

Research Article

Effects of Silver and Titanium Dioxide Nano Particles on Hatching Rates of Decapsulated and Non Decapsulated *Artemia Franciscana* Cysts

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Abstract

Wastewater and effluent of nano technology products outputs the aquatic environment, agricultural lands, aquatic and terrestrial habitats and may pose a risk to the ecosystems. We evaluated the toxic effect of nano materials on the aquatic environment, the effect of colloidal Ag nanoparticles (in concentration of 0.1, 1, 10, 32 and 100 mg/L) and the suspension of TiO₂ nanoparticles (in concentrations of 10, 32 and 100 mg/L) were examined on the hatching rate of *Artemia franciscana* cysts within 24 hours. The results showed that Ag and TiO₂ nanoparticles significantly reduced the hatching rate of *A. franciscana* cysts than those in the control group ($p < 0.05$). The results also showed that hatching rate of cysts at AgNPs treatments were significantly less than TiO₂NPs treatments and hatching rate of decapsulated cysts was significantly higher than undecapsulated cysts in AgNPs treatments ($p < 0.05$). Deposited AgNPs especially on the alveolar layer could reduce significantly hatching rate of the undecapsulated cysts compared to decapsulated cysts. Also the most important reason of decrease in cyst hatching rate is probably unfavorable effects of nanoparticles on the process of water absorption by cysts. In fact nanoparticles impact on the glycogen secretion and hydrophilic process which consequently leads to hatching failure.

Keywords: Ecotoxicology; Silver; TiO₂; Nanoparticles; *Artemia franciscana*; Cyst; Hatching rate

Introduction

Nanotechnology is the ability of development and producing the novel materials, tools and systems in nano size and application of their new properties at the nano scale [1]. Today nanoparticles (NPs) have been widely used in various field of sciences such as biological science e.g. biosensor [2], therapeutics [3], medical sciences (e.g. drug delivery systems) [4], environmental science, energy and cosmetics [5]. Among the various nanomaterials, Ag and TiO₂ Nanoparticles are two most widely used in both *industry and daily life* [6]. Estimated global annual production of Ag and TiO₂ NPs are about 55 and 3,000 tons, respectively [7]. The presence of Ag and TiO₂ NPs in the natural water was estimated from 0.03 to 0.32 and from 0.7 to 16 micrograms per liter (µg/L), respectively [8].

Due to its high nutritional value, *Artemia* (brine shrimp) has widely been considered as a live food for larval fish production and fish hatcheries. In addition, this organism could be used as a bioindicator to evaluate potential toxicity of various materials [9-11]. Possible use of *Artemia* in Ecotoxicological studies can be related to its highlight advantages characteristics and general physiological features such as adaptability to wide ranges of salinity (5-250 g L⁻¹) and temperature (6-35 °C), short life cycle, high adaptability to unfavorable environmental

conditions, high fecundity, bisexual parthenogenetic reproduction strategy (by production of nauplii and or cysts), small body size, and adaptability to divers nutrient resources as it is as a non-selective filter feeder [11].

Due to the large production and use of nanomaterials and their consequent entry into the aquatic environment as well as the limitations of available aquatic nanotoxicity data, the aim of the present work was identification and comparison the effects of Ag and TiO₂ nanoparticles (as two widely used nanomaterials) on hatching rate of both decapsulated and non-decapsulated *Artemia franciscana* cysts.

Materials and Methods

Nanomaterials and Characterization

Colloidal silver nanoparticles (AgNPs) and powdered titanium dioxide nanoparticles (TiO₂NPs) were used in the anatase form. The AgNPs, brand L (Nanocid) and TiO₂NPs were purchased from Pars Nano Nasb Co (Tehran, Iran) and US Research Nano materials, Inc. (Houston, USA), respectively.

Prior to toxicity tests, to determine the geometry and size of nanomaterials, TEM analysis was performed using a transmission electron microscope (H-7100Fa, Hitachi-Japan). In order to calculate the average size of Ag and TiO₂NPs, 700 particles were randomly selected on the images at 100,000 magnifications and measured using Axio Vision digital image processing software (Release 4.8.2.0, GmbH Carl Zeiss Micro Imaging-German).

