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Effects at molecular level of multi-walled carbon nanotubes (MWCNT) in *Chironomus riparius* (DIPTERA) aquatic larvae

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ABSTRACT

Nowadays, due to the physical, chemical, electrical, thermal and mechanical properties of carbon nanotubes (CNT), its have been currently incorporated into biomedical products and they are employed in drug delivery drug administration, biosensor design, microbial treatments, consumer products, and new products containing CNT are expected in the future. CNT are hydrophobic and have a tendency to accumulate in sediments if they are released into aquatic ecosystems. Vertebrate studies have revealed concerns about the toxicity of carbon nanotubes, but there is very limited data on the toxic effects in aquatic invertebrate species. The aim of the present study is to determine the effects of MWCNT in *Chironomus riparius* at the molecular level, understanding its mode of action and analyzing the suitability of this species to monitor and assess risk of nanomaterials in aquatic ecosystems. To evaluate possible toxic effects caused by carbon nanotube environmental dispersion with regard to aquatic compartment, we study the mRNA levels of several related genes with DNA repairing mechanisms, cell stress response, cell apoptosis and cytoskeleton by Real-Time PCR and proposed a freshwater invertebrate *C. riparius,* which is a reference organism in aquatic toxicology. The obtained results show a transcriptional alteration of some genes included in this study, indicating that different cell processes are affected and providing one the first evidences in the mechanisms of action of MWCNT in invertebrates. Moreover, this data reinforces the need for further studies to assess the environmental risk of nanomaterial to prevent future damage to aquatic ecosystems.

1. Introduction

Carbon nanotubes (CNT) are currently used in different applications (Mauter and Elimelech, 2008; De Volder et al., 2013). Their structural characteristics provide them with exceptional electrical, physicochemical and mechanical properties. The carbon based engineered nanoparticles include single-walled carbon nanotubes (SWCNT), and multi-walled carbon nanotubes (MWCNT), spherical fullerenes and dendrimers. Polymeric materials containing CNT are increasingly used in different products such as consumer products, construction, aerospace, medical products, textiles, sports articles, fertilizers, pesticides, *etc.* (Lee et al., 2010; Negri et al., 2010; Amenta and Aschberger, 2015; Vance et al., 2015). This increase of production leads to an inevitable release into the aquatic environment. CNT are dispersible in water and accumulate in soils. Environmental risk assessment of CNT in aquatic ecosystems is essential because their use and manufacturing are quickly expanding. The negative effect caused by CNT release into the environment has been explored in the last few years using organisms that inhabit terrestrial, sediment or aquatic habitats. Although various studies of CNT toxicity appear in the literature, there is an unclear description of their toxic effect. Some works have shown a lack of toxic effects (Petersen et al., 2011) but other studies have described potential impacts of CNT on aquatic and soil invertebrates (Baun et al., 2008; Ali et al., 2014). The use of nanoparticles in agrochemicals or soil remediation has a toxic effect in different organisms (Demir et al., 2011; Hayashi et al., 2012). On the other hand, adverse effects of SWCNT and MWCNT on growth, reproduction and mortality have been reported in several aquatic organisms such as the diatom *Thalassiosira pseudonana*,

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the copepods *Amphiascus tenuiremis* and *Tigriopus japonicus* and cladoceran *Daphnia magna* (Templeton et al., 2006; Roberts et al., 2007; Zhu et al., 2009; Edgington et al., 2010; Kwok et al., 2010). MWCNT can be accumulated in zooplankton (Edgington et al., 2010; Alloy and Roberts, 2011) and earthworms (Scott-Fordsmand et al., 2008; Calisi et al., 2016). In addition, other studies describe MWCNT enhancing the bioavailability and toxicity of different contaminants into organisms (Hu et al., 2013; Zhang et al., 2014; Wang et al., 2016; Zindler et al., 2016; Cano et al., 2018). It is well known that the MWCNT tend to accumulate in the digestive tract of aquatic organisms such as *D. magna* and fish (Petersen et al., 2009; Maes et al., 2014; Bjorkland et al., 2017). Furthermore, trophic transfer of MWCNT is possible in the aquatic ecosystem (Cano et al., 2018).

To analyze the environmental risk of CNT, we evaluate parameters related to genotoxicity, stress proteins and apoptosis.

Genotoxicity is considered to be one of the main parameters in chemical testing and environmental risk assessment. The information provided by the scientific world about genotoxicity of nanomaterials has grown in the last years (van Berlo et al., 2012v). The genotoxicity induced by CNT can be either direct or indirect. Direct genotoxicity is known as the result of physical interactions between the material and the genomic DNA. The indirect genotoxicity is the result of increasing the reactive oxygenated species (ROS), chromosomal aberrations, gene mutations, or apoptosis (Di Giorgio et al., 2011; van Berlo et al., 2012v; Ali et al., 2015). Genotoxic effects implicate DNA damage that can be analyzed by determining the expression of genes related to DNA repair. For example, X-ray repair cross complementing 1 (XRCC1) is a base excision repair (BER) enzyme that is involved in the core processes of single strand break repair (SSBR) (Hanssen-Bauer et al., 2012) and ataxia-telangiectasia mutated (ATM) is a protein that intervenes in response to DNA double-strand breaks (DSB) (Guleria and Chandna, 2016).

On the other hand, heat shock proteins (HSPs) are a family of highly conserved proteins, ubiquitous from prokaryotes to eukaryotes, which are induced in response to a wide variety of biotic and abiotic stressors (*e.g.* chemical, temperature, UV light, hypoxia) (Lindquist and Craig, 1988). They are powerful anti-apoptotic proteins, associated with key apoptotic factors and thereby block the cell death process at different levels (Joly et al., 2010; Wang et al., 2014).

Apoptosis is a programmed cell death pathway that is essential in the development of organisms and functions in the maintenance of homoeostasis. It has been described that the induction of *caspase-3* (*Cas3*), *caspase-8* (*Cas8*), and *caspase-9* (*Cas9*) genes causes apoptosis, and different factors can influence apoptosis and antioxidant gene expression (Ravichandran et al., 2011; Wang et al., 2012). DECAY is a caspase from *Drosophila melanogaster* related to mammalian caspase 3 and caspase 7, involved in cell apoptosis, thus it matters greatly in development and other biological processes (Dorstyn et al., 1999). Moreover, DECAY acts in response to diverse stimuli of apoptosis in a wide variety of cell types as an effector.

It has been previously reported that some carbon based nanomaterials such as fullerenes, carbon nanotubes, graphene and its derivatives, affect the organization and redistribution of the actin cytoskeleton. However, their interaction mechanism with the organism has to be further studied (Shams et al., 2014; Dong et al., 2016).

