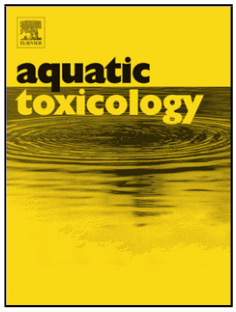


Title: Bioaccumulation of silver nanoparticles in Rainbow trout (Oncorhynchus mykiss): Influence of concentration and salinity

Author: Hamid Salari Joo Mohammad Reza Kalbassi Il Je Yu Ji Hyun Lee Seyed Ali Johari<ce:footnote id="fn0005"><ce:note-para id="npar0005">Tel: +98 9179425247.</ce:note-para></ce:footnote><ce:footnote id="fn0010"><ce:note-para id="npar0010">Tel: +82 172370867.</ce:note-para></ce:footnote><ce:footnote id="fn0015"><ce:note-para id="npar0015">Tel: +82 415409845/+82 1041480867.</ce:note-para></ce:footnote><ce:footnote id="fn0020"><ce:note-para id="npar0020">Tel: +98 9126268409.</ce:note-para></ce:footnote>

PII: S0166-445X(13)00178-1
DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2013.07.003
Reference: AQTOX 3563

To appear in: *Aquatic Toxicology*

Received date: 14-5-2012
Revised date: 4-7-2013
Accepted date: 7-7-2013

Please cite this article as: Joo, H.S., Kalbassi, M.R., Yu, I.J., Lee, J.H., Johari, S.A., Bioaccumulation of silver nanoparticles in Rainbow trout (Oncorhynchus mykiss): Influence of concentration and salinity, *Aquatic Toxicology* (2013), http://dx.doi.org/10.1016/j.aquatox.2013.07.003

Bioaccumulation of silver nanoparticles in Rainbow trout (*Oncorhynchus mykiss*): Influence of concentration and salinity

1. **Hamid Salari Joo**, MSc. Graduated, Department of Aquaculture, Marine Science Faculty, Tarbiat Modares University, Mazandaran, Noor, Iran, Tel: +98-9179425247, E-mail: h.salary1365@gmail.com

2. **Mohammad Reza Kalbassi***, (Corresponding author), Associated Professor, Department of Aquaculture, Marine Science Faculty, Tarbiat Modares University, Mazandaran, Noor, Iran, Tel: +98-911-2204336, Fax: +98-1226253499, E-mail: kalbassi_m@modares.ac.ir

3. **Il Je Yu**, Professor, Institute of Nano-product Safety Research, Hoseo University, 165 Sechul-ri, Baebang-myun, Asan 336-795, Korea, Tel: +82-172370867, E-mail: u1670916@chol.com

4. **Ji Hyun Lee**, Institute of Nano-product Safety Research, Hoseo University, Asan, Korea, Tel: +82-415409845, Mobile: +82-1041480867, E-mail: toxin@dreamwiz.com

5. **Seyed Ali Johari**, Assistant Professor, Aquaculture Department, Natural Resources Faculty, University of Kurdistan, Kurdistan, Sanandaj, Iran, Tel: +98-9126268409, E-mail: a.johari@uok.ac.ir

Abstract

With the increasing use of silver nanoparticles (Ag-NPs), their entrance into aquatic ecosystems is inevitable. Thus, the present study simulated the potential fate, toxicity, and bioaccumulation of Ag-NPs released into aquatic systems with different salinities. The Ag-NPs were characterized using inductively coupled plasma-atomic emission spectroscopy (ICP-AES), dynamic light scattering (DLS), transmission electron microscopy (TEM), energy-dispersive X-ray analysis (EDX), and UV-Vis spectroscopy. Juvenile rainbow trout were exposed to Ag-NPs in three different salinity concentrations, including low (0.4 ppt), moderate (6±0.3 ppt), and high (12±0.2ppt) salinity, for 14 days in static renewal systems. The nominal Ag-NP concentrations in the low salinity were 0.032, 0.1, 0.32, and 1ppm, while the Ag-NP concentrations in the moderate and high salinity were 3.2, 10, 32, and 100 ppm. UV-Vis spectroscopy was used during 48 hours (re-dosing time) to evaluate the stability and possible changes in size of the Ag-NPs in the water. The results revealed that the λmax of the Ag-NPs remained stable (415-420nm) at all concentrations in the low salinity with a reduction of absorbance between 380-550nm. In contrast, the λmax quickly shifted to a longer wavelength and reduced absorbance in the moderate and higher salinity. The bioaccumulation of Ag in the studied tissues was concentration-dependent in all the salinities based on the following order: liver > kidneys ≈ gills > white muscles. All the tissue silver levels were significantly higher in the high salinity than in the moderate salinity. In addition, all the fish exposed to Ag-NPs in the low, moderate, and high salinity showed a concentration-dependent increase in their hepatosomatic index (HSI). In conclusion, most Ag-NPs that enter into freshwater ecosystems (low ionic strength) remain suspended, representing a potentially negative threat to the biota in an ionic or nanoscale form. However, in a higher salinity, nanoparticles agglomerate and precipitate on the surface of the sediment.

Keywords: Rainbow trout, silver nanoparticles, salinity, bioaccumulation, UV-Vis spectroscopy.

1. Introduction

The general definition of nanoparticles (NPs) described by ISO TS 80004-1 (2010) is a "material with any external dimension on the nanoscale or having an internal structure or surface structure on the nanoscale". In recent years, nanotechnology has attracted huge investment and focused on producing materials within this size range. NPs possess unique properties due to their shape, surface structure, size, and unusual chemical and physical properties. In particular, since 40–50% of the atoms in NPs are on the surface, this produces greater reactivity than with non-nanoscale materials. Therefore, NPs have different biological effects than their micro and macro counterparts (Farré et al., 2009)

With the increasing development of manufactured nanomaterials (MNs), concern is also increasing over possible risks of exposure to NPs released from nanoproducts into the environment. Hence, the ecotoxicology of manufactured NPs has become a relatively new and emerging field (Picado, 2010). In the case of silver nanoparticles (Ag-NPs), their exceptional properties, including good conductivity, catalytic, and antibacterial effects, have made them the largest and fastest growing class of MNs in commercial applications (Pinto et al., 2010), representing 56 percentage of currently manufactured MNs (Mantovani et al., 2009). This increasing use of Ag-NPs also increases their eventual entrance into aquatic ecosystems (Meyer et al., 2010; Scown et al., 2010), and several studies have already documented that the presence of Ag-NPs in an aquatic ecosystem can have a toxic effect on aquatic organisms (Asharani et al., 2008; Kalbassi et al., 2011; Laban et al., 2010; Wise Sr et al., 2010; Wu et al., 2010; Asghari et al., 2012; Johari et al., 2013). For example, Ag-NPs can adversely affect fish and other aquatic biota by releasing silver ions or uptaking nanoscale particles. However, the commercial application of nano-Ag is still loosely regulated due to a lack of hazard data (Borak, 2009).

Silver ions ($Ag^{+}_{(aq)}$) are considered the most toxic form of silver in water (Ratte, 1999). Plus, for Ag^{+} and other forms of silver, the chemistry of the surrounding environment, such as the water hardness, pH, alkalinity, salinity, and dissolved organic carbon (DOC), affects the bioavailability of the silver species,

where salinity has the greatest impact on reducing the toxicity and bioavailability of silver ions in water (Nichols et al., 2006; Webb and Wood, 2000).

Notwithstanding, the effects of salinity on the toxicity of Ag-NPs in rainbow trout remain unknown and no study has yet focused on the toxicity, fate, and bioaccumulation of Ag-NPs in this species in saline ecosystems. In addition, it is still unclear whether the effects of Ag-NPs are similar to those of ionic silver or require special consideration in the abovementioned environments. Accordingly, this study used rainbow trout as a well-known model in aquatic toxicology and colloidal Ag-NPs in three different salinities to estimate the potential toxicity and bioaccumulation of Ag-NPs in the gills, kidneys, muscles, and liver.

