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Nanosilver toxicity in gills of a neotropical fish: Metal accumulation, oxidative stress, histopathology and other physiological effects

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ABSTRACT

The widespread and increasing use of silver nanoparticles (AgNP) as biocide results in an unknown release into the aquatic environment. In order to contribute to the understanding of their potential toxicity, the aim of this study was to analyze branchial multiple biomarkers on the neotropical fish *Prochilodus lineatus*. We exposed fish to sublethal AgNP concentrations: 0 (control), 2.5 and 25.0 μg L^{-1}. After 5 and 15 days, we analyzed in gills total Ag accumulation, oxidative stress markers (antioxidant enzymes, lipid peroxidation and antioxidant capacity against peroxyl radicals), aspartate and alanine aminotransferases activities (ALT, AST) and histopathologies (morphometric analysis, proportion of the secondary lamellae available for gas exchange, reaction indexes, and organ index -I_{gills}-) that included mucus cell count (MCc). The Ag accumulation after 15 days was five times higher than after 5 days in the case of 25.0 μg AgNP L^{-1}-exposure. Regarding oxidative stress, all enzymes activities were inhibited after 5 days at both AgNP concentrations. ALT activity decreased and a reduction in the antioxidant capacity was evidenced after 2.5 μg AgNP L^{-1} and 15 days. LPO levels and AST activity increased after the highest time of exposure and AgNP concentration, and the same occurred with I_{gills}. MCc increased after 15 days at both AgNP concentrations. The results confirmed that the presence of low AgNP concentrations, in short and subchronic exposures, generates alterations in stress biomarkers and in the structure of this vital organ that are the gills.

1. Introduction

Silver has been used as an antibiotic since the ancient time, and it has had greater applications in medicine, optics, sensing, paintings and cosmetics (Chen and Schluesener, 2008; McShan et al., 2014). The exceptional characteristics of nanosilver particles (AgNP) as biocide have made them the largest and fastest growing class of manufactured nanomaterials in commercial applications (Pinto et al., 2010). The Nanodatabase (www.nanodb.dk) listed 353 consumer products containing AgNP in 2017. The use of AgNP is proliferating, and it is expected that the environment will be increasingly exposed to these materials.

When AgNP arise the aquatic media, they release Ag^+ that is one of the most toxic metal forms for organisms in natural water systems (Ratte, 1999). As well as most nanoparticles, AgNP physico-chemistry suggests they are likely to aggregate depending on concentration and type of organic matter, and solids in suspension (Handy et al., 2008). Unfortunately, precise estimations of the emissions from silver-containing materials are hampered by lack of available information about content and form of the silver in the products (Geranio et al., 2009). The release of AgNP can happen at any stage of the product life-cycle: production, transport, storage, usage and disposal (Ribeiro et al., 2014). The predicted environmental concentrations for AgNP in the aquatic environment are in the low μg L^{-1} or ng L^{-1} (Gottschalk et al., 2013). Therefore, environmental concerns have risen due to there is evidence that AgNP induce deleterious effects in aquatic systems as well in aquatic life (Choi et al., 2010).

Many investigations have proved that fish are valuable organisms to asses toxicological effects caused by AgNP (Govindasamy and Rahuman, 2012; Lee et al., 2012; Wu and Zhou, 2012; Massarsky et al., 2013; Bacchetta et al., 2016; Martin et al., 2016). Particularly, the gills constitute a multifunctional organ (respiration, ionoregulation, acid-

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base regulation, nitrogenous waste excretion) accounting for well over 50 per cent of the total surface area of the animal. They are the major site of uptake for most waterbone toxicants and also the first, and most important, site of toxic impact for many of them. Essential physiologic processes are performed by the gills and they are sensitive to both structural and biochemical disturbance of the branchial epithelium (Schlenk and Benson, 2001).

Nanosilver can be taken up by many different cells and become internalized inside the cell. High levels of Ag accumulation from AgNP has been reported in the liver, gills, kidney, intestine and muscle of fish (Scown et al., 2010; Wu and Zhou, 2012; Bacchetta et al., 2016; Martin et al., 2016). The main deleterious effect reported by AgNP is oxidative stress due to they enter the cell through diffusion or endocytosis and generate reactive oxygen species, leading to damages to proteins and acids inside the cell, and finally inhibition of cell proliferation (McShan et al., 2014). Lipid peroxidation, impairment of antioxidant enzyme system and glutathione depletion caused by AgNP has been reported in gills and liver of many fish species (Farmen et al., 2012; Govidasamy and Rahuman, 2012; Wu and Zhou, 2012; Griffitt et al., 2013; Martin et al., 2016). Regarding a higher level of damage, it has been demonstrated that AgNP caused histopathological alterations in liver, muscle and gills of fish (Wu et al., 2010; Govindasamy and Rahuman, 2012; Wu and Zhou, 2013), including mucus cells proliferation (Lee et al., 2012). Finally, other responses such as metallothioneins induction, DNA damage and gene expression have been associated with toxicity generated by AgNP (Choi et al., 2010; Gagné et al., 2012; Martin et al., 2016).

The neotropical fish *Prochilodus lineatus* has been widely used in experimental designs for being sensitive to variations in water quality and tolerant to laboratory conditions (Camargo and Martinez, 2006; Cazenave et al., 2014; da Silva and Martinez, 2014; Vieira et al., 2016). Concern and reports about toxicity mechanism related to AgNP exposures have risen through the years; however assays that include aquatic organisms exposed to chronic conditions remain scarce. Massarsky et al. (2014) considered essential to predict environmental concentrations and thus the risk associated with AgNP. In order to contribute to the understanding of their potential toxicity, the aim of this study was to analyze multiple responses in gills of a neotropical fish (*Prochilodus lineatus*) exposed to sublethal AgNP concentrations.

2. Materials and methods

2.1. Nanosilver suspension, preparation and characterization

A colloidal suspension of 1% w/v AgNP was provided by Nanotek S.A., under the brand name nanArgen®. The main ingredient of the product (> 99.9%) is metallic silver (CAS Number 7440-22-4), with an average particle size of 20–40 nm (Material Safety Data Sheet, MSDS).