A stock suspension of 300 mg/L AgNPs was prepared by diluting of initial colloid (4000 mg/L) in double distilled water and this stock then were sonicated using a bath sonicator (U1250HD) for 30 min. In order to make a stock suspension of 300 mg/L TiO₂NPs deionized water was added drop by drop into 0.3 g of TiO₂NPs powder and mixed until a steady paste formed. Adding deionized water into this paste continued for 40 min when the mixture was reached to 1000 ml. Mixture was placed into a jar and then was sonicated for 5 min using a bath sonication system (U1250HD) following 10 more minutes sonication using a probe sonicator (misonixs-400-01, 70 amplitude = 70-75 wat). Finally, the milky suspension of TiO₂NPs was obtained. The suspension was placed in a dark place because the dilute suspension of TiO₂NPs has high *light sensitivity* [12,13].

Hatching Conditions of Artemia Cysts

The *A. franciscana* cysts (San Francisco Strain Brine shrimps-A.H.T brand- United States) were used in the present study. The cysts were incubated in conical tubes filled with the artificial seawater (ASW) adjusted at 3.5% salinity (35 g of Urmia lake salt was added to 1 L distilled water), and 30 °C (through the immersion of conical tubes in a water bath equipped with electric heater). The pH was adjusted at 8.0 ± 0.5 (by adding NaHCO₃ solution) and range of light intensity values were about 1800 lux (provided by fluorescent lamps). Air was bubbled through the incubator via a glass tube extending to the bottom of the hatching vessel to keep all the cysts in continuous motion [14]. Average water hardness obtained by flame photometer was 145 mg/L CaCO₃. In order to investigate the resistance effect resulting from *Artemia* cyst shell, against nanoparticles, cysts were hatched either decapsulated or non-decapsulated.

Toxicology Tests

Hatching rate of *Artemia franciscana* cysts in the presence of AgNPs (at the concentrations of 0.1, 1, 10, 32, and 100 mg/L) or TiO₂NPs (at the concentration of 10, 32, and 100 mg/L) were measured and compared to the control groups (without nanoparticles). In each treatment, after 24 hours of incubation (hatching time), 250 µl were sampled from each incubator in 6 replicate and hatching rates were calculated and compared to the controls. Samples were fixed using logol solution and then were examined under stereomicroscope and hatched nauplii, umbrellas and unhatched cysts were counted. Hatching rates were calculated by the Van Stappen [14] formula:

$$(H\%) = N/(N+C) \times 100.$$

In which, H is hatching rate, N is the number of hatched cysts, including the umbrella stage, and C is the number of total cysts (hatched and unhatched).

Statistical Analyses

All the measured data were incorporated in an excel database. All

statistical tests were performed using SPSS v. 13.2. Normality test was done by Kolmogorov-Smirnov and Shapiro-Wilk depending on the type of data. If the data were non-parametric, Mann-Whitney U test was used for comparison of two independent groups and Kruskal-Wallis test was used for comparison of independent groups. For the analysis of normal data, The ANOVA were used for comparison of several independent groups and the Duncan test were used for means compare, while the Samples T-Test was used for compare of two independent groups. Also, one way ANOVA was used to compare several independent groups with control, and Dunnett's test was used to compare means. In the case of normal data with non homogeneity variance, Games-Howell test was used to compare several independent groups.

Results

Properties of Nanomaterials

According to the TEM images, the shape of AgNPs was spherical and maximum diameter was about 129 nm (Figure 1). Only 2.28% of the particles were larger than 100 nm. Also 65.14% of the particles estimated to be in range of 1 to 13 nm and count median diameter (CMD) was equal to 6.47 nm (Figures 2 and 3). Geometric mean diameter (GMD) and Geometric standard deviation (GSD) of particles diameter was 12.65 nm and 1.46, respectively. The particle diameter arithmetic mean was 2.54 ± 1.14 nm.

