Ecological Indicators 76 (2017) 230–239

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Genotoxicity and oxidative stress in fish after a short-term exposure to silver nanoparticles

Carla Bacchetta[a,*], Analía Ale[a], María F. Simoniello[b], Susana Gervasio[c], Carla Davico[d], Andrea S. Rossi[a,d], Martín F. Desimone[e], Gisela Poletta[b], Gerardo López[f,g], José María Monserrat[h,i,j], Jimena Cazenave[a,d]

[a] INALI, UNL, CONICET. Paraje El Pozo, Ciudad Universitaria UNL, 3000, Santa Fe, Argentina
[b] Facultad de Bioquímica y Ciencias Biológicas, UNL. Paraje El Pozo, Ciudad Universitaria UNL, 3000 Santa Fe, Argentina
[c] INTEC, UNL, CONICET. Predio CCT CONICET, 3000, Santa Fe, Argentina
[d] Facultad de Humanidades y Ciencias, UNL, Paraje El Pozo, Ciudad Universitaria UNL, 3000, Santa Fe, Argentina
[e] Universidad de Buenos Aires, CONICET, Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Cátedra de Química Analítica Instrumental, Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina
[f] Nanotek S.A., Argentina
[g] Universidad Tecnológica Nacional, Santa Fe, Argentina
[h] Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande—FURG, Rio Grande, Brazil
[i] Programa de Pós-Graduação em Ciências Fisiológicas e Fisiologia Animal Comparada, Universidade Federal de Rio Grande e FURG, Rio Grande, Brazil
[j] Instituto Nacional de Ciência e Tecnologia Nanomateriais de Carbono, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 13 January 2017
Accepted 18 January 2017

Keywords:
Nanomaterials
Biomarkers
Bioaccumulation
Antioxidant enzymes
Lipid peroxidation
DNA damage

ABSTRACT

This study examined the effects of waterborne silver nanoparticles (AgNPs) on juvenile fish *Piaractus mesopotamicus* ("pacú"), and analyzed toxicological endpoints such as metal burdens, oxidative stress and genotoxicity in a short-term assay. Fish were individually exposed to 0 (control), 2.5, 10, and 25 μg AgNPs/L. After 24 h, silver accumulation was greater in the brain than the liver and gills at all silver concentrations. Fish exposed to higher AgNPs concentrations showed major alterations in oxidative stress markers. An increase in lipid peroxidation (LPO) levels was observed in the liver of fish exposed to 10 μg AgNPs/L with no changes in the antioxidant enzymes activities. In the case of the 25 μg AgNPs/L treatment, a hepatic activation of the enzymatic antioxidant defense occurred, and LPO levels resulted unaltered. On the other hand, the brain presented the highest LPO levels at both 10 and 25 μg AgNPs/L exposures. The AgNPs toxicity was also evidenced by the DNA damage in fish erythrocytes at higher concentrations. Summarizing, a short exposure to sublethal concentrations of AgNPs is enough to generate deleterious effects on fish, including DNA damage.

1. Introduction

There are many consumer products and applications utilizing silver nanoparticles (AgNPs). "The Nanodatabase" is an inventory of commercially available products that claim to contain engineering nanoparticles in the European consumer market. This inventory is updated daily, and currently lists 2362 products of which 353 are registered as silver containing products (The Nanodatabase, 2017). Several AgNPs applications are mainly related to the exceptionally broad spectrum of silver bactericidal activity (Kim et al., 2007; Marambio-Jones and Hoek, 2010). The low cost of manufacturing AgNPs has also made them the largest and fastest growing class of nanomaterials in product applications such as plastics, metals, textiles, and in medical and veterinary devices (Ahamed et al., 2010; Messaoud et al., 2010; Rather et al., 2011; Rhim et al., 2013). Moreover, the nanoparticle-based vaccines and the use of nanoparticles as tools for diagnosing bacterial, fungal and viral diseases, is an emerging field in fish research (Shaalan et al., 2016). However, the potential environmental impact of AgNPs has not been fully understood yet (Massarsky et al., 2014a), and their toxic properties deserve further analysis.

AgNPs may be discharged to the environment by several routes: manufacturing, incorporation into goods, and goods recycling or waste (Fabrega et al., 2011). Recent studies have reported that AgNPs may be released from biocidal plastics, textiles, paints and

* Corresponding author at: Instituto Nacional de Limnología, INALI, UNL, CONICET, Paraje El Pozo, Ciudad Universitaria UNL, 3000, Santa Fe, Argentina.
E-mail address: carlabacchetta@yahoo.com.ar (C. Bacchetta).

http://dx.doi.org/10.1016/j.ecolind.2017.01.018
1470-160X/

other home products (Benn et al., 2010; Lorenz et al., 2012; Voelker et al., 2015), reaching the aquatic environment. Such AgNPs input occurs mainly by their releasing in wastewaters which reaches sewage treated plants (Kaegi et al., 2011). According to Blaser et al. (2008) silver residues from Europe, Asia and North America reached 190–410 t/year and between 11.5–31.7% of those residues had been treated in wastewater plants and were found in receiving natural water. Although most AgNPs underwent a wastewater treatment process, a significant proportion (about 10%) was released in the effluent (Gottschalk et al., 2009). Environmental concentrations of AgNPs in surface water are still unknown but according to the estimates the concentrations may range 40–320 ng/L (Blaser et al., 2008) and 0.09-2.63 ng/L (Gottschalk et al., 2009). In a recent study, Bruneau et al. (2016) investigated the fate, bioavailability of AgNPs and their effects on fish using municipal effluents. This study consisted of juvenile rainbow trout exposure to 40 μg AgNPs/L or 4 μg $AgNO_3$/L in diluted (10%) municipal wastewater for 96 h, yielding that both forms of silver produced immunotoxic effects and AgNPs in wastewater were bioavailable to fish despite of their formation of aggregates.

The AgNPs toxicity is closely related to their transformation in biological and environmental media, including surface oxidation, silver ion release, and interaction with biological macromolecules (McShan et al., 2014). AgNPs can interact with membrane proteins and activate signaling pathways, leading to inhibition of cell proliferation (Braydich-Stolle et al., 2010; Roh et al., 2012). These nanoparticles can also enter the cell through diffusion or endocytosis causing a mitochondrial dysfunction (Bressan et al., 2013) and generating reactive oxygen species (ROS) inside the cell. Previous studies demonstrate that oxidative stress could be involved in the toxicity of AgNPs. Some authors have correlate ROS accumulation with the activation of antioxidant enzymes and the depletion of glutathione content in vitro in rat liver cells (Hussain et al., 2005), mouse germline stem cells (Braydich-Stolle et al., 2005), human cells (Piao et al., 2011; Rosarin et al., 2012), and fish cells (Massarsky et al., 2014b; Taju et al., 2014). In addition, Asharani et al. (2009) have suggested that the disruption of the mitochondrial respiratory chain by AgNPs increases ROS production and interrupts ATP synthesis, leading to DNA damage. These AgNPs deleterious effects on DNA have already been demonstrated *in vitro* with human and other mammalian cells (Ahamed et al., 2008; Singh et al., 2010). Some studies have investigated *in vivo* the effects of AgNPs in fish. Results indicate that these nanoparticles are accumulated in the gills and liver tissue affecting the ability of fish to cope with low oxygen levels and inducing oxidative stress (Bilberg et al., 2010; Scown et al., 2010). Massarsky et al. (2013) reported decreased glutathione content (GSH) in zebrafish embryos exposed to AgNPs. Such depletion has been observed by other authors along with an increase in lipid peroxidation levels in embryos and adults medaka (Wu and Zhou, 2013a,b).

The alkaline (pH 13) version of the comet assay (Singh et al., 1988) is commonly used as it is highly sensitive and detects a broad spectrum of DNA lesions. Such assay has made it possible to evaluate DNA alterations induced by xenobiotics and has been successfully applied in fish erythrocytes exposed to different genotoxic agents (Ali et al., 2008; Cavalcante et al., 2008; Frenzilli et al., 2009; Vanzella et al., 2007). One advantage of this technique lies in the fact that it can be applied regardless of both the chromosomes size and number, and the mitotic activity. The latter is particularly important in fish because the metabolic rate fluctuates considerably with temperature making it difficult to isolate mitotically active tissue (Simoniello et al., 2009). The use of enzymes increases both the sensitivity of the assay (in terms of the ability to detect a wider range of damage overall), and more importantly its specificity. Knowledge concerning mechanisms of uptake and toxic effects of nanomaterials including AgNPs in waterborne exposure scenarios (Stone et al., 2010) is still scarce. Chae et al. (2009) and Farmen et al. (2012) carried out short-term assays in which fish were exposed *in vivo* to AgNPs (between 20 and 100 μg/L) and the gene expression related to stress biomarkers in their liver and gills was analyzed, reporting changes in heat shock protein 70, metallothionein A, Na/K ATPase, glutathione S-transferase, cytochrome P450 1A, and transferrin genes. Most studies about genotoxic effects of AgNPs are restricted to *in vitro* ones in fish cells (Munari et al., 2014; Taju et al., 2014; Wise et al., 2010). *Piaractus mesopotamicus* ("pacú") is one of the most important species for Argentinian fish farming due to its fast growth rate, easy adaptation to artificial feeding and high consumer appreciation. Besides, this is a neotropical species widely distributed in South America which have been selected due to its favorable experimental properties (it can be obtained from local fish farms and has an easy adaptation to laboratory conditions), and their sensitiveness to pollutants exposure (Bacchetta et al., 2014; de Moraes et al., 2015; Sampaio et al., 2012).

