Acute toxicity of titanium dioxide (MTiO2) microparticles in Artemia salina nauplii instar I and II

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Abstract

In this study, the toxicity effects of titanium dioxide ($MTiO_2$) microparticles on *A. salina* nauplii instar I and II between 24 and 48 h was evaluated. The $MTiO_2$ were characterized by X-ray Powder Diffraction (XRPD), Fourier Transform Infrared Spectra (FT-IR), Scanning Electron Microscopy (SEM), Zeta potential (ζ), Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). $MTiO_2$ rutile phase with an average crystallite size of 114.5 ± 2.44 nm was used in toxicity tests at concentration of 12.5, 25, 50 and 100 ppm. No toxicity was observed in *A. salina* nauplii instar I at the time of 24 and 48 h. However, *A. salina* nauplii instar II toxicity was observed within 48 h of exposure. $MTiO_2$ dispersed with concentrations of 25, 50 and 100 ppm were lethal for 30%, 50% and 30% of the individuals respectively, thus showing a significant difference ($P \leq 0.05$) relative to the control group, artificial sea water (ASW). The mortality number of *A. salina* was recorded and LC_{50} value of 50 ppm was calculated. Analysis of optical and SEM revealed tissue damage and morphological changes in *A. salina* nauplii instar II. By using confocal laser scanning microscopy, cell damage was observed due to the toxicity of $MTiO_2$ at a concentration of 20, 50 and 100 ppm in nauplii instar II. At this stage, *A. salina* was more affected by the action of $MTiO_2$ with higher mortality rate, morphological alterations and cellular damage.

Introduction

Metallic particles are present in several science and industry areas and are used in the environmental protection and building engineering, medicine, agriculture and the food and cosmetic industry (Sirotkin et al., 2021). Among the metal particles titanium dioxide (TiO_2) has desirable properties in the production of new materials because they have low combustion, odorless, high resistance to corrosive agents, high density and very high refractive index (Shi et al., 2013). TiO_2 exists as rutile, anatase and brookite bulk crystalline polymorphic forms (Sadrieh et al., 2010). The rutile phase is more stable and has lower photocatalytic activity than anatase (Barbosa et al., 2018). This oxide has been used as an inorganic filter in sunscreens due to protection effect against UV radiation absorbing, scattering and reflecting (Lu et al., 2015).

TiO_2 are the most relevant nanomaterial in terms of world production volumes with reaches about 10 tons per year (Bundschuh et al., 2018), and used worldwide in several areas in the manufacture of products such as sunscreen, cosmetics, paints, food additives, medicines and construction (Ozkan et al., 2016). The daily use of these products results in the release of TiO_2 particles (in the micro and nano scale) into the environment, negatively affecting aquatic organisms (Nowack and Bucheli, 2007), through the food chain (Farré et al., 2009). Most of the studies currently are related to the toxicity of TiO_2 on a nanometric scale and few research related to the toxicity of TiO_2 microparticles has been done.

The toxicity of TiO_2 is related to their photocatalytic properties. Under UV-A radiation they become reactive, oxidizing molecules and organic substrates causing cellular damage (Fu et al., 2014). In ecotoxicological tests using aquatic organisms it was observed that TiO_2NPs were toxic to algae and

microcrustaceans (Clément et al., 2013). With *Daphnia magna*, it was observed that TiO_2 caused mortality above 50% in individuals and the toxicity was proportional to the increase of particles concentration (Hund-Rinke and Simon, 2006). In addition to *D. magna*, *A. salina* are used in toxicity tests as noted by Rekulapally et al. (2019) using different types of particles. These toxic effects were generated through the accumulation of NPs in aquatic environments (Kachenton et al., 2019), directly affecting zooplankton (Farré et al., 2009). Thus, our hypothesis is that $MTiO_2$ enters the environment causing toxic effects for biota. To assess possible toxicity of $MTiO_2$, we used *A. salina* as a study model.

The genus *Artemia* is worldwide distributed and extensively used to toxicological test (Nunes et al., 2006), being the taxon with the highest biomass and primary consumer in the food chain. (Sorgeloos et al., 1978). The life cycle of brine shrimp begins with the breaking of the cyst dormancy, small spheres with great physical and chemical resistance. Dormancy breaks when the spheres come into contact with saline water (Morgana et al., 2018).

The use of this specie as a model in toxicity tests is related to easy handling, high adaptation to laboratory conditions, low maintenance cost, short life cycle and high reproduction rate (Manfra et al., 2014). In addition, the reliability and validity of ecotoxicological tests using *A. salina* has been confirmed by several tests using different stressors like chemical compounds (Manfra et al., 2014; Pillard and Tapp. 2021) and pharmaceutical (Nunes et al., 2006). Using NPs, several studies point to acute toxicity in *A. salina*, among them are AgNPs (Lacave et al., 2017; An et al., 2019; Palácio et al., 2021), ZnONPs (Ates et al., 2013a; Khoshnood et al., 2017; Sarkheil et al., 2018), MO-NPs (Gambardella et al, 2014) and TiO_2NPs (Ozkan et al., 2016). All these studies show the toxicity of NPs, however, different results can be observed due to different experimental conditions and characteristics of NPs (Sarkheil et al., 2018) justifying ecotoxicological tests.

The aim of this work was to evaluate the acute toxicity of $MTiO_2$ with emphasis on morphological changes, cell damage and number of dead individuals. In our study, it was used $MTiO_2$ in the rutile crystalline phase for acute toxicity tests in *A. salina* nauplii instar I and instar II.

Material And Methods

Synthesis and characterization of $MTiO_2$

Titanium dioxide (TiO_2) (ViaFarma) particles was used as received without any purification. The characterization of was carried out by Barbosa et al. (2018) using techniques such as X-ray powder diffraction (XRPD), Fourier Transform Infrared Spectroscopy (FT-IR), Dynamic Light Scattering (DLS), Energy Dispersive Spectroscopy (EDS), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The particle size was obtained by measuring the size of 100–200 particles from SEM images. Sample of presented the diameter of 114.5 ± 2.44 nm. The zeta potential and DLS was

performed to check the surface and colloidal properties of $MTiO_2$. The value was negative for $MTiO_2$ (-5.29 ± 0.77) and an increase in the particles size in solution was observed (2301 ± 1097.56 nm)

Test organism

A. salina cysts were purchased from aquaculture store in Fortaleza, Ceará - Brazil. Dehydrated cysts were kept at 4°C and used in all experiments. Instar I and instar II stage nauplii (24 and 48 h post hatching) were obtained as described by (Garaventa et al., 2010). Briefly, 500 mg of cysts incubated for 24 h at 28°C under 16 h light, 8 h dark conditions and continuous aeration of the cyst suspension in ASW (30% salinity). The hatched nauplii were separated from non-hatched cysts based on their positive phototaxis and then transferred by a Pasteur pipette into beakers containing the ASW.

$MTiO_2$ LC_{50}

Current study performed acute toxicity test by determination of LC_{50} (Lethal Concentration 50). *A. salina* stage nauplii instar I and II were exposed to $MTiO_2$ at a concentration of 12.5, 25, 50 and 100 ppm in 24 and 48 h. After exposure, a correlation graph between $MTiO_2$ concentration and percentage dead nauplii was plotted.

Acute toxicity test of $MTiO_2$

Acute exposure was conducted on *A.* nauplii (instar I and II) for 24 and 48 h exposure according to (Johari et al., 2019) and Organization for Economic Cooperation and Development, testing guidelines (OECD, 2004). Briefly, four different test concentrations (12.5, 25, 50 and 100 ppm) of TiO_2 were administered in nauplii within 24 and 48 h. Negative control group was exposed to artificial seawater and positive control was exposed to potassium dichromate ($K_2Cr_2O_7$) 0.5 M. The experiment was carried out on 24-well polystyrene microplates with 2 mL. Each concentration carried out by three replicate and each replication contained ten newly hatched nauplii. The room temperature was set to 24°C and photoperiod of 12 h dark/12 h light. After that, the numbers of dead larvae were counted under a stereomicroscope Stemi 508 with attached camera ZEISS (Axiocam 208/202 mono). The test considered valid only when survival rate in the control group was ≥ 90% (OECD, 2004).