As per TEM images, TiO₂NPs were needle or pyramidal shape (Figure 3). Also 86.23% of the particles estimated to be in range of 5 to 30 nm and 5.07% of the particles were larger than 50 nm and the maximum particle diameters was measured as 81 nm. Also median value of cumulative frequency particle size, GMD and GSD of TiO₂NPs was calculated as 13.90 nm, 17.50 nm and 1.71, respectively. Also TEM images of suspended TiO₂NPs showed that large clusters had formed in water; in which 28.47%, 52.55%, and 18.98% of the clusters had diameter between 15 to 100 nm, between 100 to 500 nm and larger than 500 nm, respectively (Figure 4).

Effect of Agnps and Tio₂nps on Hatching Rate of Artemia Franciscana Cyst

Hatching rate of the decapsulated cysts was not significantly different between concentrations of 0.1, 1, 10, and 32 mg/L AgNPs ($p > 0.05$). But exposure to 100 mg/L AgNPs significantly reduced

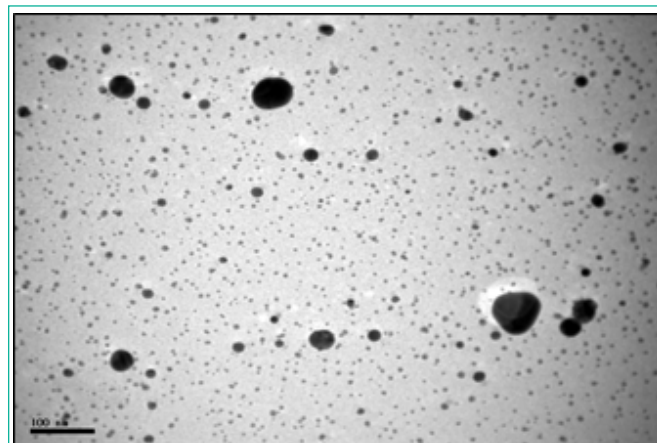


Figure 1: TEM image of silver nanoparticles (4000 mg/L).

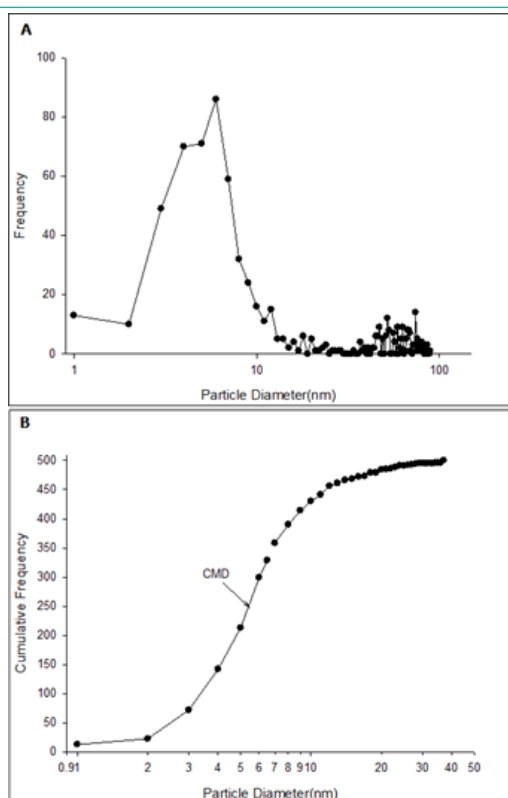


Figure 2: Size distribution of silver nano particles in undiluted suspension (4000 mg/L) based on transmission electron microscope data. A: Number Frequency, B: Cumulative Frequency. (CMD: Cumulative Median Diameter).