In the present study, we assess the toxicity of MWCNT in the larvae of *Chironomus riparius*, which is a reference organism in the aquatic toxicology. The midge *C. riparius* is widely used as a test organism in environmental toxicology to investigate the potential of chemicals and also, as a reference organism in standardized protocols, (OECD, 2010, 2011). In this work, we evaluated in *C. riparius* the expression level analysis of genes implicated in DNA repairing, cell stress response, cytoskeleton and cell apoptosis could be a useful approach to study the adverse effects of carbon nanomaterials in freshwater invertebrates.

2. Materials and methods

All experiments were performed in triplicate.

2.1. Animals and treatments

Chironomus riparius were maintained under standard laboratory conditions (Martínez-Paz, 2018). Fourth instar larvae (n = 10 per replica) were exposed to 10, 100, 1000 µg/L of MWCNT diluted in culture medium for 24 h. No food or substrate was provided to the larvae during treatment. No mortality was observed during the exposures. The treatments were of three independent experiments performed in each analysis using samples from three different egg masses. The control larvae used were exposed to the same concentration of solvent (Pluronic™ 0.015%) as the corresponding treatment. Larvae exposed to MWCNT and untreated larvae were stored at −80 °C.

2.2. Multi-walled carbon nanotubes (MWCNT)

MWCNT (Short-Purified, > 95% carbon purity, < 2% amorphous carbon, average diameter/length 6–9 nm/5 µm), produced by the chemical vapor deposition (CVD) process with CoMoCat catalyst were purchased from Sigma Aldrich (Tres Cantos, Madrid, Spain). Metal content and functionalities present in these preparations were further characterized by Total X Ray Fluorescence TXRF (Fe 0.25% and Co 0.13% and FT-IR (ATR-Ge) ν = 1691, 1557, 1413 cm^{-1}).

Reagents: nitric (65%) and sulphuric (98%) acids were purchased by Carlo Erba SDS (Sabadell, Barcelona, Spain); Pluronic™ F68 at Alfa Aesar (Thermo Fisher, Karlsruhe, Germany); MilliQ Water from water purification system Direct-Q™ (Merck-Millipore, Madrid, Spain); phosphorus pentoxide (Sigma Aldrich, Tres Cantos, Madrid, Spain). Membrane filter Omnipore®PTFE were purchased from Merck (Madrid, Spain) and Universal Indicator paper pH1-pH14 from Johnson Test Paper (Oldbury, UK).

Total reflection X-ray fluorescence analyses (TXRF), were carried out in a TXRF EXTRA-II instrument, (Rich & Seifert, Ahrensburg, DE) with a V internal standard.

Fourier Transformed Infrared (FT-IR) spectroscopy was performed in a FT-IR Bruker Vector 22 (Bruker Daltonics, Rheinstetten, DE) spectrometer with an Attenuated Total Reflection (ATR) analyzer (Ge Miracle TM Selecta). Raman Spectroscopy was realized in spectrometer NT-MDT NTEGRA Spectra, equipped with a monocromator Solar TII MS5004i (520 mm focal) and a detector CCD Andor iDUS DU-420 (1024 × 128 pixels). Excitation source: solid state laser with a λ = 532 nm and power = 22 mW attenuated by variable neutral density filter adjusted at ND 0.6.

Solid samples were measured without any additional preparation by depositing a small amount of substance in a glass sample holder and placing it under the microscope using a 20-increments air lens (Olympus MPlanFL N 20x/0.45NA). Spectra were recorded with a 30 s exposure time and 10 accumulations. An intermediate resolution diffraction grating was used (600 rows/mm, blaze 500 nm). Spectra were calibrated recording a neon lamp emission spectrum at the same conditions as the samples.

Thermogravimetric Analyses (TGA) were performed using TGA Q500 (TA instruments, Barcelona, ES). Under synthetic air, the TGA analyses were performed with a heating ramp of 50 °C min^{-1} from 20 to 900 °C (flow rate of 90 mL min^{-1}).

2.3. MWCNT oxidation

The oxidizing treatment was previously reported as a way of incorporating carboxylic groups on the borders of the CNT (Li et al., 2008; Samorì et al., 2010; Calle et al., 2018). An amount of 324 mg of pristine MWCNT was added to a mixture (3:1 v/v) of (98%) sulfuric and (65%) nitric acid (36 mL). Then, they were sonicated in a water bath at

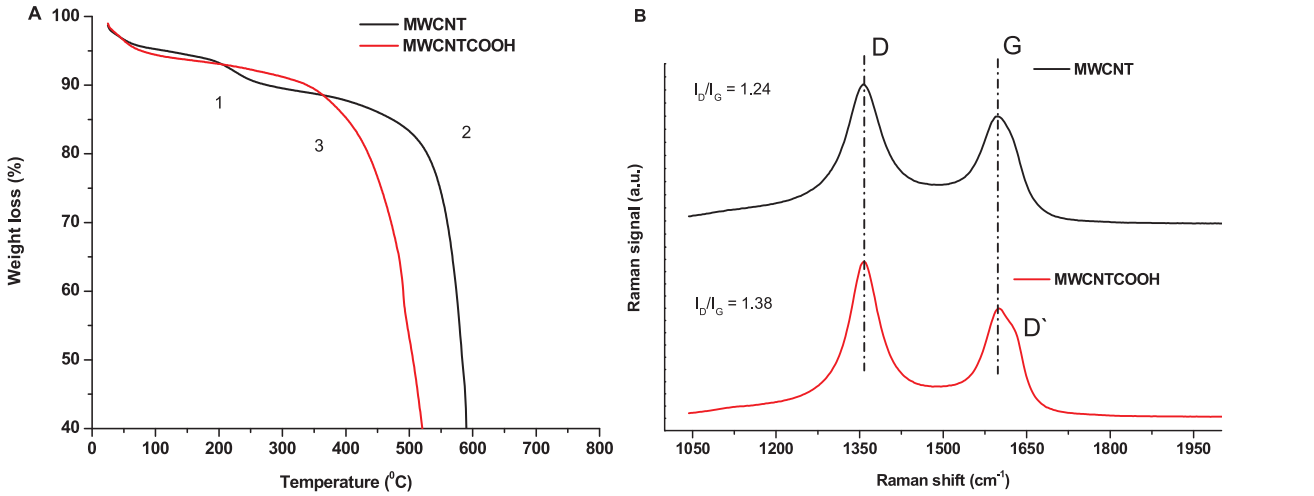

Fig. 1. TGA weight loss (A) of MWCNT (black) and MWCNTCOOH (red) in air atmosphere. Raman spectra (B) of MWCNT before (black) and after (red) oxidation with HNO_3/H_2SO_4.

22 °C for a day (Li et al., 2008; Samorì et al., 2010). After the reaction took place, Milli-Q water was slowly incorporated until the volume reached 1 L. MWCNT was centrifuged until the pH of the washing fractions was 3. Then, a 0.45 µm membrane filter was used to separate the suspension. Afterwards, the MWCNT filtrate was thoroughly washed until its pH was 6. The final oxidized material, MWCNTCOOH, was dried under vacuum at 80 °C using P_2O_5 as drying agent.

