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Comparative toxicity of single-wall and multi-wall carbon nanotubes to brine shrimp Artemia salina

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Abstract

Effects of single-wall (SWCNT) and multi-wall carbon nanotubes (MWCNT) on Nauplii and adults of *Artemia salina* were investigated in this study. Organization for Economic Co-operation and Development guideline for acute toxicity test was used to evaluate the LC50 of SWCNT and MWCNT on nauplii and adult of *Artemia salina*. The standard hatching method was first performed on the cysts of *Artemia*, and then Nauplii and adults of *Artemia* were exposed to concentrations of SWCNT and MWCNT for a 48-h exposure period. LC50 experiment was conducted on 24 and 48 h exposure time and for each toxicity test, a control group (without any nanomaterials) was considered. Probit software was then used to analyze the mortality and LC50 concentrations. Results showed that the LC50 of Nauplii in 24 and 48 h after exposure to SWCNT were 509.25 ± 49.04 and 241.03±19.69 mg/L, respectively. For adults, LC50 values showed 556.74 ± 29.84 and 416.57 ± 31.93 mg/L during a 24- and 48-h exposure time, respectively. In addition, the LC50 of MWCNT for Nauplii was 3750.51 ± 145.59 and 3319.73 ± 563.7 mg/L in 24 h and 48 h, respectively. For adults, LC50 values were calculated at 5619.67 ± 44.41 and 4062.81 ± 782.32 mg/L after 24- and 48- h, respectively. The toxicity of SWCNT and MWCNT to *Artemia* showed a time- and concentration-dependent manner in the exposure test. Taken together, as the marine environments are being faced with an ever-increasing emergence and presence of SWCNTs and MWCNTs, valuable marine food species such as *Artemia salina* are significantly vulnerable to carbon-based

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nanomaterials, and more efforts should be made to reduce the environmental concentrations of carbon nanotubes in the marine environments.

Keywords: Toxicity effects; Single-wall nanotubes; Multi-wall nanotubes; Nauplii*;* Adult *Artemia*.

1. Introduction

Nanotechnology has revolutionized our lives due to its emergence into rather all parts of public lifestyle (Seyedi *et al*., 2021). Nano-based products have received a great deal of attention because of their benefits in a diverse range of industries, including food, medicine, agriculture, electronic, and personal care products (Veisi *et al*., 2021). It is expected that the application of manufactured nanomaterials witnesses an annual growth of 15% by 2022 (Behzadi Tayemeh *et al*., 2020). Having a wide range of advantages such as antimicrobial attributes, very small size, and high absorbance surface, engineered nano-products have been increasingly used in recent decades (Palit 2019). However, nanomaterials have caused many problems in aquatic and terrestrial environments because of their toxicity to flora and fauna (Syafiuddin *et al*., 2018). As the application of these human-made nanomaterials has increased in human life, the environmental concentration of such toxic materials has caused many issues in the ecology and health of organisms (Kazemi *et al*., 2022). The adverse effects of nanomaterials in the wild depend on the type and structure of these materials, and nanoparticles, nano colloids, and carbon nanotubes cause different toxicity to the organisms. Banan *et al*. (2022) showed that silver nanoparticles caused accumulation in the liver and gut of Persian sturgeon, and induced DNA damage in the liver single cells.

There are a variety of synthesized nanomaterials that have been used in different industries. Carbon nanotubes (CNTs) are a class of engineered nanomaterials constructed using carbon layers in the structure and based on the number of carbon walls, they are categorized into two groups as single-wall carbon nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs) (Li *et al*., 2017). Such carbon-based nanomaterials are increasingly used in optic, biology, material strengthening, nano-biosensing, drug delivery systems, materials science, medicine, and food industries (Mesarič *et al*., 2015; Parang and Esmaeilbeigi, 2022). The most important reason for the toxicity of SWCNTs and MWCNTs is related to the metal impurities, including Fe, Co, Ni, Mo, and As that are added to the designed structure in the production step (Zhu *et al*., 2017). Li *et al*. (2017) used Zebrafish as a model organism to show the toxicity and bioconcentration of SWCNTs in the liver, gills, intestine, and skin tissues. These toxic nano carbons interact with other chemical substances in the water environment and cause deleterious impacts on the health of aquatic species. The metal contents and ions of MWCNTs can cross the cell membrane, and in turn, accumulate in the internal organs causing acute and chronic toxicity effects in aquatic species (Trompeta *et al*., 2019). The effects of water-dispersible oxidized multiwalled carbon nanotubes on marine alga, *Dunaliella tertiolecta* (Wei *et al*., 2010), the toxicity of single-walled carbon nanotubes to benthic organisms (Urgess *et al*., 2013), and multi-walled carbon nanotubes on the toxicity of tri phenyl tin to the marine copepod *Tigriopus japonicus* (Yi *et al*., 2019; Shirdel *et al*., 2020) have been studied. However, the effects of these nanomaterials on the brine shrimp and respective microcrustaceans in the marine environment have not been fully understood.

