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Particle Size and Agglomeration Affect the Toxicity Levels of Silver Nanoparticle Types in Aquatic Environment

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ABSTRACT In order to understand the importance of particle size and agglomeration for nano-eco-toxicological studies in aquatic environments, the acute toxicity of two different types (suspended powder and colloidal) of silver nanoparticles (AgNPs) were studied in alevin and juvenile rainbow trout. Fish were exposed to each type of AgNPs at nominal concentrations of 0.032, 0.1, 0.32, 1, 3.2, 10, 32, and 100 mgl^{-1}. Lethal concentrations (LC) were calculated using a Probit analysis. Some physical and chemical characteristics of silver nanoparticles were determined. In the case of colloidal form, particles were well dispersed in the water column and retained their size; but in the case of suspended powder, particles were agglomerated to large clumps and precipitated on the bottom. In alevins, the calculated 96 h LC50 values were 0.25 and 28.25 mgl^{-1} for colloidal and suspended powder AgNPs respectively. In the case of juveniles, the 96h LC50 of colloidal form was 2.16 mgl^{-1}, but suspended powder did not caused mortality in fish even after 21 days. The results showed that both in alevin and juvenile stages, colloidal form is much toxic than suspended powder; this shows increase of nanoparticles size due to agglomeration, will reduce the toxicity. Silver nanoparticles are toxic materials and their release into the water environment should be avoided.

Key words: *Aquatic Nanotoxicology, Agglomeration, Rainbow trout, Silver Nanoparticle, Size-Dependent Toxicity*

1 INTRODUCTION

Manufactured nanomaterials are materials with diameters ranging from 1 to 100nm, and nanotechnology is one of the rapid growing parts of the new technology. Although the applications of nanoparticles are increasing broadly in many fields, concerns about their environmental and health impacts remain unresolved. The use of nanomaterials is also likely to result in their release into aquatic environments and may pose risks to the aquatic ecosystems. The aquatic ecotoxicology of engineered nanomaterials, aquatic nanoecotoxicology, is a relatively new and evolving field.

Silver nanoparticles, have been, and continue to be, recognized worldwide as either a cure or as a preventive for bacterial, fungal, and viral diseases (Murr, 2008). Nano silver is

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about 24 percentages of commonly used nanomaterials in consumer products (Woodrow Wilson Database, 2011). Nanoscale silver is used in a range of products including water treatment, textiles, washing machines, dyes/paints and varnishes, polymers, medical applications, sinks and sanitary ceramics as well as various consumer applications such as disinfectants, cosmetics, cleaning agents, baby bottles, etc (Senjen, 2009). The extensive application of nanoscale silver might eventually lead to the release of these particles into the environment (Benn and Westerhoff, 2008). The European market for silver-containing biocidal products is planned to reach between 110 and 230 metric tons of silver annually by 2010 and a significant portion of this will be nanosilver (Blaser, *et al*., 2008). Also Blaser *et al*, (2008) assessed 68% increase of the silver load in waste water due to silver-containing biocidal products from 2010 to 2015. Recent studies by researchers has been focused on the toxicity of silver nanomaterials in aquatic environments, especially in the case of fishes (Asharani *et al*., 2008; Lee *et al*., 2007; Yeo and Kang, 2008; Bar-Ilan *et al*., 2009; Chae *et al*., 2009; Choi *et al*., 2009; Griffitt *et al*., 2009; Wu *et al*., 2009; Bilberg *et al*., 2010; Powers *et al*., 2010). Although most of those have been focused on zebra fish (Asharani *et al*., 2008; Yeo and Kang, 2008; Bar-Ilan *et al*., 2009; Choi *et al*., 2009; Griffitt *et al*., 2009; Powers *et al*., 2010) and there is only one in vivo study in regard to chronic toxicity of nanosilver in rainbow trout (Scown *et al*., 2010).

It is recognized that, when the size of a particle decreases to the nanoscale, the physical properties of the particle will change; this means nano-sized particles, have optical, electrical and magnetic properties that differ substantially from larger particles of the same compounds (Dowling *et al*., 2004). Furthermore size of particles affects the toxicity on the cells and organisms (Nowack and Bucheli, 2007; Carlson *et al*., 2008; Inoue *et al*., 2009). The smaller size of nanoparticles might allow it to enter an organism more easily than its conventional counterpart which may lead to changing the toxicological properties of particle.

One of the most commonly applied animal tests in regulatory ecotoxicology to this day is the fish acute lethality test (Schirmer, *et al*., 2008). In this study, the acute toxicity of colloidal silver nanoparticles (smaller size in aquatic environment) was compared with suspended silver nanoparticles (larger size due to agglomeration in aquatic environment) on alevin (sac fry) and juvenile rainbow trout. Alevin stage is ecotoxicologically important because at this stage of life cycle, the fish are still receiving nutrition only from the yolk sac with no alimentary relation to the environment (fish have endogenous feeding). Therefore results of this stage, will shown only external impacts of chemicals on fishes. Also early-life stages in fish are known to be the most sensitive to environmental perturbation (Weis and Weis, 1989). While juveniles have exogenous feeding, and are related to the environment via gills, skin, and alimentary canal (Handy *et al*., 2008, B).

The main objective of this study was to determine the toxicity of two different types of silver nanoparticles (colloidal and powdered forms) with different sizes and degrees of agglomeration in rainbow trout.

2 MATERIALS AND METHODS

2.1 Characterization of silver nanoparticles

The colloidal AgNPs at pH 2.4, type L (commercial name: Nanocid) were purchased from Nano Nasb Pars Co. Ltd., Tehran, Iran. The colloid product was synthesized using a novel process involving the photo-assisted reduction of Ag+ to metallic nanoparticles, registered under United States Patent Application No: 20090013825 (Rahman Nia, 2009). Briefly, 4.5g of LABS (Linear alkyl benzene sulfonate) was

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dissolved in 95 ml of distilled water and then added to a solution containing 0.32 g of silver nitrate. After mixing thoroughly, 0.2g of a hydrazine solution (0.03 M) was added, resulting in the formation of a yellowish silver colloidal solution. According to information provided by the manufacturer, the product was a water-based colloidal suspension containing 4000mgl^{-1} spherical silver nanoparticles (average size 16.6nm).

