lushchak, 2011; Tiedke et al., 2014) at the origin of DNA damages, and induction of antioxidant enzymes.

5. Conclusion

In conclusion, these results demonstrated that exposure of *Xenopus laevis* tadpoles to MWCNTs induced an increase of oxidative stress in entire larvae and DNA break-down in blood erythrocytes. However, the induction of oxidative stress and DNA damages appears to be closely related to the concentration of CNTs in the exposure media. Data also demonstrated that Comet assay is a more sensitive method than anti-oxidative enzyme activity. The presence of nanotubes in the gill baskets suggest a possible hypoxia that must be confirmed by additional work.

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We dedicate this publication to our friend and colleague Mr. FAUCON Bruno who has passed away on December 17, 2013.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.05.010.

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Supplementary data:

Figure 4:

TEM microphotographs of raw MWCNTs at low magnification.

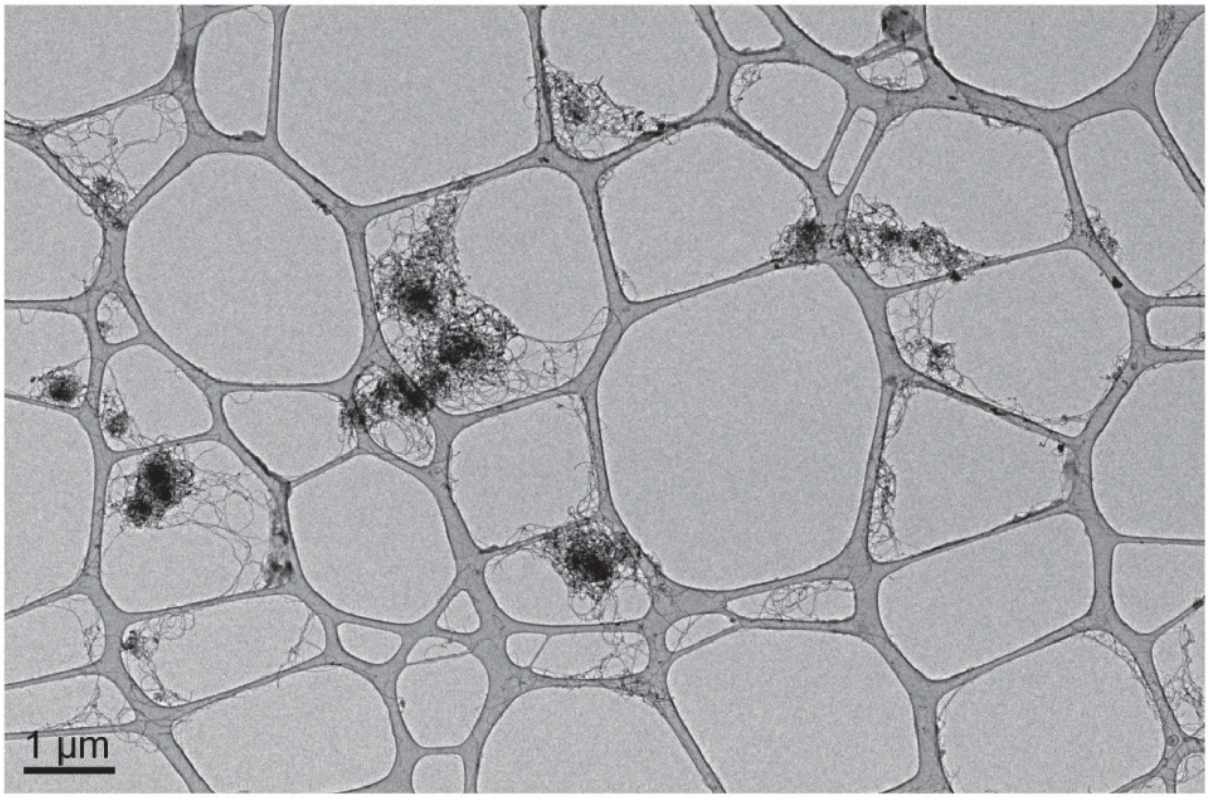

Figure 5:

TEM microphotographs of raw MWCNTs at high magnification.

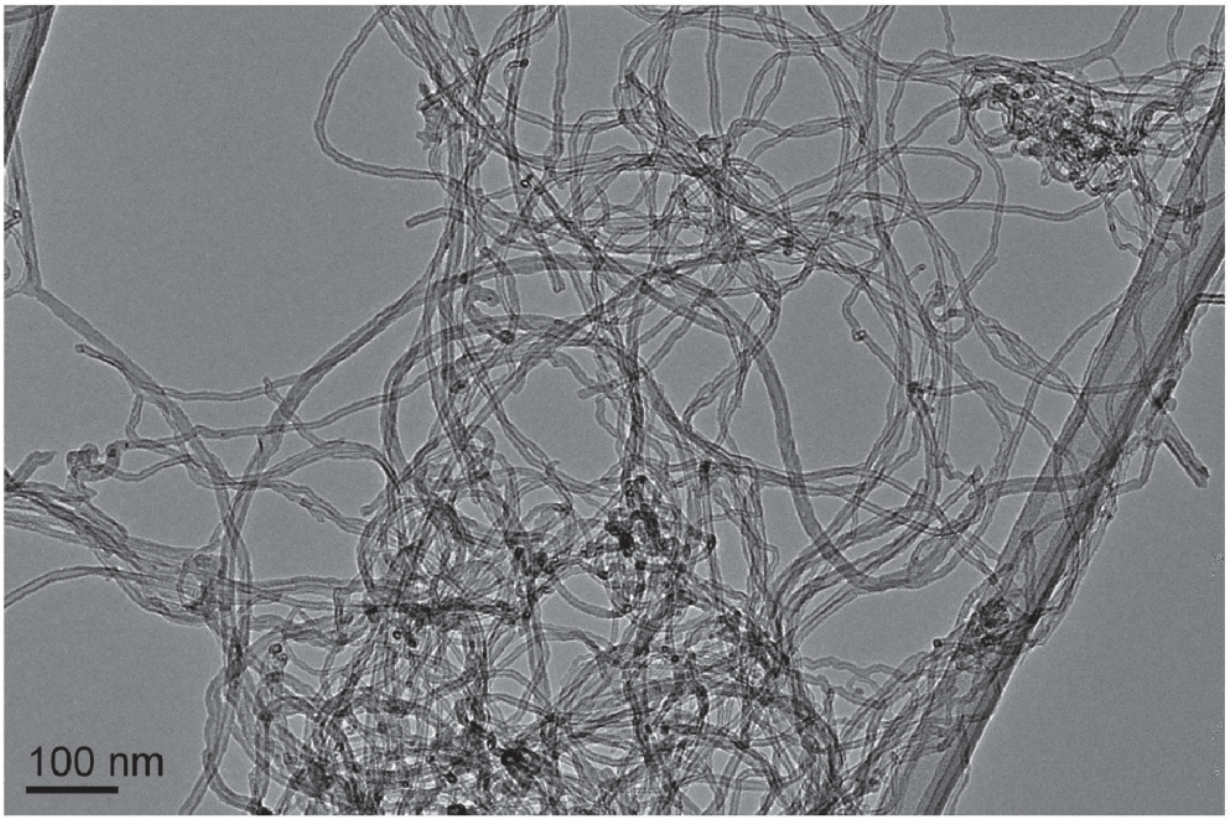

Figure 6 : Microscopic observation of Xenopus leavis DNA damages

(A) negative control and (B) larvae exposed during 4 hours to 1 mg/L of MWCNTs