Marine microcrustaceans such as *Artemia* have been largely used in toxicological exposure experiments as marine model species with favorable attributes such as small size, fast reproduction, short lifecycle, easy to handle in the lab, and working on a large number of nauplii (Tortella *et al*., 2020). Further, *Artemia* is widely used in supplying live food for larvae culture in aquaculture and fish cultivation. *Artemia salina*, known as brine shrimp, is a small saltwater crustacean that produces a large number of metabolically inactive cysts and tolerates high-saline waters (Kohler *et al*., 2021). In the environment, *Artemia* is considered as a bioindicator because of its resistance to environmental changes, salinity gradients, water quality, toxic materials, and temperature variations (An *et al*., 2019). As the production and application of CNTs are being significantly increased in the aquatic biota, the interaction and behavior of these materials to induce toxicity to living organisms in the water environment have not yet been fully understood. Moreover, as there are differences between SWCNTs and MWCNTs in terms of the chemical and physical structures and metal contents, the induced toxicity of these CNTs is important to the health and viability of food species such as *Artemia salina* in the marine environment.

Artemia in marine environments plays a crucial role in supplying live food for a diverse range of aquatic species. The vast majority of marine species such as fish, crustaceans, and mollusks rely greatly upon *Artemia* (larvae, nauplii, and adults) as a nourishing source of food (Mesarič *et al*., 2015). *Artemia* contains a number of essential ingredients, including fatty acids, proteins, vitamins, and minerals which are vital for larvae stage in marine species. However, chemical substances and toxic materials in recent years have led to devastating the population and habitat of *Artemia* worldwide (León-Silva *et al*., 2016). In addition, environmental stressors such as invasive species have caused many problems for these valuable aquatic species in terms of diversity, biology and ecology. More importantly, nano materials in aquatic systems have posed a serious pressure to saline species which are filter-feeder. *Artemia* can actively filter and absorb nano materials and therefore they face chronic and acute toxicity of nano materials. Gambardella *et al*. (2014) reported that selected metal oxide nanoparticles can cause different mortality, behavioral and biochemical responses.

The results showed inhibitory effects of nano materials on the swimming and enzyme activity of *Artemia salina*. Nano carbons, in particular, have a strong capability to be accumulated and concentrated in the body of saline brine shrimps, and in turn, lead to many abnormalities, malfunction, and mortality. Therefore, this study aims to test and compare the induced acute toxicity of SWCNTs and MWCNTs to *Artemia salina* as a saline model organism and show the potential toxicity of these nanomaterials to marine crustaceans.

2. Materials and Methods

2.1. Nano carbon characterization

CNTs were purchased from Zharfa pazhoohan company (Tehran, Iran). MWCNTs were characterized at 95% purity, outer diameter of 10-20 nm, approximate length of 30 µm, and SWCNTs showed 90% purity, outer diameter of 1-2 nm and an approximate length of 30 µm. TEM (transmission electronic microscope) analysis was conducted to show the morphograph of studied CNTs (Figure 1).

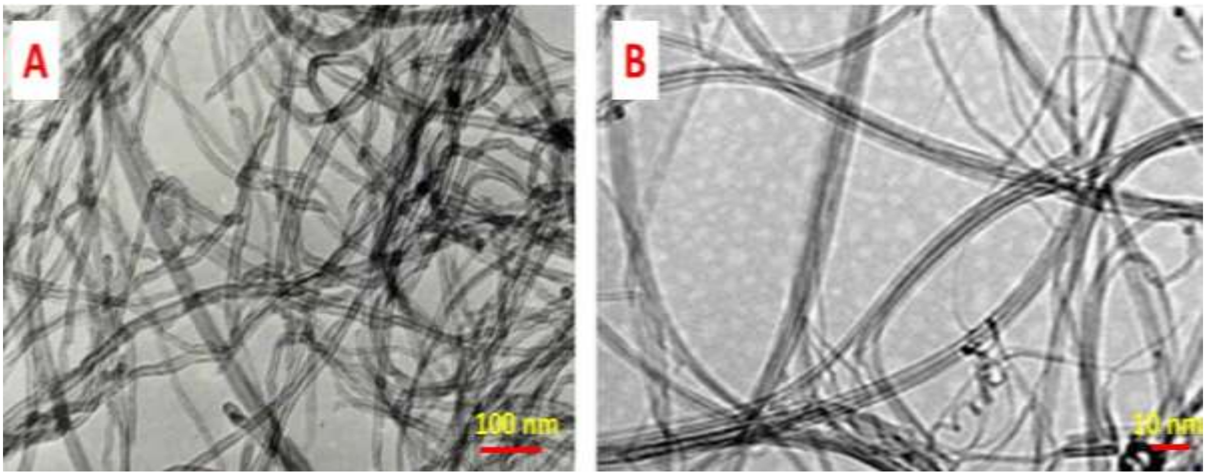

Figure 1. TEM morphograph of studied CNTs (A: MWCNTs and B: SWCNTs)

