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International standardized procedures for *in vivo* evaluation of multi-walled carbon nanotube toxicity in water

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ABSTRACT

The classical approach in ecotoxicological evaluation of chemical substances consists of conducting standardized bioassays on organism models. In this work, the potential impact of industrial multi-walled carbon nanotubes was investigated by ecotoxicological standardized procedures using aquatic organisms of different trophic levels, namely bacteria, green algae, invertebrates, fish, and amphibians. The results indicated (1) inhibition of growth in amphibians at 50 mg L^{-1} and higher, and (2) no effects on daphnia and fish up to 100 mg L^{-1}. With the exception of algae (for which Fe deficiency is measured), it seems that the observed toxicity may be due to physiological effects in relation to the ingestion of carbon nanotubes not necessarily related to their intrinsic effects.

KEYWORDS

Multi-walled carbon nanotubes; ecotoxicity; standardized bioassays; bacteria; algae; fish; amphibians

Introduction

The publication of Iijima (1991) generated unprecedented interest in the world of carbon nanostructures and led to an exponential growth in research on carbon nanotechnology. Carbon nanotubes (CNTs) can be described as graphene sheets rolled up to form cylinders that are closed at both ends. There are two main types, i.e. single-walled CNTs (SWNTs) and multi-walled CNTs (MWNTs). They have remarkable physical, i.e. mechanical, electric, and thermal and chemical properties (inertness, stability), making them a material of choice for polymer composites, electromagnetic shields, super capacitors, gas including hydrogen storage devices, batteries, structural composites, or medical applications (Eklund et al. 2007), especially of MWNTs for biomedical engineering, used in biosensors, as vehicles for drug delivery, and in gene therapy (Kostarelos, Bianco, and Prato 2009).

Most likely, during production and use, some quantities will get into the environment, especially the aquatic compartment. Even if toxicological data are available, obtained

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most often with *in vitro* systems (Guadagnini et al. 2013) and with animal models (Van der Zande et al. 2011), nevertheless ecotoxicological exposure and effect data are necessary for understanding the potential hazards these new carbon-based materials may pose for the environment. As new substances, CNTs require registration under the Toxic Substances Control Act in the USA and in the European Union according to REACh (Registration, Evaluation, Authorization and Restriction of Chemicals) regulations (EU 2008). Some (eco)toxicological and environmental properties of SWNTs and MWNTs are listed in the report ENV/JM/MONO 13/REV (2008), but more is required for the proper evaluation of the potential ecotoxicity of CNTs. Aquatic ecotoxicity assessment of CNTs is a challenge since tests have been developed for water-soluble chemical compounds. Nevertheless, standard environmental hazard assessment is generally appropriate for nanoecotoxicological research (Crane et al. 2008), especially using the test battery concept (Kahru et al. 2008; Blaise et al. 2008) in order to accumulate knowledge about their ecotoxicity (Kahru and Dubourguier 2011) toward a wider range of biological species providing valuable insight into likely exposure scenarios (Zhao and Liu 2012).

The aim of the present work is to contribute to the ecotoxicological assessment of the potential impact of MWNTs as an example of industrial CNTs in aquatic organisms belonging to different trophic levels by carrying out ecotoxicological standardized procedures. The selected species were decomposers (bacteria), primary producers (photosynthetic green algae, *Pseudokirchneriella subcapitata*), primary consumers (invertebrates *Daphnia magna*), and secondary consumers (vertebrate fish and amphibians, *Danio rerio* and *Xenopus laevis*).

Materials and methods

MWNTs preparation of suspensions

MWNT (Graphistrength® C100, Arkema, Colombes, France) suspensions in ultrapure water were prepared by sonication for 10 min at 45 kHz, 80 W (USC 300T, VWR, Fontenay sous Bois, France) just before each bioassay. The physical characteristics and transmission electron microscopic observations of the MWNTs were previously described by Mouchet et al. (2010). Figure 1 displays scanning electron micrographs of MWNTs.

Biological bioassays

Activated sludge respiration inhibition test, OECD (1984) Guideline 209

The inoculum was activated sludge of a small biological domestic wastewater treatment plant (Abidos, France). MWNTs were studied at 500 and 5000 mg L^{-1}. Dissolved oxygen concentrations were determined with an oxygen electrode (Stirrox G, WTW, Weilheim, Germany) and meter (OXI 538, WTW). The inhibitory effect was expressed as percentage of the mean respiration rate of two controls, calculated from the recorder trace as mg O_2 L^{-1} h^{-1} over a period of 10 min. The inhibition was expressed as percentage relative to the mean of the respiration rates in two controls: % inhibition $= [1 - (2R_s/(R_{c1}+R_{c2}))] \times 100$, where R_s is the oxygen consumption rate at the tested concentration of test substance, and R_{c1} and R_{c2} are the oxygen consumption rates for controls 1 and 2. The sensitivity of the test system and the method were evaluated with 3,5-dichlorophenol.

