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Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Recognizing the significance of the life stage of fish for nano-eco-toxicological studies, the acute toxicity of colloidal silver nanoparticles (AgNPs) was tested in three different life stages of rainbow trout. Fishes were exposed to colloidal AgNPs at nominal concentrations of 100, 32, 10, 3.2, 1, 0.32, 0.1, and 0.032 mg/L. The estimated 96 hr LC50 values were 0.25, 0.71, and 2.16 mg/L for the eleutheroembryos, larvae and juveniles, respectively, revealing a higher sensitivity for the early life stages. In addition, a dose-dependent blood plasma reduction of chloride and potassium, also increase of cortisol and cholinesterase were observed in the juveniles to exposed AgNPs when compared to the controls. Thus, colloidal AgNPs should be classified as "very toxic" and "toxic" to the eleutheroembryo-larva and juvenile stages, respectively, meaning that the release of nanosilver into the aquatic environment or its direct application as an antimicrobial agent in aquaculture should no longer be allowed.

Keywords: Silver, Nanoparticles, Nanotoxicology, Rainbow trout, Life stage, Blood plasma

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Introduction

The increasing commercial application of nanomaterials (NMs), with at least one dimension of ≤100 nm, is currently showing inventory listings of 1317 nanotechnology-based consumer products in 30 countries (Woodrow Wilson Database, 2011), while the production of engineered nanoparticles is expected to reach approximately 60 000 tons in 2011 (Jovanovic´ et al., 2011). The most common nanomaterial mentioned in consumer product inventories is silver, with 313 products (Woodrow Wilson Database, 2011). In addition, to their importance as antimicrobial agents (Cho et al., 2005; Mohan et al., 2007; Shahverdi et al., 2007; Zheng et al., 2008), silver nanoparticles (AgNPs) are also widely utilized in material science, chemistry and physics fields due to their particular magnetic, optical, electronic, and catalytic properties.

Fungal infections of the eggs of freshwater fish are well known as problematic disease (Shahbazian et al., 2010). Recently, colloidal silver nanoparticles have been investigated as an antibacterial and antifungal medication for incubation systems of rainbow trout (Soltani et al., 2009, 2011). Soltani et al. (2009) reported that the mean percentage of hatchability reached 48.6±1.5% in incubation systems of rainbow trout treated for 30 minutes per day with 4 mg/L colloidal AgNPs, compared with 64.7±0.2% for systems treated for 20 minutes per day with malachite green at 2 mg/L as the positive control ($P<0.05$) and 5.1± 0.2 % for systems without any added medication.

Yet, despite the potential benefits of NMs, there is now a serious debate over the possible risks associated with the use of nanomaterials. Indeed, the very properties that make nanoscale materials useful are the same properties that may pose risks to humans and the environment, as the potential toxicological and long-term environmental properties of many types of nanomaterials are not yet well understood. Therefore, risk assessment of nanomaterials needs to be considered together with their positive properties.

More than 50 papers have already been published on different aspects of the toxicity of silver compounds (except silver nanoparticles) in rainbow trout, plus the acute and chronic toxicity mechanisms of these compounds have also been studied in various fish species, including rainbow trout (for a more detailed description, the reader is invited to see: Hogstrand and Wood, 1998; Wood et al., 1999). To the end of December 2011, based on an online search of different search engines, about 137 papers have been published on the toxicity of 44 different nanomaterials in 14 different fish species, where 34 papers focused on the toxicity of silver nanoparticles in 9 different fish species, including 13 papers on zebrafish and 7 papers on rainbow trout. In summary, these papers found that silver nanoparticles could cause an increase in mortality (Asharani et al., 2008; Griffitt et al., 2008; Yeo and Yoon, 2009; Asharani et al., 2010), hatching delay (Asharani et al., 2008; Powers et al., 2011), various types of malformation, such as edema, spinal abnormalities, heart malformations, and eye defects (Lee et al., 2007; Asharani et al.,

2008; Yeo and Kang, 2008; Bar-Ilan et al., 2009; Wu et al., 2010; Yeo and Yoon, 2009; Asharani et al., 2010; Laban et al., 2010; Kannah et al., 2011), growth retardation (Wu et al., 2010; Asharani et al., 2010), changes in gene expression (Yeo and Pak, 2008; Chae et al., 2009; Choi et al., 2010; Griffitt et al., 2009; Yeo and Yoon, 2009), cellular and DNA damage (Chae et al., 2009; Choi et al., 2010), and carcinogenic and oxidative stress (Chae et al., 2009; Choi et al., 2010). The body uptake of AgNPs and concentrations in different tissues, such as the gills and liver, have also been reported (Asharani et al., 2008; Yeo and Pak, 2008; Griffitt et al., 2009; Yeo and Yoon, 2009; Scown et al., 2010; Laban et al., 2010).

Hrovat et al. (2009) proposed that initial ecotoxicological tests could always utilize rainbow trout as the reference species, and thereafter consider additional species. A similar proposal is also contained in the guidance document on aquatic ecotoxicity developed for the purpose of pesticide risk assessment, where an acute toxicity test on trout is mandatory, followed by additional tests on warm water fish (EFSA, 2002).