2.2. Artemia reproduction

For the production of *Artemia salina*, packed cysts were purchased from the Ocean Nutrition company (under USA license and produced in Thailand). To provide appropriate conditions for the hatching of cysts, 1-liter transparent plastic bottles were used as incubators. For better incubation, an aeration system was considered to supply oxygen and gas exchange. An amount of 800 ml dechlorinated water was poured into the bottle and 25g sea salt was added to provide a 25 ppt saline water. Then, 1.6 g of *Artemia* cyst was added into the bottle and temperature was adjusted at 28 °C. To supply the required light, a 20-watt fluorescent lamp with a length of 15 cm was considered to produce 2000 lux above the incubator. After 24 hours, hatched nauplii were seen on the surface of the incubator in response to the fluorescent light.

2.3. Nauplii collection

To collect the hatched nauplii, the aeration system was first rejected and collected shells on the water surface were removed. Following the methodology, nauplius that was on the bottom of the incubator were collected and transferred into a black bottle. To separate healthy and more active nauplius, in the black water highly reacted nauplii were cumulated on top of the incubator in response to the light and unhealthy or dead nauplii were at the bottom which were removed from the test.

2.4. Concentrations of SWCNTs and MWCNTs

Five concentrations were considered for SWCNTs (nauplii: 25, 50, 100, 200, and 400; adults: 30, 60, 120, 240, and 480 mg/L) and MWCNTs (nauplii: 500, 1000, 2000, 4000, and 8000; adults: 500, 1000, 2000, 4000 and 8000 mg/L).

2.5. Short-term exposure test and LC50 for nauplii

Organization for Economic Co-operation and Development (OECD) conducted an acute toxicity test using OECD TG 202 (OECD, 2004) at 24 and 48h of exposure period. Salinity and temperature were adjusted at 25 ppt and 28 ^{O}C, respectively. 50-ml beakers containing 30 nauplius were considered for each treatment and a control group was selected without adding SWCNTs and MWCNTs. At each exposure time, the number of healthy and alive nauplii was recorded in triplicates. In each exposure group and time concentrations with 50% lethality were considered as LC50 concentrations.

2.6. Short-term exposure test and LC50 for adults

Healthy nauplius were first separated and transferred into the new incubator and after 20-days maintenance, proper and active adults were selected based on the positive photoreaction for the exposure test. Adults were fed with *chlorella vulgaris* during the exposure test. For each concentration and time, a 50-beaker was selected and a number of 30 nauplii were added into the beakers. Alive adult *Artemia* were counted at 24- and 48- h exposure period triplicates.

2.7. Bioaccumulation analysis

In order to study the uptake and excretion of O-SWCNTs in *A. salina*, newly hatched larvae were treated with O-SWCNTs (50 mg/L) for 48- h and then transferred in fresh FNSW to excrete for another 24 h. Five larvae were randomly selected at 0, 48 and 72 h, and thoroughly washed with FNSW. The accumulation of O-SWCNTs in the larvae was checked under a microscope, and images were obtained using a digital camera. Moreover, a TEM was used to observe the distribution of O-SWCNTs in *A. salina*.

2.8. Behavioral changes

Instar I, II and III larvae were treated with O-SWCNT suspensions (0, 25, 50, 100, 200, 400, and 600 mg/L) as described above. After exposure for 24 h, the surviving larvae were separated, rinsed and transferred into Petri dishes with FNSW. Then, the Petri dishes were placed in a dark box for 10 min to make the larvae reach a uniform spatial distribution.

2.9. Statistical analyses

All data were analyzed using Microsoft Excel (2021) and SPSS package (21). Data were reported as mean ± SD and Probit (V. 1.50) was used to calculate the LC50. In order to compare the dead animals in different concentrations and exposure times, three-way analysis of variance considering important factors, including life cycle (Nauplii and adults), toxicity (LC10, LC20, LC50, and LC90), and exposure time (24 and 48 hours) were used in SPSS.

