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Integrative assessment of silver nanoparticles toxicity in *Prochilodus lineatus* fish

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ABSTRACT

The aim of this study was to evaluate simultaneously a battery of biomarkers in *Prochilodus lineatus* fish in order to obtain a holistic overview of silver nanoparticles (AgNP) toxicity. Juveniles were exposed to 2.5 and 25.0 μgAgNP L^{-1} for 5 and 15 days. We analyzed silver accumulation in tissues (liver, intestine and brain), morphometric and hematological parameters, oxidative stress markers (antioxidant enzymes, lipid peroxidation, and antioxidant capacity against peroxyl radicals-ACAP-), and metabolic responses in plasma (glucose, proteins, triglycerides and cholesterol), liver and muscle (glycogen, proteins, and lipids). Additionally, after 15-day exposure, the number of colony forming units (CFU) of bacteria living at the skin mucus secretion was analyzed to corroborate the AgNP biocide properties. Tissue Ag accumulation was concentration- and time-dependent: liver > intestine > brain. The hepatosomatic index increased at both periods of exposure at 25.0 μgAgNP L^{-1}. Hematological and plasma biochemical parameters increased after 5 days of exposure to the highest concentration. When fish were exposed for 15 days to 25.0 μgAgNP L^{-1}, many changes were observed: glycogen content increased in liver and muscle, muscle protein concentration decreased, all antioxidant enzymes activities were enhanced and ACAP decreased in liver. The CFU decreased in the mucus of fish exposed to both AgNP treatments when compared to the control group. The multivariate analysis revealed a clear difference in physiological profiles of individuals exposed to the different treatments. Our results showed that AgNP in short and subchronic exposures threaten the health of fish.

1. Introduction

There is a large body of evidence suggesting Nanotechnology as an expanding area of commerce due to the increasing number of products that contain nanoparticles (NP) (Mansoori et al., 2008). Scown et al. (2010) put emphasis on the vulnerability of aquatic environment as many of NP end up in it by intentional and accidental releases or via weathering of products that contain them. Particularly, the unique and efficient biocide properties of silver nanoparticles (AgNP) make them broadly used in health and fitness products (Lee et al., 2012). AgNP incorporated into spray, textiles, bandages and energetic applications have a higher release potential than those fully integrated into composites (Kennedy et al., 2014). According to the Nanodatabase (www.nanodb.dk) and The Project on Emerging Nanotechnologies (www.nanotechproject.org), a number of 378 and 442 commercial products containing AgNP were registered respectively in 2018.

The scarce understanding of the harmful effects of NP on the environment and their interaction with biotic and abiotic components has been raising concern over the last decades. AgNP can cause an effect called the Trojan horse, in which Ag^{+} ions are released inside tissues or cells exerting responses different from those of the AgNP or the silver salt (Bermejo-Nogales et al., 2016). It has been suggested that the AgNP concentrations in the aquatic environment are in the order of μg L^{-1} or ng L^{-1} (Gottschalk et al., 2013), though these concentrations are still toxic to aquatic organisms (Garner et al., 2015; Sayed and Soliman, 2017). Unfortunately, precise estimates of emanations from silver-containing materials are hindered by the lack of information about the content and form of such metal in products (Geranio et al., 2009).

Fish species have been widely used in studies for assessing the biological and biochemical impact of environmental contaminants (van der Oost et al., 2013). When AgNP come into contact with aquatic organisms, these particles easily penetrate their tissues or are retained at

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their external surfaces where they can also cause damage (Frabega et al., 2011). This metal nanoparticles are easily bioaccumulated in external and internal tissues such as gills, brain, liver, and gills of fish (Jung et al., 2014; Gagné et al., 2012; Wu and Zhou, 2012; Ale et al., 2018) exerting different toxic effects. In many species, exposure to AgNP has caused hematological (Khan et al., 2017a; Clark et al., 2018) and histological (Govindasamy and Rahuman, 2012; Lee et al., 2012; Rajkumar et al., 2016; Khan et al., 2017a; Ale et al., 2018) alterations; mobilized the store reserves in liver and skeletal muscles (Gagné et al., 2012; Massarsky et al., 2014a); and disrupted the endocrine system (Degger et al., 2015). Moreover, AgNP affect the development of organisms (Wu and Zhou, 2012), and induce metallothioneins and DNA damage (Choi et al., 2010; Martin et al., 2016). One of the most studied effects caused by AgNP is the generation of reactive oxygen species (ROS) (McShan et al., 2014), which alters the activity of antioxidant enzymes leading to oxidative damage in vital organs such as the liver and gills (Lee et al., 2012; Massarsky et al., 2013; Khan et al., 2016, 2017b).

It is scarcely known about the AgNP effects on mucus layer of fish. The mucus layer is the first line of the immune system defense against pathogens and parasites (Jovanović and Pacić, 2012). It is worth evaluating the AgNP effects on the bacteria colonies living in mucus of freshwater fish since they have antibacterial properties (Soltani et al., 2009; Bacchetta et al., 2016).

The *Prochilodus lineatus* sensitivity to variations in water quality and at the same time their tolerance to laboratory conditions made this species appropriate for the measurement of multiple biomarkers in our study (Cazenave et al., 2014; Simonato et al., 2016). It also has an important value in the regional economy and human diet. We aimed at analyzing a battery of biomarkers simultaneously in order to obtain an integrative assessment of the AgNP toxicity. We hypothesized that through the waterborne exposure, AgNP enter into the tissue of fish, bioaccumulate in their organs, and generate toxic effects. We expected to find oxidative damage; and changes in morphological and hematological parameters, in the energy reserves; and biocide effects on the bacteria community living at the mucus layer of *P. lineatus*. To the best of our knowledge, this is the first report providing a holistic overview of the toxicity caused by AgNP.

2. Materials and methods

2.1. Nanosilver preparation and characterization

Under the brand name nanArgen®, Nanotek S.A. provided a colloidal suspension of 1% w/v AgNP (10.000 ppm). According to the Material Safety Data Sheet, the average particle size is 20–40 nm. The capping agent is made of glucose oligomers, mainly nanocrystalline cellulose (CAS Number 9004-34-6). Regarding the synthesis of the nano-sized silver colloid, silver nitrate was dissolved in Millipore water to a concentration of 0.20 M, and mixed with an aqueous solution of 0.1 M polyvinyl pyrrolidone 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.