2.4. Standard solution preparation

A stock solution for the toxicity studies was prepared using MWCNTCOOH (5.5 mg) in 50 mL of culture medium with a 0.015% of Pluronic F68 (Alfa Aesar, Madrid Spain) used to obtain a stable suspension of MWCNT.

2.5. Photograph analysis

To check out the possible accumulation of MWCNT in *C. riparius*, macro-observations were performed with a digital camera (Sony NEX-5) to observe the general aspect of the exposed larvae to 10, 100 and 100 µg/L of MWCNT for 24 h and were compared to those of the non-exposed controls.

2.6. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using a guanidine isothiocyanate-based method performed with a commercial kit (TRIZOL, Invitrogen) according to the manufacturer's protocol. Subsequently, RNA was treated with RNase-free DNase (Roche) followed by phenolization (phenol: chloroform:isoamyl alcohol-extracted, 25:24:1, and isopropanol-precipitated) and resuspended in DEPC water (Martínez-Paz et al., 2017).

Reverse transcription was performed using 0.5 µg of the isolated RNA, 0.5 µg oligonucleotide dT_{20} primer (Sigma), and 100 units of MMLV enzyme (Invitrogen) in a final volume of 20 µL according to the manufacturer's instructions (Martínez-Paz et al., 2017).

A total of 25 ng of cDNA was used as a template for the PCR. The primers for qRT-PCR have been described previously (Martínez-Guitarte et al., 2007; Morales et al., 2011; Martinez-Paz et al., 2014; Aquilino et al., 2018). The reference genes were *GAPDH* and *ribosomal protein L13* (Martínez-Guitarte et al., 2007). qRT-PCR was performed following the conditions from Martínez-Paz et al. (2017).

2.7. Statistical analysis

The comparison between the control and the treated larvae were done using the variance analysis test (ANOVA) with Dunnett's multiple comparison tests. Normality and homogeneity of variances were calculated by Kolmogorov-Smirnov and Levene tests, respectively.

All statistical tests were performed using SPSS 22.0 (IBM). The results were considered significant at $p < 0.05$. The results are expressed as the mean ± standard error of the mean (SEM) of three experiments.

3. Results

3.1. MWCNT characterization

Non oxidized and oxidized carbon nanotubes were characterized by FT-IR (ATR Ge), TGA, Raman and TXRF. To evaluate metal content, derived from metal catalyst employed in the production process MWCNT were analyzed by Total X-Ray Fluorescence (TXRF): (Fe 0.09%, Co: 0.03%). MWCNT−COOH FT-IR (ATR Ge) display bands at 3300 cm^{-1} from O−H stretching, at 1712 cm^{-1} from C=O stretching (not found in no-oxidized sample), at 1560 cm^{-1} from C=C stretching, and at 1377 cm^{-1} C−O stretching, respectively (ν: 3300, 1910, 1712, 1690, 1560, 1380, 1150, 850 cm^{-1}) (Esteban-Arranz et al., 2018).

The weight loss in air atmosphere of the MWCNT before and after the oxidative treatment is depicted in Fig. 1A. The MWCNT shows two main weight losses: the first one at 250 °C (1) is related with the oxidation of the amorphous carbon present on the surface of the MWCNT. The second one found between 400 and 600 °C (2) is the result of the combustion of the graphitic structure from the MWCNT. After the treatment of the MWCNT with HNO_3 and H_2SO_4 the amorphous carbon content from the material is removed, and some oxygen functional groups such as carboxylic groups are incorporated (Samorì et al., 2010; Calle et al., 2018). The presence of these oxygen functional groups increases the number of defects in the material, which alters its combustion temperature. Therefore a single weight loss between 350 and 450 °C (3) is found, which is in line with a more defective material (Bom et al., 2002; Datsyuk et al., 2008).

The Raman spectra of MWCNT before and after oxidation are depicted in Fig. 1B. Two main bands between 1000 and 2000 cm^{-1} are detected. The D band located at 1355 cm^{-1} is related to the disorder of the hybridized carbon present in the MWCNT. Otherwise, the G-band found at ~ 1595 cm^{-1} is assigned to the sp^2 carbon atoms typical of graphene like structures. There is a minor shift of the G band in the MWCNTCOOH, corroborating the distortion of the graphitic structure

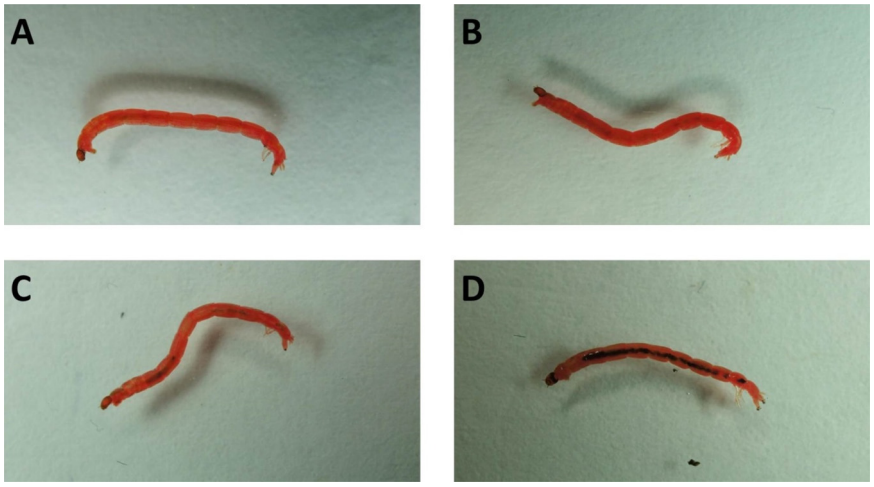

Fig. 2. The presence of MWCNT can be observed in the digestive tract of *C. riparius* larvae exposed to 0 (A), 10 (B), 100 (C) and 1000 (D) μg/L of MWCNT for 24 h.

of the MWCNT after the oxidation process (Cerpa et al., 2013). The relationship between the intensities of the D and G bands (I_D/I_G) is well known as a parameter to quantify the presence of the defects in the material (Dresselhaus et al., 2010). The I_D/I_G ratio has increased from 1.24 to 1.38 after the oxidation, showing a more defected material. These results are in agreement with the ones obtained by TGA in air, given that after the treatment with HNO_3 and H_2SO_4 more defects are created and consequently, lower temperatures are needed to burn out the material.