3. Results and Discussion

3.1. Acute toxicity of SWCNTs to Artemia salina

Figure 2 shows LC10, 20, 50 and 90 of SWCNTs (mg/L) in nauplii of *Artemia salina* during a 24- and 48-h exposure period. Based on the findings, LC50 values showed 509.25 ± 49.04 and 241.03 ± 19.69 mg/L for nauplii at 24 and 48 h exposure time, respectively (Table 1). Nauplii is a neonate of *Artemia* and is more vulnerable to toxic materials than adults. Results demonstrated that the toxicity of SWCNTs to nauplii at 24 h was greater than the respective values after 48 h exposure time. Some studies have shown that small particles, like nanoparticles and fibers, can easily pass the cell membrane and accumulate in the internal organs of organisms (Kik & Sici, 2020). Other studies have reported that SWCNTs, which are more soluble compared to MWCNTs, show less toxicity. Our results demonstrated LC50 values at 556.74 ± 29.84 and 416.57 ± 31.93 mg/Lfor adults at 24 and 48 h exposure time, respectively (Table 1). These findings are in line with those reported by Ma-Hock *et al*. (2013), however there is differences between the toxicity of nano carbons among the organisms. LC50 is one of the best methods to examine the acute toxicity of hazardous materials and this concentration can later use in investigating chronic exposure of toxic materials in long-term exposure period. Esmaeilbeigi *et al*. (2021) reported that nanomaterials can cause deleterious impacts on the DNA structure of fish species due to reactive oxygen species (ROS). This means that nano materials such as nano carbons have also toxicity effects which are agreed to the results of this study. In another study, Behzadi Tayemeh *et al*. (2020) proposed that silver nanoparticles could induce severe toxicity even at low concentrations to *chlorella vulgaris* as a microalga in a 72-exposure time. Results showed a significant difference between lethal concentrations of SWCNTs and exposure times at 24 and 48 h in nauplii group (Figure 3). SWCNTs concentrations revealed more lethal concentrations at 48 h than 24 h.

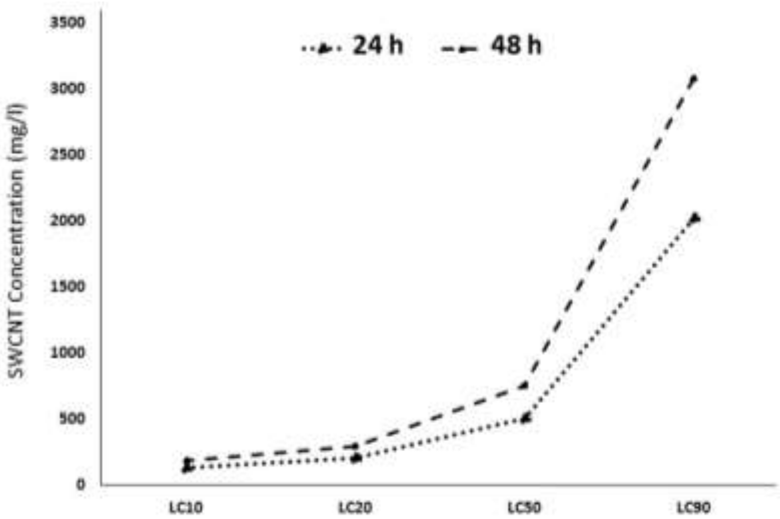

Figure 2. LC10, 20, 50, and 90 of SWCNTs (mg/L) in nauplii of *Artemia salina* during a 24- and 48-h exposure period.

Table 1. LC10, 20, 50, and 90 values of SWCNTs concentrations (mg/L) in nauplii and adults of *Artemia salina* during a 24-h and 48-h exposure period

Stage	Nauplii	Nauplii	Adult	Adult
Exposure time	24 hours	48 hours	24 hours	48 hours
LC10	128.28 ± 10.10	55.48 ± 9.15	105.04 ± 12.71	83.84 ± 9.94
LC20	205.92 ± 14.24	91.80 ± 10.19	186.12 ± 14.55	145.15 ± 9.06
LC50	509.25 ± 49.04	241.03 ± 19.69	556.74 ± 29.84	416.57 ± 31.93
LC90	2022.06 ± 231.00	1053.18 ± 44.84	2963.07 ± 170.92	2099.65 ± 413.78