As was previously described in Ale et al. (2018), AgNP were characterized in terms of Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), Transmission Electron Microscopy (TEM), release of Ag^+ ions, and quantification of total recoverable Ag in water samples.

2.2. Fish and exposure conditions

Juvenile *Prochilodus lineatus* were obtained from a local fish farm (n = 120; 6.81 ± 0.08 cm standard length; 6.58 ± 0.23 g). The acclimation period lasted two weeks, and the laboratory conditions consisted of 120-l tanks with well aerated dechlorinated water (pH 6.58; conductivity 178.0 mS/cm^3; total hardness 48.01 ppm CO_3Ca; alkalinity 60.0 ppm CO_3Ca) and *ad libitum* feeding once per day with dry commercial pellets. Laboratory conditions corresponded to 12:12 h light-dark cycles and temperature of 25 ± 1 °C. All experiments were conducted in accordance with national and institutional guidelines (CONICET, 2005) for the protection of animal welfare.

A total of 40 fish per treatment were exposed to the following concentrations: 0 (control); 2.5 and 25.0 μgAgNP L^{-1} under semi-static conditions. Those concentrations were selected according to both previous reports (Bacchetta et al., 2016, 2017) and the LC50-96h value estimated as 53.84 μgAgNP L^{-1} (confidence interval: 35.29–82.18) (data not published).

Each 10-l aquarium contained 2 fish, which were considered as a unique experimental unit (each treatment was replicated twenty times). The AgNP were directly added to the aquaria from the stock solution. The aquarium solutions were renewed every 48 h by transferring the fish to another aquarium.

After 5 days, 20 fish per treatment were sampled, and the other remaining 20 fish were dissected after 15 days. Before the dissection, fish were anesthetized and measured, weighted, and sacrificed. Mucus samples of fish exposed during 15 days were isolated before dissection and inoculated in agar. Blood was extracted by dissection of the caudal peduncle (Reichenbach-Klinke, 1980). Liver, intestine, brain and muscle were immediately frozen and stored at −80 °C until biochemical determinations were carried out. All the reagents used for the determinations were purchased from Sigma-Aldrich®.

2.3. Tissue silver content

The quantification of total recoverable Ag in tissue samples (liver, intestine and brain; n = 4 per treatment per time) was performed according to the method described by the US EPA (1991). Samples were digested by adding concentrated nitric acid, heating to 95 °C. Then, 30% hydrogen peroxide was dosed, and the samples were then diluted with 0.1 N HNO_3. In accordance with the method 200.9 (US EPA, 1994), Ag was then quantified in tissue samples using a graphite furnace atomic absorption spectrophotometer (GF AAS, Perkin Elmer AAnalyst 800) equipped with an autosampler (limit of detection on the digested sample: 1.0 μgAg L^{-1}). Three readings of each run were recorded.

2.4. Morphometric and hematological biomarkers

Condition factor (CF) and hepatosomatic index (HSI) were calculated according to Goede and Barton (1990). Red blood cells (RBC) count was performed with a Neubauer chamber. Hematocrit (Ht) values were determined by the micromethod using capillary tubes and centrifuged at 1409*g* for 10 min. Hemoglobin concentration (Hb) was measured by the cyanmethemoglobin method (Houston, 1990). Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated from primary indexes according to Cazenave et al. (2005). A drop of freshly collected blood was smeared on clean slides to estimate the total white blood cells (WBC) counts and for determination of leukocyte frequency through the methods proposed by Tavares-Dias and Ruas de Moraes (2007). All determinations were performed in 10 fish per treatment per time.

2.5. Plasma metabolites and tissue energetic reserves

Plasma levels of total protein, cholesterol, triglycerides and glucose were determined (n = 10) with enzymatic colorimetric methods using the appropriate kits and according to the protocols of the manufacturer Wiener Lab® (Rossi et al., 2017). Muscle and liver glycogen content (n = 4) was analyzed following the Anthrone method (Seifter et al., 1950); protein by Folin's method (Lowry et al., 1951) and lipid after

chloroform/methanol extraction (Folch et al., 1957). All biochemical analyses were measured in triplicate. The estimation of liver glycogen after 5 days of exposure was not possible to determinate because of the lack of tissue.

2.6. Antioxidant and oxidative damage determinations

Enzyme extracts from the tissues (n = 4) were prepared according to Bacchetta et al. (2014). The activities of antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione S-transferase (GST, EC 2.5.1.18), glutathione peroxidase (GPx, EC 1.11.1.9), and glutathione reductase activity (GR, EC 1.6.4.2) were measured according to Misra and Fridovich (1972), Beutler (1982), Habig et al. (1974), Drotar et al. (1985), and Tanaka et al. (1994), respectively.

Total antioxidant competence against peroxyl radicals (ACAP) (n = 4) was determined according to Amado et al. (2009) with modifications proposed by Monserrat et al. (2014). Lipid peroxidation (LPO) levels (n = 4) were determined by measuring the formation of thiobarbituric reactive substances (TBARS) according to Yagi (1976).

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

2.7. Bacterial colony forming units count

Bacterial communities living in the mucus of *P. lineatus* were isolated from the right flank and analyzed according to Marques et al. (2013). After 15 days of exposure, mucus of fish from each treatment (n = 10) was isolated with loops of 5 µl passed twice (10 µl in total) and transferred to test tubes containing 1300 µl of sterilized water (dilution 1/130). Then, 100 µl of each sample were inoculated in Petri dishes containing Trypto-Casein-Soy Agar (Biokar Diagnostics) and incubated at 25 °C for 7 days. The colony forming units (CFU) were counted daily.

2.8. Statistical analyses

Data are reported as mean ± standard error. Shapiro-Wilks and Levene's test were applied to evaluate normality and homogeneity of variance, respectively. Variables without normal distribution were transformed using $\log_{10}$ and tested again, prior to parametric analysis. For statistical comparisons of data among the treatments, 1-way ANOVA followed by Tukey post-test was used for normally distributed data, and the Kruskal Wallis test for non-normally distributed data (all data was normally distributed but: neutrophils percentage and GPx activity in intestine after 5 days of exposure). Differences between control and treatments means were considered significant when p-values were below 0.05. Additionally, principal component analysis (PCA) was performed in order to obtain a holistic interpretation of AgNP toxicity. Multivariate analysis was carried out taking into account four cases per treatment per time (N = 24) and 21 variables (those which showed significant differences with respect to the control group were considered). All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2015).