Thus, this study aimed to analyze several toxicological endpoints such as metal burdens, oxidative stress and genotoxicity in *P. mesopotamicus* exposed *in vivo* to waterborne AgNPs in a short-term assay. We hypothesize that AgNPs enter and are bioaccumulated in fish organs after 24 h of waterborne exposure. The presence of AgNPs leads to an activation of the antioxidant defense system and the occurrence of lipid and DNA damage if antioxidant enzymes were unable to overcome oxidative stress.

2. Materials and methods

2.1. AgNPs suspension, preparation and characterization

A colloidal suspension of 1% w/v AgNPs was provided by Nanotek S.A., which manufactures the product under the brand name nanArgen®. According to the Material Safety Data Sheet (MSDS) of nanArgen®, the main ingredient of the product (> 99.9%) is metallic silver (CAS Number 7440-22-4), with an average particle size of 50 nm. To synthesize the nano-sized silver colloid, silver nitrate was dissolved in Milli pore water to a concentration of 0.20 M and mixed with an aqueous solution of 0.1 M polyvinyl pyrrolidone (PVP) as the stabilizing agent. Next soluble nanocrystalline cellulose in a 0.02 M solution was added as a reducing agent. The reaction mixture was then placed in a pressurized reactor and held at 130 °C for 30 min. All reagents and solvents were used without any further purification. To evaluate particles size and surface charge of AgNPs suspension, dynamic light scattering (DLS) and zeta potential measurements were carried out using a Zeta-Sizer Malvern (Model Nano-ZS). Transmission electron microscopy (TEM) was used to visualize and confirm the DLS results; TEM was performed in a JEOL JEM 1010 equipment. Energy dispersive spectroscopy (EDS) was employed to chemical characterization of the nanoparticulate silver suspension. Preparation of colloidal AgNPs samples for EDS included initial filtration, dilution 1:100, centrifugation and redispersion of 50 ml aliquots in 100 ml of pure water. The EDS analysis of nanArgen® stabilized on a calcium carbonate crystal was performed as follows: no peaks were omitted in the spectrum and all elements were analyzed (normalised).

In parallel, the release of Ag+ ions from AgNPs was evaluated. For this purpose, the AgNPs suspension was filtered at different times using Vivaspin™ ultrafiltration devises (30 kDa MWCO, Sartorius Stedim Biotech Gmbh) and the filtrate analyzed by Atomic Absorption Spectrometry in a VGP 210 atomic absorption spectrometer (BuckScientific, East Norwalk, CT, USA) by the electrothermal atomization method using pyrolytic graphite tubes.

2.2. Fish and exposure conditions

Juvenile *P. mesopotamicus* (n = 80; 7.3 ± 1.5 cm standard length; 15.1 ± 10.3 g) were obtained from a local fish farm. For acclimation purpose, fish were held in 150-L tanks containing well aerated dechlorinated water for two weeks, and fed once daily with dry commercial pellets. Fish feeding was halted 24 h before the beginning of the test. All experiments were conducted in accordance with the Ethical Framework for Biomedical Research in laboratory animals, livestock and obtained from nature (CONICET, 2005) for the protection of animal welfare.

Test was conducted in 10-L glass aquaria under static conditions, and 12:12 h light–dark cycles. The test water conditions were: dechlorinated tap water, pH 7.0 ± 0.2, total hardness 49 ± 0.1 ppm CO_3Ca, and temperature 25 ± 1 °C. Fish (*n* = 20 per treatment) were individually exposed to 0 (control), 2.5, 10, and 25 μg AgNPs/L, during 24 h. Nominal concentrations of AgNPs stand for the silver content as Ag° and do not include the capping agent. Prior to blood sampling and dissection, fish were anaesthetized in benzocaine 100 mg/L as described by Parma de Croux (1990). Body weight (g) and both total and standard length (cm) were recorded for each individual. Fish were euthanized by cervical transection (AVMA, 2013) and blood was collected immediately from the caudal vein (Reichenbach-Klinke, 1980). Before freezing, the wet weight of the liver was determined. Whole blood was used for the comet assay, using in the analysis the erythrocytes, the most abundant cell line. The entire brain, gills, and liver were ground with liquid nitrogen immediately after dissection, and tissues were stored at −80 °C until biochemical measurements were determined.

2.3. Exposure media and tissue silver content

Water samples preparation for measurements of total recoverable silver were carried out according to the method 200.2 proposed by the United States Environmental Protection Agency (US EPA, 1994) with some modifications. Water samples were taken from each aquarium at the beginning (without fish) and at the end of the exposure time (after 24 h). Briefly, a 100 ml aliquot from each sample was transferred to a Griffin beaker, and 2 ml of (1 + 1) nitric acid and 1 ml of (1 + 1) hydrochloric acid were added. The volume of the samples aliquots was reduced to 20 ml by heating evaporation, and heated at 85 °C for 30 min, covering the beakers lips. Digested samples were evaporated to dryness to remove the excess of HCl, and then diluted with 0.1 N HNO_3. A cyanogens iodide solution (matrix modified) was added, mixed and let to stand for 30 min. The cyanogens iodide solution was prepared by dissolving potassium cyanide in iodine solution, and stabilized with sodium/ammonium hydroxide solution (method D 3866-02; ASTM, 2002). Tissue sample preparation was carried out according to the method 200.3 proposed by the US EPA (1991), with some modifications. Samples of brain, gills, and liver were digested by adding concentrated nitric acid, heating to 95 °C, and letting to cool. This process was repeated until all tissues were in the solution. Then, 30% hydrogen peroxide was dosed and finally concentrated hydrochloric acid was added. Similarly to water samples, digested tissues were evaporated to dryness to remove HCl excess; the samples were then diluted with 0.1 N HNO_3, and the cyanogens iodide solution was added. In accordance with the method 200.9 (US EPA, 1994), silver was then quantified in water and tissue samples using a graphite furnace atomic absorption spectrophotometer (GF AAS, PerkinElmer AAnalyst 800) equipped with an auto sampler. For this purpose, one aliquot was injected into the GF AAS, and three readings of each run were recorded. The reading mean was used to calculate the amount of silver in the aliquot of digested water and fish tissue.

2.4. Biomarkers

Tissue extracts for antioxidant enzymes measurements were obtained from brain, gills, and liver by homogenizing the tissues using 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE) according to Bacchetta et al. (2014). The activity of glutathione *S*-transferase (GST, EC 2.5.1.18) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al., 1974 Glutathione reductase activity (GR, EC 1.6.4.2) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase activity (CAT, EC 1.11.1.6) was determined according to Beutler (1982). Brain, gills, and liver lipid peroxidation (LPO) levels were determined by measuring the formation of thiobarbituric reactive substances (TBARS), according to Fatima et al. (2000). The rate LPO was expressed as nanomoles of TBARS formed per hour, per milligram of proteins (nmol TBARS/mg prot). The enzymatic activity and LPO was calculated in terms of the sample protein content using albumin from bovine serum (Sigma-Aldrich) as standard (Bradford, 1976).

The use of genotoxicity assays is of interest because they are more sensitive than cytotoxicity assays (Amaeze et al., 2015). The alkaline comet assay (pH >13) was then performed according to the method described by Singh et al. (1988), with the following modifications (Simoniello et al., 2009): blood samples were diluted 1:19 (v/v) with RPMI-1640 medium and used immediately. Then, 3 μl of each diluted blood sample (2.5×10^3 erythrocytes, approximately) was added to 200 μl of 1% low melting point agarose (LMA, Sigma) and two slides were prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly-prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and DMSO 10%; pH 10) and left at 4 °C overnight. After lysis slides were washed and excess liquid dabbed off with tissue. For each fish included in this experimental, two slides were prepared, 50 μl of Endo III enzyme solution or buffer alone as control was placed on the gel and covered with a cover slip. The Endo III enzyme was used at a final dilution of 1/3000. This dilution factor was previously established via titration experiments. All slides were incubated at 37 °C for 30 min. At the end of the incubation period, coverslips were removed and slides were placed in electrophoresis tank to continue with the comet assay (Poletta et al., 2016). The slides were then immersed in freshly-prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13), first for unwinding (10 min) and then for electrophoresis (0.7–1 V cm-1, 300 mAmp, 10 min at 4 °C). All of the steps were carried out under conditions of minimal illumination and low temperature (on ice). Once electrophoresis was completed, the slides were neutralized and dehydrated with ethanol. Slides were stained with acridine orange at the moment of analysis and one hundred randomly selected comets from each animal were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score, and were expressed in arbitrary units ($AU = n_1 + 2\,n_2 + 3\,n_3 + 4\,n_4$, where n_1, n_2, n_3 and n_4 are the number of cells in each class of damage, respectively). Control slide (with buffer alone) was incubated in parallel with the enzyme slide (with Endo III), and measure oxidative DNA damage was calculated by subtract the mean Comet score of the control (AU Comet) from the mean score of the enzyme slide (AU Endo). Net enzyme-sensitive sites are then the measure of the oxidised bases concerned (Collins, 2009). To the cytotoxicity assay, the same cell suspension used in the comet assay was mixed with fluorescent DNA-binding dyes (100 μg ml^{-1} acridine orange and 100 μg ml^{-1}ethidium bromide prepared in Ca^{2+} and Mg^{2+}free PBS) and examined by fluorescent