3.2. MWCNT uptake and accumulation

The first step in this study was to evaluate the ability of the larvae to internalize the MWCNT. Usually nanoparticles and nanotubes enter in aquatic organisms by gills and digestive tracts (Zindler et al., 2016). Analysis of the larvae treated with MWCNT showed that they were accumulated in a dose-dependent manner in the digestive tract (Fig. 2). Then the larvae are able to ingest the nanotubes and they remain in the digestive tract for at least 24 h.

3.3. Effects of MWCNT on DNA repairing genes

As DNA damage activates DNA repairing mechanisms, two genes related with this process, *XRCC1* and *ATM*, were analyzed to evaluate the ability of *C. riparius* cells to manage with nanotubes. *XRCC1* encodes a serine/threonine protein kinase recruited and activated by DNA double-strand breaks while *ATM* encodes a protein involved in base excision repair pathway. The results show that while *XRCC1* mRNA levels were not affected, *ATM* transcriptional activity decreased after 24 h of exposure (Fig. 3).

3.4. Effects of MWCNT on cell stress response genes

To evaluate the stress response in *C. riparius* after MWCNT exposure two genes coding for heat shock proteins were selected. The transcriptional activity of the small heat shock protein gene *hsp27* and *hsp70* genes was analyzed. In both cases a downregulation of mRNA levels was observed. At the highest concentration tested both genes showed a clear downregulation. Moreover, *hsp70* gene was also downregulation at 10 μg/L. However, it is necessary to remark that *hsp27* did show an increase in 10–100 μg/L, but no statistically significant (Fig. 4).

3.5. Effects of MWCNT on cell apoptosis gene

Although there is a lack of genes described in *C. riparius* related with apoptosis, recently it has been described as *decay* (a caspase gene) so we analyzed its mRNA levels to evaluate the effects of MWCNT. The highest concentration tested was able to increase the mRNA levels of this gene suggesting that the MWCNT activated apoptosis (Fig. 5).

3.6. Effects of MWCNT on cytoskeleton gene

To assess whether MWCNT had any effect on cytoskeleton, we evaluate the *actin* mRNA levels. The obtained results suggest that these nanotubes did not modify the expression of this gene in *C. riparius* larvae after 24 h exposure (Fig. 6).

4. Discussion

The use of nanotechnology has been newly extended in different areas (medicine, materials, energy, environment and biotechnology).

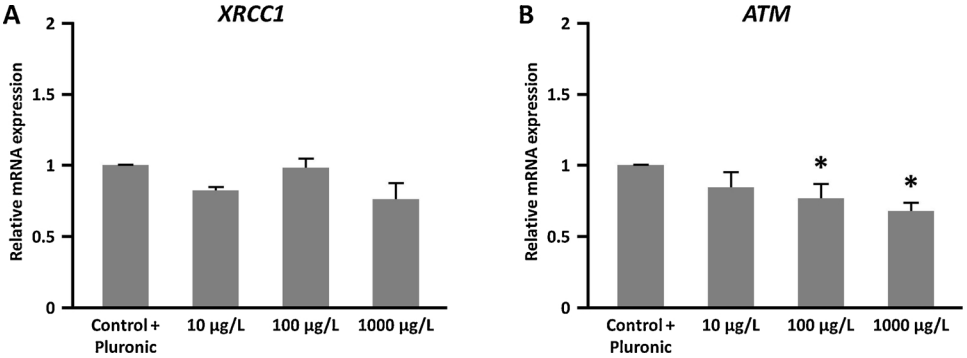

Fig. 3. Changes in mRNA levels of *XRCC1* (A) and *ATM* (B) at 24 h after MWCNT treatments and pluronic (control). Levels were normalized to control, which was set to 1, and it is shown for every treatment the mean ± SEM from three independent experiments (n = 30), each with three sample replicates. * Significant differences ($p \leq 0.05$).

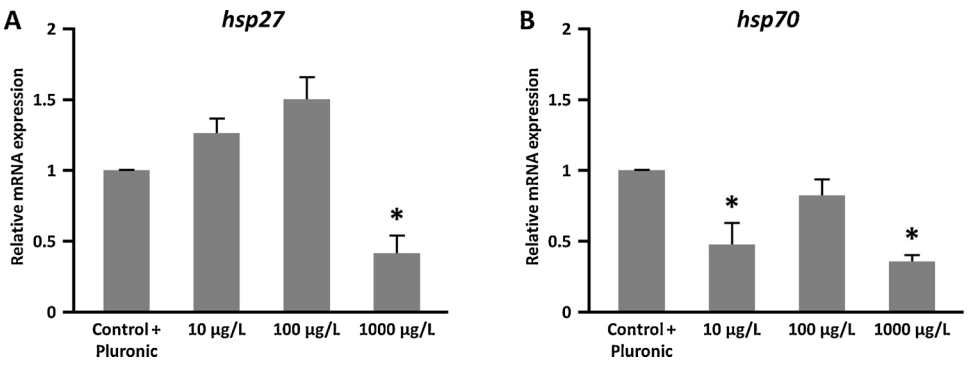

Fig. 4. Changes in mRNA levels of *hsp27* (A) and *hsp70* (B) at 24 h after MWCNT treatments and pluronic (control). Levels were normalized to control, which was set to 1, and it is shown for every treatment the mean ± SEM from three independent experiments (n = 30), each with three sample replicates. * Significant differences ($p \leq 0.05$).

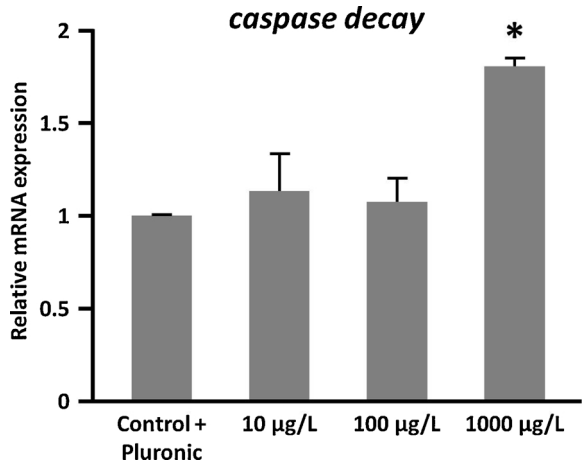

Fig. 5. Changes in mRNA levels of caspase *decay* at 24 h after MWCNT treatments and pluronic (control). Levels were normalized to control, which was set to 1, and it is shown for every treatment the mean ± SEM from three independent experiments (n = 30), each with three sample replicates. * Significant differences ($p \leq 0.05$).