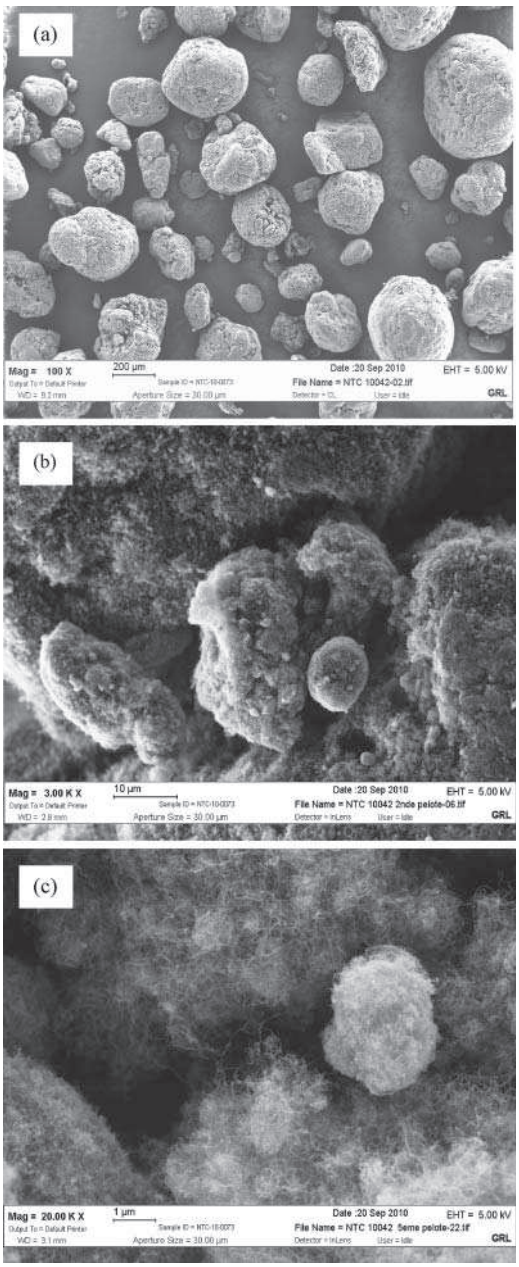

Figure 1. Scanning electron microscopy observations of MWNTs, raw MWNTs from the same sample are observable in balls at different magnifications: (a) 100 X, (b) 3000 X and (c) 20,000 X.

Algal growth inhibition test (P. subcapitata)*, OECD* (*2006*) *Guideline 201*

P. subcapitata (CCAP 278/4 stock) was obtained from the Culture Centre of Algae and Protozoa (Ambleside, UK). The cell density (measured fluorescence, Cytofluor 2350, Millipore, Molsheim, France) for the preliminary test was 1.21×10^6 cells mL^{-1}, and for the definitive test, 1.09×10^6 cells mL^{-1}. Algae were exposed under static conditions over

a time period of 72 h to MWNTs dispersed in water (EN ISO 8692, 2004) at (1) 100, 50, 10, 5, 1, and 0 mg L^{-1} in the preliminary test, and (2) 1000, 500, 230, 105, 48, 22, 10, and 0 mg L^{-1} for the definitive test. The MWNT concentrations resulting in 0% and 100% of the uninhibited cell growth rate, and growth rate inhibition causing a 50% reduction in biomass (E_bC_{50}) within 72 h (E_bC_{50}−72 h) and in growth rate (E_rC_{50}−72 h) were estimated. The sensitivity of the test system and the method were evaluated by performing an algal growth inhibition test on $K_2Cr_2O_7$ (Sigma, Lyon, France). The growth inhibition data were analyzed using an Excel sheet to calculate the effective concentration (EC_{50} value) and the 95% confidence interval. Probit analysis was used to calculate the 24-, 48-, and 72-h EC_{50} values. The no-observed effect concentration (NOEC), the highest tested concentration at which no significant inhibition of growth is observed relative to the control, was estimated by Dunnett's test. Values of pH (345 pH meter, Mettler Toledo, Viroflay, France) and dissolved O_2 (OXI 538 oxymeter, WTW) were measured.

Analytic complementary experiments have been carried out to study the ecotoxicological response of algae in relation to a potential deficiency of ionic metallic species, with the well-known property of CNTs to adsorb ionic species (Li et al. 2009; Stafiej and Pyrzynska 2007). B, Mn, Fe, Co, Ni, Cu, Zn, and Mo were measured in algal media (water dilution) with and without MWNTs after filtration (0.45 μm to remove most of MWNTs) by ICP−MS (ICP−MS 7500, Agilent, Les Ulis, France) at the end of the experiment. Detection limits were 1 μg L^{-1}. Metal traces were measured in water dilution alone, with and without Fe. The algal growth inhibition test was carried out with and without Fe to check the effect of iron deficiency.

D. magna *acute immobilization test, OECD (2004) Guideline 202*

D. magna Straus (*Cladocera*, *Crustacea*), clone 5 and clone A, were from stock breeding in the laboratory reared in Volvic® water added of 0.1 mL L^{-1} B_{12} solution (1 μg L^{-1}, Alfa Aesar, Kandel, Germany), 0.1 mL L^{-1} $Na_2SeO_3{\cdot}5H_2O$ solution (6.7 μg L^{-1}, Sigma), 1 mL L^{-1} solution of $Ca(NO_3)_2{\cdot}4H_2O$ (208 g L^{-1}, Sigma) and $MgCl_2{\cdot}6H_2O$ (28 g L^{-1}, Sigma), and unicellular green freshwater algae (*P. subcapitata* and *Chlorella vulgaris*). A stock suspension at 100 mg L^{-1} was used to realize dilutions of 100, 50, 10, 5, 1, and 0.1 mg L^{-1} of MWNTs in water (EN ISO 6341, 1996) for the preliminary test. In the definitive test, based on the results of the preliminary test, a limit test was performed at 100 mg L^{-1}. Five *D. magna* aged from 6 to 24 h were added to each test flask. Two preliminary and four definitive test replicates were prepared for each concentration. As controls, two preliminary tests and four definitive test flasks without MWNTs were prepared under the same conditions. After 24-h incubation (definitive test), mobile *D. magna* were counted and flasks were placed back for continued incubation. At 48 h, mobile *D. magna* were counted again (preliminary and definitive tests). The sensitivity of the test system and the method were evaluated every month by performing an inhibition test with $K_2Cr_2O_7$ (Sigma). At 24 h and at the end of the 48-h test period, the actual concentrations inhibiting the mobility of daphnids by 50%, i.e. EC_{50}−24 h and EC_{50}−48 h, were estimated. The NOEC was estimated when possible. Dissolved O_2 (OXI 538 oxymeter, WTW) and pH (345 pH meter, Mettler Toledo) were measured at the highest concentration and in the control at the beginning and at all concentrations, and in the control at the end of the test.

