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Determination of the acute toxic effect of ZnO-TiO_2 nanoparticles in brine shrimp (*Artemia salina*)

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Abstract: This study aims to investigate aquatic stability and toxic effects of ZnO-TiO_2 NPs (45.2 nm) on *Artemia salina*. According to probit analyzes LC_{50} values for 24, 48, 72 and 96 hours were calculated as 5.111, 0.296, 0.143 and 0.119 mg/L, respectively. According to the results of Ti amount in ZnO-TiO_2 were compared in terms of concentrations by multiple comparison test, significantly differences were observed among 0.1-0.01 mg/L 0.1-0.5 g/l and 0.1-1 mg/L ($P<0.01$). According to the statistics in terms of the dependent variables, a highly significant difference of Ti nanoparticle amount was found in terms of concentration elimination and accumulation ($P<0.01$).

Keywords: ZnOTiO_2, Nanoparticles, Nanotoxicology, *Artemia salina*, Acute toxicity, Bioaccumulation.

Introduction

Nanotechnology applications lead to changes in the basic, physical and chemical properties of conventional materials. These changes show rapid progress when conventional materials gain unseen properties on the nano-scale; therefore, new materials with excellent electrical, optical and mechanical properties are created (McWilliams, 2006; Handy et al., 2008). Recently, the application of nanomaterials have been increased in the industry thanks to its new properties that have emerged as the material's dimensions approach nano-scale, and to the developed products that have entered into the daily life rapidly (Handy et al., 2008).

The produced nanoparticles led by the fast development of nanotechnology, have created some concerns over their potential toxicity and ecotoxicity, and they also cause concern due to their hazards to health and environment (Service, 2005; Nel et al., 2005). Although the changing quantum behavior and increasing reactivity turns the produced nanoparticles to be useful in many areas, these materials can generate adverse effects on the environment and living organisms (Handy et al., 2008).

Countries practicing in nanotechnology have different inclinations towards their effects on environment. For example, despite the fact that Russia has made a great progression in nanotechnology, it has not paid much attention to its dangers on the environment and human health (Tret'yakov, 2007; Krysanov et al., 2010). However, the United States and European Union have developed special programs to overcome these problems (Service, 2005; Nelet al., 2006). The studies conducted up to this date have recorded that the factors changing the toxicity in nanoparticles can be altered depending on the chemical composition, size, shape, crystallinity, zeta potential, coating effect, surface treatment, and surface stimulation by ultra-violet (UV) rays, surface properties (charges), aggregation and impurities during production (Farre et al., 2009). When these properties are compared to the conventional (micro-scale) materials, the nanoparticles can interact better with biological systems causing serious toxicity due to their large surface area and nano-sizes.

The reason for this more effective interaction is the surface area of nanomaterials, their surface properties (40-50% of nanoparticles are on the surface), chemical reaction, physical absorptivity, and other basic

Table 1. Loading of the catalyst particle size, calculated by the Bragg equation.

Catalysts	FWHM (β)	Radyan (R)	2-Theta	D (particle size, nm)
TiO_2	0.197°	$3.43x10^{-3}$	25.580	44.01
(%1) ZnO-TiO_2	0.180°	$3.14x10^{-3}$	25.253	45.2

Full width at half maximum (FWHM), radian (R), 2 theta and particle size were measured for TiO_2 and (1 %) $AgTiO_2$.

characteristics are related to the size of nanoparticles. All of these factors have an effect on in vivo toxicity (Zhao et al., 2007). This study aimed to investigate the acute toxicity of ZnO-TiO_2 NPs on *Artemia salina.*

Materials and Methods

In this study TiO_2 (Titanium dioxide) (99.0% pure) was obtained in pure anatase from Sigma Aldrich, Germany. In order to use toxicology experiments ZnO-TiO_2 (zinc oxide titanium dioxide) nanoparticles (NPs) were synthesized in the chemistry laboratory of Faculty of Science, Karadeniz Technical University. ZnO was loaded into the TiO_2 photocatalyst with a 1% ratio. 10 g TiO_2 catalyst and 10 ml water were mixed to have slurry. 0.3355 g ZnO was added to slurry and was calcined at 400°C for 6 h. Then it was cooled in a desiccator and stored in a closed dark bottle.