The capping agent is made of glucose oligomers, mainly nanocrystalline cellulose (CAS Number 9004-34-6). NP were characterized by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS) and Transmission Electron Microscopy (TEM). A drop of the NP suspension was air dried onto a carbon film-coated grid. Afterwards, SEM was assessed in a Zeiss EVO 40 (Carl-Zeiss, Oberkochen, Germany) at 20000X. In addition, Energy Dispersive Spectroscopy (EDS) was employed for the chemical characterization of the nanoparticulate silver suspension.

The size and the shape of the particles were investigated by TEM. Briefly a drop of the sample in aqueous solution was deposited on 400-mesh carbon-coated copper grids. After one minute, the liquid was blotted with filter paper (Whatman no. 4). TEM was performed at room temperature using a JEOL 1011 electron microscope operating at 100 kV.

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

2.2. Fish and exposure conditions

Juvenile *Prochilodus lineatus* (n = 120; 6.81 ± 0.08 cm standard length; 6.58 ± 0.23 g) were obtained from a local fish farm. For acclimation purpose, fish were held in 120-l tanks containing well aerated dechlorinated water for two weeks, and fed once daily ad libitum with dry commercial pellets. The test water conditions were: pH 6.58; conductivity 178 mS/cm^3; total hardness 48.01 ppm CO_3Ca; calcium 16.0 ppm Ca^{++}, magnesium 2.0 ppm Mg^{++}, alkalinity 60.0 ppm CO_3Ca; 61.50 CO_3H^-.(levels of CO_3^{2} not detected). Fish feeding was suspended 24 h before the fish dissection. The laboratory conditions corresponded to 12:12 h light-dark cycles and temperature of 25 ± 1 ºC. All experiments were conducted in accordance with national and institutional guidelines (CONICET, 2005) for the protection of animal welfare. Aquaria were covered with a thin black plastic that prevented stress by management practices in adjacent aquaria.

The selected concentrations were based on previous studies (Bacchetta et al., 2016, 2017) and on the LC50–96 h value calculated previously (data not published), which was stated as 53.84 µg AgNP L^{-1} (confidence interval: 35.29 - 82.18). Fish (2 fish per 10-L aquarium) were exposed to the following AgNP concentrations: 0 (control), 2.5 and 25.0 µg AgNP L^{-1}. All treatments were replicated twenty times. Experiments were performed under semi-static conditions and solutions were renewed every 48 h by transferring the fish to another aquarium that contained the renewed AgNP concentration. During assays, fish were fed once a day in the morning (8 a.m., approximately). After 5 and 15 days, the animals were anesthetized and measured, weighted, sacrificed and dissected. For histopathological analyses, the right second branchial arch from each individual was fixed in paraformaldehyde (PAF) 4%. The remaining gill tissue was immediately frozen and stored at −80 ºC until biochemical determinations were determined.

2.3. Exposure media and tissue silver content

The measurement of total recoverable Ag in water samples was performed according to the method 200.2 described by the United States Environmental Protection Agency (US EPA, 1994), with modifications. Water samples were taken from each aquarium at: 0 h (beginning of the assay, without fish), 12 h, 24 h, 36 h and 48 h (re-dosing time). Briefly, a 100 ml aliquot from each sample was transferred to a Griffin beaker, and 2 ml of (1 + 1) nitric 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.

Tissues samples preparation was carried out according to the method 200.3 proposed by the US EPA (1991), with modifications. Samples of gills were digested by adding concentrated nitric acid, heating to 95 °C, and leaving to cool. This process was repeated until all tissue was in the solution. Then, 30% hydrogen peroxide was dosed.

In accordance with the method 200.9 (US EPA, 1994), Ag was then quantified in water and tissue samples using a graphite furnace atomic absorption spectrophotometer (GF AAS, Perkin Elmer AAnalyst 800) equipped with an auto sampler and which limit of detection on the digested samples was 1 µg Ag L^{-1}. Lectures were made using a calibration curve with Merck® certificated pattern. One aliquot was injected into the GF AAS, and three readings of each run were recorded. The mean of the reading was used to calculate the amount of silver in the aliquot of digested water and tissue.

2.4. Antioxidant and oxidative damage determinations

Antioxidant enzymes extracts from gills tissue were prepared from

each individual, and were homogenized with a phosphate buffer (pH 6.5) according to Bacchetta et al. (2014). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by Misra and Fridovich (1972). Catalase activity (CAT, EC 1.11.1.6) was determined according to Beutler (1982). 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). 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. Glutathione reductase activity (GR, EC 1.6.4.2) was assayed according to Tanaka et al. (1994).

Total antioxidant competence against peroxyl radicals (ACAP) was determined through reactive oxygen species (ROS) determination in sample tissues treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (37 ºC) decomposition of 2,2`-azobis (2-methylpropionamidine) dihydrochloride (ABAP; 4 mM; Aldrich). ROS concentration was measured in a fluorescence microplate reader (Synergy Biotek) with the fluorogenic compound 2, 7-dichlorofluorescin diacetate (H_2DCF-DA) at a final concentration of 40 μM, in according to the methodology reported by Amado et al. (2009) and further modifications adopted by Monserrat et al. (2014). The measure of antioxidant capacity is given by difference in the fluorescence of the samples after 30 min with and without ABAP and is calculated by the following expression: (FU 30 $min_{with\ ABAP}$ - FU 30 $min_{without\ ABAP}$) / FU 30 $min_{without\ ABAP}$. As high fluorescence levels are obtained after adding ABAP, a high difference is considered to indicate a low antioxidant capacity suggesting a low ability to neutralize peroxyl radicals (Amado et al., 2009).

Lipid peroxidation levels (LPO) were determined by measuring the formation of thiobarbituric reactive substances (TBARS), according to Yagi (1976) from the same homogenized samples described before.

The enzymatic activities, LPO and ACAP levels were calculated in terms of the sample protein content (Bradford, 1976). All assays were carried out in triplicate.