D. magna *reproduction test, OECD (2008) Guideline 211*

Daphnia were exposed to MWNTs in a semi-static test from 5 to 100 mg L^{-1}. Exposure water was prepared with Volvic® water complemented as follows: 0.1 mL L^{-1} B_{12} solution (1 μg L^{-1}, Alfa Aesar, Kandel, Germany), 0.1 mL L^{-1} $Na_2SeO_3 \cdot 5H_2O$ solution (6.7 μg L^{-1}, Sigma), 1 mL L^{-1} solution of $Ca(NO_3)_2 \cdot 4H_2O$ (208 g L^{-1}, Sigma), and $MgCl_2 \cdot 6H_2O$ (28 g L^{-1}, Sigma). The test was performed with one Daphnia per vessel and with 10 replicates for each concentration. Ten control flasks without MWNTs were prepared under the same conditions. The positive control was with $K_2Cr_2O_7$ (Sigma). For each exposure concentration, the percentage of inhibition of reproduction was recorded after 21 days. The results of the acute toxicity test were used to define the concentration range for the reproduction test. The MWNT concentrations resulting in 0% and 100% inhibition of reproduction were determined by observation, and EC_{50} was estimated by calculation using the HILL model (an Excel® macro REGTOX http://www.normalesup.org/~vindimian/fr_download.html). The lowest observable effect concentration (LOEC) and NOEC were determined using Dunnett's test.

Fish acute toxicity test (D. rerio)*, OECD (1992) Guideline 203*

The organisms used for the test were *D. rerio* (*Teleostei*, *Cyprinidae*), batch n° 10/Br/01/1 supplied by Aquatrade (Saint Forgeux, France). The sensitivity of the biological reagent was checked at least once for each new batch of fish by determining the lethal concentration at 24 h ($LC_{50}-24$ h) of $K_2Cr_2O_7$. Fish were exposed under static conditions to 1, 35, 50, and 100 mg L^{-1} of MWNTs dispersed in water (EN ISO 7346, 1998) for the preliminary test and to 100 mg L^{-1} for the definitive test. Two replicate test chambers were maintained for each treatment and each control group. For the range-finding test, MWNT suspensions were directly prepared for each concentration, and for the definitive test by weighing the respective amounts of MWNTs into 100 mL water and under adjustment to the 5 L in the test tanks. In both tests, the fish were considered dead if no reaction was observed when no respiratory movement was observed upon stimulation of their caudal peduncle. Visible anomalies were noted, as were any sublethal effects such as loss of balance, altered pigmentation, changes in swimming behavior, or respiratory malfunction. The dead fish were counted and removed from the aquaria. At 24, 48, and 72 h and at the end of the 96-h test period, the concentrations killing 50% of the fish, i.e. $LC_{50}-24$, -48, -72 and -96 h, were estimated. Dissolved O_2 (OXI 538 oxymeter, WTW) and pH (345 pH meter, Mettler Toledo) were monitored.

Amphibian (X. laevis) *bioassays*

Eggs were obtained from the Ecolab laboratory. The procedure for rearing of *X. laevis* and breeding until they reached the development stage appropriate for experimentation – stage 50 of the development table of Nieuwkoop and Faber (Nieuwkoop and Faber 1956) – is described by Mouchet et al. (2008). MWNT dilutions were made in 20 mL of ultra-pure water in glass tubes, and then sonicated (Bioblock 89863, Fisher Scientific, Illkirch, France) for 5 min before their transfer to the exposure media. Exposure was in reconstituted water (RW), i.e. distilled tap water to which nutritive salts were added as described in ISO 21427–1 (ISO 2006). The negative control condition (NC) was RW alone.

The first exposure was under static conditions for 96 h. Larvae were exposed in triplicate groups of 10 animals per flask containing either RW or test media at 10, 50, 100, and 500 mg L^{-1} MWNTs in RW. Each day, the number of dead larvae was counted and the lethal concentration at which mortality occurred for 50% of the animals (LC_{50}) was calculated. The sensitivity of the test system and the method were evaluated using $CdCl_2$ (Sigma).

The second type of exposure was performed for 12 d according to ISO 21427−1 (ISO 2006) for the amphibian micronucleus test (MNT) with a daily renewal of the exposure medium. For the positive control (PC), cyclophosphamide (Sigma) in RW at 20 mg L^{-1} was used. *Xenopus* larvae were exposed to 0.05, 0.1, 0.5, 1, 5, 10, 25, and 50 mg L^{-1} of MWNTs in RW. Larvae were exposed in groups of 20 animals in dishes containing either control media (NC and PC) or test media (0.1, 1, 10, and 50 mg L^{-1} of raw MWNTs in RW). Acute toxicity (mortality) of larvae exposed to MWNTs was examined for 12 d by visual inspection and counting. Chronic toxicity, i.e. growth inhibition, was evaluated by measuring the size of each surviving larva ($n = 20$) at the beginning of exposure (t_0) and at the end of the exposure at day 12 (t_{12}). The measurements and statistical analyses were performed according to Mouchet et al. (2011) using a Kruskall−Wallis test followed by Dunn's test to isolate the group(s) that differ(s) from others, using a multiple comparison procedure with unpaired data versus the NC group ($\alpha < 0.05$). Graphic representations are proposed based on the growth rate calculated as mentioned in Mouchet et al. (2011).

