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Bioaccumulation and oxidative stress caused by aluminium nanoparticles and the integrated biomarker responses in the common carp (*Cyprinus carpio*)

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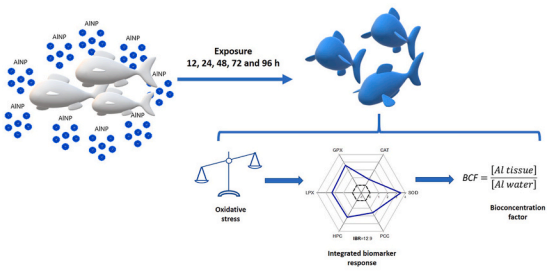
HIGHLIGHTS

- The AlNPs are one of the most widely used nanoparticles in the world.
- Exposure to 50 μg L^{-1} of AlNPs causes significant oxidative damage in liver and gill of common carp.
- The IBRv2 index revealed a high biological response caused by AlNP exposure in liver of *C. carpio*.
- Muscle and gills have a higher bioconcentration factor compared to brain and liver.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of nanoparticles (NPs) in various industries has experienced significant growth due to the advantages they offer, so the increase in their use has generated the continuous discharge of these products in numerous water bodies, which can affect the organisms that inhabit them. Previous studies have shown that Al is capable of producing oxidative stress in aquatic organisms; however, so far the impact of AlNP on hydrobionts is limited.

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Therefore, the objective of this work was to determine the oxidative stress produced by AlNP in liver, gill and blood of *Cyprinus carpio*, as well as their bioconcentration factor (BCF) in various tissues. For this purpose, the organisms were exposed to 50 μg L^{-1} AlNP for 12–96 h. Subsequently, the tissues were obtained and the activity of antioxidant enzymes, oxidative damage to lipids and proteins were determined, and the BCF was calculated for liver, brain, gill and muscle. The results showed alterations in the activity of antioxidant enzymes and increased levels of lipoperoxidation, hydroperoxides and oxidized proteins. When establishing the integrated biomarker response, it was observed that the liver is the most affected organ and these effects are related to the Al content in the tissue. Finally, it was observed that muscle and gills presented a higher BCF, compared to brain and liver. These findings show that AlNP are capable of generating oxidative stress in carp, affecting tissue function and accumulating, which represents an important risk for the health of fish such as common carp.

1. Introduction

Nanoparticles (NPs) are considered the basic components of nanotechnology and refer to particles with at least one dimension smaller than 100 nm (Keck and Müller, 2013). The use of NPs has been around for at least a century under different names, however, there has been a resurgence recently due to the ability to synthesize and manipulate such materials. The incredible surge of interest since 2000 has brought NPs into the focus of application in a wide diversity of areas, ranging from materials science to chemistry, biology, and medicine (Biswas and Wu, 2005; Stark et al., 2015). NPs are divided into several categories according to their morphology, size, and chemical properties. According to physical and chemical characteristics, some of the well-known classes of NPs are carbon-based, metal-based, ceramic-based, semiconductor-based, and polymer-based (Khan et al., 2019).

In particular, the class of metal NPs contains elements such as aluminum (Al), copper, gold, iron, silver, titanium and zinc, among others (Schrand et al., 2010; Shaw and Handy, 2011). In this regard, aluminum nanoparticles (AlNP) are one of the most widely used; in 2005, it accounted for approximately 20% of the global market (Rittner, 2002; Yang et al., 2012). The annual world production of AlNPs in 2010 was 42 000 tons, and this is constantly increasing (Nogueira et al., 2020). The AlNP are widely used, for example, as coatings, propellants and fuels in the military area, as well as coagulating agent in water treatment. They have also been applied in catalysis, structural ceramics for reinforcement, polymer modification, textile functionalization, in medicine as biosensor, biofiltration, drug delivery systems and antigen delivery for immunization (Prakash et al., 2011; Schrand et al., 2010).

Although there is a boom in the use of NPs, several researchers have pointed out the potential risk of their release into ecosystems and the importance of studying the possible adverse effects on biological systems (Biswas and Wu, 2005; Bystrzejewska-Piotrowska et al., 2009; Handy et al., 2008; Moore, 2006). Currently, studies in the area of nanotoxicity have increased, a large part of researchers consider that the toxicity and fates of NPs should be studied before paying too much attention to their applications (Sajid et al., 2015).

Several authors have explored the toxicity of AlNP, mainly in cell lines and mammals, derived from these investigations it has been found that AlNP can cause oxidative stress due to damage to lipid membranes and imbalance in the antioxidant defense system, as well as cell death, mitochondrial damage and genotoxicity (Arab-Nozari et al., 2019; Cheraghi et al., 2017; De et al., 2020; Dong et al., 2019; Shrivastava et al., 2014). For aquatic organisms, toxic concentrations between 0.1 and 900 mg L^{-1} have been described for AlNP, whose effects are characterized by the accumulation of nanoparticles in gills, liver, brain and intestine, accompanied by histopathological changes. In addition, a decrease in Sodium (Na^{+}) Potassium (K^{+}) -ATPase (NKA) activity and an imbalance in ion regulation have been observed in the gills. As well as an increase of oxidative stress and behavioral changes such as reduced swimming, feeding, decreased growth and reproduction (Benavides et al., 2016; Griffitt et al., 2011; Murali et al., 2017, 2018; Nogueira et al., 2020; Vidya et al., 2018). Based on the predictive models of the expected NPs in the environment, it is estimated that these are found at concentrations below 1 mg L^{-1} (Gottschalk et al., 2009). However, most of the reported studies on the toxicity of Al NPs in the literature are performed using higher concentrations and that is why an environmentally relevant concentration was used in the present study.

Metal intoxication, as is the case of Al, can accelerate the production of oxygen species (ROS), so it is expected that these compounds are capable of generating damage in the organism, particularly in aquatic organisms such as common carp (García-Medina et al., 2013; Lee et al., 2019). However, data on the physiological effects of nanometals (NMs) on aquatic organisms are still limited, although several authors mention that their adverse effects will be similar to those produced by dissolved metals. Thus, oxidative stress is one of the responses that has been identified (Gürkan, 2018; Horie and Tables i and 2020; Ozmen et al., 2020; Shaw and Handy, 2011) and due to the oxidative damage caused by NMs, an alteration of the enzymatic antioxidant defense system may occur.

There are some reports that NPs are toxic to aquatic life although the severity depends on a number of factors such as size, type of NPs, load and the species being exposed (Turan et al., 2019). In the case of fish, they are one of the most abundant populations in the aquatic environment, so they are susceptible to any physicochemical alteration of their habitat (Bukhari et al., 2012). In addition, some organs are more affected than others in the same species (Sajid et al., 2015), so it is important to evaluate the response in each organ. When fish come into contact with the NPs, they can be absorbed through the gills, transported to the tissues and organs through the blood, and finally deposited or excreted. Therefore, the concentrations in the different tissues will differ depending on their function and the physicochemical characteristics of the NPs (Murali et al., 2017, 2018). Establishing the bioaccumulation potential of NPs is an important factor in risk assessment. Accumulation can cause high internal concentrations to occur leading to toxicity, even when environmental concentrations are low, so determining the bioconcentration of a toxicant allows us to estimate the long-term effects (Murali et al., 2017; Wassenaar et al., 2020).

In the present work, *Cyprinus carpio* was proposed as a study model because it is an organism frequently used in toxicity studies, in addition to being one of the most widely used fish families in aquaculture and is considered a sentinel organism due to its wide geographical distribution (Farhangi and Jafaryan, 2019; Özcan Oruç and Üner, 2002; Vajargah et al., 2018). In Mexico, this species is one of the most important aquaculture products for consumption by the population. This species has an annual production of 48 thousand tons, mostly through extensive and semi-intensive farming systems, and sometimes these organisms are farmed in contaminated water bodies (CONAPESCA, n.d.; García-Medina et al., 2010). In this sense, the aim of the present work is to determine the oxidative stress produced by aluminum nanoparticles in gills, liver and blood, as well as their bioconcentration factor in gills, liver, brain and muscle in *Cyprinus carpio*.