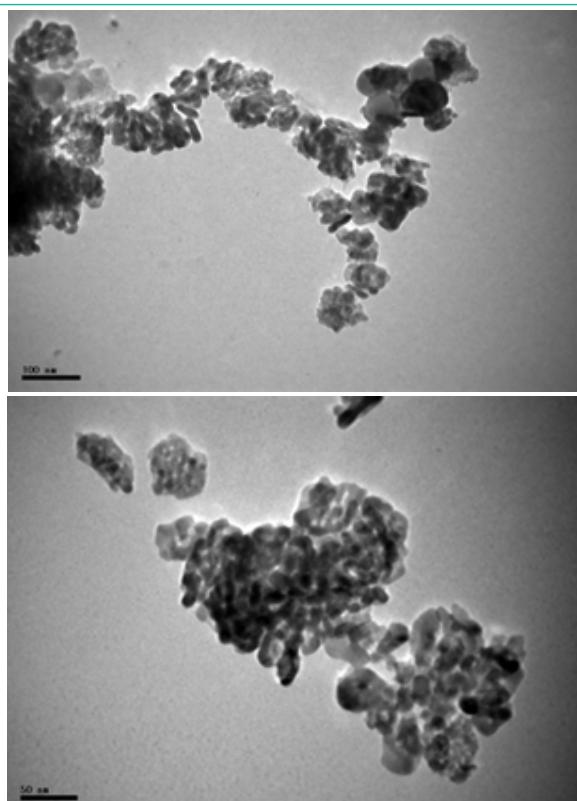


Figure 3: TEM images of titanium dioxide nanoparticles. (A): Dry TiO₂ powder. (B): Suspended TiO₂ powder (400 mg/L). Diameter).

the cysts hatching rate. In the case of uncapsulated cysts, all AgNPs concentrations were significantly different from each other ($p < 0.05$) and the concentrations of 0.1 and 100 mg/L AgNPs were showed maximum and minimum hatching rate, respectively. Moreover, hatching rate of both decapsulated and uncapsulated cysts in all AgNPs concentrations were significantly lower than the controls ($p < 0.05$). Hatching rate of decapsulated cysts was significantly higher than uncapsulated cysts in AgNPs treatments (Figure 5, A).

The results showed that hatching rates did not vary significantly between decapsulated and uncapsulated cysts. Also there were no significant differences between TiO₂NP concentrations of 10, 32, and 100 mg/L ($p > 0.05$). Hatching rate of cysts in control groups was significantly higher than all TiO₂NP treatments for both decapsulated and uncapsulated cysts ($p < 0.05$, Figure 5, B).

Statistical analysis showed that hatching rate of the decapsulated cysts of AgNPs and TiO₂NPs treatments was not significantly different in 10 and 32 mg/L treatments ($P > 0.05$); while there was a significant difference in hatching rate between 100 mg/L TiO₂NPs and 100 mg/L AgNPs.