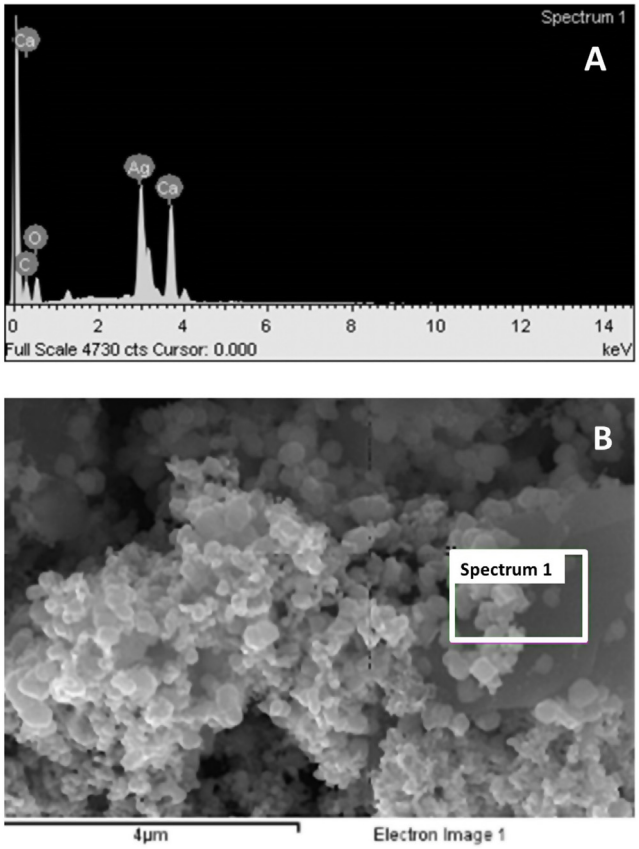

Fig. 1. (A) EDS spectrum for nanArgen® on a $CaCO_3$ crystal; (B) Area for EDS characterization of nanArgen®.

microscopy (400 x objective) to visualize and count cells with aberrant chromatin organization. A volume of 4 µl of this mixture was added to 100 µl of cell suspension. A minimum of 200 total cells was counted, recording the number of each of the following cellular states: viable cells and nonviable cells. The percentages of each of these cellular states in relation to the total cells were obtained (Mercille and Massie, 1994).

2.5. Statistical analysis

All data are reported as mean ± standard error. Shapiro-Wilks test was applied to evaluate normality while Levene test was used to test the homogeneity of variance. For statistical comparisons of data among treatments, one way analysis of variance (ANOVA) followed by a Multiple Comparison Test (Tukey) were performed. Kruskal-Wallis test was applied to those variables with non-normal distribution or variance heterogeneity. Correlation was estimated using the Pearson and Spearman rank methods (for parametric and non-parametric variables, respectively). *P*-values below 0.05 were regarded as significant. All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2015).

3. Results

3.1. Particle characterization

EDS results are shown in Fig. 1A and quantified in Table 1. The spectrum was obtained from the area depicted in Fig. 1B. Zeta potential for nanArgen® suspension resulted in a value of −12 ± 4 mV. This suggests a surface negative charge, probably due to remnants of glucose oligomers from synthesis. Particles size estimated from DLS resulted on a hydrodynamic radius of 57 ± 4 nm. This is in good agreement with TEM analysis (Fig. 2), in which the semispherical geometry and polydispersion of sizes around 50 nm can also be assessed.

Table 1
Energy dispersive spectroscopy (EDS) of nanArgen®.

Element	Weight (%)	Atomic (%)
C K	6.63	15.69
O K	33.47	59.44
Ca K	20.39	14.46
Ag L	39.51	10.41
Totals	100.00	

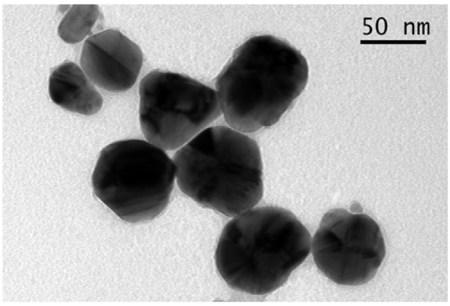

Fig. 2. TEM image of a concentrated colloidal suspension of nanArgen®.

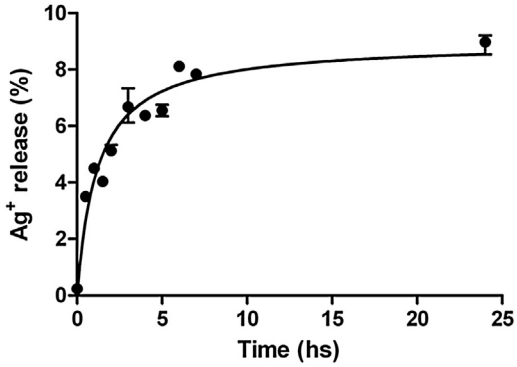

Fig. 3. Percentage of Ag+ ion release from AgNPs suspension after 24 hs. The values are expressed as means ± SD at least triplicate experiments.

As measured by Atomic Absorption Spectrometry, Ag+ ion release was determined as 3.5% of the total Ag in the AgNP suspensions at 30 min and increased to 9% after 24 h (Fig. 3).

3.2. Exposure media and tissue silver content

No mortality-related treatment was observed during the exposure period. Measured AgNPs in test water were 40–60% of nominal concentrations (Table 2). Total silver in water decreased between 0.02 and 15% after 24 h of exposure. The GF AAS data indicated that silver accumulated in all analyzed tissues (Fig. 4).

3.3. Biomarkers

Antioxidant enzyme activities are summarized in Table 3. Different concentrations of AgNPs caused significant changes in the hepatic GPx activity, with a reduction in fish exposed to 2.5 or 10 µg AgNPs/L, and an increase at the highest concentration ($p < 0.01$). In the group exposed to 25 µg AgNPs/L, a rise in GST and a decrease in GR activities were also observed. Antioxidant enzyme activities in gills and brain of AgNPs-exposed fish were not statistically different from the control group ($p > 0.05$). Analysis of TBARS showed no

Table 2
Concentration of total silver measured in test water by graphite furnace atomic absorption spectrophotometer. The values are expressed as means ± SE.

Nominal concentration ($\mu g\,L^{-1}$)	Measured concentration ($\mu g\,L^{-1}$)	Percentage of nominal concentration (%)	24h- Measured concentration ($\mu g\,L^{-1}$)	Depletion after 24h (%)
Control	0.05 ± 0.01	–	0.003 ± 0.001	–
2.5	1.15 ± 0.10	46	1.97 ± 0.07	–
10	5.14 ± 0.23	51	5.13 ± 0.88	0.02
25	13.84 ± 0.43	53	11.72 ± 1.59	15.3

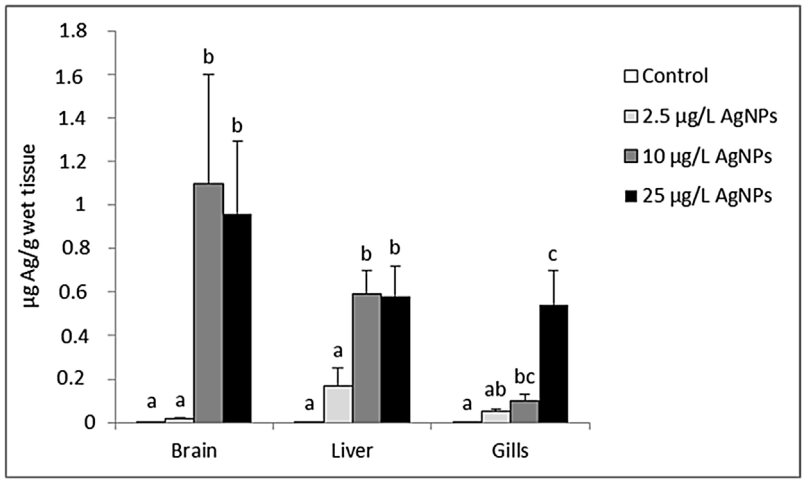

Fig. 4. Silver concentration in different tissues of *Piaractus mesopotamicus* after exposure to 2.5, 10, and 25 µg/L AgNPs, for 24 h. The values are expressed as means ± SE. Means not sharing the same letter (a, b, or c) are significantly different at $p<0.05$.

Table 3
Activity of glutathione *S*-transferase (GST), glutathione reductasa (GR), glutathione peroxidase (GPx) and catalase (CAT) in different tissues of *Piaractus mesopotamicus* after exposure to 2.5, 10, and 25 µg AgNPs/L, for 24 h. The values are expressed as means ± SE. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$.