2. Materials and methods

2.1. Ag-NP characterization

The colloidal Ag-NPs, type L (commercial name: Nanocid), purchased from Nano Nasb Pars Co. (Tehran, Iran), were synthesized using a novel process involving the photo-assisted reduction of Ag+ to metallic NPs, registered under United States Patent Application No: 20090013825 (Nia, 2011). Briefly, 4.5 g of LABS (Linear alkyl benzene suffocate) were dissolved in 95 ml of distilled water and then added to a solution containing 0.32 g of silver nitrate. After mixing thoroughly, the addition of 0.2 g of a hydrazine solution (0.03 M) formed 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). The stock solution was stored in a dark room at 25^{o}C and used within 25 days. Before use, the physicochemical properties of the colloidal product were measured and characterized. The pH was determined as 2.40 using a standard digital pH meter. To determine the silver concentration, equal volumes of the suspension and 69% HNO_3 were mixed, thereby dissolving the Ag-NPs. The silver concentration in the solution was then measured using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Model: 3410 ARL, Switzerland), where the

result showed 3980 mg/L, which was very close to the concentration declared by the manufacturer. Meanwhile, the zeta potential of a 100 mg/L suspension (nanosilver colloid diluted in double distilled water), measured by dynamic light scattering (DLS) using a Malvern Zetasizer model 3000HSa (Malvern Instruments Ltd., Worcestershire, UK), was -53.33±7.86 mV. Furthermore, the distribution of the hydrodynamic diameter of the particles in the diluted Ag-NP suspension (100 mg/L) ranged from 3.9 to 163.5 nm, where 54.1% of the particles had a hydrodynamic diameter of less than 100 nm, the remaining 45.9% ranged from 100 nm to 165 nm, and the average silver hydrodynamic diameter was 54.8 nm (Figure 1).

Desired location for Figure 1

Transmission electron microscopy (TEM) analyses of the stored undiluted Ag-NP suspension (4000 mg/L) were also performed using an H-7100FA transmission electron microscope (Hitachi, Japan) with an acceleration voltage of 125kV. The suspended silver nanoparticles were filtered through a 0.2 μm Nucleopore filter (Nucleopore Corp., Pleasanton, CA), which was then coated with carbon using a sputtering device (JEE-4x, JEOL, Akishima, Japan). Random sections of the carbon-coated filter were transferred to a carbon-coated nickel gird (200 mesh, Veco, Eerbeek, Holland) and chloroform added to dissolve the filter. Thereafter, 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 TEM preparation and imaging were both performed on the same day. The Ag-NPs 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 (2.28% of the particles had diameters more than 100 nm) and the CMD (count median diameter) for the particles was 6.47 nm (Figure 3. B). Plus, the geometric mean diameter (GMD) and geometric standard deviation (GSD) of the silver nanoparticles were 12.65 nm and 1.46, respectively (Figure 3. A). EDX (Energy-dispersive X-ray analyzer) analyses were performed using an EX200 (Horiba, Japan) (Figure 2. B). Absorption spectral measurements of the diluted Ag-NP suspension (400 mg/L) measured using a Spectra-MAX-PLUS 384

UV–visible spectrophotometer (Molecular Devices, USA) within a range of 190-1000 nm showed a peak absorbance (λmax) at approximately 415 nm (Fig. 4).

Desired location for Figure 2

Desired location for Figure 3

Desired location for Figure 4

2.2. Experimental protocol

Three different waters were used in the experiments, including (1) low salinity (de-chlorinated tap water, 0.4 ppt), (2) moderate salinity (combination of de-chlorinated tap water and Caspian Sea water, 6±0.3 ppt), and (3) high salinity (Caspian Sea water, 12±0.2 ppt). Various chemical parameters of the 3 waters, including the total ammonium, magnesium, total hardness, total alkalinity, total organic carbon, calcium hardness, sodium, and chloride, were measured (Table 1). One batch of fresh-water rainbow trout (*Oncorhynchus mykiss*) was used for all the experiments (n= 600, 25±3g). The fish were first separated into three equal groups (n=150) in 1000-liter fiberglass tanks. Two groups were then transferred to the moderate and high salinity, respectively, and allowed to adapt to the higher salinities for at least ten days, while the third group was kept in the low salinity water.

Desired location for Table 1

The study was conducted based on 14 days of static-renewal exposure (re-dosing every 48 hours) according to the Organization for Economic Cooperation and Development test guideline 204 (OECD, 1998). The fish were fed three times a day on commercial trout food, yet the feeding was stopped 24 hours prior to and during the experiments. For each experimental treatment, ten healthy fish were randomly selected from each tank and transferred to 30-liter aquariums containing the same respective levels of salinity, where the fish were allowed to acclimate for 24 h prior to the addition of Ag-NPs. In addition to a control group, four nominal concentrations of Ag -NPs were selected: 0.032, 0.1, 0.32, and 1 ppm for the low salinity, and 3.2, 10, 32, and 100 ppm for both the moderate and high salinity. All the

treatments were performed in triplicate. During the experiments, the average pH, dissolved oxygen, and water temperature were 8±0.2, 9±0.3 mgl^{-1}, and 14±1°C, respectively.

Ag-NPs have a surface plasmon resonance (SPR) that shows a λmax near 400 nm for un-agglomerated particles and shifts to longer wavelengths for agglomerated particles (Zook et al., 2011). Thus, to assess the potential impact of the salinity on the presence of un-agglomerated Ag-NPs in the water columns when renewing the concentrations, samples were collected from each water column at 0.5, 1, 4, 12, 24, and 48 h (re-dosing time) and scanned between 380 to 550 nm using a Spectra-MAX-PLUS 384 UV–visible spectrophotometer (Molecular Devices, USA). Furthermore, to determine the Ag^{+} concentrations, triplicate samples were also taken at 1, 4 and 48 h, and based on a previously reported study (Beer et al., 2012) they were analysed using an Atomic Absorption Spectrophotometer (Phonix-986, Biotech Engineering Management, England).

2.3. Tissue Ag accumulation and HSI index

To determine the effect of Ag-NPs on silver bioaccumulation, at the end of the experiments, ten fish were sampled from each treatment group and their gill, kidney, muscle, and liver tissues dissected out. All the tissue samples were freeze-dried, ground using a mortar and pestle, and then finely ground into a powder. Next, 0.1g of each dry tissue was weighed using an XS205 dual range analytical balance (METTLER TOLEDO) and digested for 45 min at 100°C using a microwave digestion system (MARSXpress Microwave Digestion System, CEM Co. USA) in 3ml of concentrated HNO_3 to break down the fish tissue and dissolve all the silver content. The digested tissues were then cooled, diluted in 25 to 1000 ml of deionized water, and the silver quantified using a graphite furnace atomic absorption spectroscope (GFAAS, Perkin Elmer Analyst Model AA800) equipped with a Perkin Elmer AS800 Auto sampler.

To determine the hepatosomatic index (HSI), the wet weight of the liver was normalized according to the total body weight of each fish and calculated as: HSI (%) = liver weight (g)/ body weight (g) × 100 - (Kjesbu et al., 1991).

160 2.4. Statistical Analysis

161 For the statistical analysis of the Ag bioaccumulation, each experimental value was compared
162 with its corresponding control and the results expressed as the mean ± standard deviation (S.D.). Multi
163 group comparisons of the means were carried out using a one-way analysis of variance (ANOVA),
164 following multiple comparison tests using Tukey's method ($P<0.001$, 99% confidence limit). Dunnett's
165 test was also used to compare the differences between the experimental groups and the control group
166 ($P<0.001$). To determine the influence of elevated salinity on the Ag bioaccumulation in the fish organs,
167 the treatments with the same Ag-NP concentrations in the moderate and high salinity were all analyzed
168 using an Independent Sample T-Test ($P<0.05$).

169 **3. Results**

170 3.1. Fish mortality and spectroscopy of Ag-NPs

171 In the experiment, no mortalities were recorded with any of the Ag-NP concentrations in the low
172 salinity during 14 days of exposure. However, all the fish exposed to 100ppm in the moderate and high
173 salinity died within 4 hours of exposure to the Ag-NPs, whereas no mortalities were recorded with the
174 other concentrations throughout the experiment. The addition of Ag-NPs to the low salinity had no
175 influence on the water clarity or visible sedimentation of the particles. However, the high concentrations
176 of Ag-NPs (except for 3.2ppm) in the moderate and high salinity formed thin layers of brownish and
177 black sediment on the bottom of the aquarium after 48 hours, although most of the sediment was formed
178 during the first four hours of the experiment. In addition, the spectrophotometry results for the water
179 samples indicated a gradual decline of Ag-NPs in the water columns until 48 h.

180 During the 48-h re-dosing time, the optical absorption spectra for the Ag-NPs in the low salinity
181 showed that all the surface plasmon resonance (SPR) absorbance at the λmax was located within a range
182 of 415-420 nm, while the absorption spectra peaks for the Ag-NPs in the moderate and high salinity were
183 mostly shifted above 420 nm (red-shift) (Table 2). This red-shift of the λmax suggested agglomeration
184 and an increasing size of the Ag-NPs at all concentrations in the moderate and high salinity.