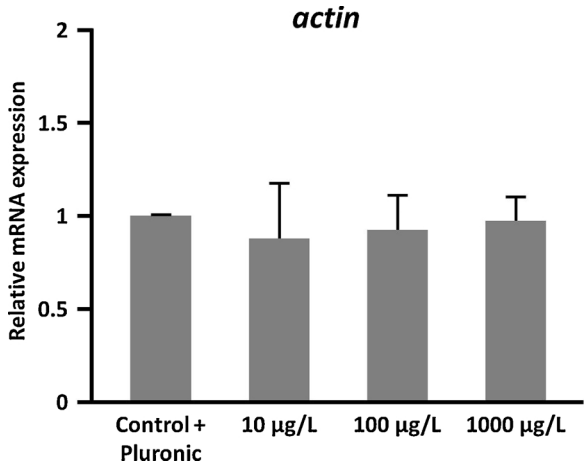

Fig. 6. Comparison of mRNA levels of *actin* at 24 h for treated and control samples. The expression levels were normalized to reference genes and are presented in relation to the values of the control, which were set to 1. * Significant differences ($p \leq 0.05$).

As a result of the increased use and production of CNT, its release in the aquatic environment has also increased (Mottier et al., 2017). *C. riparius* is a benthonic organism and it is expected that the nanoparticles and nanotubes reach the sediments. Presence of MWCNT in the digestive tract is clearly visible in exposed larvae suggesting that the entering route of MWCNT will be mostly by ingestion. MWCNT are gradually accumulated in the gut of exposed *C. riparius*, reaching an appreciable amount after only 24 h. Other works have also reported similar results in other aquatic invertebrates, such as *Daphnia magna* (Sohn et al., 2015) and *Artemia salina* (Zhu et al., 2017), and aquatic vertebrates, such as *Xenopus laevis* tadpoles (Saria et al., 2014) and *Danio rerio* (Maes et al., 2014). Accumulation of MWCNT in *C. riparius* is relevant because it is one of the most abundant dipteran in aquatic ecosystems and an important component of the diet of numerous aquatic and terrestrial species. As a consequence, a subsequent accumulation of MWCNT to higher organisms can be expected in time by their concentration in lower links of the food chain (Cano et al., 2018).

The studies of toxicity assessment of the CNT effects have grown in the last years, although few studies have focused on the molecular and cellular mechanisms (Jain et al., 2007; Roberts et al., 2007; Mauter and Elimelech, 2008; Kennedy et al., 2009; Edgington et al., 2010). The genotoxicity induced by CNTs generate reactive oxygen species (ROS) inducing oxidative stress and DNA damage in vertebrates (Di Giorgio et al., 2011; Ghosh et al., 2011; Guo et al., 2011; van Berlo et al., 2012v; Girardi et al., 2017; Cimbaluk et al., 2018) and micronuclei in cultured cells (Visalli et al., 2015; Catalán et al., 2016). On the other hand, nanoparticles reach the environment affecting invertebrates but we have poor knowledge about their effect in them. Using the comet assay, it has been shown that DNA damage in *Lymnea luteola* is treated with CNTs (Ali et al., 2015) but in general, there are few studies about of the toxic effects of CNTs related with aquatic toxicology.

We have studied the effects induced by commercial MWCNT over *C. riparius,* a sentinel organism for the assessment of pollution ecosystems (OECD, 2010), to elucidate their response to nanotubes and evaluate their ability to manage the damage that they produce. Through exposure inside a range of concentrations described in others works (Saria et al., 2014; Ali et al., 2015; De Marchi et al., 2017, 2018) and below the concentrations described in many others, we have used the transcriptional activity of several genes to analyze the response of DNA repairing mechanisms, the activation of apoptosis, the stress response, and the cytoskeleton. Downregulation of *ATM* and no variations on *XRCC1* indicate that DNA repairing mechanisms are altered differentially, in this case affecting ability to repair double strand breaks but not base mutations. At this moment, it not clear if the mechanisms that work in DNA are damaged by MWCNT although it has been described that they are able to nitrate DNA (Hiraku et al., 2016), produce ROS that mutate genes (Rubio et al., 2016), and induce micronuclei in a differential way in cell lines (Louro et al., 2016). This last study suggests that the length of the MWCNT could have some influence in genotoxicity. Anyway, downregulation of *ATM* could provide an explanation of micronuclei since this gene codes for a protein involved in DNA double strand breaks repairing. On the other hand, some publications suggest that CNT ecotoxicity is mediated by the residual catalyst metals, amorphous carbon or presence of defective sites in the carbon framework (Petersen and Henry, 2012) while inhibition of DNA repair has been described as a main mechanism for the carcinogenicity of certain metals (*e.g.* arsenic, lead and nickel) in humans (Beyersmann and Hartwig, 2008). Altogether this suggests that MWCNT works in two

ways, by affecting the DNA and altering the repairing mechanisms. This double effect in invertebrates could compromise the viability of the population. However, further research is needed to fully understand the mechanisms involved.

HSPs are powerful anti-apoptotic proteins, associating with key apoptotic factors, and have the ability to block the cell death (Joly et al., 2010; Wang et al., 2014). HSP70 and HSP90 can directly interact with several proteins of the programmed cell death machinery and they can block the apoptotic process in different points. In this sense, there are reports that account for the role of HSP27 and HSP70 in apoptotic cell death inhibition (Wang et al., 2014). Here we have observed a significant downregulation of *hsp27* and *hsp70* by MWCNT, a similar result to that obtained for HSP90 with SWCNTs in human lung fibroblasts and keratinocytes (Ong et al., 2017). This suggests an impaired stress response but, as stated before, heat shock proteins are also related with apoptosis regulation. It is possible that downregulation of both genes reflects the apoptosis activation, which could also require reduction of anti-apoptotic proteins levels. The increase of mRNA levels observed for *Decay* supports this idea because this gene code is one of the seven members of the caspase family of cysteine proteases in Drosophila. It has substrate specificity similar to the effector caspases and similarity to caspase 3, an effector caspase (Dorstyn et al., 1999). To our knowledge, the present study describes, for the first time, the capability of MWCNT to alter the expression pattern of a gene involved in the apoptosis pathway in insects. Previous studies in other organisms have shown MWCNT induced apoptosis-related gene expression and antioxidant depletion (Lee et al., 2015). It can be proposed that activation of *Decay* and downregulation of *hsp27* and *hsp70* genes reflect apoptosis progression. The downregulation of *ATM* could explain, in part, the apoptosis activation as a consequence of the impossibility of repairing DNA double strand breaks by the cell. Our results are the first evidence about the ability of MWCNT to activate apoptosis in an insect at environmental concentrations and short exposures. Additional research is needed to unravel the relevance of each of the above postulated mechanisms.

In contrast to other studies, exposure to MWCNT did not imply substantial changes in gene expression of the actin cytoskeleton. Recently, investigations have found that some carbon-based nanomaterials can affect actin cytoskeleton remarkably, including fullerenes derivatives, carbon nanotubes, graphene and its derivatives (Shams et al., 2014; Dong et al., 2016).