Optical microscopy

For analysis of $MTiO_2$ accumulation, *A. salina* nauplli instar II were collected at 24 and 48 h of experiment and washed in ASW. We do not use nauplii instar I, because at this stage the mouth and anus of *A. salina* are not developed (Ocaranza-Joya et al., 2019), preventing the accumulation of $MTiO_2$ by the animal. The nauplii were mounted on a glass slide and images were obtained using optical microscopy Primo Star with Axiocam Color camera coupled. Subsequent microscopy analyzes such as confocal laser scanning microscopy (CLSM - LM 710 Zeiss) and scanning electron microscopy (SEM - Quanta FEG 450 (FEI) were performed only with *A. salina* nauplii instar II within the 48 h exposure, because at this stage there was a higher mortality rate.

Confocal Laser Scanning Microscopy (CLSM)

To assess possible cellular damage caused by $MTiO_2$, acridine orange was used in *A. salina* nauplii instar II, exposed to different concentrations of TiO_2, in addition to the negative (ASW) and positive ($K_2Cr_2O_7$) controls. Briefly, *A. salina* was transferred to 24-well polystyrene microplates of 2 mL and then 500 µL of acridine orange at a concentration of 5 µg/mL was added to each well for 20 minutes at room temperature. After 20 minutes, *A. salina* were washed in phosphate buffer solution, pH 7.2. Stained samples were observed under CLSM with 488 nm excitation and 532–580 nm emission.

Scanning electron microscopy (SEM)

Samples of *A. salina* nauplii instar II were collected at 24 and 48 h and fixed in solution of glutaraldehyde 2.5%, formaldehyde 4.0% in cacodylate buffer 0.05 mol L^{-1}, pH 7.2 at room temperature for 24 h. Subsequently, the material was rinsed in the sodium cacodylate buffer 0.05 M three times for 45 min each wash. After the washes, the samples were increasing series dehydrated with acetone for 45 min each step. After dehydration, the material was critical point dried (EMS 850). Dried samples were placed in stubs and sputtered with 20 nm gold in metallization equipment QUORUM 150T ES. Observation and documentation were performed in scanning electron microscope (Quanta FEG 450 FEI), with 20kV beam acceleration.

Statistical analysis

For the statistical analyses, the data were recorded daily as the mean and standard deviation. The normality of the data and the averages significance was performed through the R environment, using the *psych* package. The normality of the data was assessed using the Shapiro-Wilk test as a function of $n < 30$, through the *shapiro.test* function. The t-test was used to verify the significance of differences between the means of each treatment (12.5 ppm, 25 ppm, 50 ppm, 100 ppm and ($K_2Cr_2O_7$) as a function of the control treatment (ASW), through the *t.test* function. The nonlinear regression analyzes were performed using the Sigma Plot 11.0 software package.

Results

LC_{50} of $MTiO_2$

No mortality was observed for nauplii instar I exposed to TiO_2 between 24 and 48 h, with no correlation between exposure time and increase in $MTiO_2$ concentration (Table 1). Same result was observed for nauplii instar II with 24 h exposure a $MTiO_2$ (Table 2). However, a correlation between $MTiO_2$ concentration and percentage of mortality was observed, revealing a dose-dependent effect at 48 h experiment for *A. salina* nauplii instar II (Fig. 1). The polynomial nonlinear regression ($P \leq 0.05$) demonstrates a strong relationship ($R^2 = 0.94$) between concentration of the TiO_2 and number of dead nauplii (Fig. 1). In addition, the LC_{50} of $MTiO_2$ was 50 ppm for nauplii instar II in 48 h exposure (Fig. 1).

Acute toxicity test of $MTiO_2$

A. salina nauplii instar I submitted to concentration of 12.5, 25, 50 and 100 ppm of $MTiO_2$ no mortality rate was observed in 24 and 48 h experiment, not being observed significant difference ($P \leq 0.05$) in relation to the control (ASW) (Table 1). $K_2Cr_2O_7$ was lethal for all nauplii instar I in 24 h and 48 h of experiment (Table 1). Similar result was observed in *A. salina* nauplii instar II within the 24 h exposure interval.

A. salina nauplii instar II within the 48 h exposure, a toxic effect was observed for TiO_2 at the concentration of 25, 50 and 100 ppm (Table 2). *A. salina* nauplii instar II submitted to concentration of 25, 50 and 100 ppm of TiO_2 exhibited mortality of 30, 50 and 30 % respectively (Table 2). The negative control (ASW) and positive control ($K_2Cr_2O_7$) presented 0% and 100% mortality (Table 2).

$MTiO_2$ accumulation

The accumulation of $MTiO_2$ in *A. salina* was observed by optical microscopy at the time of 24 and 48 h. The control group showed no morphological damage (Fig. 2A). With 24 h exposure was observed $MTiO_2$ accumulation in the gut of the nauplii instar II, no apparent damage, presenting similar morphology to the control. Low $MTiO_2$ accumulation was observed in the gut of animals exposed to 12.5 and 25 ppm (Fig. 2B, C). At higher concentrations (50 and 100 ppm) $MTiO_2$ was observed in the animal's gut (Fig. 2D, E) and swelling in the cephalothorax (Fig. 2E). Individuals exposed to positive control ($K_2Cr_2O_7$) showed underdeveloped body (Fig. 2F).

The $MTiO_2$ aggregation in *A. salina* is directly associated with the consumption of NPs and may present a large accumulation gut even in low concentrations (12.5 and 25 ppm). In *A. salina* nauplii instar II within 48 h of exposure, the control (ASW) showed no damage in its morphology with appendages and translucent gut (Fig. 3A). At the minimum concentration used (12.5 ppm), $MTiO_2$ were observed only in the gut region (Fig. 3B). A similar result was observed at 25 and 50 ppm (Fig. 3C, D). However, at the maximum concentration tested (100 ppm), $MTiO_2$ dispersed throughout the animal's body were observed causing morphological alteration with tissue degradation (Fig. 3E). Individuals exposed to $K_2Cr_2O_7$ was observed incomplete development with abnormalities in the appendages and abdomen (Fig. 3F). The images corroborate the statistical data, which point to toxicity of $MTiO_2$ in high concentrations, time of exposure and stage of life of the *A. salina*.

Cell damage analysis

To assess cell damage, *A. salina* nauplii instar II was submitted to different concentrations of $MTiO_2$ with acridine orange in the interval of 48 h. In artificial sea water (ASW) *A. salina* showed low fluorescence emission because the cells were not damaged (Fig. 4A). In addition, image of the ASW without acridine orange was performed, which presented an emission pattern similar to the ASW with acridine orange (Supplementary material). In low concentration of TiO_2 (12.5 ppm) the result was similar to ASW (Fig.

4B). At 25 ppm concentration, many fluorescent spots were observed in the abdomen of *A. salina* (Fig. 4C), due to cell damage. In moderate concentration of $MTiO_2$ there was an increase in fluorescence emission related to cell damage, observed in *A. salina* exposed to a concentration of 50 ppm (Fig. 4D). In high concentrations of $MTiO_2$ (100 ppm) fluorescence emission was observed throughout the animal's body (Fig. 4E), indicating cell damage. Positive control showed strong emission due to cell damage caused by $K_2Cr_2O_7$ (Fig. 4F).

Morphological changes

Morphological changes in *A. salina* nauplii instar II were observed under scanning electron microscopy and morphological changes were observed in high concentrations of $MTiO_2$. Overview of *A. salina* in ASW (Fig. 5A), with normal development of the swimming legs (Fig. 5B) and posterior region (Fig. 5C). *A. salina* submitted to $MTiO_2$ at a concentration of 12.5ppm (Fig. 5D) no damage to the swimming legs (Fig. 5E). Posterior region showed wrinkling cuticle (Fig 5F). At the 25 ppm slight body wrinkling was observed (Fig. 5G), no damage on swimming legs (Fig. 5H) and posterior region with wrinkling cuticle (Fig. 5I).

