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Polluted water from a storage dam (Villa Victoria, méxico) induces oxidative damage, AChE activity, embryotoxicity, and behavioral changes in *Cyprinus carpio* larvae

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ABSTRACT

The Villa Victoria dam is one of the most important storage reservoirs in Mexico since it distributes water to more than 20 million inhabitants in the Metropolitan Zone of Mexico City. In this dam, the common carp (*Cyprinus carpio*) is an important food resource for the inhabitants, so the aim of this work was to evaluate the oxidative damage (lipoperoxidation, oxidized proteins, antioxidant enzymes activity and gene expression), AChE, embryotoxicity and behavioral changes in *C. carpio* embryos and larvae exposed to water from Villa Victoria dam for 24, 48, 72 and 96 h. The embryotoxicity was evaluated trough the General Morphology Score (GMS) and the teratogenic index. Behavioral changes in basal locomotor activity and thigmotaxis were evaluated in a DanioVision, Noldus ™. An increase in lipid and protein oxidation as well as modification of CAT, SOD and GPx enzymatic activity was observed during the exposure times. The GMS indicated a low development in the embryos, the teratogenic index was less than 1, however teratogenic effects as yolk edema, fin malformation, head malformation and scoliosis were observed. In parallel, an increase in AChE activity and gene expression was observed reflecting changes in distance traveled of the basal locomotor activity and thigmotaxis at the sampling points. In conclusion, pollutants in water from Villa Victoria dam caused oxidative damage, changes in SOD, CAT, GPx and AChE activity as well as embryotoxicity and modifications in the behavior of *C. carpio* larvae. This study demonstrates the need to implement restoration programs for this reservoir since, contamination in the Villa Victoria dam could eventually endanger aquatic life and human health.

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1. Introduction

Dams are natural or artificial reservoirs whose purpose is the storage and distribution of water (Lin et al., 2022; Mohamed Khir Alla and Liu, 2021). These aquatic bodies play an important role in the development of human activities such as electricity generation, recreational activities, storage and supply of fresh water, as well as livestock activities and cultivation of aquatic species for human consumption (Lin et al., 2022; Mohamed Khir Alla and Liu, 2021).

In Mexico, the most important dam system for water distribution is the Cutzamala System, which consists of 4 derivative dams and 3 storage and distribution dams: Valle de Bravo, El Bosque and Villa Victoria (CONAGUA, 2005, 2010, 2020; H. Ayuntamiento Constitucional de Villa Victoria, 2021).

The Villa Victoria dam is one of the most important water bodies, not only in the state, but also in the Metropolitan Zone of the Valley of Mexico, as it is the main supplier of water to the Cutzamala system (CONAGUA, 2005, 2010, 2020), which contributes to the water supply of nearly 20 million people. This reservoir is fed mainly by the Río de la Compañía, springs and streams, has a capacity of 186 cm^3 and is located at an altitude of 2545 mas. In recent years, decreases in water volume of over 70% have been reported between 2018 and 2022, due to the deforestation of forested areas that are replaced by agricultural zones (CONAGUA, 2005, 2020; H. Ayuntamiento Constitucional de Villa Victoria, 2021). There are also 3 treatment plants; however, they are below 50% of their functionality, affecting the availability and supply of the dam. Few studies have been conducted on contamination of the reservoir. However, in 2022 our working group identified the presence of metals, such as aluminum and iron that exceed the maximum permissible limits for the protection of aquatic life, as well as drugs like carbamazepine, diclofenac, metformin and fertilizers and pesticides, among other pollutants (Table 1; García-Medina et al., unpublished results).

This water body is home to different aquatic species, with fish being the most representative and commercially important. Among these are the white charal (*Chirostoma* sp), which is endemic and consumed by humans, as well as tilapia (*Oreochromis* sp) and common carp (*Cyprinus carpio*) (CONAGUA, 2020; CONAPESCA, 2018). In recent years, residents have reported a decline in the common carp and other species population, reducing fishing and affecting their economy.