Desired location for Table 2

During the 48-hour spectroscopy, while the λmax of the Ag-NPs remained stable in the low salinity, there was a decreasing absorbance at all concentrations (Fig.5A). The full spectra within a range of 380-550nm (over 48 hours) revealed that the absorbance of the agglomerated Ag-NPs decreased more rapidly in the high salinity (especially at 100 and 32ppm) than in the moderate salinity, indicating that the Ag-NPs were more unstable and agglomerated faster in the high salinity than in the moderate salinity. However, the Ag-NPs that initially showed a rapid rate of agglomeration at all concentrations in the moderate and high salinity disappeared during the first four hours and transitioned to a much slower rate of disappearance thereafter (Figs.5B,C). Moreover, in spite of receiving the lowest concentrations of Ag-NPs, the Atomic Absorption Spectroscopy results revealed that the highest release rates of Ag^{+} occurred in the low salinity (Table 3).

Desired location for Figure 5

Desired location for Table 3

3.2. Tissue silver burden and hepatosomatic index

The fish that died with 100 ppm Ag-NPs in the moderate and high salinity were not analyzed for their tissue silver level. After 14 days of exposure to the Ag-NPs, the Ag contents in the fish tissues increased significantly for all the Ag-NP concentrations in the low salinity and the 3.2, 10, and 32 ppm concentrations in the moderate and high salinity (Figs.6A,B,C). An increased Ag accumulation was also observed in all the examined tissues, including the muscles, gills, kidneys and liver, when compared with the control groups after 14 days of exposure (Dunnett's, $P<0.001$). Furthermore, although concentration-dependent increases in the Ag level were observed in the muscles, livers, kidneys and gills, the liver showed a higher Ag content than the other tissues for all treatments (Tukey, $P<0.001$). Meanwhile, the amounts of Ag in the white muscles were lower than those in the gills, kidneys, and liver (Tukey, $P<0.001$), and a comparison of the silver level between the gills and the kidneys showed no significant difference for the waters used in this study (order: liver > kidneys ≈ gills > white muscles).

Desired location for Figure 6

In aquatic toxicology studies, the hepatosomatic index (HSI) is commonly applied. All the fish exposed to Ag-NPs in the low, moderate, and high salinity exhibited an increasing concentration-dependent liver weight to body weight ratio (Fig. 7), yet there were no significant differences between the treatments when compared with their respective control groups (ANOVA, $P>0.05$).

Desired location for Figure 7

3.3. Influence of salinity on Ag bioaccumulation

The present study of rainbow trout showed that the environmental salinity had a dramatic effect on the tissue Ag bioaccumulation during 14 days of exposure. The mean Ag level in the liver, kidneys, gills, and muscles of the fish exposed to Ag-NPs for 14 days in the moderate and high salinity was found to be salinity-dependent (Table 4), and at the same Ag-NP concentrations, the tissue silver accumulation in the fish in the high salinity was significantly greater than that in the fish in the moderate salinity (Independent Sample T-Test, $P< 0.05$).

Desired location for Table 4

4. Discussion

4.1. Agglomeration and aggregation of Ag-NPs

The present study provides a range of data on the potential fate of Ag-NPs with increasing salinity from freshwater to brackish aquatic ecosystems. The present data also indicated that the bioaccumulation of Ag in fish tissues was influenced by salinity during 14 days of chronic exposure. Therefore, these results enhance current knowledge on the environmental health impact of MNs.

The high (100 %) mortality in the groups exposed to 100 ppm nominal Ag-NPs in the moderate and high salinity was presumably due to suffocation caused by the deposition or adhesion of the Ag-NPs to the gills, as previously shown in the case of rainbow trout and Atlantic salmon exposed to nano Ag and SWCNT in freshwater (Farmen et al., 2012; Smith et al., 2007).

The physical and chemical properties of exposed aquatic media, such as the salinity or ionic strength, pH, divalent cations, and organic material, are the most important parameters affecting the stability of dispersed nanoparticles in an aqueous environment (Stebounova et al., 2011). Depending on the chloride (Cl^-) concentration, the release of silver ions into aquatic systems leads to the formation of different aqueous forms of silver ($AgCl_0$, $AgCl^-$, $AgCl^-_2$, $AgCl^-_3$, and $AgCl^{3-}_4$) (Wood et al., 2004). Based on the aqueous forms of Ag, the chloride concentrations were low for AgCl precipitation in the moderate and high salinity used in the current experiment. Hence, it is likely that the formation of the brownish/black sediment observed with the high concentrations of Ag-NPs (10, 32, and 100ppm) in the moderate and high salinity was due to agglomeration/aggregation and then precipitation of the Ag-NPs out of the water column.

It is already well known that dispersed Ag-NPs cause a λmax near 420 nm, which then shifts to longer wavelengths for agglomerated particles. In the low salinity, the λmax of the Ag-NPs remained unchanged (415-420 nm) during the 48-h spectroscopy monitoring. This agrees with previous studies showing that the presence of organic matter in freshwater leads to a stable λmax and the dispersion of Ag-NPs in the water column (Chinnapongse et al., 2011). Plus, the UV–Vis absorbance reductions in the low salinity may have indicated partial solubility of the Ag-NPs into Ag ions (Shaw and Handy, 2011), absorption of the particles into the fish bodies, or binding of the Ag-NPs to secreted mucus from the fish and deposition out of the water column.

The present results showed that the λmax for all the treatments containing Ag-NPs in the moderate and high salinity changed from 420nm towards higher wavelengths, plus all the λmax in the high salinity were longer than those in the moderate salinity (Table 2). These results are also consistent with previous studies showing that due to the presence of electrolytes in the saline water and a high ionic strength, the Ag-NP hydrodynamic diameters increased with a reduction in the electrical double layer and zeta potential. Therefore, in water containing 20 mmol/l NaCl the λmax (420nm) increased to red-shift

absorbance wavelengths, indicating the presence of Ag-NP agglomerates in the water column (Jiang et al., 2009; Stebounova et al., 2011).

The reduction of the spectrophotometry absorbance (380-550nm) in the moderate and high salinity during 48h occurred much faster during the initial hours of the experiment (0.5-4h). However, the reduction of the optical absorption was faster in the high salinity than in the moderate salinity. Therefore, these results suggest that the agglomeration of the Ag-NPs and their subsequent deposit occurred during the initial hours of the experiment. The Ag-NPs agglomerated to a larger size in the high salinity than in the low salinity (Table 2), resulting in a quicker deposition of the nanomaterials. All these results agree with previous studies (Chinnapongse et al., 2011; Stebounova et al., 2011; Zook et al., 2011).

4.2. Tissue Ag accumulation

Earlier studies have already shown that the bioavailability of aqueous silver speciation ($AgCl_X$) is directly related to the chloride concentration in water. When reducing the amount of chloride, this decreases the uptake of negatively-charged aqueous forms of silver salts (Web and Wood, 2000; Wood et al., 2004; Ratte, 1999). Thus, in the present study, since the chloride concentrations in the waters were low (1.7-5.2 mg/l), the bioaccumulation of silver in the tissues of the fish were likely mainly due to the uptake of Ag-NPs and released silver ions.

Although the control fish were not exposed to Ag-NPs in the experiments, there were detectable low levels of Ag in the control fish tissues. These results were similar to the Web and Wood study (2000).

The tissues with the highest blood flow in fish are the liver, kidneys, gills, pyloric ceca, intestines, spleen, and red muscles, while the tissues receiving an intermediate blood flow include the white muscles, skin, and gonads (Barron et al., 1987). Therefore, all these organs can be specifically affected by exposure to toxic materials. Due to the central location of the liver in the circulatory system of fish and the absence of a basement membrane in fish livers, xenobiotic exchange between blood and hepatocytes is maximized in fish livers, making the liver an early target for many toxicants via both intestinal and brachial routes (Di Giulio and Hinton, 2008). In the present results, the levels of silver in the liver for the low, moderate,

and high salinity were higher than those in the kidneys, gills, and white muscles for all the groups exposed to Ag-NPs. These results also agree with previous studies that found higher levels of Ag in the liver rather than other organs of rainbow trout exposed to different sizes of Ag-NPs. The aggregated Ag-NPs may be up taken by drinking and/or feeding on aggregated material (Scown et al., 2010).

As a biomarker, the hepatosomatic index offers a significant advantage over the condition factor, since it is directly related to the toxic effects on the liver upon contaminant exposure (Di Giulio and Hinton, 2008). The present results showed that the HSI increased in a concentration-dependent manner after 14 days of exposure to Ag-NPs in all the fish groups in the waters used in the experiments. Thus, Ag-NPs would appear to exacerbate the liver burden, potentially resulting in liver damage, such as lipidosis.