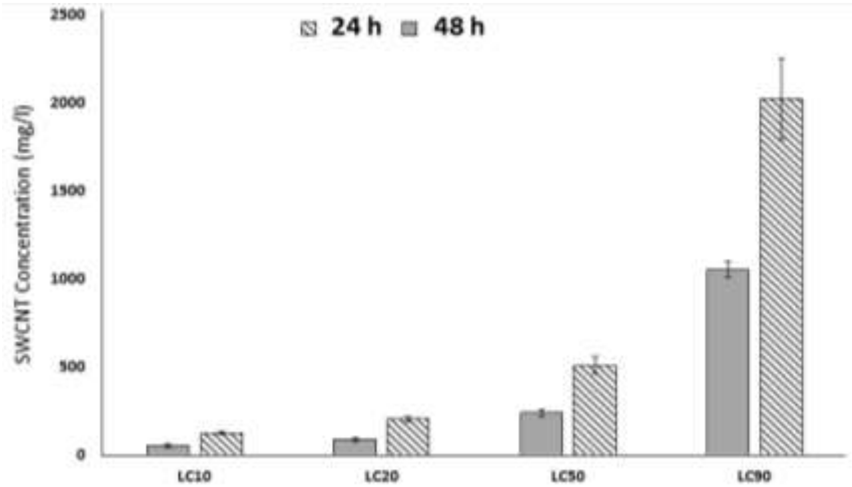

Figure 3. Comparison between different lethal concentrations and SWCNTs nanoparticles (mg/L) in nauplii of *Artemia salina* during a 24- and 48-h exposure period.

Figures 4 and 5 depict the LC10, 20, 50, and 90 of SWCNTs (mg/L) in adults of *Artemia salina* during a 24- and 48-h exposure period. Findings showed LC50 values at 556.74 ± 29.84 and 416.57 ± 31.93 mg/L for adults at 24 and 48 h exposure time, respectively. It is apparent that adults of *Artemia* showed mortality rates in higher concentrations of SWCNTs compared to nauplii. This means that adults are more tolerant to toxic nanomaterials than nauplius. Nauplii at the first hours of hatching are very vulnerable to water changes and toxic materials and they may die even at lower concentrations of nanomaterials. SWCNTs induced significant cytotoxicity to blood cells by binding with proteins, and in turn, can produce ROS as a leading cause of DNA damage and carcinogenesis (Assefi *et al*., 2021). *Artemia* is classified as one of the most resistant aquatic species to environmental changes, including salinity, acidity, temperature, water quality, and chemicals substances; however, SWCNTs can cause deleterious impacts on the survival of these species, leading to increase the mortality rate. Our findings revealed that the toxicity of SWCNTs had a time-dependent response as the toxicity increased with the elevation of exposure times.

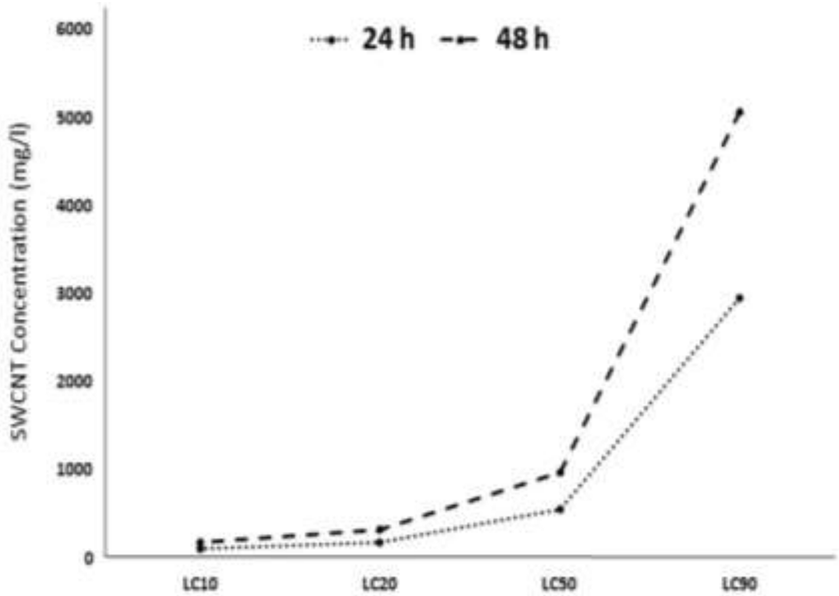

Figure 4. LC10, 20, 50, and 90 of SWCNTs (mg/L) in adults of *Artemia salina* during a 24- and 48-h exposure period.