The MNT was performed according to ISO 21427−1 (ISO 2006). At the end of 12 d of exposure − stage 54 (Nieuwkoop and Faber 1956) − larvae are anaesthetized by immersion in a MS222 solution (0.2 g L^{-1}, Sigma) and a blood sample was obtained from each larva by cardiac puncture. The number of erythrocytes containing one micronucleus (MN) or more (micronucleated erythrocytes) was determined under a microscope in a total of 1000 erythrocytes per larva. The statistical method was described in Mouchet et al. (2008). Values of pH (345 pH meter, Mettler Toledo) were measured three times during the 12 days of exposure just before the renewal of the exposure medium ($pH_{24\ h}$) and just after ($pH_{0\ h}$). Al, Fe, and Mo were measured in RW with and without MWNTs after filtration (0.45 µm to remove most of MWNTs) by ICP−MS (ICP−MS 7500, Agilent).

Results

Activated sludge respiration inhibition test

The method was applied with respect to the following criteria: (1) the difference in respiration rates between the two controls was below 15% (Table 1) and (2) EC_{50} of the control

Table 1. Results of respiration and inhibition rate of microorganisms of activated sludge after 3 h in the presence of MWNTs.

	MWNTs (mg L^{-1})			
	C1	C2	500	5000
Respiration rate (O_2 mg L^{-1} h^{-1})	52.5	45.0	54.0	28.0
% inhibition	–	–	0	42.6

C1, C2: control 1, 2

test with the reference 3,5-dichlorophenol was between the validity specified range of 5 and 30 mg L^{-1} (14 mg L^{-1}).

MWNTs did not affect the respiration rate of activated sludge in the conditions of the test up to a concentration of 500 mg L^{-1} at 54 and 28 mg O_2 mg L^{-1} h^{-1}, respectively, for 500 and 5000 mg L^{-1} of MWNTs. Inhibition percentage was 0% and 42.6% for 500 and 5000 mg L^{-1}, respectively. EC_{50} (3 h) was, therefore, higher than 5000 mg L^{-1}.

Algal growth inhibition test (P. subcapitata)

The study was performed in compliance with the following quality criteria: (1) biomass in the control cultures increased exponentially by a factor of 102 higher than 16 within the 72-hour test period which corresponds to a specific growth rate of 0.92 d^{-1}; (2) the mean coefficient of variation for section-by-section specific growth rates (days 0−1, 1−2, and 2−3, for 72-h tests) in the control cultures did not exceed 35%; and (3) the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.

In both tests, inhibition percentage of cell growth and growth rate increase with the increase in MWNT concentrations (Table 2). Total inhibition was observed to 500 mg L^{-1} of MWNTs for the cell growth and to 1000 mg L^{-1} for the growth rate. The MWNTs concentration causing a 50% reduction in cell growth (E_bC_{50}) was estimated at 34 (23−47) mg L^{-1}, and the growth rate (E_rC_{50}) was estimated at 120 (87−160) mg L^{-1}. The NOEC was also estimated at 10 mg L^{-1} for the growth rate inhibition and less than 10 mg L^{-1} for the cell growth. It has to be emphasized that the endpoint used for regulatory purposes is the growth rate and not the cell growth (biomass increase). It was observed that the majority of MWNT particles did not remain in suspension between the beginning and the end of the tests but gathered at the lower part of each flask. Microscopic observations confirmed that the algae appeared normal at the end of the test: The normal shape of *P. subcapitata* algae is a crescent-shaped cell with an average length of 5−10 μm. An increase in the pH was globally observed in both tests for a given concentration between the beginning and the end of the exposure in accordance with classical measures with

Table 2. Average percentage inhibition of cell growth (I_{Ai}) and growth rate ($I_{\mu i}$) of the freshwater algae *Pseudokirchneriella subcapitata* exposed to MWNTs for 72 h: (a) preliminary test; (b) definitive test.

	Nominal concentration of MWNTs (mg L^{-1})	I_{Ai} (%)	$I_{\mu i}$ (%)
(a)	0	0	0
	1	0	0
	5	0	0
	10	0	1
	50	27	7
	100	54	13
(b)	0	0	0
	10	13	2
	22	26	6
	48	54	14
	105	90	44
	230	99	73
	500	100	82
	1000	100	102

Table 3. Measured pH and O_2 concentrations in the preliminary test (a) and in the definitive test (b) of the exposure of the freshwater algae *Pseudokirchneriella subcapitata* to MWNTs.

	Nominal Concentration of MWNTs mg L^{-1}	pH		Dissolved O_2 (mg L^{-1})	
		T_0	$T_{72\ h}$	T_0	$T_{72\ h}$
(a)	0	7.89	7.91	9.0	9.6
	1	7.92	9.42	9.1	9.7
	5	7.94	9.29	9.1	9.5
	10	7.94	9.40	9.0	9.7
	50	7.95	8.83	9.0	9.4
	100	7.96	8.62	9.0	9.3
(b)	0	7.96	7.98	8.8	8.8
	10	7.90	8.90	9.0	8.9
	22	7.91	8.60	9.0	8.8
	48	7.89	8.30	9.0	8.7
	105	7.90	8.04	9.0	8.6
	230	7.91	7.97	9.0	8.5
	500	7.96	7.97	9.0	8.5
	1000	8.03	7.99	8.9	8.4

algae (Table 3). This may be associated with consumption of the dissolved CO_2 due to the growth of algae. Above 230 mg L^{-1} of MWNTs, pH becomes stable during 72 h. Evolution of dissolved O_2 concentration during 72 h is not significant.

Table 4 highlights the decrease of Fe concentrations under MWNT exposure. 11.65 μg kg^{-1} of Fe was measured in the presence of Fe and the absence of MWNTs, whereas no Fe was measured in the presence of both Fe and MWNTs. Zn concentrations also decreased in less proportion from 3.4 μg L^{-1} in the presence of Fe and the absence of MWNTs. Other elements were not affected by the treatment. The results of cell growth and growth rate inhibition without Fe check the effect of iron deficiency and demonstrate iron absorption by MWNTs. Indeed, both inhibition rates lead to 88.88% and to 57.22% in Fe-deprivation medium, whereas there is no inhibition of growth in presence of Fe.

