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Toxic Effects of Multiwalled Carbon Nanotubes on the Zebrafish (*Danio rerio*) and the Brine Shrimp (*Artemia salina*): a Morphological, Histological, and Immunohistochemical Study

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HIGHLIGHTS

Histological changes in zebrafish gills after exposed to MWCNTs;

Zebrafish gills from treated groups with MWCNTs displayed strong staining for HSP 70 and the Bax;

MWCNTs promoted mortality to *Artemia salina* nauplii;

MWCNTs were uptake by *Artemia sallina* nauplii;

Abstract: We describe the toxicity of multiwalled carbon nanotubes (MWCNTs) in two aquatic species: *Danio rerio* and *Artemia salina*. Fish were exposed to 25 mg L^{-1}, 50 mg L^{-1}, and 100 mg L^{-1} MWCNTs for 7 days. After exposure, the gills from *D. rerio* showed lamellar fusion and epithelial lifting in the secondary lamellae, and vasodilation in the primary lamellae. In the gills, heat shock protein 70 and Bax (pro apoptotic member) were stained more intensely in the treated groups than in the control group. The nauplii of *A. salina* were exposed to 25 µg L^{-1}, 50 µg L^{-1}, and 100 µg L^{-1} MWCNTs for 96 h. After exposure, the nauplii from the control group presented 20 % mortality, whereas the 25 µg mL^{-1}, 50 µg mL^{-1}, and 100 µg L^{-1} groups had 46.7 %, 70 %, and 100 % mortality, respectively. The nauplii exposed to 25 µg mL^{1} and 50 µg mL^{1} MWCNTs had black aggregates in the intestine because of the uptake of nanomaterials. In addition, the 25 µg mL^{-1} and 50 µg mL^{-1} MWCNT groups had black aggregates attached onto the body surface, appendages of the second pair, and mandibles.The results revealed the toxic effects of MWCNTs on the two aquatic organisms, and both species showed great potential for toxicological evaluation of MWCNTs in aquatic environments.

Keywords: Apoptosis; Biomarkers; Gills; Nanotoxicology.

INTRODUCTION

Carbon nanotubes (CNTs) are basically formed by cylindrical structures of sp2 carbon atoms. CNTs can be classified according to the number of graphene layers, and two classes are identified: single-walled CNTs (SWCNTs), with a single layer of graphene, and multiwalled CNTs (MWCNTs), with two or more layers of graphene [1-2]. These structures and features provide an unusual and advantageous combination of highly desirable properties in many industrial products and a variety of applications in nanotechnology, electronics, clinical science, and other fields of science [3]. Due to the low cost of synthesis and a non-critical diameter, the production and application of MWCNTs are more favorable compared with those of SWCNTs [4]. Also, the inert and resistant character of MWCNTs increases the accumulation of discarded MWCNTs in the environment, mainly the aquatic environment. Some factors, such as functionalization, purity, size, length, diameter, poor or no dispersion in water, surface chemistry, and no treatment for disposal, enhance the toxicity of CNTs in nature [5]. Because of the large production and wide application of these nanocomposites in various sectors of society, assessing the impact of CNTs on the environment is essential [1,6-10].

Fish represent a rich source of human food and, ecologically, contribute to the functional diversity of aquatic ecosystems. Fish populations are susceptible to various environmental impacts, such as industrial and domestic waste [11-12]. Morphological and molecular changes in key organs of fish homeostasis, such as the gills, liver, and cranial kidney, have been used as biomarkers of aquatic toxicity [11-12]. Gills, in particular, are excellent biomarkers because they are in constant direct contact with water. Therefore, histopathological changes in fish gills play a key role in determining the quality of the aquatic environment [13].

Apoptosis (programmed cell death) and heat shock proteins (HSPs), which are molecular chaperones that interfere with protein synthesis under stress conditions, have also been used as biomarkers of environmental impacts in fish [14]. Apoptosis is important for the maintenance of homeostasis in multicellular organisms [15-16]. It is coordinated by several regulators, such as members of the Bcl-2 family (anti- and pro-apoptotic proteins), p53, cytochrome C, Apaf-1 and initiator, and effector caspases [15]. Bax is a pro-apoptotic protein from the Bcl-2 family that is transported from the cytoplasm to the outer membrane of the mitochondria, where it promotes the production of cytochrome C, which is an important factor in triggering programmed cell death [16]. Heat shock proteins are expressed constitutively, but environmental stress, such as changes in temperature and xenobiotics, can increase the expression of these proteins [17-19]. Among HSPs, HSP70 is the most recorded one and is mainly related to the response to thermal shocks, toxic substances, and environmental changes [19-21].