At the concentration 50 ppm, wrinkling was observed on the surface of the body of *A. salina* (Fig. 5J) and cavities were observed in the abdomen. No damage was observed in the swimming legs (Fig. 5K), however posterior region showed wrinkling cuticle (Fig. 5L). At the maximum concentration tested (100 ppm), *A. salina* showed the damage body with remarkable surface wrinkling and absence swimming setae (Fig. 5M). Significant damage was also observed on the swimming legs (Fig. 6N). The posterior region showed abnormalities with cuticle rupture (Fig. 5O). The positive control ($K_2Cr_2O_7$) cuticle wrinkling was not observed on the animal's body (Fig. 5P). however, absence of swimming setae was observed in swimming legs (Fig. 5Q). The posterior region showed morphological alteration such as deformations on the surface of the animal's body (Fig. 5R).

Discussion

$MTiO_2$ LC_{50}

The toxicity of $MTiO_2$ is associated with the concentration and exposure time of these particles with *A. salina*. No LC_{50} values were observed for nauplii instar I at 24 and 48 h intervals. The mortality rate is directly proportional to the increase in the concentration of $MTiO_2$ and linear regression graph was observed for nauplii instar II. The value of LC_{50} were reported to be around 50 ppm within 48 h of exposure, however, in the 24 h interval no LC_{50} value was observed. Same result observed by Ates et al. (2013b) in *A. salina* nauplii exposed to $MTiO_2$ at a concentration of 100 $mg.L^{-1}$.

Usually the LC_{50} values are related to the exposure time, above 48 h as noted by Ozkan et al. (2016) in which the LC_{50} value for *A. salina* exposed to TiO_2NPs with size of 44.1 nm was 18.77 mgL^{-1} in the 96 h

interval. Other authors observed similar results, such as Sarkheil et al. (2018) using ZnONPs with size of 32.28 nm in *Artemia franciscana* in the 96 h interval and Khoshnood et al. (2017) using TiO_2NPs with size of 20nm in the same time interval. The TiO_2 used in our study showed an average diameter of 114.5nm showing toxicity in nauplii within 48 h of exposure. The toxicity of TiO_2 in a shorter period of time may be associated with their characteristics such as size, charge, composition and hydrodynamic radius (Boran et al., 2016; Murdock et al., 2008). In addition, smaller NPs are more toxic for *A. salina*, as they have a large surface area available for interaction with biological organisms (Kvıtek et al., 2008). Also the increased nanoparticles toxicity is related to the *A. salina* exposure time to the contaminant, as they are filter animals that can ingest and accumulate particles with a diameter of 50 µm (Hund-Rinke and Simon, 2006; Zhu et al., 2017).

In addition to microparticles, other contaminants also showed toxic effects on *A. salina* as described by several authors. In a study using plant extracts of different species, the LC_{50} was 1000 $\mu g/mL^{-1}$ in experiment time of 24 and 48 h (Mayorga et al., 2010). Assay evaluating the toxicity of organophosphorous insecticide in *A. salina*, it was observed that the highest toxicity was observed in nauplii instar II (48 h after incubation) with LC_{50} of 0.16 $mg.L^{-1}$ for insecticide chlorpyrifos (Sánchez-Fortún et al., 1996). These results are similar to our study showing toxicity in *A. salina* in the short period of exposure to the contaminant

Acute toxicity test of $MTiO_2$

No acute toxicity was observed in nauplii instar I. At this stage of life, the animal is less sensitive, due to incomplete mouth development and not actively eating (Sorgeloos et al., 1978). A similar result was observed by (Ocaranza-Joya et al., 2019) in which nauplii instar I was less sensitive to potassium dichromate, due to incomplete development of the mouth and anus.

A. salina nauplii instar II was more sensitive to $MTiO_2$ within 48 h of exposure only at high concentrations of $MTiO_2$ (25, 50 and 100ppm). This occurs due to the exposure time to the contaminant, because the 24 h interval, no acute toxicity of $MTiO_2$ was observed in *A. salina* nauplii instar II. In study using AgNPs, it was observed that *A. salina* nauplii instar II was more sensitive to contaminates in the 48 h exposure interval (Lacave et al., 2017). Many authors have described a similar result for acute toxicity only at high concentrations and time of exposure to the contaminant (Ates et al., 2013a; Khoshnood et al., 2017; Sarkheil et al., 2018). However, exposure of *A. salina* nauplii instar II to high concentrations of metal oxide nanoparticles (CeO_2, SnO_2 and Fe_3O_4) did not induce any lethal effect after 48 h of exposure (Gambardella et al., 2014). These adverse toxic effects observed in different studies may be the result of different experimental conditions, as well as the properties of NPs (Sarkheil et al., 2018). In addition, many factors can alter the results of toxicity tests on *Artemia* spp. such as environmental factors (chemical composition of seawater, oxygen, hatching temperature and duration of the photoperiod), maintenance conditions and origin of the cysts (Libralato, 2014).

$MTiO_2$ accumulation

The $MTiO_2$ accumulation was observed mainly in the gut, in addition, was also observed TiO_2 dispersed on the surface of the *A. salina* body when exposed to a concentration of 100 ppm. In a study using silver nanoparticles (AgNPs) and silver nanowires (AgNWs), accumulations of these nanomaterials were observed in the gut of *A. salina* (An et al., 2019). Result observed by (Mehmet Ates et al., 2013) using TiO_2NPs. In this study, the aggregation of $MTiO_2$ was higher in *A. salina* nauplii instar II due to the complete development of the mouth allowing it to feed actively (Sorgeloos et al., 1978). At this stage of development, *A. salina* feeds on NPs by filtration.

The uptake of NPs through the digestive tract and subsequent aggregation in the animal's gut causes obstruction, leading to mortality (Rekulapally et al., 2019). In another study, *A. salina* submitted to TiO_2NPs and AgTiO_2NPs showed changes in the ocular surface, malformations in the body, loss of the antenna and gut enlargement of (Ozkan et al., 2016). In addition, TiNPs induce gut necrosis of epithelial cells and severe edema, indicated by swelling of the cells with enlarged cytoplasms (Kachenton et al., 2019). All these interactions and NPs accumulation in the animal's intestine leads to oxidative stress with the generation of reactive oxygen species (Ates et al., 2020).

Cell damage analysis

The cell damage observed in *A. salina* was directly proportional to the $MTiO_2$ concentration used. Using CLSM it was possible to observe apoptosis of the cells, characterized by the emission of green fluorescence using acridine orange. The use of acridine orange allows to reveal cells in apoptosis because fluorophyll binds to the DNA of cells in apoptosis emitting green fluorescence at a wavelength of 488–535 nm (Damas-souza., 2019). Acridine orange permeates all living cells and is absorbed when damage occurs in the cytoplasmatic membrane, binding to DNA. Normal living cells have a light emission of fluorophyll, however, early apoptotic cells have a bright green nucleus (Ribble et al., 2005).

At a concentration of $MTiO_2$ 25 and 50 ppm, fluorescence emission was observed, indicating cell damage. At the maximum concentration tested (100 ppm) a high fluorescence emission was observed, due to the marked damage caused by the $MTiO_2$ to the cells. Result observed by Arulvasu et al. (2014) using AgNPs in *A. salina* at 12 nM concentration.

Morphological changes

Analysis using SEM revealed morphological changes and damage to the body of *A. salina* nauplii instar II. After exposure to different concentrations of $MTiO_2$, the individuals presented cuticle wrinkling. At the 50 ppm concentration, the appearance of cavities in the animal's body was observed. At the maximum concentration tested (100 ppm) damage was observed with rupture in the animal's body, loss of swimming setae and mandible, structures responsible for mobility and feeding respectively.

Other studies using different NPs showed similar results with our research. In study using graphene oxide in *A. salina*, irreversible damage such as ruptures and holes in the animal's body were observed (Zhu et

al., 2017). In another study using α-Fe_2O_3 nanoparticles, was observed with damage to the animal's body (Wang et al., 2017).