The compounds taken up from the gastrointestinal tract (GIT) are transported first to the liver by the hepatic portal vein (Di Giulio and Hinton, 2008). In the present study, the kidneys exhibited a much lower presence of Ag than the liver, yet this is inconsistent with Scown's study (2009), where the highest accumulation of intravenously injected titanium dioxide was in the kidney tissue of rainbow trout after 21 days, showing 15 times higher than that in the liver. However, due to the acidic environment of the gastrointestinal tract, silver ions can be released from the surface of ingested Ag-NPs and cross the intestinal barrier of fish (Birgit et al., 2009; Clearwater et al., 2002). Therefore, it would appear that Ag-NPs and Ag ions are absorbed by the intestine and rapidly enter the bloodstream, thereby reaching the liver more than the kidneys.

It is already well known that the gills are the primary organ directly in contact with ambient pollutants or NPs. Thus, negatively charged mucus or thiol groups in the organic matter secreted from fish gills can trap a lot of cationic NPs, such as Ag-NPs (Navarro et al., 2008; Shaw and Handy, 2011).

Due to their low blood perfusion rates (very low metabolic demand), the white muscles are less exposed to pollutants (Di Giulio and Hinton, 2008). The current results also showed that the lowest level of Ag accumulation was in the white muscles, agreeing with previous studies of carp and gulf toadfish

(*Opsanus beta*) exposed to titanium dioxide nanoparticles and silver, respectively (Sun et al., 2007; Wood et al., 2004).

4.3. Increasing salinity and Ag bio-uptake

Despite using the same concentrations of Ag-NPs in the moderate and high salinity, the tissue silver levels with the high salinity (except for the muscles in the 3.2 ppm concentrations) were significantly higher than those with the moderate salinity. While this is inconsistent with previous literature on fish exposed to silver and various silver complexes in different salinities (Hogstrand et al., 1996; Webb and Wood, 2000), it is consistent with Wood's (2004) study, where increased tissue silver was related to a higher salinity than the isosmotic point (plasma osmolality) in gulf toad fish.

5. Conclusion

The present study evaluated the effects of increasing salinity on the stability of Ag-NPs (dispersion) and their accumulation in an aquatic organism model at different salinities. According to UV-Vis absorbance and Atomic Absorption Spectroscopy data, most Ag-NPs that enter freshwater ecosystems (low ionic strength) remain suspended in an ionic or nanoscale form and could have negative effects on the biota. However, in a higher salinity, nanoparticles agglomerate and precipitate on the surface of the sediment. Thus, since the entry of Ag-NPs into estuaries represent the greatest threat to benthic organisms, the arrival of Ag-NPs in freshwater, estuarine, and ocean ecosystems could have a devastating effect on the biota. Thus, further studies are needed to explore the potential toxicity mechanisms, such as the hematological, histological, and molecular effects on aquatic organisms in fresh and saline waters.

Acknowledgement

The authors gratefully acknowledge the support of the Tarbiat Modares University of I. R. Iran, who funded this research through a M.Sc. thesis project. This research was also partially supported by the

Green Nano Technology Development Program through the National Research Foundation of Korea (NRF) funded by the Korean Ministry of Education, Science and Technology (No. 2012-006648).

References

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C., Yu, I.J. 2012. Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*. Journal of Nanobiotechnology 10:14. DOI: 10.1186/1477-3155-10-14.

Asharani, P., Lian Wu, Y., Gong, Z., Valiyaveettil, S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19, 255102.

Barron, M., Tarr, B., Hayton, W., 1987. Temperature-dependence of cardiac output and regional blood flow in rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol. 31, 735-744.

Beer, Ch., Foldbjerg, R., Hayashib, Yuya., Sutherland, D.S., Autrup, H., 2012. Toxicity of silver nanoparticles—Nanoparticle or silver ion?.Toxicology Letters. 208, 286–292.

Birgit, G., Teresa, F., Charles, T., Vicki, S., 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. Environ Health Global Access Sci Source. 8 (Suppl 1):S2.

Borak, J., 2009. Nanotoxicology: Characterization, Dosing, and Health Effects. J Occup Environ Med. 51(5):620.

Chinnapongse, S.L., MacCuspie, R.I., Hackley, V.A., 2011. Persistence of singly dispersed silver nanoparticles in natural freshwaters, synthetic seawater, and simulated estuarine waters. Sci Total Environ. 409, 2443 – 2450.

Clearwater, S.J., Farag, A., Meyer, J., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp Biochem Physiol C Toxicol Pharmacol. 132, 269-313.

Di Giulio, R.T., Hinton, D.E., 2008. The toxicology of fishes. CRC.

Farmen, E., Mikkelsen, H., Evensen, Ø., Einset, J., Heier, L., Rosseland, B., Salbu, B., Tollefsen, K., Oughton, D., 2011. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low [mu] g/L concentrations of Ag nanoparticles. Aquat Toxicol. 108, 78-84.

Farré, M., Gajda-Schrantz, K., Kantiani, L., Barceló, D., 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. Anal Bioanal Chem. 393, 81-95.

Hogstrand, C., Galvez, F., Wood, C.M., 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Environ Toxicol Chem**.** 15, 1102-1108.

ISO TS 80004-1. 2010. Nanotechnologies—Vocabulary—Part 1. Core Terms. International Organization for Standardization, Geneva.

Jiang, J., Oberdörster, G., Biswas, P., 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. J Nanopart Res. 11, 77-89.

Johari, S. A., Kalbassi, M. R., Soltani, M., Yu, I. J., 2013. Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*). Iranian Journal of Fisheries Sciences.12(1), 76-95.

Kalbassi, M.R., Salari-Joo, H., Johari, A., 2011. Toxicity of Silver Nanoparticles in Aquatic Ecosystems: Salinity as the Main Cause in Reducing Toxicity. Iran J Toxicol. 5 (1&2), 436-443.

Kjesbu, O., Klungsøyr, J., Kryvi, H., Witthames, P., Walker, M.G., 1991. Fecundity, atresia, and egg size of captive Atlantic cod (*Gadus morhua*) in relation to proximate body composition. Can J Fish Aquat Sci. 48, 2333-2343.

Laban, G., Nies, L.F., Turco, R.F., Bickham, J.W., Sepúlveda, M.S., 2010. The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. Ecotoxicology. 19, 185-195.

Mantovani, E., Porcari, A., Meili, C., Widmer, M., 2009. Framing nano project: A multistakeholder dialogue platform framing the responsible development of nanosciences & nanotechnologies. Mapping Study on Regulation and Governance of Nanotechnologies. AIRI/Nanotec IT, January.

Meyer, J.N., Lord, C.A., Yang, X.Y., Turner, E.A., Badireddy, A.R., Marinakos, S.M., Chilkoti, A., Wiesner, M.R., Auffan, M., 2010. Intracellular uptake and associated toxicity of silver nanoparticles in Caenorhabditis elegans. Aquat Toxicol. 100, 140-150.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L., Behra, R., 2008. Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. Environ Sci Tech. 42, 8959-8964.

Nia, J.R., 2011. Preparation of colloidal nanosilver. Google Patents.

Nichols, J.W., Brown, S., Wood, C.M., Walsh, P.J., Playle, R.C., 2006. Influence of salinity and organic matter on silver accumulation in Gulf toadfish (*Opsanus beta*). Aquat Toxicol. 78, 253-261.

OECD, 1998. OECD guidelines for testing of chemicals. OECD Publishing.

Picado, A., 2010. Aquatic ECOtoxicology of NANOmaterials -from Ecotoxicology to nano (eco)toxicology-what is the matter?. In: Seminar Microscopy of the Nanoworld, BIOEM, Cantanhede, Dezembro 9-10.

Pinto, V.V., Ferreira, M.J., Silva, R., Santos, H.A., Silva, F., Pereira, C.M., 2010. Long time effect on the stability of silver nanoparticles in aqueous medium: Effect of the synthesis and storage conditions. Colloid Surface Physicochem Eng Aspect. 364, 19-25.

Ratte, H.T., 1999. Bioaccumulation and toxicity of silver compounds: a review. Environ Toxicol Chem. 18, 89-108.

Scown, T.M., Santos, E.M., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol Sci. 115, 521.

Shaw, B.J., Handy, R.D., 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. Environ Int. 37(6):1083-97.