The sensitivity of an organism to external stimuli varies throughout its life time, and in the case of teleosts, the embryos and larvae are generally the most sensitive stages in the life cycle (Laale and Lerner, 1981; McKim, 1985; von Westernhagen, 1988; Lele and Krone, 1996). Woltering (1984) noted that the most vulnerable stage was the eleutheroembryo phase, between hatching and the ability to feed on external food, and characterized by nutrition from the rest of the yolk sac and the absence of exogenous feeding (Belanger et al., 2010). The larvae stage is the next in sensitivity, the time of metamorphosis to a developed fish, the juvenile stage.

Accordingly, the main objective of this study was to determine the sensitivity of different life stages of rainbow trout to colloidal silver nanoparticles in an acute toxicity test. Also, various hematological parameters of exposed juveniles were surveyed to evaluate the acute effects of colloidal nanosilver on plasma.

Materials and methods

Characterization of silver nanoparticles

The colloidal silver nanoparticles (AgNPs), type L (commercial name: Nanocid®), were purchased from Nano Nasb Pars Co. (Tehran, Iran). The same type of particle was also previously used as a direct antifungal medication for rainbow trout eggs during the incubation period (Soltani et al., 2011). 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, 4.5 g of LABS (Linear alkyl benzene sulfonate) was dissolved in 95 ml of distilled water and then added to a solution containing 0.32 g of silver nitrate. After mixing thoroughly, 0.2 g 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 4000 mg/L spherical silver nanoparticles (average size 16.6nm).

Also, prior to using the colloid product in the present study, some of its properties were measured as follows. The pH was determined as 2.40 using a standard digital pH meter. Dynamic light scattering (DLS), was performed in three replicate, and each replicate with six run, using a Malvern Zetasizer model 3000HS$_a$ (Malvern Instruments Ltd., Worcestershire, UK), and results showed that the particle zeta potential of a 100 mg/L suspension (nanosilver colloid diluted in double distilled water) was -53.33±7.86 mV. A zeta potential range from ±40 to ±60 mV is a sign of good stability for colloids (ASTM, 1985). Furthermore, the DLS showed that the particle size distribution in the diluted AgNP suspension (100 mg/L) ranged from 3.9 to 163.5 nm, where 54.1% of the particles were under 100 nm and the remaining 45.9% ranged from 100 nm to 165 nm (Figure 1). Also, the average silver hydrodynamic diameter was 54.8 nm.

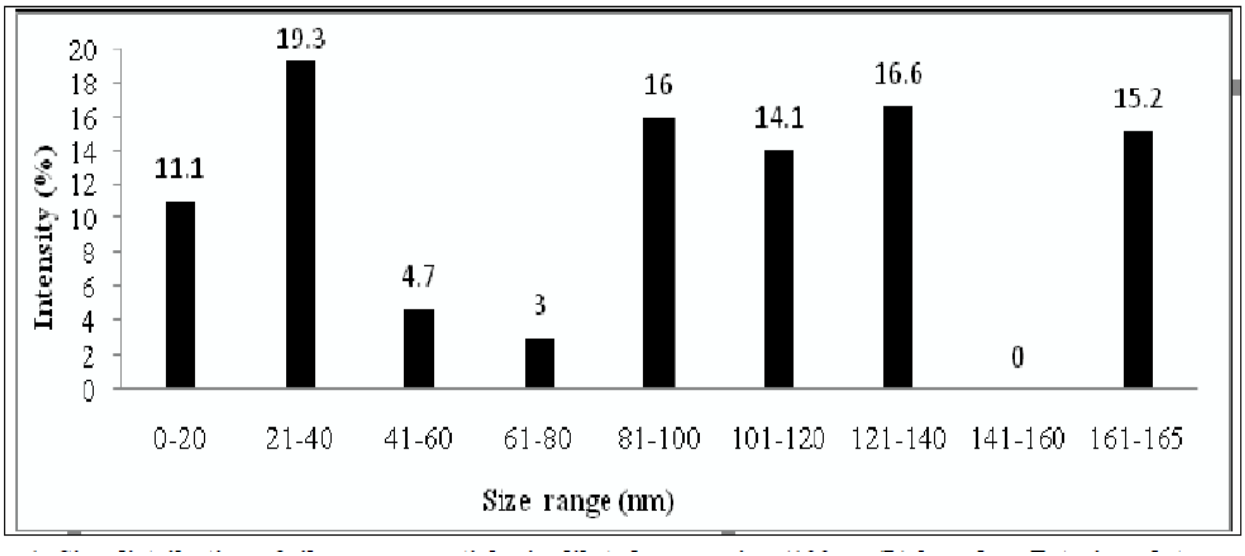

Figure 1: Size distribution of silver nanoparticles in diluted suspension (100 mg/L) based on Zetasizer data.

TEM analyses of the undiluted AgNP suspension (4000 mg/L) were performed using an H-7100FA transmission electron microscope (Hitachi, Japan) with an acceleration voltage of 125kV. 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). The silver nanoparticles observed by TEM were spherical in shape, with a maximum diameter of 129 nm (Figure 2. A): 65.14% of the particles had diameters between 1 and 13 nm (just 2.28% of the particles had diameters more than 100nm) and the CMD (count median diameter) for the particles was 6.47nm (Figure 3). Also the geometric mean diameter (GMD) and geometric standard deviation (GSD) of silver nanoparticles were 12.65nm and 1.46 respectively. EDX analyses were performed using an EX200 Energy-dispersive x-ray analyzer (Horiba, Japan) and confirmed that only elemental silver was present in the AgNP suspension (Figure 2. B).