2.5. Alanine aminotransferase and aspartate aminotransferase

Samples of gills were homogenized in phosphate buffer with sucrose (pH 7.4) according to Bacchetta et al. (2011). Alanine aminotransferase (ALT, L-alanine-2-oxaloglutarate aminotransferase; EC 2.6.1.2) and aspartate aminotransferase (AST, l-aspartate-2-oxaloglutarate aminotransferase; EC 2.6.1.1) activities were estimated according to Reitman and Frankel (1957). Each sample was measured by triplicate and the enzymatic activity was calculated in terms of protein content (Bradford, 1976).

2.6. Histopathological analysis

The samples kept in PAF 4% were washed with tap water and dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Then, cuts of 7 μm thickness were made for staining with hematoxylin and eosin. The examination of histopathological lesions in gills tissue was performed in ten sections randomly selected per fish. For that purpose, the freely available software Image J was employed to analyze photomicrographs in order to accomplish the morphometric analysis. The following measures were taken: secondary lamellar length (SLL) and width (SLW), interlamellar distance (ID; at the apex, medium and basis), and basal epithelial thickness (BET) (Nero et al., 2006a). Besides, the proportion of the secondary lamellae available for gas exchange (PAGE) was calculated according to Nero et al. (2006b) as: PAGE = 100 × (mean BET) / (mean BET + mean SLL).

For recording histopathologies in gills, a semiquantitative method proposed by Bernet et al. (1999) was employed. Alterations in gills were classified in the major patterns: (1) circulatory disturbances that result from pathological condition of blood and tissue fluid flow; (2) regressive changes which reveal functional reduction or loss of the organ; (3) progressive changes that lead to an increased activity of cells or organs; and (4) inflammatory processes, which generally are associated with other reactions patterns. Based on that, indexes were calculated according the extension of the pathological change (score value) and the pathological importance (importance factor). Depending on the occurrence of the alterations in the sample (percentage of tissue with a certain kind of alteration), a score value from 1 to 6 was assigned. Regarding the importance factor, which informs if the pathology is reversible or not after the remove of the stressor, a number from 1 to 3 was assigned.

The sum of the multiplied importance factors and score values of each reaction pattern is expressed by the reaction index (Irp). It reveals the quality of the lesions of an organ, and was calculated as:

$$\mathrm{Irp} = \sum_{\mathrm{alt}} (\mathrm{a\ org\ rp\ alt}\ x\ \mathrm{w\ org\ rp\ alt})$$

Where: org, rp = constant, and the other abbreviations are explained above. The sum of the five reactions of an organ is equivalent to the I_{org}.

The organ index (I_{gills}) sums the multiplied importance factors and score values of the alteration of the reaction patterns. It was determined as:

$$\mathrm{Igills} = \sum_{\mathrm{rp}} \sum_{\mathrm{alt}} (\mathrm{a\ org}\ x\ \mathrm{w\ org\ alt})$$

Where: org is the organ (gills, constant), rp: reaction pattern, alt: alteration, a: score value, and w- importance factor.

Additionally, gill sections were stained with periodic acid (PAS) and Alcian blue (AB) at pH 2.5 to identify the mucus cells. All stained sections were observed in the conditions previously described. Mucus cells were quantified (MCc) in ten randomly selected sections of the gill filament according to Sabóia-Moraes et al. (1996) with modifications proposed by Paulino et al. (2014). The stained sections were observed and the images were captured under the Leica Microsystem (Leica DFC 2900), at 400× magnification.

2.7. Statistical analyses

Data were first tested for normality and homogeneity of variance using Shapiro Wilks and Levene tests, respectively. Variables that had not a normal distribution were transformed using $\log_{10}$ and tested again, prior to parametric analysis. For statistical comparisons of data among treatments, 1-way ANOVA followed by Tukey post-test was used, and the Kruskal Wallis test for non-normally distributed data. Differences between control and treatments means were considered significant when $p < 0.05$. All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2015).

3. Results

3.1. Particle characterization

TEM images show that silver nanoparticles are roughly spherical and the average particle size is ca. 29 ± 8 nm (Fig. 1A). SEM analysis further confirms the TEM observations and shows the characteristic silver nanoparticles (Fig. 1B). EDS spectrum reveals strong signal in the silver region and confirms the composition of the AgNP (Fig. 1C). Silver (72.39%) was the major constituent element compared to carbon (9.76%) and oxygen (17.85%) as shown in Table 1. EDS profile showed strong signal for silver along with weak oxygen peak which may have originated from the polyvinyl pyrrolidone molecules that are on the surface of AgNP, as the stabilizing agents. Indeed, these silver nanoparticles are highly stable and only 8% of the silver is released in aqueous media after 24 h, as determined by Atomic Absorption Spectroscopy (Fig. 1D).

