Biocompatible polymer-assisted dispersion of multi walled carbon nanotubes in water, application to the investigation of their ecotoxicity using *Xenopus laevis* amphibian larvae

Floriane Bourdiol [a,c,e], *Florence Mouchet* [c,d,e], *Annie Perrault* [c,d,e], *Isabelle Fourquaux* [f], *Lucien Datas* [g], *Christian Gancet* [e,h], *Jean-Charles Boutonnet* [e,h], *Eric Pinelli* [c,d,e], *Laury Gauthier* [c,d,e], *Emmanuel Flahaut* [a,b,e,*]

[a] *Université de Toulouse, INP, UPS, Institut Carnot CIRIMAT (Centre Inter-universitaire de Recherche et d'Ingénierie des Matériaux), UMR CNRS 5085, 31062 Toulouse cedex 9, France*
[b] *CNRS, Institut Carnot CIRIMAT, F-31062 Toulouse, France*
[c] *Université de Toulouse; INP, UPS; EcoLab (Laboratoire d'Ecologie Fonctionnelle et Environnement); ENSAT, Avenue de l'Agrobiopole, 31326 Castanet Tolosan, France*
[d] *CNRS; EcoLab; 31326 Castanet Tolosan, France*
[e] *Laboratoire Commun NAUTILE (CNRS-UPS-INPT-ARKEMA France), Laboratoires EcoLab/CIRIMAT/GRL, France*
[f] *CMEAB, Centre de Microscopie Electronique Appliquée à la Biologie, Université Paul Sabatier, Faculté de Médecine Rangueil, 133 route de Narbonne, 31062 Toulouse cedex 4, France*
[g] *TEMSCAN – CIRIMAT, CNRS, Université de Toulouse, UPS, 118 route de Narbonne, 31400 Toulouse, France*
[h] *Laboratoire d'Ecotoxicologie, Département Analyse, Groupement de Recherches de Lacq, ARKEMA France, BP 34, 64170 Lacq, France*

A B S T R A C T

Carbon nanotubes (CNTs) tend to readily agglomerate and settle down in water, while the adsorption of compounds present in natural aquatic media could enhance their dispersion and stabilization in the water column. We designed a new exposure protocol to compare the biological responses of *Xenopus laevis* larvae exposed in semi-static conditions to size-reduced agglomerates of multi-walled carbon nanotubes (MWCNTs) in suspension in the water column and/or to larger agglomerates. Suspensions were prepared using a combination of a non-covalent functionalization with a non-toxic polymer (either carboxymethylcellulose, CMC, or gum arabic, GA) and mechanical dispersion methods (mainly ultrasonication). The ingestion of agglomerates which have settled down was incriminated in the disruption of the intestinal transit and the assimilation of nutrients, leading to acute and chronic toxicities at the highest tested concentrations. Rise in mortality, decrease in the growth rate and induction of genotoxicity from low concentrations (1 mg/L in the presence of CMC) were evidenced in presence of suspended MWCNTs in the water column. The biological responses seemed to be modulated when GA, a potential antioxidant, was used. We hypothesized that MWCNTs should interfere mainly at the surface of the gills, acting as a potential respiratory toxicant and generally inducing indirect effects.

* *Corresponding author.*
E-mail address: flahaut@chimie.ups-tlse.fr (E. Flahaut).

1. Introduction

Due to their nanoscale and outstanding physicochemical, electrical, mechanical, optical and thermal properties, the development of materials and applications including carbon nanotubes (CNTs) is spreading in various areas, ranging from plastics and composites markets to medicine and pharmaceutical applications. They are already integrated to sporting goods and are likely to be very soon part of our everyday life. Companies developing applications of CNTs have increasingly focused on the production of multi-walled carbon nanotubes (MWCNTs). Thus, taking in consideration the future demand, major players such as Bayer MaterialScience, Nanocyl or Arkema France, are planning to increase the global production capacity of MWCNTs, that should reach about 14,000 tons per year by 2016 [1]. Taking into consideration the entire life cycle of CNTs and CNT-containing composites (i.e. from the cradle to the grave, including their use), CNTs should be released into the environment from point sources, such as factories, landfills and wastewater effluents, but also from nonpoint sources, such as during the normal use of CNT-containing products, storm water runoff and wet deposition from the atmosphere [2], and so end-up in the aquatic compartment, well known as the main receptacle of pollutants. As well as other nanoparticles (NPs), such as nano-TiO_2 and nano-Ag, CNTs should be considered as potential emergent contaminants and deserve special attention concerning the risk assessment to aquatic organisms which could be exposed to them.

Due to their high specific surface area and aspect ratio (length to diameter ratio, L/D), raw CNTs tend to form large ropes where they are tightly bound by van der Waals interaction existing between tubes, but also to form larger agglomerates, and actually settle down in deionised water (DW). Furthermore, the presence of monovalent (i.e. Na^+) and especially divalent cations (i.e. Ca^{2+} and Mg^{2+}) in aqueous media facilitate this phenomenon by suppressing electrostatic repulsion [3]. On the contrary, it has been shown that the adsorption of natural organic matter (NOM) or/and surfactants present in natural systems onto raw CNTs effectively covers their initial hydrophobic surface, but also that their negative surface charge and their large molecular size are respectively responsible for charge repulsion and steric hindrance [4]. This results in the enhancement of the dispersibility and the stabilization of CNTs at environmentally relevant concentrations [3], depending on water physico-chemical properties (i.e. pH, ionic strength, total organic carbon content, salt concentration). Saleh et al. [3] concluded that raw MWCNTs are relatively stable at pH and electrolyte conditions typical of aquatic environment. Due to the fact that individual suspended CNTs are more mobile, and that the formation of agglomerates followed by the settling down of CNTs initiates partitioning, the fate and the transport of CNTs would be largely influenced by the presence in the water column of dispersing compounds. Moreover, Schwyzer et al. [5] reported that the dispersion state of the CNTs during the release is crucial for their environmental fate. Indeed they compared the stability of CNT suspensions (50 mg/L) in the presence of NOM (20 mg/L) or artificial surfactant (from 20 mg/L to 5 g/L) over several days of horizontal shaking (to simulate natural conditions). On the one hand, when dry CNT powder was directly added to the NOM or surfactant solutions, 20 days of shaking were not enough to separate and effectively suspend agglomerated raw CNTs. On the other hand, when small volumes of pre-dispersed CNT stock suspensions (produced in the presence of a dispersant such as sodium dodecyl sulphate (SDS)) were added into the same solutions, shaking the CNT suspensions in the same conditions allowed stabilizing up to 65% of the added CNTs after 5 days of sedimentation. They concluded that for raw CNTs the sediment is likely to be the major sink and only minor amounts could be present as suspended particles, while for stabilized CNTs the water column and the sediment could comparatively be affected. The enhanced stability resulting from the adsorption of NOM is likely to lead to an increased residence time in the water column and increased exposure times for pelagic organisms [6]. Thus, from an environmental point of view, and more precisely regarding the evaluation of the toxicity of MWCNTs with aquatic organisms, such as the amphibian model *Xenopus laevis*, it is very relevant to compare their exposure to both raw and stabilized CNTs in laboratory conditions.

There are two distinct approaches for dispersing CNTs which are often combined to obtain stable and homogeneous suspensions of well-individualised CNTs. Indeed, mechanical methods such as sonication and high shear mixing debundle partially or totally CNTs but could also fragment them, thus decreasing their aspect ratio [7], while surface functionalization of CNTs is designed to alter their surface energy. There are two ways of functionalizations: the covalent route, which consists in the grafting of functional groups at the surface (mainly through acid treatments), and the non-covalent one, which involves the adsorption of a surfactant (i.e. amphiphilic molecules) or a polymer on the surface of CNTs. In our context, the non-covalent functionalization is more relevant than the covalent one because the π-system of the CNTs is not disturbed, and thus this method preserves their intrinsic properties, which is important to correctly and realistically evaluate their potential toxic effect.

In order to maximise the exposure to individual CNTs/bundles and to limit agglomeration, it is required to prepare homogeneous and stable suspensions of MWCNTs. A combination of mechanical dispersion methods and a non-covalent functionalization via a dispersing agent was chosen. Two water soluble anionic (i.e. negatively charged) polymers were compared: sodium carboxymethylcellulose (CMC) and gum arabic (GA). They have been selected because they are non-toxic to organisms [8], colourless, and more generally because they are best suited (cheap, mass-produced, water-processable and safe) and widely used in many industrial products, and are thus likely to be present in the aquatic compartment [8].

CMC (E-number 466) is an etherified derivate of natural cellulose (i.e. made by swelling cellulose with NaOH and then reacting it with monochloroacetic acid), widely used in food industry, but also for cosmetic and pharmaceutical applications (e.g. creams, lotions, toothpaste formulations), for its good binding, thickening, suspending and stabilizing properties. Since its polymeric structure acts as a film-forming agent, it is also used to improve moisturizing effects. It plays

an important role in textile industry as a coating agent, in resins, emulsion paints, adhesive and printing inks, and in coating colours for the pulp and paper industry.

GA (E-number 414), also called acacia gum, is a natural, edible, gummy complex polysaccharide. It is defined by the FAO/WHO Joint Expert Committee for Food Additives (JECFA) as "a dried exudation obtained from the stems and branches of *Acacia senegal* (L.) Willdenow or *Acacia seyal* (fam. *Leguminosae*)" [9]. GA consists mainly of high-molecular weight heteropolysaccharides and their calcium, magnesium, and potassium salts. Size exclusion chromatography coupled with multi-angle light scattering and refractometry allowed to isolate three major molecular species [10]: an arabinogalactan (AG or AraG; *ca*. 90% of total mass), an arabinogalactan-protein (AGP or AraGP; *ca*. 10% of total mass) and a glycloprotein (GP). AGP provides excellent interfacial properties for GA, which are attributed to 'wattle blossom-type' structure, in which hydrophilic carbohydrate blocks are linked to common hydrophobic polypeptide chain [11]. This structure contributes to the gum amphiphilicity, that favours its absorption to air/water or oil/water surfaces [12] and confers it good emulsification characteristics [13]. Also, the great flexibility of AG structure allows these molecules to be easily deformed at interfaces [14]. Therefore, GA is mainly used as emulsifier/stabilizer. It is among the oldest and commercially-well established anionic polysaccharides. The Ancient Egyptians commonly used it more than 4000 years ago as an adhesive for mineral pigments in paints, as a fixative for ink, as a binder in cosmetics, and as an agent in the mummification process, but also as a pain reliever base [15]. Nowadays, it is used as an emulsifier, stabilizer, thickener, carrier, bulking and glazing agent [9]. It is primarily employed in the food industry (e.g. in soft drinks, syrups, gummy candies and marshmallows) for its nutritional and surface properties [16], but also in the textile, pottery, lithography, explosive, cosmetics and pharmaceutical industries (e.g. microencapsulation or complex coacervation processes) [17]. Finally, in folk medicine, GA is recommended for the treatment of both internal and external inflammation (respectively intestinal mucosa and surfaces). Recent toxicological studies have investigated the antioxidative properties of GA and reported its protective effect against cardiotoxicity [18], hepatotoxicity [19] and nephrotoxicity [20] induced in mice, at least partly through inhibition of the production of oxygen free radicals.