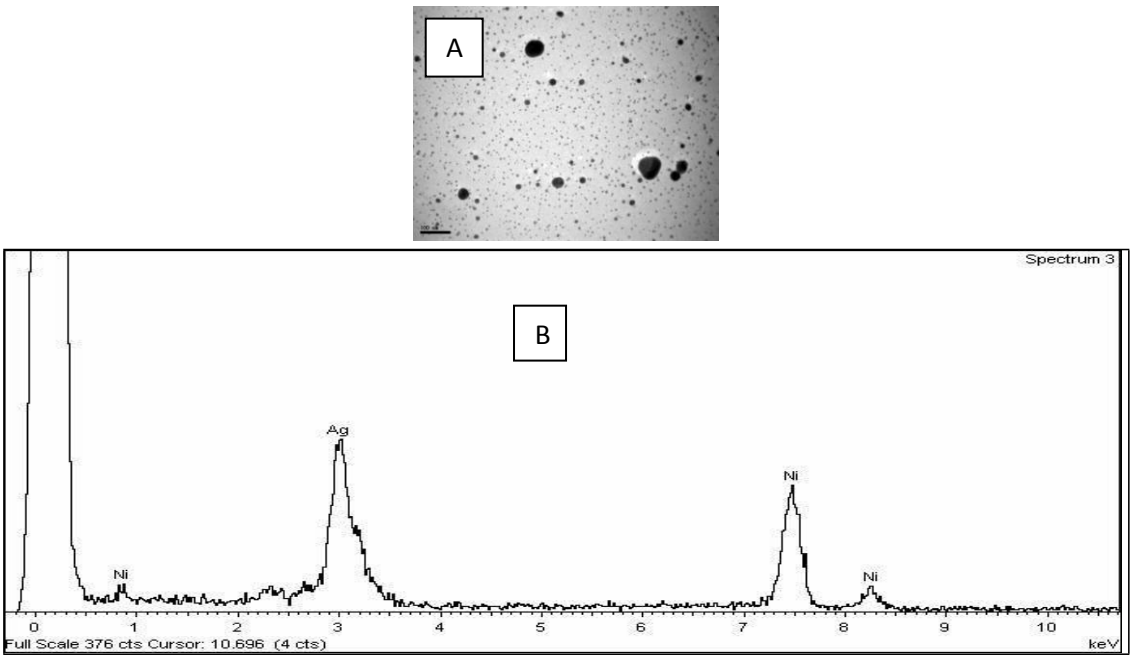

Figure 2 :A: Transmission Electron Microscope (TEM) morphology of silver nanoparticles and B: Energy Dispersive X-ray (EDX) spectrometer pattern (Ni signals in EDX spectrometer are from TEM grid).

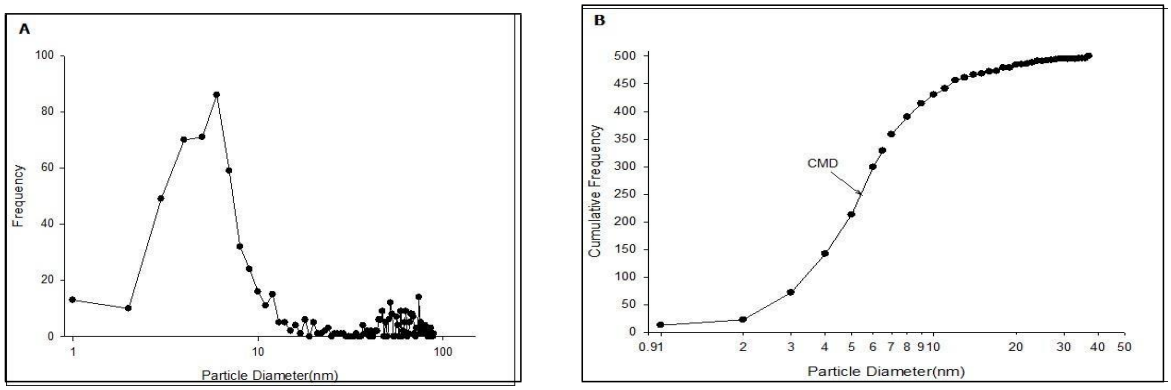

Figure 3: Size distribution of silver nanoparticles in undiluted suspension (4000 mg/L) based on transmission electron microscope (TEM) data. A: Number Frequency and B: Cumulative Frequency (CMD: Cumulative median diameter).

Absorption spectral measurements of the diluted AgNP suspension (400 mg/L) were conducted using a Spectra-MAX-PLUS 384 UV–visible spectrophotometer (Molecular Devices, USA) within a range of 190-1000 nm. A strong surface plasmon resonance was centered at approximately 410 nm, (Figure 4) which was similar to previous results for AgNPs (Petit et al., 1993; Kong and Jang, 2006; Shahverdi et al., 2007; Bhui et al., 2009).