In the case of uncapsulated cysts, it was found that hatching rate

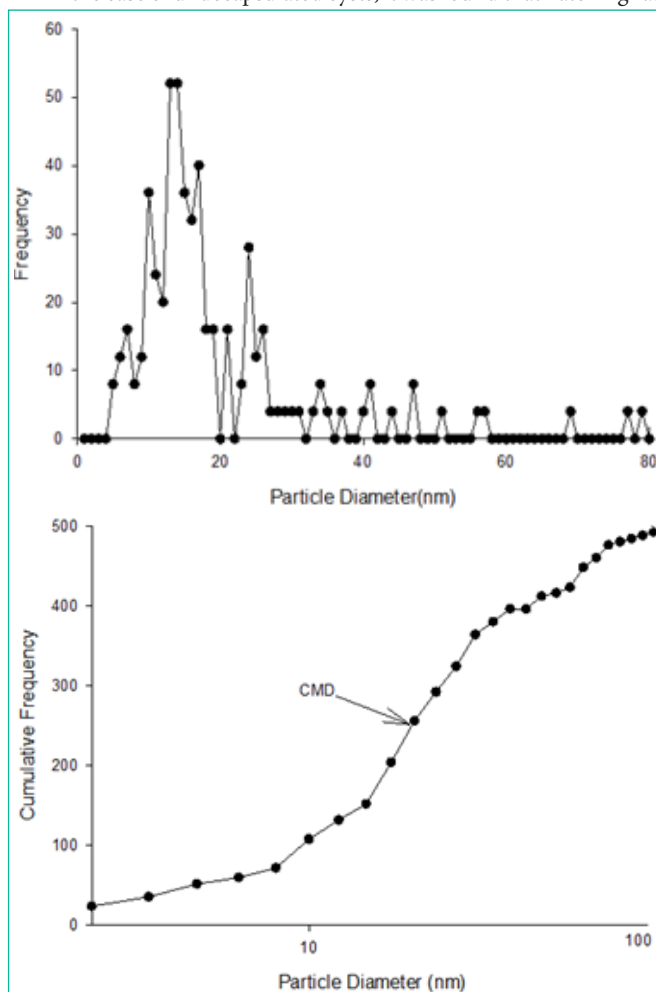
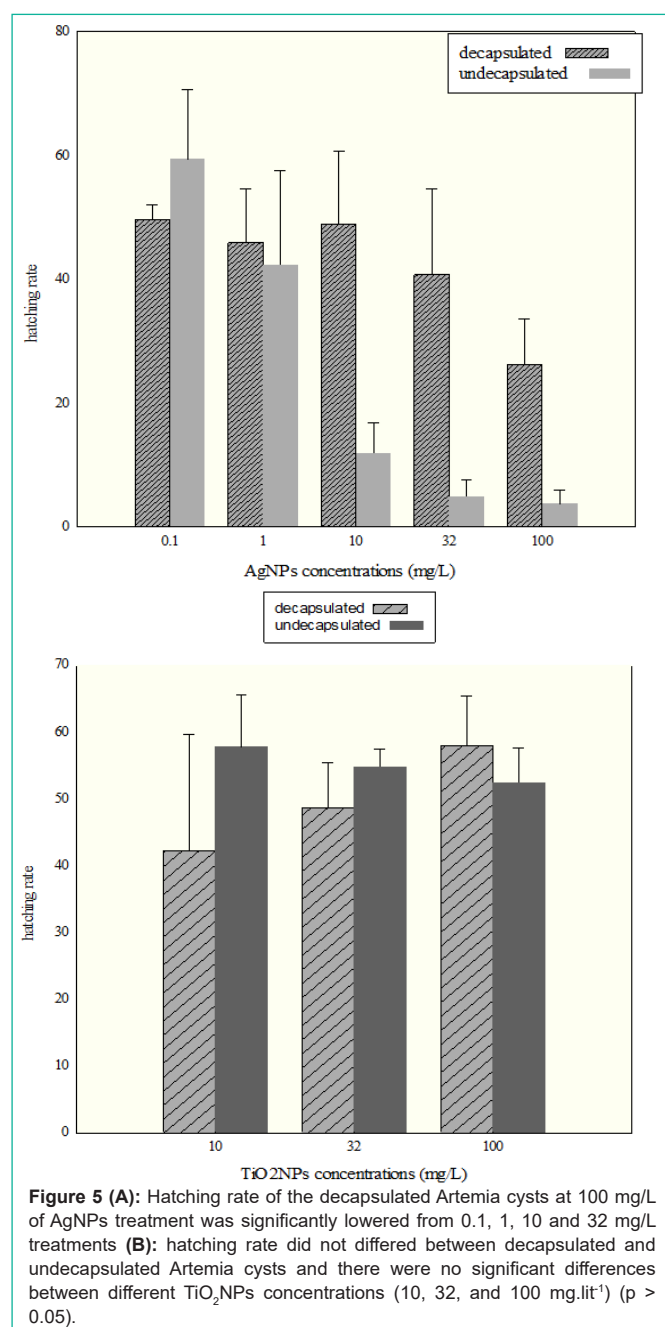


Figure 4: Size distribution of nano particles of titanium dioxide in suspension (400 mg/L) based on transmission electron microscope data. A: Number Frequency, B: Cumulative Frequency. (CMD: Cumulative Median Diameter).



of the cysts incubated with TiO₂NPs was higher than those incubated with AgNPs in all equal concentrations (10, 32 and 100mg/L) ($p < 0.05$, Figure 6).