The powdered type AgNPs and dispersant reagent (bacterial polysaccharide) were purchased from Xuzhou Hongwu Nanometer Material Co. Ltd., Jiangsu, China. The water solubility of this type of AgNPs was very low and particles were settling on the bottom of vessel; so it was necessary to disperse particles in water column via dispersant reagent. A stock suspension of 500 mgl^{-1} dispersed particles was prepared according the manufacturer recommendations. Briefly, 100mg suspending reagent was added to 1L of deionized water, stirring on magnet stirrer, and 500 mg of powdered silver nanoparticles was added to with continues stirring for 24 hours. The pH of final mixture was determined as 7.7.

The hydrodynamic sizes and also surface charge (zeta potential) of the colloidal and suspended powder silver nanoparticles were measured in four replicate runs, each run 6 measurements, using zetasizer (Malvern Instruments Inc, UK, Model: 3000HS$_a$).

To determine the concentrations of silver in the stocks of colloidal and suspended powder AgNPs, equal volumes of each stock and 69% HNO3 were mixed resulting in dissolution of the silver particles. The concentrations of silver in each digested solution were then measured using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Model: 3410 ARL, Switzerland).

TEM analyses of the dry powder, suspended powder and colloidal type AgNPs were performed using an H-7100FA transmission electron microscope (Hitachi, Japan) with an acceleration voltage of 125kV. For each type the diameters of 700 randomly selected particles were measured at a magnification of 100,000 using Axio Vision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany). EDX analyses of the dry powder, suspended powder and colloidal type AgNPs were performed using an EX200 Energy-dispersive x-ray analyzer (Horiba, Japan).

To determine the crystalline phase of dry powdered AgNPs, X-ray diffraction (XRD) was performed with a Philips X'Pert-MPD X-ray Diffraction System (Netherland) (Tube: Cu kα, λ: 1.54056 A°, Step Size: 0.02 °/s, Voltage: 40kV, Current: 40mA). Also X-ray fluorescence (XRF) chemical analysis of dry powdered AgNPs was performed using a Philips PW2404 X-ray Fluorescence Spectrometer (Netherland).

2.2 Rainbow Trout

Alevin rainbow trout (n=480) from the same brood stock were randomly selected 2 days after hatching, and exposed in 1L cylindrical glass beakers containing the desired concentration of the test chemical at 10±0.5 °C with a semi-statistic exposure regime and aerated using 2 cm air stones. The beakers were covered with a special dark plastic due to the light sensitivity of the embryos.

Juvenile rainbow trout with a weighing 15.47±0.83 g (mean ± S.E.) obtained from Marzan Ghezel Trout Farm, (Mazandaran, Iran.) and were maintained in the aquatic organisms laboratory in 1000 L tanks supplied by a semi-static system with dechlorinated tap water under a 12/12 hour light/dark cycle and were fed pelleted feed (Chineh, Iran), at 1% of their body weight at 10-14°C. After one week of adaptation, the juveniles (n=480) were transferred to 90L cylindrical tanks (10

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fish/tank) in triplicates and allowed to adapt for 24 h prior to the start of the experiments. Each tank was continuously aerated using a 5cm spherical air stone. To minimize risk of the Ag particle absorption to food or fecal material, and also keeping a constant water quality, feeding of fish were stopped 48h prior to the experiments.

All the animals were treated humanely as regards the alleviation of suffering, and all the laboratory procedures involving the animals were reviewed and approved by an Animal Care and Use Committee in accordance with the Animal Welfare Act and Interagency Research Animal Committee guidelines (Nickum *et al*., 2004).

The tap water was dechlorinated by adding 1mgl^{-1} sodium thiosulfate followed by vigorous aeration for at least 48 hours in 1000L reservoir tanks. The dechlorinated tap water was then used as the water source of experiments, and some of its chemical characteristics, including the ammonium, sulfide, magnesium, total hardness, potassium, calcium hardness, and chloride were measured using a Palintest photometer (Model: 8000, UK), while the sodium was measured using a Philips atomic absorption spectrophotometer (Model: PU9400X). The means of the chemical characteristics for the dechlorinated tap water are shown in Table 1. Also, the pH and dissolved oxygen recorded daily and were 8.02±0.14 and 8±0.21mg l^{-1}, respectively. The means of water temperature in the alevin beakers and juvenile tanks were 10±0.5°C and 12±2°C, respectively.

2.3 Exposing to silver nanoparticles

Logarithmic series of each type of AgNPs were used according to the OECD guideline for chemicals testing (OECD, 2000). The selected concentrations were 100, 32, 10, 3.2, 1, 0.32, 0.1, and 0.032 mg l^{-1} for both colloidal and suspended powder AgNPs. 10 healthy alevin and/or ten juvenile rainbow trout were transferred directly to each prepared concentration in triplicate (30fish/treatment). Control groups (without chemicals) were also included for each treatment. In addition to control groups, three dispersant controls including 20 mg l^{-1} bacterial polysaccharide were also used to make sure this dispersant is not lethal for fish; this amount is equal to the concentration of dispersant which was added with maximum concentration of suspended powder AgNPs. Fish were exposed to the materials for continuously 4 days in a semi-static exposure regime (100% water change after 48 hour with re-dosing after change). The aeration and water flow in the tanks dispersed each dose around the tank in less than one minute and helped to maintenance of suspension during the exposure. To determine the actual silver concentrations in the exposure tanks, water samples were collected from the middle of the water column one hour after dosing the tank. The samples were then placed in brown glass vessels, acidified with HNO_3 to reduce the pH to less than 2, and kept at 4ºC. Prior to taking measurements, the water samples were digested with 69% HNO_3 and the concentrations of silver measured using a Philips model PU9400X atomic absorption spectrophotometer. The means of the actual silver concentrations in suspended powder and colloidal AgNPs treatments are shown in Table 2.

In each treatment and during 4 day, dead fish were removed every 24 hours and considered as mortality rate. The LC10, LC50, and LC90 values (with 95% confidence limits) were determined using entering the number of dead fish in each concentration/time to the EPA Probit analysis program (version 1.5). In all cases the standard deviations (SD) were calculated using Microsoft Office Excel.