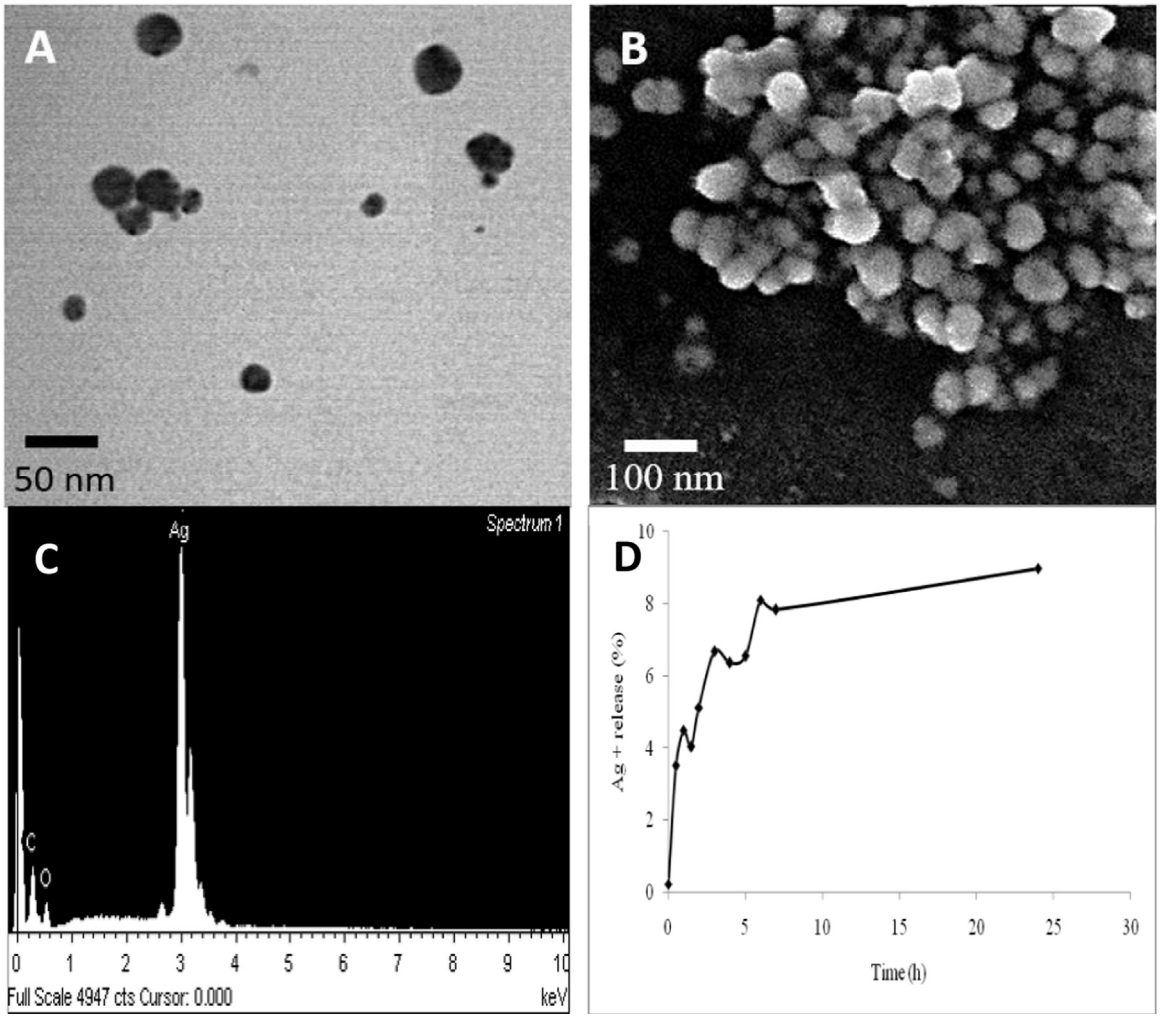

Fig. 1. Transmission electron microscopy (A), Scanning electron microscopy (B), Energy Dispersive Spectroscopy spectrum (C) of AgNP. Percentage of Ag^{+} ion release from AgNP suspension after 24 h, the values are expressed as means ± S.D. (at least triplicate experiments) (D).

Table 1
Energy dispersive spectroscopy (EDS) of silver nanoparticles.

Element	Weight %	Atomic %
C K	9.76	31.25
O K	17.85	42.93
Ag L	72.39	25.82
Totals	100.00	

3.2. Exposure media and tissue silver content

After 48 h (re-dosing time) measured Ag in exposure media were 95–100% of nominal AgNP concentrations (no significant differences between the beginning and the re-dosing time were founded) (Table 2).

No mortality was observed during the exposure periods. In gill tissues, total Ag increased at both AgNP concentration after both times of exposures ($p_{5\ days} = 0.0045$; $p_{15\ days} = 0.0019$). The Ag accumulation in gills after 15 days was five times higher than after 5 days in the case of 25.0 µg AgNP L^{-1}-exposure (Fig. 2).

3.3. Antioxidant and oxidative damage determinations

Gills showed a significant decrease in antioxidant enzymes activities after 5 days of exposure to both AgNP concentrations ($p_{SOD} = 0.0094$, $p_{CAT} = 0.0357$, $p_{GST} = 0.0275$, $p_{GR} = 0.0019$, $p_{GPx} = 0.0011$), but they returned to control levels after 15 days. The total antioxidant capacity diminished (high ΔFU) in fish exposed for 15 days to 2.5 µg AgNP L^{-1} ($p = 0.0060$). LPO levels increased in the case of 25.0 µg AgNP L^{-1} exposure ($p = 0.0467$) after 15 days (Table 3).

Table 2
Concentration of total silver measured in test water by GF AAS (µg Ag L^{-1}). The values are expressed as means ± S.E.

	0 h	12 h	24 h	36 h	48 h
Control	< DL	< DL	< DL	< DL	< DL
2.5 µg L^{-1}	2.47 ± 0.14	2.56 ± 0.23	2.55 ± 0.16	2.50 ± 0.13	2.46 ± 0.10
25.0 µg L^{-1}	25.13 ± 0.16	23.04 ± 0.69	23.78 ± 0.69	24.45 ± 0.37	22.55 ± 0.99

< DL: under the detection limit.

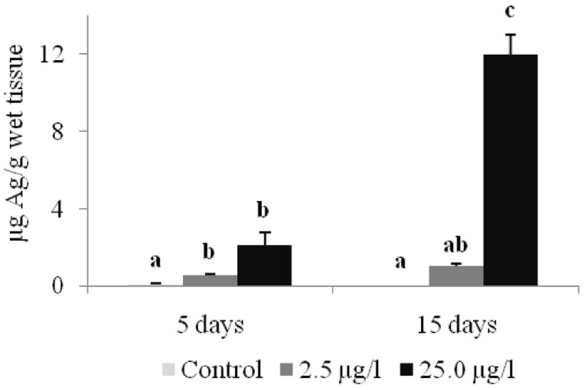

Fig. 2. Silver concentration in gills of *Prochilodus lineatus* after exposure to 2.5 and 25.0 µg AgNP L^{-1} for 5 and 15 days. The values are expressed as means ± S.E. Means not sharing the same superscript (a, b, or c) in each column are significantly at $p < 0.05$.