2. Materials and methods

2.1. Characteristics of aluminum metal nanoparticles

Aluminum nanoparticles (AlNPs) were obtained from Sun Innovations (ITEM SN1201; size range 2–50 nm, average size 18 nm).

2.1.1. Particle size distribution

Prior to measurements, the aluminum powder sample (1000 mg) were introduced in 100 mL of distilled water (stock solution). The suspension was then dispersed by ultrasound (GT-sonic, Mod. P3) for 10 min at a frequency of 40 KHz to avoid sedimentation of the particles in the sample.

The particle sized was evaluated by Mastersizer 3000-Hydro EV (Malvern Instruments Ltd., GB), a wet dispersion laser diffraction particle size analyzer. Before measurement, the 50 μg L^{-1} suspension was prepared by taking 1 mL of the stock solution and adding 200 mL of water (distilled or reconstituted) and then poured into the beaker (500 mL) of the equipment for measurements. The particle size analysis was reported as in histograms of volume percentage. Measurements were performed in triplicate for reconstituted water ($NaHCO_3$: 174 mg L^{-1}, $MgSO_4$: 120 mg L^{-1}, KCl: 8 mg L^{-1}, $CaSO_4{\cdot}2H_2O$:120 mg L^{-1}) and distilled water.

2.1.2. Zeta potential measurements

The zeta potential (ZP) and polydispersion index (PDI) was measured by analyzing 0.1 g of aluminum powder sample in 10 mL of water using the Zetasizer Nano ZS (Malvern Instruments Ltd., GB). Before zeta potential measurements all samples were sonicated (GT-sonic, Mod. P3) for 5 min at 40 KHz, and then the sample was poured into a cell (DTS1060C). This equipment analyzes by means of dynamic light-scattering (DLS). Nine measurements were performed for each type of water (deionized and reconstituted, a total of 18 samples were analyzed).

2.1.3. Atomic force microscopy

For Atomic force microscopy (AFM) analysis, an Innova equipment (Veeco Instruments Inc, USA) was used to evaluate the particle size of the aluminum particles. One sample was dispersed in distilled water (pH = 7) and the second sample was dispersed in reconstituted water (pH = 7.8) and sonicated (GT-sonic, Mod. P3) by 20 min at 40 KHz, then centrifuged at 6000 RPM for 5 min and the supernatant was taken for analysis. Samples were deposited in glass slides and mounted onto an AFM. AFM height and deflection images were obtained in contact mode in air at ambient temperature. For image processing the software Nanoscope Analysis v2.0 (Bruker Nano, Santa Barbara, CA, United States) was used. All sample images were acquired in a scan size of 5 μm and shown in 2D for height images.

2.2. Carp maintenance

Juvenile carp (n = 100) weighing 40 ± 10 g and 13 ± 2 cm in length were transported from the Tiacaque aquaculture center in the State of Mexico to the Aquatic Toxicology Laboratory of the National School of Biological Sciences of the National Polytechnic Institute in Mexico City. The organisms were distributed according to their size in 100 L glass tanks (8–12 carps per container) and acclimatized for a period of three weeks. Filtration systems were installed to keep the water clean and to favor circulation and aeration. They were kept at room temperature (24 °C), with natural light-dark cycles and fed twice a day with high quality food (Nutripec®, 35% protein, 8% fat).

2.3. Experimental design

After acclimatization, 2 experimental groups were formed in plastic aquaria of 120 L capacity; the first group corresponds to the control group (reconstituted water), while the second was exposed to 50 μg L^{-1} AlNP which is equivalent to the maximum permissible limit of aluminum for the protection of aquatic life according to Mexican regulations (DOF, 1989; García-Medina et al., 2013) and according to Pakrashi et al. (2012), this concentration is within the range considered environmentally relevant for AlNPs (50 μg L^{-1} to 100 μg L^{-1}). For each group, 5 fish were exposed in duplicate (n = 10) at different exposure times. Water was maintained at 20 ± 2 °C, 90–100% oxygen saturation, 7.5–8.0 pH, 6.470 ± 0.5 mg L^{-1} dissolved oxygen, ammonia concentration 0.3270 ± 0.02 mg L^{-1} and nitrate 0.2670 ± 0.01 mg L^{-1} over the experimental period. The organisms were maintained with constant aeration in static systems and natural light-dark cycles during exposure. The exposure times were 12, 24, 48, 72 and 96 h. After this time, the organisms were euthanized in an ice bath with 2% lidocaine (Collymore et al., 2016; Gasca-Pérez et al., 2019) and gills, liver and blood were extracted. All procedures were performed in accordance with the Official Mexican Norm NOM- 062-ZOO-1999, technical specifications for the production, care and use of laboratory animals (DOF, 2001) and the Guide for the Care and Use of Laboratory Animals, additional considerations and recommendations for aquatic organisms (Maso and Matthews, 2012). The protocol was submitted for evaluation and approved by the Bioethics Committee of the Escuela Nacional de Ciencias Biológicas, IPN.

The tissues homogenate (gills and liver) was prepared in 1:4 (w:v) ice-cold phosphate buffer (PBS, 0.138 M NaCl, 0.0027 M KCl at pH 7.4) using a homogenizer (LabGEN™ 125, Cole-Parmer) at 15 000 rpm for 5 min. The blood samples were diluted 1:5 (w:v) with ice-cold PBS and homogenized for 30 s at 5000 rpm. The homogenate was divided into two parts, one of the fractions was centrifuged at 14 691×*g* for 15 min at - 4 °C and supernatant was separated. The homogenate and the supernatant were stored in an ultra-refrigerator at −70 °C. The homogenized samples were used to determine lipid hydroperoxides (hydroperoxide content - HPC) and lipoperoxidation degree (malondialdehyde content - MDA), and the supernatant was used to determine total and oxidized proteins (PCC) and the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

2.4. Determination of antioxidant enzyme activity

2.4.1. Superoxide dismutase activity

SOD enzyme activity was determined using the Ransod kit (Randox, catalogue no. SD125). The kit measures the degree of inhibition of the reaction between superoxide radicals with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I·N.T.) to form a red formazan dye. The superoxide radical is generated by the reaction between xanthine and xanthine oxidase (XOD). The method consisted of adding 7.5 μL of the supernatant to 200 μL of the substrate (R1a: Xanthine, I·N.T) and 40 μL of the enzyme (R2: XOD) in a 96 wells plate. The plate was mixed, and absorbance was measured in an ELx 800 reader (BioTek) at 490 nm at 30 s and 210 s. The readings were interpolated in the calibrating curve included in the kit. The data are expressed in U SOD per mg of protein.

2.4.2. Catalase activity

The determination of CAT activity was carried out by the method of Radi et al. (1991). The method consisted in adding 20 μL of the supernatant to 980 μL of the isolation buffer (0.3 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM HEPES, 5 mM potassium phosphate monobasic (KH_2PO_4) and 200 μL of the 20 mM peroxide solution (H_2O_2) prepared at the time. Subsequently, at 0 and 60 s, absorbance was determined at 240 nm in a spectrophotometer (Metash UV-5200). The results were expressed as mM H_2O_2 per mg protein.