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Table 1 Chemical characteristics of dechlorinated tap water used for toxicity tests in all experiments

Variable	NH_4^+	S^{2-}	Mg^{2+}	Cl^-	Na^+	K^+	Calcium Hardness	Total Hardness
mg l^{-1}	0.1±0.01	Not detectable	39±1.15	2.4±0.2	13.8±0.11	3.9±0.1	26±3.78	150±3.60

Table 2 Comparison of nominal silver concentrations versus actual concentrations. Actual concentrations were measured one hour after dosing of fish tanks with silver nanoparticles (ND = not detectable)

Nominal AgNP Conc. (mg l^{-1})	Actual Ag Conc. in exposure tanks of colloidal AgNPs (mg l^{-1}, mean± SD)	Actual Ag Conc. in exposure tanks of suspended powder AgNPs (mg l^{-1}, mean± SD)
100	108.1±2.26	10.5±1.76
32	37.3±1.67	1.9±0.45
10	13.3±0.42	1±0.14
3.2	3.7±0.14	0.3±0.07
1	1.3±0.1	0.1±0.03
0.32	0.37±0.07	0.02±0.02
0.1	0.1±0.03	ND
0.032	0.03±0.02	ND

3 RESULTS

3.1 Particle characterization

Base on the results of zetasizer instrument (Figure 1), hydrodynamic size distribution of AgNPs in the suspended powder stock solution, ranged from about 100 to over 300 nm; with a mean average size (ZAve) of 0.2 µm (196.1 nm). In the colloidal solution, hydrodynamic size distribution of AgNPs ranged from 3.9 to 163.5 nm and the zeta average (mean particles size) was 54.8 nm; also in this case, totally tree classes of particles were distinguishable: 10-50nm (33.6%), 50-100nm (20.5%), and 100-165nm (45.9%). Also according to zetasizer instrument information, zeta potential of colloidal and suspended powder AgNPs had an average of -53.33±7.86 mV and +1.03±0.13 mV respectively. A zeta potential range from ±40 to ±60 mV is a sign of good stability for colloids (ASTM, 1985).

The colloidal AgNPs observed by TEM were spherical in shape (Figure 2. A), with a maximum diameter of 129 nm: 65.14% of the particles had diameters between 1 and 13 nm (Figure 3. A), just 2.28% of the particles had diameters more than 100nm, and the CMD (count median diameter) for the particles was 6.47nm (Figure 4. A). Also the geometric mean diameter (GMD) and geometric standard deviation (GSD) of colloidal silver nanoparticles were 12.65nm and 1.46 respectively.

The dry powdered AgNPs observed by TEM were spherical in shape (Figure 2. B), with a maximum diameter of 161 nm: 85.97% of the particles had diameters between 1 and 45nm (Figure 3. B), just 1.34% of the particles had diameters more than 100nm, and the CMD for the particles was 17.97nm (Figure 4. B). Also the GMD and GSD of dry powdered silver nanoparticles were 14.39nm and 1.31 respectively.

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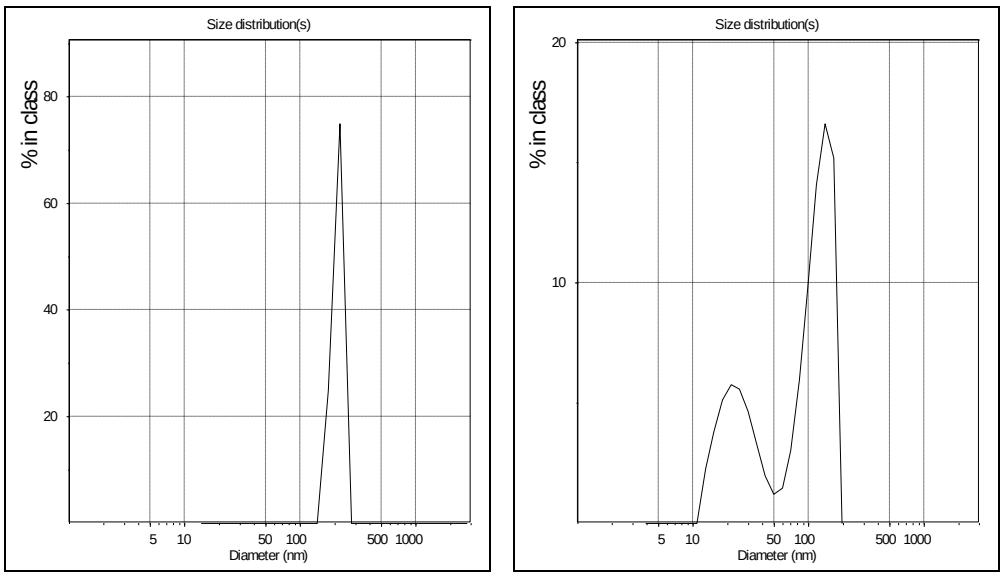

Figure 1 Size distribution in stock solutions of suspended powder (left) and colloidal (right) AgNPs, Determined by dynamic light scattering method (Zetasizer)