Studies in ecotoxicology have frequently used *in vivo* assays with model organisms, with a focus on the vertebrate *Danio rerio* and the invertebrate *Artemia salina*. The zebrafish (*D. rerio*) is a small tropical freshwater fish of the order Cypriniformes, originating in South Asia. In the context of evaluating aquatic environments, it is an established experimental model in several research fields, such as genetics, toxicology, pharmacology, and ecotoxicity [3,22-23]. Zebrafish have high fecundity (200 to 300 eggs per spawn), external fertilization, high genetic similarity with mammals (about 70 %), and high sensitivity when exposed to chemical products [24]. The brine shrimp (*A. salina*) is a small crustacean that has been commonly used in aquaculture to feed fish in the early stages of development. Due to its easy handling, transparency, and low cost, *A. salina* has been used with great success in research, from environmental to pharmacological studies, as a bioindicator model for several toxicological tests [25-27].

The goal of our study was to evaluate the toxicity of MWCNTs on the zebrafish and brine shrimp larvae (nauplii), which are two experimental models widely used in toxicological evaluations. We recorded several biomarkers after exposure to MWCNTs in *D. rerio* and different responses in *A. salina* after exposure to the nanocomposites.

MATERIAL AND METHODS

Chemical characterization of MWCNTs and experimental design

The MWCNT (CAS No. 659258, purity > 90%, D × L: 110-170 nm × 5-9 μm) was purchased from Sigma-Aldrich, Brazill. It was dispersed in water plus sonification, following the manufacturer's recommendations. The chemical characterization of MWCNTs can be found in our previous work [28].

For the experimental design, 24 healthy specimens of *D. rerio* of both sexes (1:1) (Wild type AB strain, with an average length of 3.0 cm and an average body weight of 2.5 g) were acclimated for 2 weeks in the

laboratory. The fish were kept in a 14:10 light/dark cycle with dechlorinated water inside a recirculation system. The following water parameters were maintained: temperature 28.0 °C, pH 7.4, and 0.001 ppm ammonia. Moreover, 10 % of the water was exchanged daily. The animals were fed twice a day, in the morning and afternoon, using a commercial feed (Alcon Basic fish feed).

After the acclimatization period, the animals were divided into four groups that were kept in 3 L tanks (n = 6 per group, 3 males and 3 females): a negative control group (G1) and 25 mg L^{-1} (G2), 50 mg L^{-1} (G3), and 100 mg L^{-1} (G4) MWCNT groups, which were exposed for 7 days in the static system. We chose these concentrations and this exposure time based on a study that determined the toxicity of MWCNTs on fish gills [4]. After the experiment, the fish were euthanized by an anesthetic overdose of eugenol (600 mg L^{-1}). The present study was approved by the Animal Use Ethics Committee of the Federal University of São João del-Rei (CEUA-UFSJ Protocol 013/2016).

Histological and immunohistochemical assays

Samples of gills were obtained by dissection and fixed in Bouin's liquid for 24 h at room temperature. Next, the samples were subjected to routine histological techniques, embedded in paraffin, and sectioned into sections of 5 µm thickness. Then, the histological sections were stained with hematoxylin and eosin (HE), and the periodic-acid Schiff technique (PAS) was used for the identification of neutral glycoproteins in globet cells.

Histological gill sections fixed in Bouin's liquid were used to identify Bax and HSP70 by immunohistochemistry [14]. Histological sections were deparaffinized, hydrated, and maintained in PBS. The sections were subjected to antigen recovery with sodium citrate buffer (10 mM, pH 6.0) for 20 min at 100 °C and then subjected to endogenous peroxidase blockade with 3 % H_2O_2 in PBS for 30 min at room temperature. Nonspecific antigens were blocked with 2 % bovine albumin in PBS buffer for 30 min at room temperature. Next, the histological sections were treated with primary mouse antibodies (anti-Bax, Clone P-19, 1:200, or anti-HSP70 Clone F-3, 1:100) in a humid chamber overnight at 4 °C. After that, the gill sections were incubated with biotin-conjugated goat anti-mouse IgG secondary antibody (1:200) for 45 min, followed by incubation with peroxidase-conjugated streptavidin in a humid chamber for 45 min. The complex primary-secondary antibodies plus streptavidin were revealed using diaminobenzidine, and then histological sections were counterstained with hematoxylin. For the negative control, some sections did not receive primary antibody.