3. Results

3.1. Nanosilver characterization

The particles were roughly spherical, and the average size was determined as 29 ± 8 nm through the TEM analysis, and the SEM one confirmed this observation. EDS spectrum determined the composition of the AgNP: 72.39% silver, 9.76% carbon, 17.85% oxygen. Only 8% of silver was released after 24 h. After 48 h (re-dosing time) Ag in exposure media was 95–100% of nominal AgNP concentrations. Further details are shown in Ale et al. (2018).

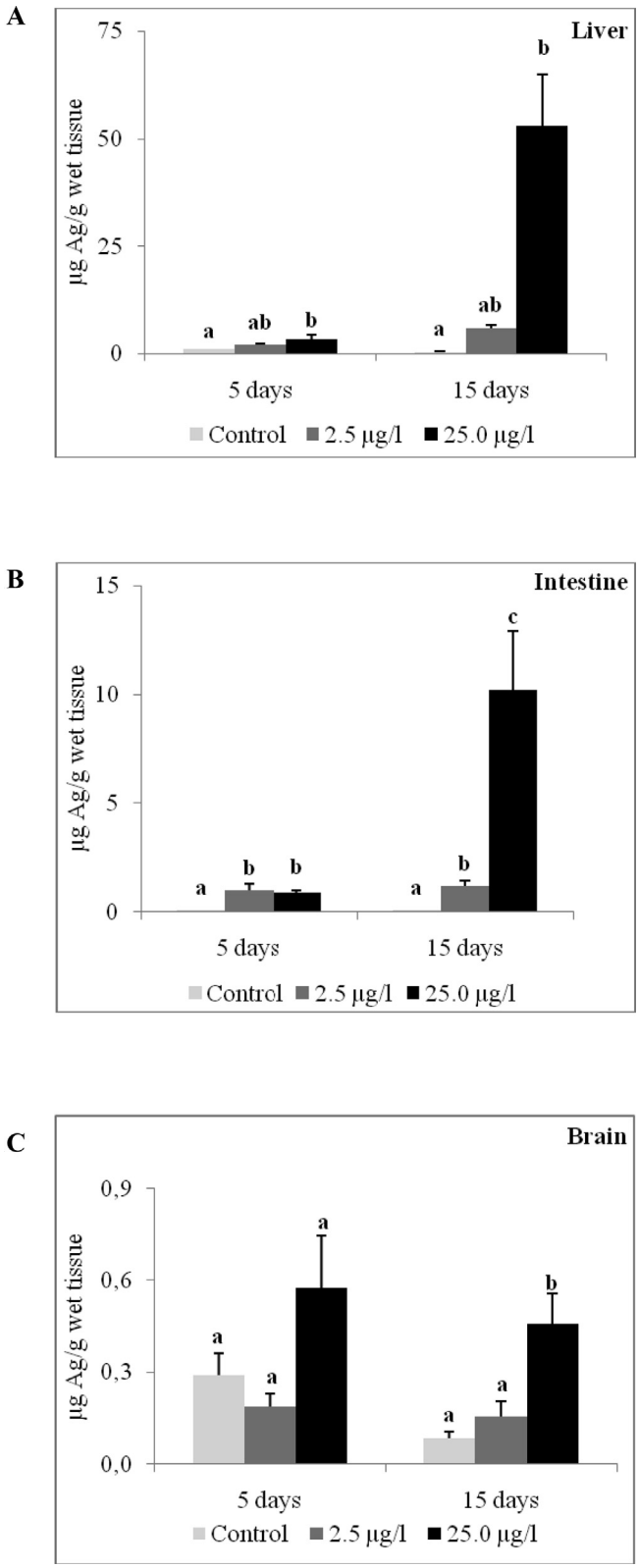

Fig. 1. Silver concentration in (A) liver, (B) intestine and (C) brain of *Prochilodus lineatus* after exposures to 2.5 and 25.0 µgAgNP L^{-1} for 5 and 15 days. The values are expressed as means ± SE. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$.

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

	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}
HSI	0.44 ± 0.04^a	0.41 ± 0.05^a	0.75 ± 0.04^b	0.48 ± 0.03^a	0.49 ± 0.04^a	0.73 ± 0.07^b
CF	2.14 ± 0.06	2.15 ± 0.03	2.09 ± 0.04	2.07 ± 0.07	1.95 ± 0.07	1.92 ± 0.06
RBC (10^6 μl $^{-1}$)	3.81 ± 0.16^a	3.83 ± 0.16^a	4.61 ± 0.17^b	2.06 ± 0.09	2.00 ± 0.14	2.23 ± 0.12
Ht (%)	43.77 ± 1.83	44.79 ± 1.65	47.03 ± 1.55	40.85 ± 1.56	39.07 ± 2.66	41.29 ± 1.39
Hb (g dL^{-1})	9.55 ± 0.35	8.90 ± 0.24	10.09 ± 0.43	9.34 ± 0.28	8.35 ± 0.58	9.25 ± 0.37
MCV (μm^3)	118.48 ± 8.59	118.32 ± 8.93	116.55 ± 8.06	193.84 ± 10.85	194.32 ± 15.63	172.96 ± 11.39
MCH (pg)	25.13 ± 1.37	23.50 ± 0.94	21.46 ± 2.04	46.45 ± 1.53^a	41.09 ± 2.04^{ab}	38.80 ± 2.99^b
MCHC (%)	21.72 ± 0.54^a	19.76 ± 0.41^a	25.20 ± 0.86^b	23.89 ± 0.79	21.45 ± 1.01	22.39 ± 0.32
WBC (μL)	7687 ± 992	6290 ± 1460	7532 ± 1777	1709 ± 227^a	2914 ± 484^a	6947 ± 722^b
Lymphocytes (%)	89.21 ± 8.67	83.495 ± 7.26	73.86 ± 10.40	93.06 ± 5.30^a	88.1 ± 6.22^a	69.84 ± 8.68^b
Neutrophils (%)	0.28 ± 0.62	0.56 ± 1.24	1.12 ± 1.17	0	0	0
Eosinophils (%)	5.62 ± 5.29	5.66 ± 5.56	0.81 ± 1.26	0	0.90 ± 1.34	0.72 ± 0.66
Monocytes (%)	4.89 ± 4.09^a	10.30 ± 4.24^a	24.21 ± 10.97^b	6.94 ± 5.29^a	11.00 ± 6.43^a	29.44 ± 8.66^b