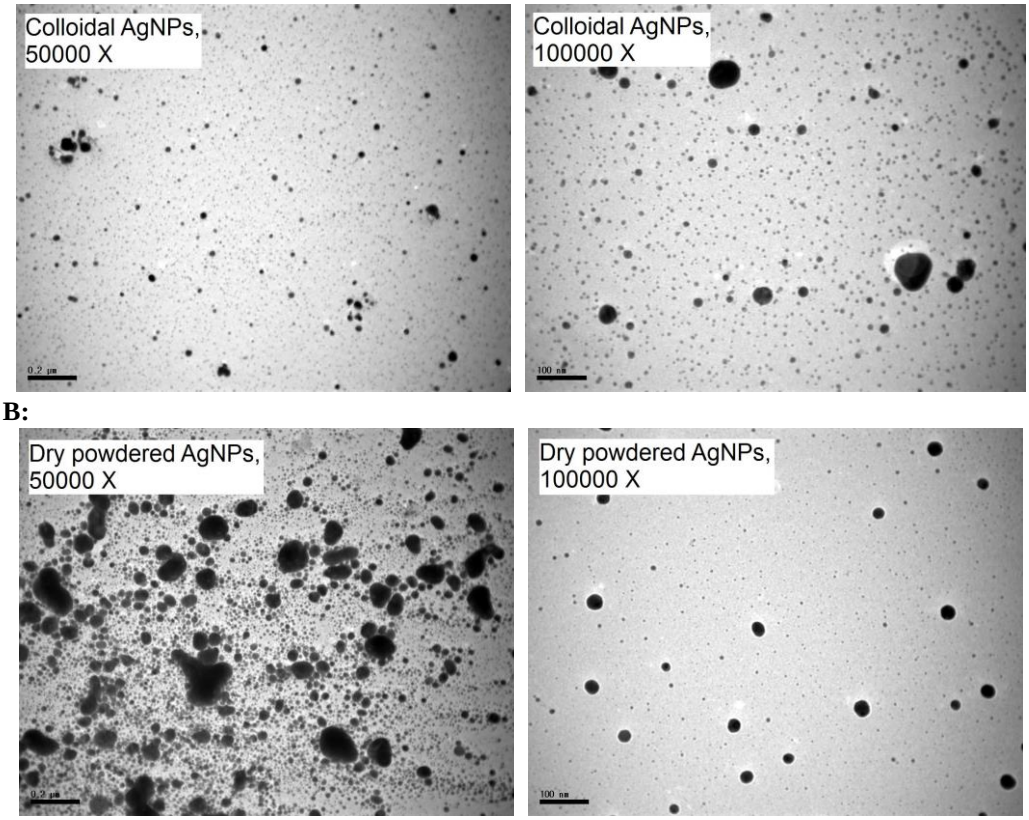

Figure 2 TEM micrographs of colloidal (above) and dry powdered (below) silver nanoparticles. Scale bars are 200 and 100 nm in left and right images respectively

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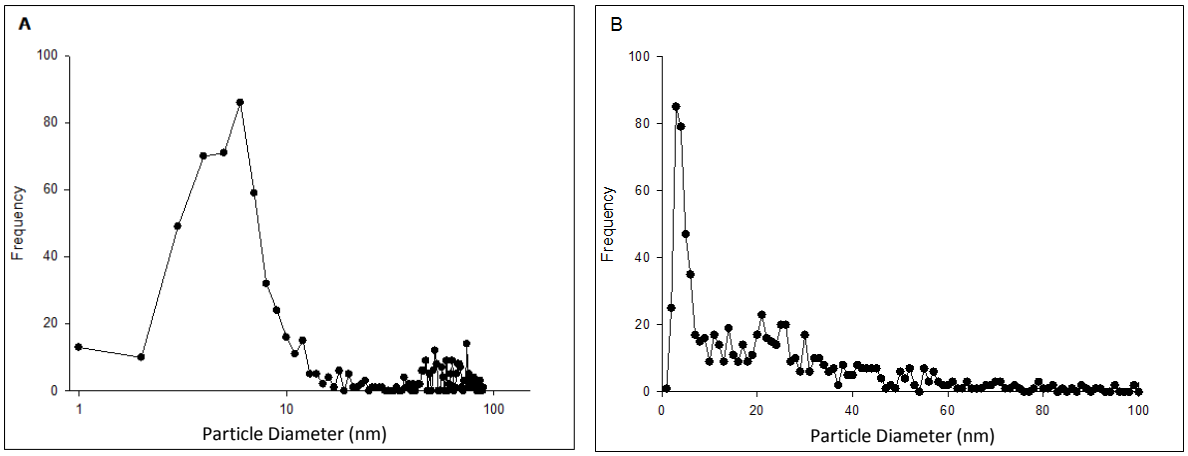

Figure 3 Size distribution of particles in colloidal (A) and dry powdered (B) AgNPs based on number frequency determined from transmission electron microscope data

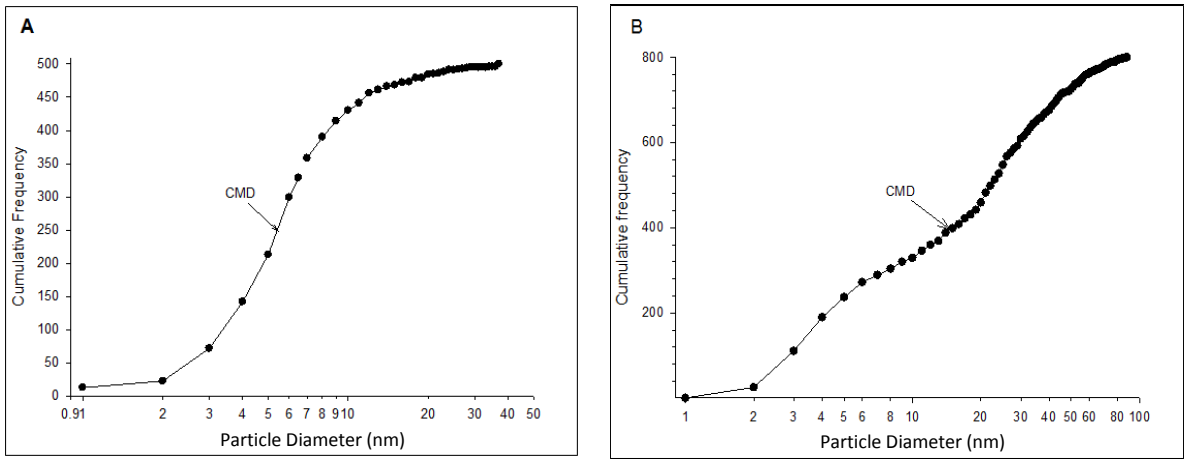

Figure 4 Size distribution of particles in colloidal (A) and dry powdered (B) AgNPs based on cumulative frequency determined from transmission electron microscope data. (CMD: Cumulative median diameter)

In the case of suspended powder AgNPs, TEM images showed that in an aqueous environment the nanoparticles clumping and form large aggregates, many of which are larger than 100nm (Figure 5). This result is consistent with the results of hydrodynamic size distribution which was obtained from the dynamic light scattering method (zetasizer).

As seen in figures 6, EDX analyses were confirmed that only elemental silver was presented in both colloidal and dry powdered AgNPs. According to the ICP-AES results, the concentrations of Ag ions in the acid digested stocks of colloidal and suspended powder AgNPs were 3980 and 447.2 mgl^{-1}, respectively. The XRD pattern of powdered type AgNPs is shown in Figure 7; as can be seen, all diffraction peaks correspond to the characteristic face centered cubic (FCC) silver lines. Also results of the XRD pattern confirms the crystallinity of powdered type AgNPs and presence of elemental crystalline silver, also other phases, except

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metallic silver, were not present in the sample. In addition the XRF results showed that purity of powdered type AgNPs was 97.86%.