Brine shrimp samples and lethality assay

The *A. salina* cysts were incubated in seawater prepared from commercial 35 gL^{-1} red sea salt under constant aeration and temperature. After 48 h, the cysts hatched, and the nauplii were used in the toxicity test. The lethality test with nauplii was performed using a control group (seawater medium) and treatment groups with 25 µg mL^{-1}, 50 µg mL^{-1}, or 100 µg mL^{-1} MWCNTs in 24-well plates. Ten nauplii per well were added to the plates and kept at room temperature for 96 h. Next, healthy and dead nauplii were counted and photographed under a stereo microscope (SMZ 161) to obtain the mortality rate. The experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Gill histology

The study of biomarkers aims to assess the impact that aquatic organisms suffer at the molecular, cellular, tissue, physiological, and behavioral levels as a result of exposure to chemical substances and stressors present in the aquatic environment. The potential toxicity of carbon-based nanomaterials has been of great concern. Initially, much skepticism was expressed about the clinical or biological use of CNTs as drug delivery systems, because these fiber-like materials are biopersistent and, therefore, have a pathogenicity similar to that of asbestos or silica [29]. In addition, the nanocompounds might accumulate in aquatic organisms, leading to a biomagnification effect and migration along food webs [30]. In aquatic animals, due to their size, nanoparticles can easily be internalized into the body, either through the respiratory system or through the digestive system. The structure and modification of carbon nanocompounds enable these nanomaterials to enter the body of aquatic animals, promoting nanotoxicity [30]. For example, CNTs and fullerenes present low solubility in water, but when surface functionalization of these nanoparticles occurs, an increase in dispersion has been recorded. Most metal oxide nanoparticles used in several industrial fields are soluble in water, but they also present a low solubility [31]. We observed that MWCNTs

promoted injury in zebrafish gills and mortality in brine shrimps. Moreover, we found an increase in biomarkers (HSP70 and Bax) in zebrafish gills after exposure to MWCNTs for 7 days.

Due to their large contact surface, fish gills have been widely used as biomarker organs in the study of xenobiotics and environmental impact [14,32-36]. Simultaneously, clusters of MWCNTs are less toxic than dispersed MWCNTs, since the presence of larger clusters has the obvious consequence of substantially decreasing the surface area available for the interaction between cells and MWCNTs. In aquatic environments, pristine or non-functionalized MWCNTs tend to agglomerate, provoking less toxicity than dispersed MWCNTs. Also, the MWCNT composition can lead to acute toxicity, due to the presence of residual catalytic metals, which accumulate, causing ROS, besides interacting with proteins and DNA and rupturing membranes [29, 37-38]. Nevertheless, previous study using inductively-coupled plasma optical emission spectroscopy (ICP OES) found a < 95 % presence of metals in MWCNTs, indicating low or no acute toxicity [3]. The rapid response of gills can be explained by the contact of the pollutant with the blood, which is harmful to the vital function of the organ: gas and ion exchange. As a result, breathing difficulties may be responsible for inducing vasodilation [33]. The zebrafish gills from the control group were organized in branchial arches, from which the primary lamellae emerged, which were supported by hyaline cartilage and lined externally by the simple squamous epithelium (Figure 1A-B). From the primary lamellae, the secondary lamellae emerged, which were also covered by the simple squamous epithelium and supported by pillar cells. Both lamellae displayed highly vascularized. Mitochondria-rich cells were identified mainly at the base of the secondary lamellae (Figure 1A-B). Moreover, globet cells were scattered between the epithelial cells.