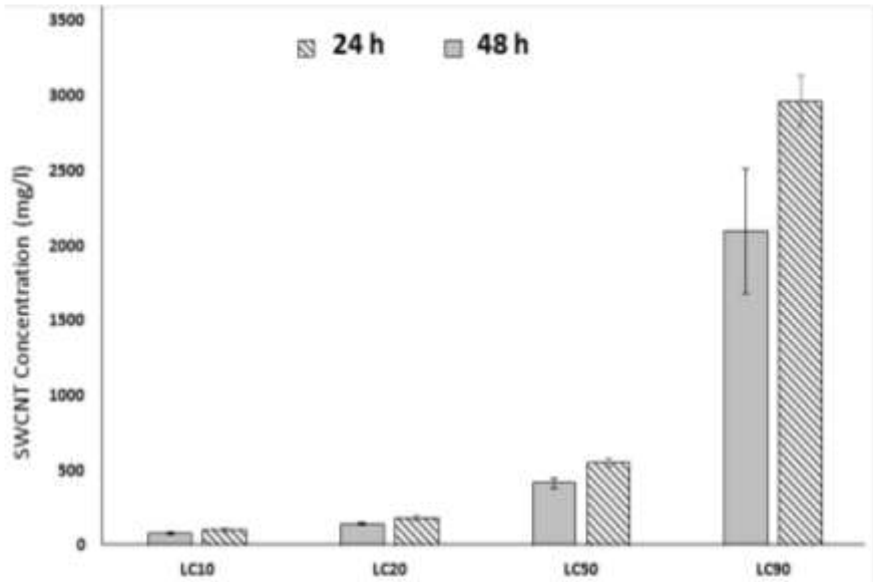

Figure 5. Comparison between different lethal concentrations and SWCNTs (mg/L) in adults of Artemia salina during a 24- and 48-h exposure period.

3.2. Acute toxicity of MWCNTs to Artemia salina

Table 2 represents the LC10, 20, 50, and 90 values of MWCNTs concentrations (mg/L) in *Nauplii* and adults of *Artemia salina* during a 24-h and 48-h exposure period. Based on the results, the LC50 values for *nauplii* were at 3750.78 ± 145.59 and 3319.73 ± 563.7 mg/L after 24 and 48 exposure times. MWCNTs are constructed from a number of carbon layers in the structure and therefore may contain more metal impurities and toxic materials. Zhu *et al*. (2017) studied the developmental toxicity of MWCNTs on *Artemia salina* cysts and larvae. They examined the uptake, accumulation, excretion, and toxic responses, and results showed that the hatchability of capsulated and decapsulated cysts decreased significantly in exposure to 600 mg/LMWCNTs over a 36-h exposure time. MWCNTs caused swimming inhibitory effects and mortality rate in studied *Artemia* nauplius.

It is possible that in our experiments, adults tolerated carbon nanomaterials higher than the nauplii, therefore the mortality rate for nauplii was higher than the adults. In recent years, the release and penetration of MWCNTs into the aquatic environments have considerably increased due to emissions from wastewater treatment plants, industrial activities, and petrochemical developments. Many studies have reported that the toxicity of MWCNTs is mainly due to the metal catalyst impurities in carbon-based nanomaterials (Jia *et al*., 2005; Ma-Hock *et al*., 2013; Yi *et al*., 2019; Zhao *et al*., 2020; Asadi *et al*., 2022).

Our findings demonstrated that there was no significant difference between 24- and 48- h exposure time to MWCNTs in nauplii (Figure 6 and Figure 7). This finding is different compared to what we obtained in SWCNTs as showed different toxicity at 24 and 48 h. Results revealed LC50 values at 5619.67 ± 44.41 and 4062.81 ± 782.32 mg/L for adults. In comparison with the SWCNTs, MWCNTs demonstrated more values for the LC50, meaning that in higher concentrations MWCNTs could cause death in the tested *artemia*. For this change, one possibility can be the complicated structure of MWCNTs compared to SWCNTs, which makes less solubility in the water media and harder to cross the cell membrane of organisms. However, Trompeta *et al*. (2019) suggested that the toxicity effects of MWCNTs are mainly due to the attachment of carbon-based nanomaterials on the surface of the body, leading to the reduction of swimming activity, feeding rate, and finally death. The comparison between different lethal concentrations and MWCNTs (mg/L) in adults of *Artemia salina* during a 24- and 48-h exposure period is shown in Figure 8 and Figure 9.

According to the outcomes, there was no significant difference between 24- and 48-h exposure times in adult group. In a study that was conducted by Mesarič *et al*. (2015), the responsibility of high surface adsorption properties of carbon-based nanomaterials for mortality, swimming inhibition, and biochemical responses of *Artemia salina* larvae were examined. They outlined that the attachment of MWCNTs onto the body surface could cause a concentration-dependent inhibition of larval swimming. These findings were in line with our results as the mortality of *Artemia* increased with the concentration of nanomaterials. An *et al*. (2019) compared the toxicity of particulate and wire-based nanomaterials on *Artemia salina*. They suggested that both silver nanoparticles (AgNPs) and silver nanowires (AgNWs) caused ROS in the body and reduced superoxidase dismutase (SOD) activity in tested *artemia*. ROS activity is one of the responses of the organisms to toxic substances, and in our experiments, it is highly possible that nauplii *Artemia* could not persist ROS and thus died in exposing to single- and multi-wall nanocarbons.