Smith, C.J., Shaw, B.J., Handy, R.D., 2007. Toxicity of single walled carbon nanotubes to rainbow trout,(*Oncorhynchus mykiss*): respiratory toxicity, organ pathologies, and other physiological effects. Aquat Toxicol. 82, 94-109.

Stebounova, L.V., Guio, E., Grassian, V.H., 2011. Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. J Nanopart Res. 13:233–244.

Sun, H., Zhang, X., Niu, Q., Chen, Y., Crittenden, J.C., 2007. Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles. Water Air Soil Pollut. 178, 245-254.

Webb, N.A., Wood, C.M., 2000. Bioaccumulation and distribution of silver in four marine teleosts and two marine elasmobranchs: influence of exposure duration, concentration, and salinity. Aquat Toxicol. 49, 111-129.

Wise Sr, J.P., Goodale, B.C., Wise, S.S., Craig, G.A., Pongan, A.F., Walter, R.B., Thompson, W.D., Ng, A.K., 2010. Silver nanospheres are cytotoxic and genotoxic to fish cells. Aquat Toxicol. 97, 34-41.

Wood, C.M., McDonald, M.D., Walker, P., Grosell, M., Barimo, J.F., Playle, R.C., Walsh, P.J., 2004. Bioavailability of silver and its relationship to ionoregulation and silver speciation

438 across a range of salinities in the gulf toadfish (*Opsanus beta*). Aquat Toxicol. 70, 137-
439 157.
440 Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T., Jiang, G., 2010. Effects of silver nanoparticles on the
441 development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using
442 the partial-life test. Aquat Toxicol. 100, 160-167.
443 Zook, J.M., Long, S.E., Cleveland, D., Geronimo, C.L.A., MacCuspie, R.I., 2011. Measuring silver
444 nanoparticle dissolution in complex biological and environmental matrices using UV–
445 visible absorbance. Anal Bioanal Chem. 401(6):1993-2002.
446
447
448
449
450

Figure legends

Fig. 1. Size distribution of silver nanoparticles in stock suspension (100 mg/L) based on Zetasizer data.

Fig. 2. A: TEM morphology of silver nanoparticles and B: EDX spectrometer pattern (Ni signals in EDX spectrometer are from TEM grid).

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

Fig. 4. UV–VIS absorption spectra of AgNP colloid in stock solution.

Fig. 5. Decrease in UV-Vis absorbance of Ag-NPs in low (A), moderate (B) and high (C) salinity during 48 hours (scanned between 380-550nm).

Fig. 6. Silver concentration in white muscles, gills, kidneys, and livers of rainbow trout after 14 days of exposure to different concentrations of Ag-NPs (0.032, 0.1, 0.32, and 1 for low salinity and 3.2, 10, and 32ppm for both moderate and high salinity). (A) Low, (B) moderate, and (C) high salinity. Significant difference from control group (Dunnet's. $P < 0.001$) is denoted by asterisk (*). (a, b…) Significant difference between liver and other organs (muscles, gills, kidneys) within treatments (Tukey, $P < 0.001$). Data are expressed as mean±S.D., n=30 fish/treatment).

Fig. 7. Hepatosomatic index (HSI) at various concentrations of Ag-NPs in 6±0.3 and 12±0.2 ppt waters (0, 3.2, 10, 32ppm) and 0.4 ppt water (0, 0.032, 0.1, 0.32, 1ppm) during 14 days of exposure. Data are expressed as mean ± S.E. n = 10 fish/treatment.

469 **Table 1.** Various chemical properties of experimental waters (low, moderate, and high salinity) used in
470 experiments.

Parameter (mg^{-1})	Water Salinity		
	Low	Moderate	High
Magnesium	41	51	72
Total alkalinity	326	254.5	183
Total ammonium	0.1	0.2	0.5
Chloride	1.7	3.9	5.2
Sodium	13.8	1857.5	3084.8
$CaCO_3$	102	138	176
HCO_3	320	290	231
Total organic carbon	7.6±0.8	8.9±0.4	9.4±0.1

471

471 **Table 2**. UV-Vis absorption peak (λmax) positions for Ag-NPs at different times (during 48h) and water
472 salinities (low, moderate, and high).

Low salinity			Moderate salinity			High salinity		
Ag-NP Concentration (ppm)	Time (hour)	λmax (nm)	Ag-NP Concentration (ppm)	Time (hour)	λmax (nm)	Ag-NP Concentration (ppm)	Time (hour)	λmax (nm)
1	0.5	415	100	0.5	425	100	0.5	440
	1	415		1	425		1	445
	4	415		4	425		4	460
	12	420		12	430		12	465
	24	420		24	435		24	455
	48	420		48	480		48	405
0.32	0.5	415	32	0.5	440	32	0.5	440
	1	415		1	445		1	440
	4	415		4	445		4	445
	12	415		12	465		12	455
	24	420		24	470		24	450
	48	415		48	470		48	435
0.1	0.5	415	10	0.5	435	10	0.5	435
	1	420		1	435		1	435
	4	420		4	440		4	435
	12	415		12	440		12	435
	24	415		24	445		24	435
	48	415		48	450		48	430

	0.5	415		0.5	430		0.5	430
	1	415		1	435		1	430
	4	420		4	435		4	430
0.032	12	415	3.2	12	435	3.2	12	430
	24	415		24	435		24	430
	48	415		48	435		48	430

490

491 **Table 3.** Ag^+ concentration (ppm) detected by Atomic Absorption Spectrophotometer at 1, 4, and 48

492 hours after inoculating Ag-NPs into low, moderate, and high salinities.

Low salinity			Moderate salinity			High salinity		
Ag-NP Concentration (ppm)	Time (hour)	Ag^+ (ppm)	Ag-NP Concentration (ppm)	Time (hour)	Ag^+ (ppm)	Ag-NP Concentration (ppm)	Time (hour)	Ag^+ (ppm)
1	1	0.204	100	1	0.321	100	1	0.735
	4	0.204		4	0.345		4	0.809
	48	0.267		48	0.307		48	0.811
0.32	1	0.161	32	1	0.361	32	1	0.565
	4	0.166		4	0.322		4	0.565
	48	0.165		48	0.322		48	0.649
0.1	1	0.167	10	1	0.321	10	1	0.482
	4	0.167		4	0.401		4	0.607
	48	0.170		48	0.281		48	0.523
0.032	1	0.128	3.2	1	0.361	3.2	1	0.482
	4	0.128		4	0.401		4	0.649
	8	0.131		48	0.403		48	0.565

493

Table 4. Silver concentration (mg/g dry wt.) in tissues of trout exposed to different Ag-NP concentrations (3.2, 10, and 32 ppm) in moderate and high salinity for 14 days.

Organ	Salinity	Ag-NPs (ppm)			
		Control	3.2	10	32
Liver	Moderate	1.98±0.01	47.01±0.78	126.02±2.1	119.08±0.7
	High	2.08±0.06	76.1±1.02*	135.3±0.6*	277±1.4*
Kidneys	Moderate	1.68±0.03	9.63±0.1	12.95±0.16	12.8±0.08
	High	1.73±0.05	12.01±0.01*	30.7±1.7*	13.7±0.14*
Gills	Moderate	0.98±0.02	10.96±0.05	9.4±0.14	15.9±0.5
	High	0.94±0.01	12.03±0.0*	9.98±0.1*	22.4±0.9*
Muscles	Moderate	0.17±0.0	1.53±0.002	1.51±0.003	2.43±0.05
	High	0.18±0.0	1.50±0.01	2.0±0.02*	5.4±0.04*

Data are expressed as mean ± SD. n=10 fish/treatment. (*) Asterisk represents significant difference between moderate and high salinity at equal Ag-NP concentrations in same organs, (independent sample t-test, $p<0.05$).