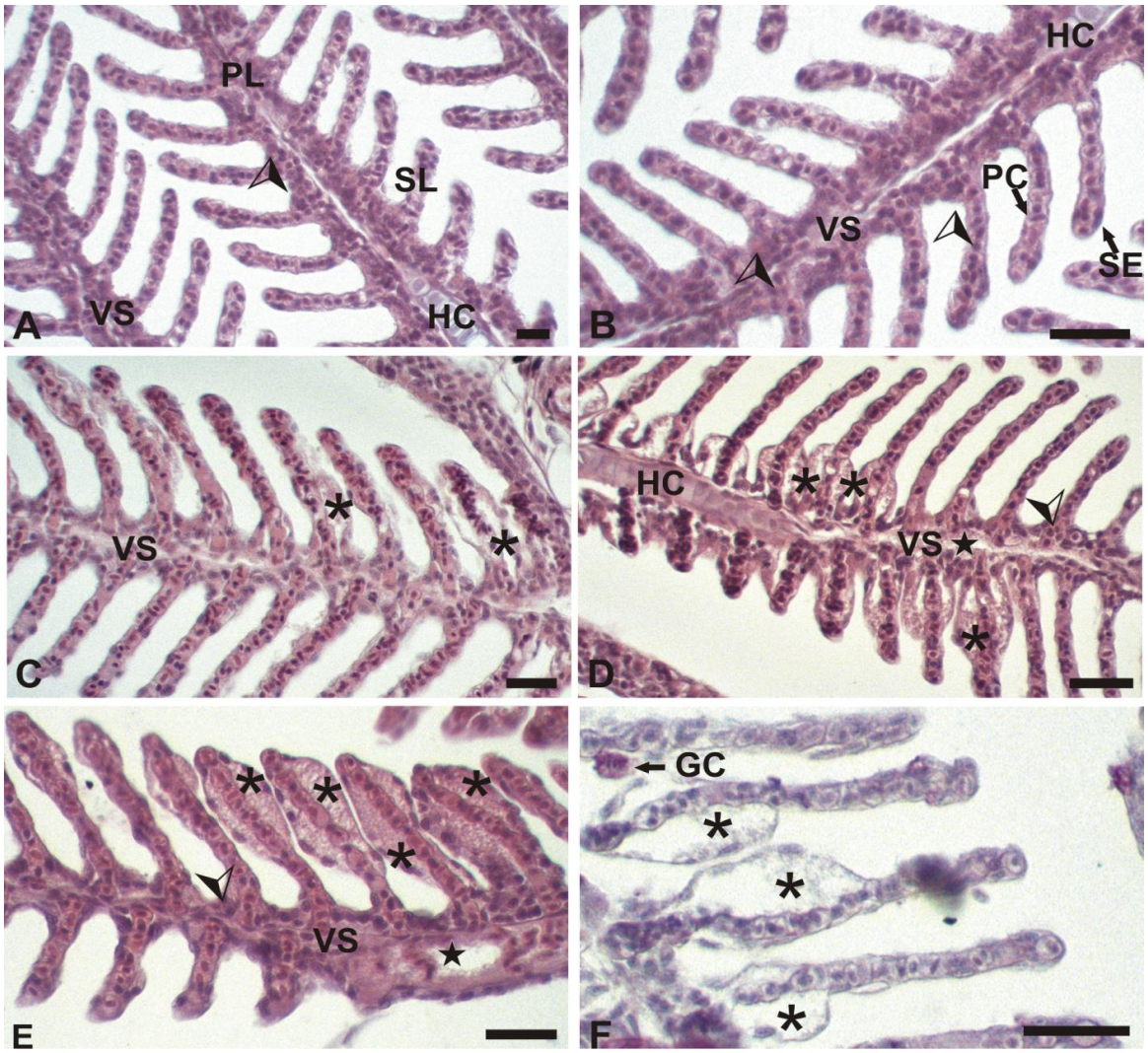

Figure 1. Histological analysis of *D. rerio* gills exposed to different concentrations of MWCNTs. A-B: G1, control group; C: G2, 25 mg L^{-1}; D: G3, 50 mg L^{-1}; E-F: G4, 100 mg L^{-1}. Primary lamella (PL), secondary lamella (SL), venous sinus (VS), hyaline cartilage (HC), mitochondria-rich cells (arrowheads), squamous cells (SE), pillar cells (PC), and goblet cells (GC). HE (A-E) and PAS (F). A-E: bars = 50 µm; F: bar = 25 µm. Note the epithelial lifting present in the secondary lamellae, increasing with concentration (asterisks) and a vasodilation in the primary lamellae (stars).