3.4. Alanine aminotransferase and aspartate aminotransferase

Transaminases activities measured in fish gills are summarized in Table 3. There were no differences in enzymes activities between control and 5 days-exposed fish. Regarding the 15-days exposure, ALT activity decreased in the gills of fish exposed to the lowest concentration ($p = 0.0019$) and AST showed an increase in its activity in the case of 25.0 µg AgNP L^{-1} ($p = 0.0213$).

3.5. Histopathological analysis

All results are summarized in Table 4. Regarding the morphometric analysis, SLL length decreased after 2.50 µg AgNP L^{-1} and 5 days ($p = 0.0001$). After 15 days in the case of the highest concentration there was an increase of the SLW ($p < 0.0001$) and ID ($p_{apex} = 0.0320$, $p_{basis} < 0.0001$) values. ID-medium increased but after 5 days ($p < 0.0001$) at 25.0 µg AgNP L^{-1}. However, no changes were identified with respect to the PAGE index.

Gill alterations included hemorrhage, aneurisms, hypertrophy and the presence of fusions (Fig. 3). These observed pathological changes were classified into two reaction patterns: circulatory disturbances (rp_1), and progressive changes (rp_3). The calculated reaction pattern indexes (Irp_1; Irp_3) did not show significant differences among treatments. The I_{gills} increased significantly in the case of the highest concentration and time of exposure ($p = 0.0407$).

In the case of the MCc, a dose-dependent increase of the number was detected (2.50 and 25.0 µg L^{-1}) after 15 days of exposure ($p < 0.0001$) (Table 4, Fig. 3).

4. Discussion

4.1. Particle characterization

The AgNP employed were spherical in shape with size ranging around 29 nm as observed in SEM/TEM. In addition, these silver nanoparticles are highly stable and only small amount of Ag^+ ions were released to the aqueous media, highlighting the effect of the PVP stabilizing agent. Indeed, the silver ions release reaches the equilibrium after 24 h without further increase after that time. The size of particles obtained by the analysis coincided with the described by the manufacturer. It is important to highlight the silver nanoparticles of different sizes and with or without different stabilization agents show different degrees of toxicity in different organisms, under different exposures times and conditions (Taju et al., 2014). This type of heterogeneous AgNP population may be present in commercial products like the used in this study, and this situation is the real scenario regarding the release of NP to the environment. Scown et al. (2010) studied different size of AgNP in *Oncorhynchus mykiss* and stated the smallest ones (10 nm) were the most bioaccumulated. Further studies should be developed in order to understand this major concern with respect to the environmental risk assessment of NP and their toxicity specifically related to the nano-size of particles and their intrinsic properties (Ribeiro et al., 2014).

4.2. Ag accumulation

The measurement of silver concentration in the exposure media did not show differences in 48 h (re-dosing time) in comparison to nominal AgNP concentrations. This result agrees with Ribeiro et al. (2014) who described the AgNP as reasonably stable for two weeks and registered 91% nominal concentration. In this study has occurred a similar situation, where test water samples represented 95–100% of nominal concentrations. However, some authors registered the 40–50% of the nominal concentration after different time of exposure (Scown et al., 2010; Griffitt et al., 2013; Bacchetta et al., 2017). It is known that the states of aggregation and dissolution in environmental and biological media are dependent on how nanosilver is prepared, what types of surface coating are used, and condition media (McShan et al., 2014). So it could be expected different results among studies.

There was observed a dose and time-dependent Ag bioaccumulation in the gill of exposed fish, as it was expectable. Gills are considered one of the tissues with highest blood flow (Barron et al., 1987), so they could be specifically affected by toxics. High levels of Ag were founded in gills in *Danio rerio* exposed to AgNP after short times of exposure (Griffitt et al., 2008; Bacchetta et al., 2016), and Farmen et al. (2012) found accumulation of Ag in gills of *Salmo salar* after 48 h and 100 µg AgNP L^{-1}. Wu and Zhou (2012) analyzed increased levels in *Oryzias*

Table 3
Oxidative stress parameters and transaminases activities in gills of *Prochilodus lineatus* after exposure to 2.5 and 25.0 µg AgNP L^{-1} for 5 and 15 days. The values are expressed as means ± S.E. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$.

	5 days			15 days		
	Control	2.5 µg L^{-1}	25.0 µg L^{-1}	Control	2.5 µg L^{-1}	25.0 µg L^{-1}
SOD	141.65 ± 19.96 ^{a}	72.05 ± 9.38 ^{b}	76.57 ± 10.20 ^{b}	95.72 ± 15.56	130.48 ± 15.29	99.65 ± 7.98
CAT	12.87 ± 1.62 ^{a}	7.41 ± 0.82 ^{b}	9.54 ± 1.14 ^{ab}	13.67 ± 3.28	10.58 ± 1.54	12.57 ± 2.91
GST	292.6 ± 41.59 ^{a}	159.21 ± 18.05 ^{b}	181.17 ± 27.12 ^{b}	146.37 ± 19.54	201.70 ± 42.02	191.94 ± 7.37
GR	62.69 ± 8.81 ^{a}	30.65 ± 3.14 ^{b}	44.67 ± 7.22 ^{b}	53.05 ± 16.43	49.28 ± 10.51	49.22 ± 4.18
GPx	355.53 ± 34.36 ^{a}	196.15 ± 19.49 ^{b}	230.40 ± 37.65 ^{b}	277.67 ± 50.11	289.48 ± 48.78	223.85 ± 15.83
ACAP	0.25 ± 0.02	0.37 ± 0.11	0.35 ± 0.05	0.62 ± 0.06 ^{a}	2.48 ± 0.69 ^{b}	0.56 ± 0.09 ^{a}
LPO	0.28 ± 0.09	0.31 ± 0.07	0.47 ± 0.04	0.88 ± 0.06 ^{a}	1.15 ± 0.16 ^{ab}	1.48 ± 0.26 ^{b}
ALT	3.5 ± 0,42	3.51 ± 0.66	4.63 ± 0.54	5.26 ± 0.14 ^{a}	3.76 ± 0.34 ^{b}	5.02 ± 0.2 ^{a}
AST	29.09 ± 3.08	33.03 ± 2.59	30.13 ± 3.77	35.14 ± 2.75 ^{a}	40.29 ± 3.27 ^{ab}	47.82 ± 2.46 ^{b}