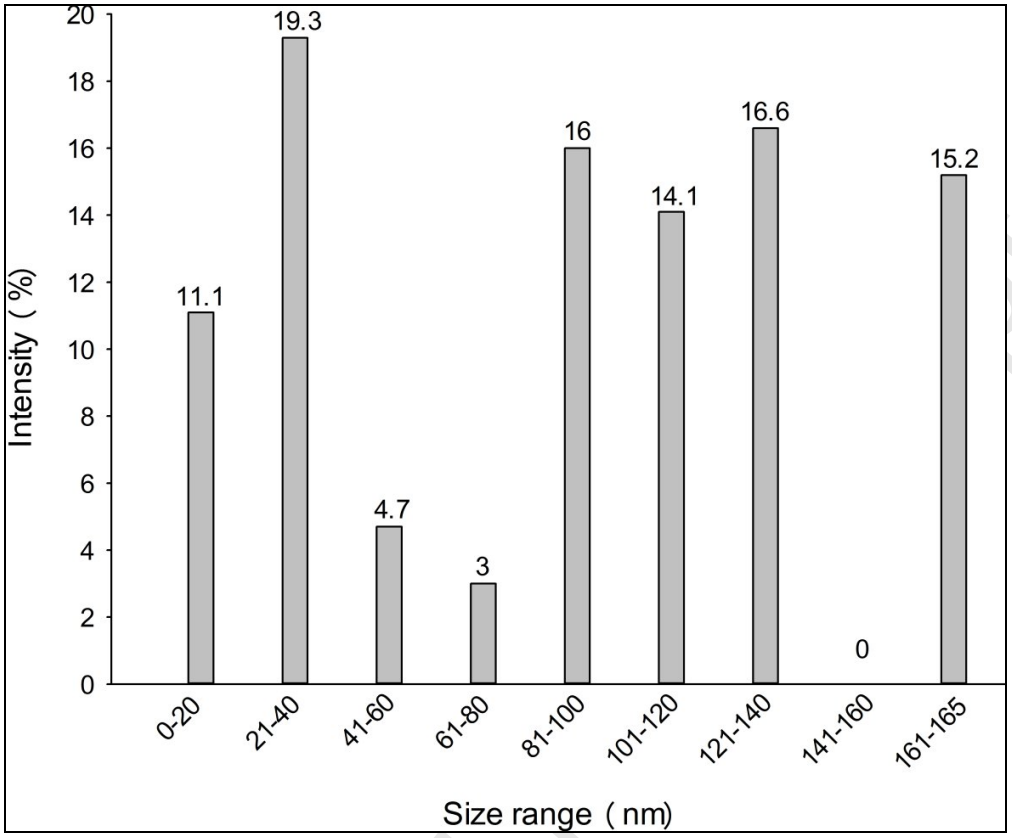
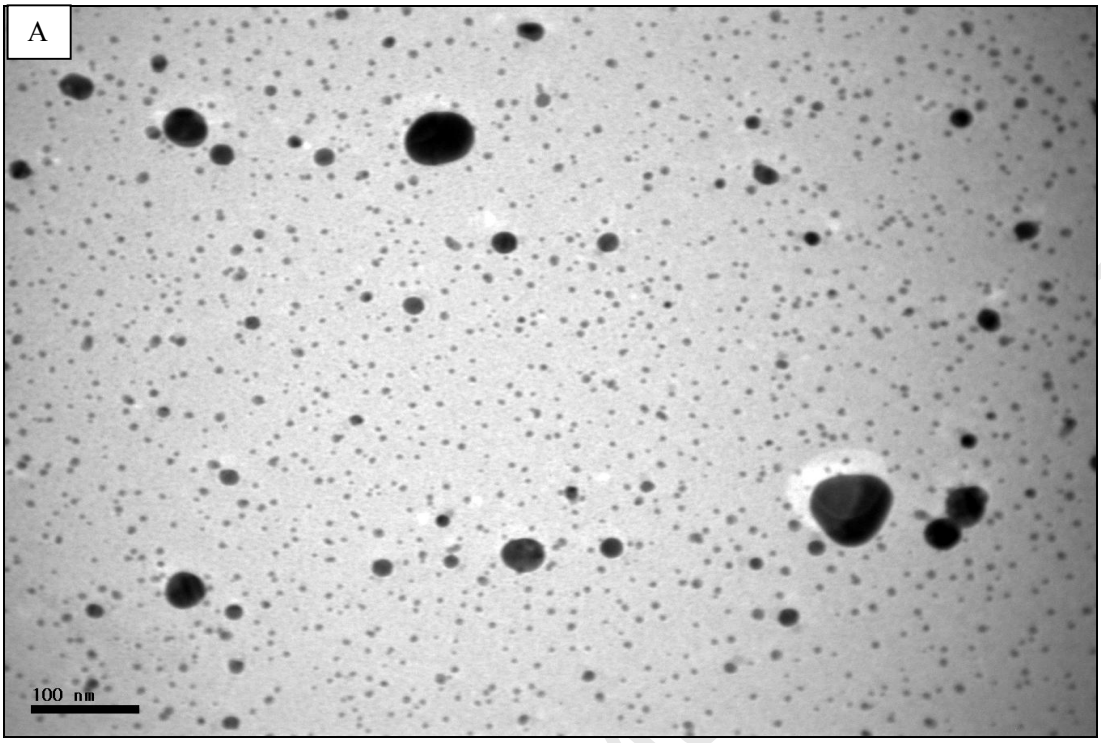


Fig. 1. Size distribution of silver nanoparticles in stock suspension (100ppm) based on Zetasizer data.



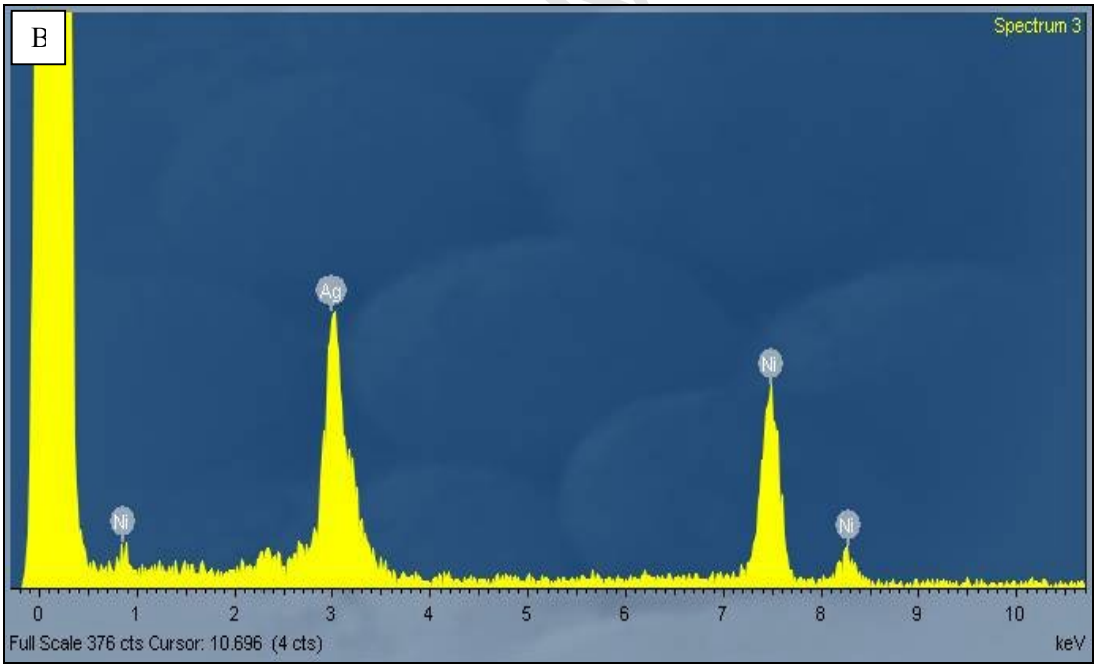
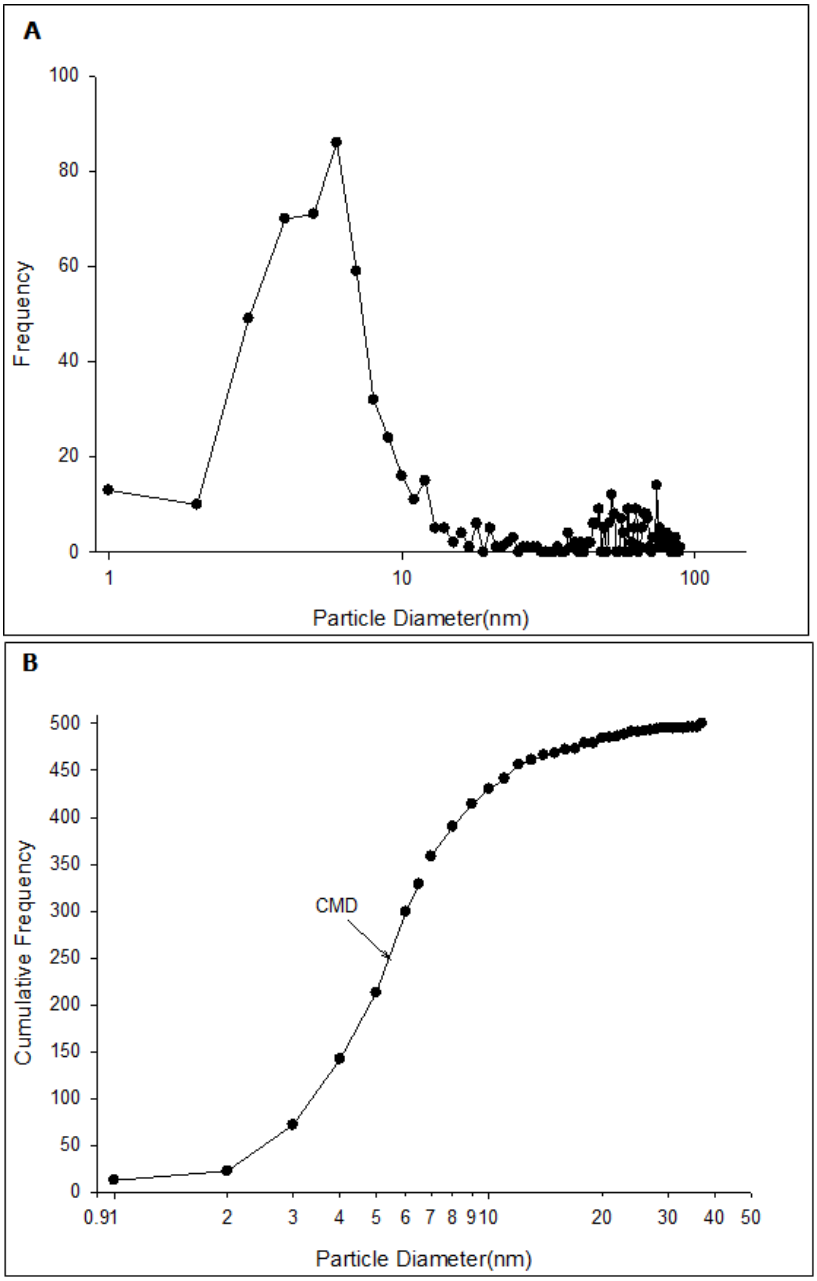