The common carp (*C. carpio*) has proved to be an excellent bioindicator organism in ecotoxicological studies since, like *Danio rerio*, it presents a transparent chorion and is sensitive to contaminants present in the water (Gutiérrez-Noya et al., 2020; Luja-Mondragón et al., 2019; Pérez-Coyotl et al., 2019) (Fig. 1). Several studies have shown that common carp, in adult and embryonic stages, are highly sensitive, evidencing alterations related to oxidative stress, embryotoxicity, genotoxicity, teratogenesis, and central nervous system and behavioral alterations produced by contaminants in the water (Gutiérrez-Noya et al., 2020; Luja-Mondragón et al., 2019; Pérez-Coyotl et al., 2019; Rao, 2016).

Table 1
Pollutants found in Villa Victoria Dam (García-Medina et al., unpublished results).

Pollutant	Concentration (mg/L)
Metals	
Aluminum	0.35–0.74
Iron	0.24–0.40
Lead	0.003
Cadmium	0.0003
Chromium	0.008–0.031
Pharmaceutical products	
Paracetamol	Detected
Albendazole	Detected
Metformin	Detected
Carbamezepine	Detected
Diclofenac	Detected
Caffeine	Detected
Testosterone	Detected
Pesticides	
Dimethoate	Detected
Diazinon	Detected

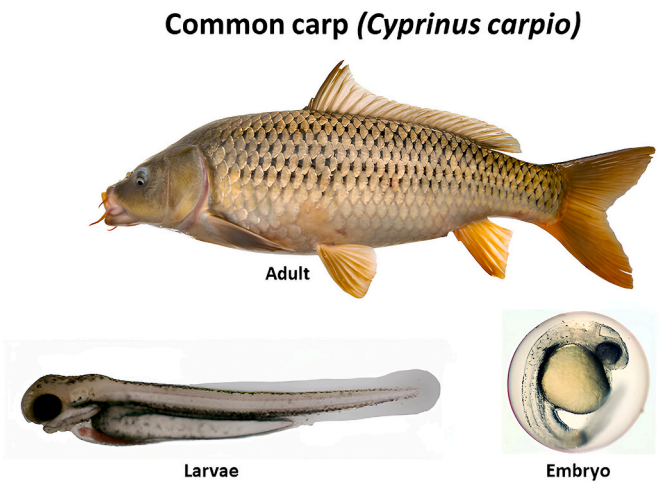

Fig. 1. Common carp (*Cyprinus carpio*) adult, larvae and embryo.

Oxidative damage is an early warning biomarker to assess the effects of pollutants on aquatic organisms (Capriello et al., 2021; Gopi et al., 2019; García-Medina et al., 2010). Oxidative stress is defined as an imbalance between oxidative species, including reactive oxygen species (ROS), and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). This toxicological mechanism of action is very important, as several studies have shown that pollutants such as metals, hydrocarbons, polychlorinated biphenyls and emerging drug-type pollutants, among others, are capable of producing ROS and other radical species leading to the oxidation of biomolecules as important as lipids, proteins, carbohydrates and nucleic acids of the exposed organism.

In addition to the modification of antioxidant enzymes, contaminants in water can generate oxidative damage, which manifests as lipoperoxidation and increased oxidized protein content and can affect the activity of enzymes related to the central nervous system and muscle in fish larval fish, such as acetylcholine (ACh), among others (Gobi et al., 2016; Marinho et al., 2019).