D. magna *acute immobilization test*

The study was performed in compliance with the following quality criteria: (1) the immobilization in the control did not exceed 10% at the end of the test, (2) daphnids in the control were not trapped at the surface of the water, and (3) the dissolved oxygen concentration remained above 3 mg L^{-1} over the test period. No immobilization

Table 4. Measured metal species in μg kg^{-1} using ICP−MS in alga medium in presence or absence of Fe and MWNTs.

			B	Mn	Fe	Co	Ni	Cu	Zn	Mo
Alga medium	−Fe	−MWNTs	53.2	110.4	<1	<1	<1	<1	1.6	2.7
Alga medium	+Fe	−MWNTs	39.5	122.8	11.6	<1	<1	<1	3.4	3.3
Alga medium	+Fe	+MWNTs	43.8	94.8	<1	<1	<1	<1	<1	3.2

ICP−MS: Inductively coupled plasma−mass spectrometry.
B: Boron − Mn: Manganese − Fe: Iron − Co: Cobalt − Ni: Nickel − Cu: Copper − Zn: Zinc − Mo: Molybdenum.
Measured values correspond to mean value from two replicates.

Table 5. Dissolved O_2 and pH measured at the beginning (T_0) and at the end ($T_{48\ h}$) of the exposure of *Daphnia magna* to MWNTs for the definitive test of immobilization.

MWNT Concentrations mg L^{-1}	pH		Dissolved O_2 (mg L^{-1})	
	T_0	$T_{48\ h}$	T_0	$T_{48\ h}$
0	7.95	7.77	8.4	8.1
100	7.95	7.73	8.5	8.3

effect is observed, regardless of the MWNT concentration in both tests, at 24 and 48 h. After the 24-h and 48-h test periods, the actual concentrations inhibiting the mobility of daphnids, i.e. EC_{50}−24 h and EC_{50}−48 h, were estimated to be both higher than 100 mg L^{-1}. Neither pH nor concentrations of dissolved O_2 were impacted in the presence of 100 mg L^{-1} of MWNTs during 48 h (Table 5). The appearance of the test suspensions was visually checked at the beginning and at the end of the test: as flasks were continuously maintained under axial rotation by use of a cylindrical roller device, MWNTs remained in suspension.

D. magna *reproduction test*

The study was performed in compliance with the quality criteria: (1) the mortality in the controls (parent females) did not exceed 20% at the end of the test, and (2) the average cumulative number of living young produced per surviving parent female was higher than 60 in the controls at the end of the test. The percentage of inhibition of the reproduction was dose dependent (Table 6). Inhibition percentage ranged from 0.30 (to 10 mg L^{-1} of MWNTs) to 21.06% (at 100 mg L^{-1} of MWNTs). The EC_{50} value was 317.75 mg L^{-1}, and LOEC and NOEC were 100 and 47 mg L^{-1}, respectively. After filling and between each renewal, there was sedimentation of the MWNTs at the bottom of the flasks.

Fish acute toxicity test (D. rerio)

The study was performed in compliance with the following quality criteria: (1) the mortality in the control did not exceed 10% at the end of the test; (2) the concentration of dissolved oxygen in the test vessels remained above 60% of the air saturation value at the end of the test; (3) the pH did not vary by more than 1 unit. The results indicated no mortality effect regardless of the MWNTs concentration in both tests and irrespective of exposure time, 24, 48, 72 or 96 h (Table 7). LC_{50} were then higher than 100 mg L^{-1} at each time. pH (Table 7) and saturation in oxygen (Table 8) were stable both in the preliminary and

Table 6. Results of the *Daphnia magna* reproduction test. Percentage of inhibition of reproduction measurement after 21 days of exposure.

MWNT concentrations (mg L^{-1})	0	5	10	22	47	100
Mean	193.70	197.67	193.11	186.40	169.70	152.90
Standard deviation	21.78	64.40	61.74	15.60	27.08	46.73
% Inhibition	–	2.05	0.30	3.77	12.39	21.06

Table 7. Measured pH in (a) preliminary test at the beginning (0 h) of the experiment and at the end of exposure (96 h) and in (b) definitive test, each having a total exposure time of 24 h in fish experiment.

		pH	
(a)	MWNT concentrations (mg L^{-1})	0 h	96 h
	0	7.81	7.81
	1	7.76	7.81
	35	7.83	7.83
	50	7.86	7.87
	100	7.85	7.87

		pH				
(b)	MWNT concentrations (mg L^{-1})	0 h	24 h	48 h	72 h	96 h
	0	7.76	7.77	7.46	7.78	7.83
	100	7.82	7.75	7.76	7.80	7.85

definitive tests. Thanks to the stirring device, it was observed that many of the MWNT particles remained in suspension within each tank.

Amphibian (X. laevis) *bioassays*

No mortality was observed until 72 h of exposure whatever the MWNT concentration. Very low mortality was observed from 50 mg L^{-1} at 96 h of exposure and was not significant compared to the negative control (0 mg L^{-1}). EC_{50} was then estimated to be higher than 500 mg L^{-1}.

Results of *Xenopus* exposure for 12 days in semi-static conditions indicated 20% mortality at 50 mg L^{-1} of MWNTs. No mortality was observed at lower concentrations. Growth inhibition results (Figure 2) were significantly evidenced in larvae exposed to 25 (no growth of larvae) and 50 mg L^{-1} of MWNTs (four times less). MN induction in *Xenopus* larvae after 12 days of exposure to the referent genotoxic CP (Figure 3) was significant

Table 8. Measured O_2 concentrations (a) in the preliminary test at the beginning of the experiment (0 h) and at the end of exposure (96 h) and (b) in the definitive test.