After 7 days of exposure, the gills of the animals treated with MWCNTs showed significant histopathological changes. In groups G2, G3, and G4, we found moderate to severe gill changes, such as hypertrophy and hyperplasia of the lamellar epithelium, secondary lamella fusion, and epithelial lifting (Figure 1C-F). Moreover, we also observed vasodilation of the vascular axis of the primary and secondary lamellae in groups treated with MWCNTs when compared with the control group. The aneurysm was also recorded in secondary lamellae of animals exposed to MWCNTs. The increase of epithelial lifting was apparently concentration dependent (Figure 1C-F). These results corroborate studies that have used gills as a biomarker of cytotoxic agents [4;14,32-36]. Epithelium displacement (epithelial lifting) has often been recorded as a histopathological parameter in the gills of animals exposed to cadmium [32]. These responses act as defense mechanisms because they reduce the vulnerable surface area of the gills and/or increase the diffusion barrier to the pollutant [14;34]. In addition to functioning as a defense mechanism, lamellar fusion can damage the organ because of cell hyperplasia, which consequently reduces gas exchange and leads to respiratory problems and hypoxia, resulting in the rupture of epithelial cells [13]. After exposure to 5-50 mg L^{-1} graphene for 48 h, the gills presented severe hyperplasia [39], in agreement with the current findings. The fact that MWCNTs and graphene are similar in structural organization could explain why they cause similar cellular changes in gills [39]. It is believed that the rupture of the pillar cells, and the loss of the support function, promotes lamellar aneurysms. Vascular changes such as aneurysms have been used as biomarkers in gills after exposure to environmental stress or xenobiotics [14; 40]. The gills of *Capoeta fusca* presented lamellar fusion, lamellar synechiae, and edema after being subjected to nickel oxide nanoparticles for 28 days [41]. The *Clarias garepinus* gills displayed aneurism, subepithelial edema, epithelium lifting, lamellar fusion, and hyperplasia of the interlammellar epithelium after exposure to silver nanoparticles for 15 days [42]. Once nanoparticles reach the aquatic environment, they are subject to transformations that can alter their original state, and these physical and chemical changes are important for understanding the toxicological potential of nanoparticles. Transformations may involve processes such as dissolution, adsorption, aggregation, and sedimentation, which will certainly influence the fate of the nanoparticles and therefore their toxicity [43]. The gills possibly present these changes in an attempt to preserve some physiological functions [14;34].

The goblet cells are responsible for the synthesis and secretion of mucus, and their increase in fish gills may be related to stress conditions [14]. Thus, the increase in these cells suggests an additional protection mechanism, as the thick mucus layer protects the lamellar surfaces against infectious and toxic agents and suspended particles [14,35]. The goblet cells were identified in all experimental groups using the PAS technique (Figure 1F). In the histopathological evaluation, there were no morphological or quantitative changes in the goblet cells of gills from zebrafish exposed to MWCNTs when compared with the control group. On the other hand, *C. fusca* exposed to nickel oxide nanoparticles displayed an increase in globlet cells in gills when compared with the control group [41]. In addition, an increase in globet cell degeneration was observed in fathead minnow and African catfish gills exposed to silver nanoparticles [42,44].

HSP70 and Bax as biomarkers of MWCNT toxicity

In the control group, the staining for HSP70 was found mainly in the simple epithelium lining the secondary lamellae (Figure 2A). In the groups treated with MWCNTs, the epithelial cells showed abundant labeling for HSP70 in their cytoplasm (Figure 2B), which was more intense in the 100 mgL^{-1} group (Figure 2C) than in the control group. Besides, in the treated groups, as compared with the control group, we observed poor HSP70 labeling in the pillar cells (Figure 2B-C). The mitochondria-rich cells were strongly stained for HSP70 in the treated groups when compared to control group (Figure 2B-C).HSP70 has been widely used as a biomarker of cytotoxicity or environmental stress [18, 45-46]. Under stress conditions, it acts as a molecular chaperone that maintains homeostasis in protein synthesis and also protects the cell from damage [47-48]. The gills of *Hypostomus francisci* collected in an urban river with anthropic influence presented strong staining for HSP70 [14]. In this work, when *H. francisci* was kept in an environment with good water quality, the HSP70 staining in gills decreased [14]. In a different context, HSP70 is regarded as a potential indicator of cellular changes. For example, *Astyanax lacustri* exposed to samples of water collected at sites close to a tailings dam rupture in Mariana, MG, Brazil, showed an increase or decrease in HSP70 in gills depending on the pollutants present in the water samples [49]. The group exposed to river water near the disaster site showed an increase in HSP70 in gills. On the other hand, fish exposed to water sampling from downstream of dam, displayed a decrease in HSP70 in gills [49]. An increase in the expression of HSP70 and HSP90 was described in ovaries, with a significant decrease in steroidogenesis after exposure to zinc and lead in *Cirrhinus cirrhosis* [50]. Carp (*Cyprinus carpio*) exposed to di-n-butyl phthalate (DBP) (1 mg L^{-1}), which is a compound commonly used in the plastic industry and which can disrupt the endocrine system, promoted a time-dependent increase in HSP70 in gills after 96 h of exposure [46]. In the present

study, it was found that MWCNTs caused a dose-dependent increase in HSP70 in the gills of *D. rerio* after 7 days. The relation between HSPs and nanotoxicology using fish gills as biomarkers has been little explored.