Test organisms: *Artemia salina* cysts (Salt Lake Aqua Feed Premium, Great Salt Lake, Utah USA) were incubated in artificial sea water acquired by using artificial ocean salt and then was filtered with a 30 μm millipore cellulosic filter. *Artemia* in cyst form were hydrated in distilled water at 4°C for 12 h, and washed to separate the sunken cysts from the floating cysts. The sunken cysts were washed in deionized water and collected using a Buchkner funnel. Three grams of pre-cleared cysts were incubated in conic plastic bottles containing 1.5 L seawater at 30±1°C and hatched over 24 h. Continuous light was provided with fluorescent lamps. *Artemia* count was done followed by Sorgelos (1980). In abbreviation, 100 ml solution containing hatched *Artemia* nauplii larvae was placed in a clean beaker. This solution was constantly mixed to maintain homogeneity, and 1 ml of the stock solution was diluted to 100 ml with seawater. This solution was also continuously mixed; 0.1 ml of this solution was taken and placed on a petri dish for counting. The nauplii count was determined with this volume under a stereo microscope (Leica S8APO). This count was done separately for each elimination, accumulation, and LC_{50} tests.

Preparing the aqueous suspension (test solutions) of ZnO-TiO_2NPs: In order to prepare the stock solutions in desired concentrations, the test materials ZnO-TiO_2 NPs powders were dispersed in deionized water. Then, this solution was vortexed for 30 seconds, and ultrasound water bath was used (Bandelin, sonorex) to increase dispersion and to provide maximum distribution of nanoparticles. After these steps, the determined concentrations were obtained by diluting the stock solution.

Particle distribution and characterization of ZnO-TiO2NPs: ZnO-TiO_2 NPs size distribution and morphology were characterized by SEM (scanning electron microscope), hydrodynamic dimension was characterized by Master Sizer (Malvern Hydro 2000 μ) and the particle size measurement was performed by XRD (X-ray diffractometer). Three different size distributions were taken in different times at Master Sizer and the size changes were recorded. The first measurement was when the stock solutions were prepared, the next one was conducted 24 h later and the third one was carried out 96 h later. The dry size measurement of nanoparticles (in powder form) was determined with XRD (Table 1). SEM images were taken to determine the aggregation behavior of nanoparticle.

Experimental setup: Nanoparticles have different physical and chemical properties than those of conventional materials. As they are prone to aggregate and sink in the aqueous medium, a motion system must be used while studying their toxic behavior. For this reason, a new test system that provides motion but does not affect the test subjects must be developed. For this purpose, 0.5-L conic polyethylene containers were used in exposure tests. A hole was made so that thin plastic tubes could enter. The lid was sealed with silicone and parafilm to prevent liquid discharge. To control the air supply and to ensure easy collection of the *Artemia*, a valve was placed. Aeration for accumulation and elimination test was provided by an air pump. In this way, constant mixing was obtained without harming the organisms and providing constant oxygen to *Artemia*. In order to use the air pump in more than one

Table 2. LC_{50} values of ZnO-TiO_2 NPs effects on *Artemia salina* (nauplii).

Hours	24 hour	48 hour	72 hour	96 hour
LC_{50} values	5.111 mg/L	0.296 mg/L	0.143 mg/L	0.119 mg/L

bottle, a thin tube was put in the motor's exit, the free end of this tube was connected by a T joint, and a check valve was placed in order to prevent return flow. At the same time, a plastic lid was placed on top of the system to prevent water evaporation. The water was not replaced during the experiment and any kind of intervention was avoided.

Determining the Acute Toxicity of ZnO-TiO2NPs: Acute exposure test for *Artemia* nauplii were carried out according to OECD (2004). The determined concentrations of ZnO-TiO_2 NPs were applied on the *Artemia* culture. The control groups were formed without the testing compounds. The experiments were carried out in conic plastic containers (0.5 and 2 L) with three repeats. The exposure tests for accumulation and elimination were carried out in 1000 ml, while the LC_{50} experiments were carried out in 100 ml volume. For LC_{50}, 50 individuals that were most active under the microscope and with the healthy extremities were chosen. In all concentrations, the dying nauplii were counted under the stereomicroscope at 24, 48, 72 and 96 h. The experiments were carried out in three replicates. To prevent the sedimentation of nanoparticles, aeration throughout the experiment. The experiments were carried out in a 16:8-hlight/dark cycle and a temperature of 25.0±1.5°C. The pH of the medium was measured before and after the experiments, and the mean value was calculated as 7.80±0.2. The *Artemia* nauplii were not fed during the exposure tests. After the acute toxicity tests, the changes that took place in the larvae (nauplii) exposed to nanoparticles were recorded with the help of analyses under the phase contrast microscope (Nikon Eclipse 80 i). Every exposed group was compared to the control group and the potential anomalies were recorded by taking pictures.