SOD: superoxide dismutase (U SOD mg $prot^{-1}$); CAT: catalase (U mg $prot^{-1}$); GST; gutathione S-transferase (mU mg $prot^{-1}$); GR: glutathione reductase (mU mg $prot^{-1}$); GPx: glutathione peroxidase (mU mg $prot^{-1}$); ACAP: antioxidant capacity against peroxyl radicals (ΔFU, fluorescence units); LPO: lipid peroxidation (nmol TBARS mg $prot^{-1}$); ALT: alanino-aminotransferase (mU mg $prot^{-1}$); AST: aspartate-aminotransferase (mU mg $prot^{-1}$).

Table 4
Morphometric analysis and histopathological indexes in gills of *Prochilodus lineatus* after exposure to 2.5 and 25.0 μg AgNP L^{-1} for 5 and 15 days. The values are expressed as means ± S.E. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$.

	5 days			15 days		
	Control	2.5 μg L^{-1}	25.0 μg L^{-1}	Control	2.5 μg L^{-1}	25.0 μg L^{-1}
SLL (μm)	31.39 ± 0.67 [a]	27.85 ± 0.82 [b]	31.72 ± 0.63 [a]	32.54 ± 0.54	33.54 ± 0.73	34.76 ± 0.70
SLW (μm)	4.22 ± 0.15	3.88 ± 0.09	3.76 ± 0.09	3.85 ± 0.09 [a]	3.79 ± 0.11 [a]	4.42 ± 0.10 [b]
ID (μm)						
Apex	7.49 ± 0.36 [a]	7.13 ± 0.26 [a]	7.87 ± 0.25 [b]	8.63 ± 0.41 [a]	9.91 ± 0.52 [ab]	10.19 ± 0.43 [b]
Medium	6.17 ± 0.28 [a]	6.77 ± 0.26 [a]	7.78 ± 0.23 [b]	7.61 ± 0.31	8.58 ± 0.34	8.60 ± 0.35
Basis	8.60 ± 0.35 [a]	8.40 ± 0.22 [b]	7.73 ± 0.19 [b]	8.29 ± 0.23 [a]	8.89 ± 0.30 [a]	10.22 ± 0.30 [b]
BET (μm)	7.62 ± 0.45 [ab]	8.23 ± 0.65 [a]	6.52 ± 0.17 [b]	5.72 ± 0.18 [ab]	5.92 ± 0.25 [a]	6.65 ± 0.28 [b]
PAGE	81.12 ± 0.90	77.21 ± 1.71	82.48 ± 0.54	84.81 ± 0.48	84.39 ± 0.72	85.07 ± 0.52
Irp_1	9.50 ± 1.19	9.50 ± 1.44	9.25 ± 0.75	12.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00
Irp_3	13.00 ± 4.33	13.50 ± 4.50	20.50 ± 2.60	1.00 ± 0.71	1.50 ± 1.50	10.00 ± 3.65
I_{gills}	22.50 ± 3.23	23.00 ± 3.14	29.75 ± 1.93	12.33 ± 0.33 [a]	13.50 ± 1.50 [ab]	22.00 ± 3.65 [b]
MCc	45.75 ± 4.01	36.00 ± 3.94	35.75 ± 11.97	8.50 ± 0.65 [a]	20.50 ± 1.89 [b]	107.25 ± 11.52 [c]

SLL: secondary lamellar length; SLW: secondary lamellar width; ID: interlamellar distance; BET: basal epithelium thickness; PAGE: proportion of the secondary lamellae available for gas exchange; Irp_1 and Irp_3: reaction indexes (reaction patterns 1 and 3, respectively), I_{gills}: organ index; MCc: mucus cells count.

latipes gills after 14 days and 0.24 mg AgNP L^{-1}.

It results remarkable that considering the low AgNP concentrations used in this study, the Ag from nanoparticles widely bioaccumulated in gill tissue. Handy et al. (2008) explained that NP can be trapped in the mucus layer of the gills of be absorbed by the gill epithelial cells. The mucus layer surrounding the gill epithelia could act as a barrier and reduce NP uptake by the gills. In this study the major Ag accumulation was observed in gills exposed to 25.0 μg AgNP L^{-1}, and at the same time it was analyzed the highest increase of mucus cells (Table 4). So, it would be interesting further studies that separate the Ag quantification in tissue from the trapped Ag in mucus cells.

There are other hypotheses that explain potential routes of AgNP absorption. Gaiser et al. (2009) studied the potential uptake of NP via gastrointestinal tract and explained that ingestion is a viable route of uptake into different epithelial cells. So, much of the uptake of the AgNP into the fish may have occurred as a consequence of the fish