In order to adapt current methodology applied to assess the potential ecotoxicity of chemical contaminants with the amphibian model *X. laevis* to those of CNTs, we have designed a new protocol to prepare MWCNT exposure medium characterized by a different initial dispersion state. Larvae were exposed to a range of concentration of raw MWCNT suspensions prepared by mechanical dispersion, but also of chemically stabilized MWCNT suspensions obtained by a combined mechanical dispersion and non-covalent functionalization with a biocompatible anionic polymer (CMC or GA). Physical and chemical properties such as the length of CNTs [21], the presence of structural defects [22], and the dispersion state of CNTs [21] were reported to play a role in the toxicity of CNTs. In an attempt to explain the observed effects and their potential relation to these parameters, we have used various methods to characterize the suspensions before and during the biological assays, as well as multi-scale biological observations.

2. Materials and methods

2.1. MWCNTs and dispersing agents

MWCNTs (Graphistrength batch 09215) were produced by catalytic chemical vapour deposition (CCVD) by Arkema France, using a fluidized bed process. As a precaution for safer work conditions, MWCNTs were not supplied as dry powder but as a suspension at 10 g/L in deionised water, which was used to prepare the range of exposure concentrations (0.1, 1, 10 and 50 mg/L) of raw or stabilized CNTs. The carbon content of dried MWCNTs was measured by flash combustion (heating up to 1000 °C during about 1 s, after preheating at 925 °C; measurement accuracy ≈ ±2%). The metal content (i.e. catalyst residues) was determined by Atomic Absorption Spectroscopy (AAS, measurement accuracy ≈ ±0.1%). According to the theory of Brunauer, Emmett and Teller (BET), the specific surface area was measured after degassing the sample for 4 h at 120 °C in N_2 and adsorption of nitrogen gas at the temperature of liquid nitrogen (Micrometrics Flow Sorb II 2300; measurement accuracy ≈ ±3%). According to the supplier, MWCNTs had 5–15 walls, their length ranged from 0.1 to 10 μm, and their mean agglomerate size ranged between 200 and 500 μm (laser scattering granulometer, $d(v; 0.5)$).

CMC ([9004-32-4]; carboxymethylcellulose sodium salt) was supplied by Fluka (Sigma Aldrich). This complex polysaccharide is characterized by an ultra low viscosity (15–50 mPa s), a nominal molecular weight ranging between ca. 15–50 kDa, a degree of polymerization of 60–90, a degree of substitution (DS) of 0.60–0.95 (i.e. 6–9.5 carboxymethyl groups per 10 anhydrous units), a density of 1.59 g/cm^3 and a pH ranging from 5.5 to 8.5 (10 mg/mL in water). GA [9000-01-5] was supplied by Sigma Aldrich. Compared to CMC, GA is characterized by a nominal molecular weight of ca. 250 kDa, a density of 1.35 g/cm^3, and an amount of insoluble residue ⩽0.20%.

2.2. Xenopus rearing and breeding

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. Twenty-four hours later, the pairs were separated and viable eggs were maintained in an aquarium also containing normal tap water filtered through active charcoal at 20–22 °C, until they reached a development stage appropriate for experimentation (i.e. stage 50; [23]). The larvae were fed every day on dehydrated aquarium fish food.

2.3. Exposure conditions

The micronucleus test (MNT) was performed according to the International Standard 21427-1 [24]. Larvae were exposed, during 12 days, to different conditions including (i) control conditions (negative control [NC] and positive control [PC])

which allow checking the responsiveness of the amphibian larvae (strain control), and (ii) test media composed of the different MWCNT concentrations ranging from 0.1 to 50 mg/L, corresponding to weak and potential accidental ones. The NC was composed of reconstituted water (RW, distilled tap water to which nutritive salts were added [294 mg/L $CaCl_2$, $2H_2O$; 123.25 mg/L $MgSO_4$, $7H_2O$; 64.75 mg/L $NaHCO_3$; 5.75 mg/L KCl]), whereas the PC was composed of cyclophosphamide monohydrate (CP, [6055-19-2], Sigma, France) in RW at 20 mg/L. As the influence of the dispersion state of MWCNTs was investigated, larvae were exposed to raw MWCNTs (MWCNTs in RW) and stabilized MWCNTs in RW in the presence of CMC or GA, depending on the exposure protocol used. In order to check their potential toxicity against *X. laevis*, the organisms were also exposed to the anionic polymer alone dispersed in RW (CMC control [CMCC] and GA control [GAC]). To limit the number of dispersing agent control conditions to only one, MWCNTs were dispersed at the same concentration of dispersing agent (i.e. 50 mg/L), which is the effective concentration to correctly disperse CNTs at the maximum concentration used in our experiments (i.e. 50 mg/L of MWCNTs).

Two experiments were conducted on separated *X. laevis* hatches. In the first one raw and CMC-stabilized MWCNTs were tested simultaneously (experiments I and II), while in the second experiment only GA-stabilized MWCNTs were tested (experiment III). Larvae were submitted to a natural light–dark cycle at 22.0 ± 0.5 °C. They were exposed in groups of 20 animals in crystallizing dishes which contained either a control solution (NC or PC or CMCC or GAC) or a test suspension (0.1 mg/L or 1 mg/L or 10 mg/L or 50 mg/L of raw or dispersed MWNTs). Every 24 h during the exposures the larvae were removed and placed in fresh control solutions or fresh test suspensions (to ensure exposure consistency) [24]. Concerning the replacement of test and dispersing agent control media, 20 mL test tubes (TT) containing the appropriate amount of MWCNTs and/or dispersing agent were beforehand prepared according to the dispersion protocol presented in Fig. 1 and described below. Therefore, everyday, the media were replaced by transferring the content of the test tubes into the corresponding crystallizing dishes before adjusting the final volume to 2 L with RW, reintegrating larvae and feeding them with dehydrated aquarium fish food.

2.4. *Preparation of raw and stabilized MWCNT suspensions*

The minimal but effective dispersing agent concentration, and the ratio between MWCNTs and dispersing agent of stocks suspensions were investigated aiming at simplifying the dispersion protocol applied earlier by our team for the assessment of the potential ecotoxicity of GA-stabilized Double-walled carbon nanotubes (DWNTs; [25]), without decreasing its effectiveness. Studies related to the evaluation of the ecotoxicity of DWNTs dispersed with CMC were achieved in parallel to the present work. They showed that 10 mg/L of dispersing agent was not enough to stabilize 50 mg/L of DWNTs in the absence of organism and food (data not shown), but 50 mg/L of CMC (0.005%) turned out to be an effective concentration (the initial DWNT concentration and the average one for a 24 h period was respectively estimated at 43.31 mg/L and 42.836 ± 0.429 mg/L). This concentration was thus chosen to disperse MWCNTs whatever the exposure concentration (i.e. from 0.1 to 50 mg/L).

The dispersion protocol applied to prepare the test tubes containing a given amount of MWCNTs and/or dispersing agent for larvae exposure in the MNT is presented in Fig. 1. A mechanical dispersion with a rotor–stator homogeniser (Ultra-Turrax DI 25 Basic (UT), 50/60 Hz, 600 W, 9500 rpm, 10 min) and a tip sonicator (Vibra Cell 75042, 20 kHz, 500 W, 40% power with 5 s on/5 s off pulse, 30 min) was used to prepare the raw MWCNT test tubes, while the non-covalent functionalization with a dispersing agent (CMC or GA) was combined with the mechanical dispersion to prepare the stabilized MWCNT test tubes (as well as dispersing agent control test tubes). The question of the possible shortening of MWCNTs during this process will be discussed later. Thus, the first step of this dispersion protocol consists in preparing a suspension which contains only MWCNTs or both MWCNTs and the dispersing agent in the same weight ratio (MWCNTs were added after the total dissolution of the dispersing agent). This first stock suspension (SS1; concentration of MWCNTs and/or dispersing agent of 5 g/L) was dispersed with the UT and then with the tip sonicator. The test tubes called "TT10" and "TT50" were prepared by sampling, with graduated glass pipettes, under constant sonication, the corresponding volume of SS1. These test tubes will be used to renew the exposure media corresponding to the higher MWCNT concentrations (i.e. respectively 10 and 50 mg/L). To prepare the stock suspension 2 (SS2), 15 mL of SS1 (sampling under constant sonication) was transferred in DW to adjust the concentration of MWCNTs and/or dispersing agent to 200 mg/L. SS2 was sonicated using the tip sonicator (same conditions as the sonication of SS1) before preparing the test tubes "TT0.1" and "TT1" corresponding to the lower MWCNT concentrations. When MNT included the CMCC and GAC, a stock suspension of CMC or GA was prepared and followed the same dispersion protocol applied to SS1 in order to prepare the test tubes "TTCMC" or "TTGA". The volume of the test tubes (except "TT50", "TTCMC" and "TTGA") was adjusted to 20 mL with DW or a solution of dispersing agent. New exposure media were daily prepared by sonicating during 10 min one test tube per condition using an ultrasonication water bath (Bioblock T570, 35 kHz, 160 W) and transferring the content into the crystallizing dishes before simply adjusting the volume to 2 L with RW. No additional stirring step was required.