Elimination and accumulation experiment for ZnO-TiO_2 NPs: The predetermined amounts of *Artemia* cysts were weighed and hatched using artificial seawater. After adding 3 g *Artemia* eggs to 1.5 L seawater, nauplii were obtained. For the elimination and accumulation tests, before determining the *Artemia* number, and to clear away the unhatched eggs, the egg-nauplii mixture was poured into a large container after 48 hours. The mixture was left to settle to separate the unhatched eggs. Following a wait time, a thin layer of eggs were visible on the top layer. These eggs were collected by using plankton net. After the eggs were collected, 100 ml of the 12 L *Artemia* stock solution was taken, and then 1 ml from 100 ml was obtained to be counted under the stereo microscope in order to calculate how many creatures were there for each concentration. For elimination, 376 creatures were counted in 1ml, and 50 individuals were counted for accumulation, then the exposure tests took place.

To determine the actual metal concentrations in the collected *Artemia*, analyses were done with Inductively Coupled Plasma Optical Emission Spectroscopy (XSERIES 2 ICP-MS). The *Artemia* collected after the exposure was washed with deionized water on plankton net. The clean samples were filtered with a 0.45 mm Whatman filter paper. The collected samples were stored in a glass bottle at -20°C until the analysis. The instrumental analyses were done according to the protocols of Arslan et al. (2000; 2011). Each sample was weighed on a precision scale such that the samples were 0.1 g and the weighed samples were placed on Teflon crucibles. These Teflon crucibles were washed with pure water and dried at 80°C for 1 h in the drying oven. 2 ml concentrated nitric acid (HNO_3) and 0.5 ml hydrogen fluoride (HF) was added on the weighed samples. They were left to wait on a hot plate at 60°C in a fume cupboard for 2 h. After the samples cooled down, the remains in the crucible were covered with a lid and stored until analysis. After the analysis, 3 ml 65% of HNO3 were added to the sample and 12 ml pure water was added, diluting it 5 times. Then 2ml of these samples were taken, passed through 0.22 µm (pore size) filter and 6.6 ml deionized water was added, dropping the nitric acid ration to 2%. In the final dilution, 10 µl were taken, 10 ml 2% HNO_3 was added, diluting it 100 times before the analysis.

Statistical analyses: The data are expressed as mean ± standard error (SE) of the triplicates. The LC_{50} value and the related 95% confidence limits were calculated using the probit method. Significant differences between controls and treated samples about accumulation and elimination were determined using one-way ANOVA

followed by the Bonferroni non-parametric post hoc tests, where P<0.05 is considered to be significantly different.

Results

Acute Toxicity Test : In order to determine the acute toxic effect of samples in *A. salina* with the static test system, 0.01, 0.1, 0.5 and 1 mg/L of ZnO-TiO_2 NPs were tested in the specified concentrations, taking the 24, 48, 72 and 96 hours into consideration, the LC_{50} values were determined with probit analysis (Table 2).

As shown in Table 2 above, for ZnO-TiO_2 NPs, the LC_{50} values that were determined in 24 h period were much higher than that of other periods of time. This shows that the toxic effect of NPs in a 24 h period was quite low. The toxic effect of NPs increased with time.

Morphological Changes: *Artemia salina* (nauplii) larvae were subjected to ZnO-TiO_2 NPs acutely for 24 h in a static test system. The *Artemia* in the control group had no visible change in the digestive system and the intestines were almost empty. No missing extremities (antennae) or malformations were observed. On the other hand, the *Artemia* subject to nanoparticles showed changes in the eye formation, enlargement of the intestine malformations in the outer Shell and extremity loss and malformation (antennae) were the first observed changes under the microscope (Fig. 1).