These NPs are widely used and inevitably released in aqueous environments, causing ecological and health risks (Wang et al., 2015). As new nanomaterials and products containing particles at the nanoscale are manufactured, many inevitably reach environmental repositories. (Ates et al., 2013a). In addition, titanium dioxide particles (TiO_2) have been used around the world in a variety of products, including sunscreen, cosmetics, paints, food additives, drugs and building materials (Ozkan et al., 2016) can be disposed of in the environment. Thus, $MTiO_2$ can also be toxic to the environment, particularly to aquatic organisms.

Conclusions

The acute toxicity of $MTiO_2$ was evaluated using *A. salina* nauplii instar I and instar II as model by using essays with 24 and 48 h. Toxicity of TiO_2 microparticles was observed only in concentrations of 25, 50 and 100 ppm in nauplii instar II within 48 h of exposure, with high mortality rate, cell damage and morphological alteration. The toxicity in *A. salina* is directly related to filtration of microparticles, therefore, in nauplii instar I model, the $MTiO_2$ showed no toxicity due to the incomplete development of the mouth and anus. With this study it was possible to observe the dynamics and interaction of microparticles with *A. salina* and the physicochemical characteristics of microparticles directly affect toxicity in biological systems.

Declarations

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Author Contribution

All authors contributed to the study conception and design. Developed research (Emilio de Castro Miguel and Antonio Gomes Souza Filho). Write the manuscript (Sergimar kennedy de Paiva Pinheiro). Revision of the manuscript (Thaiz Batista Azevedo Rangel Miguel and Saulo Pireda). Synthesis of TiO_2 microparticles (Pierre Basílio Almeida Fechine). Performed the experiments (Ana Kamila Medeiros Lima). All authors read and approved the final manuscript version.

Data Availability statement

The data that support this study are available in Figshare at DOI 10.6084/m9.figshare.15169032

Conflicts of interest

The authors declare no conflit of interest

References

1. An, H.J., Sarkheil, M., Park, H.S., Yu, I.J., Johari, S.A., 2019. Comparative toxicity of silver nanoparticles (AgNPs) and silver nanowires (AgNWs) on saltwater microcrustacean, *Artemia salina*. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 218, 62–69. doi:10.1016/j.cbpc.2019.01.002
2. Arulvasu, C., Jennifer, S.M., Prabhu, D., Chandhirasekar, D., 2014. Toxicity effect of silver nanoparticles in brine shrimp artemia. Sci. World J. 2014. doi:10.1155/2014/256919
3. Ates, M., Danabas, D., Ertit Tastan, B., Unal, I., Cicek Cimen, I.C., Aksu, O., Kutlu, B., Arslan, Z., 2020. Assessment of Oxidative Stress on *Artemia salina* and *Daphnia magna* after exposure to Zn and ZnO nanoparticles. Bull. Environ. Contam. Toxicol. 104, 206–214. doi:10.1007/s00128-019-02751-6
4. Ates, Mehmet, Daniels, J., Arslan, Z., Farah, I.O., 2013b. Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: assessment of nanoparticle aggregation, accumulation and toxicity. Env. Monit Assess 185, 3339–3348. doi:10.1007/s10661-012-2794-7.
5. Ates, Memet, Daniels, J., Arslan, Z., Farah, I.O., Rivera, H.F., 2013a. Comparative evolution of impact of Zn and ZnO nanoparticles on brine shrimp (*Artemia salina*) larvae: effects of particles size and solubility on toxicity. Environ. Sci. Process. Impacts 15, 225–233. doi:10.1016/j.dcn.2011.01.002.
6. Barbosa, J.S., Neto, D.M.A., Freire, R.M., Rocha, J.S., Fechine, L.M.U.D., Denardin, J.C., Valentini, A., de Araújo, T.G., Mazzetto, S.E., Fechine, P.B.A., 2018. Ultrafast sonochemistry-based approach to coat TiO_2 commercial particles for sunscreen formulation. Ultrason. Sonochem. 48, 340–348. doi:10.1016/j.ultsonch.2018.06.015
7. Boran, H., Boyle, D., Altinok, I., Patsiou, D., Henry, T.B., 2016. Aqueous Hg^{2+} associates with TiO_2 nanoparticles according to particle size, changes particle agglomeration, and becomes less bioavailable to zebrafish. Aquat. Toxicol. 174, 242–246. doi:10.1016/j.aquatox.2016.02.017
8. Bundschuh, M., Filser, J., Lüderwald, S., McKee, M.S., Metreveli, G., Schaumann, G.E., Schulz, R., Wagner, S., 2018. Nanoparticles in the environment: where do we come from, where do we go to? Environ. Sci. Eur. 30, 1–17. doi:10.1186/s12302-018-0132-6
9. Clément, L., Hurel, C., Marmier, N., 2013. Toxicity of TiO_2 nanoparticles to cladocerans, algae, rotifers and plants - Effects of size and crystalline structure. Chemosphere 90, 1083–1090. doi:10.1016/j.chemosphere.2012.09.013
10. Damas-Souza, D.M., Nunes, R., Carvalho, H.F., 2019. An improved acridine orange staining of DNA/RNA. Acta Histochem. 121, 450–454. doi:10.1016/j.acthis.2019.03.010
11. Farré, M., Gajda-Schrantz, K., Kantiani, L., Barceló, D., 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. Anal. Bioanal. Chem. 393, 81–95. doi:10.1007/s00216-008-2458-1

12. Fu, P.P., Xia, Q., Hwang, H.M., Ray, P.C., Yu, H., 2014. Mechanisms of nanotoxicity: Generation of reactive oxygen species. J. Food Drug Anal. 22, 64–75. doi:10.1016/j.jfda.2014.01.005
13. Gambardella, C., Mesari, T., Milivojevi, T., Sep, K., Gallus, L., Carbone, S., Ferrando, S., Faimali, M., 2014. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemical responses. Env. Monit Assess 4249–4259. doi:10.1007/s10661-014-3695-8
14. Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of Artemia sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519. doi:10.1007/s10646-010-0461-8
15. Hund-Rinke, K., Simon, M., 2006. Ecotoxic effect of photocatalytic active nanoparticles (TiO2) on algae and daphnids. Environ. Sci. Pollut. Res. 13, 225–232. doi:10.1065/espr2006.06.311
16. Johari, S.A., Rasmussen, K., Gulumian, M., Ghazi-Khansari, M., Tetarazako, N., Kashiwada, S., Asghari, S., Park, J.W., Yu, I.J., 2019. Introducing a new standardized nanomaterial environmental toxicity screening testing procedure, ISO/TS 20787: aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using Artemia sp. nauplii. Toxicol. Mech. Methods 29, 95–109. doi:10.1080/15376516.2018.1512695
17. Kachenton, S., Jiraungkoorskul, W., Kangwanrangsan, N., Tansatit, T., 2019. Cytotoxicity and histopathological analysis of titanium nanoparticles via *Artemia salina*. Environ. Sci. Pollut. Res. 26, 14706–14711. doi:10.1007/s11356-018-1856-y
18. Khoshnood, R., Jaafarzadeh, N., Sh, J., Farshchi, P., Taghavi, L., 2017. Acute toxicity of TiO_2, CuO and ZnO nanoparticles in brine shrimp, *Artemia franciscana*. Iran. J. Fish. Sci. 16, 1287–1296.
19. Kvıtek, L., Soukupova, J., Vec, R., Prucek, R., Holecova, M., Zbor, R., 2008. Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). J. Phys. Chem. C 112, 5825–5834. doi:10.1021/jp711616v
20. Lacave, J.M., Fanjul, Á., Bilbao, E., Gutierrez, N., Barrio, I., Arostegui, I., Cajaraville, M.P., Orbea, A., 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI-coated silver nanoparticles to zebrafish. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 199, 69–80. doi:10.1016/j.cbpc.2017.03.008
21. Libralato, G., 2014. The case of Artemia spp. in nanoecotoxicology. Mar. Environ. Res. 101, 38–43. doi:10.1016/j.marenvres.2014.08.002
22. Lu, P.J., Huang, S.C., Chen, Y.P., Chiueh, L.C., Shih, D.Y.C., 2015. Analysis of titanium dioxide and zinc oxide nanoparticles in cosmetics. J. Food Drug Anal. 23, 587–594. doi:10.1016/j.jfda.2015.02.009
23. Manfra, L., Tornambè, A., Savorelli, F., Rotini, A., Canepa, S., Mannozzi, M., Cicero, A.M., 2014. Ecotoxicity of diethylene glycol and risk assessment for marine environment. J. Hazard. Mater. 284, 130–135. doi:10.1016/j.jhazmat.2014.11.008
24. Mayorga, P., Pérez, K.R., Cruz, S.M., Cáceres, A., 2010. Comparação de bioensaios com os crustáceos *Artemia salina* e *Thamnocephalus platyurus* para abordagem de extratos de plantas com toxicidade. Brazilian J. Pharmacogn. 20, 897–903. doi:10.1590/S0102-695X2010005000029