Enzyme[a]	Control	2.5 $\mu g\,L^{-1}$	10 $\mu g\,L^{-1}$	25 $\mu g\,L^{-1}$
Brain				
GST	310.38 ± 23.72	265.08 ± 15.55	265.82 ± 16.96	279.58 ± 27.32
GR	100.44 ± 13.64	80.85 ± 9.90	81.57 ± 13.59	84.34 ± 13.94
GPx	715.58 ± 152.33	523.82 ± 79.72	485.64 ± 40.66	488.22 ± 64.80
CAT	13.77 ± 1.26	10.59 ± 2.36	10.00 ± 1.19	12.41 ± 1.67
Liver				
GST	762.20 ± 23.24 [a]	690.69 ± 99.88 [a]	693.63 ± 104.75 [a]	1044.44 ± 43.21 [b]
GR	57.11 ± 5.73 [b]	65.02 ± 12.43 [b]	63.93 ± 5.73 [b]	33.61 ± 2.51 [a]
GPx	4.05 ± 0.35 [b]	2.84 ± 0.38 [a]	2.92 ± 0.46 [a]	5.28 ± 0.18 [c]
CAT	114.25 ± 10.73	90.67 ± 16.42	122.42 ± 21.91	109.36 ± 8.26
Gills				
GST	221.37 ± 23.71	198.09 ± 5.13	224.85 ± 47.44	152.60 ± 26.60
GR	79.09 ± 4.96	63.81 ± 10.31	70.91 ± 21.54	55.49 ± 11.53
GPx	0.49 ± 0.03	0.44 ± 0.01	0.68 ± 0.21	0.41 ± 0.07
CAT	12.82 ± 1.43	9.77 ± 0.37	17.33 ± 5.54	9.63 ± 1.78

[a] Activity expressed in mU mg prot^{-1} (GST, GR and GPx) or U mgprot^{-1} (CAT).

evidence of LPO in liver and gills from any of the treatments, except for the liver of fish exposed to 10 µg AgNPs/L, which showed significantly higher levels than the control group ($p<0.05$) (Fig. 5). In addition, no significant correlations between silver accumulation and TBARS levels were found neither in liver nor gills ($p>0.005$). On the contrary, brain TBARS levels progressively increased in AgNPs-exposed fish, exhibiting a dose-dependent response and a significant positive correlation with total silver concentration ($p = 0.0014$). Such increases resulted statistically significant in the 10 and 25 µg AgNPs/L treatments ($p < 0.01$).

Cell viability in blood samples was determined previously to the comet assay as recommended by Singh (2000) and it was greater than 95% in all treatments, except for those from fish exposed to 25 µg AgNPs/L (90%). Significant increases were found in both the Comet and Endo in fish exposed to 10 µg/L AgNPs ($p < 0.01$; Fig. 6A) and in those exposed to 10 and 25 µg/L AgNPs in Endo sites ($p < 0.05$; Fig. 6B).

4. Discussion

All the actual measurements of silver concentration in the exposure media were approximately 50% lower than the nominal concentrations, which are in the range of recovery values described by other authors. For instance, Scown et al. (2010) and Farmen et al. (2012) reported silver recovery percentages of 35 and 55%, respectively, in assays with 10–35 nm size at nominal concentration of 100 µg AgNPs/L. These data suggest that particle aggregation and sedimentation in the water column would result in a lower AgNPs

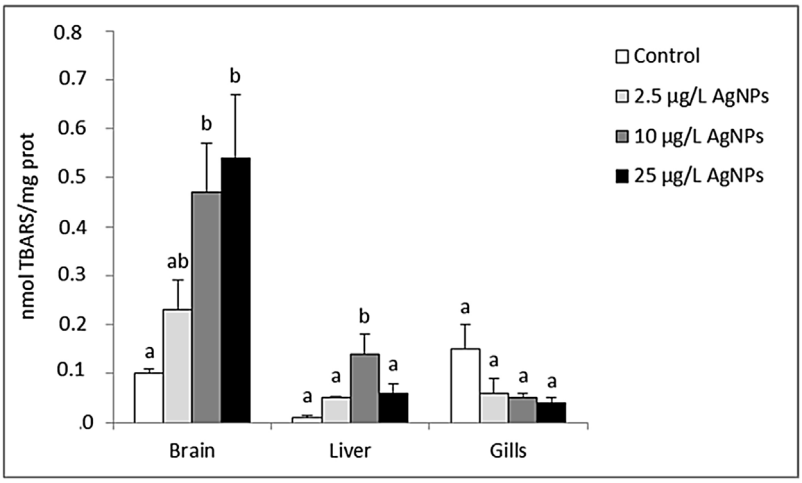

Fig. 5. TBARS levels in different tissues of *Piaractus mesopotamicus* after exposure to 2.5, 10, and 25 µg AgNPs/L, for 24 h. The values are expressed as means ± SE. Means not sharing the same letter (a or b) are significantly different at $p<0.05$.

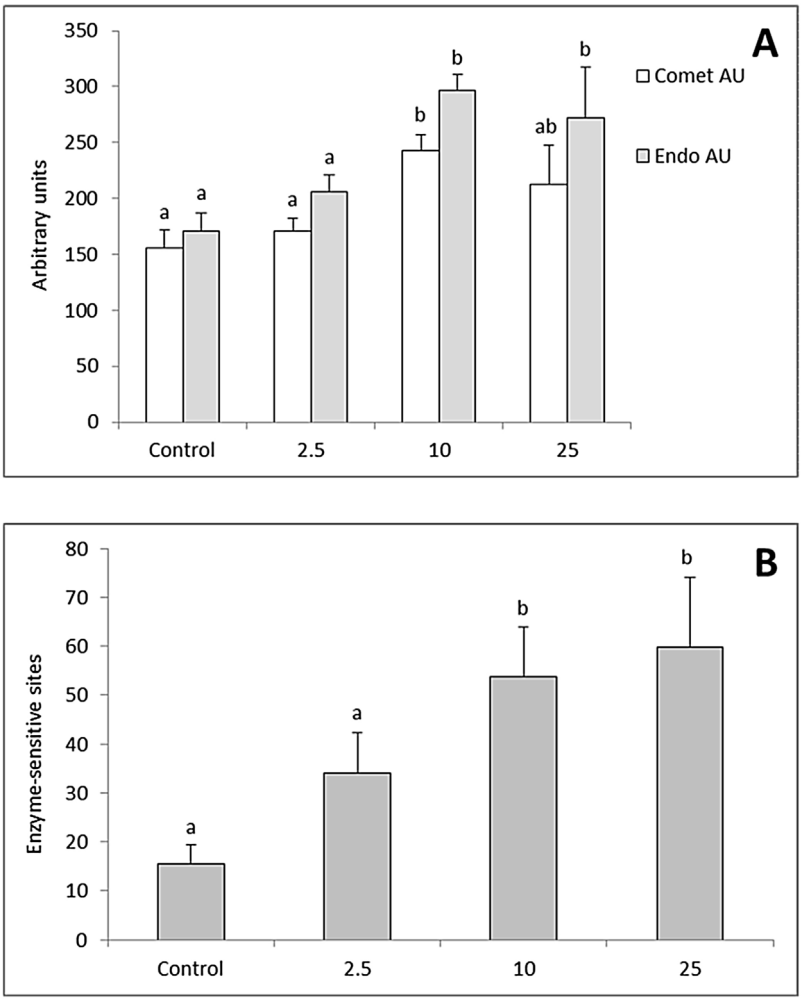

Fig. 6. (A) Single DNA damage score expressed in arbitrary units measured by the comet assay and modified comet assay with Endonuclease III in erythrocytes of *Piaractus mesopotamicus* after exposure to 2.5, 10, and 25 µg AgNPs/L, for 24 h. (B) Oxidative DNA damage measured by Endo sites in erythrocytes of *Piaractus mesopotamicus* after exposure to 2.5, 10, and 25 µg AgNPs/L, for 24 h. The values are expressed as means ± SE. Means not sharing the same letter (a or b) are significantly different at $p < 0.05$.

concentration, therefore the bioavailability and the potential bioaccumulation of AgNPs in fish might also change. It is worth noting that the AgNPs concentrations used here are similar to those in previous *in vivo* studies, but our levels are higher than the surface water concentrations of 0.09–2.63 or 40–320 ng/L predicted by Gottschalk et al. (2009) and Blaser et al. (2008), respectively. Further research is necessary to fully understand the behavior and fate of soluble engineered nanoparticles in natural water bodies