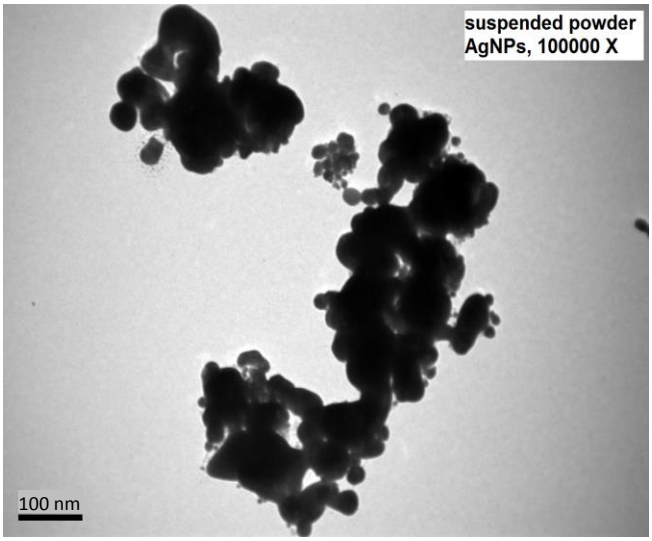

Figure 5 TEM micrograph of suspended powder silver nanoparticles. This image shows Clumping of nanoparticles and formation of large aggregations (scale bar: 100 nm)

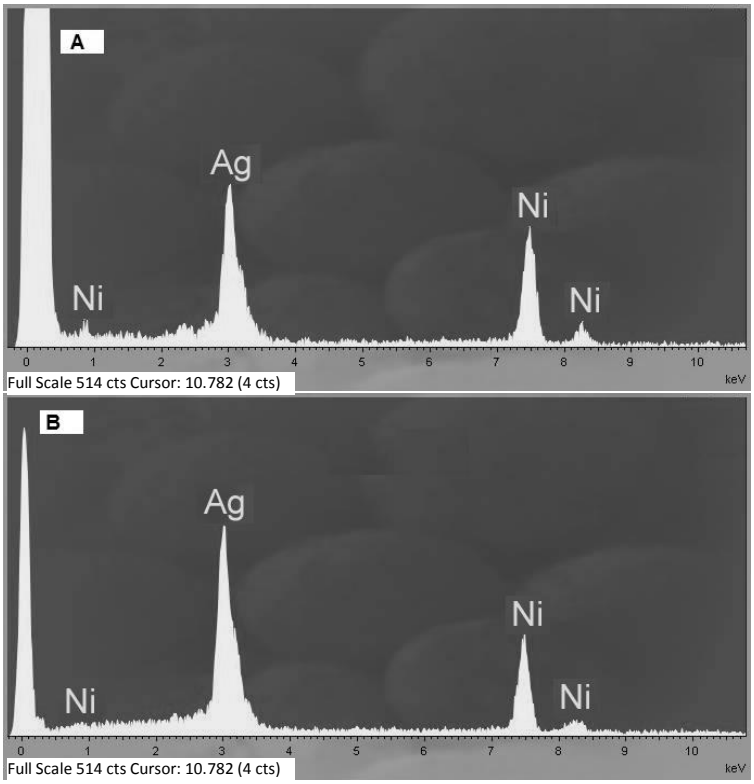

Figure 6 EDX spectrometer patterns of colloidal (A) and dry powdered (B) silver nanoparticles. (Ni signals in EDX spectrometer are from TEM grid)

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3.2 Determination of lethal concentrations (LC)

The mortality of controls during the experiment was one alevin in one of the control groups. No mortality was observed in dispersant controls. Results of LC determinations are shown in Table 3. The lowest concentrations of colloidal and suspended powder AgNPs which were caused 100% mortality in alevins after 96 hour were 1 and 100 mgl^{-1}, respectively. In alevins, calculated median lethal concentration (LC50) values for the colloidal AgNPs were 2.75, 0.44, 0.35 and 0.25 mgl^{-1}, after 24, 48, 72 and 96 hour, respectively. While these values for the suspended powder AgNPs were 186.42, 69.37, 36.93, and 28.25 mgl^{-1}, respectively (Table 3). According to these results it is clear that compared to the suspended powder AgNPs, much less concentrations of the colloidal AgNPs are toxic to alevins.

The Mortality of controls and dispersant controls of juveniles was zero during the experiments. The lowest concentration of colloidal AgNPs which were caused 100% mortality in juveniles was 3.2 mgl^{-1} after 96 hour. Also as shown in Table 4, in juveniles the calculated LC50 values of colloidal AgNPs were 3.76, 3.13, 2.39 and 2.16 mgl^{-1} at 24, 48, 72 and 96 hour, respectively. Survey on survival rates of juveniles, which exposed to suspended powder AgNPs, showed that even in maximum concentration (100 mgl^{-1}) and after 21 day exposure, no mortality was observed. In toxicity test of the suspended powder AgNPs on juveniles, rapid aggregation was occurred so that large clumps were observable in the water column. Also rapid sedimentation of these clumps was observed in exposure tanks and because juveniles were swim in water column, most of these particles were away from fish contacts.