2.5. *Characterization of raw and stabilized MWCNT suspensions*

The effectiveness of the protocol and the dispersing agent concentration chosen (50 mg/L) was checked by regularly examining the stability (and so the agglomeration and settling down) of MWCNTs at the maximal concentration, without introducing neither larvae nor food (NLNF). Besides MWCNT dispersion state characterization of the test media was achieved during the MNT to investigate the influence of the presence of larvae and food. As larvae were exposed daily

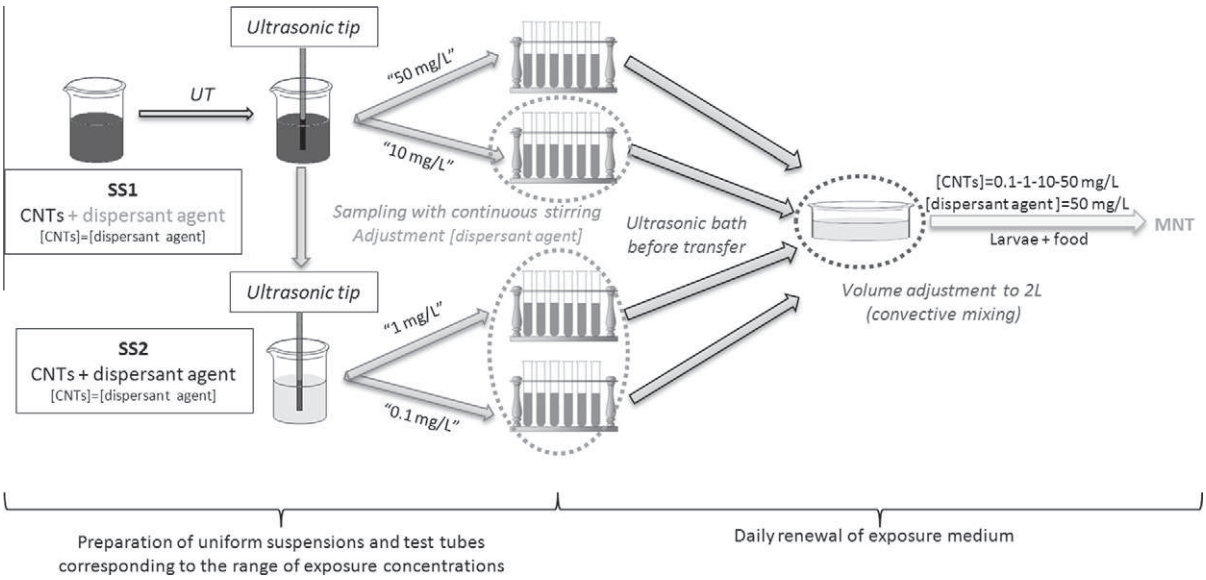

Fig. 1 – Preparation of test tubes corresponding to the range of raw and stabilized MWCNT concentrations and renewal of exposure media related to the micronucleus assay. UT, Ultra-Turrax (rotor–stator homogeniser); SS1, stock suspension 1; SS2, stock suspension 2; TT0.1, TT1, TT10, TT50: test tubes prepared to renew exposure media where MWCNT final concentration is respectively 0.1, 1, 10 and 50 mg/L; MNT, micronucleus test.

to (fresh) concentrations, each monitoring was achieved only during 24 h. A more detailed study was achieved in the case of MWCNTs dispersed with CMC. It included a monitoring of the MWCNTs in the entire range of exposure conditions in presence of larvae but absence of food and vice versa. To monitor the agglomeration *vs.* time, a regular visual examination of raw and stabilized MWCNT suspensions was performed and completed by quantifying the optical density (OD; Perkin Elmer Lambda 2 Spectrophotometer; $\lambda = 550$ nm) of 3 mL water column samples taken at half height. The MWCNT concentration of each sample was deduced from the average of three successive measurements using a calibration curve which was obtained by the OD measurement of calibration suspensions of known raw or stabilized MWCNT concentrations ranging from 0 (i.e. respectively DW or dispersing agent suspension at 50 mg/L) to 60 mg/L and prepared according to the same dispersion protocol applied to the test tubes. Before the OD measurement, calibration suspensions and samples were placed into an ultrasonic bath during 5 min. The data were background corrected and the deviation during the measurement of each batch was regularly checked by controlling the absorption signal of DW or the solution of dispersing agent in DW.

Transmission electron microscopy (TEM) observations and Raman spectra of (i) raw and stabilized MWCNT suspensions, but also of (ii) starting material (i.e. non-sonicated) and MWCNTs after a long period of sonication (tip sonicator; 2 h) were performed with a JEOL JEM-1400 (120 kV). CNTs called "non-sonicated MWCNTs", "mechanical dispersion" and "long-time sonication" were sonicated in ethanol for 5 min (as it is usually done), whereas raw and stabilized MWCNT suspension were directly sonicated (in water) to avoid their alteration. Then a drop of the resulting suspension (whatever its nature) was placed over a holey copper grid. Images were recorded on a CCD (Coupled Charge Device) camera.

Raman spectra of MWCNTs were recorded on a Horiba Jobin Yvon LabRAM HR800 Raman micro-spectrometer at 633 nm (red laser excitation, He/Ne), equipped with a thermoelectrically cooled CCD. Five spectra were averaged for each sample, after baseline correction, and the D-bands were normalized with the G-band intensity of the corresponding spectra.

2.6. *Toxicity measurements*

Acute toxicity (mortality rate, %) of larvae exposed to CNTs was examined for 12 days according to the standardized recommendations [24] by visual inspection.

Chronic toxicity (potential growth inhibition compared to the NC) was evaluated after 12 days of exposure. Larvae were anesthetized (MS222, Sandoz, France) before the beginning (*d*0) and at the end (*d*12) of the exposure in order to measure the size of each larva using the Mesurim image analysis software [26]. Statistical analyses were performed using SigmaStat 3.5. First of all, the homogeneity of the larvae size was verified at the beginning of the test. A one way analysis of variance (ANOVA) or a non-parametric Kruskal–Wallis analysis of variance by ranks (if all the data were not drawn from normally distributed populations with the same standard deviations) was applied to the *d*0-data. It revealed that there was not a statistically significant difference between the groups. Then the effect on the growth of larvae of the different test conditions was evaluated at the end of the test. Treated and controlled groups were compared using ANOVA or a Kruskal–Wallis test, followed respectively by a Dunnet's test or a

Dunn's test when the analysis of variance revealed a statistically significant difference. Finally, for each experimental condition, the growth rate of larvae (r, %) was calculated with Eq. (1), from the average length (μ) of the selected larvae ($d0$, 20 larvae per condition), and the survival larvae ($d12$), which was estimated for each condition (μX) including the NC (μNC). Graphic representations are proposed, based on the calculated growth rates.

$$r = \frac{(\mu X_{d12} - \mu X_{d0}) - (\mu NC_{d12} - \mu NC_{d0})}{\mu NC_{d12} - \mu NC_{d0}} \times 100 \quad (1)$$

Genotoxicity was evaluated on each larva after intracardiac puncture under a binocular at the end of the exposure time. A blood sample was obtained from each anesthetized larva (MS222, Sandoz, France). Smears were fixed with methanol and stained using Groat's hematoxylin following the standard recommendations [24]. The number of erythrocytes that contained one micronucleus or more (micronucleated erythrocytes [MNE]) was determined in a total sample of 1000 erythrocytes per larva under light microscope. Based on median values and quartiles [27], the number of micronucleated erythrocytes per thousand, MNE ‰ is presented with their 95% confidence limits expressed by the median $\pm 1.57 \times$ interquartile range (IQR; upper quartile − lower quartile)/$\sqrt{n}$. 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.

2.7. *Larvae macro-observations and histological optical and TEM preparations*

After puncturing, the general aspect of the larvae exposed to CNTs was visually compared with that of the NC group under the binocular. Histological preparations from intestine, liver and gills were prepared for optical and transmission electron microscopy (TEM) observations at the "Centre de Microscopie Electronique Appliquée à la Biologie" of the Medical University of Rangueil (Toulouse, France). After their sampling, these organs were promptly fixed in gluteraldehyde solution (2% on 0.1 M Sörensen buffer at 4 °C), then post-fixed in osmium tetroxide (1%), and finally dehydrated by bathing in ethanol solutions of increasing alcohol concentrations (30–100°) before embedding in epoxy resin (Embed812-Araldite502 resin). A substitution step in propylene oxide/resin mix is required before placing the biological samples in moulds filled with pure liquid resin and polymerization (60 °C, 48 h). Sets of halfthin (about 1.0 μm thin) and ultrathin sections (about 70 nm) were sliced from the blocks using an ultramicrotome (Ultracut Reichert) equipped with a diamond knife. Note that the first slice is never suitable for microscopic observations. The selected halfthin sections were placed on glass slices, dried, and stained with methylene blue. While the selected ultrathin ones were collected on collodion-coated copper grids, and stained with uranyl acetate and lead citrate, or only with uranyl acetate in order to enhance the detection of MWCNTs. A thin layer of carbon was deposited onto the microtomies (to prevent their deterioration) before their observation in the same conditions as those used for the characterization of raw and stabilized MWCNTs.

3. Results

3.1. *Characterization of raw and stabilized MWCNT suspensions*

The carbon content of dried MWCNTs was *ca.* 95 wt.%. The final product still contained catalyst residues, including aluminium (1.32 wt.%) and iron (0.85 wt.%). It is assumed that the complement to 100% is likely to correspond to oxygen. The specific surface area measured by BET was 270 m^2/g. From our own TEM observations, the outer diameter ranged from a few nm to 20 nm.

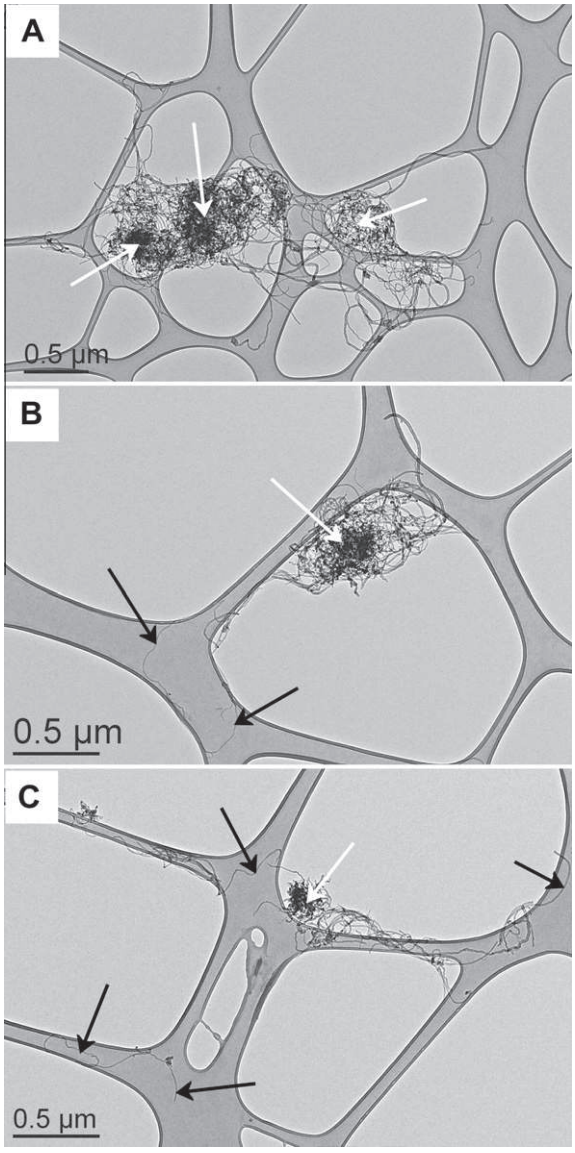

Fig. 2 – Comparison of TEM microphotographs of raw MWCNTs after (A) any mechanical dispersion (non-sonicated MWCNTs), (B) mechanical dispersion (dispersion protocol), (C) long-time sonication. Note the correlation between the increase in sonication-time and (i) the size reduction of the agglomerates (white arrows) and (ii) the increase in the number of individualized MWCNTs (black arrows).