		Dissolved O_2 (mg L^{-1})	
(a)	MWNT concentrations (mg L^{-1})	0 h	96 h
	100	8.0	9.2
	50	8.3	9.3
	35	8.3	9.2
	1	8.1	9.1
	0	8.2	9.1

		Dissolved O_2 (mg L^{-1})				
(b)	MWNT concentrations (mg L^{-1})	0 h	24 h	48 h	72 h	96 h
	100	95	99	96	97	98
	100	95	99	95	98	98
	0	95	98	93	98	98
	0	95	98	93	97	97

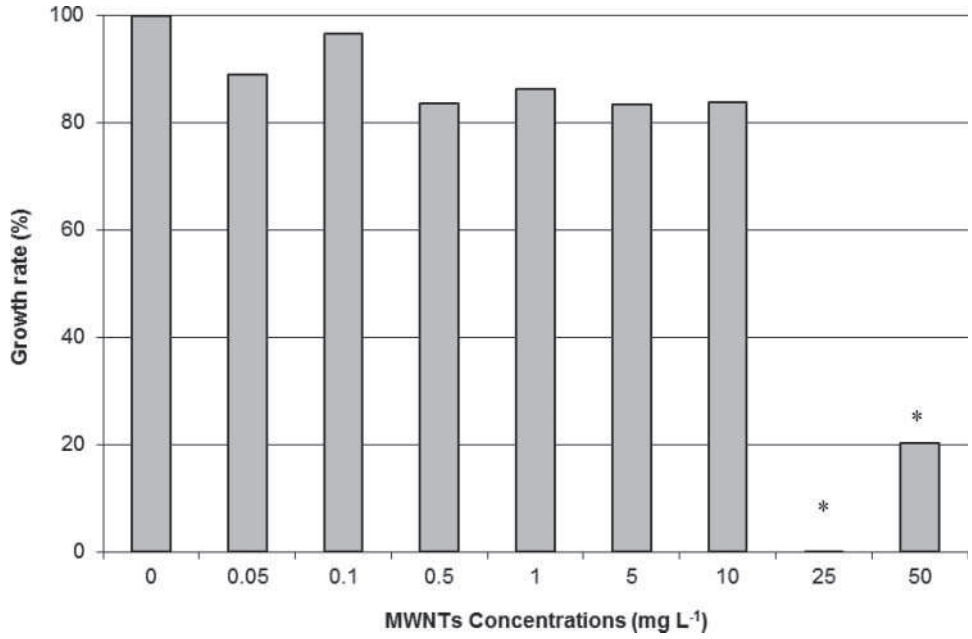

Figure 2. Growth inhibition measurement of *Xenopus* larvae after 12 days of semi-static exposure to MWNTs.
Note: *indicates a significant lower length compared to the control (0 mg L^{-1}). Growth rate is calculated as a percentage based on the length measurement of larvae at the beginning of the exposure and at the end.

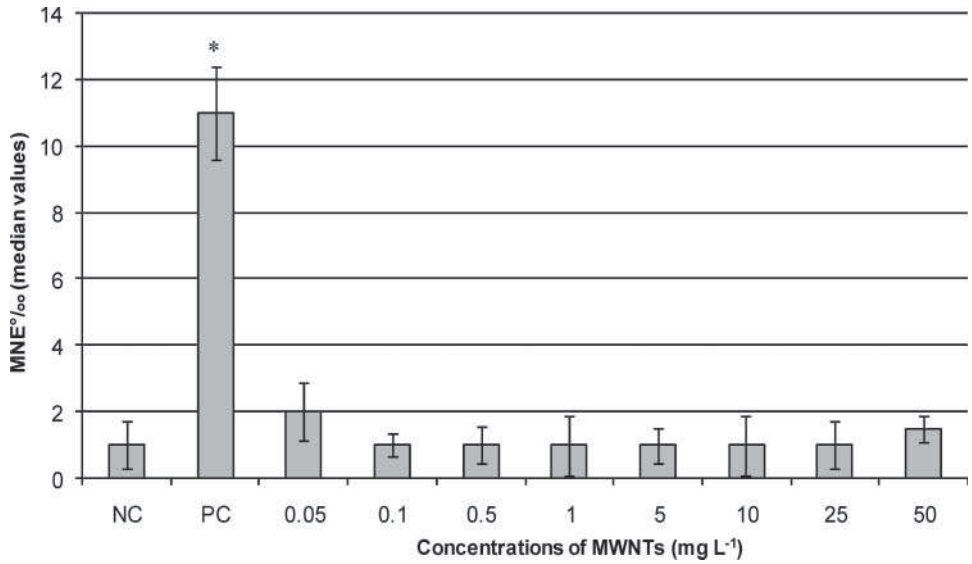

Figure 3. Micronucleus induction measurement (median ± IC 95%) in erythrocytes of *Xenopus* larvae after 12 days of semi-static exposure according to the concentration of MWNTs.
Note: *indicates a genotoxic condition compared to the negative control NC (0 mg L^{-1} of MWNTs).PC: Positive Control, Cyclophosphamide, genotoxic of reference to 20 mg L^{-1}.MNE°/oo: Micronucleated erythrocytes.

Table 9. Measured pH values during the 12 days of exposure of *Xenopus* larvae to different concentrations of MWNTs. $pH_{0\ h}$ was measured just after the renewal of the exposure medium. $pH_{24\ h}$ was measured just before the renewal.