Accumulation and Elimination of ZnO-TiO_2 NPs: In order to determine whether there was a significant difference between (A) the amount of *Artemia* absorbing ZnO-TiO_2 NPs in 24 h exposure (E) and the amount of nanoparticles extracted from the intestines 24 h later, Tukey multi-comparison test was applied. In this test, the titanium (Ti) and zinc oxide (ZnO) amounts in ZnO-TiO_2 were determined separately and every concentration value was compared with others in terms of accumulation and elimination. When Ti in ZnO-TiO_2 were compared in terms of concentrations, among concentrations 0.1-0.01 mg/L, 0.1-0.5 mg/l, and 0.1-1 mg/L were found a highly significant difference (P<0.01). In comparing ZnO, concentration between 0.01-0.1 mg/L and 0.1-1 mg/L is present with a significant difference (P<0.05) (Fig. 2). According to the statistics done in terms of the dependent variable, a highly significant difference of Ti nanoparticle amount in terms of concentration, elimination and accumulation (P<0.01) (Fig. 3). After 24 h exposure in ZnO-TiO_2, the amount of Ti accumulation in 0.01, 0.1, 0.5, and 1 mg/L were 2.963, 7.149, 2.536, 0.915 ppb respectively. The Ti amount in elimination was measured as 0.508, 2.027, 0.591 and 0.514 ppb. The accumulation

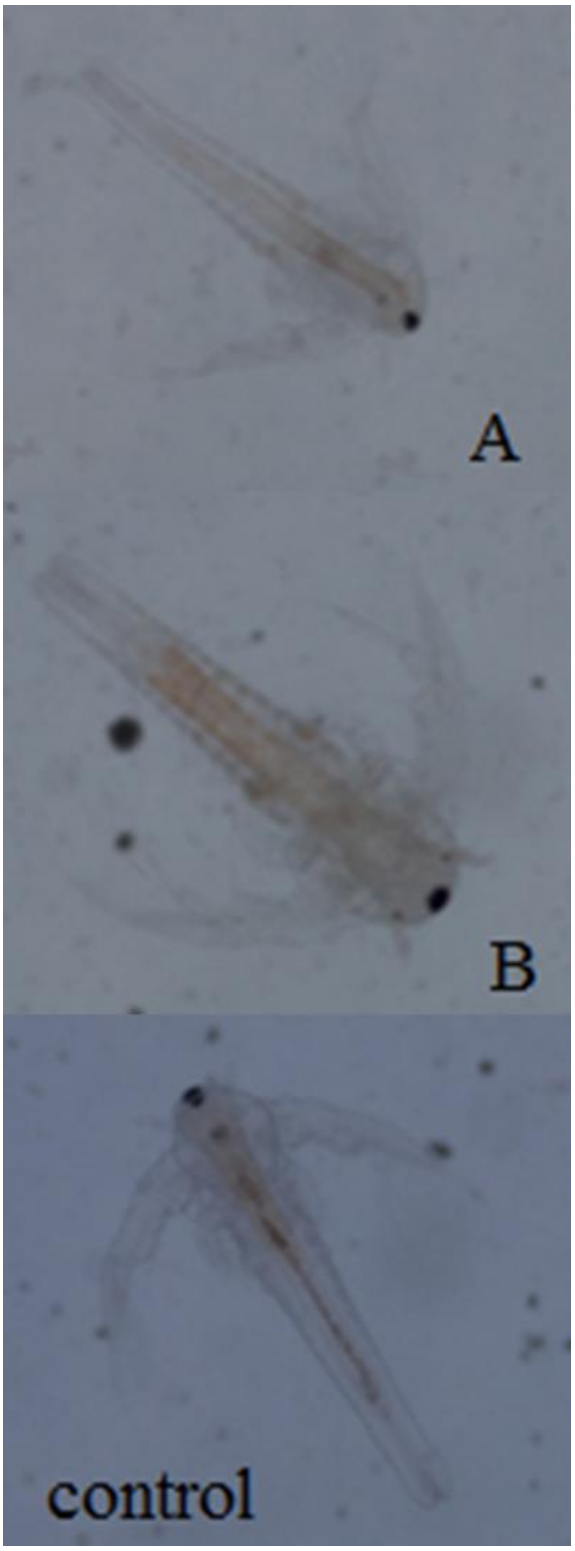

Figure 1. Morphological changes of *Artemia salina* exposed to NPs. Specimen not exposed to any nanoparticle (control), loss of antennae and deformation of antennae (a) and intestinal enlargement and outer shell anomalies (b).

Table 3. After exposing *Artemia salina* to different concentration of ZnO-TiO_2 NPs for 24 h, percent excretion of ZnO and Ti in seawater.