3.2. Tissue silver content

Ag accumulation in tissue increased at both exposure times and AgNP concentrations according to the following sequence: liver > intestine > brain (Fig. 1). The liver followed a dose-dependent response pattern at both periods ($p_{5\ days} = 0.0130$; $p_{15\ days} = 0.0001$) and total Ag concentration was sixteen-fold higher after 15 days than after short period of exposure at 25.0 μgAgNP L^{-1} (Fig. 1A). When compared to control group, increased intestine Ag content was evidenced at both concentrations and exposure times; in the case of 25 μgAgNP L^{-1} the accumulation was eleven-fold higher after the 15-day period, in comparison to the 5-day one ($p_{5\ days} = 0.0002$; $p_{15\ days} < 0.0001$) (Fig. 1B). Finally, the brain showed a significant increase in Ag concentration after 15 days of exposure to 25.0 μgAgNP L^{-1} when compared to control group ($p = 0.0041$) (Fig. 1C).

3.3. Morphometric and hematological biomarkers

Results are summarized in Table 1. All significant changes occurred at the highest AgNP concentration tested. After both periods of exposure an increased HSI was detected ($p_{5\ days} > 0.0001$, $p_{15\ days} = 0.0012$). RBC ($p = 0.0021$) and MCHC ($p = 0.0005$) increased after 5-day exposure. After 15 days, MCH decreased ($p = 0.0391$), WBC increased ($p = 0.0001$) and the percentage of lymphocytes decreased ($p = 0.0004$). Monocytes levels showed a rise after both periods of exposure tested ($p_{5\ days} = 0.0032$; $p_{15\ days} = 0.0006$).

3.4. Plasma metabolites and tissue energetic reserves

Results are shown in Table 2. Significant changes were observed at the highest AgNP concentration tested. Plasma glucose, protein and triglycerides levels increased after 5 days of exposure ($p < 0.0001$, $p = 0.0055$ and $p = 0.0072$, respectively). Only triglycerides remained elevated after 15 days ($p = 0.0002$). Cholesterol levels did not show differences among treatments. Liver and muscle glycogen levels increased ($p_{liver} = 0.0001$, $p_{muscle} = 0.0002$) and muscle protein content decreased ($p = 0.0014$) after 15 days of exposure.

3.5. Antioxidant and oxidative damage determinations

Antioxidant enzyme activities, TBARS and ACAP levels in liver, intestine and brain are summarized in Table 3. In liver there was an increase of all antioxidant enzymes activities when fish were exposed to 25.0 μgAgNP L^{-1} for 15 days ($p_{SOD} = 0.0002$, $p_{CAT} = 0.0022$, $p_{GST} = 0.0002$, $p_{GR} = 0.0070$, $p_{GPx} = 0.0157$). Moreover, ACAP decreased in this group of fish ($p = 0.0276$) and TBARS showed no difference when compared to control group. When fish were exposed 5 days to 2.50 μgAgNP L^{-1}, an increment of CAT activity ($p = 0.0034$) and LPO levels ($p = 0.0039$) were observed. Intestine showed only decreased SOD activity ($p = 0.0411$) after 5 days exposure to the higher concentration of AgNP. Finally, in brain SOD and GST activities augmented after 15 days exposure to 25.0 μgAgNP L^{-1} ($p = 0.0120$, $p = 0.0036$ respectively). Both tissues, intestine and brain, did not

Table 2
Plasma metabolites and tissue energetic reserves (liver and muscle) of *Prochilodus lineatus* exposed to 2.5 and 25.0 μgAgNP L^{-1} for 5 and 15 days. The values are expressed as means ± SE. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$. NM: not measured, wt: wet tissue.

	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}
Plasma						
Glucose (g L^{-1})	0.38 ± 0.03^a	0.51 ± 0.05^a	0.91 ± 0.06^b	0.45 ± 0.06	0.42 ± 0.05	0.63 ± 0.03
Proteins (g dL^{-1})	3.43 ± 0.12^a	3.42 ± 0.03^a	4.08 ± 0.15^b	10.99 ± 1.66	15.64 ± 2.10	15.92 ± 1.48
Triglycerides (g L^{-1})	1.66 ± 0.17^a	2.07 ± 0.32^{ab}	3.14 ± 0.09^b	0.65 ± 0.16^a	0.23 ± 0.02^a	2.29 ± 0.61^b
Cholesterol (g L^{-1})	4.29 ± 0.11	3.96 ± 0.22	4.63 ± 0.07	3.29 ± 0.49	2.77 ± 0.40	2.85 ± 0.16
Liver						
Glycogen (μmol g wt^{-1})	NM	NM	NM	246.44 ± 5.63^a	247.54 ± 41.13^a	733.96 ± 86.32^b
Proteins (mg g wt^{-1})	144.59 ± 16.06	142.47 ± 13.50	117.20 ± 5.50	170.14 ± 35.06	132.78 ± 14.20	121.31 ± 3.36
Lipids (μmol g wt^{-1})	12.928 ± 0.36	10.57 ± 1.66	11.26 ± 1.09	8.82 ± 0.89	10.76 ± 0.65	9.14 ± 0.21
Muscle						
Glycogen (μmol g wt^{-1})	0.37 ± 0.06	0.35 ± 0.04	0.37 ± 0.06	0.55 ± 0.03^a	0.63 ± 0.03^a	1.46 ± 0.17^b
Proteins (mg g wt^{-1})	86.01 ± 9.64	75.14 ± 9.12	71.08 ± 11.83	143.66 ± 6.82^a	126.97 ± 15.17^a	58.02 ± 13.54^b
Lipids (μmol g wt^{-1})	5.78 ± 0.76	5.24 ± 0.28	5.41 ± 0.54	4.13 ± 0.50	3.53 ± 0.32	3.40 ± 0.17

Table 3
Activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx), and ACAP and LPO levels in liver, intestine and brain of *Prochilodus lineatus* after exposures to 2.5 and 25.0 μgAgNP L^{-1} for 5 and 15 days. Enzyme activities are expressed in U SOD mg $prot^{-1}$ (SOD), U mg $prot^{-1}$ (CAT), and mU mg $prot^{-1}$ (GST, GR and GPx). ACAP is expressed in ΔFU (fluorescence units) and LPO in nmol TBARS/mg prot. The values are expressed as means ± SE. Means not sharing the same superscript (a, b, or c) in each column are significantly different at $p < 0.05$.