2.4.3. Glutathione peroxidase activity

The activity of the GPX enzyme was determined using a Ransel kit (Randox, catalogue no. RS504). This method is based on GPX catalyzing the oxidation of glutathione (GSH) by cumene hydroperoxide. Oxidized glutathione (GSSG) in the presence of glutathione reductase (GR) and NADPH is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$ (Paglia and Valentine, 1967). The method consisted in adding 10 μL of the supernatant to 500 μL of substrate (R1a: GR ≥ 0.5 U L^{-1}, NADPH 0.34 mM reconstituted in buffer R1b: Phosphate buffer 0.05 mM, EDTA 4.3 mM, pH 7.2) and 40 μL of

cumene hydroperoxide 0.18 mM (R2) in a quartz cell. Reagents were mixed and absorbance was measured in a spectrophotometer at 340 nm at 60, 120 and 180 s. The $\Delta A_{120\text{-}180}$ was multiplied by an 8412 factor (provided by manufacturer) and the data are expressed as UL^{-1} of GPX per mg of protein.

2.5. Biomarkers of oxidative damage

2.5.1. Determination of hydroperoxide content

Lipid hydroperoxide levels were established by HPC quantification. To 200 μL of the homogenate, were added 200 μL of 15% trichloroacetic acid and centrifuged at 846×*g* for 10 min. From the supernatant obtained, a 200 μL aliquot was taken and 800 μL of the reaction solution (4 mM butylated hydroxytoluene (BHIT), 0.25 mM ferrous sulfate, 0.1 mM xylenol orange and 250 mM sulfuric acid) was added and incubated for 1 h at room temperature. Finally, the absorbance of the solution was measured at 560 nm in a spectrophotometer (Metash UV-5200) and the values obtained were extrapolated into a calibration curve from a 200 μM cumene reference solution. Results were expressed as nanomoles (nM) of cumene per mg of protein per g of tissue (Jiang et al., 1992).

2.5.2. Determination of lipoperoxidation degree

Lipoperoxidation (LPX) degree were established by MDA quantification. To 300 μL of homogenate supernatant, 700 μL of phosphate buffer solution was added. Subsequently, 2 mL of 0.375% thiobarbituric acid solution in 15% trichloroacetic acid was added. It was incubated at 90 °C for 15 min in a water bath. At the end of the time it was centrifuged at 94×*g* for 10 min and the absorbance was determined at 535 nm. The results are expressed as nM MDA per mg protein (Buege and Aust, 1978).

2.5.3. Determination of oxidized protein content

Quantification of oxidized proteins was carried out using the method of Levine et al. (1994) with modifications. 100 μL of the supernatant was taken and 150 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) was added, incubated for 1 h in darkness at room temperature. Subsequently, 500 μL of 6% trichloroacetic acid was added, allowed to stand for 15 min and centrifuged at 850×*g* for 5 min at 4 °C. Three washes were performed with 1 mL of ethyl acetate-ethanol 1:1, until the sample was clarified and between each wash it was centrifuged at 14 690×*g* for 5 min, the precipitate was dissolved in 1 mL of 6 M guanidine. The absorbance was read at 366 nm and the results were expressed as nM of reactive carbonyls (CO) per mg protein.

All oxidative stress biomarkers were expressed as total protein content per gram of tissue (gill and liver) or per milliliter of blood. Total protein concentration in the samples was determined according to the method of Bradford (1976).

2.6. Integrated biomarker response

The biomarker results (CAT, SOD, GPX, HPC, LPX and PCC) were combined to estimate the Integrated biomarker response index (IBRv2) according to the method outlined by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013) and described in detail in Vieira et al. (2018). Mean IBR values were calculated for each sampling time and tissue, which was then used to evaluate biomarker responses.

2.7. Quantification of Al and determination of the bioconcentration factor

For the quantification of aluminum in water and tissue, 3 fish were exposed in duplicate (n = 6) to 50 μg L^{-1} AlNPs for 96 h, after the exposure time the brain, gills, liver and muscle were extracted. Water samples were taken in triplicate at 0 and 96 h. To 0.5 g or mL of the sample (water or tissue), 2 mL of concentrated nitric acid was added, followed by autoclave digestion at 120 °C and 15 lb pressure, the samples were filtered with Whatman filter papers (grade 40, 125 mm) and diluted with 25 mL deionized water (Fernández-Dávila et al., 2012). Samples were read on graphite furnace atomic absorption spectrometry (Agilent, 240 FS AA) and interpolated on an aluminum type curve. The bioconcentration factor (BCF) was calculated by relating the concentration of aluminum in the tissue and that found in the water.

2.8. Statistical analysis

Data normality and homoscedasticity were verified by Shapiro-Wilk and Bartlett tests, respectively. The data obtained for each biomarker of oxidative stress were as follows processed using a Kruskal-Wallis non-parametric ANOVA analysis, and the significant differences in each group were compared using Dunn's test for multiple comparison with a $p < 0.05$.

For the quantification of aluminum in tissues and water, the mean ± standard deviation value was presented. Finally, a Pearson correlation analysis was performed between biomarkers of oxidative stress and aluminum levels in gills and liver at 96 h. The SigmaPlot 12.3 program was used for statistical analysis.

3. Results

Fig. 1A corresponds to the histogram selected from the measurement of particles dispersed in distilled water. The particle size distribution is bimodal, on average, the first peak showed a particle size of less than 100 nm (43 ± 1.82 nm), representing 56.83% of nanometric particles in relation to the total number of particles counted, and a second peak with average values of 0.38 ± 2.24 μm, representing 43.17% of the total number of particles.

Fig. 1B corresponds to the measurement of particles dispersed in reconstituted water. It can be observed that the particle size distribution is trimodal, the first peak shows a particle size of less than 100 nm (63 ± 2.39 nm), representing 42.1% of the nanometer particles in relation to the total number of particles counted, a second peak with values of 0.57 ± 2.53 μm, and a third peak with values of 2.93 ± 2.26 μm representing 35.3% and 22.6% of the total number of particles, respectively.

The ZP (Table 1) showed a wide distribution when the particles were dispersed in distilled water, being −9.03 ± 1.27 and a PDI index of 0.19 ± 0.10, in the case of reconstituted water these values increased, for ZP it was −9.40 ± 1.64 and PDI of 0.59 ± 0.12.

Fig. 2A, C presents the AFM analysis when using distilled water, the AlNP suspension presents a distribution of 87.95% of nanoparticles smaller than 100 nm, the numerical mean size (NMS) is 64.65 ± 21.43 nm. While for AlNP in reconstituted water (Fig. 2B, D) presented a lower distribution of nanoparticles smaller than 100 nm (65.66%), observing several aggregates, the NMS was 100.23 ± 49.67 nm.

Fig. 3A shows the enzymatic activity of SOD, where a significant increase ($p < 0.05$) of the activity is observed from 12 to 96 h in the gills of the common carp, when comparing the proportion of increase with respect to the control, it was observed that at 12 h there was an increase of up to five times more than the control, for the rest of the times the increase was around double. For the liver, there was no significant difference between the control and the exposed ones, however, a tendency to reduce the activity by about 50% at 12 h was observed ($p = 0.0535$). In blood at 48, 72 and 96 h, a significant decrease of about 75% in SOD enzyme activity was observed with respect to the control.

Fig. 3B shows the enzyme activity of CAT. In the gills, a significant decrease in enzyme activity of 38% at 12 and 96 h and around 75% at 24, 48 and 72 h can be seen with respect to the control. For the liver, fluctuations in enzyme activity can be seen at 12 and 96 h there was a significant reduction of 60 and 80%, respectively. While at 24 and 72 h the significant increase was around double the activity presented in the control group. In blood, the behavior of the enzyme activity is similar between the exposed group and the control at all times.