and to quantify their release during the use or washing of consumer products. In addition, the development of analytical procedures and methods to identify and quantify specifically engineered nanoparticles requires further investigation in order to validate current models (Gottschalk et al., 2013). The bioavailability and bioaccumulation of AgNPs are likely to be dependent on the size, shape, chemical composition, charge, surface structure and area, solubility, and aggregation state of the particle or material (Navarro et al., 2008). Furthermore, AgNPs used in commercial products are likely to be coated with organic compounds which are designed to increase colloidal stability of the AgNPs by reducing the surface energy (Ju-Nam and Lead, 2008). As a result, capping agents prevent interactions with the surrounding environment and avoid interactions between nanoparticles reducing aggregation rates (Kvítek et al., 2008). AgNPs used in the present work are stabilized with polyvinyl pyrrolidone (PVP). According to El Badawy et al. (2010), water pH value, ionic strength, and type of electrolyte had no influence on the aggregation of PVP-stabilized AgNPs (for review see Sharma et al., 2014), and its seems to be more stable in solution than nanoparticles capped with other compounds such as citrate (Farkas et al., 2011). Our results indicate that, in spite of the short time of exposure, silver does accumulate in *P. mesopotamicus* brain, liver and gill tissues. Tissue accumulation was higher in brain than liver and gills at all silver concentrations (order: brain□liver□gills) and followed a dose-dependent response pattern only in gills, since in the other tissues silver concentration were similar between 10 and 25 AgNPs/L treatments. This might be related to both an increased aggregation and lowered bioavailability of AgNPs at higher silver concentrations. In mammals, it has been shown that AgNPs could come across the blood-brain barrier (BBB) and be accumulated in the brain (see Karmakar et al., 2014). This translocation mechanism has been demonstrated in an *in vitro* BBB model comprising primary rat brain microvessel vascular endothelial cells (Tang et al., 2009, 2010). However, a few studies reporting AgNPs accumulation in fish brain are available and most of them have been carried out in fish embryos using TEM and hyperspectral images to detect silver presence (Asharani et al., 2008; Kwok et al., 2012). In our study, the levels of silver found in the brain of fish exposed to 10 and 25 μg AgNPs/L were almost twofold higher than those in both gills and liver. Other nano-sized particles can be selectively transported to the brain via the olfactory neuron in mammals (Oberdörster et al., 2004). As described by Tjälve et al. (1995), soluble $54Mn^{2+}$ dosed into the olfactory chamber of pike was taken up by the olfactory receptor cells and transported at a maximal rate of 2.90 ± 0.21 mm/h into the olfactory bulb. It is possible that AgNPs are also mobilized in this way to the brain. In addition, Oberdörster (2004) suggest that colloidal fullerenes may need to be transported to lipid-rich regions (e.g., brain) before the colloid dissociates freeing individual redox-active fullerenes in fish. The molecular weight cut off in the vertebrate kidney (about 60 kDa) suggests that nanoparticles would not pass through the glomerular filter, leaving the liver as the most likely route of excretion for nanometals (Handy et al., 2008). In the present study, silver levels in the liver of fish exposed to 10 μg AgNPs/L were much greater than those in the gills. These results also agree with recently published studies that found higher levels of silver in the liver rather than other organs of fish exposed to different sizes of AgNPs (Jung et al., 2014; Salari Joo et al., 2013; Wu and Zhou, 2013a,b). Comparing silver accumulation in organs with silver levels in water evidenced a clearer dose-response relationship between AgNPs and gill tissue accumulation. There are several mechanisms that explain how AgNPs exposure increases the gill metal contents. For example, nanoparticles can be trapped in the mucus layer of the gill or absorbed by its epithelial cells (Handy et al., 2008; Wu and Zhou, 2013b). The decrease in the AgNPs absorption into the gill epithelia caused by their fixing to the mucus could account for the lack of changes in the antioxidant activity and lipid peroxidation levels found in our assay. Similar results were recently published in zebrafish exposed to 25, 50 and 100 μg/L of nanArgen® AgNPs for 24 h (Bacchetta et al., 2016). These authors found bioaccumulation of silver in gills, but no changes in total antioxidant capacity and lipid peroxidation. According to as previously stated, routes of uptake may be analyzed from another perspective. Gaiser et al. (2009) demonstrate the potential for uptake of nano and larger silver particles by fish via the gastrointestinal tract, and by human intestinal epithelial cells, therefore suggesting that ingestion is a viable route of uptake into different organism types. These authors explained that much of the uptake of the AgNP into the fish may have occurred as a consequence of the fish eating agglomerated NP material, rather than uptake via the water through the gills. As reviewed by Bergin and Witzmann (2013), studies with mammals showed that larger nanoparticles remain within the submucosa or gastrointestinal tract, while smaller nanoparticles enter the bloodstream and accumulate in target organs where they may cause deleterious effects.

Some *in vitro* studies have revealed that AgNPs exposure induces oxidative stress to fish cells by increasing levels of both ROS and LPO; and reducing gluthatione levels and antioxidant enzymes activities (Farkas et al., 2010, 2011; Rosarin et al., 2012; Taju et al., 2014). In the present study, hepatic GPx and GR responses at lower concentrations were different to those observed in fish exposed to 25 μg AgNPs/L. The exposure to 2.5 and 10 μg AgNPs/L caused a decrease of the GPx activity. On the contrary, fish exposed to the highest concentration of AgNPs (25 μg/L) showed an enzyme activity increase, which may be related to the increase of LPO levels (though it was not statistically significant). Besides, a low GR activity was observed in fish exposed to 25 μg AgNPs/L. Studies regarding the influences of various factors on fish GR activity have gained great attention over the past years due to the important antioxidant capacity of such enzyme (Aksakal et al., 2011). For instance, Tekman et al. (2008) showed that metal ions inhibit rainbow trout hepatic GR *in vitro* at very low concentrations (0.0655-0.804 mM). However, no *in vivo* assays have reported this response in the liver of fish exposed to AgNPs. Thus, our results suggest that the highest concentration of AgNPs significantly increased the activity of liver antioxidant enzymes which are crucial in detoxifying oxyradicals and preventing oxidative stress. The hepatic antioxidant mechanisms observed at 25 μg AgNPs/L were effective against oxidative damage. Nevertheless, high hepatic LPO levels in fish exposed to 10 μg AgNPs/L indicated that antioxidant defenses were unable to cope with pro-oxidant damage, eventually leading to cell injury. These results are in accordance with studies carried out by Gagné et al. (2012) and Choi et al. (2010) in which lower (0.6 – 6 μg/L) and higher (60 – 120 mg/L) AgNPs concentrations and were used respectively.

According to our results, brain was the most affected organ, with the highest silver bioaccumulation and LPO levels. A dose-dependent response in LPO and a positive correlation between such biomarker and the increasing silver concentrations in AgNPs-treated fish were observed in our study. Taking into account the metal burden measurements, the unchanged antioxidant enzymes activities, and the LPO occurrence, we suggest that AgNPs exposure results in ROS accumulation and functional damage in brain. Massarsky et al. (2014b) showed that AgNPs increased LPO in fish hepatocytes after 48 h of exposure, and proposed that the nanoparticles generate ROS extracellularly and/or within close proximity to the cell membrane. According to these and our results, some authors have stated that the cell membrane/wall is the main biological target of nano-sized silver (Choi et al., 2010; Zhang et al., 2016) and other metallic nanoparticles (He et al., 2014). To our knowledge, this is the first report of oxidative damage in fish brain after a short exposure to AgNPs.

Nanoparticles induce oxidative stress leading to DNA damage and apoptosis in mammalian cells (Ahamed et al., 2008). In this study, few apoptotic or necrotic blood cells were observed through the viability assay, though the higher AgNPs concentration the higher citotoxicity values. Considering the low cytotoxicity in erythrocytes, we decided to assess the potential AgNPs genotoxicity in this species. Increased DNA damage was observed in erythrocytes of *P. mesopotamicus* after being exposed to 10 μg AgNPs/L. In a recent study, we have reported that the same species at 25 μg AgNPs/L showed increased micronucleus frequency (MNF) in erythrocytes (Davico et al., 2015), revealing that the comet assay was more sensitive to AgNPs than MNF in the same cell population. Massarsky et al. (2014b) exposed rainbow trout erythrocytes and hepatocytes to AgNPs and silver ions concentrations of 3.1–31 μg/ml for 48 h and found that erythrocytes were more sensitive than hepatocytes to both types of silver. These authors also stated that this is likely due to erythrocytes susceptibility to the LPO arising from the high content of poly-unsaturated fatty acid in their cell membrane, and high cytoplasmic oxygen and iron concentrations which continuously produce ROS. As we have discussed earlier, oxidative stress has specific effects, including oxidative damage on DNA. Thus, the modified version of the comet assay using Endonuclease III enzyme to detect oxidative DNA damage showed a significant increase at 10 and 25 μg AgNPs/L when Endo sites were compared to control group. Perhaps, the high variability among individuals at the highest concentration was generated by higher levels of breaks that saturate the assay and cannot be evaluated, as it was suggested by Huk et al. (2015).

One of the major concerns regarding environmental risk assessment of nanoparticles is whether toxicity is specifically related to the nano-size of particles and consequently to their intrinsic properties, or it is also related to the time needed for the ion dissolution and release from the nanoparticles. According to our results, the amount of Ag+ ions release from AgNPs was low and estimated at less than 9%, suggesting that these particles contribute minimal amounts of Ag+. Assuming the reported percentage of dissolution, the measured exposure concentrations of 1.2-13.8 μg Ag/L would have contributed amounts of Ag+ between 0.01 and 1.1 μg Ag/L. However, the possibility cannot be excluded that AgNPs release Ag+ ions in the aquarium and after being absorbed into the body (Santoro et al., 2007). Thus, AgNPs can penetrate into cells and undergo subsequent ionization or dissolution. This Trojan-horse type antimicrobial mechanism is also found in several other engineered nanoparticles (Palza, 2015; Weber et al., 2006). For instance, Gliga et al. (2014) reported that AgNPs (size 10 nm) could release silver ions both intracellularly and extracellularly, but only the intracellular silver release caused cytotoxicity to human lung cells. Consequently, particulate nanosilver can contribute significantly to the overall antimicrobial properties of AgNPs through this Trojan-horse pathway (Zhang et al., 2016). In addition, unstable nanoparticulate suspensions may dissolve quicker and contribute more Ag+. Tejamaya et al. (2012) investigated the stability of AgNPs with three different capping agents in different media chemistry and media concentration. These authors found that PVP was the most stable, proposing it as the capping agent of choice for chronic exposure studies. In a study comparing various surfactants and polymers, sodium dodecyl sulfate (SDS), polyoxyethylenesorbitan monooleate (Tween 80), and PVP were found to act as the best stabilizers resulting also in a considerable enhancement of the antibacterial activity of AgNPs (Kvítek et al., 2008).

Risk estimates for AgNPs based on available predictive models are derived using a PEC (predicted environmental concentration)/predicted no effect concentrations (PNEC) approach and suggest little current risk (Kennedy et al., 2014). If proven true, the PEC's seem to indicate that primary toxicity to the tested organisms are not of high concern, however, bioconcentration up trophic levels, reproductive and chronic studies are perhaps more urgently needed to ensure a thorough risk assessment (McGillicuddy et al., 2017).