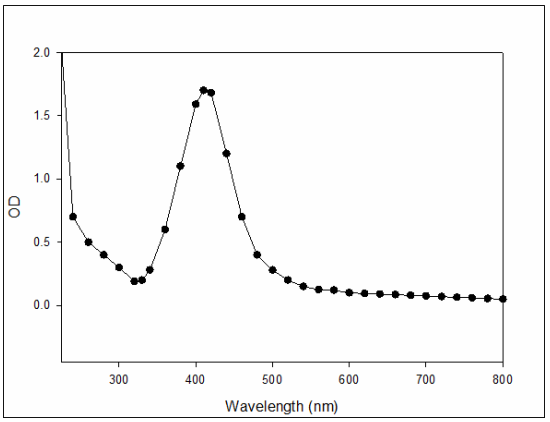

Figure 4: UV–VIS absorption spectra of AgNP colloid.

To determine the concentration of silver in the stock nanosilver suspension, equal volumes of the suspension and 69% HNO_3 were mixed, resulting in dissolution of the silver nanoparticles. The concentration of silver in this solution was then measured using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Model: 3410 ARL, Switzerland). Based on the ICP-AES results, the concentration of Ag ions in the stock AgNP suspension was 3980 mg/L.

Fish

Eleutheroembryo-stage rainbow trout (n=240) from the same brood in the holding stock were randomly selected 2 days post-hatching (32 day post fertilization ,dpf), and exposed in a 1L cylindrical glass beaker containing the desired concentration of colloidal AgNPs with a semi-static (renewal) exposure regime and aerated using 2cm air stones. The beakers were covered with a special dark plastic due to the light sensitivity of the embryos.

In addition, rainbow trout larvae (n=240) with a total weight of 154 ± 2.16 mg (mean ± SD) from the same brood in the holding stock were randomly selected 22 days post-hatching (52dpf). The larvae were then exposed to the colloidal silver nanoparticles in the same 1L cylindrical glass beaker based on a light regime of 12 h light/day.

Juvenile rainbow trout (n=240) with a mean total body weight of 15.47 ± 0.83 g (mean ± SD) were obtained from Marzan Ghezel Trout Farm (Mazandaran, Iran). The fish were kept in 1000L tanks with a semi-static supply system and 12 h light/day, and were fed pellet feed (Chineh, Iran) at 1% of their body weight. The water temperature was between 10 and 14°C. After 7 days of adaptation, the fish were transferred to 24 cylindrical tanks (10fish/tank) with a total volume of 90L, in a triplicate design, and allowed to adapt for 24 h prior to starting the experiments. Each tank was continuously aerated using a 5cm spherical air stone.

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 larva and juvenile feeding was stopped 48 h prior to the experiments to minimize the risk of AgNP absorption in the fecal material or food and minimize the dissolved organic carbon (DOC) in the exposure tanks in order to avoid an overestimate of the toxicity in the renewal exposure systems (Welsh et al., 2008).

The tap water was dechlorinated by adding 1 mg/L 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, 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 values for the dechlorinated tap water are shown in Table 1. Also, during the experiments, the mean and SD of the water pH and dissolved oxygen in the exposure tanks were 8.02±0.14 and 8±0.21 mg/L, respectively. The mean water temperature in the eleutheroembryo beakers was 10±0.5 °C, while in the larva beakers and juvenile tanks it was 12±2 °C.

Table 1:Chemical characteristics of dechlorinated tap water used in experiments.

Parameter	NH_4^+	S^{2-}	Mg^{2+}	Cl^-	Na^+	K^+	Calcium Hardness	Total Hardness
mg/L	0.1±0.01	0.00	39±1.15	2.4±0.2	13.8±0.11	3.9±0.1	26±3.78	150±3.60

Dosing and toxicity tests

In all the experiments, logarithmic series of AgNP concentrations were used according to the Organization for Economic Co-operation and Development (OECD) guideline for chemical testing (OECD, 2000). The selected concentrations were 100, 32, 10, 3.2, 1, 0.32, 0.1, and 0.032 mg/L. The aeration and water flow in the tanks dispersed each dose around the tank in less than one minute and maintained a 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 are shown in Table 2.

Table 2:Comparison of nominal silver concentrations versus actual concentrations. Actual concentrations were measured one hour after dosing fish tanks with silver nanoparticles.

Nominal AgNP Concentration (mg/L)	0.032	0.1	0.32	1	3.2	10	32	100
Measured Ag Concentration (mg/L, mean± SD)	0.03±0.02	0.10±0.03	0.37±0.07	1.30±0.1	3.70±0.14	13.3±0.42	37.30±1.67	108.10±2.26

For every life stage experiment, 10 healthy rainbow trout were transferred directly to each prepared concentration in triplicate. Control groups (10 fish in 3 replicates) were also included for each treatment. The fish were exposed to the AgNPs based on a semi-static exposure regime (100% of water changed after 48 h followed by re-dosing). The mortalities were recorded at 24, 48, 72, and 96 hours post-exposure.

Blood plasma analysis of juveniles

To evaluate the toxicity effect of AgNPs on the blood plasma parameters, 54 juvenile rainbow trout with a mean total body weight of 13.82 ± 0.56 g (mean ± SD) were divided into 18 experimental tanks (three fish/tank), in a triplicate design (three tanks/treatment), and kept there for 24 hours prior to the experiment. The fish were then exposed to AgNPs at high nominal concentrations of 8, 6, 4, 2, and 1 mg/L, plus control groups were included. On the basis of preliminary test results, 3 hours post-exposure was determined as the best time for sampling the fish, as this was just prior to any mortality. The fish were anaesthetized with 100mg/L clove oil and whole blood (about 1 ml) collected via the caudal vein into heparinised syringes (heparin sodium, Rotexmedica, Germany), centrifuged at 2000 rpm for 10 min, and the plasma removed and stored at -20°C until analysis.