ACh is a neurotransmitter involved in the transmission of nerve impulses in the cholinergic system that promotes movement in fish larvae. Once the impulse is generated, the neurotransmitter must be removed from the synapse, either by reuptake, as in the case of adrenaline, or by degradation of the neurotransmitter, as in the case of ACh which is hydrolyzed to choline and acetate by the enzyme acetylcholinesterase (AChE) (Senger et al., 2011; Gobi et al., 2018; Marinho et al., 2019). Inhibition of AChE results in the accumulation of the neurotransmitter ACh at the synapse, leading to hyperstimulation of related receptors. Recent studies have shown that ROS such as H_2O_2 promote oxidation of mRNA proteins, modifying signaling pathways for ache gene activation and expression, which could lead to AChE inhibition (Senger et al., 2011; Yen et al., 2011; Marinho et al., 2019).

Because of this, AChE activity and the gene expression has been used as a biomarker to assess neurotoxicity and neuromuscular effects by water pollutants such as organophosphorus pesticides, heavy metals, and drugs in the water (Basnet et al., 2019; Wang et al., 2019; Zhou et al., 2019; Capriello et al., 2021; Fitzgerald et al., 2021).

Embryotoxicity is also an excellent biomarker of damage to know the effects of aquatic pollution since these can be reflected in alterations of morphological development in fish embryos (Luja-Mondragón et al., 2019; Pérez-Coyotl et al., 2019). ROS are involved in the embryonic

development of fish, participating in the processes of embryogenesis, cell division, oocyte maturation and blastocyst formation These reactive species are essential as second messengers in the regulation of multiple signaling pathways that control key cellular functions, such as proliferation, differentiation, and apoptosis in embryogenesis. However, when individuals in early stages of development are exposed to contaminants in water, oxidative stress can be generated, inducing changes in enzyme levels, and oxidation of lipids, proteins and genetic material, leading to cell death (García-Medina et al., 2010; Pérez-Coyotl et al., 2019; Xia et al., 2020).

Although apoptosis regulates cellular mechanisms of embryonic development in fish, an imbalance in cell death can negatively affect cell homeostasis, tissue and organ formation and growth, modify chorion permeability, and affect hormonal signaling pathways of embryonic development leading to malformations and organism death (Luja-Mondragón et al., 2019; Cano-Viveros et al., 2022).

There are parameters such as the General Morphological Score (GMS), the frequency of teratogenic effects and the teratogenic index (TI) that allow the evaluation of developmental stages and alterations produced by pollutants in aquatic bodies (Luja-Mondragón et al., 2019; Gutiérrez-Noya et al., 2020).

The assessment of behavioral changes in fish larvae proves to be an integral biomarker of pollution with many advantages (Boyda et al., 2021; Fan et al., 2021; Chen et al., 2022; Faria et al., 2022). Once a fish larva hatches, it begins to have behavioral patterns on which its reproduction and depredator scape depends. In ecotoxicology, behavioral studies in fish larvae represent an important tool to understand the effects of contaminants on the muscular and central nervous system and the potential risk of population survival (Beyer et al., 2014; De Loera -González et al., 2016; Faria et al., 2022; Idalia et al., 2015; Nassour et al., 2021; Shen et al., 2020; Velki et al., 2017). The contaminants present in surface waters can affect the motor, visual and neuronal functions of fish larvae, directly altering their basal locomotor activity, orientation or thigmotaxis which, consequently, would put at risk their biological survival activities such as feeding, reproduction, orientation and escape from predators (Qiang et al., 2016; Velki et al., 2017; Shen et al., 2020; Fan et al., 2021; Chen et al., 2022; Faria et al., 2022).

Behavioral testing has proven to be a tool that aids ecotoxicological studies as it is an integral biomarker in biological models sensitive to contaminants. However, studies focused on behavioral changes in fish have been conducted mainly in *Danio rerio* larvae and there is still a gap with respect to behavioral changes in other indicator and commercially important fish such as the common carp *Cyprinus carpio* (Gutiérrez-Noya et al., 2020; Shen et al., 2020; Capriello et al., 2021; Chen et al., 2022). For this reason, the aim of this study was to evaluate the oxidative damage, activity and gene expression of AChE, embryotoxicity, and behavioral changes in *Cyprinus carpio* larvae exposed to water from the Villa Victoria dam, State of Mexico.