MWNT concentrations (mg L^{-1})	$pH_{0\ h}$	$pH_{24\ h}$
0	8.00	7.31
0.05	8.04	7.30
0.1	8.04	7.26
0.5	8.05	7.27
1	8.14	7.22
5	8.12	7.33
10	8.27	7.19
25	8.14	7.43
50	8.09	7.29

compared to the NC. This result validates the bioassay. The results of MN induction in larvae exposed to MWNTs indicated no genotoxicity compared to the NC group. Median values were distributed without MWNTs' dose−effect relation. pH values (Table 9) were slightly lower after 24 h of exposure ($pH_{24\ h}$) compared to just after the renewal of the exposure medium ($pH_{0\ h}$). It may be in relation to the excretion process of larvae for 24 h and the acidification of the exposure media. There was no pH modification in relation to MWNT concentrations. Metals were dosed in water exposure in the presence or absence of 50 mg L^{-1} of MWNTs, without larvae, after 24 h of contact (Table 10). 12.8 ± 0.6 μg L^{-1} of Mo and 131.5 ± 5.4 μg L^{-1} of Al were measured in medium in the presence of MWNTs, whereas concentration of Fe was under the quantification limit (<10 μg L^{-1}).

Discussion

The aim of the present work is not to compare the biological effects between different biological models but to contribute to a better understanding of the ecotoxicity of CNTs and their environmental exposure assessment to provide valuable insight into likely exposure scenarios at different levels of the trophic chain. Synthetic results of the biological effects in organisms after MWNT exposure are presented in Table 11. The results are as follows: no toxicity in activated sludge (bacteria) at 500 mg L^{-1} of MWNTs, no acute toxicity in fish and daphnia up to 100 mg L^{-1} of MWNTs, inhibition of growth in amphibian larvae at 25 mg L^{-1} of MWNTs, and a notable effect at high concentrations of MWNTs with the growth inhibition test in algae (EC_{50} = 120 mg L^{-1} and NOEC = 10 mg L^{-1}).

Observed toxicity in this present work is in accordance with much of the data published in the literature relative to the potential toxicity of raw CNTs in aquatic organisms.

Table 10. Measured metal species (Al, Fe, Mo) in μg L^{-1} using ICP−AES in amphibian mediums in the presence or absence of 50 mg L^{-1} of MWNT after 24 h of contact.

	Fe	Mo	Al
−MWNTs	<10 μg L^{-1}	<10 μg L^{-1}	<50 μg L^{-1}
+MWNTs	<10 μg L^{-1}	12.80 ± 0.58 μg L^{-1}	131.50 ± 5.36 μg L^{-1}

Fe: Iron − Mo: Molybdenum − Al: aluminum.
Calculated values correspond to mean value from 4 replicates (± standard error).
Quantification limit (QL) is 10 μg L^{-1} for Fe and Mo, and 50 μg L^{-1} for Al.

Table 11. Synthetic results of the biologic effects in organisms after exposure to MWNTs.

Bioassay	Organism	Biological endpoint	Exposure time	Measure	EC_{50} mg L^{-1}	NOEC mg L^{-1}	LOEC mg L^{-1}
OECD 209	Bacteria activated sludge	Inhibition percentage	3 h	0% at 500 mg L^{-1} 42.6% at 5000 mg L^{-1}	>5000		
OECD 201	Fresh algae *Pseudokirchneriella subcapitata*	Cell growth − biomass	72 h		34	<10	
		Growth rate − cell multiplication	72 h		120	10	
OECD 202	Daphnid	Mobility	24 h		>100		
			48 h		>100		
OECD 211	*Daphnia magna*	Reproduction	21 days		317.75	47	100
ISO 21 427−1	Amphibian *Xenopus laevis*	Mortality	96 h		>500		
		Mortality	12 days	20% at 50 mg L^{-1}			
		Growth inhibition	12 days	Significant inhibition from 25 mg L^{-1}			
		Micronuclei (MN) induction	12 days	No significant MN induction			

An LOEC of 10 mg L^{-1} was observed in daphnia (Roberts et al. 2007), in marine copepod (Templeton et al. 2006) and in amphibians (Mouchet et al. 2008). Zhu and collaborators (Zhu et al. 2009) calculated EC_{50} to 8.72 and 1.30 mg L^{-1} for immobilization of daphnia exposed to raw SWNTs and MWNTs, respectively, and to 22.57 and 2.42 mg L^{-1} for mortality exposed to raw SWNTs and MWNTs, respectively. Kennedy and collaborators (Kennedy et al. 2008) calculated EC_{50} for mortality after 48 h of raw MWNTs exposure in daphnia at 50.9 mg L^{-1}. No toxicity was evidenced for hydra and crustaceans up to 100 mg L^{-1} of raw SWNTs (Blaise et al. 2008), whereas toxicity to algae exposed to SWNTs was observed at 10 mg L^{-1}. Cheng, Flahaut, and Cheng (2007) observed hatching delay in fish eggs exposed to 120 mg L^{-1} of raw SWNTs and 240 mg L^{-1} of raw DWNTs. Neither mortality nor growth inhibition was observed in urodele amphibian larvae up to 1 g L^{-1} of raw DWNTs (Mouchet et al. 2007), whereas mortality was observed at 50 mg L^{-1} and growth inhibition from 10 mg L^{-1} in anuran amphibian larvae exposed to raw DWNTs (Mouchet et al. 2008, 2011) as well as growth inhibition at 50 mg L^{-1} for raw MWNTs exposure (Mouchet et al. 2010). Kahru and Dubourguier (2011) calculated on the basis of 34 median values a $L(E)C_{50}$ between 1 and 10 mg L^{-1} for SWNTs and MWNTs ($L(E)C_{50}$ derived from 77 individual values. The majority of the published results indicate that exposure to CNTs generally leads to biological disorder at different levels but usually above unrealistic concentrations of 10 mg L^{-1}. In surface water in Europe, for the simulation results of the predicted environmental concentrations, Gottschalk et al. (2009) indicated lower CNTs concentrations, with 0.004 ng L^{-1} (most frequent value) and 0.0035 ng L^{-1} as the range of the lower quantile and 0.021 g L^{-1} as the upper quantile. Nevertheless, it could be hypothesized that CNT concentrations accumulate into the environment over time.