5. Conclusions

Due to the various AgNPs antibacterial proprieties, their production, use, and waste into the environment are exponentially increasing. Thus, all *in vivo* assays that contribute to a better understanding of the impact caused by nanoparticles are greatly appreciated. Our results evidenced that AgNPs, do accumulate in gills, liver and brain eventually leading to oxidative damage in brain and liver. Blood erythrocytes of fish exposed to higher concentrations also showed DNA damage. In evidence of our results, it is likely that oxidative stress works as a toxicological mechanism of AgNPs in fish. The neotropical fish *P. mesopotamicus* proved to be a useful indicator sensitive to AgNPs, even after a short time exposure. In a recent study, with the same AgNPs and under similar experimental conditions (Bacchetta et al., 2016), no oxidative stress responses at higher concentrations were found in zebrafish (50 and 100 μg AgNPs/L). The fact that *P. mesopotamicus* showed more sensitiveness than the standard test species used in toxicological research is worthy of attention. Considering the variability of AgNPs production as well as their routes of entry into the environment, our findings on the impact of AgNPs in native fish are an important contribution to scientific knowledge about nanoparticles ecotoxicology.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP2013-2015 N° 11220120100323CO) and Universidad Nacional del Litoral (CAID-UNL). Authors are thankful to Dr. Andrew Collins (University of Oslo, Oslo Norway) for providing the enzyme Endonuclease III, and to S. Mancini for helping with the characterization of nanoparticles used in this work during her research stay funded by the argentinean Program nanopymes, at the University of Vigo, Spain, under the lead of Dra. Isabel Pastoriza Santos. J.M. Monserrat is a research fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

References

ASTM (American Standard Testing Materials), 2002. Standard Test Method for Silver in Water, PA, USA.

AVMA (American Veterinary Medical Association), 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition, IL, USA.

Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S., Schlager, J., Hong, Y., 2008. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicol. Appl. Pharm. 233, 404–410, http://dx.doi.org/10.1016/j.taap.2008.09.015.

Ahamed, M., AlSalhi, M.S., Siddiqui, M.K.J., 2010. Silver nanoparticle applications and human health. Clin. Chim. Acta 411, 1841–1848, http://dx.doi.org/10.1016/j.cca.2010.08.016.

Aksakal, E., Ekinci, D., Erdoğan, O., Beydemir, S., Alım, Z., Ceyhunf, S.B., 2011. Increasing stocking density causes inhibition of metabolic-antioxidant enzymes and elevates mRNA levels of heat shock protein 70 in rainbow trout. Livest. Sci. 141 (1), 69–75, http://dx.doi.org/10.1016/j.livsci.2011.07.006.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., 2008. Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71, 1823–1831, http://dx.doi.org/10.1016/j.chemosphere.2008.02.007.

Amaeze, N.H., Schnell, S., Sozeri, O., Otitoloju, A.A., Egonmwan, R.I., Arlt, V.M., Bury, N.R., 2015. Cytotoxic and genotoxic responses of the RTgill-W1 fish cells in combination with the yeast oestrogen screen to determine the sediment quality of Lagos lagoon. Nigeria. Mutagenesis 30 (1), 117–127, http://dx.doi.org/10.1093/mutage/geu032.

Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveettil, S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19, 255102, http://dx.doi.org/10.1088/0957-4484/19/25/255102, 8pp.

Asharani, P., Hande, M.P., Valiyaveettil, S., 2009. Anti-proliferative activity of silver nanoparticles. BMC Cell Biol. 10 (65), http://dx.doi.org/10.1186/1471-2121-10-65.
Bacchetta, C., Rossi, A., Ale, A., Campana, M., Parma, M.J., Cazenave, J., 2014. Combined toxicological effects of pesticides: a fish multi-biomarker approach Ecol. Indic 36, 532–538, http://dx.doi.org/10.1016/j.ecolind.2013.09.016.
Bacchetta, C., López, G., Pagano, G., Muratt, D.T., de Carvalho, L.M., Monserrat, J.M., 2016. Toxicological effects induced by silver nanoparticles in zebra fish (*Danio rerio*) and in the bacteria communities living at their surface. Bull. Environ. Contam. Toxicol. 97, 456–462, http://dx.doi.org/10.1007/s00128-016-1883-7.
Benn, T., Cavanagh, B., Hristovski, K., Posner, J.D., Westerhoff, P., 2010. The release of nanosilver from consumer products used in the home. J. Environ. Qual. 39, 1875–1882, http://dx.doi.org/10.2134/jeq2009.0363.
Bergin, I.L., Witzmann, F.A., 2013. Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps. Int. J. Biomed. Nanosci. Nanotechnol. 3, 1–2, http://dx.doi.org/10.1504/IJBNN.2013.054515.
Beutler, E., 1982. Catalase. In: Beutler, E. (Ed.), Red Cell Metabolism, a Manual of Biochemical Methods. Grune and Stratton Inc., New York, pp. 105–106.
Bilberg, K., Malte, H., Wang, T., Baatrup, E., 2010. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). Aquat. Toxicol. 96, 159–165, http://dx.doi.org/10.1016/j.aquatox.2009.10.019.
Blaser, S.A., Scheringer, M., MacLeod, M., Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. Sci. Total Environ. 390, 396–409, http://dx.doi.org/10.1016/j.scitotenv.2007.10.010.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Braydich-Stolle, L., Hussain, S., Schlager, J.J., Hofmann, M.C., 2005. In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. Toxicol. Sci. 88, 412–419, http://dx.doi.org/10.1093/toxsci/kfi256.
Braydich-Stolle, L., Lucas, B., Schrand, A.M., Murdock, R.C., Lee, T., Schlager, J.J., Hussain, S.M., Hofmann, M.C., 2010. Silver nanoparticles disrupt GDNF/Fyn kinase signaling in spermatogonial stem cells. Toxicol. Sci. 116, 577–589, http://dx.doi.org/10.1093/toxsci/kfq148.
Bressan, E., Ferroni, L., Gardin, C., Rigo, C., Stocchero, M., Vindigni, V., Cairns, W., Zavan, B., 2013. 2013: Silver nanoparticles and mitochondrial interaction. Int. J. Dentistry 31274, 7, http://dx.doi.org/10.1155/2013/312747.
Bruneau, A., Turcotte, P., Pilote, M., Gagné, F., Gagnon, C., 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. Aquat. Toxicol. 174, 70–81, http://dx.doi.org/10.1016/j.aquatox.2016.02.013.
CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), 2005. Marco Ético de Referencia para las Investigaciones Biomédicas en Animales de laboratorio, de granja y obtenidos de la naturaleza, Buenos Aires, Argentina. http://web.conicet.gov.ar/documents/11716/0/OCR+RD+20050701-1047.pdf.
Cavalcante, D.G., Martínez, C.B., Sofia, S.H., 2008. Genotoxic effects of Roundup_ on the fish *Prochilodus lineatus*. Mutat. Res. 655, 41–46, http://dx.doi.org/10.1016/j.mrgentox.2008.06.010.
Chae, Y.J., Pham, C.H., Lee, J., Bae, E., Yi, J., Gu, M.B., 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 94, 320–327, http://dx.doi.org/10.1016/j.aquatox.2009.07.019.
Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.-Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat. Toxicol. 100, 151–159, http://dx.doi.org/10.1016/j.aquatox.2009.12.012.
Collins, A.R., 2009. Investigating oxidative DNA damage and its repair using the comet assay. Mutat. Res. 681, 24–32, http://dx.doi.org/10.1016/j.mrrev.2007.10.002.
Davico, C., Bacchetta, C., López, G., Cazenave, J., Poletta, G., Simoniello, M.F., 2015. Evaluación de genotoxicidad a través de la frecuencia de Micronúcleos en eritrocitos de *Piaractus mesopotamicus* (pacúes) expuestos in vivo a nanopartículas de plata. Acta Toxicol. Argent. 23 (2), 73–78.
Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., 2015. InfoStat Versión 2015 Grupo InfoStat. FCA, Universidad Nacional de Córdoba, Argentina, http://www.infostat.com.ar.
Drotar, A., Phelps, P., Fall, R., 1985. Evidence for glutathione peroxidase activities in cultured plant cells. Plant Sci. 42, 35–40, http://dx.doi.org/10.1016/0168-9452(85)90025-1.
El Badawy, A.M., Luxton, T.P., Silva, R.G., Scheckel, K.G., Suidan, M.T., Tolaymat, T.M., 2010. Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. Environ. Sci. Technol. 44, 1260–1266, http://dx.doi.org/10.1021/es902240k.
Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531, http://dx.doi.org/10.1016/j.envint.2010.10.012.
Farkas, J., Christian, P., Gallego Urrea, J.A., Roos, N., Hassellöv, M., Tollefsen, K.E., Thomas, K.V., 2010. Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat. Toxicol. 96, 44–52, http://dx.doi.org/10.1016/j.aquatox.2009.09.016.
Farkas, J., Christian, P., Gallego Urrea, J.A., Roos, N., Hassellöv, M., Tollefsen, K.E., Thomas, K.V., 2011. Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. Aquat. Toxicol. 101 (1), 117–125, http://dx.doi.org/10.1016/j.aquatox.2010.09.010.
Farmen, E., Mikkelsen, H.N., Evensen, Ø., Einset, J., Heier, L.S., Rosseland, B.O., Salbu, B., Tollefsen, K.E., Oughton, D.H., 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low (g/L concentrations of Ag nanoparticles. Aquat. Toxicol. 108, 78–84, http://dx.doi.org/10.1016/j.aquatox.2011.07.007.
Fatima, M., Ahmad, I., Sayeed, I., Athar, M., Raisuddin, S., 2000. Pollutant-induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. Aquat. Toxicol. 49, 243–250, http://dx.doi.org/10.1016/S0166-445X(99)00086-7.
Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. 681, 80–92, http://dx.doi.org/10.1016/j.mrrev.2008.03.001.
Gagné, F., André, C., Skirrow, R., Gélinas, M., Auclair, J., van Aggelen, G., Turcotte, P., Gagnon, C., 2012. Toxicity of silver nanoparticles to rainbow trout: a toxicogenomic approach. Chemosphere 89, 615–622, http://dx.doi.org/10.1016/j.chemosphere.2012.05.063.
Gaiser, B.K., Fernandes, 1, Jepson, T.F., Lead, M., 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. Environ. Health 8 (1), S2, http://dx.doi.org/10.1186/1476-069x-8-s1-s2.
Gliga, A.R., Skoglund, S., Wallinder, I.O., Fadeel, B., Karlsson, H.L., 2014. Size dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. Part. Fibre Toxicol. 11 (11), http://dx.doi.org/10.1186/1743-8977-1111-1111.
Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag CNT, fullerenes) for different regions. Environ. Sci. Technol. 43, 9216–9222, http://dx.doi.org/10.1021/es9015553.
Gottschalk, F., Sun, T.Y., Nowack, B., 2013. Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. Environ. Poll. 181, 287–300, http://dx.doi.org/10.1016/j.envpol.2013.06.003.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first stepin mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Handy, R.D., Henry, T.B., Scown, T.M., Johnston, B.D., Tyler, C.R., 2008. Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis. Ecotoxicology 17, 396–409, http://dx.doi.org/10.1007/s10646-008-0205-1.
He, X., Aker, W.G., Leszczynski, J., Hwang, H.-M., 2014. Using a holistic approach to assess the impact of engineered nanomaterials inducing toxicity in aquatic systems. J. Food Drug Anal. 22 (1), 128–146, http://dx.doi.org/10.1016/j.jfda.2014.01.011.
Huk, A., Collins, A.R., El Yamani, N., Porredon, C., Azqueta, A., de Lapuente, J., Dusinska, M., 2015. Critical factors to be considered when testing nanomaterials for genotoxicity with the comet assay. Mutagenesis 30 (1), 85–88, http://dx.doi.org/10.1093/mutage/geu077.
Hussain, S.M., Hess, K.L., Gearhart, J.M., Geiss, K.T., Schlager, J.J., 2005. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. Toxicol. In Vitro 19, 975–983, http://dx.doi.org/10.1016/j.tiv.2005.06.034.
Ju-Nam, Y., Lead, J.R., 2008. Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. Sci. Total Environ. 400, 396–414, http://dx.doi.org/10.1016/j.scitotenv.2008.06.042.
Jung, Y.-J., Kim, K.-T., Kim, J.Y., Yang, S.-Y., Lee, B.-G., Kim, S.D., 2014. Bioconcentration and distribution of silver nanoparticles in Japanese medaka (*Oryzias latipes*). J. Hazard. Mater. 267, 206–213, http://dx.doi.org/10.1016/j.jhazmat.2013.12.061.
Kaegi, R., Voegelin, A., Sinnet, B., Zuleeg, S., Hagendorfer, H., Burkhardt, M., Siegrist, H., 2011. Behavior of metallic silver nanoparticles in a pilot wastewater treatment plant. Environ. Sci. Technol. 45, 3902–3908, http://dx.doi.org/10.1021/es1041892.
Karmakar, A., Zhang, Q., Zhang, Y., 2014. Neurotoxicity of nanoscale materials. J. Food Drug Anal. 22 (1), 147–160, http://dx.doi.org/10.1016/j.jfda.2014.01.012.
Kennedy, A.J., Diamond, S., Stanley, J.K., Coleman, J., Steevens, J.A., Chappell, M.A., Laird, J., Bednar, A., 2014. Nanomaterials ecotoxicology: a case study with nanosilver. In: Hull, M., Bowman, D. (Eds.), Nanotechnology Environmental Health and Safety. , second edition. Elsevier Inc., pp. 117–151.
Kim, J.S., Kuk, E., Yu, K.N., Kim, J.-H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., Hwang, C.-Y., Kim, Y.-K., Lee, Y.-S., Jeong, D.H., Cho, M.-H., 2007. Antimicrobial effects of silver nanoparticles. Nanomedicine: NBM 3, 95–101, http://dx.doi.org/10.1016/j.nano.2006.12.001.
Kvítek, L., Panáček, A., Soukupova, J., Kolar, M., Vecerova, R., Prucek, R., Holecová, M., Zboril, R., 2008. Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). J. Phys. Chem. C 112 (15), 5825–5834, http://dx.doi.org/10.1021/jp711616 v.
Kwok, K.W.H., Auffan, M., Badireddy, A.R., Nelson, C.M., Wiesner, M.R., Chilkoti, A., Liu, J., Marinakos, S.M., Hintona, D.E., 2012. Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (*Oryzias latipes*): Effect of coating materials. Aquat. Toxicol. 120 (-121), 59–66, http://dx.doi.org/10.1016/j.aquatox.2012.04.012.
Lorenz, C., Windler, L., von Goetz, N., Lehmann, R.P., Schuppler, M., Hungerbühler, K., Heuberger, M., Nowack, B., 2012. Characterization of silver release from commercially available functional (nano)textiles. Chemosphere 89, 817–824, http://dx.doi.org/10.1016/j.chemosphere.2012.04.063.
Marambio-Jones, C., Hoek, E.M.V., 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. J. Nanopart. Res. 12, 1531–1551, http://dx.doi.org/10.1007/s11051-010-9900-y.
Massarsky, A., Dupuis, L., Taylor, J., Eisa-Beygi, S., Strek, L., Trudeau, V.L., Moon, T.W., 2013. Assessment of nanosilver toxicity during zebrafish (*Danio rerio*) development. Chemosphere 92, 59–66, http://dx.doi.org/10.1016/j.chemosphere.2013.02.060.