Nanoparticles	Concentrations (mg/L)			
	0.01 mg/L	**0.1 mg/L**	**0.5 mg/L**	**1 mg/L**
Titanium (%)	83.0	72.0	76.7	44.0
Zinc oxide (%)	57.5	9.0	89.2	84.5

Table 4. Dimension measurement of ZnOTiO2 NPs aqueous suspensions in MasterSizer (µm).

ZnO-TiO_2 concentrations	stock solution	24 hour	96 hour
0.01 mg/L	4.548	1.236	0.806
0.1 mg/L	6.709	6.158	6.055
0.5 mg/L	5.990	-	5.195
1 mg/L	6.990	-	4.134

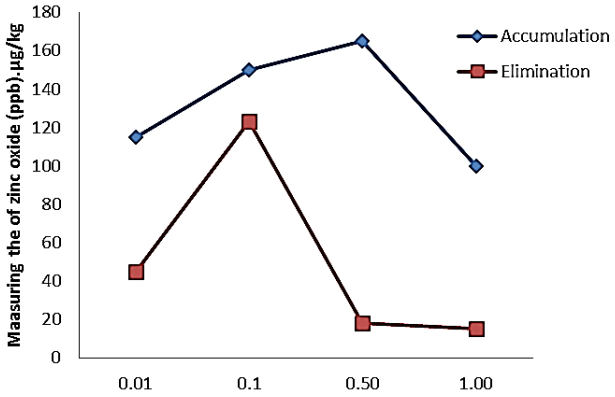

Figure 2. The amount of ZnO uptake into *Artemia salina* when exposed to ZnO-TiO_2 NPs for 24 h; and the amount of excreted ZnO 24 h after being placed into clean seawater.

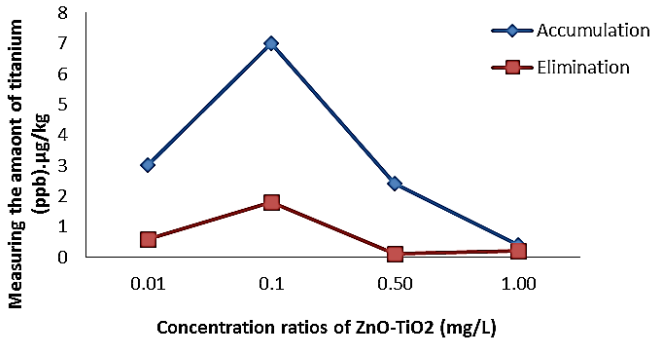

Figure 3. The amount of Ti uptake into *Artemia salina* when exposed to ZnO-TiO_2 NPs for 24 h; and the amount of excreted Ti 24 h after being placed into clean seawater.

and elimination amount of ZnONPs were 120.2, 158.1, 165.8, 104.6 ppb respectively and the elimination was 51.16, 128, 17.94 and 16.25 ppb. In terms of ZnO-TiO_2 excretion percentage, the values for the concentrations above for Ti are 83.0, 72.0, 76.7, and 44.0, 57.5, 9.0, 89.2, and 84.5 for ZnO. Only in 0.1 mg/L of ZnO concentration, a low excretion of ZnO in Artemia was observed. When ZnO and Ti were compared among each other, the percentage of ZnO excretion was found to be above the average (Table 3).

Characterization of ZnO-TiO_2 NPs: In Master Sizer measurement, the mean predicted hydrodynamic dimensions were taken (Table 4). Also there are SEM images of ZnO-TiO_2NPs (Fig. 4). When the table is analyzed, a decrease in dimension with respect to time can be observed. The highest hydrodynamic dimension measured for 1 mg/L ZnO-TiO_2 is 6.990 µm and the smallest dimension measured in is 0.01 mg/L ZnO-TiO_2 at 0.806 µm.