Toxicity obtained from algae in the present work appears to be in relation to the effect of Fe deficiency on algal growth. Indeed, algal growth experiments in the absence of iron indicated 88.8% of cell growth inhibition (biomass) and 57.2% of growth rate inhibition (cell multiplication) compared to the absence of inhibition when iron was present. Moreover, among the 8 micro-nutrients that are present in the algal culture medium (boron, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum), iron is strongly removed by the MWNTs from the filtrated culture medium (<1 μg kg^{-1}). When compared to the initial algal medium in which iron concentration is 11.65 μg kg^{-1}, this leads to suspect iron adsorption by MWNTs. Iron ion adsorption has already been demonstrated in chemical studies using different types of CNTs (Li et al. 2009). In contrast to the present growth inhibition of *P. subcapitata* due to iron deprivation, Schwab et al. (2011) indicated that observed inhibition of *C. vulgaris* and *P. subcapitata* is in relation to light masking by CNTs, which can adhere to algal surfaces and hence restrict light accessibility to the cells, resulting in the inhibition of growth. Long et al. (2012) indicated that their MWNTs significantly inhibited the algal growth of *Chlorella* sp. with a negligible contribution of metal catalyst residues in the MWNTs and nutrient elements adsorbed by MWNTs. These authors hypothesize that the toxicity of algae could mainly be explained by the combined effects of oxidative stress, agglomerations and physicals interactions, and shading effects, with the quantitative contributions from these mechanisms depending on the MWNT size and concentration. In any case, comparison of results between these different works must be limited because of the diversity of studied CNTs. Nevertheless, Verneuil et al. (2014) indicated that only direct exposure to 50 mg L^{-1} of MWNTs (the same type of

MWNTs as in the present study) led to growth inhibition of *Nitzschia palea* after 48 h and suggested that EPS (extracellular polymeric substances) provide considerable protection against MWNTs, without alteration of the photosynthesis.

Concerning the absence of genotoxicity in erythrocytes of *Xenopus* larvae, the present results are in agreement with the previous ones obtained on amphibian larvae in the same conditions of exposure to MWNTs (Mouchet et al. 2010) and DWNTs (Mouchet et al. 2007, 2008). The majority of the time, if acute and chronic toxicities are generally observed after CNTs exposure of different biological models, genotoxicity, especially via micronucleus induction mechanism, is not demonstrated. Kim et al. (2011) indeed obtained no genotoxicity of raw MWNTs according to OECD test guidelines 471 (bacterial reverse mutation test), 473 (*in vitro* chromosome aberration test with and without S9), and 474 (*in vivo* micronuclei test). Di Sotto et al. (2009) and Szendi and Varga (2008) also reported that MWNTs had no mutagenic effect in bacteria systems. In the same way, Wirnitzer et al. (2008) indicated no genotoxicity of raw MWNTs testing for chromosome aberrations in V79 cells and for gene mutations in the *Salmonella* microsome test. Nevertheless, genotoxic effects may be produced either by direct interaction of particles with genetic material or by secondary damage from particle-induced reactive oxygen species. In this context, some authors demonstrated oxidative stress by MWNTs (Reddy et al. 2010; Srivastava et al. 2011), and, for example, in *Xenopus* larvae after MWNT exposure (Saria et al. 2014).

Exposure media for the different organisms would play a role in the observed toxicity of the present work. Nevertheless, characterizations of the MWNT suspension in exposure medium do not appear essential to place it in relation with biological effects because they are observed at very high and unrealistic concentrations. The effects obtained in the present work are globally weakly marked, probably in relation with the MWNTs' limited bioavailability in the water column for organisms because of CNTs' sedimentation at the bottom of containers. As displayed in Figure 1, raw MWNTs appeared as large rather spherical agglomerates of bundles without free or isolated nanotubes. Observed effects in organisms may be in connection with exposure to these agglomerates inducing respiratory and/or intestinal clogging in relation with their absorption and not necessarily related to the intrinsic effects of CNTs (Mouchet et al. 2010 and 2011; Petersen et al. 2011). This result is also in accordance with the observation of CNTs in the guts of aquatic organisms such as *Lumbriculus variegatus* (Petersen, Huang, and Weber 2008), *Arenicola marina* (Galloway et al. 2010), *D. magna* (Zhu et al. 2009), *Hyalella azteca*, *Leptocheirus plumulosus*, and *Ceriodaphnia dubia* (Kennedy et al. 2009). Li and Huang (2011) describe the ingestion of CNTs in *C. dubia* followed by excretion in exposure media. Many of these studies tend to highlight that ingested CNTs by organisms may enter the ecological pyramid via their move up through the food chain. Moreover, excreted CNTs may contribute to maintaining a pressure contamination of CNTs in media.

Nevertheless, a few $\mu g\ L^{-1}$ of Mo and Al (and no Fe) were measured in the water medium of amphibian exposure after 24 h containing the higher concentration of MWNTs (50 mg L^{-1}). It would suggest that they may contribute, especially Al, to the toxicity observed in amphibians to high concentrations of MWNTs, although no genotoxicity was observed. This result encourages us to investigate the potential release of metal impurities at lower concentrations and in function of time in the different water exposures.

Conclusion

The present knowledge concerning the ecotoxic effects of CNTs is rather limited and deserves to be documented more extensively. First, the ecotoxicological hazard assessment needs approaches and measurement tools using standardized test methods. Then, adaptation of well-known protocols is necessary. This work is thus a contribution to the assessment of the potential ecotoxicity of CNTs within the aquatic compartment; it could be helpful for regulatory purposes. The results indicate that MWNT effects are weakly marked and expressed at unrealistic nominal concentrations (approximately 10 mg L^{-1}), in relation with probable MWNT ingestion. Considering their increasing use in commercial products, this study emphasizes the need to further study their ecotoxicity and highlights that assessing the risks of CNTs requires a better understanding of their toxicity, bioavailability, and behavior in relation with their intrinsic physicochemical properties.

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Disclosure statement

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