Fig. 2. A: TEM morphology of silver nanoparticles and B: EDX spectrometer pattern (Ni signals in EDX spectrometer are from TEM grid).

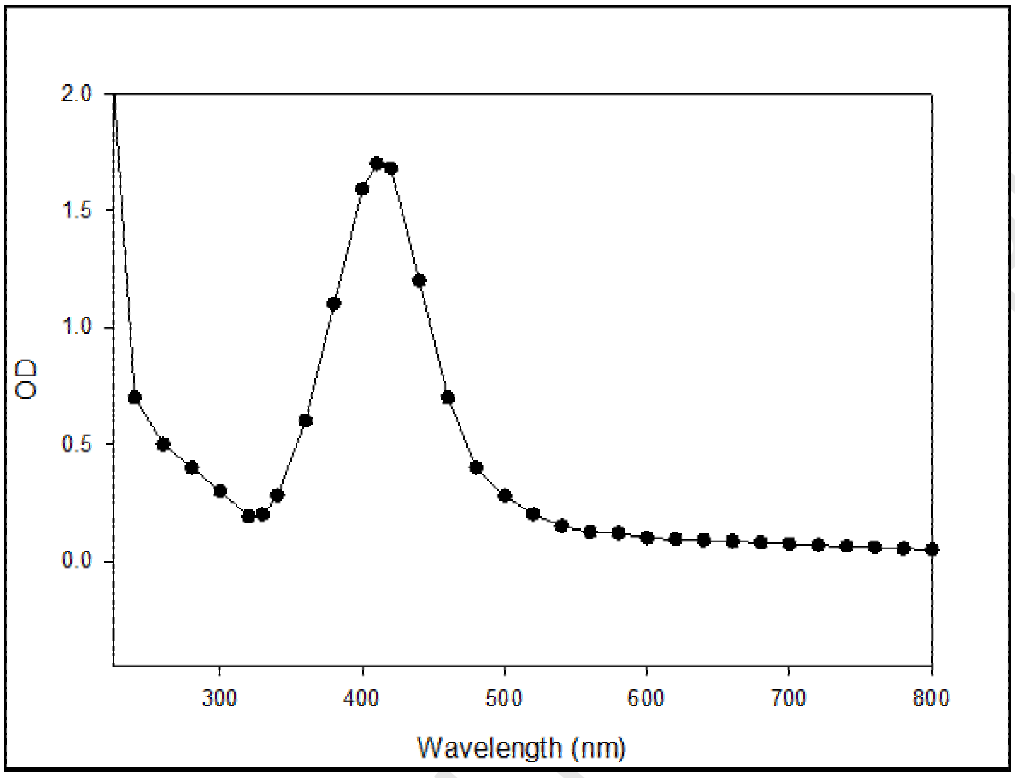


504

505 **Fig. 3.** Size distribution of silver nanoparticles in undiluted suspension (4000ppm) based on transmission
506 electron microscope data. A: Number Frequency and B: Cumulative Frequency (CMD: Cumulative
507 median diameter).

508

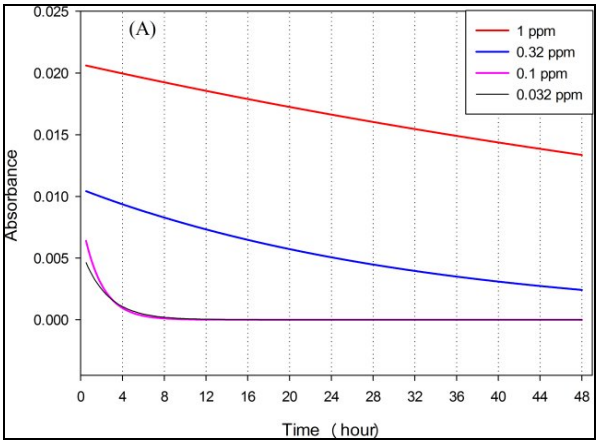
509

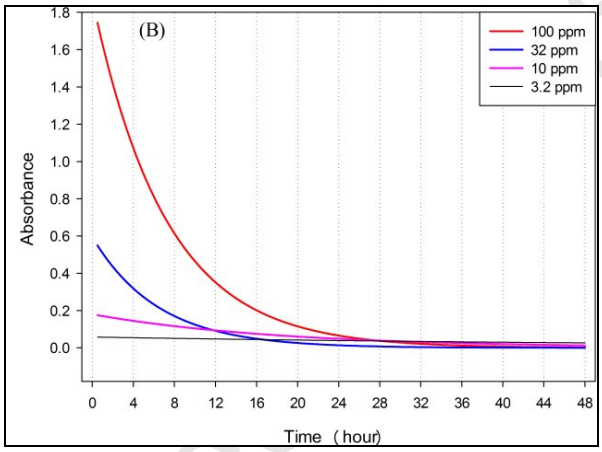


510

511 **Fig. 4.** UV–VIS absorption spectra for AgNP colloid in stock solution.