Discussion

Artemia spp. is one of the most widespread saltwater organisms suitable for ecotoxicity testing. The most cited testing methods chronologically ordered belong to, Michael et al., Harwig and Scott, Sorgloos et al., Persoone and Vanhaecke, Persoone and Wells, Solis et al., Libralato et al., Manfra et al., Gambardella et al. [11,15-22] and also *Artemia franciscana* is one of the model organism in ecotoxicity testing [23], because it is widely used as a nutritious live food source to the larvae of a variety of marine organism [24] and is easy to maintain under laboratory conditions [10,25]. Regarding the toxic

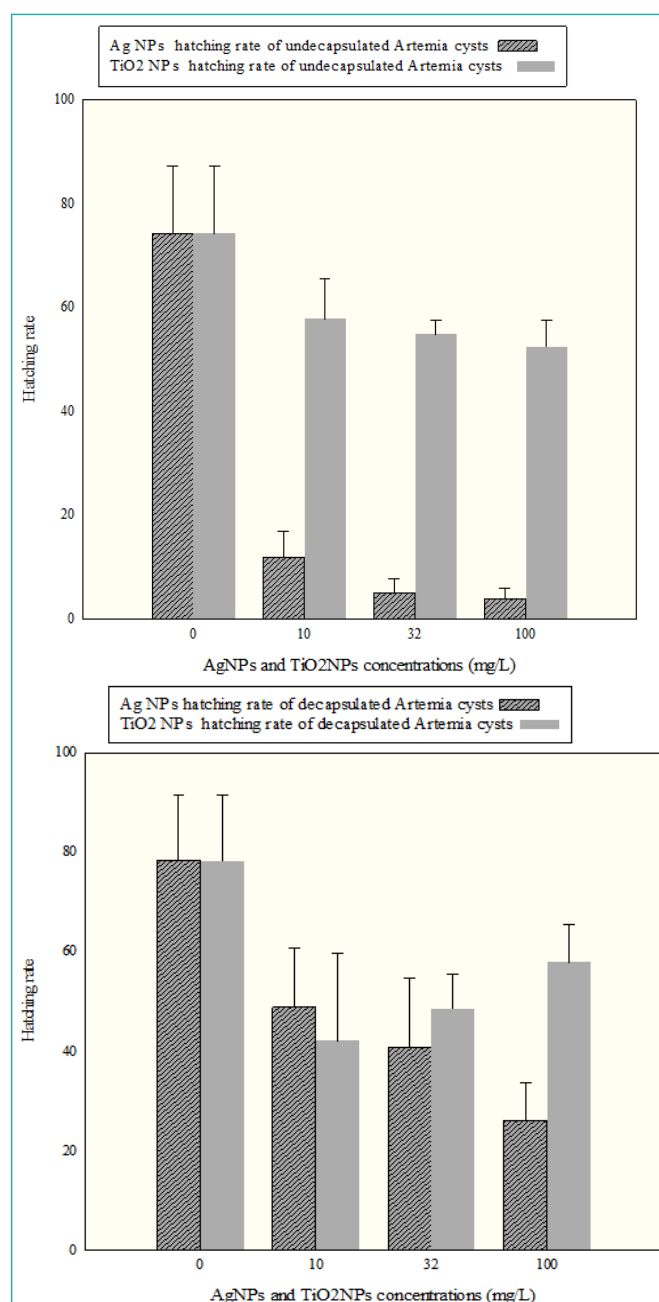


Figure 6: Comparison of toxicity effect silver nano particles with TiO₂ nanoparticles on cyst hatching rate of *Artemia franciscana*. (A): hatching rate of the uncapsulated Artemia cysts (B): hatching rate of the decapsulated Artemia cysts.

effects of Ag-NPs, some studies concerning rainbow trout sperm cells [26], *Daphnia magna* nauplii [27] *Artemia salina* nauplii, [22,28-32], in addition, toxic effects of TiO₂ NPs were investigated on zebrafish [33,34], *Daphnia magna* [35], and *Artemia salina* nauplii [36,37].

Results of the present study revealed that exposure to AgNPs and TiO₂NPs significantly reduce hatching rate of the *Artemia franciscana* cysts, also exposure to the AgNPs caused higher reduction of hatching rate when compared to TiO₂NPs; Similarly Ozkan et al. [37] were found AgTiO₂ to be more toxic to *Artemia salina* nauplii than TiO₂; In previous study concerning the toxicity of nano silver on *Artemia* sp. cyst was found to be similar [38]; which this could be due to the effect

from Ag⁺ ions that released from AgNPs on the ion regulatory of cysts. Similar to our study, Guadagnolo et al. [39] have been reported the effect of ion regulatory impairment on increased mortality in rainbow trout eggs.