The enzymatic activity of GPX is presented in Fig. 3C, in the gills it can be seen that the activity increased significantly three times higher than that observed in the control group at 12 h, for the rest of the times

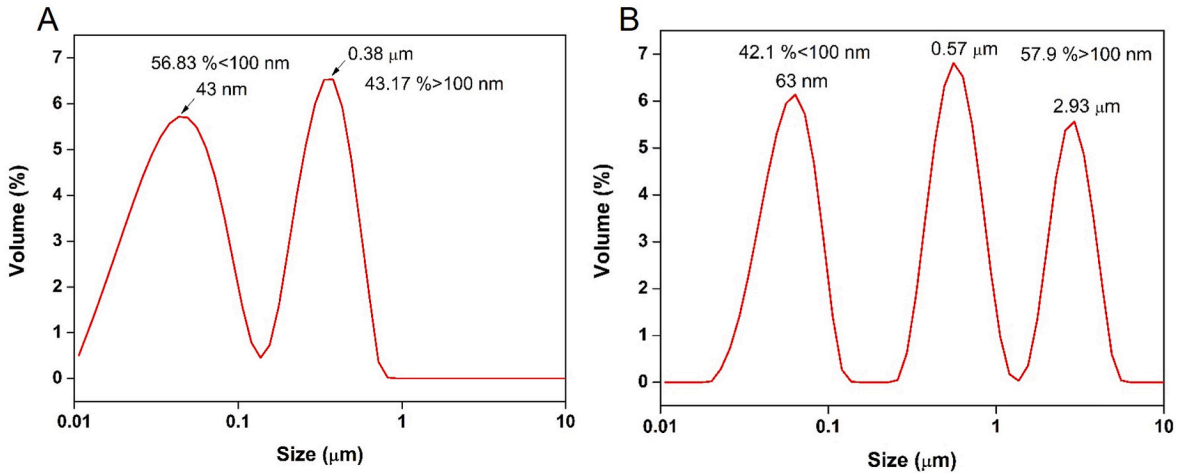

Fig. 1. Particle size histograms of Al-NP dispersed in distilled water (A) and reconstituted water (B).

Table 1
Results of zeta potential and polydispersion index analysis.

# Measure	DW/ZP (mV)	DW/PDI	RW/ZP (mV)	RW/PDI
1	−8.50	0.252	−10.10	0.442
2	−7.30	0.106	−9.70	0.506
3	−10.23	0.164	−13.10	0.634
4	−9.10	0.219	−8.91	0.589
5	−8.62	0.314	−9.07	0.414
6	−7.43	0.345	−8.85	0.745
7	−11.28	0.122	−7.13	0.622
8	−9.63	0.095	−9.48	0.588
9	−9.21	0.102	−8.22	0.736

DW: distilled water; RW: reconstituted water; ZP: Zeta potential; PDI: polydispersion index.

the behavior was similar in both batches. On the other hand, when comparing the activity in the liver between the control group and the organisms exposed to AlNP at all exposure times, a statistically significant increase was observed, this increase was around a range of two and four times more than that of the controls. In the blood samples obtained in the evaluated groups it can be observed that the enzyme activity is similar between 12 and 72 h. In contrast, for fish exposed for 96 h, there was a significant increase of about twice that found in the control group.

Fig. 4A shows the levels of hydroperoxides in each of the tissues studied, for the gills only a significant increase of three times more than that presented in the control group at 12 h was observed. For the liver and blood, a significant increase was observed at all exposure times with respect to the control group; this increase was found within a range of ten and twenty times more than the control group.

The degree of lipoperoxidation is shown in Fig. 4B, where an increase in MDA levels is observed depending on the tissue and time. In the gills, a significant increase can be seen with respect to the control at 12, 24 and 48 h, presenting a maximum of five times more than the control group at 48 h. In the case of the liver, a significant increase is observed at all exposure times tested, with a maximum of seven times more than the control group at 72 h. Blood samples at 24 h showed almost a twofold increase in LPX levels in AlNP-exposed fish compared to control fish.

Finally, the reactive carbonyl content (Fig. 4C) showed a significant increase of almost two times more in fish exposed to AlNP compared to controls at 12 and 24 h in the gills. In the liver, a significant increase was observed throughout the exposure time, with a maximum of almost fivefold increase compared to the control group at 48 h. In contrast, blood showed no significant difference between fish exposed to AlNPs and controls at all exposure times.

The IBRv2 was estimated for each tissue and exposure time. Fig. 5 shows the results of this analysis, where it can be seen that the tissue with the highest value was the liver, followed by the gills and finally the blood. The time where a greater affectation was observed was at 12 h for the liver and blood, while for the gills it was at 96 h.

The Al concentration in water at 0 h was 69.05 ± 12.65 and 96 h was 51.95 ± 10.06 μg L^{-1}. Table 2 shows the BCF for each tissue analyzed. It can be seen that the main organ that accumulates the greatest amount of aluminum is the muscle, followed by the gills, brain and liver.

Table 3 shows the Pearson correlation matrix, which shows that there is a significant and positive correlation between the biomarkers of oxidative stress and the aluminum levels in the gill and liver at 96 h.

4. Discussion

Intoxication by metals, as is the case of Al, can accelerate the production of ROS, so it is expected that these compounds are capable of generating damage to the organism, particularly in aquatic organisms such as common carp (García-Medina et al., 2013; Lee et al., 2019). On the other hand, data on the physiological effects of nanometals (NM) in aquatic organisms is still limited, several authors mention that they have well-known adverse effects such as dissolved metals, so oxidative stress is one of the responses that have been identified (Gürkan, 2018; Ozmen et al., 2020; Shaw and Handy, 2011) and due to oxidative damage caused by NMs, disruption of the enzymatic antioxidant defense system (SOD, CAT and GPx) may occur.

Antioxidant defense system enzymes can be induced by increased ROS production as a protective mechanism against oxidative stress or inhibited when a deficiency of the system occurs (Wang et al., 2012). In this regard, when carp were exposed to 50 μg L^{-1} of AlNP, significant changes in the activity of antioxidant enzymes were observed in the tissues studied. For SOD, the enzyme responsible for dismutation superoxide to peroxide, it is clearly seen that there is an induction from the first times of exposure in the gills and liver and a reduction in activity after 48 h in blood. An increase in SOD indicates a generation of superoxide radical anion, whereas a reduction can occur through direct oxidative damage of SOD molecules, or through the expression of the SOD gene altered by oxidative stress, mechanisms (Varadinova et al., 2016). If oxidative stress is not strong, or very prolonged, SOD activity increases, and if oxidative stress persists or its level is very high, protein damage becomes profound and SOD activity may decrease, as observed in gills and blood. On the other hand, CAT converts hydrogen peroxide (H_2O_2) into water and oxygen, acting like SOD in the first line of enzymatic defense against oxidative stress induced by pollutants, such as metals (Borković-Mitić et al., 2013). In CAT activity, there are different changes among the tissues and times observed. In the gills, a significant reduction was observed from the first hours. For the blood samples at 24 and 48 h, a tendency in the reduction of activity was