Massarsky, A., Trudeau, V.L., Moon, T.W., 2014a. Predicting the environmental impact of nanosilver. Environ. Toxicol. Pharm. 38, 861–873, http://dx.doi.org/10.1016/j.etap.2014.10.006.

Massarsky, A., Abraham, R., Nguyen, K.C., Rippstein, P., Tayabali, A.F., Trudeau, V.L., Moon, T.W., 2014b. Nanosilver cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) erythrocytes and hepatocytes. Comp. Biochem. Physiol. C 159, 10–21, http://dx.doi.org/10.1016/j.cbpc.2013.09.008.

McGillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., Dockery, P., Prendergast, M., Rowan, N., Morris, D., 2017. Silver nanoparticles in the environment: sources, detection and ecotoxicology. Sci. Total Environ. 575, 231–246, http://dx.doi.org/10.1016/j.scitotenv.2016.10.041.

McShan, D., Ray, P.C., Yu, H., 2014. Molecular toxicity mechanism of nanosilver. J. Food Drug. Anal. 22, 116–127, http://dx.doi.org/10.1016/j.jfda.2014.01.010.

Mercille, S., Massie, B., 1994. Induction of apoptosis in nutrient-deprived cultures of hibridoma and mieloma cells. Biotechnol. Bioeng. 44, 1140–1154, http://dx.doi.org/10.1002/bit.260440916.

Messaoud, M., Chadeau, E., Brunon, C., Ballet, T., Rappenne, L., Roussel, F., Leonard, D., Oulahal, N., Langlet, M., 2010. Photocatalytic generation of silver nanoparticles and application to the antibacterial functionalization of textile fabrics. J. Photochem. Photobiol. A 215, 147–156, http://dx.doi.org/10.1016/j.jphotochem.2010.08.003.

Munari, M., Sturve, J., Frenzilli, G., Sanders, M.B., Brunelli, A., Marcomini, A., Nigro, M., Lyons, B.P., 2014. Genotoxic effects of CdS quantum dots and Ag2S nanoparticles in fish cell lines (RTG-2). Mutat. Res. 775 (-776), 89–93, http://dx.doi.org/10.1016/j.mrgentox.2014.09.003.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.-J., Quigg, A., Santschi, P.H., Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17, 372–386, http://dx.doi.org/10.1007/s10646-008-0214-0.

Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., Cox, C., 2004. Translocation of inhaled ultrafine particles to the brain. Inhal. Toxicol. 46, 437–445, http://dx.doi.org/10.1080/08958370490439597.

Oberdörster, G., 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health Persp. 112 (10), 1058–1062, http://dx.doi.org/10.1289/ehp.7021.

Palza, H., 2015. Antimicrobial polymers with metal nanoparticles. Int. J. Mol. Sci. 16 (1), 2099–2116, http://dx.doi.org/10.3390/ijms16012099.

Parma de Croux, M.J., 1990. Benzocaine (Ethyl-p-Aminobenzoate) as an anaesthetic for *Prochilodus lineatus*, valenciennes (Pisces, curimatidae). J. Appl Ichthyol. 6 (3), 189–192.

Piao, M.J., Kang, K.A., Lee, I.K., Kim, H.S., Kim, S., Choi, J.Y., Choi, J., Hyun, J.W., 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol. Lett. 201, 92–100, http://dx.doi.org/10.1016/j.toxlet.2010.12.010.

Poletta, G.L., Simoniello, M.F., Mudry, M.D., 2016. Biomarkers of oxidative damage and antioxidant defense capacity in *Caiman latirostris* blood. Comp. Biochem. Physiol. C 179, 29–36, http://dx.doi.org/10.1016/j.cbpc.2015.08.003.