	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}
Liver						
SOD	169.18 ± 24.97	98.57 ± 17.14	113.54 ± 4.15	138.15 ± 25.36^{a}	102.20 ± 7.33^{a}	352.83 ± 34.96^{b}
CAT	47.09 ± 2.90^{a}	56.49 ± 11.18^{b}	41.75 ± 4.07^{a}	54.49 ± 5.86^{a}	49.67 ± 5.56^{a}	108.36 ± 29.59^{b}
GST	154.76 ± 35.46	176.62 ± 14.13	180.74 ± 23.52	257.29 ± 26.29^{a}	253.56 ± 32.47^{a}	616.85 ± 148.74^{b}
GR	52.44 ± 6.77	42.29 ± 3.54	41.49 ± 4.23	38.30 ± 4.45^{a}	41.41 ± 3.35^{a}	133.75 ± 30.94^{b}
GPx	204.39 ± 30.72	171.59 ± 31.25	165.59 ± 18.26	215.06 ± 30.07^{a}	167.52 ± 22.39^{a}	605.13 ± 144.21^{b}
TBARS	0.45 ± 0.06^{a}	0.69 ± 0.05^{b}	0.42 ± 0.03^{a}	0.43 ± 0.07	0.40 ± 0.06	0.45 ± 0.03
ACAP	2.32 ± 0.28	2.46 ± 0.21	2.48 ± 0.14	2.01 ± 0.14^{a}	2.47 ± 0.06^{ab}	3.21 ± 0.42^{b}
Intestine						
SOD	152.13 ± 33.93^{a}	85.71 ± 9.28^{ab}	50.79 ± 10.58^{b}	121.7 ± 10.32	113.48 ± 21.90	73.97 ± 8.71
CAT	11.89 ± 2.33	8.35 ± 1.47	6.83 ± 0.27	10.35 ± 2.35	10.59 ± 2.30	7.92 ± 1.14
GST	310.95 ± 71.26	266.93 ± 22.89	282.99 ± 37.07	237.92 ± 19.83	361.96 ± 99.08	268.50 ± 70.27
GR	52.94 ± 14.80	38.05 ± 6.09	59.33 ± 10.12	34.84 ± 5.31	39.50 ± 4.96	39.11 ± 6.34
GPx	349.45 ± 77.67	269.20 ± 46.25	364.77 ± 58.03	205.04 ± 12.28	230.21 ± 48.15	194.51 ± 26.94
TBARS	0.89 ± 0.07	0.66 ± 0.07	0.95 ± 0.10	1.99 ± 0.20	1.57 ± 0.13	1.74 ± 0.12
ACAP	1.20 ± 0.15	1.01 ± 0.19	1.23 ± 0.09	1.65 ± 0.30	1.77 ± 0.42	1.60 ± 0.31
Brain						
SOD	132.57 ± 8.60	157.29 ± 11.43	157.34 ± 15.56	106.06 ± 12.15^{a}	104.32 ± 6.06^{a}	190.14 ± 30.52^{b}
CAT	8.83 ± 0.24	10.06 ± 2.46	8.12 ± 1.32	6.8 ± 0.70	5.48 ± 0.07	7.52 ± 1.14
GST	162.53 ± 30.80	212.61 ± 45.95	159.87 ± 27.11	118.13 ± 16.55^{a}	105.93 ± 12.07^{a}	187.46 ± 31.23^{b}
GR	54.85 ± 2.28	71.21 ± 7.71	57.65 ± 14.21	45.03 ± 5.74	28.03 ± 5.04	45.73 ± 8.28
GPx	216.70 ± 34.90	297.71 ± 60.93	226.35 ± 30.99	170.95 ± 4.78	149.79 ± 4.22	220.88 ± 38.50
TBARS	0.40 ± 0.12	0.24 ± 0.03	0.50 ± 0.12	0.18 ± 0.004	0.13 ± 0.00	0.16 ± 0.02
ACAP	0.55 ± 0.08	0.58 ± 0.07	0.42 ± 0.07	0.53 ± 0.05	0.75 ± 0.02	0.68 ± 0.07

present differences in ACAP or LPO levels when compared to control group.

3.6. Bacterial colony forming units count

Significant differences were analyzed among the treatments regarding the CFU count of bacteria (Fig. 2). After 1 day, the mucus inoculated from the control fish, showed less amount of CFU than exposed fish ($p = 0.0118$). From day 2 to day 7, the amount of CFU decreased significantly ($p < 0.0001$) in AgNP-exposed fish in comparison to the control group.

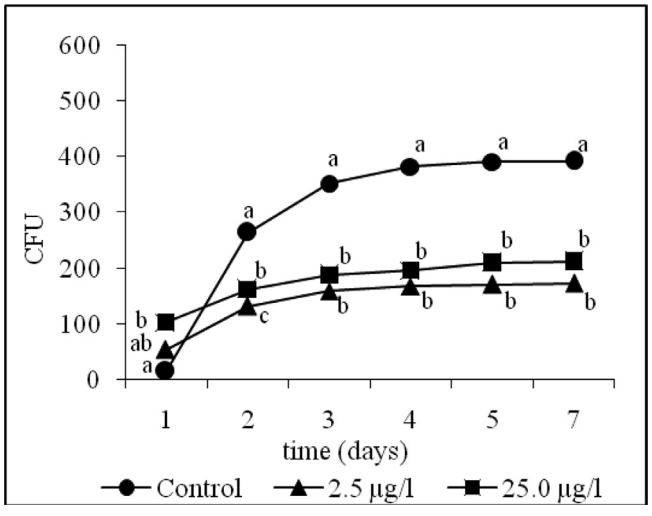

Fig. 2. Bacteria growth (CFU/day) obtained from samples of the mucus of *Prochilodus lineatus* after exposures to 2.5 and 25.0 μgAgNP L^{-1} for 15 days. The values are expressed as means ± SE. Means not sharing the same superscript (a, b, or c) are significantly different at $p < 0.05$. CFU: Colony forming units.