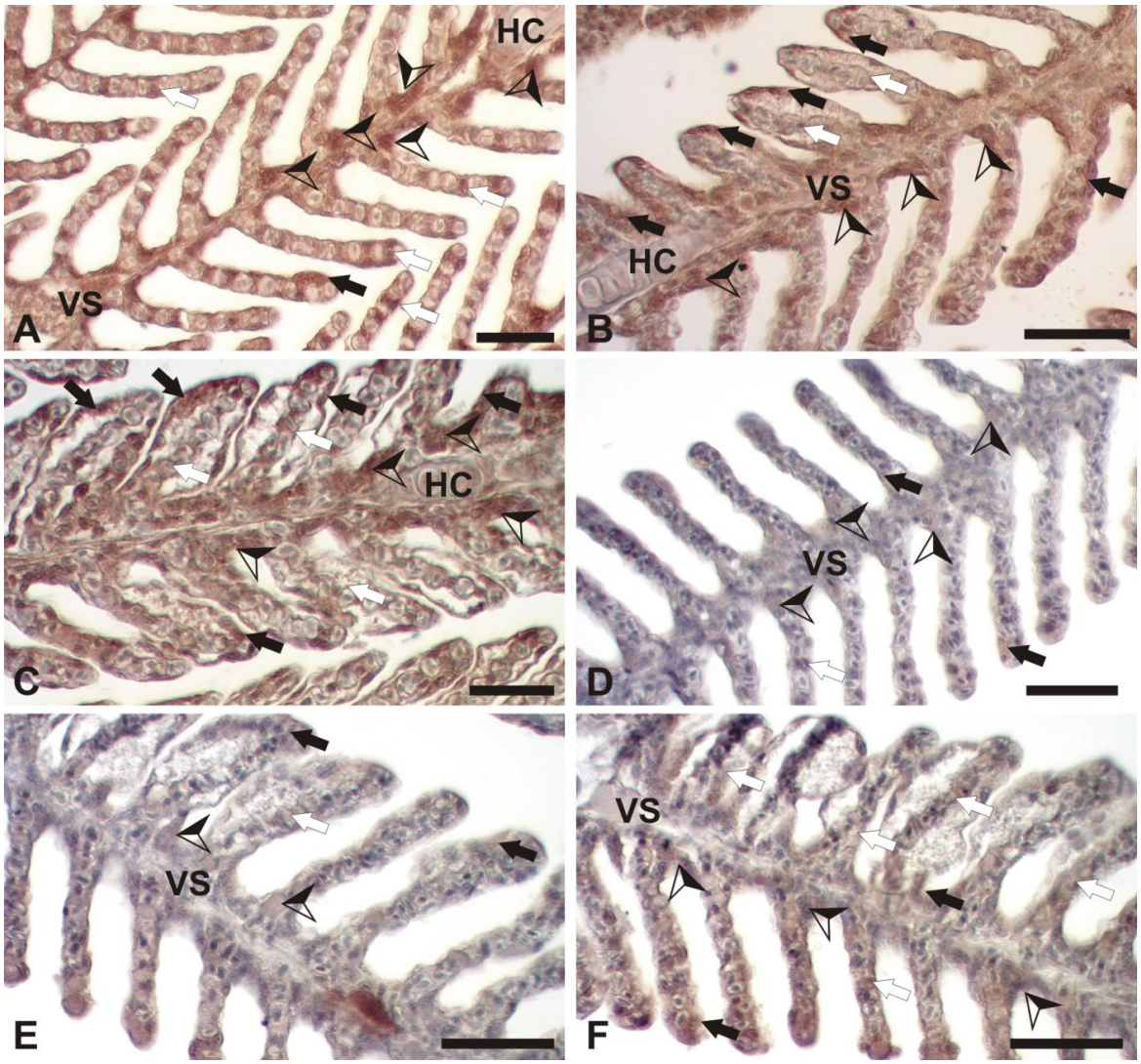

Figure 2. Immunohistochemistry for HSP70 (A-C) and Bax (D-F) in *D. rerio* gills in different experimental groups. A and D: G1, control group; B and E: G2, 25 mg L^{-1} MWCNTs; C and F: G4, 100 mg L^{-1} MWCNTs. Arrows indicate positive HSP70 and Bax staining in the simple epithelium (black) and pillar cells (white). A-F: bars = 50 µm. Venous sinus (VS), hyaline cartilage (HC), mitochondria-rich cells (arrowheads). The sections were counterstained with hematoxylin.

In all experimental groups, it was possible to identify the marking for Bax (Figure 2D-F). The labeling of positive Bax cells was predominantly in the cytoplasm of the epithelial cells lining the secondary lamellae. Qualitatively, the marking was intense in the gills of the animals exposed to MWCNTs, and it was concentration dependent (Figure 2E-F). The squamous cells with epithelial lifting also showed Bax marking (Figure 2E-F). Pillar cells did not exhibit Bax labeling in all experimental groups. In *Oryzias latipes,* an increase in the gene expression of apoptosis-related proteins, such as caspase-3, -8, and -9, has been observed in gills after 4 days of exposure to 100 mg L^{-1} MWCNTs [4]. In this work, the authors described that acute exposure was more efficient in inducing the expression of these proteins than chronic exposure to MWCNTs for 14 days.