Rather, M.A., Sharma, R., Aklakur, M., Ahmad, S., Kumar, N., Khan, M., Ramya, V.L., 2011. Nanotechnology: a novel tool for aquaculture and fisheries development: a prospective mini-review. Fisheries and Aquaculture Journal 2011, FAJ–16, http://astonjournals.com/faj (accessed 05.04.11).

Reichenbach-Klinke, H.H., 1980. Enfermedades De Los Peces. Acribia, Zaragoza.

Rhim, J.-W., Park, H.-M., Ha, C.-S., 2013. Bio-nanocomposites for food packaging applications. Prog. Polym. Sci. 38, 1629–1652, http://dx.doi.org/10.1016/j.progpolymsci.2013.05.008.

Roh, J.-Y., Eom, H.-J., Choi, J., 2012. Involvement of *Caenorhabditis elegans* MAPK signaling pathways in oxidative stress response induced by silver nanoparticles exposure. Toxicol. Res. 28, 19–24, http://dx.doi.org/10.5487/TR.2012.28.1.019.

Rosarin, F.S., Arulmozhi, V., Nagarajan, S., Mirunalini, S., 2012. Antiproliferative effect of silver nanoparticles synthesized using amla on Hep2 cell line. Asian Pac. J. Trop. Med. 2012, 1–10, http://dx.doi.org/10.1016/S1995-7645(12)60193-X.

Salari Joo, H., Kalbassi, M.R., Yu, I.J., Lee, J.H., Johari, S.A., 2013. Bioaccumulation of silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*): Influence of concentration and salinity. Aquat. Toxicol. 140-141, 398–406, http://dx.doi.org/10.1016/j.aquatox.2013.07.003.

Sampaio, F.G., Boijink, C.L., dos Santos, L.R.B., Oba, E.T., Kalinin, A.L., Luiz, A.J.B., Rantin, F.T., 2012. Antioxidant defenses and biochemical changes in the neotropical fish pacu, Piaractus mesopotamicus: responses to single and combined copper and hypercarbia exposure. Comp. Biochem. Physiol. C 156, 178–186, http://dx.doi.org/10.1016/j.cbpc.2012.07.002.

Santoro, C.M., Duchsherer, N.L., Grainger, D.W., 2007. Antimicrobial efficacy and ocular cell toxicity from silver nanoparticles. Nanobiotechnology 3 (2), 55–65, http://dx.doi.org/10.1007/s12030-008-9007-z.

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., van Aerle, R., Tyler, C.R., 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol. Sci. 115 (2), 521–534, http://dx.doi.org/10.1093/toxsci/kfq076.

Shaalan, M., Saleh, M., El-Mahdy, M., El-Matbouli, M., 2016. Recent progress in applications of nanoparticles in fish medicine: a review. Nanomedicine: NBM 12, 701–710, http://dx.doi.org/10.1016/j.nano.2015.11.005.

Sharma, V.K., Siskova, K.M., Zboril, R., Gardea-Torresdey, J.L., 2014. Organic-coated silver nanoparticles in biological and environmental conditions: fate, stability and toxicity. Adv. Colloid. Interface. Sci. 204, 15–34, http://dx.doi.org/10.1016/j.cis.2013.12.002.

Simoniello, M.F., Loteste, A., Kleinsorge, E.C., Campana, M., Parma, M.J., 2009. Alcaline comet assay for genotoxic effects detection in a neotropical fish *Prochilodus lineatus* (Pisces Curimatidae). Bull. Environ. Contam. Toxicol. 83, 155–158, http://dx.doi.org/10.1007/s00128-009-9771-z.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individuals cells. Exp. Cell Res. 175, 184–191, 0.1016/0014-4827(88)90265-0.

Singh, S., D'Britto, V., Prabhune, A., Ramana, C., Dhawan, A., Prasad, B., 2010. Cytotoxic and genotoxic assessment of glycolipid-reduced and-capped gold and silver nanoparticles. New J. Chem. 34 (2), 294–301, http://dx.doi.org/10.1039/B9NJ00277D.

Stone, V., Nowack, B., Baun, A., Brink, N., Kammer, F., Dusinka, M., Handy, R., Hankin, S., Hassellov, M., Joner, E., Fernandes, T., 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physicochemical characterization. Sci. Total Environ. 408, 1745–1754, http://dx.doi.org/10.1016/j.scitotenv.2009.10.035.

Taju, G., Abdul Majeed, S., Nambi, K.S.N., Sahul Hameed, A.S., 2014. In vitro assay for the toxicity of silver nanoparticles using heart and gill cell lines of *Catla catla* and gill cell line of *Labeo rohita*. Comp. Biochem. Physiol. C 161, 41–52, http://dx.doi.org/10.1016/j.cbpc.2014.01.007.

Tanaka, K., Sano, T., Ishizuka, K., Kitta, K., Kawamura, Y., 1994. Comparison of properties of leaf and root glutathione reductases from spinach. Physiol. Plant. 91, 353–358, http://dx.doi.org/10.1111/j.1399-3054.1994.tb02960.x.

Tang, J., Xiong, L., Wang, S., Wang, J., Liu, L., Li, J., Yuan, F., Xi, T., 2009. Distribution, translocation and accumulation of silver nanoparticles in rats. J. Nanosci. Nanotechnol. 9, 4924–4932, http://dx.doi.org/10.1166/jnn.2009.1269.

Tang, J., Xiong, L., Zhou, G., Wang, S., Wang, J., Liu, L., Li, J., Yuan, F., Lu, S., Wan, Z., Chou, L., Xi, T., 2010. Silver nanoparticles crossing through and distribution in the bloodebrain barrier in vitro. J. Nanosci. Nanotechnol. 10, 6313–6317, http://dx.doi.org/10.1166/jnn.2010.2625.

Tejamaya, M., Römer, I., Merrifield, R.C., Lead, J.R., 2012. Stability of citrate PVP, and PEG coated silver nanoparticles in ecotoxicology media. Environ. Sci. Technol. 46, 7011–7017, http://dx.doi.org/10.1021/es2038596.

Tekman, B., Ozdemir, H., Senturk, M., Ciftci, M., 2008. Purification and characterization of glutathione reductase from rainbow trout (*Oncorhynchus mykiss*) liver and inhibition effects of metal ions on enzyme activity. Comp. Biochem. Physiol. C 148 (2), 117–121, http://dx.doi.org/10.1016/j.cbpc.2008.04.005.

The Nanodatabase, 2017 URL http://nanodb.dk/en/(accessed 04.01.17).

Tjälve, H., Mejare, C., Borg-Neczak, K., 1995. Uptake and transport of manganese in primary and secondary olfactory neurons in pike. Pharm. Toxicol. 77, 23–31, http://dx.doi.org/10.1111/j.1600-0773.1995.tb01909.x.

US EPA (United States Environmental Protection Agency), 1991. Method 200.3. Sample preparation procedure for spectrochemical determination of total recoverable elements in biological tissues. Cincinnati, OH, USA.

US EPA, 1994 Methods for the determination of metals in environmental samples Cincinnati, OH, USA.

Vanzella, T.P., Martinez, C.B.R., Cólus, I.M.S., 2007. Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species. Mutat. Res. 631, 36–43, http://dx.doi.org/10.1016/j.mrgentox.2007.04.004.

Voelker, D., Schlich, K., Hohndorf, L., Koch, W., Kuehnen, U., Polleichtner, C., Kussatz, C., Hund-Rinke, K., 2015. Approach on environmental risk assessment of nanosilver released from textiles. Environ. Res. 140, 661–672, http://dx.doi.org/10.1016/j.envres.2015.05.011.

Weber, K.A., Achenbach, L.A., Coates, J.D., 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat. Rev. Microbiol. 4 (10), 752–764, http://dx.doi.org/10.1038/nrmicro1490.

Wise Sr., J.P., Goodale, B.C., Wise, S.S., Craig, G.A., Pongan, A.F., Walter, R.B., Thompson, W.D., Ng, Ah-Kau, Aboueissa, A., Mitani, H., Spalding, M.J., Mason, M.E., 2010. Silver nanospheres are cytotoxic and genotoxic to fish cells. Aquat. Toxicol. 97 (1), 34–41, http://dx.doi.org/10.1016/j.aquatox.2009.11.016.

Wu, Y., Zhou, Q., 2013a. Dose- and time-related changes in aerobic metabolism, chorionic disruption, and oxidative stress in embryonic medaka (*Oryzias latipes*): Underlying mechanisms for silver nanoparticle developmental toxicity. Aquat. Toxicol. 124-125, 238–246, http://dx.doi.org/10.1016/j.aquatox.2012.08.009.

Wu, Y., Zhou, Q., 2013b. Silver nanoparticles cause oxidative damage and histological changes in medaka (*Oryzias latipes*) after 14 days of exposure. Environ. Toxicol. Chem. 32 (1), 165–173, http://dx.doi.org/10.1002/etc.2038.

Zhang, C., Hu, Z., Deng, B., 2016. Silver nanoparticles in aquatic environments: physiochemical behavior and antimicrobial mechanisms. Water Res. 88, 403–427, http://dx.doi.org/10.1016/j.watres.2015.10.025.

de Moraes, F.D., de Figueiredo, J.S.L., Rossi, P.A., Venturini, F.P., Moraes, G., 2015. Acute toxicity and sublethal effects of phenol on hematological parameters of channel catfish *Ictalurus punctatus* and pacu *Piaractus mesopotamicus*. Ecotoxicol. Environ. Contam. 10 (1), 31–36, http://dx.doi.org/10.5132/eec.2015.01.05.