The plasma sodium (Na^+) and potassium (K^+) were analyzed using a Jenway flame photometer (UK). The chloride (Cl^-) and cholinesterase were analyzed using a Technicon Auto Analyzer (USA), where the chloride was measured using a colorimetric technique and the Chemenzyme chloride reagent (Iran), while the cholinesterase was measured using an enzymatic technique and Parsazmun kits (Iran). The total thyroxin (TT4) and cortisol were measured using a radioimmunoassay technique with an LKB gamma counter (Finland), plus total T4 RIA and cortisol RIA kits from Immunotech (France).

Statistical analysis

The LC10, LC50, and LC90, values (with 95% confidence limits) were calculated using the EPA Probit analysis program (version 1.5). In all cases, the standard deviations (SD) and standard errors of the means (SE) were calculated using Excel 2007. The statistical analyses of the blood plasma parameters were performed using SPSS Statistics 17.0 software. All the data were tested for

normality (Kolmogorov–Smirnov test) and analyzed using one-way analysis of variance (ANOVA). The significant means were compared using Tukey's test and $p<0.05$ was considered statistically significant.

Results

Toxicity

No mortality was observed in any life stage of the control fish during the experimental period. The lowest AgNP concentrations causing 100% mortality (within a period of 96 hours following exposure) of the juveniles was 3.2 mg/L and 1 for both larvae and eleutheroembryos was 1 mg/L, respectively. A dose-dependent increase in the mortality rate after exposure to the silver nanoparticles was observed for all the life stages, where the sensitivity of the eleutheroembryos was higher than that of the larvae, and the sensitivity of the larvae was higher than that of the juveniles. Plus, all 3 life stages exhibited a time-dependent decrease in the concentration that caused mortality, meaning that in time lapse experiments, lower concentrations also created toxic effects.

The average values of the lethal concentrations and their 95% confidence limits are shown in Table 3 and Figure 5. The 96h LC50s were estimated to be 0.25, 0.71, and 2.16 mg/L AgNPs for the eleutheroembryos, larvae, and juveniles, respectively. Since the 96h LC50 is a valid standard for estimating toxicity, the toxicity of silver nanoparticles to eleutheroembryos was estimated as 3-fold the level of toxicity to larvae and 9-fold the toxicity to juveniles. Plus, the magnitude of the maximum acceptable toxicant concentration (MATC), no observable effect concentration (NOEC), and lowest observable effect concentration (LOEC) of silver nanoparticles during 96 h of exposure for the 3 tested life stages of rainbow trout are all summarized in Table 4.

The fish exposed to the AgNPs showed signs of gill stimulation and increased mucus secretion from the gills when compared with the control fish. In addition, the higher AgNP concentrations (>1mg/L) resulted in visible long strands of a mucus-nanosilver mixture on the surface of the gills (Figure 6.A). While some of the strands remained attached to the gills (Figure 6. B.), others separated and were found at the bottom of the tanks (Figure 6.C).

Blood plasma parameters for juveniles

The blood plasma parameters for the juveniles exposed to the AgNPs for 3 h are shown in Figure 7. An increase in the AgNPs concentration caused enhanced cortisol and cholinesterase levels, yet reduced chloride and potassium content. Meanwhile, the total thyroxin and sodium exhibited no regular dose-dependent relationship.

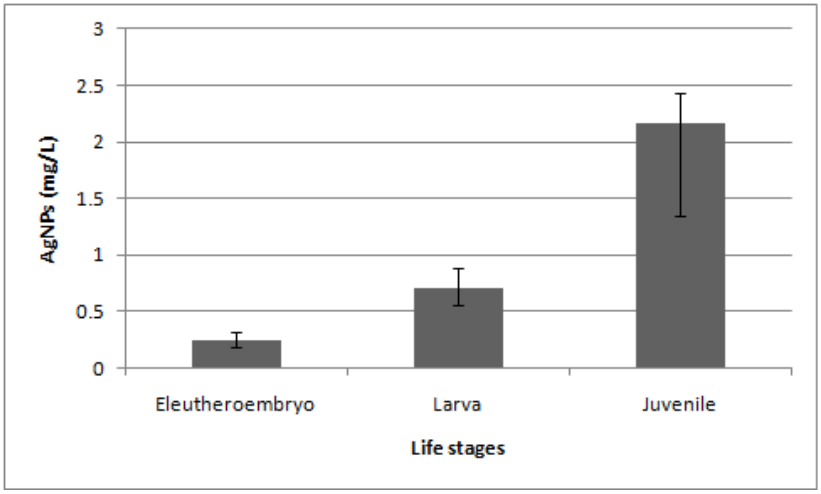

Figure 5: LC50s and 95% confidence limits for AgNPs after exposing different life stages of rainbow trout for 96 h