In the present study, interestingly, a qualitative increase in Bax coincided with an increase in HSP70 in the gills of *D. rerio*. HSP70 has been associated with apoptotic pathways [51]. In fish, an increase in HSP70 and apoptosis have been described in stress conditions. This relationship is believed to be associated with the role of HSP70 in inhibiting apoptosis in some biological systems to maintain cell viability even under conditions of cellular stress [51-52]. An increase in HSP70 and caspase-3 has been described in the gills of *H. francisci* specimens captured in an urban river with anthropic influence [14]. In this work, there was a decrease in HSP70 and caspase-3 when compared to animals that were kept in an environment with good water quality for 30 days. We believe that a similar association might occur for Bax and HSP70 in zebrafish

gills after exposure to MWCNTs, which may promote toxicity and may lead to epithelial lifting of the squamous epithelium. Thus, MWCNTs might increase the expression of Bax and apoptosis in zebrafish gills. On the other hand, the HSP70 could acts inhibit the apoptosis and consequently, the maintenance of cell viability for gill functions.

MWCNT-induced toxicity in brine shrimps

The use of *A. salina* has been proposed in nanotoxicology studies because of several biological characteristics, such as a well-described short lifecycle, easy handling, and potential interactive effects with nanomaterials [53]. The morphology of the brine shrimp nauplii from the control group had a brownish-orange colour and an eye (photoreceptor) present in the head region (Figure 3A). It also presented three pairs of appendages: the first pair of antennae (sensory function), the second pair of antennae (filtering and locomotor function) and the third pair of antennae, the mandibles (food uptake function) (Figure 3A). In abdomen region, a transparent intestine was observed (Figure 3A). On the other hand, the nauplii exposed to 25 µg mL^{1} and 50 µg mL^{1} MWCNTs had black aggregates in the intestine because of the uptake of nanomaterials (Figure 3B-C). Regarding lethality, the control group presented a mortality rate of 20 %. The brine shrimps treated with 25 µg mL^{-1}, 50 µg mL^{-1}, and 100 µg mL^{-1} MWCNTs displayed mortality rates of 46.7 %, 70 %, and 100 %, respectively. The results showed a concentration-dependent mortality in *A. salina*. An animal mortality rate of less than 20 % was recorded in the nauplii exposed to metal oxide nanoparticles (MO-NPs) for 48 h [54]. The *A. franciscana* exposed to polystyrene nanoparticles (PS-NPs) for 48 h (short term) or 14 days (long term) showed reduced growth and survival of nauplii [55]. In this work, HSP70 expression was increased in brine shrimps mainly after long-term exposure to 1 µg mL^{-1} PS-NPs [55]. The instar I, II and III of *Artemia salina* exposed to 600 mg L^{-1} single-walled oxidized carbon nanotubes (O-SWCNTs) for 72 h displayed the mean mortality rates of 36.1 %, 57.9 % and 45.2 %, respectively [56].