512





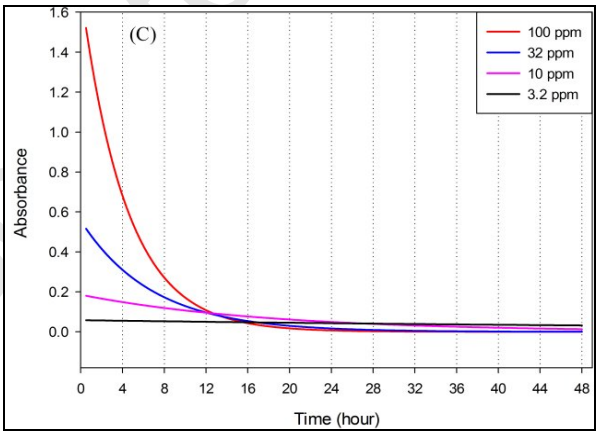
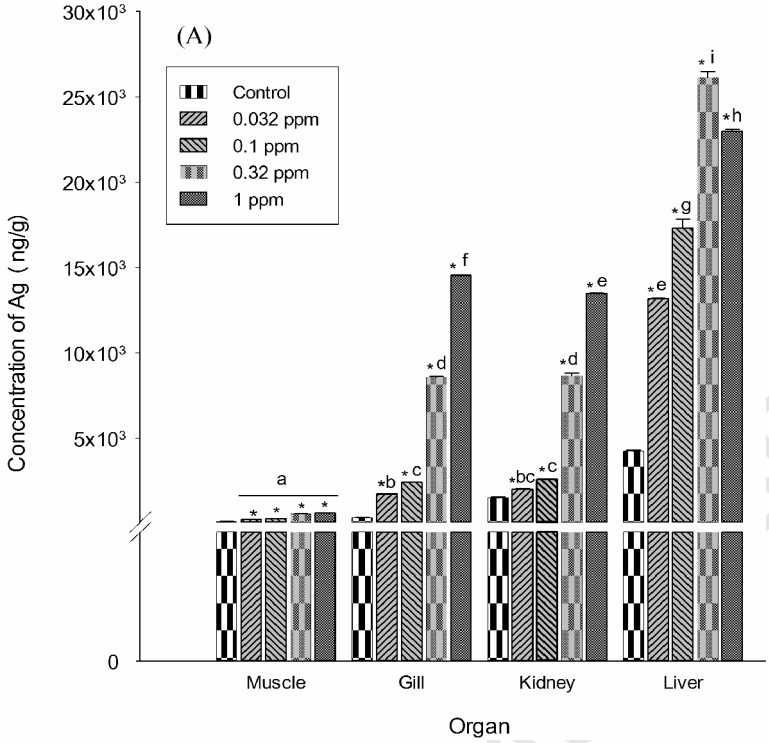
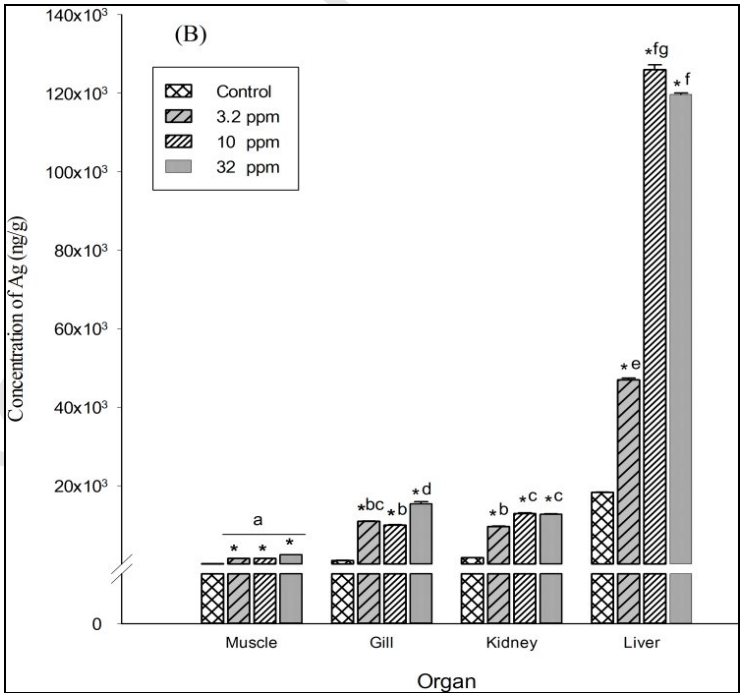


Fig. 5. Decrease in UV-Vis absorbance of Ag-NPs in waters with low (A), moderate (B), and high (C) salinity during 48 hours (absorbance scanned between 380-550nm).



517

518



519

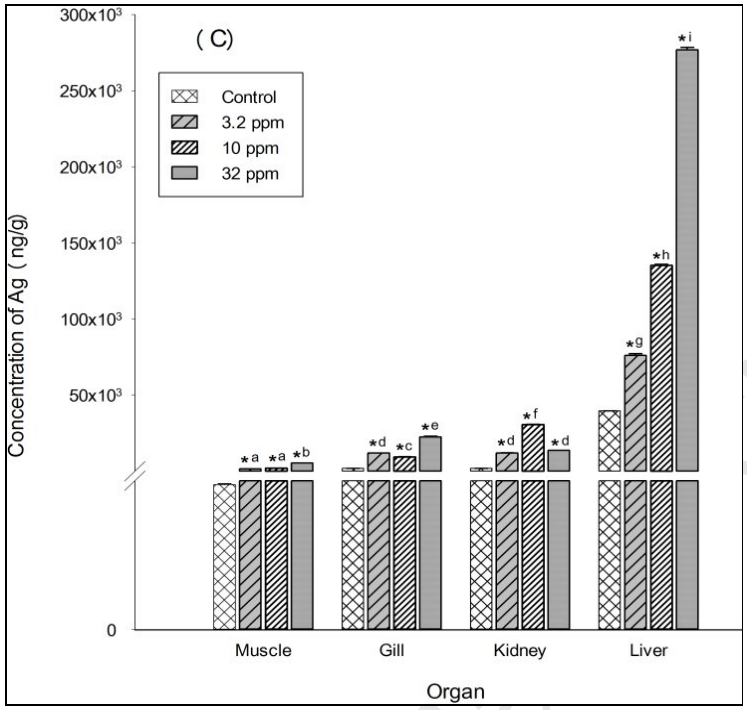
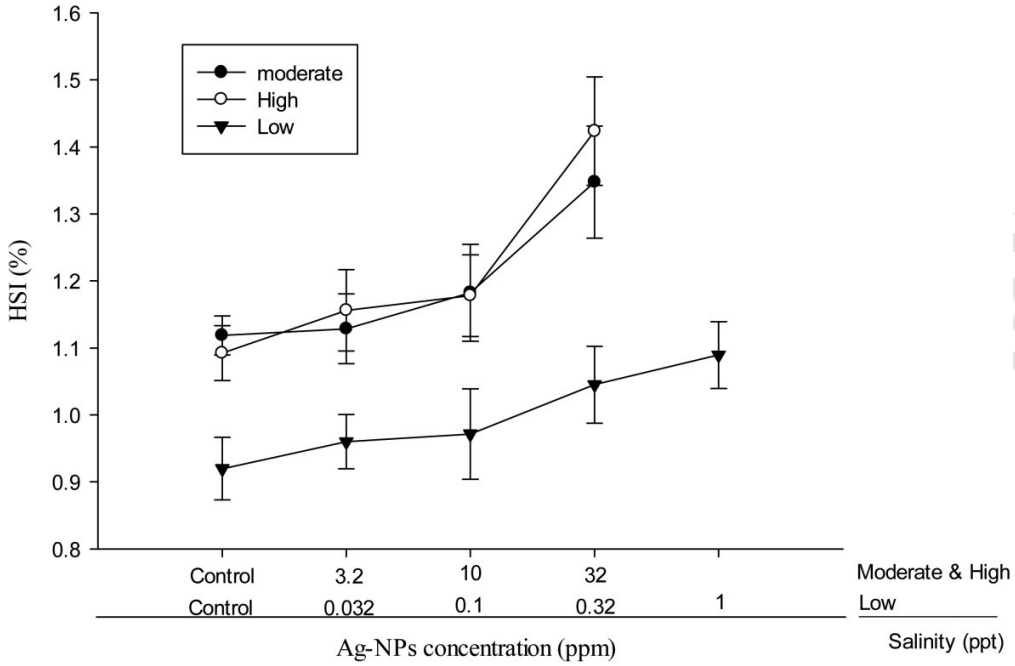


Fig. 6. Silver concentrations in white muscles, gills, kidneys, and liver of rainbow trout after 14 days of exposure to different concentrations of Ag-NPs. Data are expressed as mean ± S.D., n=30 fish/treatment. (A): low salinity with addition of 0.032, 0.1, 0.32, and 1ppm Ag-NPs. (B) and (C): moderate and high salinity, respectively, with addition of 3.2, 10, and 32ppm Ag-NPs. Asterisks show significant difference from control group (Dunnet's. $P<0.001$). Symbols "a" to "i" show significant difference between liver and other organs (muscles, gills, kidneys) within treatments (Tukey, $P<0.001$).



527

528 **Fig.7.** Hepatosomatic index for fish after 14 days of exposure to various concentrations of Ag-NPs (0,
529 0.032, 0.1, 0.32, and 1ppm in low salinity; 0, 3.2, 10, and 32ppm in moderate and high salinity). Data are
530 expressed as mean ± S.E. n = 10 fish/treatment.

531

532

Highlights:

>We studied Influence of concentration and salinity on Bioaccumulation of silver nanoparticles in Rainbow trout (*Oncorhynchus mykiss*)

> The Ag-NPs were characterized using standard methods.

> the organism were exposed to Ag-NPs in three different salinity concentrations, for 14 days in static renewal systems

> The bioaccumulation of Ag in the studied tissues was concentration-dependent in all the salinities and its order were liver > kidneys ≈ gills > white muscles respectively.

In conclusion, the most Ag-NPs that enter into freshwater ecosystems (low ionic strength) have potential to remain suspend and cause a negative effect potential on the biota in an ionic or nanoscale form. However, in a higher salinity, nanoparticles agglomeration and precipitation could be occurred on the surface of the sediments.