5. Conclusions

Our data shows the presence of MWCNT in the digestive tract in exposed larvae and its effects at the molecular level, showing it is able to affect transcription of genes involved in DNA repair mechanisms, cell stress response and cell apoptosis after 24 h of exposure at low concentrations. The effects of MWCNT are demonstrated on a suite of genes related to DNA damage, which are essential for the proper maintenance and survival of the cell under chemical stress conditions. However, further research is needed to fully understand the mechanisms involved and the extent of the damage produced to the cell by MWCNT. The results obtained at 1000 µg/L suggest that the threshold to manage MWCNT damage has been overcome so viability of the population can be compromised. Apoptosis activation in fourth instar larvae, probably by DNA damage and other effects of MWCNT, would interrupt the development eliminating part of the population. Consequently, freshwater ecosystem would be altered in balance by the central role of this species in the food chain.

Disclosure Statement

The authors declare no conflicts of interest.

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References

Ali, D., Yadav, P.G., Kumar, S., Ali, H., Alarifi, S., Harrath, A.H., 2014. Sensitivity of freshwater pulmonate snail Lymnaea luteola L., to silver nanoparticles. Chemosphere 104, 134–140.
Ali, D., Ahmed, M., Alarifi, S., Ali, H., 2015. Ecotoxicity of single-wall carbon nanotubes to freshwater snail Lymnaea luteola L.: impacts on oxidative stress and genotoxicity. Environ. Toxicol. 30, 674–682.
Alloy, M.M., Roberts, A.P., 2011. Effects of suspended multi-walled carbon nanotubes on daphnid growth and reproduction. Ecotoxicol. Environ. Saf. 74, 1839–1843.
Amenta, V., Aschberger, K., 2015. Carbon nanotubes: potential medical applications and safety concerns. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 7, 371–386.
Aquilino, M., Sánchez-Argüello, P., Martínez-Guitarte, J.L., 2018. Genotoxic effects of vinclozolin on the aquatic insect *Chironomus riparius* (Diptera, Chironomidae). Environ. Pollut 232, 563–570.
Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17, 387–395.
Beyersmann, D., Hartwig, A., 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch. Toxicol. 82, 493–512.
Bjorkland, R., Tobias, D., Petersen, E.J., 2017. Increasing evidence indicates low bioaccumulation of carbon nanotubes. Environ. Sci. Nano 4, 747–766.
Bom, D., Andrews, R., Jacques, D., Anthony, J., Chen, B., Meier, M.S., Selegue, J.P., 2002. Thermogravimetric analysis of the oxidation of multiwalled carbon nanotubes: evidence for the role of defect sites in carbon nanotube chemistry. Nano Lett. 2, 615–619.
Calisi, A., Grimaldi, A., Leomanni, A., Lionetto, M.G., Dondero, F., Schettino, T., 2016. Multibiomarker response in the earthworm Eisenia fetida as tool for assessing multiwalled carbon nanotube ecotoxicity. Ecotoxicology 25, 677–687.
Calle, D., Negri, V., Munuera, C., Mateos, L., Touriño, I.L., Viñegla, P.R., Ramírez, M.O., García-Hernández, M., Cerdán, S., Ballesteros, P., 2018. Magnetic anisotropy of functionalized multi-walled carbon nanotube suspensions. Carbon 131, 229–237.
Cano, A.M., Maul, J.D., Saed, M., Irin, F., Shah, S.A., Green, M.J., French, A.D., Klein, D.M., Crago, J., Cañas-Carrell, J.E., 2018. Trophic transfer and accumulation of multiwalled carbon nanotubes in the presence of copper Ions in Daphnia magna and Fathead Minnow (*Pimephales promelas*). Environ. Sci. Technol. 2, 794–800.
Catalán, J., Siivola, K.M., Nymark, P., Lindberg, H., Suhonen, S., Järventaus, H., Koivisto, A.J., Moreno, C., Vanhala, E., Wolff, H., Kling, K.I., Jensen, K.A., Savolainen, K., Norppa, H., 2016. *In vitro* and *in vivo* genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology 10, 794–806.
Cerpa, A., Köber, M., Calle, D., Negri, V., Gavira, J.M., Hernanz, A., Briones, F., Cerdán, S., Ballesteros, P., 2013. Single-walled carbon nanotubes as anisotropic relaxation probes for magnetic resonance imaging. MedChemComm 4, 669–672.
Cimbaluk, G.V., Ramsdorf, W.A., Perussolo, M.C., Santos, H.K.F., Da Silva De Assis, H.C., Schnitzler, M.C., Schnitzler, D.C., Carneiro, P.G., Cestari, M.M., 2018. Evaluation of multiwalled carbon nanotubes toxicity in two fish species. Ecotoxicol. Environ. Saf. 150, 215–223.
Datsyuk, V., Kalyva, M., Papagelis, K., Parthenios, J., Tasis, D., Siokou, A., Kallitsis, I., Galiotis, C., 2008. Chemical oxidation of multiwalled carbon nanotubes. Carbon 46, 833–840.
De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Soares, A.M.V.M., Freitas, R., 2017. Physiological and biochemical responses of two keystone polychaete species: *Diopatra neapolitana* and *Hediste diversicolor* to multi-walled carbon nanotubes. Environ. Res. 154, 126–138.
De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R., 2018. Toxic effects of multi-walled carbon nanotubes on bivalves: comparison between functionalized and nonfunctionalized nanoparticles. Sci. Total Environ. 622–623, 1532–1542.
De Volder, M.F.L., Tawfick, S.H., Baughman, R.H., Hart, A.J., 2013. Carbon nanotubes: present and future commercial applications. Science 339, 535–539.
Demir, E., Vales, G., Kaya, B., Creus, A., Marcos, R., 2011. Genotoxic analysis of silver nanoparticles in Drosophila. Nanotoxicology 5, 417–424.
Di Giorgio, M.L., Bucchianico, S.D., Ragnelli, A.M., Aimola, P., Santucci, S., Poma, A., 2011. Effects of single and multi walled carbon nanotubes on macrophages: cyto and genotoxicity and electron microscopy. Mutat. Res-Gen. Tox. En. 722, 20–31.
Dong, Y., Sun, H., Li, X., Li, X., Zhao, L., 2016. Impact of carbon nanomaterials on actin polymerization. J. Nanosci. Nanotechnol. 16, 2408–2417.
Dorstyn, L., Read, S.H., Quinn, L.M., Richardson, H., Kumar, S., 1999. DECAY, a novel Drosophila caspase related to mammalian Caspase-3 and Caspase-7. J. Biol. Chem. 274, 30778–30783.
Dresselhaus, M.S., Jobrio, A., Souza Filho, A.G., Saito, R., 2010. Defect characterization in graphene and carbon nanotubes using Raman spectroscopy. Philos. Trans. Math. Phys. Eng. Sci. 368, 5355–5377.