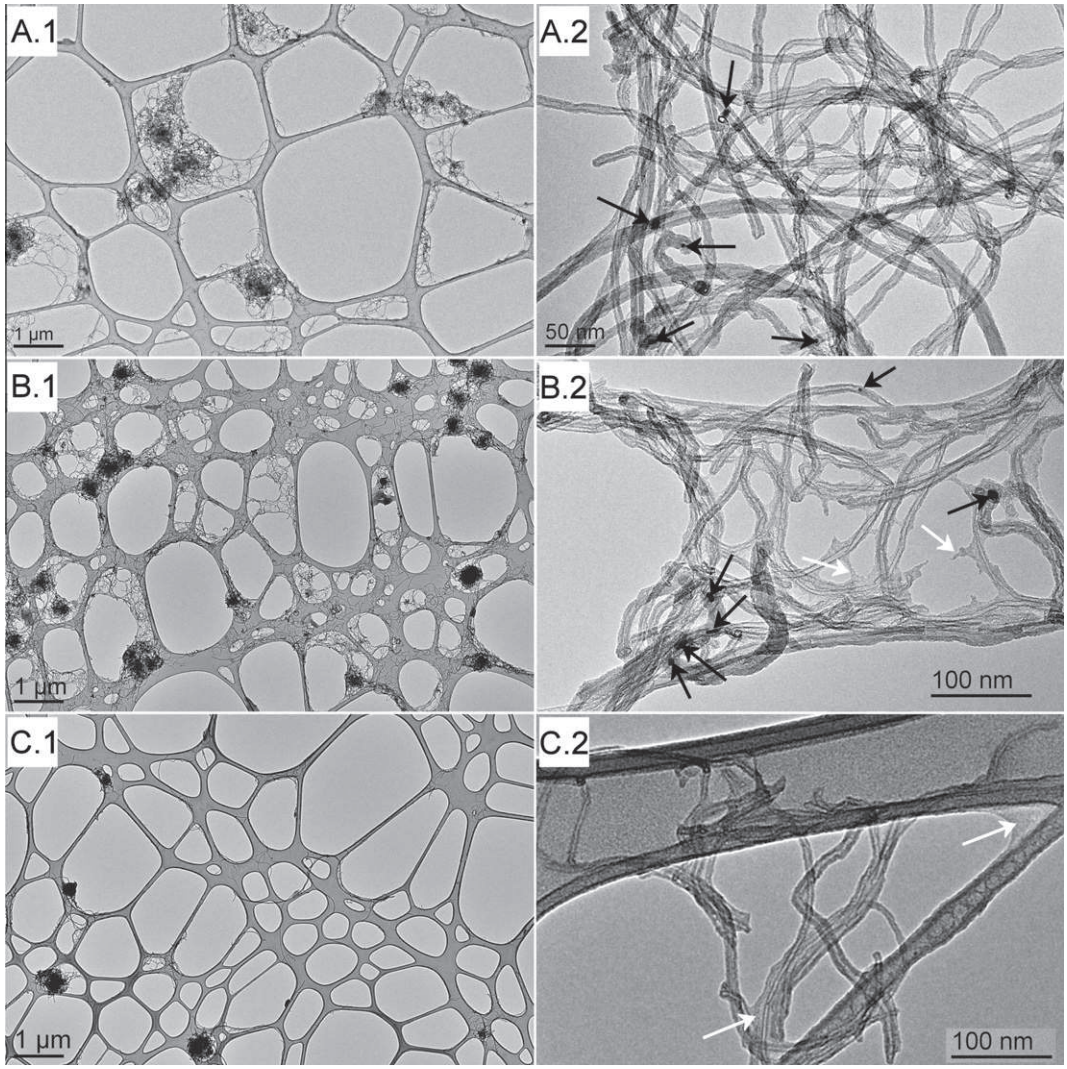

Fig. 3 – Comparison of TEM microphotographs of (A) raw MWCNTs (mechanical dispersion) and (B) CMC-stabilized or (C) GA-stabilized MWCNTs (mechanical dispersion and non-covalent functionalization) at (1) low magnification and (2) high magnification. Black arrows indicate the presence of catalyst nanoparticles, while white ones indicate the presence of dispersing agent (CMC or GA) wrapping MWCNTs and nanofibers.

TEM observations of raw and stabilized MWCNT suspensions (Figs. 2 and 3) revealed the presence of both agglomerates and individualized MWCNTs, but also some by-products of their synthesis such as nanofibres (i.e. distorted MWCNTs with much thicker walls and larger diameters) and metal (or a metal compound) nanoparticles (Fig. 3, black arrows) as catalyst residues encapsulated within graphitic shells or MWCNTs. We have noticed both a significant decrease of the size of agglomerates (Fig. 2, white arrows) and the presence of individualized MWCNTs (Fig. 2, black arrows) when a mechanical dispersion (UT and tip sonicator) was applied to MWCNT suspensions. The more extended the sonication (tip sonicator), the more pronounced this phenomenon. Moreover, when only mechanical dispersion (as defined in the established dispersion protocol) was used the size of agglomerates could reach several μm (Fig. 3, A.1), while they did not exceed 1 μm when a dispersing agent was added (Fig. 3, B.1, C.1). Finally, MWCNTs dispersed in presence of CMC or GA (Fig. 3, B.1, C.1) seemed to be more individualized than MWCNTs dispersed only with UT and tip sonicator (Fig. 3, A.1). Non-sonicated MWCNTs were so tangled that it was impossible to estimate their length, and thus not possible to compare it to the one of sonicated MWCNTs. Higher magnification TEM images of samples containing CMC and GA evidenced the wrapping of MWCNTs by the dispersing agent (Fig. 3, B.2 and C.2, white arrows).

No difference was noticed between Raman spectra of MWCNTs before (Fig. 4A, "Non-sonicated MWCNTs") and after the application of a more or less extended mechanical dispersion (Fig. 4A, "mechanical dispersion" and "extended sonication"). Raman spectra of raw MWCNTs (mechanical dispersion; Fig. 4B, "Raw MWCNTs") and those of stabilized MWCNTs (mechanical dispersion in presence of a dispersing agent; Fig. 4B, "MWCNTs + CMC" and "MWCNTs + GA") practically overlaid and exhibited nearly identical $I_{D/G}$ peak intensity ratios. The only difference was that D and G-bands position of stabilized MWCNT spectra were slightly up-shifted. In fact,

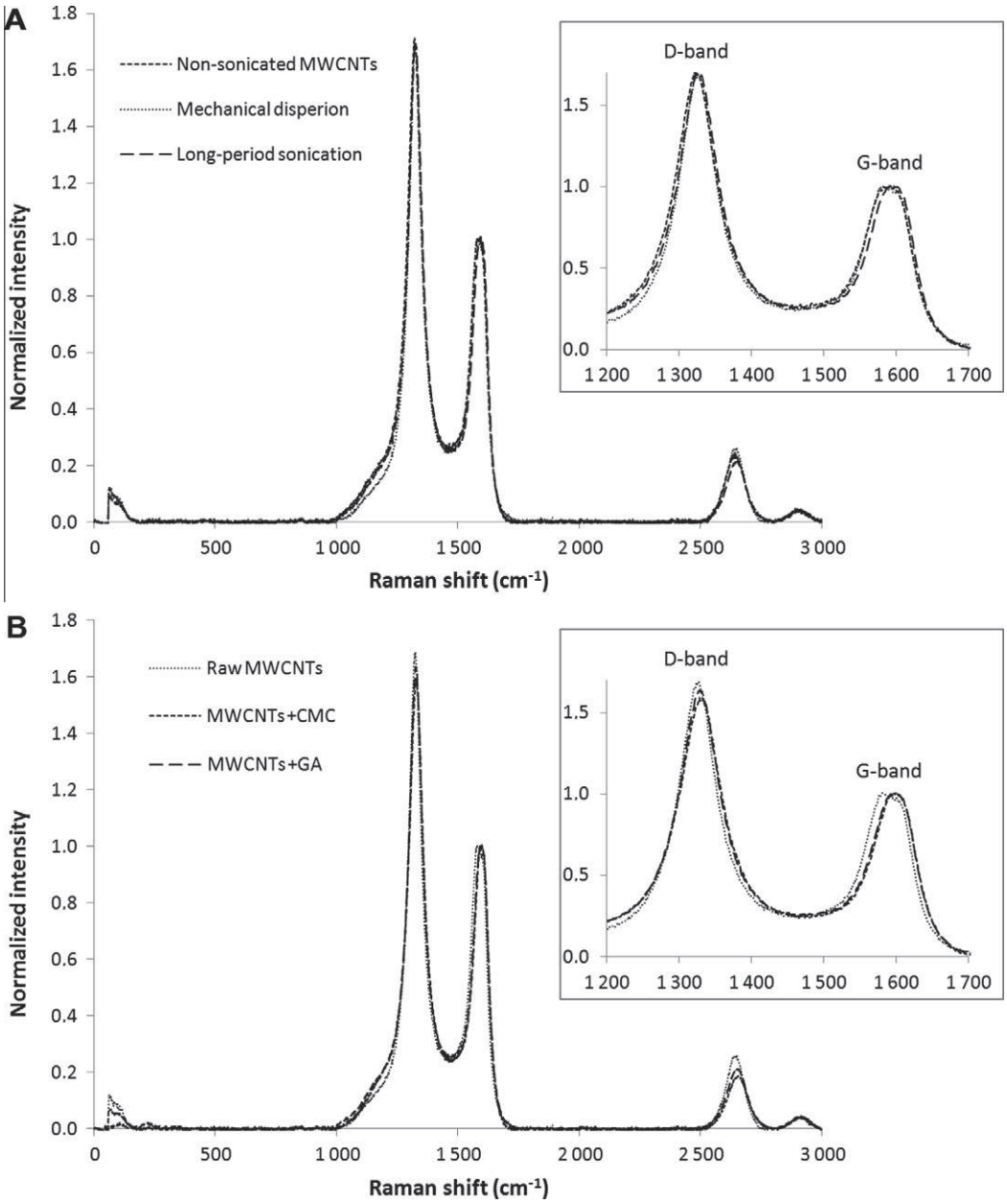

Fig. 4 – Raman spectra of raw and stabilized MWCNTs. Influence of (A) the mechanical dispersion and (B) the non-covalent functionalization with an anionic polymer. D-band normalized with respect to the intensity of the G-band intensity of the same spectra. Non-sonicated MWCNTs: MWCNTs before any mechanical dispersion and non-covalent functionalization; Mechanical dispersion/Raw MWCNTs: MWCNTs after the application of the mechanical dispersion; Long-time sonication: MWCNTs after a prolonged sonication using the tip sonicator; MWCNTs + CMC/GA: MWCNTs after the application of the mechanical dispersion combined with the non-covalent functionalization (carboxymethylcellulose/gum arabic).

the irradiation of the samples by the Raman laser leads to a local heating of CNTs, resulting in a shift toward lower frequencies, but their dispersion provides better heat dissipation and thus results to less down-shifted spectra.