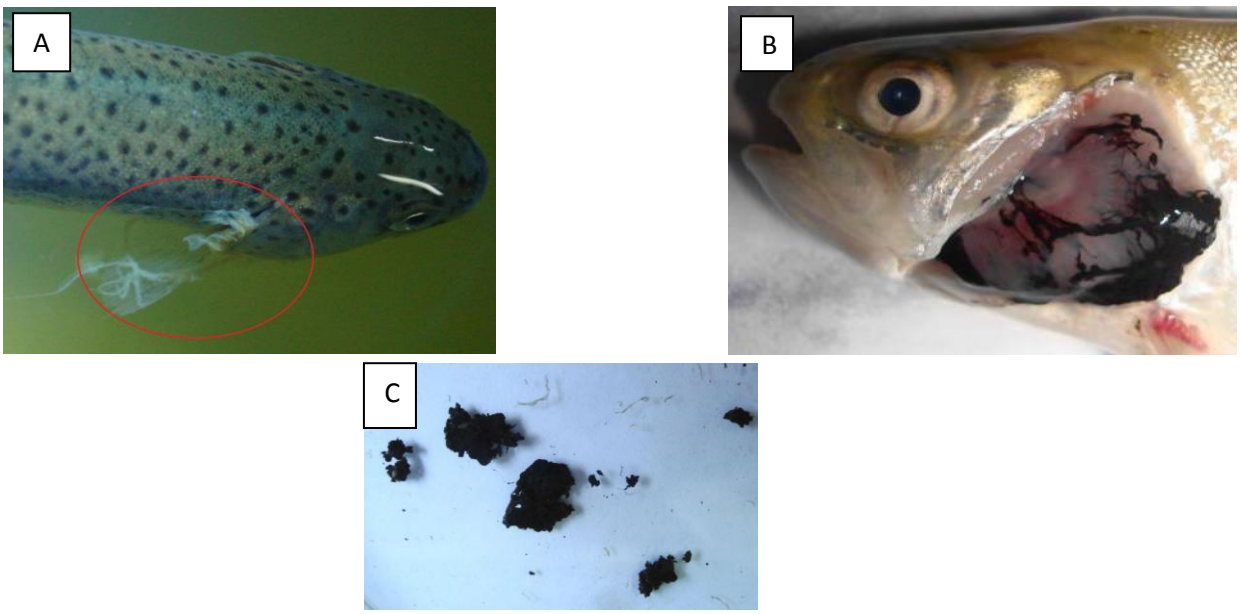

Figure 6: Long strands of mucus-nanosilver mixture connected to fish gills (A), partial strands still attached to gills (B), and detached strands at bottom of tank (C).