3.7. Multi-biomarker approach

Two components were extracted (Fig. 3) from the principal component analysis (PCA). The components explained 54.6% of the global inertia in the data. The first (PC1) and the second (PC2) axes accounted for 32.7% and 21.9% of the variance, respectively. The significance of the correlation coefficients obtained was stated according to Legendre and Legendre (1979). The coefficients higher than 0.63 were considered a good interpretation of the variables. Positive loadings in PC1 were shown for monocytes, muscle glycogen, and Ag accumulation in all tissues; while PC2 shown only MCH. Negative correlations were shown in PC1: lymphocytes and muscle proteins; and also in PC2: RBC, WBC, and triglycerides in plasma. Overall, PC1 evidenced a marked difference among the treatments (Control, 2.5 and 25.0 μgAgNP L^{-1}), meanwhile PC2 showed differences between times of exposure (5 and 15 days).

4. Discussion

We assessed a battery of biomarkers simultaneously in the fish *Prochilodus lineatus* in order to obtain an integrated overview of AgNP toxicity. First, we analyzed Ag accumulation in different organs. The major bioaccumulation occurred in the liver, followed by intestine and brain of fish exposed to 25.0 μgAgNP L^{-1} during 15 days. These results are in agreement with several studies involving different types of AgNP, form and surface coating, sizes of particles, concentrations, and periods of exposure (Jung et al., 2014; Gagné et al., 2012; Wu and Zhou, 2012). Different forms of Ag are distributed throughout the circulatory system and mainly accumulated in the liver (Panyala et al., 2008). Thus, the xenobiotic exchange between blood and hepatocytes is maximized, making the liver an early target for many toxicants (Di Giulio and Hinton, 2008).

Other tissues accumulate much less Ag than the liver. For example, gills (Ale et al., 2018) and intestine (present work) of *P. lineatus* accumulated four-fold less Ag than the liver. Johari et al. (2015) exposed *O.*

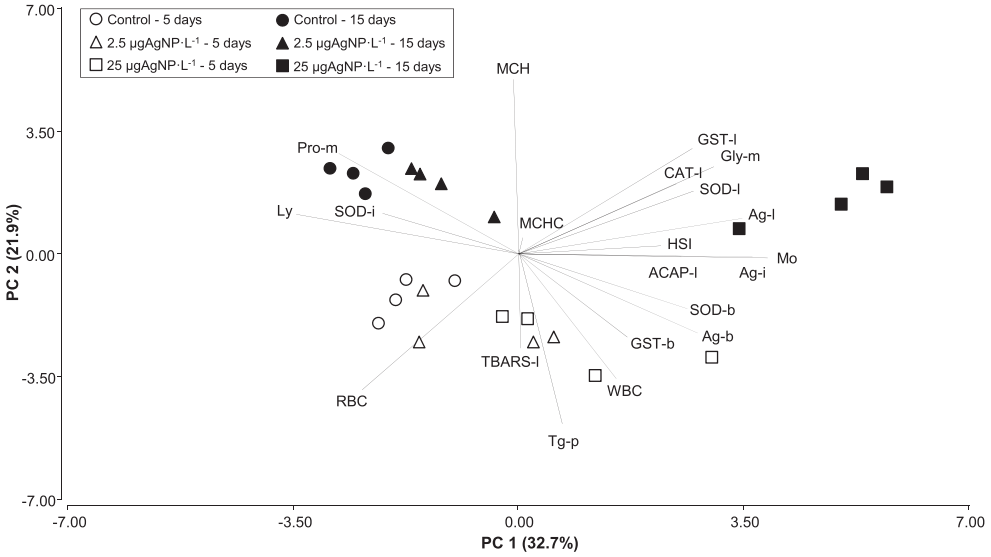

Fig. 3. Representation of the biomarkers (in letters) and individuals (in forms, n = 4 per treatment per time) onto the first factorial plane of the principal component analysis (PCA). All biomarkers abbreviations are explained in the text except for: Ag (total Ag level), Mo (monocytes), Ly (lymphocytes), Gly (glycogen), Pro (proteins), Tg (triglycerides). The small letters in reference to the biomarkers mean: l (liver), i (intestine), b (brain), m (muscle), p (plasma).

mykiss to colloidal and powered AgNP to 0.1–1 mg L^{-1} for 21 days and found a dose-dependent increase of Ag accumulation in liver, gills and intestine. They stated that the amount of water drank by freshwater fish could increase several folds under stress, pointing out that one of the principal AgNP routes of entry could be the gastrointestinal tract.

In this study, an increase of Ag accumulation in the brain of fish exposed to the highest AgNP concentration for 15 days was observed. Bacchetta et al. (2017) also found high Ag levels in the brain of *Piaractus mesopotamicus* exposed to 10.0 and 25.0 µgAgNP L^{-1}. Pretto et al. (2010) found high levels of cadmium in the brain of *Rhamdia quelen*, and stated that saturation of hepatic storage capacity stimulates metal accumulation in other organs. The presence of Ag in the brain can generate neurological damage in fish, such as alterations in embryonic development and larval behavior (Powers et al., 2011).

Although Ag bioaccumulation results allow us to hypothesize about the possible uptake routes and target organs, the AgNP toxic effects on the fish health have not been revealed yet. In this context, we have proposed an assessment of multiple biomarkers responses. One of them is the hepatosomatic index (HIS) which represents a measurement of both liver growth status and energy reserves (Chellappa et al., 1995). Besides, the condition factor (CF) can provide an insight into the overall fish health (Murray et al., 2017). In the present study no significant difference was observed in the CF, while HSI increased after both exposure times at the highest AgNP concentration. In agreement with our results, juvenile rainbow trout exposed to 0.28 mg L^{-1} and 47.60 mg AgNP L^{-1} (on average) for 28 days showed no significant differences in CF (Murray et al., 2017). Nevertheless, no pattern of the effect of AgNP exposure on HSI has been discerned among different studies: in rainbow trout exposed to 32 mg L^{-1} to 32000 mg L^{-1} AgNP for 14 days, HSI significantly increased (Joo et al., 2013) while it decreased when they were exposed to 3000 mg L^{-1} AgNP for 8 weeks (Massarsky et al., 2014b). Considering the low AgNP concentrations used in this study, we can state that the *P. lineatus* is more sensitive to this nanometal than other species. High HSI values indicate that the liver may increase its capacity to metabolize xenobiotics under AgNP exposure to 25.0 µgAgNP L^{-1} for both 5 and 15 days.