3.2. *Stability of MWCNT suspensions*

Absorbance calibration curves used to determine the concentration of MWCNTs in the water column (not shown) were all characterized by a good correlation coefficient (i.e. r^2 = 0.9985–0.9998). In media free from larvae and food where 50 mg/L of MWCNTs (i.e. maximum exposure concentration) were stabilized with CMC or GA, we have respectively measured during a 24 h-period a mean MWCNT concentration of 50.17 ± 0.23 mg/L or 51.04 ± 0.19 mg/L (Fig. 5C, "NLNF"). Regarding the whole range of exposure conditions "MWCNTs + CMC", the mean concentration was 0.25 ± 0.05 mg/L, 1.52 ± 0.06 mg/L and 10.14 ± 0.14 mg/L respectively for 0.1, 1 and 10 mg/L (Fig. 6, "NLNF"). These results indicated that the combination of a mechanical dispersion and a non-covalent functionalization

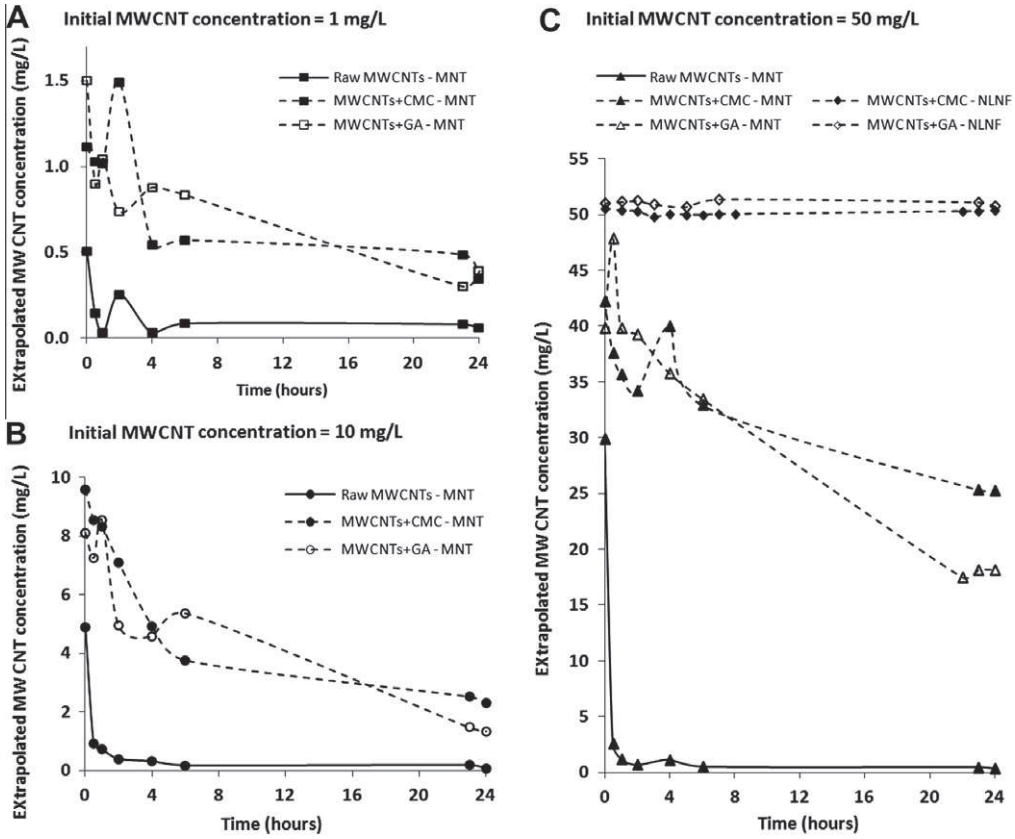

Fig. 5 – Variation with time (24 h) of the MWCNT concentration in the water column when larvae and food are both (A–C; MNT) present or (C; NLNF) absent. Comparison between raw MWCNTs and CMC- or GA-stabilized MWCNTs. The initial MWCNT concentrations are (A) 1 mg/L, (B) 10 mg/L and (C) 50 mg/L. Raw MWCNTs, multi-walled carbon nanotubes; MWCNTs + CMC, CMC-stabilized MWCNTs; MWCNTs + GA, GA-stabilized-MWCNTs; MNT, micronucleus test; NLNF, neither larvae nor food.

with one of the chosen anionic polymers was efficient enough to maintain during at least 24 h the initial MWCNT concentration in the water column.

Fig. 5 compares the stability of the suspensions (i.e. sedimentation) of raw MWCNTs to CMC- or GA-stabilized MWCNTs during MNT (real test conditions). It shows that, whatever the concentration (0.1 mg/L not shown), raw MWCNTs settled down almost instantly, and the concentration in the water column was close to zero after a few hours of exposure (Fig. 5, "Raw MWCNTs"). However, the concentration of stabilized MWCNTs only slowly decreased (Fig. 5, "MWCNTs + CMC" and "MWCNTs + GA") and after 24 h the concentration of MWCNTs still present in the water column ranged from 23% to 50% and from 11% to 39% of the initial value respectively in the case of CMC and GA. These results indicate that CMC seems to be a more efficient dispersing agent. Furthermore, we have observed few peaks of concentration during the first hours of the study. This phenomenon could be explained by interferences with free food particles (see next paragraph).

The sedimentation of CMC-stabilized MWCNTs, while food or larvae was added to the test medium, was compared to the previous results. On the one hand, when larvae were added (Fig. 6, "Larvae" and "MNT"), MWCNT concentration in the water column decreased progressively whereas when there was neither food nor larvae (Fig. 6, "NLNF") it remained stable. The higher the MWCNT initial concentration, the more visible this phenomenon. On the other hand, whatever the test condition and the sampling time, the OD (and thus the extrapolated MWCNT concentrations) measured when only food was added (in excess) to the medium (Fig. 6, "Food") was always higher than the OD measured in media free from food (Fig. 6, "NLNF" and "Larvae") or when both food and larvae were present (Fig. 6, "MNT"). The lower the MWCNT initial concentration, the more important the difference between the extrapolated concentration and the target one. For example, the ratio between mean concentration when food was added (Fig. 6, "Food") and mean concentration in media exempt from food and larvae (Fig. 6, "NLNF") was 1.07 for the condition "50 mg/L" and 27.41 for the condition "0.1 mg/L". Furthermore, we observed some abrupt rises or drops, which were similar to these noticed in Fig. 5. Taking in consideration the fact that food was added in excess in media without larvae (Fig. 6, "NLNF"), and was only partly ingested by them in MNT media (Fig. 6, "MNT"), these abrupt rises could be assimilated to artefacts due to the presence of free non-ingested food particles which were dispersed during sonication of the samples (just before measuring the absorbance). The presence of these solid particles increased the OD of samples, and thus led to the overestimation of the extrapolated MWCNT concentrations especially for to lowest ones (i.e. 0.1 and 1 mg/L).

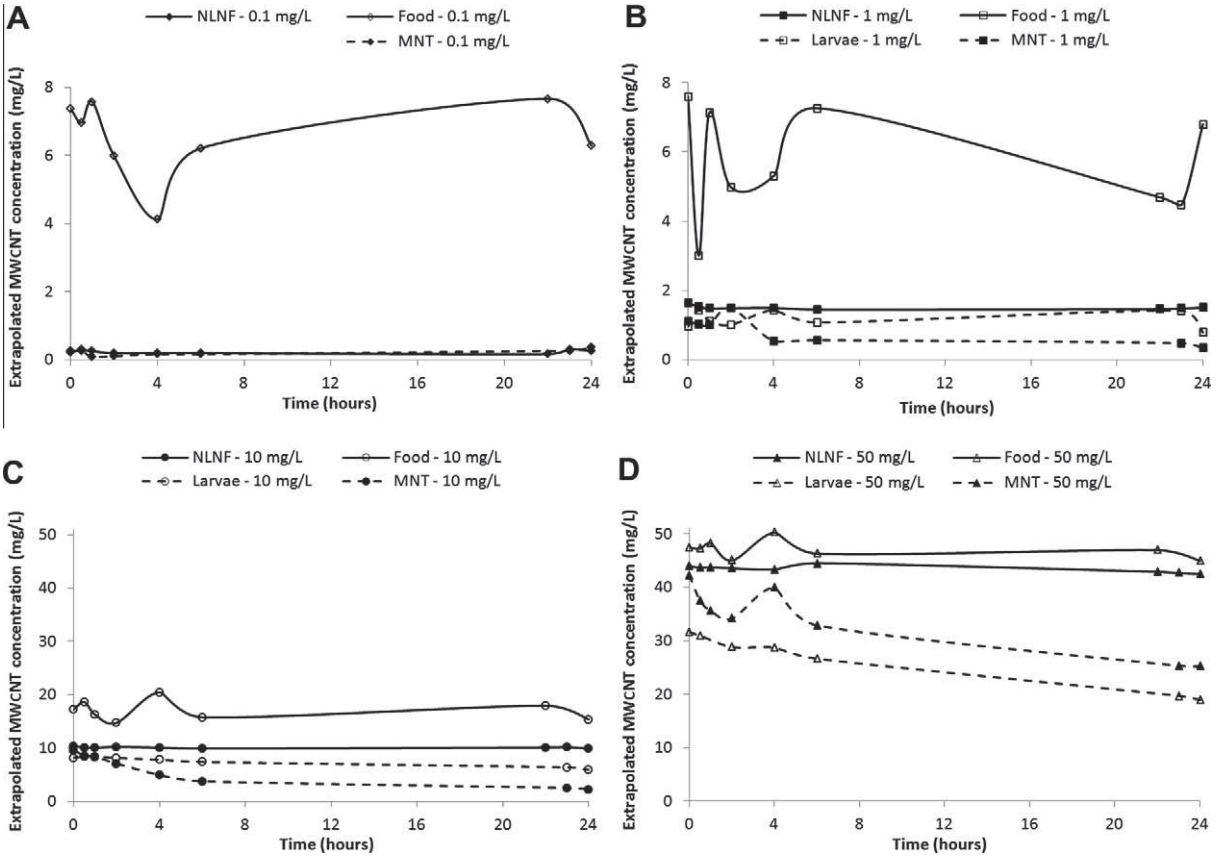

Fig. 6 – Variation with time (24 h) of the CMC-stabilized MWCNT concentrations in the water column. Comparison between exposure media corresponding to the MNT (presence of larvae and food) and media free from larvae (presence of food) or free from food (presence of larvae) or free from both larvae and food. The initial MWCNT concentrations are (A) 0.1 mg/L (no data for "Larvae"), (B) 1 mg/L, (C) 10 mg/L and (D) 50 mg/L. NLNF, neither larvae nor food, exposure medium containing only MWCNTs and CMC; Food, exposure medium containing MWCNTs, CMC and food; Larvae, exposure medium containing MWCNTs (no data for "0.1 mg/L"), CMC and larvae; MNT, micronucleus test exposure medium containing MWCNTs, CMC, larvae and food.