25. Morgana, S., Estévez-Calvar, N., Gambardella, C., Faimali, M., Garaventa, F., 2018. A short-term swimming speed alteration test with nauplii of Artemia franciscana. Ecotoxicol. Environ. Saf. 147, 558–564. doi:10.1016/j.ecoenv.2017.09.026
26. Murdock, R.C., Braydich-Stolle, L., Schrand, A.M., Schlager, J.J., Hussain, S.M., 2008. Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scattering technique. Toxicol. Sci. 101, 239–253. doi:10.1093/toxsci/kfm240
27. Nowack, B., Bucheli, T.D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environ. Pollut. 150, 5–22. doi:10.1016/j.envpol.2007.06.006
28. Nunes, B.S., Carvalho, F.D., Guilhermino, L.M., Van Stappen, G., 2006. Use of the genus Artemia in ecotoxicity testing. Environ. Pollut. 144, 453–462. doi:10.1016/j.envpol.2005.12.037
29. Ocaranza-Joya, V.S., Manjarrez-Alcivar, I., Ruizgonzález, L.E., Guerrero-Galván, S.R., Vega-Villasante, F., 2019. Sensitivity of different stages of *Artemia franciscana* to potassium dichromate. Panam. J. Aquat. Sci. 14, 8–12.
30. OECD, 2004. OECD Guideline for testing of chemical, Guideline for Testing of Chemicals, Section 2. doi:10.1787/9789264069947.
31. Ozkan, Y., Altinok, I., Ilhan, H., Sokmen, M., 2016. Determination of TiO_2 and $AgTiO_2$ Nanoparticles in *Artemia salina*: Toxicity, morphological changes, uptake and depuration. Bull. Environ. Contam. Toxicol. 96, 36–42. doi:10.1007/s00128-015-1634-1
32. Palácio, S.M., de Almeida, J.C.B., de Campos, É.A. et al. Silver nanoparticles effect on *Artemia salina* and *Allium cepa* organisms: influence of test dilution solutions on toxicity and particles aggregation. Ecotoxicology 30, 836–850 (2021). https://doi.org/10.1007/s10646-021-02393-7
33. Pillard, D.A., Tapp, K.L. Influence of feeding and organism age on the acute toxicity of sodium bromide to *Artemia salina*. Ecotoxicology 30, 914–918 (2021). https://doi.org/10.1007/s10646-021-02417-2
34. Rekulapally, R., Chavali, L.N.M., Idris, M.M., Singh, S., 2019. Toxicity of TiO_2, SiO_2, ZnO, CuO, Au and Ag engineered nanoparticles on hatching and early nauplii of Artemia sp. PeerJ 2019. doi:10.7717/peerj.6138
35. Sadrieh, N., Wokovich, A.M., Gopee, N. V., Zheng, J., Haines, D., Parmiter, D., Siitonen, P.H., Cozart, C.R., Patri, A.K., McNeil, S.E., Howard, P.C., Doub, W.H., Buhse, L.F., 2010. Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano and submicron-size TiO_2 particles. Toxicol. Sci. 115, 156–166. doi:10.1093/toxsci/kfq041
36. Sánchez-Fortún, S., Sanz, F., Barahona, M. V., 1996. Acute toxicity of several organophosphorous insecticides and protection by cholinergic antagonists and 2-PAM on *Artemia salina* larvae. Arch. Environ. Contam. Toxicol. 31, 391–398. doi:10.1007/BF00212678
37. Sarkheil, M., Johari, S.A., An, H.J., Asghari, S., Park, H.S., Sohn, E.K., Yu, I.J., 2018. Acute toxicity, uptake, and elimination of zinc oxide nanoparticles (ZnONPs) using saltwater microcrustacean, *Artemia franciscana*. Environ. Toxicol. Pharmacol. 57, 181–188. doi:10.1016/j.etap.2017.12.018

38. Shi, H., Magaye, R., Castranova, V., Zhao, J., 2013. Titanium dioxide nanoparticles: A review of current toxicological data. Part. Fibre Toxicol. 10. doi:10.1186/1743-8977-10-15
39. Sirotkin, A. V., Bauer, M., Kadasi, A., Makovicky, P., Scsukova, S., 2021. The toxic influence of silver and titanium dioxide nanoparticles on cultured ovarian granulosa cells. Reprod. Biol. 21, 100467. doi:10.1016/j.repbio.2020.100467
40. Sorgeloos, P., Remiche-Van Der Wielen, C., Persoone, G., 1978. The use of Artemia nauplii for toxicity tests-A critical analysis. Ecotoxicol. Environ. Saf. 2, 249–255. doi:10.1016/S0147-6513(78)80003-7
41. Wang, C., Jia, H., Zhu, L., Zhang, H., Wang, Y., 2017. Toxicity of α-Fe_2O_3 nanoparticles to Artemia salina cysts and three stages of larvae. Sci. Total Environ. 598, 847–855. doi:10.1016/j.scitotenv.2017.04.183
42. Wang, L.F., Habibul, N., He, D.Q., Li, W.W., Zhang, X., Jiang, H., Yu, H.Q., 2015. Copper release from copper nanoparticles in the presence of natural organic matter. Water Res. 68, 12–23. doi:10.1016/j.watres.2014.09.031
43. Zhu, S., Luo, F., Chen, W., Zhu, B., Wang, G., 2017. Toxicity evaluation of graphene oxide on cysts and three larval stages of *Artemia salina*. Sci. Total Environ. 595, 101–109. doi:10.1016/j.scitotenv.2017.03.224

Tables

Table 1 – Acute toxicity of TiO_2 in *A. salina* nauplii instar I and percentage of individuals dead within 24 and 48 h. At this stage of development, no toxicity of $MTiO_2$ was observed at 12.0, 25, 50 and 100 ppm concentrations. Mean ± standard deviation (σ) obtained from the triplicate in each treatment. Significant difference between ASW and *A. salina* exposed to TiO_2 are symbolized by an asterisk * (t-test, $P < 0.05$).

***A. salina* - nauplii instar I**						
24 hours of exposure				48 hours of exposure		
Treatments	Mean ± SD of living individuals	*P-value*	% Mortality	Mean ± SD of living individuals	*P-value*	% Mortality
ASW	10.0 ± 0.0	-	0%	10.0 ± 0.0	-	0%
$K_2Cr_2O_7$	0.0 ± 0.0*	***P ≤ 0.01***	100%	0.0 ± 0.0*	***P ≤ 0.01***	100%
12.5 ppm	10.0 ± 0.0	*P = NA*	0%	10.0 ± 0.0	*P = NA*	0%
25 ppm	10.0 ± 0.0	*P = NA*	0%	10.0 ± 0.0	*P = NA*	0%
50 ppm	10.0 ± 0.0	*P = NA*	0%	10.0 ± 0.0	*P = NA*	0%
100 ppm	10.0 ± 0.0	*P = NA*	0%	10.0 ± 0.0	*P = NA*	0%

Table 2 – Acute toxicity of $MTiO_2$ in *A. salina* nauplii instar II and percentage of individuals killed within 24 h and 48 h. At 24 h of exposure to $MTiO_2$, no toxicity was observed in nauplii instar II. However, in 48 h of exposure the toxicity was directly proportional to the concentration of $MTiO_2$. Mean ± standard deviation (σ) for all concentrations. Significant difference between ASW and *Artemia salina* exposed to TiO_2 are symbolized by an asterisk * (t-test, P <0.05).