Our findings revealed that SWCNTs are more toxic than MWCNTs to the nauplii and adults of *Artemia salina* may be because of the simpler structure and the simplicity of passing through the cell membrane. However, MWCNTs showed the largest concentrations to induce the LC50 compared to SWCNTs, meaning that due to their more complicated structure, it is harder to cross the cell membrane and cause toxicity. Therefore, MWCNTs toxicity is mainly because of inhibitory effects on the behavior and activity of micro crustacean such as *Artemia.*

Table 2. LC10, 20, 50, and 90 values of MWCNTs concentrations (mg/L) in nauplii and adults of *Artemia salina* during a 24-h and 48-h exposure period

Stage	Nauplii	Nauplii	Adult	Adult
Exposure time	24 hours	48 hours	24 hours	48 hours
LC10	1121.68 ± 124.24	984.73 ± 74.75	1366.00 ± 131.82	1133.55 ± 111.88
LC20	1694.78 ± 82.65	1491.83 ± 108.73	2216.04 ± 155.51	1756.63 ± 135.51
LC50	3750.78 ± 145.59	3319.73 ± 563.7	5619.67 ± 44.41	4062.81 ± 782.32
LC90	12731.85 ±1137.64	1053.18 ± 3225.02	2348.49 ±1530.92	1485.64 ± 6816.00

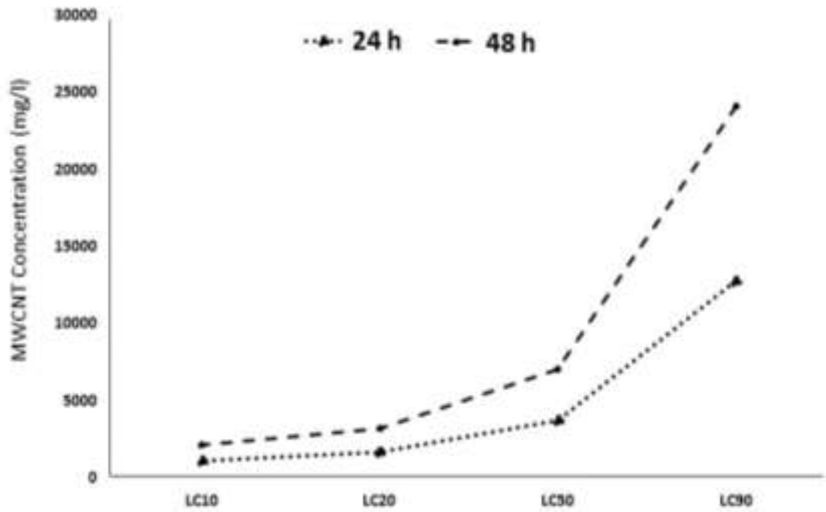

Figure 6. LC10, 20, 50, and 90 of MWCNTs (mg/L) in nauplii of *Artemia salina* during a 24- and 48-h exposure period.

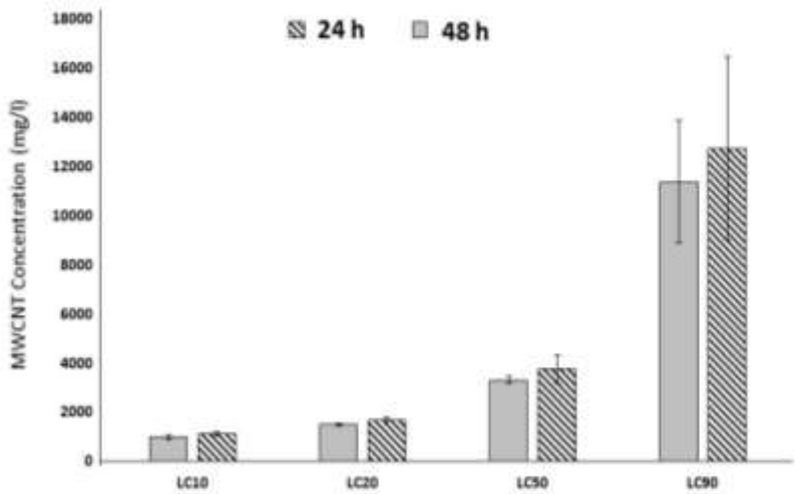

Figure 7. Comparison between different lethal concentrations and MWCNTs (mg/L) in nauplii of *Artemia salina* during a 24- and 48-h exposure period.