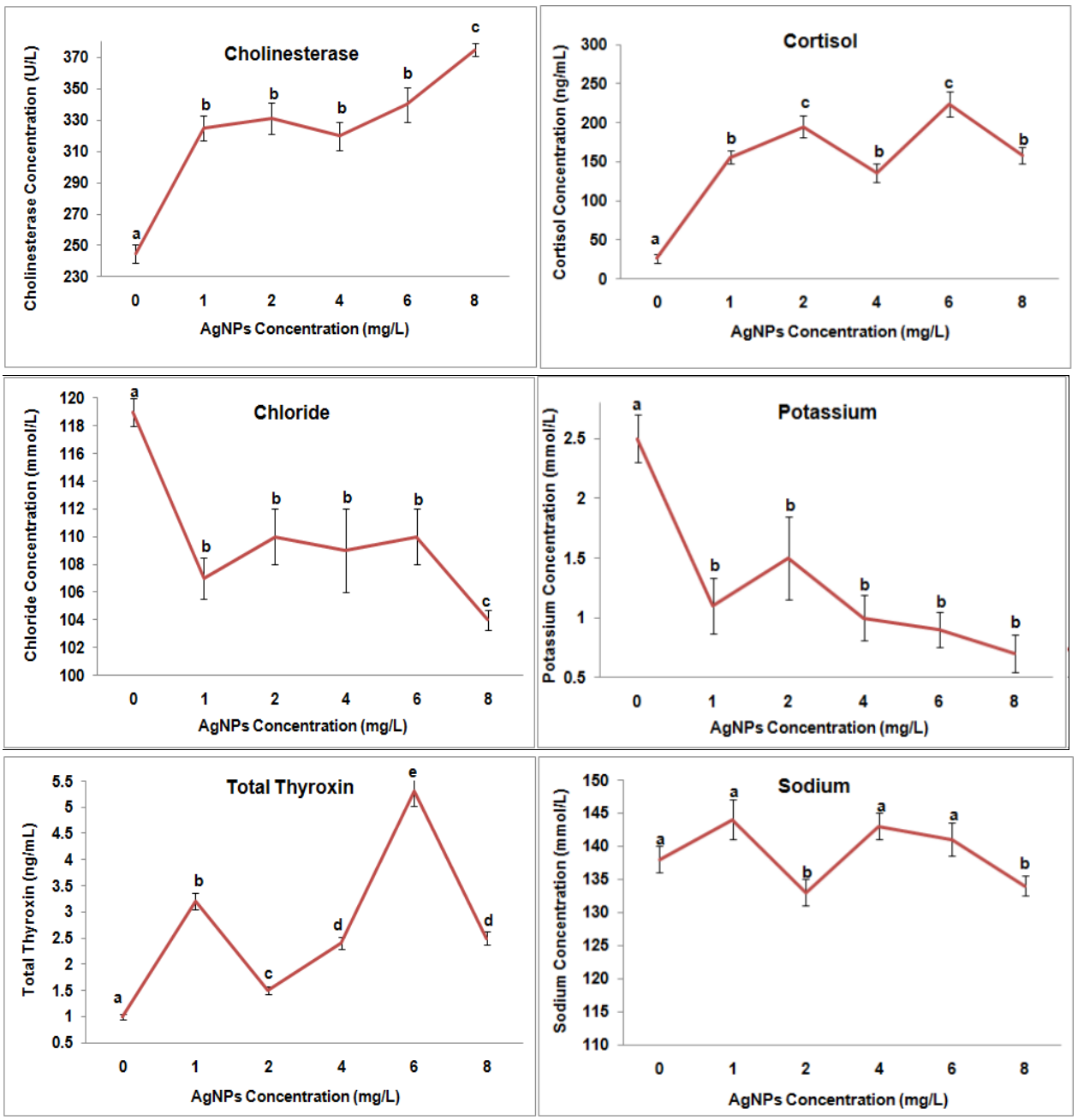

Figure 7: Concentrations of cholinesterase, cortisol, chloride, potassium, total thyroxin, and sodium in blood plasma of rainbow trout juveniles after 3 h of exposure to silver nanoparticles. Values are means ± SD. Values for each parameter with different superscripts (a,b,c,d,e) are significantly different at $P < 0.05$.

Table 3: Lethal-concentration values with lower and upper 95% confidence limits (CL) of AgNPs for each life stage of rainbow trout during 96h.

Toxicity	Time (h)/stage	Eleutheroembryo	Larva	Juvenile
Average LC10 (95%CL) (mg/L)	24	0.90 (0.06-1.80)	0.69 (0.48-0.84)	2.63 (2.15-2.93)
	48	0.16 (0.10-0.22)	0.60 (0.41-0.74)	2.56 (2.15-2.78)
	72	0.12 (0.07-0.16)	0.53 (0.36-0.66)	1.74 (0.89-2.10)
	96	0.08 (0.04-0.11)	0.36 (0.20-0.48)	1.60 (0.51-1.99)
Average LC50 (95%CL) (mg/L)	24	2.75 (1.20-7.16)	1.09 (0.92-1.29)	3.76 (3.44-4.24)
	48	0.44 (0.34-0.56)	1.02 (0.85-1.22)	3.13 (2.91-3.33)
	72	0.35 (0.27-0.45)	0.96 (0.79-1.16)	2.39 (1.87-2.66)
	96	0.25 (0.18-0.32)	0.71 (0.56-0.88)	2.16 (1.34-2.43)
Average LC90 (95%CL) (mg/L)	24	4.67 (2.46-10.76)	1.70 (1.41-2.46)	5.39 (4.67-7.22)
	48	1.21 (0.90-1.88)	1.73 (1.41-2.53)	3.84 (3.57-4.41)
	72	1.02 (0.75-1.610)	1.72 (1.38-2.54)	3.28 (2.93-4.49)
	96	0.75 (0.55-1.23)	1.42 (1.11-2.22)	2.91 (2.60-4.06)

Table 4: Magnitude of MATC, LOEC, and NOEC of silver nanoparticles (AgNPs) for each life stage of rainbow trout during 96h. MATC: maximum acceptable toxicant concentration, LOEC: lowest observable effect concentration, NOEC: no observable effect concentration.

	AgNPs (mg/L)		
	MATC	**NOEC**	**LOEC**
Eleutheroembryo	0.02	0.01	0.08
Larva	0.07	0.32	0.36
Juvenile	0.21	1.00	1.60

Discussion

In many countries, the registration of synthetic chemicals involves passing a number of tests for environmental risk assessment and hazard classification. Currently, the hazard assessment of chemicals for fish is based on international standards and guidelines (such as the OECD guidelines) with global toxicity endpoints, such as mortality, growth, reproduction impairment, and biochemical changes in exposed fish.

Colloidal silver is one of the most beneficial products of nanotechnology that is effective against many types of pathogenic microorganism, including bacterial fish pathogens (Soltani et al., 2009). Plus, Soltani et al. (2011) showed a positive effect of the direct use of silver nanoparticles in reducing fungal infection in trout eggs. However, the commercial application of colloidal silver nanoparticles (AgNPs) to fish is currently lacking clear safety regulations and toxicology data.

Korwin-Kossakowski(2008) recognized that "the time interval following hatching is the most sensitive stage in the life historyof fish"and named it the "compensatory development phase". He also suggested that the transition to exogenous feeding is of even greater importance than hatching in the development of fish, giving this stage a special importance in ecotoxicolgical studies. Indeed, the results of this study showed a different AgNP toxicity in the different life stages of rainbow trout, where the eleutheroembryo stage was the most sensitive. In a review by Grosell et al. (2002), they concluded that smaller freshwater animals are more sensitive to silver, as they exhibit a larger surface area to body mass ratio and larger mass specific gill surface area, which may affect the ion exchange, osmoregulation, and chemical absorption of the body.

The gills of fish are the principal site of gaseous exchange, osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous waste. Increased mucus secretion by fish gills is a common response to aqueous pollutants (Mallat, 1985) and waterborne metals. While this is a short-term defense mechanism designed to prevent the toxicant reaching the sensitive gill epithelium, mucus accumulation on the gill surface can cause impairment of the gill functions. In a study by Smith et al. (2007), rainbow trout exposed to sub-lethal concentrations of single walled carbon nanotubes (SWCNTs) showed signs of gill stimulation and elevated mucus secretion. Also, Federici et al. (2007) reported that rainbow trout exposed to 1 mg/L aqueous TiO_2 nanoparticles showed signs of mucus secretion on the gills. Thus, it would seem that the gill mucus precipitates the nanoparticles to prevent direct exposure of these materials to the gill epithelium. This was also observed in the current study, indicating a strong gill response by the trout to the nanoparticle material.