2. Material and methods

2.1. Water sample collection at the Villa Victoria dam, edo. of Mexico

Water was collected from the Villa Victoria dam at rain season (august 2021), in accordance with the procedure described in the Mexican official guideline (NMX-AA-003-1980) for the collection of water samples in aquatic systems. The samples were taken at a depth of 1 m with a Van Dorn bottle and the temperature and pH of each site were recorded. 20 L of water were collected at 6 reference sampling points (SP): SP 1 = pier, SP 2 = wastewater discharge, SP 3 = animal breeding area, SP 4 = agriculture zone and breeding area, SP 5 = agriculture area, and SP 6 = dam curtain (Fig. 2). The water samples were transferred in a plastic container in an icebox to the Laboratory of Aquatic Toxicology of the National School of Biological Sciences of the National Polytechnic Institute-Zacatenco.

2.2. Cyprinus carpio larvae procurement

Cyprinus carpio embryos were collected at the fish farm of Centro Carpícola Tiacaque, Edo. De México and placed in Petri dishes with Egg Water solution (60 μg/L of Instant Ocean ® salts) and in an ice box for transport to the Aquatic Toxicology laboratory of the National School of Biological Sciences (IPN). Subsequently, the plates with the embryos were placed in an incubation chamber at a temperature of 26 ± 1 °C and light/dark photoperiods of 12/12 h until 8 days post fertilization (dpf). In this study, each test was performed using 24–50 larvae per exposure