Table 1 – Results of acute toxicity (mortality rate, %) in *Xenopus* larvae exposed during 12 days to raw or CMC-stabilized or GA-stabilized MWCNTs. NC, negative control; CMCC, carboxymethylcellulose control (50 mg/L); GAC, gum arabic control (50 mg/L); MWCNTs, multi-walled carbon nanotubes; MWCNTs + CMC, CMC-stabilized MWCNTs; MWCNTs + GA, GA-stabilized MWCNTs.

	NC	CMCC	GAC	MWCNT concentration (mg/L)			
				0.1	1	10	50
Raw MWCNTs	0%			0%	0%	0%	20%
MWCNTs + CMC	0%	0%		0%	15%	30%	30%
MWCNTs + GA	0%		0%	0%	0%	0%	20%

3.3. *Acute and chronic toxicities*

The results (Table 1) showed no toxicity in control conditions (NC, CMCC and GAC), but mortality was observed from 1 mg/L of CMC-stabilized MWCNTs while acute toxicity was observed only at the maximum concentration (50 mg/L) of raw MWCNTs or GA-stabilized MWCNTs. Furthermore, whatever the initial dispersion state of MWCNTs, their presence at high concentration (50 mg/L) in the test media led to a significant negative effect ($P < 0.05$; Fig. 7, I.A, II.A, III.A) on the larvae growth rate. A significant growth inhibition was also observed at 10 mg/L of both CMC-stabilized and GA-stabilized

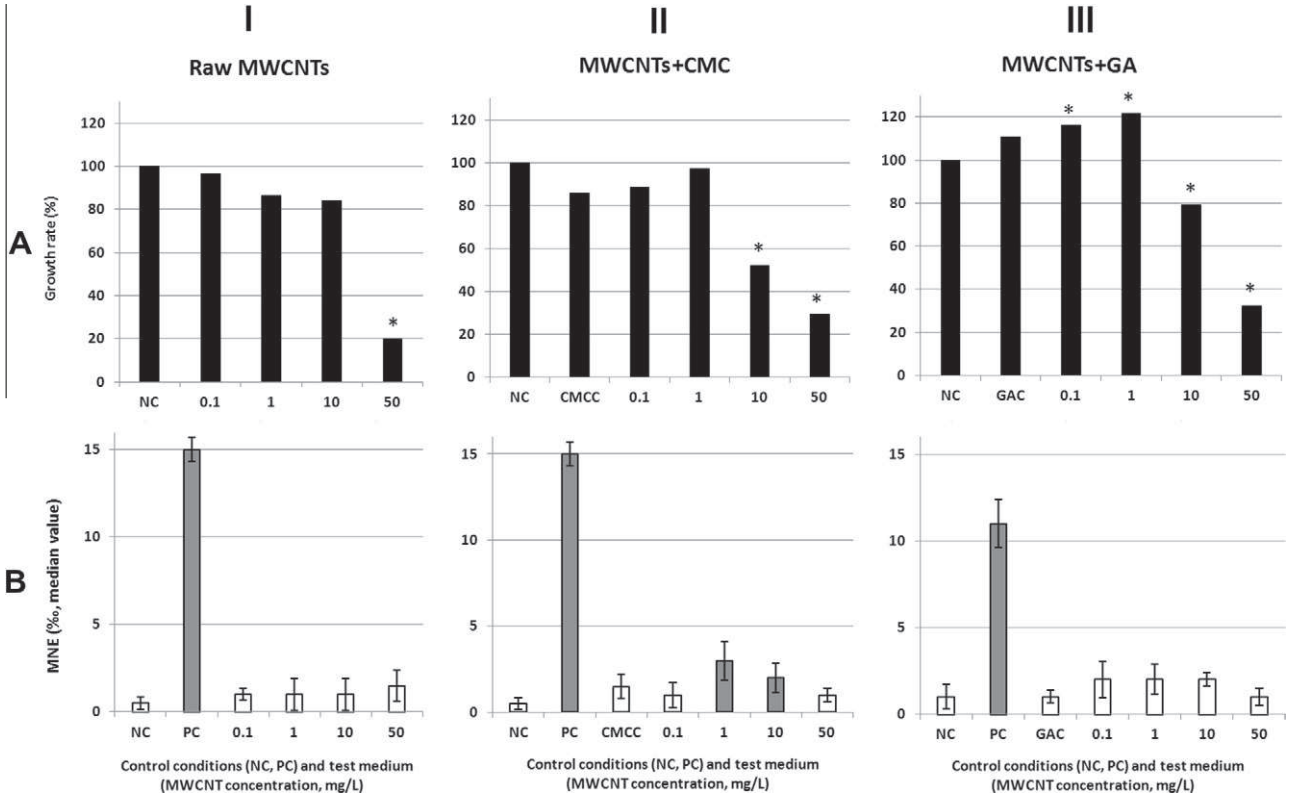

Fig. 7 – Results of (A) chronic toxicity in terms of growth inhibition and (B) micronucleus assay in erythrocytes of *Xenopus* larvae exposed during 12 days to (I) raw MWCNTs or (II) CMC-stabilized MWCNTs or (III) GA-stabilized MWCNTs. *Corresponds to a significantly different size of larvae at the end of the test compared with the NC group (mean value, $P < 0.05$). Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE‰) and their 95% confidence limits. White bars, absence of genotoxicity; Grey bars, significant response compared to the NC group; NC, negative control; PC, positive control; CMCC, carboxymethylcellulose control (50 mg/L); GAC, gum arabic control (50 mg/L); Raw MWCNTs, multi-walled carbon nanotubes in reconstituted water (RW); MWCNTs + CMC, CMC-stabilized MWCNTs; MWCNTs + GA, GA-stabilized MWCNTs.

MWCNTs. On the contrary, larvae exposed to low GA-stabilized MWCNT concentrations (i.e. 0.1 and 1 mg/L) significantly gained weight ($P < 0.05$; Fig. 6, III.A).

3.4. *Genotoxicity*

Concerning the MNT where larvae were exposed to raw MWCNTs and CMC-stabilized MWCNTs, the median value of MNE‰ for the NC and the PC were respectively 0.5 ± 0.35 and 15 ± 0.70 (Fig. 7, I.B and II.B). Concerning the MNT where larvae were exposed to MWCNTs dispersed with GA, the median value of MNE‰ for the NC and the PC were respectively 1 ± 0.7 and 11 ± 1.4. Thus, whatever the experiment, the PC group showed significantly higher MNE‰ as compared to NC group. Furthermore, the absence of genotoxicity was observed for both the CMCC and the GAC groups. No genotoxicity via micronucleus induction in erythrocytes of *Xenopus* larvae was observed whatever the concentration of MWCNTs, their state of dispersion (raw *vs.* dispersed) and the nature of the dispersing agent, except at 1 and 10 mg/L of CMC-stabilized MWCNTs (Fig. 7, II.B).

3.5. *Macro and optical observations of larvae and selected organs*

During the MNT, larvae exposed to the highest concentrations of raw MWCNTs and stabilized MWCNTs were respectively partially covered by CNTs and more grey-colored than those of control groups. From the second day of exposure to the end of the MNT, the presence of black-colored excrements was noticed at the bottom of dishes in treated media. The presence of MWCNTs in these excrements that induced their black-coloration is discussed further below. At the end of the MNT, while the digestive tract of larvae reared in the control media was normally brown-colored, those of larvae exposed to low MWCNT concentrations contained black masses and those of larvae exposed to higher concentrations was entirely black, and even seemed to be bloated in some larvae which died before the end of the assay.

The visual inspection under binocular revealed the presence of black masses around the gills of larvae only exposed to stabilized MWCNTs from 1 mg/L (Fig. 8). Finally, observing halfthin sections of digestive track, liver and gills, we did not notice any histological difference between treated and

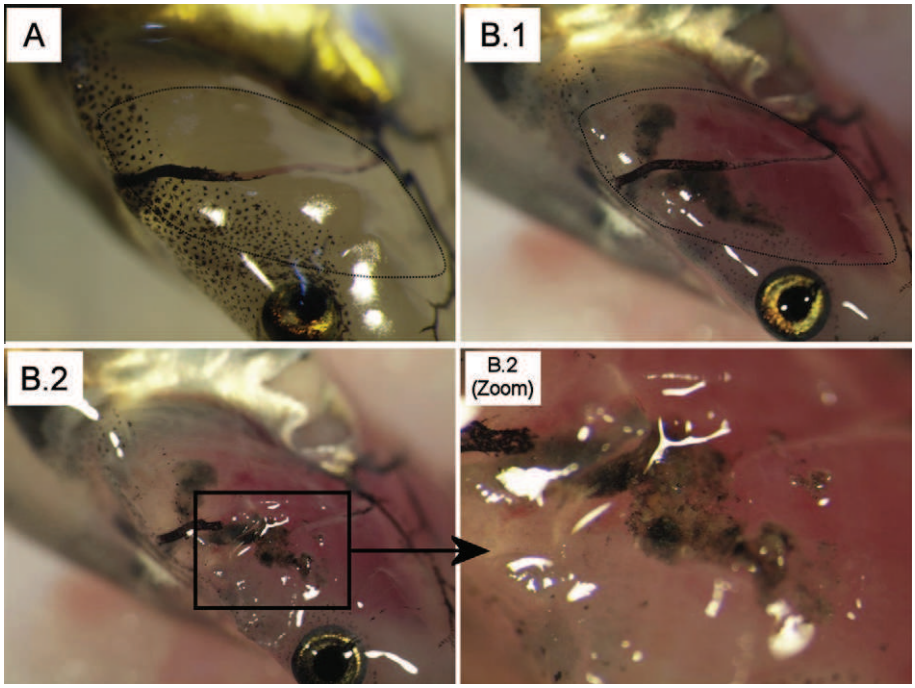

Fig. 8 – Evidence of the presence under superficial tissues of black masses located around the gills of larvae exposed to 1 mg/L of CMC-stabilized MWCNTs. Gills of a NC larvae (A) were compared to those of a treated larvae (B). The gills are outlined by the dotted lines. (B.2) Note that the black masses were not removed by getting rid of superficial tissues.

control larvae, corresponding to tissue damage or malformation.

3.6. TEM observations of ultrathin sections of intestine tract, liver and gills

The presence of agglomerated or isolated MWCNTs in the lumen of the intestine confirmed the previous macro and photonic observations. TEM observations did not allow us to undoubtedly evidence that they passed through the intestinal epithelial barrier. The presence of isolated MWCNTs in enterocytes is discussed further below. Finally, the observations of gills and liver ultrathin sections showed neither detectable surface cell-MWCNT interactions nor MWCNT internalization/translocation.