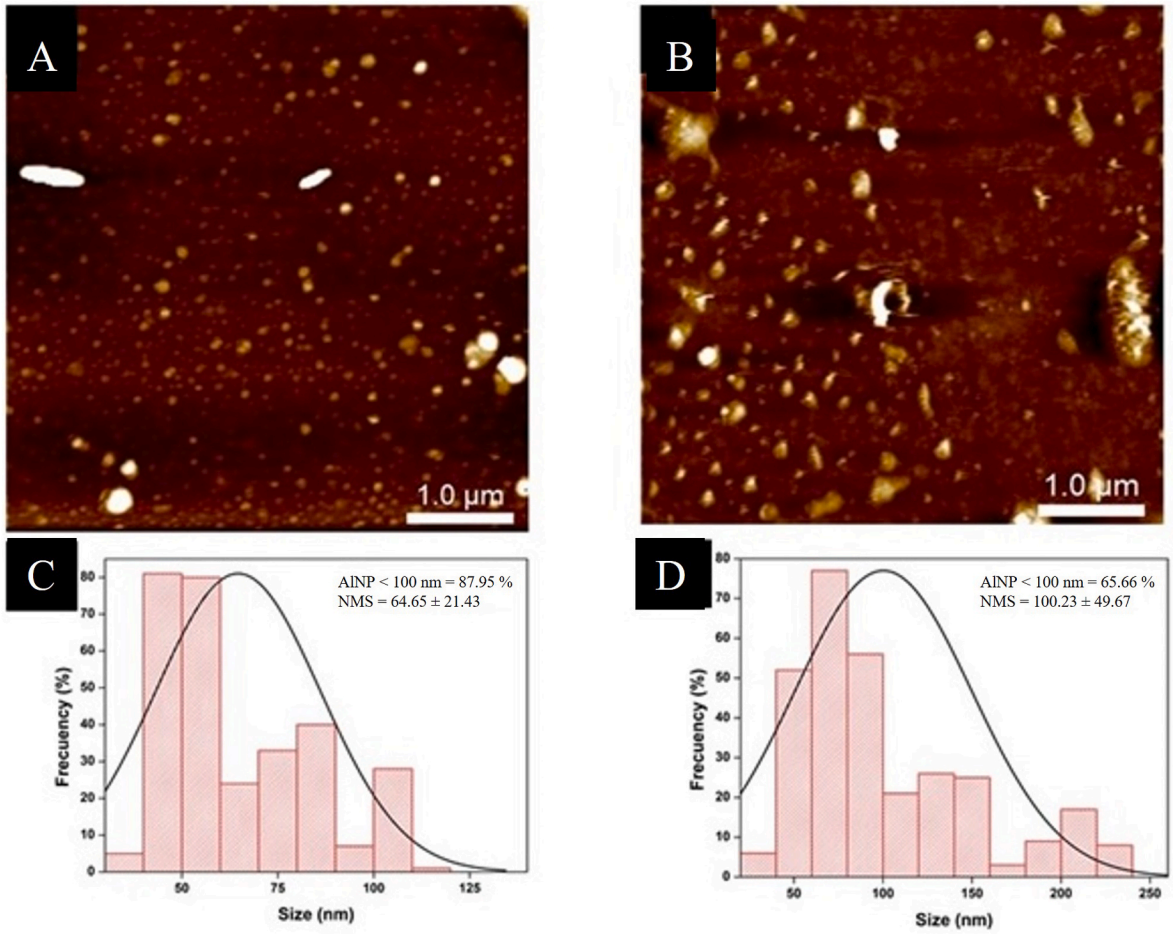

Fig. 2. AFM images in contact mode and particle size distribution histograms of aluminum nanoparticles (Al-NP). Dispersed in distilled water (A, C) and dispersed in reconstituted water (B, D).

found. Regarding CAT activity, different changes were observed among the tissues and times evaluated. Thus, in the gills a significant reduction of enzyme activity was observed from the first hours of exposure; while in the blood this reduction was present at 24 and 48 h. It is likely that the decrease in catalase activity is due to the inactivation of the enzyme by the overproduction of ROS (Pigeolet et al., 1990). On the other hand, in the gills an increase in SOD was observed, so there is an accumulation of FR that may lead to a depletion of the antioxidant enzyme system, observing a time-dependent reduction. In the liver, SOD activity showed an increase, with a maximum peak at 72 h. This increase may be due to the function of the antioxidant enzyme system. This increase may be due to an adaptive function to face the ROS produced. As is known, the liver has a greater capacity to cope with oxidative stress than other tissues, due to its high activity of antioxidant enzymes (SOD, CAT), since it is a site of multiple oxidative reactions and maximum generation of free radicals (Atli et al., 2006).

Finally, GPx has the ability to catalyze the reduction of organic hydroperoxides by thiols, through ping-pong kinetics where the enzyme is oxidized by hydroperoxide and then gradually reduced by thiols (Brigelius-Flohé and Flohé, 2020). In the gills AlNPs generated a significant increase at 12 h, whereas in the liver at all exposure times a significant increase could be appreciated and for the blood the activity was much higher compared to the control only at 96 h. The increased GPx activity may be the result of an adaptive response to the accumulation of lipid hydroperoxides, which may act as a signal for GPx bioactivation in order to convert this highly toxic free radical to less toxic compounds (Islas-Flores et al., 2014). Modification of antioxidant enzyme activity has been widely reported for metals (Kroon et al., 2017) and to a lesser extent in their NPs, for example, Gürkan (2018) demonstrated that NPs, including Al, have the potential to cause oxidative damage by activating or inhibiting several antioxidant enzymes in aquatic organisms such as the macroinvertebrate *Carcinus aestuarii*. The effects observed in this crustacean depend on the tissue studied, in particular the gills and hepatopancreas are more affected. Similarly, De et al. (2020) found that low doses of AlNP in its oxide form presented changes in SOD and CAT activity in various tissues of the Swiss albino mouse, particularly in the liver. In addition, these authors observed that the degree of damage is greater when the metal is in its nanoparticle form than in its non-nanoparticle form, possibly because the NPs present a greater reactivity due to their large surface area. Changes in enzymatic activity result in an accumulation of ROS and free radicals (FR), which can affect biomolecules such as lipids and proteins (Birnie-Gauvin et al., 2017; Javed et al., 2017).

Lipoperoxidation levels depend mainly on the availability of polyunsaturated fatty acids and antioxidant defense. Fish contain rich sources of these types of fatty acids (Javed et al., 2017), and metals such as Al can be deposited in fish tissues and contribute to the generation of ROS, leading to an accumulation of oxidative lipid degradation products. Lipid hydroperoxides are produced during the initial stage of lipid peroxidation; they are the intermediate products of the LPX process that are converted to aldehydes, such as MDA and 4-hydroxynonenal, by free radicals (Kato and Osawa, 2010). As had been mentioned, MDA is one of the major end products of LPX and they are usually inducers of oxidative stress by interacting with proteins and nucleic acids, causing severe molecular damage to their structure and functions (Esterbauer et al., 1991; Gaschler and Stockwell, 2017). In the case of these two