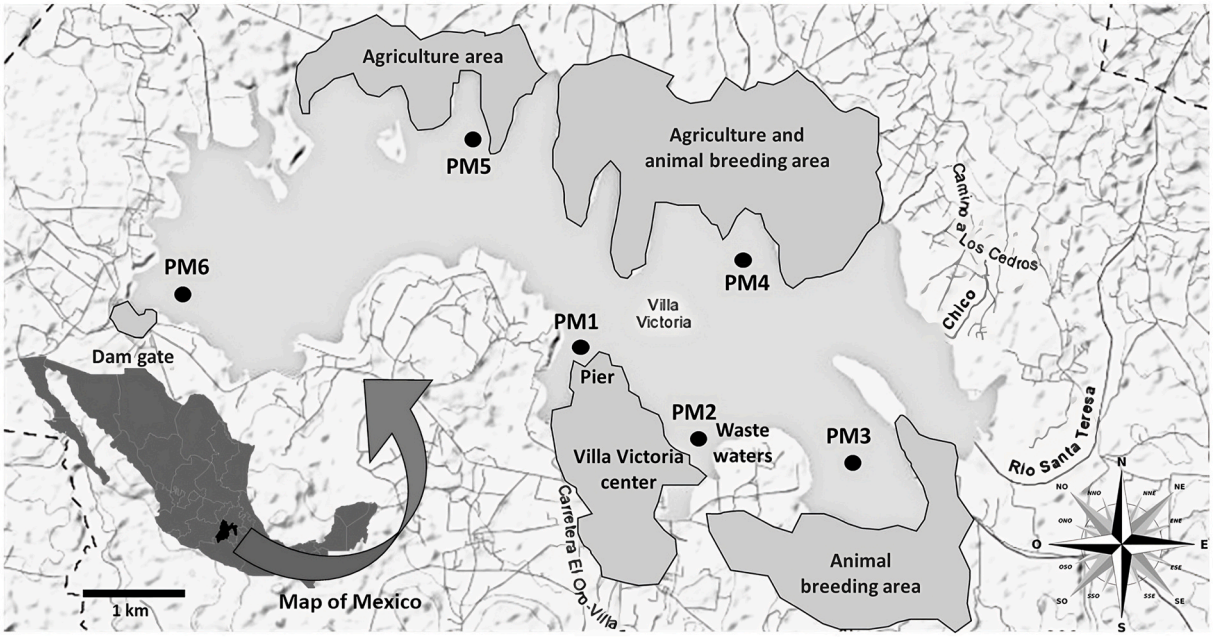

Fig. 2. Map of the sampling points (SP) of the Villa Victoria dam (19°27′37.0″N 99°59′27.8″W). SP 1 = wharf (19°27′20.4″N 100°00′00.9″W), SP 2 = wastewater discharge (19°26′44.1″N 99°59′16.5″W), SP 3 = livestock area (19°26′47.8″N 99°58′10.0″W), SP 4 = agriculture and livestock area (19°28′06.8″N 99°58′53.5″W), SP 5 = agriculture area (19°28′53.0″N 100°00′53.0″W) and SP 6 = dam gate (19°27′48.4″N 100°03′00.4″W).

time in triplicate per sampling point (1440 larvae in total).

2.3. Determination of oxidative damage and antioxidant enzyme activity in C. carpio larvae

Oxidative damage assessment was conducted by placing larvae (8 dpf) of *C. carpio* in aquaria with 2 L of water from the 6 sampling points and a control group with Egg Water solution (60 μg/L of Instant Ocean ® salts). After 24, 48, 72 and 96 h of exposure, 50 larvae (per triplicate) from each exposure time and sampling point were homogenized with 1.5 mL of Sigma-Aldrich ® phosphate salt buffer (PBS, pH = 7.4). Aliquots of the samples (0.7 mL) were transferred to 2 mL Eppendorf tubes and centrifuged at 15,000 rpm for 15 min at 4 °C. The homogenized samples were used to determine the degree of lipoperoxidation (LPX) while the supernatant of the samples was used for the assessment of carbonyl protein content (CCP) as well as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

2.3.1. Determination of lipid peroxidation degree

The degree of lipoperoxidation (LPX) was assessed following the method reported by Buege and Aust (1978). 200 μL of the larval homogenate were placed in tubes with 300 μL of PBS (pH = 7.4) and 1 mL of reaction solution (0.37 g Merck ® thiobarbituric acid, 15% Baker® trichloroacetic acid and 0.2 N Baker ® hydrochloric acid). The tubes were placed in boiling water for 15 min and subsequently centrifuged at 1000 rpm for 10 min. Samples were read at 535 nm and results were expressed as nM malondialdehyde (MDA) per milligram of total protein per gram of tissue.

2.3.2. Determination of carbonyl protein content

The method described by Levine et al. (1994) was followed to obtain the carbonyl protein content. 100 μL of the larval supernatant were taken and 150 μL of dinitrophenylhydrazine Sigma-Aldrich® (10 mM) in Baker® hydrochloric acid (2 M) were added. The samples were incubated for 1 h and then 500 μL of trichloroacetic acid (6%) Baker® were added to centrifuge at 10,000 rpm for 5 min. Three washes were performed with ethanol-ethyl acetate and consecutively 1 mL of guanidine (6 M) Sigma-Aldrich® and formic acid (50%) TSQ® were added. The samples were read at 366 nm and the results were expressed as nM of reactive carbonyls per milligram of total protein per gram of tissue.

2.3.3. Determination of catalase activity

Catalase activity (CAT) was estimated according to the method developed by Radi et al. (1991). 20 μL of larval supernatant were placed in quartz cells with 980 μL of PBS (pH = 7.4) and 200 μL of Sigma-Aldrich® hydrogen peroxide (20 mM). Samples were read at 0 and 60 s at 240 nm. Results are expressed as mM H_2O_2 per milligram of protein per gram of tissue.

2.3.4. Determination of superoxide dismutase activity

Superoxide dismutase (SOD) enzyme activity was assessed with the aid of the commercial Ransod kit (Randox). 7.5 μL of sample supernatant was taken and placed in a 96-well plate with 200 μL of substrate and 40 μL of xanthine oxidase (Ransod®). Absorbance readings were obtained at 30 s and 3 min on an Elx800 reader (BioTek) and related to the calibration curve included in the kit. Data were presented in U SOD per milligram of protein per gram of tissue.