4. Discussion

Whatever the initial dispersion state of MWCNTs to which were exposed *X. laevis* larvae, the suspensions were prepared using a mechanical dispersion including the use of a sonication probe. Several studies highlighted the role of ultrasonication in the expansion and the peeling or fractionation of MWCNT layers, leading to their shortening (length reduction) and/or their thinning (diameter reduction) [28], and thus to the removal of catalyst residues. On the contrary, Chowdhury et al. [29], who compared the size reduction of MWCNTs using the combination of scanning electron microscopy (SEM) and dynamic light scattering (DLS), concluded that the conjunction of wet milling and high energy sonication was ineffective and led to dispersion rather than CNT fragmentation and size reduction. Concerning the present study, the comparison between the TEM images of non-sonicated and mechanically dispersed MWCNT suspensions over a moderate or an extended time allows us to draw a parallel between the sonication-time and the mechanical dispersion efficiency (i.e. size reduction of agglomerates and increase in the number of individualized CNTs), but the MWCNTs in non-sonicated suspensions were so tangled that it was impossible to estimate their length and to discuss their possible fragmentation induced by tip sonication. Besides, the Raman spectra did not show any evidence of structural defects related to the sonication of MWCNTs. However, as the $I_{D/G}$ ratio was already rather high in the starting material (*ca.* 1.7), it is possible that the creation of additional structural defects or fragmentation by sonication could not have been detectable (i.e. no measurable increase in $I_{D/G}$ ratio). Thus we could not exclude that the mechanical dispersion treatment applied to prepare the test media could have damaged and/or cut MWCNTs, but we assume that their potential alteration is not significant enough to modulate the biological responses. The dispersion protocol was defined in a such way that mechanical dispersion applied to prepare stabilized-MWNCT media was similar to the one applied to prepare raw MWCNT media, and thus could not be a parameter affecting the comparison between the biological responses related to each test.

Apart from OD measurements, Raman spectrometry analysis and TEM imaging, scanning electron microscopy (SEM) [30], dynamic light scattering (DLS) [31] are techniques usually used to characterize CNT suspensions in water, but none of them can provide a reliable particle size distribution of a whole sample [6]. An alternative could be the combination between a field-flow fractionation technique (FFF), which allows to separate CNTs according to their size, and the

multi-angle light scattering (MALS) using a shape model to determine CNT length [32]. Finally, cryo-SEM does not require drying of the sample but preserves the nanostructure of CNT suspensions, and thus could be a promising technique [33]. Nevertheless to our knowledge, none of them have been pointed out to characterize CNT suspensions or to measure their concentration in such complex media as natural waters.

After 12 days of exposure to a large range of raw MWCNT concentrations (from 0.1 to 50 mg/L), no genotoxicity was evidenced in erythrocytes of *X. laevis*, but only mortality and a significant growth inhibition at 50 mg/L. Mouchet et al. [34] previously observed similar biological responses, using the same organism.

In our study, the stock suspensions and the test tubes of raw MWCNTs, as well as the test media were prepared using only mechanical dispersion. On the one hand, the dispersion with an UT and a tip sonicator of the stock suspensions partially promoted the de-agglomeration of CNTs, which led to a size reduction of agglomerates and the partial individualisation of MWCNTs (Figs. 2 and 3). On the other hand, the bath sonication of test tubes and the volume adjustment of the exposure medium helped to re-disperse them and to homogenize the final exposure suspensions. The mechanical dispersion alone was obviously not enough to stabilize the suspensions during 24 h. After only a few hours of exposure, the CNT concentration in the water column drastically dropped to zero, so that during the amphibian exposures, due to their grazing behaviour, the larvae were probably mostly exposed to MWCNTs deposited on the bottom of crystallising dishes. Macro observations of larvae revealed that they absorbed MWCNTs. As previously shown by Mouchet et al. [34], the higher the MWCNT concentration, the darker the digestive tract. TEM observations of ultrathin sections of the digestive tracts confirmed the presence of agglomerated and isolated MWCNTs in the lumen of larvae. Black-colored excrements which looked similar to those found in the treated media that we are discussing here, were collected in the media of larvae exposed to another nature of raw MWNCTs (i.e. synthesized by ethanol-CCVD). The presence of MWCNTs which are responsible for their darkening was clearly evidenced during TEM observations of the collected excrements (not shown). Thus macro and TEM observations showed that MWCNTs were absorbed, and moved within the digestive track (i.e. in the lumen) before being at least partially (if not totally) excreted in the exposure medium.

As revealed by the visual inspection and the measurement of the MWCNT concentrations with time, the concentration of the stabilized MWCNTs remained stable in the water column exempt of larvae and food for at least 24 h. Nevertheless, the interaction with living organisms destabilized the suspension, leading to the sedimentation of agglomerates with time (i.e. before the renewal of the media, 24 h). The destabilization could be induced by the replacement of the dispersing agent by organic exudates (e.g. proteins [35], polysaccharides excreted by larvae), but also by the non-ingested food. The implication of the last one is still questionable because it darkens the exposure medium and may induce an overestimation of the MWCNT concentration when quantified by absorbance spectroscopy. The lower the concentration, the higher the overestimation. The intestine of larvae exposed to CMC- and GA-stabilized MWCNTs showed similar aspect compared to the one of organisms exposed to raw MWCNTs. The presence of MWCNTs was also revealed in the lumen by TEM observations, and black-colored excrements were also found at the bottom of the dishes. Thus, even if the agglomerates in stabilized-MWCNT media were less abundant than those in raw MWCNT ones, they were obviously ingested by larvae. Besides, a significant growth inhibition of larvae was observed when they were exposed to stabilized MWCNTs at 10 and 50 mg/L, but also to raw MWCNTs at 50 mg/L, as mentioned before. The link between these biological responses and the absorption of MWCNTs is discussed below.

CNTs have also been detected as compact masses in the guts of *Daphnia magna* exposed to mechanically suspended MWCNTs [36] or to MWCNTs dispersed with NOM [6,33]. The authors reported that according to TEM images there was no evidence that MWCNTs were absorbed into cellular tissues and that the microvilli seemed to prevent MWCNT absorption by *D. magna* from crossing the gut lumen. They concluded that the toxicity of MWCNTs is a mechanical effect of MWCNTs attributable to the clogging of the daphnids' gut tract, leading to a feeding inhibition and a deficit of nutrients intake [37].

By working from intestinal ultrathin sections contrasted thanks to a treatment with both uranyl acetate and lead citrate, MWCNTs could barely be distinguished among the biological matrix, contrarily to those located in the lumen (Fig. 9B). Slightly defocusing the microscope (Fig. 9A) allowed to observe isolated MWCNTs into or onto the microvilli of larvae exposed to raw or stabilized-MWNT suspensions, but none could be observed in deeper layers. Staining these ultrathin sections only with uranyl acetate resulted in a sufficient contrast between carbon-based background biological matrix and the CNTs, and allowed us to detect isolated MWCNTs into enterocytes and in the basal membrane region (Fig. 10A). Nevertheless, on the one hand the isolated MWCNTs looked randomly distributed, parallel to each other and oriented and along the direction of the cutting, and on the other hand accumulations of MWCNTs were found on both opposite sides of the preparation (Fig. 10B) by moving along the cutting direction. We would point out the fact that the studied organs were fixed and then included in an epoxy-resin before slicing 70 nm-thin sections with a diamond knife. Ajayan et al. [38] investigated the cutting with an ultramicrotome equipped with a diamond knife of 50 nm to 1 μm-thin sections from a polymer resin-CNT composite blocks made from CNTs randomly arranged in an epoxy-resin. TEM analysis showed that even if embedded CNTs were longer than the thickness of the slices, the cutting did not produce transverse sections of CNTs, but that the stress and the shear forces applied during the cutting lead to their pulling out or their deformation from the matrix and finally to their unidirectional orientation on the section. Thus these researchers developed a simple technique to produce slices of this composite where CNTs were aligned. We hypothesized that the isolated MWCNTs detected in the apical region (i.e. microvilli), into enterocytes and in the basal region were actually probably dragged along the surface during the sample cuttings. The internalization of MWCNTs could not be discussed from TEM observations of ultrathin

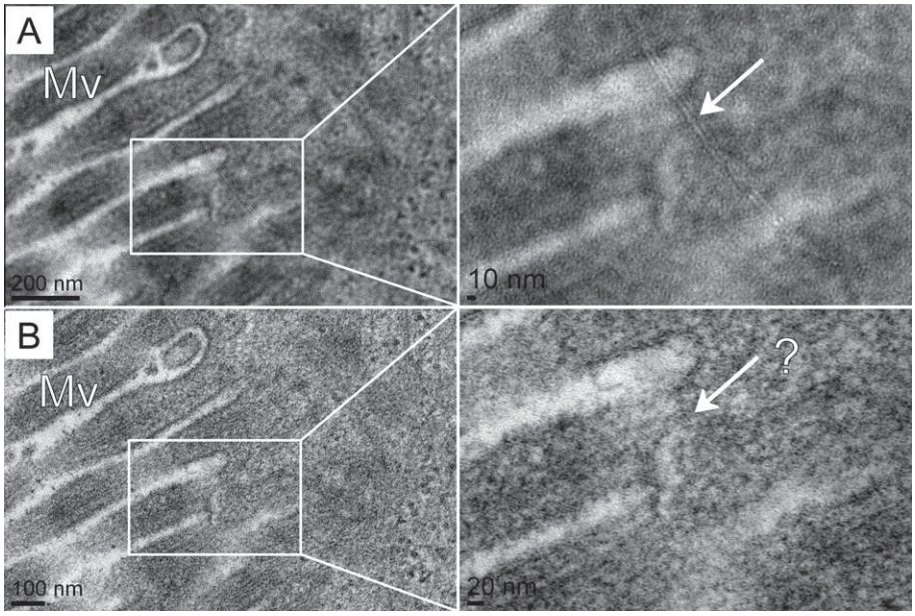

Fig. 9 – Transmission electron micrographs of an ultrathin section of digestive tract of *Xenopus laevis* larvae exposed to 10 mg/L of CMC-stabilized MWCNTs. Observation of MWCNTs (white arrows) above the villosities. Stained with both uranyl acetate and lead citrate. (A) MWCNTs walls revealed thanks to white fringes by slightly defocusing the microscope. (B) MWCNTs are barely visible when the focus is well adjusted. Mv, microvilli.

sections prepared by cutting the embedded organs that produced artifacts concerning CNT localization.

The overall results suggest that, after the agglomeration of MWCNTs followed by their sedimentation, and their ingestion by larvae, they could disturb the absorption and the assimilation of nutrients leading to a significant growth inhibition of larvae exposed to MWCNT concentrations higher that those expected in the aquatic compartment [39]. However, the question of the efficiency of the intestinal barrier of *X. laevis* larvae to prevent systemic distribution of MWCNTs is still unclear.