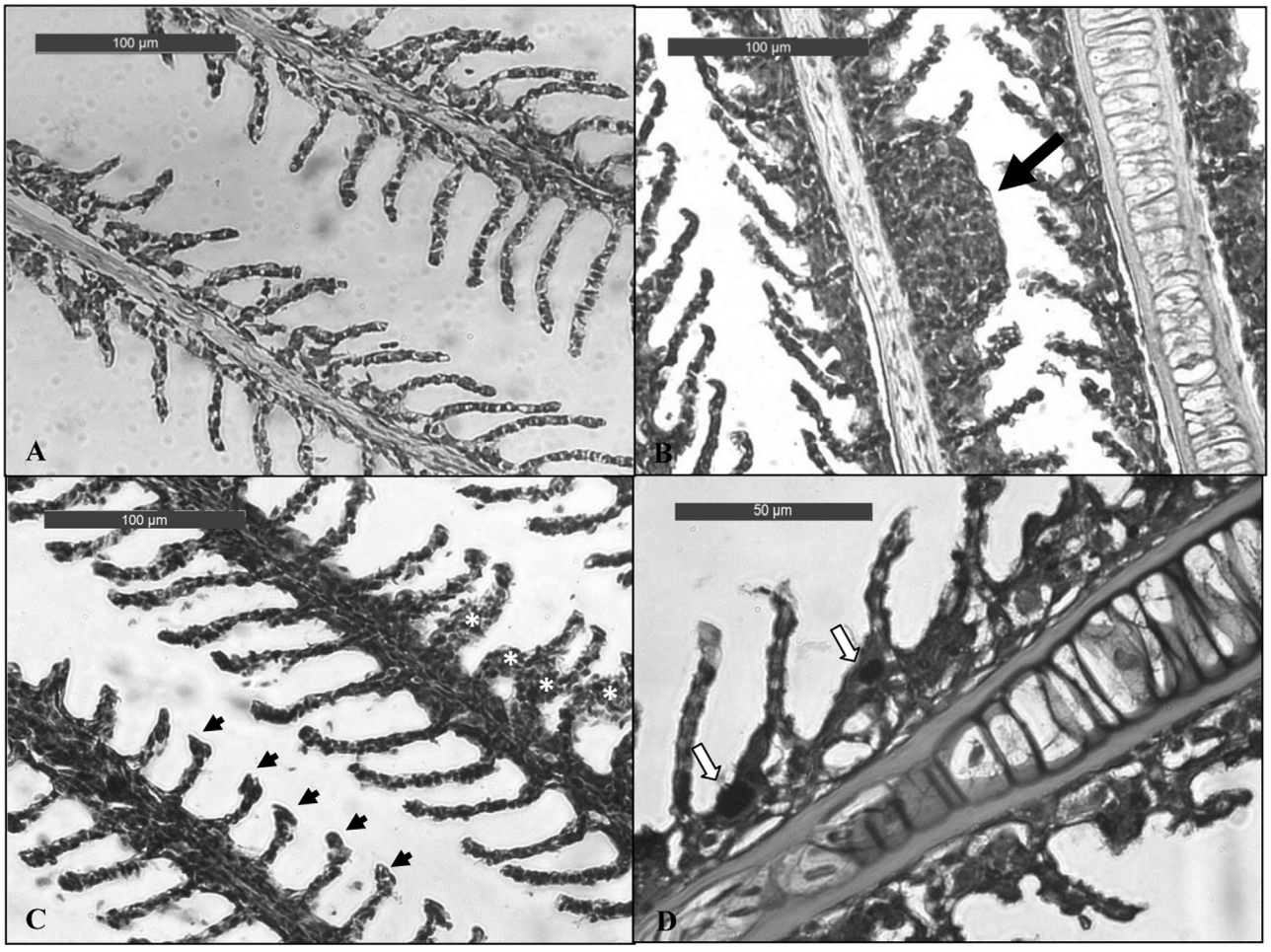

Fig. 3. Cross-sections of gills of *Prochilodus lineatus* after exposure to 2.5 and 25.0 μg AgNP L^{-1} for 5 and 15 days. Normal gill structure showing primary and secondary lamellae without lesions (A); total fusion (black arrow) of gill lamellae (B); lamellar aneurysm (arrow head) and hemorrhages (asterisks) (C); mucus cells (white arrows) (D).

eating agglomerated NP material, rather than uptake via the water through the gills. Bergin and Witzmann (2013), studied mammals and 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.

4.3. Oxidative stress

There is large evidence that one of the main mechanisms of AgNP toxicity is that they cause oxidative stress through the generation of reactive oxygen species, cause damage to the cell membrane and generate lipid peroxidation (McShan et al., 2014; Taju et al., 2014). In gills of *Prochilodus lineatus* was founded an inhibition of all enzymatic activities after 5 days of both concentrations of AgNP exposures. Similar results were reported by other authors. Lee et al. (2012) reported a decrease of GST activity under short but at higher concentrations to AgNP. Taju et al. (2014) exposed gill cells of *Labeo rohita* and *Catla catla* to low concentrations of AgNP for 96 h (0, 2, 4, 8, 16, 32 and 64 μg ml), and found both a reduction of SOD and CAT activities, and an increase in LPO levels. Besides, the authors founded depletion of GSH, which may alter the redox status of the cell and present a stressful and toxic situation since GSH plays a critical role in maintaining cellular redox homeostasis. In a recent research, Martin et al. (2016) exposed *Perca flavescens* to 1 and 100 μg AgNP L^{-1} for 96 h and 10 days and found increased LPO levels, with the highest ones after 10 days. In this study, after 5 days and 25.0 μg AgNP L^{-1} the enzymes suffered an inhibition of their activities, but at the 15 days-exposure the enzymatic antioxidant system did not activate so LPO levels increased leading to oxidative damage. Kennedy et al. (2014) explained in a review a mechanism of toxicity by AgNP because of the strong affinity of the Ag^{+} for sulfhydryl, amino, carboxylate, imidazole, and phosphate groups, although competition with enzyme cofactors may also contribute. This mechanism involves denaturing of proteins and enzymes leading cellular disruption like structural abnormalities.

There is little research that includes ACAP levels. Amado et al. (2009) emphasize the importance of understanding how antioxidants interact with ROS through the determination of the total antioxidant capacity, instead of the measurement of limited number of antioxidants. As was mentioned before, ACAP resulted decreased in gills after 2.5 μg AgNP L^{-1} and 15 days of exposure, what actually indicate that nanosilver impairs the ability of gills to cope ROS and therefore a decreased capacity of cells to neutralize peroxyl radicals. As evidenced by augmented LPO levels, this reduction of antioxidant capacity could let this organ susceptible to suffer oxidative stress. Monserrat et al. (2014) exposed *Jenynsia multidentata* to endosulfan and found a loss of antioxidant capacity in the liver after 8 days. Guerreño et al. (2016) exposed two fish species to azinphosmethyl pesticide, and analyzed an induction of ROS production in gills. Thus, this biomarker resulted sensitive in terms of toxics and complements the measurement of antioxidant enzymes activities. Choi and Hu (2008) exposed nitrifying bacteria to different AgNP sizes and analyzed the relationship between nitrification inhibition and intracellular ROS accumulation. The authors found an overall increase in intracellular ROS concentration, and even stated that Ag^{+} ions generated less ROS than that by AgNP. The degree of inhibition by AgNP was strongly correlated with intracellular ROS concentration. These results agree with the obtained in the present study in order to testify that AgNP accumulate and generate ROS production, which was evidenced in the case of gill cells exposed to 2.5 μg AgNP L^{-1} for 15 days. As the increased LPO levels in the case of 25.0 μg AgNP L^{-1} exposure, this situation may be related to the lack of activation by the antioxidant enzymatic system after 15 days. In other words, the cell detoxifying mechanisms were not activated, so peroxyl radicals accumulated into the cells.