Edgington, A.J., Roberts, A.P., Taylor, L.M., Alloy, M.M., Reppert, J., Rao, A.M., Mao, J., Klaine, S.J., 2010. The influence of natural organic matter on the toxicity of multi-walled carbon nanotubes. Environ. Toxicol. Chem. 29, 2511–2518.
Esteban-Arranz, A., Compte-Tordesillas, D., Muñoz-Andrés, V., Pérez-Cadenas, M., Guerrero-Ruiz, A., 2018. Effect of surface, structural and textural properties of graphenic materials over cooperative and synergetic adsorptions of two chloroaromatic compounds from aqueous solution. Catal. Today 301, 104–111.
Ghosh, M., Chakraborty, A., Bandyopadhyay, M., Mukherjee, A., 2011. Multi-walled carbon nanotubes (MWCNT): induction of DNA damage in plant and mammalian cells. J. Hazard. Mater. 197, 327–336.
Girardi, F.A., Bruch, G.E., Peixoto, C.S., Bosco, L.D., Sahoo, S.K., Gonçalves, C.O.F., Santos, A.P., Furtado, C.A., Fantini, C., Barros, D.M., 2017. Toxicity of single-wall carbon nanotubes functionalized with polyethylene glycol in zebrafish (*Danio rerio*) embryos. J. Appl. Toxicol. 37, 214–221.
Guleria, A., Chandna, S., 2016. ATM kinase: much more than a DNA damage responsive protein. DNA Repair 39, 1–20.
Guo, Y.Y., Zhang, J., Zheng, Y.F., Yang, J., Zhu, X.Q., 2011. Cytotoxic and genotoxic effects of multi-wall carbon nanotubes on human umbilical vein endothelial cells in vitro. Mutat. Res. 721, 184–191.
Hanssen-Bauer, A., Solvang-Garten, K., Akbari, M., Otterlei, M., 2012. X-ray repair cross complementing protein 1 in base excision repair. Int. J. Mol. Sci. 13, 17210–17229.
Hayashi, Y., Engelmann, P., Foldbjerg, R., Szabó, M., Somogyi, I., Pollák, E., Molnár, L., Autrup, H., Sutherland, D.S., Scott-Fordsmand, J., Heckmann, L.H., 2012. Earthworms and humans *in Vitro*: characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. Environ. Sci. Technol. 46, 4166–4173.
Hiraku, Y., Guo, F., Ma, N., Yamada, T., Wang, S., Kawanishi, S., Murata, M., 2016. Multi-walled carbon nanotube induces nitrative DNA damage in human lung epithelial cells via HMGB1-RAGE interaction and Toll-like receptor 9 activation. Part. Fibre Toxicol. 13, 16.
Hu, C., Cai, Y., Wang, W., Cui, Y., Li, M., 2013. Toxicological effects of multi-walled carbon nanotubes adsorbed with nonylphenol on earthworm *Eisenia fetida*. Environ. Sci. Processes Impacts 15, 2125–2130.
Jain, A.K., Kumar Mehra, N., Lodhi, N., Dubey, V., Mishra, D.K., Jain, P.K., Jain, N.K., 2007. Carbon nanotubes and their toxicity. Nanotoxicology 1, 167–197.
Joly, A.L., Wettstein, G., Mignot, G., Ghiringhelli, F., Garrido, C., 2010. Dual role of heat shock proteins as regulators of apoptosis and innate immunity. J. Innate Immun. 2, 238–247.
Kennedy, A.J., Gunter, J.C., Chappell, M.A., Goss, J.D., Hull, M.S., Kirgan, R.A., Steevens, J.A., 2009. Influence of nanotube preparation in Aquatic Bioassays. Environ. Toxicol. Chem. 28, 1930–1938.
Kwok, K.W., Leung, K.M., Flahaut, E., Cheng, J., Cheng, S.H., 2010. Chronic toxicity of double-walled carbon nanotubes to three marine organisms: influence of different dispersion methods. Nanomedicine 5, 951–961.
Lee, J., Mahendra, S., Alvarez, P.J.J., 2010. Nanomaterials in the construction industry: a review of their applications and environmental health and safety considerations. ACS Nano 4, 3580–3590.
Lee, J.W., Choi, Y.C., Kim, R., Lee, S.K., 2015. Multiwall carbon nanotube-induced apoptosis and antioxidant gene expression in the gills, liver, and intestine of *Oryzias latipes*. Biomed Res. Int., 485343 2015.
Li, S., Wu, W., Campidelli, S., Sarnatskaïa, V., Prato, M., Tridon, A., Nikolaev, A., Nikolaev, V., Bianco, A., Snezhkova, E., 2008. Adsorption of carbon nanotubes on active carbon microparticles. Carbon 46, 1091–1095.
Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.
Louro, H., Pinhão, M., Santos, J., Tavares, A., Vital, N., Silva, M.J., 2016. Evaluation of the cytotoxic and genotoxic effects of benchmark multi-walled carbon nanotubes in relation to their physicochemical properties. Toxicol. Lett. 262, 123–134.
Maes, H.M., Stibany, F., Giefers, S., Daniels, B., Deutschmann, B., Baumgartner, W., Schäffer, A., 2014. Accumulation and distribution of multiwalled carbon nanotubes in Zebrafish (*Danio rerio*). Environ. Sci. Technol. 48, 12256–12264.
Martínez-Guitarte, J.L., Planelló, R., Morcillo, G., 2007. Characterization and expression during development and under environmental stress of the genes encoding ribosomal proteins L11 and L13 in *Chironomus riparius*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 147, 590–596.
Martinez-Paz, P., Morales, M., Martin, R., Martinez-Guitarte, J.L., Morcillo, G., 2014. Characterization of the small heat shock protein *Hsp27* gene in *Chironomus riparius* (Diptera) and its expression profile in response to temperature changes and xenobiotic exposures. Cell Stress Chaperones 19, 529–540.
Martínez-Paz, P., 2018. Response of detoxification system genes on *Chironomus riparius* aquatic larvae after antibacterial agent triclosan exposures. Sci. Total Environ. 624, 1–8.
Martínez-Paz, P., Morales, M., Urien, J., Morcillo, G., Martínez-Guitarte, J.L., 2017. Endocrine-related genes are altered by antibacterial agent triclosan in *Chironomus riparius* aquatic larvae. Ecotoxicol. Environ. Saf. 140, 185–190.
Mauter, M.S., Elimelech, M., 2008. Environmental applications of carbon-based nanomaterials. Environ. Sci. Technol. 42, 5843–5859.
Morales, M., Planello, R., Martinez-Paz, P., Herrero, O., Cortes, E., Martinez-Guitarte, J.L., Morcillo, G., 2011. Characterization of *Hsp70* gene in *Chironomus riparius*: expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 150–158.