2.3.5. Determination of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined using the Ransel kit (Randox). Aliquots of 10 μL of sample were added in a quartz cell with 500 μL of substrate and 40 μL of cumene solution (Randox®). Absorbance was obtained at 340 nm at 1, 2 and 3 min. GPx concentration was calculated using the following formula: $U/L = 8412\ x\ \Delta A\ 340$ nm/min. The results were expressed as IU of GPx per milligram per gram of tissue.

2.3.6. Determination of total protein content

The results of biomarkers of oxidative damage and enzyme activity were normalized to total protein content following the method developed by Bradford (1976). 5 μL of sample were taken and placed in a 96-well plate with 45 μL of deionized water and 200 μL of Bradford's reagent (Sigma-Aldrich® brilliant blue G). Samples were incubated for 5 min and read on an Elx800 plate reader (BioTek) at 595 nm. The data were fitted with a protein curve with Sigma-Aldrich® bovine albumin.

2.4. AChE activity and gene expression in Cyprinus carpio larvae

The determination of AChE activity was evaluated only at 96 h of exposure because oxidative damage and antioxidant enzymes presented greater effects at this time. The activity was measured using the commercial Acetylcholinesterase Activity Assay kit (MAK119-1 KT), Sigma-Aldrich ®. 10 μL of supernatant was added to a 96-well plate with 190 μL of reagent and shaken for 1 min. Readings were performed on an Elx800 plate reader (BioTek) with a wavelength of 405 nm. Results were presented as IU of AChE per milligram per gram of tissue.

To know the gene expression of *ache*, after 96 h of exposition to the Villa Victoria SP, the samples (30 g of larvae per SP in triplicate) were placed in RNAlater® stabilizing reagent and stored at −20 °C according to the provided instructions, the RNA was isolated using the RNeasy® kit. Subsequently, reverse transcription was performed using the QuantiTect® kit to generate cDNA. Aliquots from each completed reverse transcription reaction were then introduced into the PCR-Real-Time mix of the QuantiTect® SYBR® kit, with all utilized kits originating from the QIAGEN brand. The cycler employed for the experiment was the Rotor-Gene Q by QIAGEN, programmed as per the protocol. To quantify the concentration of RNA and DNA, a Thermo Scientific NanodropTM 2000 spectrophotometer was employed. The data were normalized respect the control group and specific primers utilized show in Table 2.

2.5. Embryotoxicity and teratogenic index

C. carpio embryos at 4 h post-fertilization were placed in 24-well plates according to the OECD, Fish, Acute Toxicity Test (2019) with 2 mL of water from each of the Villa Victoria dam water sampling points with a control group per triplicate (72 larvae per SP per time). Observations were made in an Optika XDS-2 inverted microscope for 12, 24, 48, 72 and 96 h and, in parallel, morphological alterations were sought in each embryo. During the observation times, the general morphological score (GMS) used by Cano-Viveros et al. (2022) and compared with the final score of the control groups. The frequency of teratogenic effects was determined by observing the following alterations: coagulation, pericardial edema, low pigmentation, head and tail malformation, delayed development, delayed hatching, and scoliosis. The teratogenic index was calculated from the LC50 and EC50 used by Cano-Viveros et al. (2022) and Vasamsetti et al. (2020) where IT = LC50/EC50. The experiment was considered valid when <10% of control embryos showed coagulation (Hermsen et al., 2011).

2.6. Behavioral analysis

To measure the behavioral assay, *Cyprinus carpio* larvae of 8 dpf were placed in 24-well plates with 2 mL of Villa Victoria dam SP with a

Table 2
Primer sequences utilized for qPCR targeting the acetylcholinesterase (ACHE) and β-actin genes in *Cyprinus carpio* larvae.