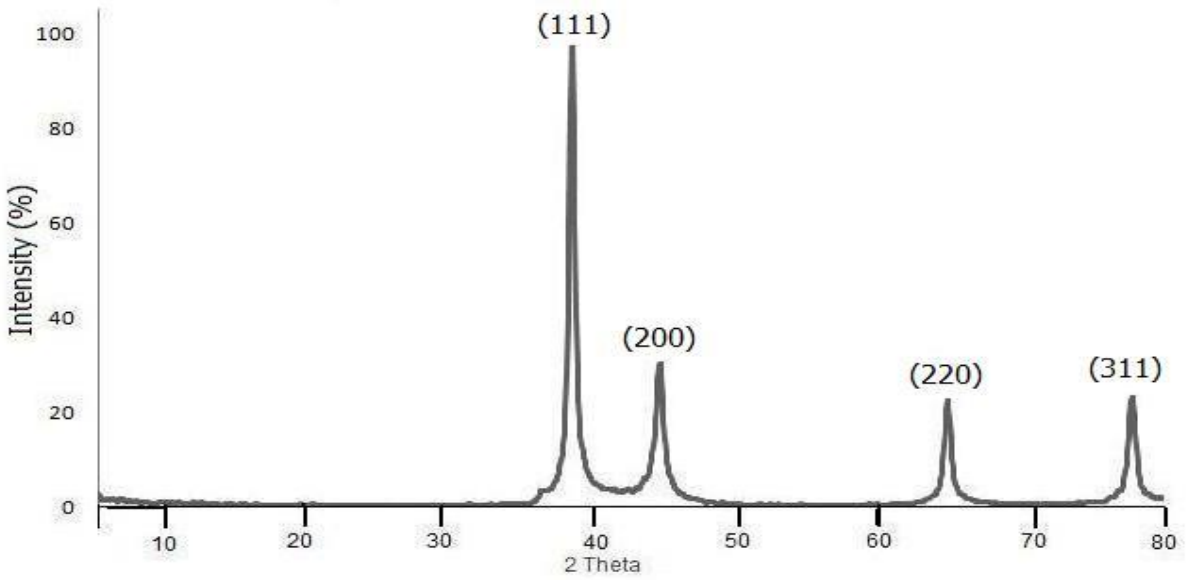

Figure 7 X-Ray diffraction pattern of powdered AgNPs

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Table 3 Lethal-concentration values with lower and upper 95% confidence limits (CL) of colloidal and suspended powder silver nanoparticles (AgNPs) for rainbow trout alevin during 96h

Toxicity	Time (h)/AgNP type	Colloidal	Suspended powder
Average LC10 ($mg\ l^{-1}$)	24	0.90 (0.06-1.80)	18.60 (7.41-26.70)
	48	0.16 (0.10-0.22)	11.93 (5.63-20.12)
	72	0.12 (0.07-0.16)	8.95 (4.42-17.01)
	96	0.08 (0.04-0.11)	7.10 (3.94-11.68)
Average LC50 ($mg\ l^{-1}$)	24	2.75 (1.20-7.16)	186.42 (93.42-448.91)
	48	0.44 (0.34-0.56)	69.37 (27.94-174.30)
	72	0.35 (0.27-0.45)	36.93 (14.62-83.39)
	96	0.25 (0.18-0.32)	28.25 (7.97-35.46))
Average LC90 ($mg\ l^{-1}$)	24	4.67 (2.46-10.76)	1868.42 (490.83-5815.19)
	48	1.21 (0.90-1.88)	403.18 (327.82-1734.55)
	72	1.02 (0.75-1.610)	152.25 (39.53-561.43)
	96	0.75 (0.55-1.23)	112.32 (30.74-217.91)

Table 4 Lethal-concentration values with lower and upper 95% confidence limits (CL) of colloidal and suspended powder silver nanoparticles (AgNPs) for rainbow trout juvenile during 96h

Toxicity	Time (h)/AgNP type	Colloidal	Suspended powder
Average LC10 ($mg\ l^{-1}$)	24	2.63 (2.15-2.93)	>100
	48	2.56 (2.15-2.78)	>100
	72	1.74 (0.89-2.10)	>100
	96	1.60 (0.51-1.99)	>100
Average LC50 ($mg\ l^{-1}$)	24	3.76 (3.44-4.24)	>100
	48	3.13 (2.91-3.33)	>100
	72	2.39 (1.87-2.66)	>100
	96	2.16 (1.34-2.43)	>100
Average LC90 ($mg\ l^{-1}$)	24	5.39 (4.67-7.22)	>100
	48	3.84 (3.57-4.41)	>100
	72	3.28 (2.93-4.49)	>100
	96	2.91 (2.60-4.06)	>100

3.3 Observable effects of silver particles on fish

In the case of alevins, agglomeration of the colloidal AgNPs was happened in contact with mucoproteins of the fish. These agglomerated particles were trapped under the gill operculum and inside the mouth of alevins (Figure 8). Also about suspended

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powder AgNPs rapid sedimentation was occurred in experimental beakers; and since alevins don't have active swimming and stay in the bottom of the beakers, the sediments of AgNPs were in close contact with the fish. In the case of juveniles similar to alevins, the colloidal AgNPs were agglomerated in contact with mucus of the gill and at higher concentrations (>1 mgl^{-1}) the mucus secretion increased and lengthy filamentous mixtures of mucus-nanosilver were connected to the gill (Figure 9).

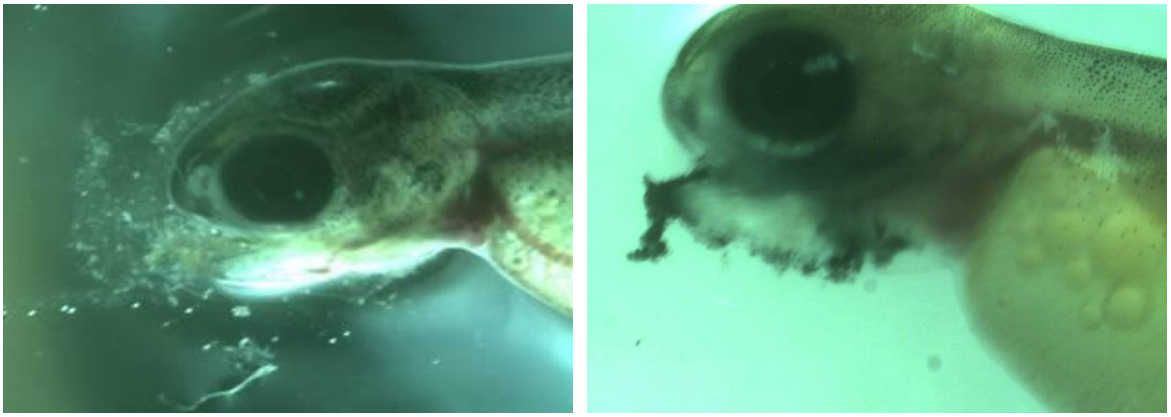

Figure 8 Agglomerated colloidal (left) and suspended powder (right) AgNPs in contact with fish mucus