***A. salina* - nauplii instar II**						
24 hours of exposure				48 hours of exposure		
Treatments	Mean ± SD of living individuals	*P-value*	% Mortality	Mean ± SD of living individuals	*P-value*	% Mortality
ASW	10.0 ± 0.0	-	0%	9.33 ± 1.15	-	6.67%
$K_2Cr_2O_7$	0.0 ± 0.0*	***P ≤ 0.01***	100%	0.0 ± 0.0*	***P ≤ 0.01***	100%
12.5 ppm	9.67 ± 0.58	*P = 0.4226*	3.33%	8.0 ± 0.0	*P = 0.1835*	20%
25 ppm	10.0 ± 0.0	*P = NA*	0%	7.0 ± 1.73*	***P = 0.0198***	30%
50 ppm	10.0 ± 0.0	*P = NA*	0%	5.0 ± 0.0*	***P = 0.0228***	50%
100 ppm	10.0 ± 0.0	*P = NA*	0%	7.0 ± 1.0*	***P = 0.0474***	30%

Supplementary Material

The Supplementary Material is not available with this version

Figures

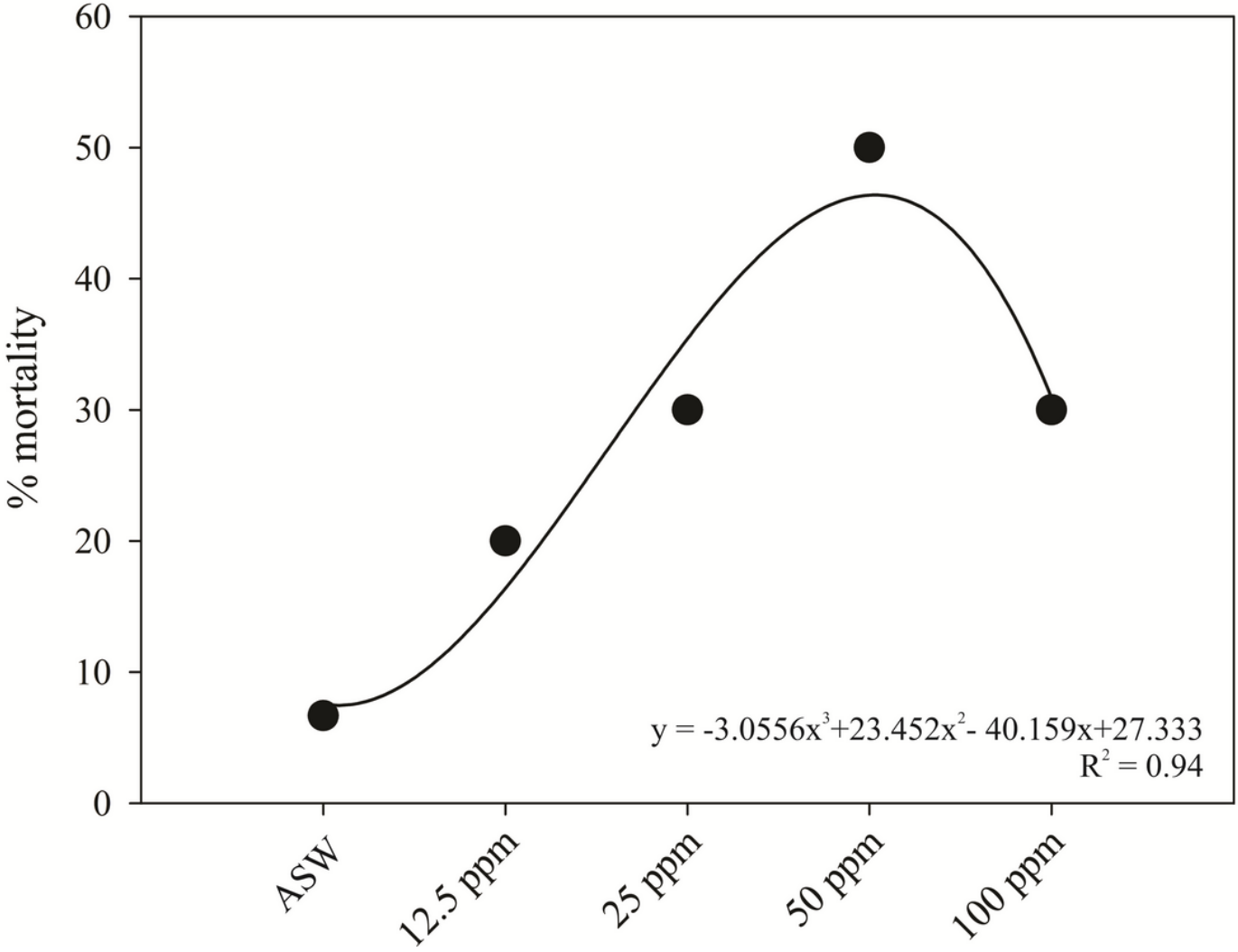

Figure 1

Polynomial nonlinear regression demonstrating the dose-dependent effect with gradual increase shows relationship between $MTiO_2$ concentrations ranged from 0 to 100 ppm and percentage of mortality (%) for *A. salina* nauplii instar II, in the 48 h exposure time.