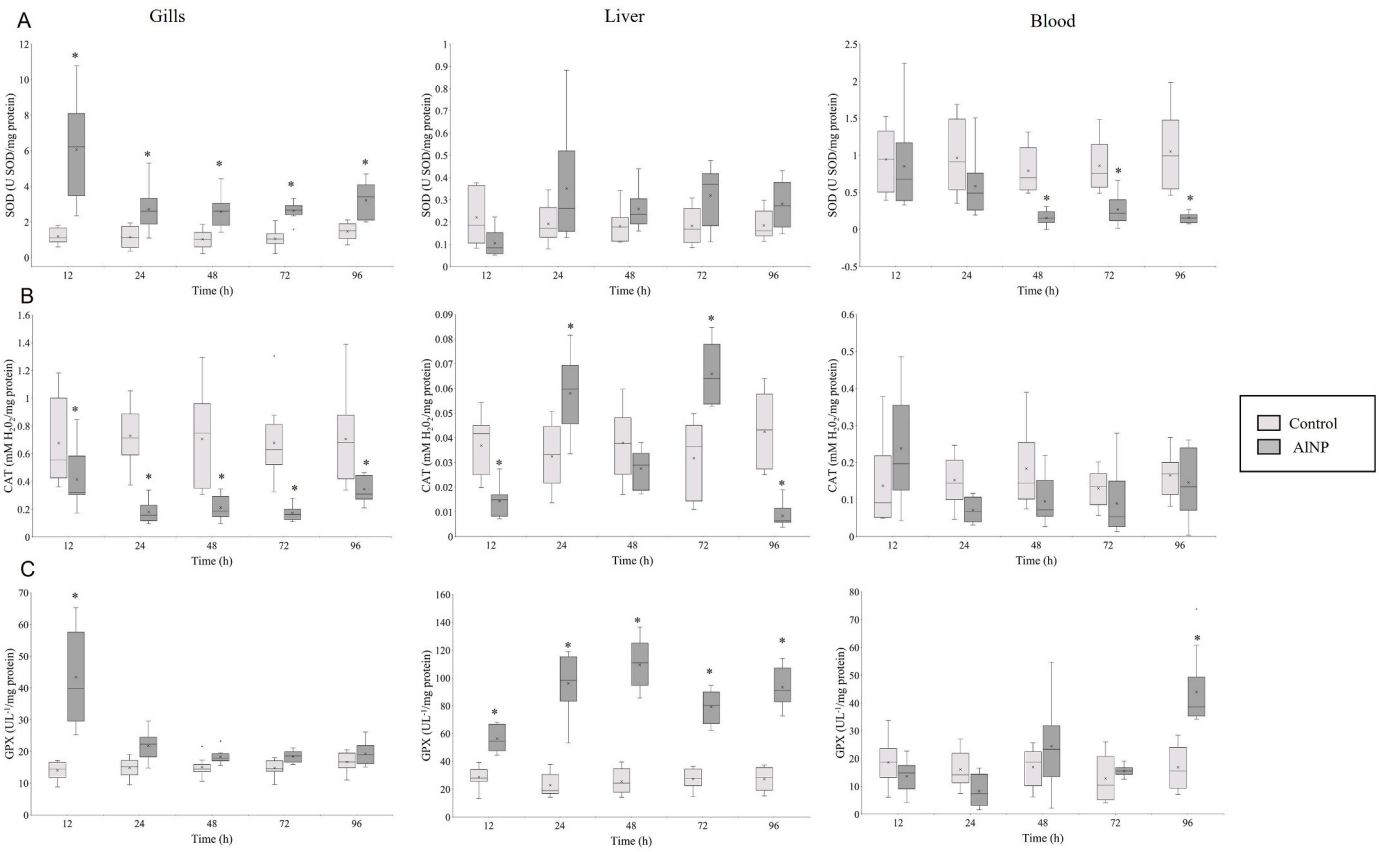

Fig. 3. Activity of the enzymes superoxide dismutase, SOD (A); catalase, CAT (B); and glutathione peroxidase in gill, liver and blood of *Cyprinus carpio* exposed to 50 μg L^{-1} of AlNP for 12, 24, 48, 72 and 96 h. The horizontal line in each box shows the medians i.e. the second quartile, or the 50th percentile, box limits are indicating the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles. *, significantly different from the control group (Dunn post hoc, $p < 0.05$).

biomarkers of oxidative lipid damage, it was observed that in the liver there was a significant increase over time in the group exposed to 50 μg L^{-1} of AlNP. On the other hand, a significant increase of HPC at 12 h and of MDA from 12 to 48 h was observed in the gills. In blood, the increase in HPC remained above the controls from 12 to 96 h, and for the concentration of MDA an increase was observed only at 24 h. These effects are in agreement with those reported by other authors for AlNPs in different biological models, when there is an imbalance between ROS production and antioxidant enzyme activity, lipid peroxidation is observed with increased MDA levels (Arab-Nozari et al., 2019; Canli and Canli, 2019; De et al., 2020; Gürkan, 2018; Nogueira et al., 2020).

NPs have a strong ability to affect mitochondria (Unfried et al., 2007), which is the alternate mechanism of oxidative stress generated by a non-prooxidant process and has also been widely described for Al in its non-nanometer form (Kumar et al., 2009; Kumar and Gill, 2014; Sharma et al., 2013). Moreover, several studies have shown that NPs of varied sizes and chemical composition preferentially localize in mitochondria while promoting ROS production (Adeyemi et al., 2020; Arab-Nozari et al., 2019; Piao et al., 2011; Zhao et al., 2016). Once NPs gain access to mitochondria, they stimulate ROS production through disruption of the electron transport chain, structural damage and depolarization of the mitochondrial membrane (Adeyemi et al., 2020; Arab-Nozari et al., 2019; Mirshafa et al., 2018). ROS generated in mitochondria has a great ability to escape from the microsomal microenvironment and modify proteins, leading to the formation of carbonyl groups which is the most common oxidative modification of proteins, and therefore, is considered a biomarker of cellular oxidative damage (Levine and Stadtman, 2001; Stadtman, 1993). In common carp, after exposure to 50 μg L^{-1} of AlNP, the levels of oxidized proteins in the liver increased significantly at all times studied. Whereas, in the gills, the significant increase with respect to the control was presented at 12 and 24 h. These results coincide with those observed by Mirshafa et al. (2018) who studied isolated rat brain mitochondria exposed to different concentrations of AlNP, where they found an increased protein carbonylation compared to the control. Similarly, Arab-Nozari et al. (2019) indicate that AlNPs can generate an increase in the oxidation of isolated rat brain mitochondrial proteins, even much more than that observed for inorganic aluminum. In the case of aquatic organisms, information regarding the effects of AlNPs is still very limited; however, it has been shown that this metal in its non-nanometric form can increase the levels of reactive carbonyls in several *Cyprinus carpio* tissues (García-Medina et al., 2010, 2013; Razo-Estrada et al., 2013), so it is possible that AlNPs present similar effects.

The IBRv2 index was used to integrate the response of the six oxidative stress biomarkers. The biomarkers evaluated showed changes in response, either greater or lesser according to the different tissues and exposure times. Moreover, the graphical spatial arrangement of such biomarkers allows to visualize more clearly which biomarker is more sensitive to pollutant exposure (Vieira et al., 2014), and in this case also allowed to follow the evolution of the oxidative stress process in each tissue over time. The tissue with a higher overall IBRv2 was the liver, by observing the graphs at 12 h this index is higher than the rest of the times and is characterized by a greater response in SOD enzyme activity and oxidative damage to lipids. While at 24 and 48 h IBRv2 is reduced and it is the antioxidant enzymes that contribute to the higher index; in the case of 72 h CAT and LPX are the ones that represent a greater weight in comparison with the rest of the biomarkers, on the other hand at 96 h CAT continues to be the biomarker that contributes most to the index. In