Discussion

In toxicology studies concerning the toxic effects of nanoparticles and determining their bioaccumulation, fish species have been generally used. In this study, *A. salina* larvae (nauplii), a crustacean species, were used since it was easier to test for research purposes. *Artemia* are species that eat other species smaller than 50 µm, and they feed by filtration (Hund-Rinke and Simon, 2006; Zhu et al., 2010; Ateş et al., 2012). Nano-sized TiO_2 is used in many areas such as sunscreen, cosmetics, paint, surface coating air-pollution prevention and medical applications. The use of ZnO particle in sunscreens, electronic applications, transparent UV protection film, chemical sensors, and ceramic absorbing material and for personal care products has begun (Auffanet al., 2011; Pendashteet al., 2013). When the increase of industrial and non-

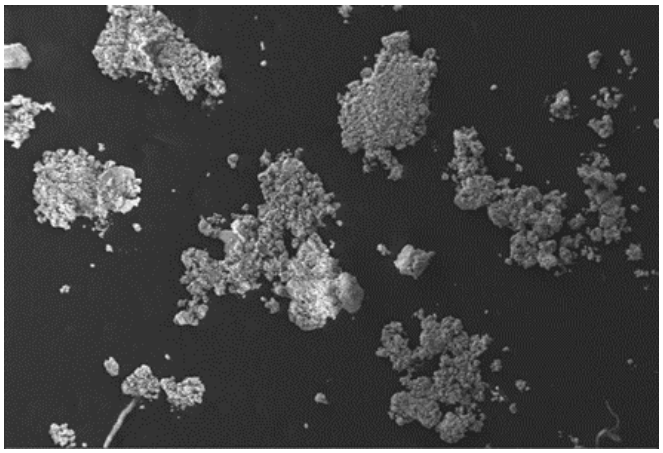

Figure 4. SEM images of $ZnOTiO_2$ NPs in dry form.

industrial zinc is considered, the potential toxic effects in aqueous media must be studied. In this study, the acute toxic effect, accumulation and elimination of ZnO loaded on titanium (ZnO-TiO_2, 45.2 nm) on crustacean shrimp were assessed. It was observed that the accumulation and elimination of Ti and ZnO nanoparticle within ZnO-TiO_2 change with respect to concentration had changed. Statistical meaning in accumulation and elimination and in ZnO-TiO_2, Ti amount was recorded at 0.1 mg/L - 001 mg/L and 0.1 mg/L - 1 mg/L, for ZnO amount it was 0.01 mg/L - 0.1 mg/L and 0.1 mg/L - 1 mg/L. When we look at the excretion ratio of ZnO and Ti nanoparticles from *Artemia*, it is seen that the excretion ratio for ZnO and Ti are approximately equal and a high ratio of excretion takes place. Only in 0.1 mg/L of ZnO elimination displayed low results. Generally, the elimination of ZnO tends to increase with increasing concentration. When the accumulation in ZnO and Ti in *Artemia* are considered, significant differences between these nanoparticles are observed. For example, in 0.01 mg/L concentration zinc accumulation is 120 ppb, while titanium build up is 2.963 ppb and there is a high amount of absorption difference, the difference is apparent in all concentrations. When these accumulation data are assessed, ZnO is most effectively absorbed at 0.5 mg/L with 165.8 ppb, and for Ti this is at 0.1 mg/L with 7.149 ppb. When the toxic effect of ZnO-TiO_2 with respect to time and concentration, the acute toxic effect increases with time in 24 hour period to 5.111 mg/L and in 48 hours it decreases 17 times to 0.296 mg/L. In short, with passing time, it is seen that less amount of ZnO-TiO_2NPs concentrations is sufficient for acute toxic effect. A study that assessed ZnO nanoparticle, $ZnCl_2$ in solutions and in macrotose form in ZnO in landlocked invertebrate isopod (*Porcello scaber*), it was seen that the zinc bioaccumulation factor and Zn absorption efficiency did not change with respect to source, all results from the three zinc sources are similar. The absorbed amount and bioaccumulation is independent of dosage and aside from some exceptions, they are alike in all concentrations. When these results are compared with this study, differences in results can be seen. In our study, the intake changes with respect to concentration, while in Pipan-Tkalec et al. (2009) no relation with dosage has been seen. In a study where the ZnO toxicity on zebra fish embryo and larvae development and the aggregation of ZnO in a culture is studied, and in a 48 hour period, the micro-scale ZnO aggregation acute toxic effect has been documented as increased ovulation and pericardial edema (Zhu et al., 2009; Özkan et al., 2015b). Toxicological evaluation of TiO_2, $AgTiO_2$ and $ZnOTiO_2$ composite in honey bee was made. The results showed that, the toxic effect of this nanoparticle increased along with the increase in the concentration and the exposure time. In their studies where they characterized 30 nm sized ZnO nanoparticles in aqueous suspension, they assessed the toxicity of the ZnO nanoparticle 96 h after the zebra fish's embryo larvae fertilization. In 50 and 100 mg/L ZnO nanoparticle and Zn^{+2} exposure, the mortality rate was the recorded as 28.3%±14.5 and 65.0±8.9. Between the concentrations of 0.598-6.305 mg/L, Zn^{+2} did not cause embryo mortality. In concentrations increased by 1-10 mg/L, the embryos exposed to nano ZnO have decreased ovulation rates, at 0.598 mg/L the embryos were not affected by Zn^{+2}, ovulation rates decreased with increasing concentration Bai et al. (2010). It was shown that acute toxicity in zebra fish due to ZnO nanoparticles bulk ZnO and Zn^{+2} are dependent on dosage (Xiongiet al., 2011). When the concentration surpasses 2 mg/L, zebra fish mortality increases with increasing particle concentration. For ZnO nanoparticle and bulk ZnO, 100% mortality was observed at 30 mg/L. The LC_{50} values calculated at 96 hours for ZnO nanoparticle is 4.92 mg/L and 3.31 mg/L for bulk ZnO. The value for Zn^{+2} is recorded as 8062 mg/L. For the toxicity effect of ZnO and ZnO nanoparticle in *Daphnia magna*, the EC_{50} value after 48 hour exposure was found as 1 mg/L (Wiench et al., 2009). The effect of ZnO nanoparticle as ion and in bulk on the development of *Corophium volutator* living in sediment, productivity and survival and its chronic effects were studied. It was found that zinc was influential of the