Few studies have considered hematological parameters for analysing the AgNP toxicological mechanisms (Shaw and Handy, 2011). Shaluei et al. (2013) reported several blood changes in *Hypophthalmichthys molitrix* exposed to AgNP (0.02–0.04 mg L^{-1}), indicating an acute anemia caused by AgNP. In our study, RBC and MCHC values increased after 5 days of exposure to 25.0 µgAgNP L^{-1} and only MCH decreased in *P. lineatus* after 15 days. However, mean values recorded in *P. lineatus* for these variables are within the normal physiological range for this species (Parma de Croux, 1994). Similarly, Clark et al. (2018) found no consistent changes in the circulating blood cells in rainbow trout exposed to low AgNP concentrations, concluding that AgNP generated moderate disturbances to red blood cells, which did not affect the physiological capability of fish.

As regards WBC, their count was increased after 25.0 µgAgNP L^{-1} for 15 days. This result agrees with Imani et al. (2015), who observed elevated levels of WBC in rainbow trout exposed to AgNP (0.1–0.4 mg L^{-1}) for 8 days. Leucocytosis is a normal fish reaction to substances altering their normal physiological processes (Takashima and Hibiya, 1995). Shaluei et al. (2013) stated that AgNP had an immediate stimulatory effect on the immune system, which might be associated with a decrease in fish nonspecific immunity. An increase of WBC could be related to primary infections caused by a toxicant and secondary infections contracted after feeble conditions. Moreover, lymphopenia and monocytosis were observed after 25 µgAgNP L^{-1} for 15 days. Lymphocytes are the most important cells in immune response and their decrease can be caused by stressful conditions. As monocytes are active macrophages (Takashima and Hibiya, 1995), monocytosis in *P. lineatus* could indicate a response against infections and also inflammatory processes.

An increase in all plasma parameters was observed after 5 days of exposure to 25.0 µgAgNP L^{-1}, and only triglycerides (Tg) remained elevated after chronic exposure. All enhanced plasma metabolites provide an increase of energy sources so that fish may be able to overcome emergency situations (Barton, 2002). Under stressful conditions, primary and secondary responses occur in fish. The release of the stress hormones like cortisol into the bloodstream leads to changes in blood and tissue chemistry (Begg and Pankhurst, 2004). This primary response mobilizes and elevates glucose production, which is mostly mediated by both stimulation of liver gluconeogenesis and liver and muscle glycogenolysis pathways (Iwama et al., 1999; Nelson and Cox, 2005). Several researchers have reported elevated plasma cortisol caused by AgNP exposure in fish (Johari et al., 2013; Shaluei et al., 2013; Murray et al., 2017). Although in the present study cortisol levels were not measured, an increase in glycemia was observed in fish exposed to 25.0 µgAgNP L^{-1}[f] for 5 days. This result was not accompanied by changes in muscle glycogen levels. Thus, the liver gluconeogenesis and glycogenolysis pathways are likely to be responsible for this plasma glucose change. Unfortunately, liver glycogen levels were not analyzed at that period of exposure. Similarly to our results, Farmen et al. (2012) found an increase of plasma glucose in *Salmo salar* exposed to 100 µgAgNP L^{-1} for 48 h; and Shaluei et al. (2013) found higher glycemia in *H. molitrix* exposed to 0.02 and 0.04 mgAgNP L^{-1} for 3 and

7 days. Massarsky et al. (2014a) also found increased glycogenolysis when rainbow trout hepatocytes were exposed to 10 μgAgNP mL^{-1}. This stimulation was receptor-independent and suggests that AgNP could affect the hormone-regulated cell signaling pathways. In the present study, an increase of glycogen levels in liver and muscle of *P. lineatus* was observed when fish were exposed to 25.0 μgAgNP L^{-1} for 15 days. Differences in experimental conditions and AgNP concentrations could explain these contrasting results. It is important to highlight that a rise in liver glycogen content can increase tissue wet mass by incorporating water into the glycogen complex (MacKay and Bergman, 1932), which might explain the higher HSI observed in *P. lineatus* exposed to 25.0 μgAgNP L^{-1}.

We found significantly higher plasma protein levels in fish exposed to the lowest AgNP concentration for 5 days, suggesting the occurrence of an inflammatory process. Silver nanoparticles can bind to different tissues causing cell activation, ROS production and finally inflammation (Xia et al., 2006; Gagné et al., 2012). This is consistent with the increased levels of TBARS observed in the liver of *P. lineatus* exposed to 2.5 μgAgNP L^{-1} for 5 days. Other authors also found changes in plasma protein levels when fish were exposed to AgNP. Vignesh et al. (2013) reported an increase of serum proteins in *Labeo rohita* exposed to 50 μgAgNP L^{-1} for 21 days. In contrast, Monfared et al. (2015) observed decreased serum total proteins in *Oncorhynchus mykiss* exposed to high AgNP concentrations (3–1000 mg L^{-1}) for eight weeks. Discrepancies among these results can be attributed to several factors such as AgNP concentrations, exposure time, and inter-species differences.

In fish exposed to 25.0 μgAgNP L^{-1} for 15 days, spontaneous amino acids use in catabolic reactions may account for the decreased protein content of muscle to relieve stress (Vignesh et al., 2013). Tissue protein content depends on the dynamic equilibrium between its synthesis and degradation rates. Higher cortisol levels induced by stress increase the proteolysis in the myocytes (Schreck et al., 2016). In addition, hepatic protein levels showed a tendency to decrease after both periods of exposure. Protein catabolism releases free amino acids which are considered one of the main gluconeogenic precursors in mammals and fish (Pretto et al., 2014). Both lower muscle protein and higher plasma amino acids levels could have increased hepatic gluconeogenesis and glycogenesis. We observed increased liver glycogen levels and a slightly higher plasma glucose in fish exposed to 25.0 μgAgNP L^{-1} during 15 days. Other metals like cadmium also generate a strong proteolysis that results in decreased muscle protein levels (Pretto et al., 2014). The hypertriglyceridemia observed in fish exposed to 25.0 μgAgNP L^{-1} during 15 days was accompanied by normal hepatic and muscle lipid content. Plasma Tg are the result of hepatic export system and removal mechanisms in peripheral tissues; but further analysis will be necessary to understand AgNP effects on such metabolic processes.