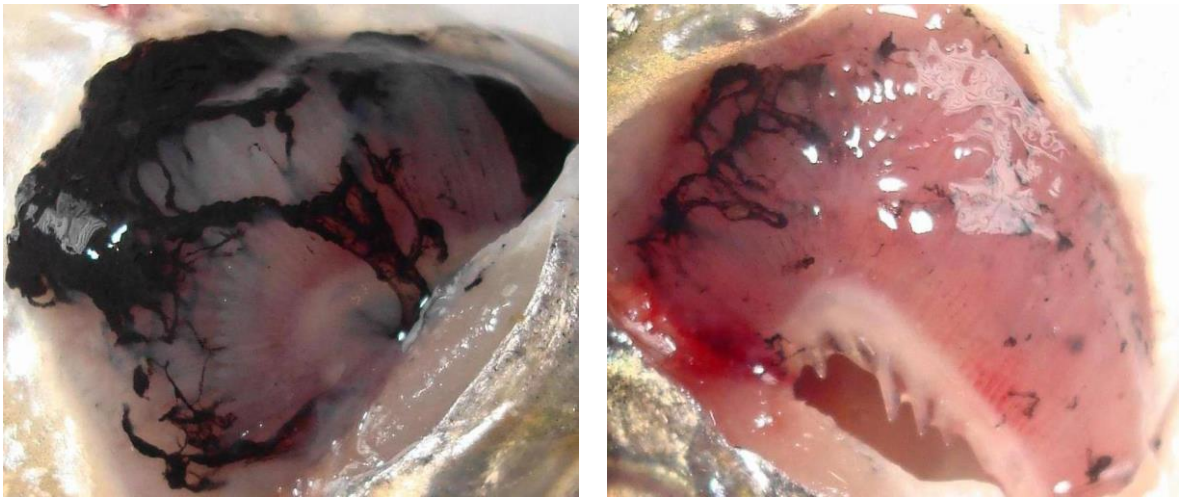

Figure 9 Secreted fish mucus rapidly agglomerates colloidal AgNPs on the surface of the gills; example fish from 10 mg l^{-1} (left) and 3.2 mg l^{-1} (right) concentrations after 24 h.

4 DISCUSSION

Particle size is a critical parameter in the assessment of environmental, health and safety aspects of nanoscale materials (OECD, 2010b). Nanoparticles (NPs) often exhibit special physical and chemical properties and activities due to their small size and homogeneous composition, structure or surface characteristics, which are not present at the larger scale. The size of the nanoparticle implies that it has a large surface area to come in contact with the cells and hence, it will have

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a higher percentage of interaction than bigger particles. In particular NPs possess a much higher specific surface area than their larger counterparts of the same material, and the proportion of atoms on the surface versus the interior of the particle is also much larger for NPs (Handy *et al*., 2008, A).

The intrinsic properties of nanomaterials, such as enhanced reactivity and unique surface structures, can result in higher dissolution rates, reduction and oxidation reactions, or increased generation of reactive oxygen species (ROS), all of which can in turn affect toxicity in a size-dependent manner (Auffan *et al*., 2009).

Although there are almost no ecotoxicological data that have systematically investigated particle-size effects, such studies are important as they inform the need for additional hazard assessments for materials in nano form.

In the present study we investigated the toxicity of two different types of silver nanoparticles on alevin and juvenile of rainbow trout. In the first type, the colloidal AgNPs, nano particles were dispersed well in aqueous environment and the agglomeration was very low (results from TEM microscopes); while in the second type, which was prepared by suspending powdered AgNPs in the water, the nanoparticles were agglomerated and formed larger size clumps. When comparing the colloidal and suspended powder AgNPs, for alevin and juvenile rainbow trout, the colloidal form (smaller size, well dispersed) were comparatively more toxic than suspended powder form (bigger, agglomerated). Kashiwada (2006) showed a particle-size effect on the accumulation of fluorescent NPs in the Japanese medaka, with the smaller particles accumulating more quickly. Also Palaniappan and Pramod (2010) reported that LC50 values of nano TiO_2 and micro TiO_2 were 30 and 100ppm, respectively for zebrafish; which show the positive effect of reduction of particle size on increasing of toxicity. On the other hand Bar-Ilan *et al*, (2009) investigated the size-dependent toxicity of nanosilver on zebrafish embryonic development using 3, 10, 50, and 100 nm silver nanoparticles. In their study LC50 values (93.31μM for 3nm particles to 137.26 μM for 100nm particles) indicated that toxicity is loosely size-dependent, although only at certain concentrations and time points; although mortality was similar across sizes, but the smaller size groups (3 and 10nm) of nanosilver produced a higher incidence of sub-lethal effects than the larger sizes (50 and 100nm).

According to the results of actual concentration measurements of silver in the samples were taken from middle of water column (Table 2), it is clear that the nominal concentrations of silver in the samples from colloidal AgNPs is approximately equal to its actual concentrations with an excellent correlation (R^2=0.99); but in the case of the samples from suspended powder AgNPs, actual concentrations of silver were approximately 10 to 16 time less than nominal concentrations. So in the case of suspended powder AgNPs, it seems that aggregation on the one hand causing sedimentation of particles, makes large amount of silver go out of the water column; and on the other hand making larger clumps with smaller surface area which release less amount of silver ions to the water. Griffitt *et al*, (2009) infer that metallic nanoparticles added to water tend to aggregate simultaneously to form larger particles, dissolve to release soluble metal ions, and sediment out of the water column. In the present study dispersion stability of suspended powder AgNPs was very low and despite application of suspending reagent and strong aeration of tanks, sedimentation was observable; moreover aggregations of particles accelerate the precipitation of AgNPs. While colloidal AgNPs were remained in a stable suspension for up to

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several hours and there were no sign of sedimentation. Sedimentation would have remove much of the suspended powder AgNPs from the water column and make them non-bioavailable for juveniles, but cause increasing potential effect of these particles on alevins. So should be more attention to the toxicity on benthic organisms in the case of materials which quickly sediment in the bottom.

Acute aquatic toxicity is normally determined using a fish 96 hour LC50, a crustacea species 48 hour EC50 and/or an algal species 72 or 96 hour EC50 (EC, 2008). In general and based on 96 hour LC50, results of this study showed that tested AgNPs were significantly more toxic for alevins compare to juveniles. This shows that alevin stage is more sensitive than juvenile toward both colloidal and suspended powder AgNPs; and in the case of juveniles, suspended powder AgNPs had no acute effect even at a concentration of 100 mgl^{-1}. Immature or young neonatal organisms often appear to be more susceptible to chemical agent than are adult organisms. This may be due to differences in degree of development of detoxification mechanisms between young and adult organisms (Rand *et al*., 1995). Differences in rate of excretion of toxic chemicals may also be involved in age-dependent toxicity effects. In general the influence of body size on toxicity must be more consider (Rand *et al*., 1995).