longevity of *C. volutator* and that the specific development significantly decreased on the 23rd day of the exposure (Fabrega, 2012). The result due to zinc dissolving in sediment and the bulk form's long time contact can be considered as a possible reason of the decreasing development. The sublethal effects of zinc on slow development causes delayed sexual maturity and reduced fertility within the range of 0.2-1 mg/L concentrations. When all of these results are considered, it is seen that zinc in low or high amounts has a toxic effect. It is seen that this toxic effect is in different ratios in different studies. The difference in the toxic effect can be firstly due to the size of the zinc, and can change with respect to concentration/dosage, exposure time (acute/chronic), the mass of the living being and lifestyle, the form of the zinc nanoparticle (bulk, powder or ion). Özkan et al. (2015a) reported aquatic stability and toxic effects of TiO_2 and $AgTiO_2$ NPs were investigated on *A. salina*. According to these results, the mortality rate in nauplii increased significantly with increasing concentration and duration of exposure.

The mean hydrodynamic sizes of ZnO-TiO_2NPs were measured. Generally the aggregation of nanoparticles tends to increase with respect to time and concentration, but this study displayed quite the opposite results; a decrease in aggregation was observed. Fresh stock solution was measured in 24 and 96 h, and the results were 0.01 mg/L, 4.548, 1.236 and 0.806 10 mg/L for ZnO-TiO_2 NPs, 4.919, 0.416 and 0.267 0.1 mg for TiO_2 and 2.162, 1.786 and 1.437 µm for $AgTiO_2$ (Xiong et al., 2011). It is recorded that TiO_2 and ZnONPs create regular aggregations in suspensions and the nanoparticle aggregate size increases with concentration. Ateş et al. (2012), recorded that the aggregation decreased in fresh stock solution, and that the nanoparticles ranged between 8-40 nm. The DLS measurements of nanoparticles showed that the form sudden aggregations in water. It was reported that the hydrodynamic size range as between 1.833-210 and the aggregate sizes increased with respect to TiO_2 concentration (Baiet al., 2010). Study noted that the aggregation of nanoparticles is an important factor in assessing the toxicity. The formation of these aggregates cause changes in particle size distribution of nanoparticles in water. The exposure time and concentration may also affect the nanoparticles' size distribution. In this study nano ZnO distribution in E3 medium decreased with high concentration and aggregation. An increase in aggregation in nano ZnO with increased exposure time was recorded. Generally the reason that that nanoparticles' dimensions decrease with time is the use of ultrasound baths which prevent aggregation and provide even distribution, also providing homogeneity with aeration has an effect in providing homogeneity. Additionally, in all concentrations of ZnO-TiO_2, that was studied under the microscope showed anomalies to some extent. *Artemia* exposed to toxic effects displayed such changes as enlarged intestines, eye shrinkage, paling of the eye, and change in the shape of the eye socket and deformations in the outer shell.

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