Mottier, A., Mouchet, F., Pinelli, E., Gauthier, L., Flahaut, E., 2017. Environmental impact of engineered carbon nanoparticles: from releases to effects on the aquatic biota. Curr. Opin. Biotech. 46, 1–6.
Negri, V., Cerpa, A., Lopez-Larrubia, P., Nieto-Charques, L., Cerdan, S., Ballesteros, P., 2010. Nanotubular paramagnetic probes as contrast agents for magnetic resonance imaging based on the diffusion tensor. Angew. Chem. Int. Ed. Engl. 49, 1813–1815.
OECD, 2010. Sediment-water chironomid life-cycle toxicity test using spiked water or spiked sediment. Test Guideline No. 233, OECD Guidelines for the Testing of Chemicals. OECD, Paris.
OECD, 2011. Chironomus sp., acute immobilisation test. Test Guideline No. 235, OECD Guidelines for the Testing of Chemicals. OECD, Paris.
Ong, L.C., Tan, Y.F., Tan, B.S., Chung, F.F., Cheong, S.K., Leong, C.O., 2017. Single-walled carbon nanotubes (SWCNTs) inhibit heat shock protein 90 (HSP90) signaling in human lung fibroblasts and keratinocytes. Toxicol. Appl. Pharm. 329, 347–357.
Petersen, E.J., Henry, T.B., 2012. Methodological considerations for testing the ecotoxicity of carbon nanotubes and fullerenes: review. Environ. Toxicol. Chem. 31, 60–72.
Petersen, E.J., Akkanen, J., Kukkonen, J.V., Weber, W.J.Jr., 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. Environ. Sci. Technol. 43, 2969–2975.
Petersen, E.J., Zhang, L., Mattison, N.T., O'Carroll, D.M., Whelton, A.J., Uddin, N., Nguyen, T., Huang, Q., Henry, T.B., Holbrook, R.D., Chen, K.L., 2011. Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. Environ. Sci. Technol. 45, 9837–9856.
Ravichandran, P., Baluchamy, S., Gopikrishnan, R., Biradar, S., Ramesh, V., Goornavar, V., Thomas, R., Wilson, B.L., Jeffers, R., Hall, J.C., Ramesh, G.T., 2011. Pulmonary biocompatibility assessment of inhaled single-wall and multiwall carbon nanotubes in BALB/c mice. J. Biol. Chem. 286, 29725–29733.
Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Lin, S., Ke, P.C., Rao, A.M., Klaine, S.J., 2007. *In vivo* biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. Environ. Sci. Technol. 41, 3025–3029.
Rubio, L., El Yamani, N., Kazimirova, A., Dusinska, M., Marcos, R., 2016. Multi-walled carbon nanotubes (NM401) induce ROS-mediated HPRT mutations in Chinese hamster lung fibroblasts. Environ. Res. 146, 185–190.
Samorì, C., Sainz, R., Ménard-Moyon, C., Toma, F.M., Venturelli, E., Singh, P., Ballestri, M., Prato, M., Bianco, A., 2010. Potentiometric titration as a straightforward method to assess the number of functional groups on shortened carbon nanotubes. Carbon 48, 2447–2454.
Saria, R., Mouchet, F., Perrault, A., Flahaut, E., Laplanche, C., Boutonnet, J.C., Pinelli, E., Gauthier, L., 2014. Short term exposure to multi-walled carbon nanotubes induce oxidative stress and DNA damage in *Xenopus laevis* tadpoles. Ecotox. Environ. Saf. 107, 22–29.
Scott-Fordsmand, J.J., Krogh, P.H., Schaefer, M., Johansen, A., 2008. The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earthworms. Ecotox. Environ. Saf. 71, 616–619.
Shams, H., Holt, B.D., Mahboobi, S.H., Jahed, Z., Islam, M.F., Dahl, K.N., Mofrad, M.R., 2014. Actin reorganization through dynamic interactions with single-wall carbon nanotubes. ACS Nano 8, 188–197.
Sohn, E.K., Chung, Y.S., Johari, S.A., Kim, T.G., Kim, J.K., Lee, J.H., Lee, Y.H., Kang, S.W., Yu, I.J., 2015. Acute toxicity comparison of single-walled carbon nanotubes in various freshwater organisms. Biomed Res. Int., 323090 2015.
Templeton, R.C., Ferguson, P.L., Washburn, K.M., Scrivens, W.A., Chandler, G.T., 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. Environ. Sci. Technol. 40, 7387–7393.
van Berlo, D., Clift, M.J., Albrecht, C., Schins, R.P., 2012v. Carbon nanotubes: an insight into the mechanisms of their potential genotoxicity. Swiss Med. 142, w13698.
Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F.Jr., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotech. 6, 1769–1780.
Visalli, G., Bertuccio, M.P., Iannazzo, D., Piperno, A., Pistone, A., Di Pietro, A., 2015. Toxicological assessment of multi-walled carbon nanotubes on A549 human lung epithelial cells. Toxicol. In Vitro 29, 352–362.
Wang, X., Guo, J., Chen, T., Nie, H., Wang, H., Zang, J., Cui, X., Jia, G., 2012. Multi-walled carbon nanotubes induce apoptosis via mitochondrial pathway and scavenger receptor. Toxicol. In Vitro 26, 799–806.
Wang, X., Chen, M., Zhou, J., Zhang, X., 2014. HSP27, 70 and 90, anti-apoptotic proteins, in clinical cancer therapy (Review). Int. J. Oncol. 45, 18–30.
Wang, X., Qu, R., Liu, J., Wei, Z., Wang, L., Yang, S., Huang, Q., Wang, Z., 2016. Effect of different carbon nanotubes on cadmium toxicity to *Daphnia magna*: the role of catalyst impurities and adsorption capacity. Environ. Pollut. 208 (Pt. B), 732–738.
Zhang, L., Hu, C., Wang, W., Ji, F., Cui, Y., Li, M., 2014. Acute toxicity of multi-walled carbon nanotubes, sodium pentachlorophenate, and their complex on earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. 103, 29–35.
Zhu, X., Zhu, L., Chen, Y., Tian, S., 2009. Acute toxicities of six manufactured nanomaterial suspensions to Daphnia magna. J. Nanopart. Res. 11, 67–75.
Zhu, S., Luo, F., Tu, X., Chen, W.C., Zhu, B., Wang, G.X., 2017. Developmental toxicity of oxidized multi-walled carbon nanotubes on *Artemia salina*cysts and larvae: uptake, accumulation, excretion and toxic responses. Environ. Pollut. 229, 679–687.
Zindler, F., Glomstad, B., Altin, D., Liu, J., Jenssen, B.M., Booth, A.M., 2016. Phenanthrene bioavailability and toxicity to *Daphnia magna* in the presence of carbon nanotubes with different physicochemical properties. Environ. Sci. Technol. 50, 12446–12454.