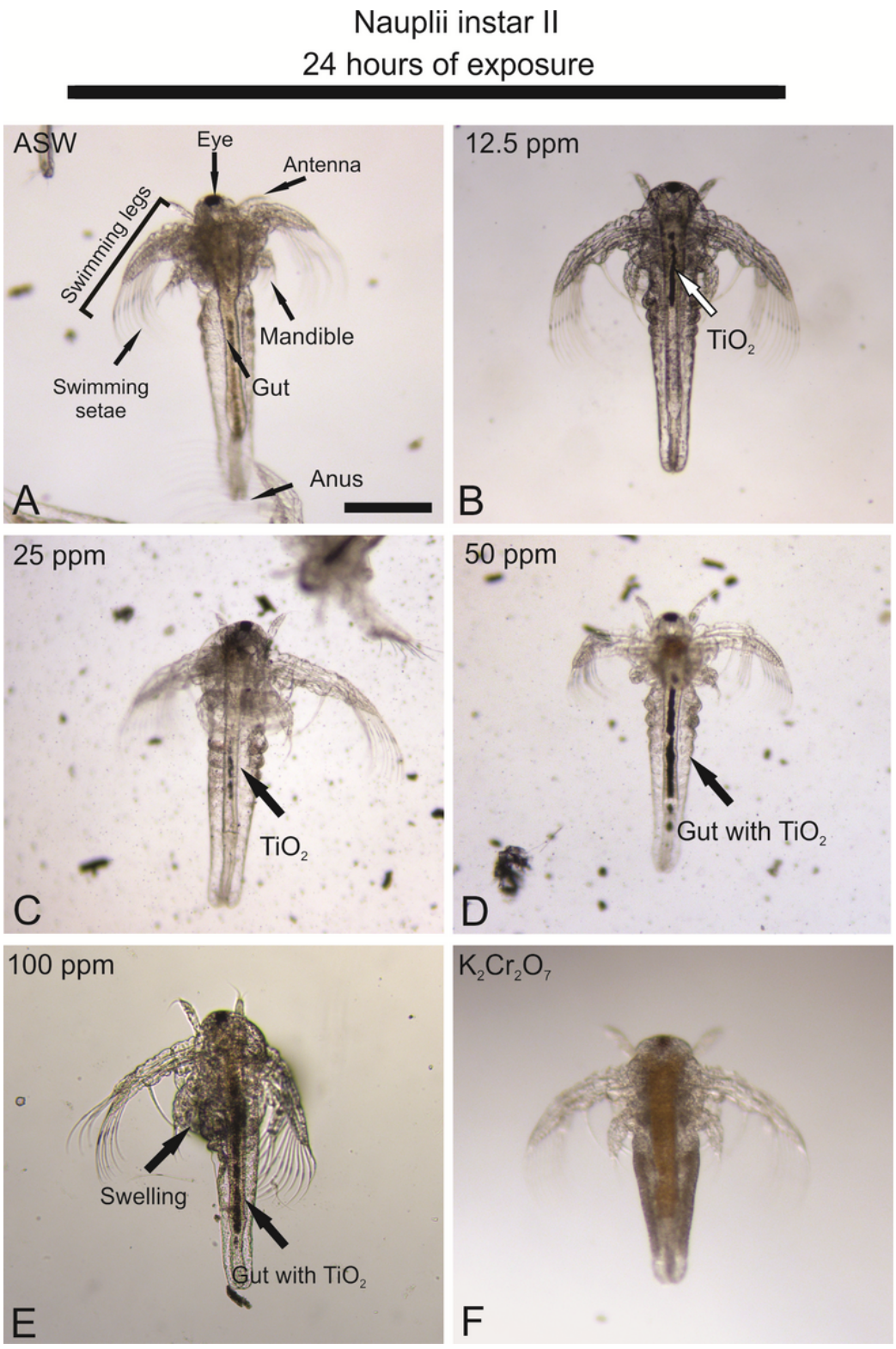

Figure 2

Optical microscopy of *A. salina* nauplii instar II, submitted to different concentrations of $MTiO_2$ within 24 h of exposure.

A – *A. salina* in ASW; B – *A. salina* nauplii exposed to a 12.5 ppm of $MTiO_2$ concentration; C – *A. salina* nauplii exposed to a concentration of 25 ppm; D – Nauplii submitted to 50 ppm of $MTiO_2$ concentration

with accumulation of $MTiO_2$ in the gut; E – Nauplii submitted to concentration of 100 ppm $MTiO_2$ with swelling in the cephalothorax; F – Nauplii instar II in $K_2Cr_2O_7$ (Positive control);

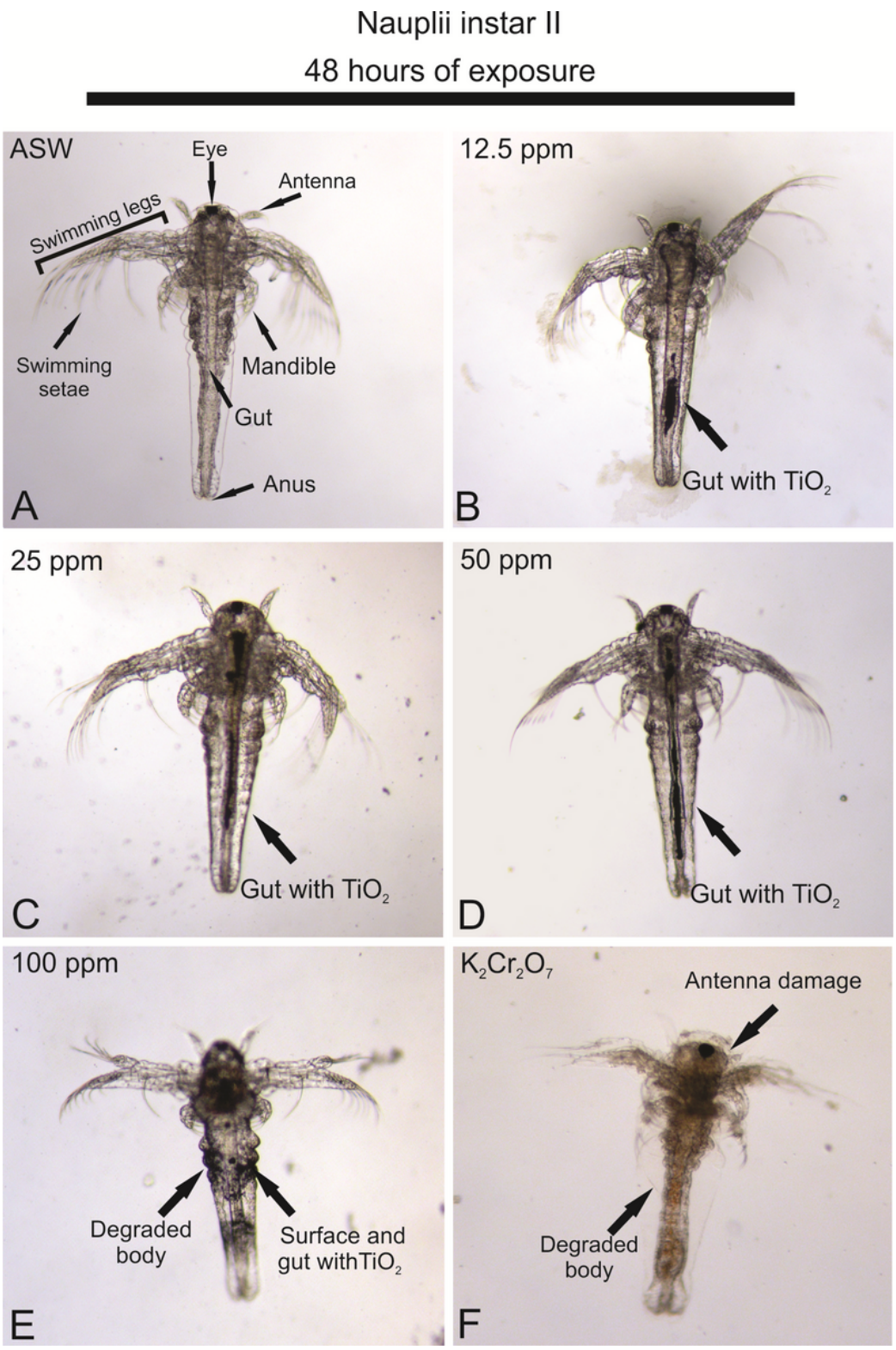

Figure 3

Optical microscopy of *A. salina* nauplii instar II, submitted to different concentrations of $MTiO_2$ within 24 h of exposure.

A – *A. salina* nauplii in ASW; B – $MTiO_2$ accumulation in the gut of the brine shrimp. At a concentration of 12.5 ppm, no damage was observed to the animal's body; C – Gut with accumulation of $MTiO_2$; D – Gut with aggregation of $MTiO_2$; E – $MTiO_2$ dispersed throughout the animal's body. Due to the toxic effect of $MTiO_2$ the animal's body begins to degrade; F – *A. salina* nauplii in $K_2Cr_2O_7$. Bars: 200 µm.