Massarsky et al. (2013) found in *Danio rerio* embryos exposed to AgNP an increase of ROS through a lack of antioxidant enzymes activation. However, a reduction of glutathione level was observed, which was not considered in the present study. The measurement of this non enzymatic biomarker may contribute to the explanation of the high LPO levels and the decrease of ACAP after 15 days of AgNP-exposure.

4.4. Transaminases

An inhibition of ALT activity after 2.5 μg AgNP L^{-1} and an induction of AST activity after 25.0 μg AgNP L^{-1} were observed in the case of 15-days exposure. De Smet and Blust (2001) explained that in gills of fish, an elevated activity of transaminases is associated with an increased protein breakdown, suggesting a functional response to deal with extra energy requirements to cope with the stress. Experiences using *Danio rerio* and *Cyprinus carpio* exposed to different kinds of pollutants resulted in an increase of both AST and ALT enzyme activities and these changes resulted in alterations in the gills, indicating a functional and structural damage (David et al., 2004; Zhang et al., 2016).

MacInnes et al. (1977) explained that aminotransferases inhibition in liver of Cd-exposed fish could be due to the metal interaction with the biosynthesis of pyrodoxal phosphate, a molecule that is an essential requirement for the normal functioning of the enzymes. Moreover, the authors also suggested that in higher metal concentrations the activity may therefore increase in order counter the energy crisis during stress. There is little information about the role of the enzymes in gill tissue, however those conclusion may explain the ALT inhibition in the case of 2.5 μg AgNP L^{-1} exposure, and the AST induction in the case of the highest one. In addition, the results of the present study support that changes in aminotransferases activities when it is accompanied with histological changes in the gills are important parameters for assessing the influence of exposure to toxic substances in fish.

4.5. Histopathology

The I_{org} presented an increase in gill of fish exposed to 25.0 μg AgNP L^{-1} for 15 days. According to the other results, these exposure conditions seemed to be the most harmful ones for fish. Bernet et al. (1999) enhanced the value of the organ index, because an increased of it represents high degree of damage. Gills histopathological responses were according with other biomarkers such as the high levels of Ag in tissue and the increase of LPO levels. This finding is result, mainly, of the increased number of fusions and hypertrophies observed in AgNP-exposed fish. Schlenk and Benson (2001) enhanced the importance of gills as a target organ of toxic compounds due to gills exhibit large surface which are in direct and permanent contact with irritant. It has been reported histological damage (mild congestion of blood vessels, fusion and hyperplasia) at 50 mg L^{-1} concentration in *Oreochromis mossambicus* gills after high AgNP concentrations (25, 50 and 75 mg L^{-1}) for 8 days (Govindasamy and Rahuman, 2012).

The gills have mucus cells located in epidermal layer of the skin, which are responsible of many protection functions such as excretion of wastes, disease resistance and communication (Shephard, 1994). After 15 days of both AgNP concentrations exposures, the amount of mucus cells significantly increased in gills filaments. The findings agree with Lee et al. (2012), who exposed *Cyprinus carpio* to 50 and 100 μg AgNP L^{-1} and observed an increase of mucus cells after 40 and 96 h. The authors explained the increase of their number as a necessary response of the body to counteract the effects of exogenous chemicals, such as nanoparticles. Thus, it is important to highlight the sensitivity of this biomarker after both acute and subchronic exposure conditions to a broad range of AgNP concentrations. On the other hand, Shephard (1993) reported a review about mucus of fish and explained that results difficult to assess the status of mucus layers in healthy unstressed fish, particularly on the gills. The author suggested that particulate inorganic material may be removed from water and in this way mucus may protect the gill surface from abrasion. In addition, he reported many

cases of increased MCc in fish stressed by the exposition to low pH, acid conditions of the environment, and heavy and essential metals. He explained that mucus layers could be an effective growth medium for parasitic fungal spores. Moreover when the thickness of the mucus-supported unstirred layer is increased, supply of oxygen can become limiting and respiratory stress occurs. MCc is considered as a useful sensitive biomarker widely employed with other kinds of pollutants such as metals, organochlorines and herbicides (Paulino et al., 2012, 2014).

5. Conclusions

As result of the wide AgNP benefits, principally due to their antibacterial proprieties, their production, use, and deposition in environment are exponentially increasing. Thus, in vivo assays that contribute to a better understanding of the impact generated by nanoparticles are valuable. According to our results, native species such as *Prochilodus lineatus* and subchronic exposure conditions to low concentrations of AgNP provided relevant information about the toxic effects generated by AgNP. This study also corroborated the importance of using fish as test species for their sensitivity to xenobiotics, and focused on the gill as a target organ for evaluating toxicity mechanisms. AgNP resulted widely biocumulative, disruptive for the antioxidant defense system, and caused damage to lipids and histophatologies, as well as led to the proliferation of mucus cells. Different harmful effects have been analyzed at both short and subchronic times of exposures, so we highlight the importance of using a large amount of biomarkers and relevant AgNP concentrations while toxicity approach is attempted. Additional studies that include chronic and sublethal conditions of AgNP exposure, and the evaluation of other relevant biomarkers such as glutathione levels (among other non enzymatic parameters), mucus cells Ag uptake, and ionic disruption should be develop in further studies. We consider this report as relevant in order to contribute to a better understanding about how this emerging pollutant is affecting environments.

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