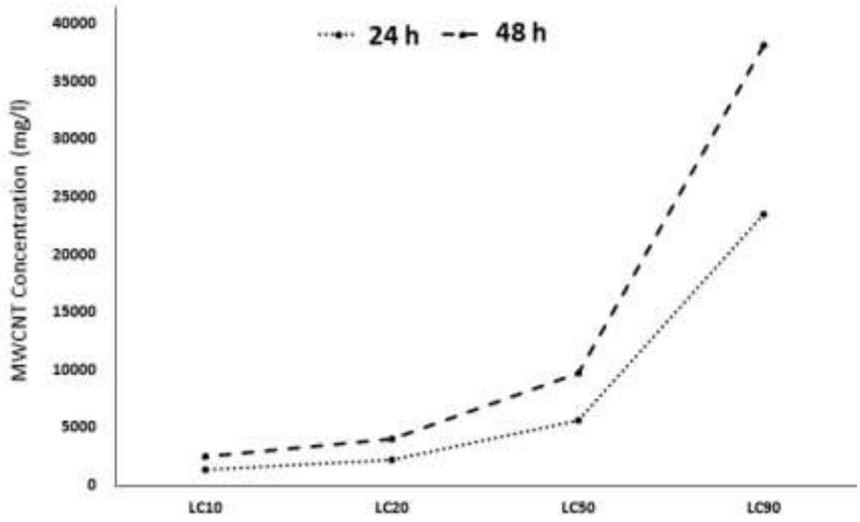

Figure 8. LC10, 20, 50, and 90 of MWCNTs (mg/L) in adults of *Artemia salina* during a 24- and 48-h exposure period.

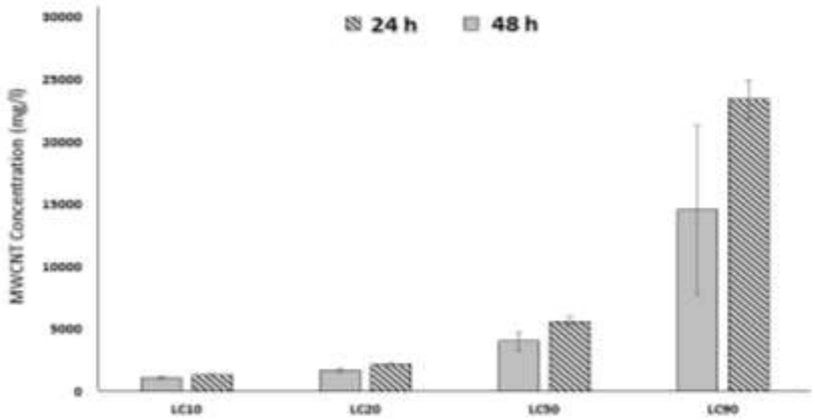

Figure 9. Comparison between different lethal concentrations and MWCNTs (mg/L) in adults of *Artemia salina* during a 24- and 48-h exposure period.

Conclusion

This study investigated the acute toxicity of SWCNTs and MWCNTs nanomaterials to nauplii and adults of *Artemia salina* during a 24-h and 48-h period. The LC10, LC20, LC50, and LC90 of SWCNTs and MWCNTs (mg/L) to the tested animals were calculated using the Probit analysis method. *Artemia salina* showed more vulnerability to SWCNTs concentrations and LC50 values were comparatively lower than the respective values in MWCNTs treatments. Nauplii of *Artemia* revealed more sensibility to SWCNTs and MWCNTs compared to adults. Results showed a significant difference in the LC50 values between 24 and 48 h exposure period in nauplius, but there was no significant difference between the two exposure times in adults. The toxicity of SWCNTs and MWCNTs showed a time-relevant response in tested animals. Carbon-based nanomaterials possess a serious hazard to aquatic species and in higher concentrations can cause destructive effects on the aquatic biota. The results of this study could be suitable for chronic exposure studies in

Artemia salina. *Artemia salina*, as a food species in marine biota, plays a leading role in supplying nutritive food for saline-water species, therefore, the health status of this valuable source of food is important for the ecology and diversity of marine ecosystems. Together, as the aquatic ecosystems are being faced with an ever-increasing emergence and presence of SWCNTs and MWCNTs, more efforts should be made to reduce the environmental concentrations and preserve valuable aquatic species. It is recommended that future studies test the chronic toxicity of SWCNTs and MWCNTs through multiple generations of *Artemia* to examine the teratogenic effects on the offspring and the capability of nanocarbons to transfer through consecutive generations. Also, it is suggested that future researchers use transfer trophy studies using larger predators like fish to simulate the natural occurrence of nanocarbons through different levels in the food chain.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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