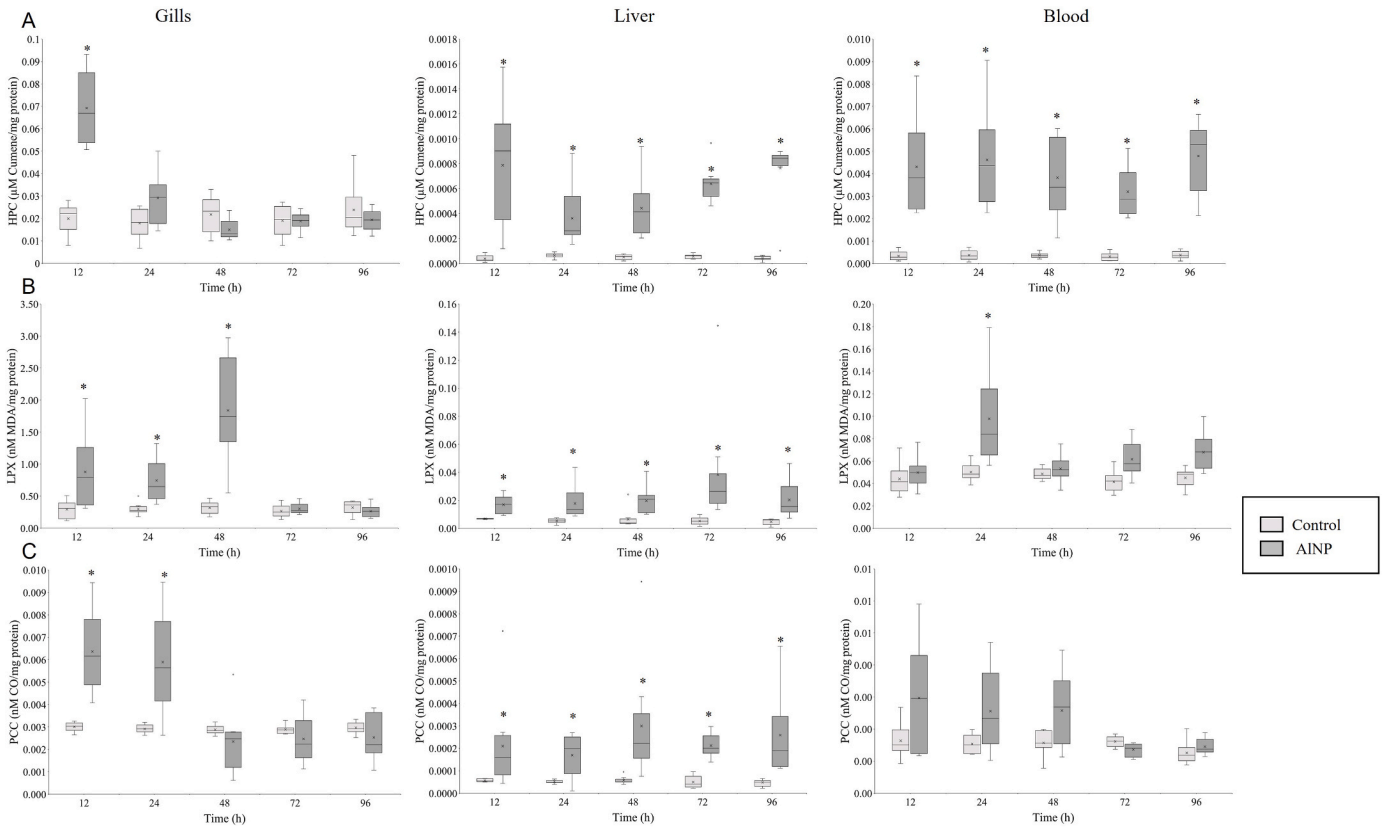

Fig. 4. Biomarkers of oxidative damage: hydroperoxide levels (A); lipid peroxidation (B) and carbonyl protein content (C) in gill, liver and blood of *Cyprinus carpio* exposed to 50 µg L^{-1} of AlNP. Box: IQR. Whiskers:1.5x IQR. *, indicates the significant difference from its controls by means of the non-parametric ANOVA test, post hoc Dunn test ($p < 0.05$).

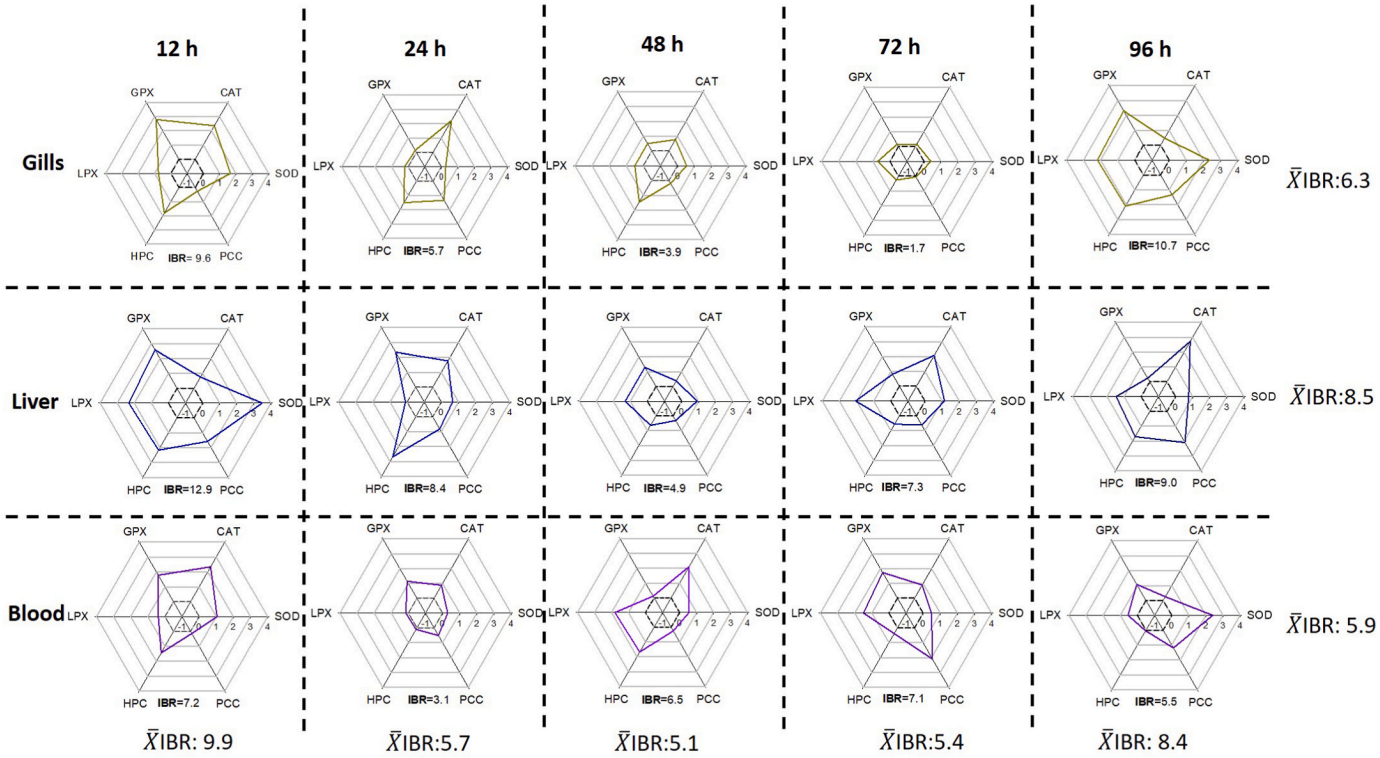

Figura 5. The star plot for the calculated IBRv2 index of the gill, liver and blood *Cyprinus carpio* exposed to 50 µg L^{-1} AlNP for 12, 24, 48, 72 and 96 h.

Table 2
Aluminium accumulation and bioconcentration factor (BCF) in tissues of *C. carpio* exposed to AlNPs for 96 h.

Tissues	Accumulated Aluminium (μg g^{-1} of wet weight)[a]	BCF
Gills	0.1166 ± 0.0875	2.2447
Liver	0.0414 ± 0.0207	0.7970
Brain	0.0875 ± 0.0672	1.6841
Muscle	0.2530 ± 0.0797	4.8703

[a] Mean±standard deviation, n = 6.

Table 3
Pearson correlation between biomarkers of oxidative stress and Al levels in gill and liver of common carp exposed to AlNPs for 96 h.

Oxidative stress biomarkers	Al levels	
	Gills	Liver
LPX	0.915*	0.986**
HPC	0.909*	0.719
PCC	0.854	0.999**
SOD	0.943*	0.993**
CAT	0.957*	0.927*
GPX	0.868	0.913*

*p < 0.001, **p < 0.05.

the gills an overall IBRv2 was found to be lower than in the liver, but higher than in the blood and, as in the liver, the 12 and 96 h exposure times were the ones in which a higher value was presented than the rest of the times, only at 12 h the highest response was found in GPX activity, while at 96 h SOD, GPX and lipid damage contributed in a similar magnitude to the index, and if IBRv2 at this time is compared with the rest of the tissues, it presented a higher value than the rest of the tissues. Finally, blood IBRv2 values were lower than those observed in liver and gill, except for 48 h, where the index was higher than the rest of the tissues and was characterized by a greater response to CAT activity and levels of MDA and lipid hydroperoxides. In addition, when IBRv2 was evaluated in this tissue over time, it was observed that the maximum value was obtained at 72 h, where oxidation to proteins was the biomarker with the greatest weight.