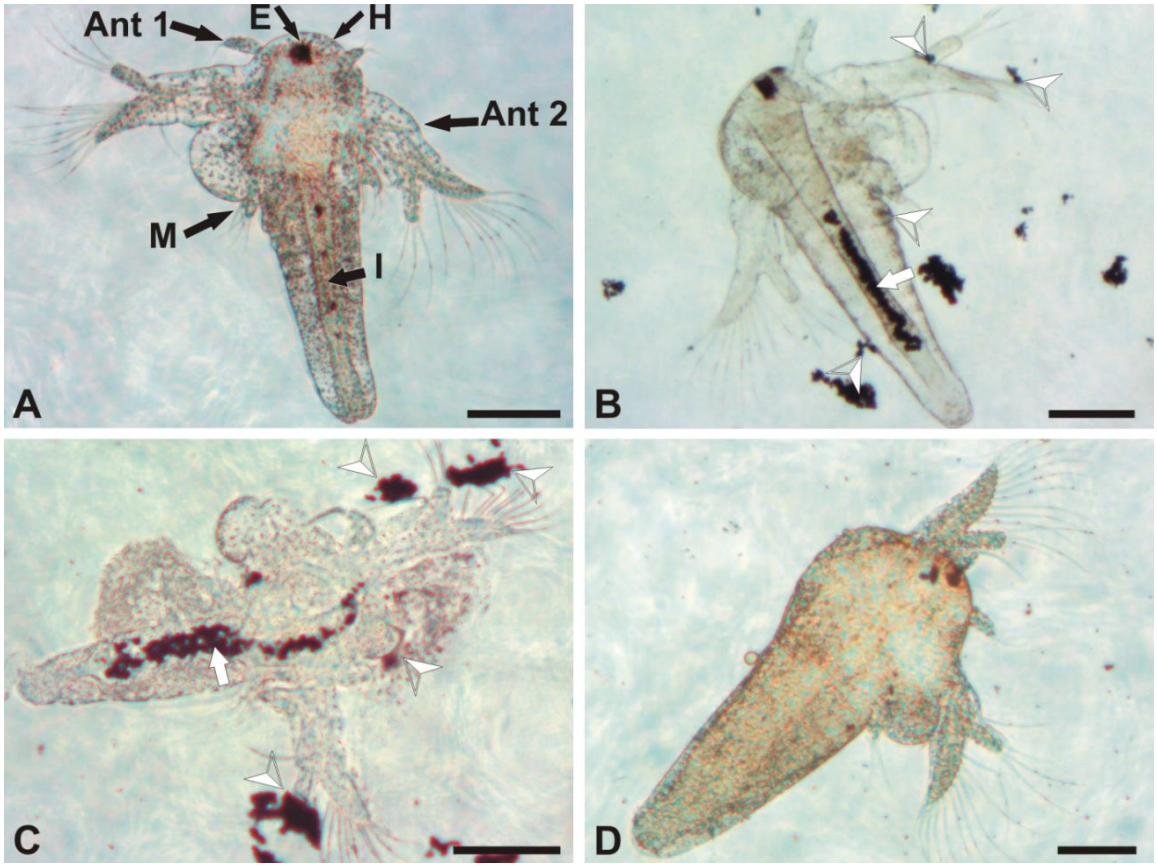

Figure 3. Intake and distribution of MWCNTs in *A. salina*. A) In the control group, the nauplius intestine (I) was empty. B-C) In the nauplii exposed to 25 µg mL^{-1} and 50 µg mL^{-1} MWCNTs, the intestine was filled with MWCNTs (white arrows), as shown by a dark line. D) The nauplii exposed to 100 µg mL^{-1} MWCNTs had an empty intestine, which could be explained by the immediate death after contact with the MWCNTs. Head (H), antenna 1 (Ant 1), antenna 2 (Ant 2), eye (E), mandible (M), intestine (I). MWCNTs adhered to the body (arrowheads). A-D: bars = 200 µm.

The nauplii treated with a concentration of 50 µg mL^{-1} MWCNTs showed a swelling in the intestine region when compared with the control group. The *A. salina* treated with *Penicillium daleae* displayed an enlarged

intestine and body malformations [57]. The 25 µg $mL^{-1}$and 50 µg mL^{-1} MWCNT groups showed sub-lethal effects, with the black aggregates attached onto the body surface, appendages of the second pair (Figure 3B-C), and mandibles. Regarding swimming behavior, the nauplii exposed to MWCNTs reduced the swimming movements when compared to control group. Some types of nanomaterials have been shown to cause disturbances in the swimming behavior of brine shrimps [54]. The brine shrimp larvae presented changes in swimming behavior and inhibition of cholinesterase enzyme after exposure to MO-NPs for 48 h [54]. An increase in antioxidant enzymes and ROS was described in brine shrimps after exposure to 600 mg L^{-1} O-SWCNTs, and these nanomaterials were also observed in the intestine, lipid vesicles, and phagocytes [56]. *A. salina* was unable to eliminate the nanoparticles as quickly as the formation of large aggregates in the intestine, and this fact might be linked to the survival rates [56]. In general, observed accumulation of nanomaterials within the *A. salina* intestine, lipid vesicles, and phagocytes could indicate a potential bio-cumulative effects and transfer along the food chain [56,58].

CONCLUSION

In summary, we conclude that the concentrations of MWCNTs used were not lethal to zebrafish as there were no deaths during the toxicological test. However, we observed structural and molecular changes in the gills. The most interesting finding was the increase in HSP70 and apoptosis via Bax in the gills of zebrafish subjected to different concentrations of MWCNTs for 7 days. Furthermore, MWCNTs presented toxicity to brine shrimps, which take up MWCNTs by filtration, potentially impairing the animals' homeostasis and leading the death. These results are useful for elucidating the effects of nanomaterials on invertebrates and vertebrates in aquatic environments.

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Conflicts of Interest: The authors declare no conflict of interest.

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