LC50 data provides a good baseline for toxicity tests. According to European Union legislation (EC, 1999; EC, 2008) any substance with a 96 hour LC50 in the range of 10 to 100 mgl^{-1} and 1 to 10 mgl^{-1} has to be classified as "harmful" and "toxic to aquatic organisms", respectively. Also any substances with a 96 hour LC50 lower than 1 mgl^{-1} has to be classified as "very toxic or hazardous to aquatic organisms". Base on this legislation and results of this study, tested colloidal AgNPs can be classifying as "toxic" to juveniles and "very toxic: to alevin rainbow trout; also regarding suspended powder AgNPs they can be classifying as "harmful" to alevins and "no acute effect" to juveniles.

Although flow through test methods can solve some problems regarding dose stability and dispersion stability, since this method can create a waste disposal problem, the semi-static test method, which can reduce waste disposal risk (OECD, 2010a), was employed in this study and 100% water change after 48 hour was done to maintain exposure concentrations. One of the important factors which can completely permute results of ecotoxicological studies on nanomaterials, and therefore should be lionized, is the water quality in which fish exposed to nanoparticles. Instances such as salinity, pH, hardness, bivalent and monovalent ions (especially Sulfide, calcium and magnesium), and dissolved organic carbon (DOC) of water can have a big effect on agglomeration and toxic effects of nanoparticles in aquatic environments (OECD, 2010a). Recently, Kalbassi *et al*, (2011) showed that water salinity could be largely decrease toxicity of silver nanoparticles for rainbow trout larva.

nother distinct example about effect of water quality on chemical properties of nanoparticle in this study was the buffer effect of utilized water on pH of colloidal AgNPs. The primary pH of 4000 mgl^{-1} AgNPs colloid was 2.40, when this colloid was diluted with deionized water to 100 mgl^{-1} the pH was increased to 3.83; but when it was diluted with dechlorinated tap water to 100 mgl^{-1}, the pH increased to 7.78. Therefore water quality assessment must carry out routinely in any ecotoxicological experiments on nanomaterials.

Fish are exposed to chemicals in solution or in suspension in water at both their gills and gastrointestinal epithelia. Between the aquatic environment and the external surface of the fish is an unstirred layer, usually with polyanionic mucus secretions (Handy *et al*., 2008, B). This unstirred layer tends to be more viscous and

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move more slowly than bulk water, thereby holding nanoparticles at the external surface of the organism (Handy *et al*., 2008, B). The various ligands present on the cell surface also are predominantly anionic. Nanoparticles should generally diffuse across the mucous layer more slowly than single molecules such as electrolytes and metal ions, and cationic nanoparticles might bind to strands of mucoproteins hindering their uptake (Handy and Shaw, 2007; Handy *et al*., 2008, B). Cell surfaces also might present ligands for nanosilver (e.g., gill epithelium is predominantly anionic) (Handy and Eddy, 2004). The role of mucous secretion from fish's body is very sensible in this study. The mucus layer on the gills and body surface can connect particles to each other and may be an effective barrier to entry of the nanoparticles into the body cells. Agglomeration can abrogate the properties associated with nano-sized particles by reducing its effective surface area (Greulich *et al*., 2009).

In conclusion**,** results of this short-term toxicity test can demonstrate some differences between well dispersed and agglomerated silver nanoparticles; although both acute and chronic ecotoxicity testing should be undertaken in order to build mathematical relationships between acute and chronic toxicity for categories of nanomaterials (OECD, 2010b). Totally it is recommended that the release of untreated silver particle waste into the environment be controlled.

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تأثیر انباشتگی و اندازه ذرات بر سمیت فرمهای مختلف نانو ذرات نقره در محیط‌های آبی

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چکیده به منظور بررسی تأثیر اندازه ذرات و انباشتگی آن‌ها در مطالعات نانو زیست سم‌شناسی در محیط‌های آبی، سمیت حاد دو نوع متفاوت نانو ذرات نقره (شامل پودر معلق و کلوئید) در لاروهای کیسه زرده‌دار و ماهیان نوجوان قزل‌آلای رنگین کمان مورد بررسی قرار گرفت. ماهی‌ها در معرض غلظت‌های اسمی ۰/۰۳۲، ۰/۱، ۰/۳۲، ۱، ۳/۲، ۱۰ و ۱۰۰ میلی‌گرم در لیتر نانو ذرات نقره قرار گرفتند. غلظت‌های کشنده با استفاده از تجزیه و تحلیل پروبیت محاسبه گردیدند. مشخصات فیزیکی و شیمیایی نانو ذرات نقره مورد استفاده نیز بررسی گردید. در مورد نانو نقره کلوئیدی، ذرات به‌خوبی در ستون آب پراکنده شده بودند و ابعاد آن‌ها نیز حفظ شده بود؛ اما در مورد نانو نقره پودری معلق، ذرات دچار انباشتگی شدند که منجر به افزایش اندازه و نهایتاً ته‌نشینی آن‌ها در کف آب گردید. غلظت کشنده میانی (LC50) در طی ۹۶ ساعت، در مورد لاروهای کیسه زرده‌دار، برای نانو نقره کلوئیدی و پودر معلق به‌ترتیب ۰/۲۵ و ۲۸/۲۵ میلی‌گرم در لیتر محاسبه گردید. در مورد ماهیان نوجوان، غلظت کشنده میانی ۹۶ ساعته برای نانو نقره کلوئیدی ۲/۱۶ میلی‌گرم در لیتر به‌دست آمد، اما نانو نقره پودری معلق، حتی طی ۲۱ روز در معرض قرارگیری ماهیان نوجوان، باعث مرگ آن‌ها نگردید. نتایج این مطالعه نشان داد که در لاروهای کیسه زرده‌دار و ماهیان نوجوان قزل‌آلا، نانو نقره کلوئیدی نسبت به محلول پودر معلق شده بسیار سمی‌تر بوده است، هم‌چنین پدیده انباشتگی موجب افزایش ابعاد نانو ذرات و در نتیجه کاهش سمیت آنها شد. نانو ذرات نقره کلوییدی موادی بسیار سمی برای ماهیان محسوب گردیده و از رهایش آن‌ها به محیط زیست آبی باید کاملاً اجتناب نمود.

کلمات کلیدی: خطرات زیست محیطی، سمیت وابسته به اندازه، قزل‌آلای رنگین کمان، نانو ذرات نقره، نانو سم‌شناسی آبزیان

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