As can be appreciated IBRv2 can be a practical tool to assess susceptibility to contaminants using multiple biomarker responses, as several authors have done, generally this index has been used in several field studies (Beliaeff and Burgeot, 2002; Samanta et al., 2018). However, in recent years it has been used in laboratory experiments (Hou et al., 2016; Iturburu et al., 2018), for example, Xia et al. (2017) used this tool to explain the effect of titanium dioxide nanoparticles (TiO2 NPs) in different tissues of the bivalve mollusk species *Chlamys farreri* in which was observed that antioxidant enzymes were the biomarkers with the highest weight and that depending on the tissue and exposure time the degree of modification was different, concluding that this index allowed affirming that exposure to TiO2 NPs at environmentally relevant concentrations produces oxidative stress, as observed with AlNPs in this study. Given that high IBR values reveal a greater biological response to AlNPs and consequently an impairment in the health status of common carp, it is important to establish the concentration levels of AlNPs in the tissues, particularly in the most affected ones such as liver and gills.

On the other hand, Keck and Müller (2013) proposed a nanotoxicological classification system consisting of four classes depending on their size and biodegradation. In the case of classes I (particles from 100 nm to 1000 nm and biodegradable), II (>100 nm, but not biodegradable) and III (<100 nm, but biodegradable) represent a minimal and medium risk to the health of organisms. Class IV are particles with a particle size smaller than 100 nm, which can access all cells and are not biodegradable, present a high risk of toxicity, as occurs with the nanoparticles studied. In the case of the AlNPs used, since they contain a metallic element, it is not possible for them to biodegrade and their size varies depending on the solvent used. A decrease in the percentage distribution of particles <100 nm was observed in reconstituted water (42.10%) compared to distilled water (56.83%). Additionally, the ZP was established for AlNPs dispersed in both distilled water and reconstituted water, where values between −10 and + 10 mV were found, these can be considered as approximately neutral (Clogston and Patri, 2011). The PDI allowed verifying the formation of agglomerates; this phenomenon causes the particles to disperse in larger sizes and decreases the surface area of the particles, thus modifying their toxicity. The PDI values for distilled water presented an average of 0.19, so they are considered moderately monodisperse (PDI 0.1–0.4), whereas for reconstituted water it was 0.59, i.e. polydisperse (PDI >0.4) (Moradi-Sardareh et al., 2018). To confirm the particle size, AFM analysis was performed and, as with the previous results, a change in the particle size distribution was observed, as well as an increase in NMS with the reconstituted water, in general, this behavior is normal since divalent cations, mainly calcium and magnesium, are present in the reconstituted water, leading to high ionic strength and increased sedimentation (Nogueira et al., 2020).

One of the objectives of this research is to establish the bioaccumulation of AlNPs in common carp, although from the studies of the characterization of the NPs it was found that there is a tendency to polydispersion and an increase of the particle size under the exposure conditions. However, with the addition of the AlNP forms in the water, a 24.8% decrease in Al content in the water column was observed at 96 h compared to the initial time. This decrease may be due to the absorption of the toxicant or to its precipitation; however, an increase in Al concentrations in the tissues was also observed, which could be due to the absorption of AlNPs, presenting a value greater than unity in the BCF at 96 h in several tissues. The tissue with the highest BCF was muscle, followed by gills, brain and finally liver. The accumulation in muscle, which is a soft tissue, is usually a site of deposition of metals such as Al, and in general, indicates that the liver and other excretory organs (gills and kidneys) were exceeded in their capacity to remove these types of toxicants (Sivakumar et al., 2012; Souza I. da et al., 2018). On the other hand, an elevated BCF in the gills indicates that there is an uptake of AlNPs from the medium. This tissue may act as a defense mechanism to prevent the uptake of contaminants that are bioavailable from the external medium, so the distribution of contaminants in the gills may help to understand the uptake mechanism of compounds in fish (Sivakumar et al., 2012). In this regard, it is known that AlNPs affect gill tissue due to a decrease in Na+, K + -ATPase activity, histopathological lesions such as aneurysm, hyperplasia and cell necrosis, as well as NP aggregates on the gill surface (Abdel-Khalek et al., 2020; Griffitt et al., 2011; Vidya et al., 2018). A correlation analysis between Al levels and biomarkers of oxidative stress showed a high correlation in biomarkers of oxidative damage to lipids and the activity of SOD and CAT enzymes, confirming that AlNPs are responsible for the oxidative stress caused in this tissue. The BCF of Al in the brain was also determined to be 1.6841, confirming the findings of other researchers that the brain is a recognized target in a wide variety of biological models (Anane et al., 1995; De et al., 2020; Morsy et al., 2016; Zaitseva et al., 2019). For example, Fernández-Dávila et al. (2012) reported a BCF of 0.909 at 96 h in grass carp, additionally these authors demonstrated that Al produces neurotoxicity due to increased oxidative damage of lipids and proteins, as well as an imbalance in CAT and SOD activity, and in the neurotransmitters, dopamine, noradrenaline, and adrenaline. Shrivastava et al. (2014) found that the accumulation of AlNPs may alter the synthesis and release of certain neurotransmitters and generate oxidative stress. The accumulation of Al in brain tissues could be due to dysfunction of the liver, the main organ for detoxification of any toxicant (Sivakumar et al., 2012).

Although the liver presented the lowest BCF value than the rest of the tissues, the accumulation of aluminum NPs, mainly in its oxide form, has been reported in several biological models (De et al., 2020; Morsy et al.,

2016; Murali et al., 2017; Zaitseva et al., 2019). This tissue is the main organ involved with the homeostasis of the organism; it is responsible for eliminating toxicants that enter the systemic circulation through the gastrointestinal system and gills, biotransforming them and then excreting them through urine, bile and gills, so dysfunctions in this tissue significantly affect the overall health of the organism (Wolf and Wheeler, 2018). On the other hand, the presence of metals in this tissue, such as Al, can cause significant damage to its structure and function, for example, it has been shown that AlNPs in their oxide form can penetrate inside the cytoplasm and nucleus after being absorbed by the intestinal membrane and cause toxic effects in fish (Murali et al., 2017). To establish the relationship between tissue Al levels and oxidative stress biomarkers, a correlation analysis was performed, where it can be observed that for most biomarkers a high Pearson correlation coefficient (>0.8) is present, except for oxidized proteins. These findings show that AlNP can generate oxidative stress in the liver of *Cyprinus carpio* and affect the function of this organ, modifying the bioavailability of the NPs in other tissues, such as muscles and brain.

In conclusion, AlNPs at a concentration of 50 $\mu g\ L^{-1}$ produce changes in the homeostasis of exposed organisms, reflected in an increase in biomarkers of oxidative damage and an imbalance in the activity of antioxidant enzymes in the liver, gill and blood of *Cyprinus carpio*. By establishing the IBRv2, which allows us to have an integrated evidence on the biological effects of AlNP, it is evident that the liver is the most affected organ and these effects are related to the Al content in the tissue. In addition, it was observed that muscle and gills presented a higher BCF, compared to brain and liver. Bioaccumulation in freshwater fish, such as common carp, has important environmental, ecological and social consequences, as well as implications for humans and other fish that consume this organism (Ali et al., 2019; Ali and Khan, 2018). It is of vital importance to evaluate the impacts of NPs on aquatic populations, since the maximum permissible limits (MPL) for the protection of aquatic life have not been established, the concentration used is equivalent to the MPL of aluminum recommended by Mexican standards and guidelines, so that, according to the results obtained in this research, it would be considered an important risk for the health of fish such as common carp.

Credit author statement

Sandra García-Medina: Conceptualization, Writing – original draft, Formal analysis. Marcela Galar-Martínez: Resources, Methodology, Writing - review & editing. Leobardo Manuel Gómez-Oliván: Writing - review & editing. Selene Cano-Viveros: Investigation, Visualization. Karina Ruiz-Lara: Investigation, Visualization. Hariz Islas-Flores: Data curation, Supervision. Eloy Gasca-Pérez: Investigation, Supervision. Ricardo Pérez-Pastén-Borja: Writing, Visualization. Benjamín Arredondo Tamayo: Investigation, Visualization Josué Hernández-Varela: Investigation, Visualization José Jorge Chanona-Pérez: Writing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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