It is widely known that AgNP have the potential to alter cellular antioxidant defense systems increasing oxidative stress by reactive oxygen species production (McShan et al., 2014; Taju et al., 2014). In this study, an induction of all antioxidant enzymes was registered in the liver of fish exposed to 25.0 μgAgNP L^{-1} for 15 days. Such activation of the antioxidant defense in the liver prevented its oxidative damage. Govindasamy and Rahuman (2012), who exposed *Oreochromis mossambicus* to 50 mgAgNP L^{-1} for 8 days, suggested the SOD-CAT system as a first defense against oxygen toxicity, revealing a strategy to prevent oxidative damage. On the contrary, inhibition of antioxidant enzymes activities in the liver of *Oryzias latipes* embryos (Wu and Zhou, 2012) and *Cyprinus carpio* (Lee et al., 2012), as well as in hepatocytes of *Oncorhynchus mykiss* (Massarsky et al., 2014b) was observed.

Regarding the ACAP, their levels decreased in liver of fish exposed to 25.0 μgAgNP L^{-1} for 15 days. This result indicates an increase of ROS species which the antioxidant enzymatic system could not prevent. Bermejo-Nogales et al. (2016) found an increase of ROS in a fish hepatoma cell line exposed to 100 μgAgNP mL^{-1} and stated that nanoparticles disrupt the mitochondrial electron transport chain and cause deleterious effects at various levels by oxidizing cell constituents, such as lipids, proteins, and DNA.

Only SOD activation was observed in fish intestine after 5 days, evidencing its low sensitivity to AgNP concentrations. However, Atli et al. (2006) considered the intestine as a sensitive organ to oxidative stress since they observed an inhibition of CAT in *O. niloticus* after Ag^{+} exposure (1.0–1.5 mgL^{-1}). It is well-known that toxic effects caused by this ion differ from the nanoparticle form, and though nanoparticles are directly related to silver ions, it remains uncertain the ion release influence on AgNP toxicity (Vignesh et al., 2013).

Finally, an induction of both SOD and GST activities was found in the brain of *P. lineatus* at 25.0 μgAgNP L^{-1} for 15 days. The brain is very susceptible to free radicals oxidative damage as it not only contains high amounts of unsaturated lipids but also utilizes about 20% of total oxygen demand of the body (Halliwell and Gutteridge, 1999). Moreover, this organ has a weak antioxidant defense system. According to Atli et al. (2006), stimulation of CAT activity could be associated with an effective antioxidant defense system that compensates for the inhibition of other antioxidant enzymes. Even though CAT activity of the exposed fish was not different to the control group in our study, the activation of SOD and GST enzymes could be enough to prevent oxidative damage. Bacchetta et al. (2017) exposed *Piaractus mesopotamicus* for 24 h to 10.0 and 25.0 μg L^{-1} using the same nanosilver brand of our study, and found no differences in brain enzyme activities, but LPO levels increased. Lee et al. (2012) also observed lack of activation of the enzymatic antioxidant system in *C. carpio* exposed to AgNP for 48 and 96 h. However, the 15-day exposure seems to be a better approximation to real scenarios, and the activation of antioxidant enzymes generated at this time may reveal a warning sign of neurological damage.

Teleost skin secretes mucus which involves immune functions. Despite the presence of a number of antimicrobial factors, fish mucus is still colonized by bacteria which developed a beneficial biofilm comprising the microbial community (Wilson et al., 2008; Benhamed et al., 2014). The present results demonstrated the antibacterial properties of AgNP since the number of colony forming units (CFU) isolated from the mucus of *P. lineatus* decreased significantly. Similarly, AgNP altered both intestinal and epithelial microbiota in the zebrafish (Merrifield et al., 2013; Bacchetta et al., 2016) and inhibited bacterial growth in the body surface of the estuarine polychaeta *Laeonereis acuta* (Marques et al., 2013; Cordeiro et al., 2014).

We also integrate the results obtained from bioaccumulation and biological responses applying a multivariate analysis. A single biomarker is not enough to reflect the health status of an organism, that is why using a large battery of biomarkers is more appropriate to assess the toxicological effects of xenobiotics (Beliaeff and Burgeot, 2002). According to the principal component analysis, the first principal component (PC1) clearly separated fish exposed to 25.0 μgAgNP L^{-1} for 15 days from other treatments fish. This grouping was mainly defined by the following biomarkers: Ag accumulation in all tissues, metabolic reserves and leukocyte frequency. The second principal component (PC2) separated the 5-day from the 15-day AgNP exposed fish. The most significant variables in this case were hematological parameters and plasma triglycerides. Carrying out chronic studies reflecting real environmental scenarios becomes vital as a subchronic exposure to AgNP revealed different physiological profile of fish. Although, oxidative stress markers are widely reported for explaining the AgNP toxicity mechanisms, they were no significant variables in the PCA. Instead, the main parameters to determine group formation were hematological biomarkers and energetic reserves. We believe that it is worth including hematological, immunological and metabolic responses in order to widen the scope of AgNP impact on environment. Summarizing, this holistic approach provides a more objective assessment of key biomarkers.

5. Conclusions

Our work demonstrated that AgNP are absorbed and

bioaccumulated in all tissues of fish undergoing waterborne exposure. The highest AgNP concentration at the longest exposure time yielded the most deleterious toxicological effects. AgNP threaten the bacterial community living on fish surface mucus, regarded as the main biological barrier and immunological defense. Our results proved that though the liver of *P. lineatus* has a high silver accumulating capacity, it is still able to prevent oxidative damage by activating the antioxidant enzyme system. The proposal of an integrative overview including immunological, hematological and metabolic biomarkers to throw light on AgNP environmental risks represents a challenge for further investigations.

Acknowledgments

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