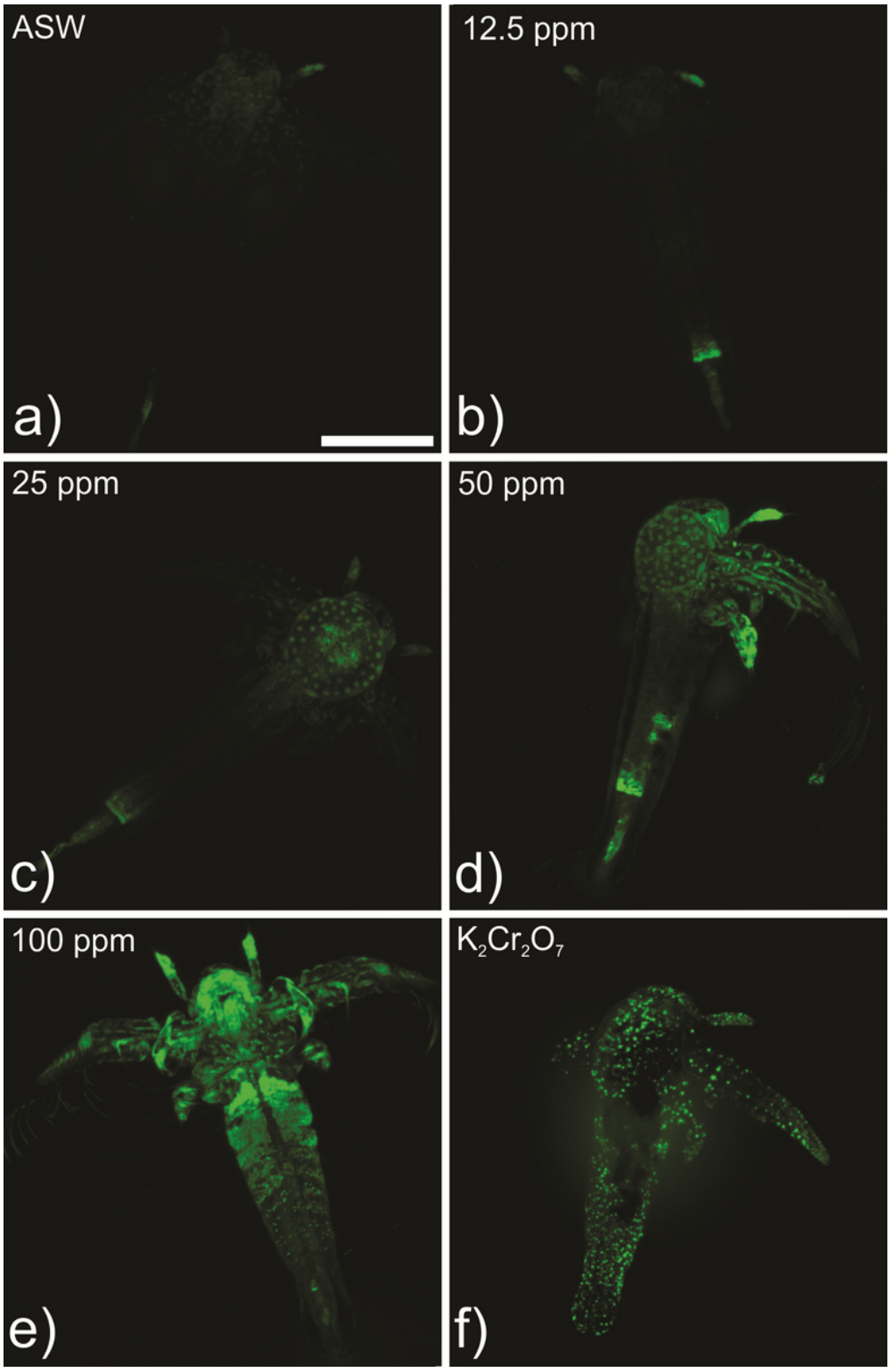

Figure 4

Confocal Laser Scanning Microscopy (CLSM) in *Artemia salina* nauplii instar II, submitted to different concentrations of $MTiO_2$ with acridine orange stained within 48 h of exposure.

A – ASW presented low emission is related to damage to the animal's body; B - *A. salina* submitted to the concentration of 12.5 ppm of $MTiO_2$; C - *A. salina* submitted to a concentration of 25 ppm of $MTiO_2$; D -

At a concentration of 50 ppm $MTiO_2$, a higher fluorescence emission was observed due to cell damage; E - At the maximum tested concentration (100 ppm), fluorescence emission was observed throughout the animal's body, due to the accumulation and damage caused by $MTiO_2$; F - *A. salina* submitted to $K_2Cr_2O_7$ presented strong emission due to cell damage caused by the reagent. Bars: 200µm.

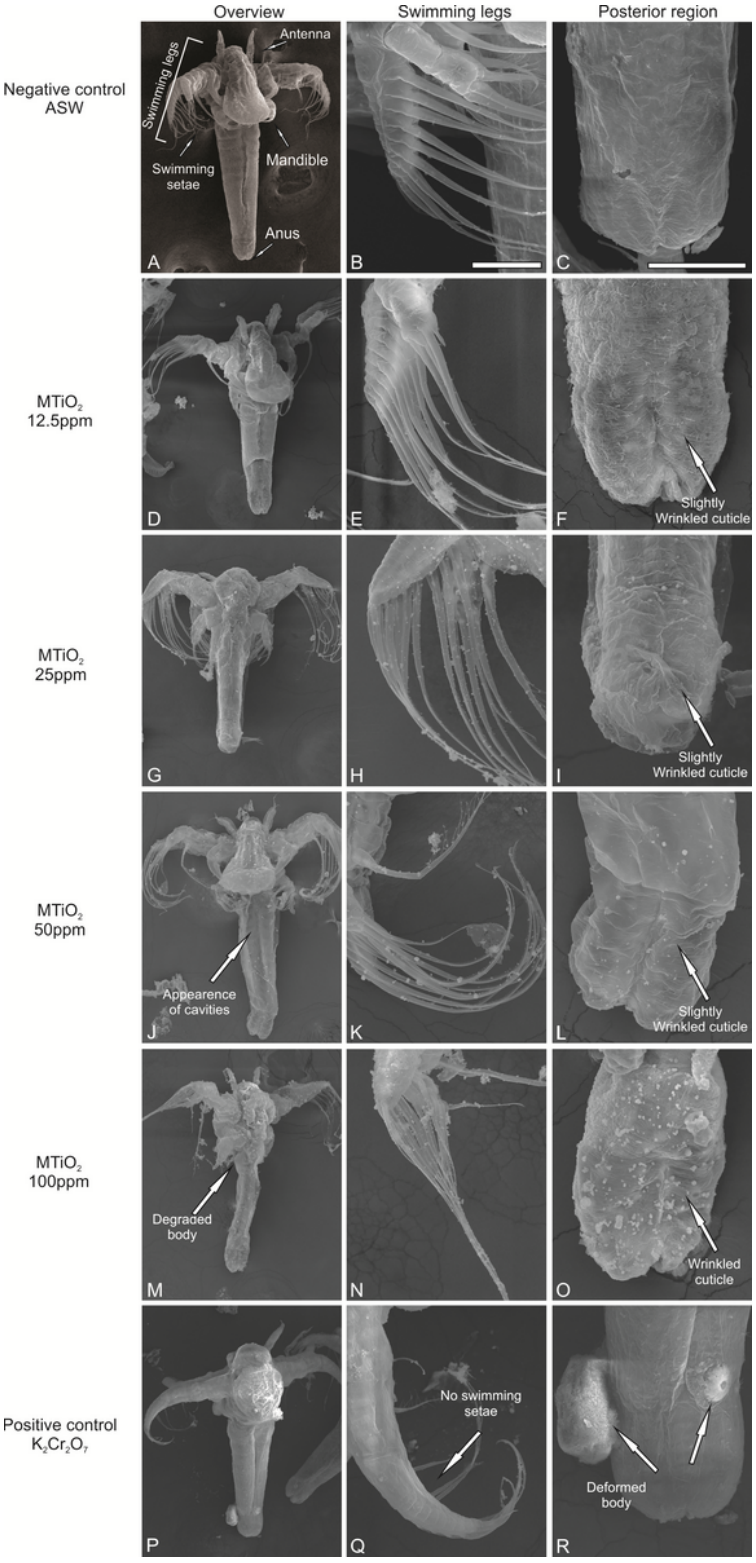

Figure 5

Scanning electron microscopy (SEM) in *Artemia salina* nauplii instar II, submitted to different concentrations of $MTiO_2$ within 48 h of exposure.

A – Overview of *A. salina* nauplii instar II in ASW; B – Swimming legs; C – Posterior region; D - Overview of *A. salina* submitted to 12.5 ppm concentration, no apparent damage; E - Swimming legs no damage; F – Slightly wrinkled posterior region; G - Overview of *A. salina* submitted to 25 ppm concentration, no apparent damage; H - Swimming legs no damage; I – Slightly wrinkled posterior region; J - Overview of *A. salina* submitted to 50 ppm concentration with cavities in the body; K - Swimming legs no damage; L - Slightly wrinkled posterior region; M - Overview of *A. salina* submitted to 100 ppm concentration. Observe the degraded animal´s body; N - Swimming legs no damage; O - Wrinkled posterior region; P - Overview of *A. salina* nauplii instar II in $K_2Cr_2O_7$; Q – Swimming setae with damage; R – Deformed posterior region. Bars: A, D, G, J, M and P - 150µm; B, E, H, K, N and Q - 50µm; C, F, I, L, O and R - 50µm