When exposed tadpoles to double-wall carbon nano tubes Mouchet et al [40] found that aggregation of nano tubes had negative effect on oxygen uptake by embryos and also caused delayed hatching due to indirect effect of hypoxia. So the negative effects of silver ions on cysts, deposition of silver nano particles on the chorion could cause physical blockage and consequent hypoxia and fetal asphyxia. Deposited AgNPs especially on the alveolar layer could reduce significantly hatching rate of the uncapsulated cysts compared to decapsulated cysts.

In the present study, as shown in Figure 5 (A), minimum hatching rate of the *Artemia franciscana* cysts was observed in 100 mg/L AgNPs which same results was also reported by Arulvasu et al. [41] that revealed that as the concentration of the silver nanoparticles increases (from 2 nM to 10nM and 12nM) the effect of toxicity increases and the percentage of hatching of the *Artemia* cysts decreased from 56% to 31.6% and 21.6%. The effects on *Artemia* cysts were also consistent with those reported that silver nano particles at high concentrations of 50 to 100 mg/L could cause mortality and delayed hatching in zebrafish embryos [42].

Present study also revealed that TiO₂NPs reduced the hatching rate in *Artemia* cysts ($p < 0.05$), however no significant difference was observed between all TiO₂NP concentrations ($p > 0.05$). Because TiO₂NPs induced the formation of free radicals which has been reported in Hund-Rinke and Simon [43]; the ROS production is especially relevant in the case of nanoparticles with photocatalytic properties such as TiO₂. Therefore, there was ROS production potential of the suspensions of TiO₂NPs in the present study and it likely could lead to significant reduction in the percentage of hatching in *Artemia* cysts. Aggregation of TiO₂NPs in aqueous suspensions has been reported previously (e.g. Adams et al. [8]; Zhu et al. [10,44]). In present study, there was no significant differences between 100 mg/L TiO₂NPs and control group for decapsulated *Artemia* cysts (Figure 5 and 6). Also, the result showed that as the concentration of TiO₂NPs increased from 10 to 100 mg/L, hatching rate did not differ significantly.

Similar to the present study, Ates et al. [45] and Ozkan et al. [37] were found that the aggregate sizes increased with respect to TiO₂ concentrations. Barelds et al. [46] were exposed the *Artemia salina* nauplii to four different concentrations of TiO₂NPs (0.01, 0.1, 1 and 10 mg/L) and illustrated that the aggregation increased sharply when the TiO₂NPs concentration increased from 0.01 to 10 mg/L. Their study also demonstrated that bioavailability of TiO₂NPs for *Artemia salina* nauplii were reduced due to their aggregation and consequently out of reach. In another study acute exposure of *Artemia* nauplii and adults was conducted in sea water in a concentration range from 10 to 100 mg/L TiO₂NPs for 24 and 96 h [45,47]. These results suggested that suspensions of the TiO₂NPs were nontoxic to *Artemia*, most likely due to the formation of benign TiO₂ aggregates in water. This is consistent with previous findings which suggest it can be caused by possible aggregation of nano-TiO₂ of different sizes ranged from 100 to 500 nm (Figure 3).

Conclusion

The present work demonstrated that exposure of both decapsulated and uncapsulated *Artemia* cysts to either AgNPs or TiO₂NPs was significantly reduced the hatching rate than controls and the toxicity of AgNPs were higher than TiO₂NP because TiO₂NPs rapidly aggregate. Regarding to the increasingly growing use of nanomaterials and future advances in medical sciences and technologies, many of them will release into the marine environment. Hence it can be concluded that the uncontrolled release of these nanoparticles, could impose a large negative consequence on the hatching percentage of organisms like *artemia* which lives in deltaic places and salt marshes area which is prone to entering the nanomaterial infected sewages. Accordingly, this study would provide the useful baseline information to researchers for future studies.

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