According to the mechanism proposed by Strano et al. [40], the mechanical dispersion may help the formation of gaps or spaces at the bundle ends by providing high local shear, while the wrapping of the surface of the CNTs with a dispersing agent (π–π stacking and van der Waals interactions) initiates the elimination of the hydrophobic interface between the tubes and the aqueous medium [41]. Then, under mechanical dispersion, the adsorption and the diffusion of the dispersing agent propagate in this space along the CNTs, thereby separating them from the agglomerates.

Unlike most of the *in vitro* or *in vivo* (eco)toxicological studies, where organisms or cells were exposed to mostly individual CNTs [42], we have chosen not to centrifuge neither the stock suspensions nor the test tubes and separate the supernatant from the agglomerated CNTs. It allowed us to keep exposure concentrations at the target values, and to be closer to real exposure conditions where part of the CNTs is agglomerated. Moreover, in comparison with mechanical dispersion alone the combination with non-covalent functionalization of an anionic polymer allowed to significantly increase the dispersion of MWCNTs in water [43], and thus their residence time in the water column. In spite of the negative influence of the presence of organisms and food, leading to the exposure to bulky agglomerates which have settled down, larvae were also exposed to smaller agglomerates and individualized MWCNTs which remained in suspension in the water column during all the exposure.

As mentioned before, raw MWCNTs induced acute and chronic toxicities only at 50 mg/L and no genotoxicity. Repair mechanisms are activated in response to environmental toxicants-induced oxidative stress, but the MNT only evidences non-repairable DNA damages in erythrocytes. Thus, raw MWCNTs-induced oxidative stress should not be excluded.

Concerning the exposure to CMC-stabilized MWCNTs, mortality and a significant growth inhibition were evidenced at lower concentrations (i.e. from respectively 1 mg/L and from 10 mg/L), and a genotoxic response was measured at 1 and 10 mg/L. The mitotic index of larvae exposed to 50 mg/L of CMC-stabilized MWCNTs was significantly lower than those of the negative control (Dunn's method, $P < 0.05$). Thus the fact that any significant induction of MNE was not emphasized at 50 mg/L could be explained by a potential disruption of the erythrocytes mitosis, and 1 mg/L of CMC-stabilized MWNCTs could be considered as the threshold level above which genotoxic response was induced after 12 days of exposure.

According to observations of gill and liver sections, we were not able to detect neither tissue damages nor the presence of MWCNTs, but the presence of black masses in the gills of larvae exposed to both CMC- and GA-stabilized MWCNTs from 1 mg/L was observed under the binocular. Similarly, the presence of precipitated CNTs as black granular masses were also noticed in the gills of rainbow trouts exposed to lower concentrations (from 0.1 to 0.5 mg/L) of SDS-dispersed SWNTs [44]. According to the overall results, the authors concluded that SWNTs were respiratory toxicants

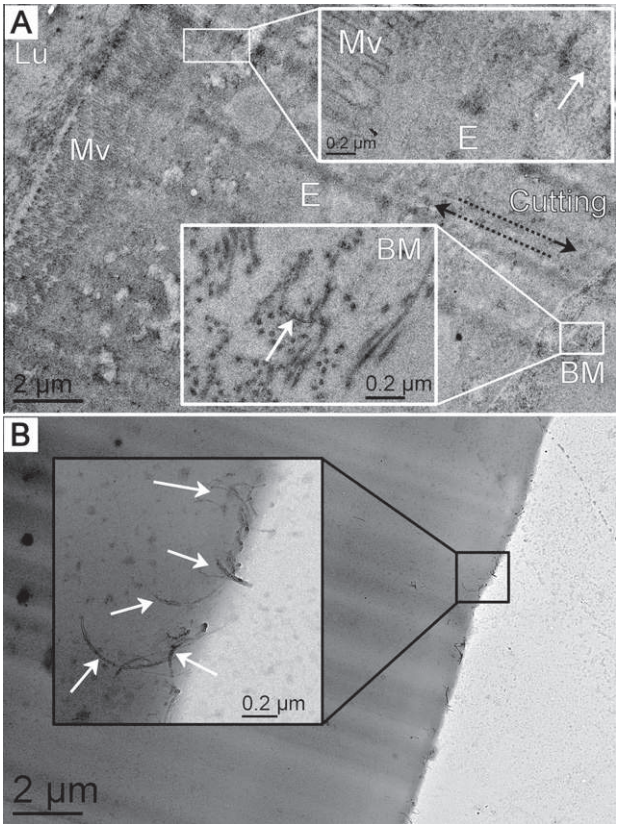

Fig. 10 – Transmission electron micrographs of an ultrathin section of digestive tract from *Xenopus laevis* larvae exposed to GA-stabilized MWCNTs. Presence of (A) isolated MWCNTs beyond the intestinal brush border but also of (B) accumulated MWCNTs on the section border. Stained only with uranyl acetate. Cutting: direction and opposite direction of the cutting during sample preparation. BM, basal membrane; E, enterocytes; Lu, intestinal lumen; Mv, microvilli.

acting at the surface of trout gills, leading to oxidative stress and moderated hypoxia, but also that they mediated systemic pathologies, linked to genotoxicity or cell cycles, in other organs where SWNTs were not histologically evidenced. In the present study, neither ventilation rate nor oxidative stress in the gills of *X. laevis* larvae was assessed. However, the interaction of these potential biological effects should not be excluded in our experiments, and may have influenced our biological responses, especially in the case of CMC-stabilized MWCNTs exposure conditions.

Nevertheless, when MWCNTs were stabilized with GA, acute and chronic toxicities were not induced at low concentrations and no genotoxic response was evidenced (i.e. results similar to raw MWCNTs). The determination of the MWCNT concentration during the MNT revealed that their residence time in the water column was lower when they were stabilized with GA, particularly at high concentration, than with CMC. Thus the difference between CMC and GA in terms of dispersion efficiency in the presence of larvae and food could partially modulate the biological responses. Furthermore a significant trend to growth stimulation was observed with GA at low concentrations (0.1 and 1 mg/L). Youn et al. [45] reported similar observations about the toxicity of SWNTs coated with GA towards a freshwater green algae, when they increased the polymer concentration without changing the SWNT concentration (0.5 or 1 mg/L). Their results suggest that CNTs stimulated the algal defence mechanisms leading to oxidative stress and the release of reactive oxygen species (ROS), but the negative effects on the organisms could be mitigated by the antioxidant potential of GA [46], through the significant enhancement of GSH synthesis.

Several physicochemical parameters of CNTs [21,22] are incriminated in the direct or indirect production of ROS and associated with oxidative stress [47]. ROS are known to easily disperse away from the original production site, and could be produced by many pathways in living organisms, leading to significant macromolecule damages, such as DNA. Regarding the different biological responses, the hypothesis of MWCNT-induced ROS in the gills of larvae (or other tissues and organs), following the interaction between gills, and individualized MWCNTs and/or small agglomerates in suspension in the water column, is possible. In our experiments, this potential indirect DNA damaging system may have contributed to the MN induction in larvae exposed to 1 and 10 mg/L of CMC-stabilized MWCNTs, while the use of GA as dispersing agent could have mitigated their genotoxicity leading to a non-significant induction of MN at the same concentrations.

5. Conclusion

The mechanical dispersion (including in particular the use of a sonication probe) of MWCNT suspension induced the deagglomeration and the partial individualization of CNTs, but the addition of a stabilizing agent was needed to keep the dispersions stable for at least 24 h. Thus a new CNT dispersion protocol, which combined mechanical dispersion and the non-covalent functionalization of MWCNTs by an anionic polymer (i.e. CMC or GA) was established to prepare stabilized MWCNT suspensions under (supposed) mimicked environmental conditions (i.e. presence in the water column of both reduced-size agglomerates and individualized MWCNTs). This protocol facilitates the progress of the ecotoxicity assay and ensures a proper dispersion of the CNTs along 12 days of exposure under semi-static conditions. Compared to the fast agglomeration and sedimentation of raw MWCNTs, it turned out to be efficient in test media exempt of larvae and food, at least over 24 h. Even if the interaction with living organisms and food destabilized the suspensions, the agglomeration and the sedimentation of stabilized MWCNTs was significantly delayed, thus allowing a longer period of exposure in the water column. UV–vis spectroscopy appeared to be a fast and convenient method to quantify and characterize the agglomeration and the sedimentation of MWCNTs in the exposure media, even if its relevance if limited when the initial concentration close to 1 mg/L and below, or in the case of turbid water (e.g. presence of food particles or other colloids in suspension). Considering this level of concentration as the environmentally realistic one, efforts are necessary to find a better way to quantify and characterize CNTs in real complex aquatic media.

During the biological assays (MNT), in the case of exposure to raw MWCNT, *X. laevis* larvae were exposed to bulky agglomerates, while in the case of stabilized MWCN, they

were exposed to agglomerates, which have eventually settled down, as well as to individualized CNTs and to significantly size-reduced agglomerates remaining in suspension in the water column. Thus, the exposure of larvae to stabilized MWCNTs and the comparison of the biological responses with those obtained from exposure to raw MWCNT aimed to study the influence of the partitioning of these CNTs between the water column and the bottom of the dishes, according to the larvae grazing behavior and their moving in the water column. The agglomeration and the sedimentation of CNTs over time, followed by the ingestion of significant amounts of MWCNTs when larvae were grazing at the bottom of the dishes, were suspected to induce a toxicity resulting, through intestinal transit mechanical disruption, in significant acute and chronic toxicities at high MWCNT concentrations. The overall results suggest that, on the one hand the presence of individualized MWCNTs and/or size-reduced agglomerates in suspension in the water column tends to increase both the growth inhibition and the mortality of larvae, and could be responsible for irreversible DNA damages. On the other hand the biological responses could be modulated by the nature of the dispersing agent itself. The partitioning of CNTs influenced by the presence of dispersants/stabilizers in natural water, but also the nature of the latter (i.e. mixtures of amino acids, fulvic and humic substances, proteins, lipids, etc.) make extremely difficult the accurate prediction of the ecotoxicological effects of CNTs on the aquatic organisms.

We evidenced that MWCNTs absorbed by the larvae passed through the digestive system before being excreted. In this study, we demonstrated that the presence of CNTs observed across the microvilli was possibly only an artifact of the ultra-thin sections preparation (at least, we could not demonstrate that they reached this location during the experiment itself). TEM observation of organ sections should thus be analyzed with caution regarding their common use as evidences of CNT internalization. According to our histological observations, no potential entry point was actually evidenced, but the MWCNT toxicity is suspected to be a consequence of CNT effects on the external epithelial surfaces. For instance, cellular events and mechanisms linked to the interaction between MWCNTs and gills membrane, in absence/presence of GA, are worth further investigations.

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