The primary mechanism of acute silver ion toxicity in fish is related to a severe ionoregulatory disturbance at the gills, causing a depletion of certain plasma ions, such as sodium and chloride (Galvez et al., 1998). Also, increased plasma glucose and cortisol have been reported due to an increase of silver ions (Webb and Wood, 1998; Hogstrand et al., 1999). However, no

published data has been found on the effects of AgNPs on plasma ions or the parameters of cortisol, glucose, and thyroxin. In the studies by Smith et al. (2007) and Federici et al. (2007), single walled carbon nanotubes and TiO_2 nanoparticles were not found to have any observable effects on the sodium and potassium concentrations in rainbow trout blood plasma. Thus, the dose-dependent plasma depletion of chloride and potassium ions found in the current study may have been partly due to the effects of the Ag ions on the fish plasma, although no regular effect was observed in the case of sodium. Plus, the increased cortisol level may have been a stress response of the fish to higher concentrations of AgNPs.

Cholinesterases (ChEs), a class of serine hydrolases, are catalyzers of choline esters. Certain pesticides, such as organophosphorus and carbamates, are known to selectively inhibit cholinesterase activity (Valbonesi et al., 2003). Notwithstanding, an increase of brain cholinesterase has been reported in *Oncorhynchus mykiss* (Dethloff et al., 1999) and *Sparus auratus* (Romani et al., 2003) exposed to sub-lethal copper concentrations. Plus, when investigating the impact of *in vivo* exposure to metals (Cu, Cd, Hg, and Zn) on the acetylcholinesterase (AChE) activity in fish, the results showed both a decrease and increase of AChEs during metal exposure, depending on the tissue sampled and the duration of the exposure (Dethloff et al., 1999). However, there has been no report on any changes in the cholinesterase activity during exposure to silver compounds. Yet, the present study found elevated ChE levels in the blood of the trout juveniles exposed to colloidal AgNPs when compared to the control levels during 3 h post-exposure. Therefore, it would appear that exposure to colloidal AgNPs can affect the ChE levels, although more studies are required to confirm the potential use of ChEs as a reliable biomarker for toxicity assessments of silver nanoparticles.

According to acute toxicity results of published papers, 96 hour LC50 of silver nitrate ($AgNO_3$) for different life stages of rainbow trout ranged from 5.3–20.2 µg/L (Davies et al., 1978; Nebeker et al., 1983; Buhl and Hamilton, 1991; Hogstrand et al., 1996; Grosell et al., 2000;). However based on acute toxicity results of the present study 96 hour LC50 of silver nanoparticles (AgNPs) for different life stages of rainbow trout ranged from 0.18-2.43 mg/L. As is clear from these values, µg/L concentrations of $AgNO_3$ are toxic for rainbow trout while in the case of AgNPs, mg/L concentrations are toxic; thus it seems that silver nanoparticles are less toxic when compare to ionic silver (as $AgNO_3$). This is consistent with the results of Laban et al. (2010) whom showed that AgNPs are 3 times less toxic than Ag nitrate for fathead minnow embryos during a 96h toxicity test. According to the toxicity results on the same colloidal silver nanoparticles which also used in the present study (Shahbazzadeh et al., 2011), Nanocid® was also toxic for normal cell lines of fibroblast, and mesenchymal stem cells (IC50s were 6.68 and 6. 33 mg/L, respectively).

According to European Union legislation (EC, 2008) and European Union

Council Directive 67/548/EEC of 27 June 1967 (EC, 1999), any substance with a 96hr LC50 (for fish) of less than 1mg/L must be classified as very toxic to aquatic organisms with long-term adverse effects for the aquatic environment. Therefore, according to the results of the current study, colloidal AgNPs should be classified as "very toxic" for the eleutheroembryo and larva stages of rainbow trout. Furthermore, according to the same Council Directive (EC, 1999), any substance with a 96hr LC50 (for fish) in the range from 1 to 10 mg/L must be classified as toxic to aquatic organisms with long-term adverse effects for the aquatic environment. Thus, according to the results of the current study, colloidal silver nanoparticles should also be classified as "toxic" for the juvenile stage of rainbow trout.

Conclusion

The direct exposure of different life stages of rainbow trout to colloidal AgNPs revealed that the fish were more sensitive to colloidal silver nanoparticles during the early life stage than in later stages. Furthermore, colloidal silver nanoparticles should be classified as "very toxic" and "toxic" to the eleutheroembryo-larva stages and juvenile stage of rainbow trout, respectively, meaning that the release of colloidal silver nanoparticles into the environment or their direct application as an antifungal/bacterial medication during the hatching period of eggs or larval stages should be avoided. Instead, indirect techniques, like the use of filters containing silver nanoparticles, are better for incubation system water treatment. Further studies are also recommended on the potential use of a fish blood plasma index, such as ChEs, as a reliable biomarker for toxicity assessments of silver nanoparticles.

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