Gene	Forward	Tm°
ache	Forward: GGGGAAGCTGTGAGGACAA	56 °C
	Reverse: GCAGAGGAGGGGGTGTTAAG	56 °C
β-actin	Forward: CACGTCGACTCCGGAAAGT	60 °C
	Reverse: ATGGTGATACCACGCTCACG	60 °C

control group (Egg water). Plates were placed in the observation chamber DanioVision Noldus ™ and the temperature was regulated at 26 ± 1 °C with a temperature control unit. 24, 48, 72 and 96 h after exposure larvae were acclimated in dark conditions for 15 min and the locomotor activity was recorded during 5 min. The basal locomotor activity (BLA), defined here as the distanced traveled by the larvae during a 5 min period in the dark (Bedrossiantz et al., 2023; Faria et al., 2021), was analyzed using the multi-tracking module of EthoVision software. For the evaluation of the thigmotaxis, which refers to the inclination to steer clear of the central area of an arena and instead remain near or travel along the edges of the surroundings, serves as an indicator for measuring anxiety levels in fish larvae. Each well of the 24-well plate was divided into two sections that were equivalent in size to the body length of the larvae (6 mm) using the EthoVision software. Thigmotaxis is presents as time (s) that larvae stay on the periphery of the wall in the outer zone according to Schnörr et al. (2012) and Karaman et al. (2023).

2.7. Statistical data analysis

For the determination of oxidative damage, enzyme activity and behavioral changes, normality and homoscedasticity were determined by Shapiro-Wilk and Bartlett's test. Data were analyzed by one-way and two-way ANOVA test, Kruskal-Walli's test and Dunnett's post hoc for multiple testing at $P < 0.05$ with GraphPad Prism 8.0.1 statistical software.

The R studio software was used to establish the correlation between variables at 96 h, establishing the Spearman correlation coefficient. Subsequently, a chord diagram was made, as well as a correlation matrix where the significance of Spearman's coefficient is presented.

(Kruskal and Wallis, 1952; Madansky and Madansky, 1988; McKight and Najab, 2010; Shingala and Rajyaguru, 2015; Siraj-Ud-Doulah, 2019).

3. Results

3.1. Oxidative damage to Cyprinus carpio larvae

Oxidative damage in *C. carpio* larvae by exposure to water from Villa Victoria dam was determined through lipoperoxidation and oxidized proteins during 24, 48, 72 and 96 h of exposure.

The determination of lipoperoxidation degree showed an increase in the concentration of nM malondialdehyde (MDA) in the exposed larvae. At 24 h of exposure, an increase with respect to the control groups was observed in SP 2 (58%), SP 3 (84%), SP 4 (93%), SP 5 (94%) and SP 6 (83%) ($P < 0.05$) (Fig. 3-A). Subsequently, it was observed that SP 2, 5 and 6, at 48 h and SP 1, 2, 3, 5 and 6 at 72 h, continued to increase above 50% (Fig. 3-A). Finally, at 96 h, larvae exposed to SP 2, 3 and 6 water showed an increase of 83, 73 and 82 %, respectively, of lipoperoxidation compared to the control group ($P < 0.05$) (Fig. 3-A).

With respect to oxidized proteins, it was observed that *C. carpio* larvae exposed to water from the Villa Victoria dam presented an increase in the concentration of nM of carbonyl proteins at 48 h with a 72% increase in SP 5 (Fig. 3-B). Likewise, at 96 h of exposure compared to the control groups, there were significant differences ($P < 0.05$) of 83 and 79 % in SP 3 and 4, respectively (Fig. 3-B).

3.2. Determination of the enzymatic activity of SOD, CAT, and GPx

The SOD activity was evaluated in *C. carpio* larvae exposed to 6 points of water from the Villa Victoria dam. A tendency to increase the activity of this enzyme was evidenced through the exposure times, but it was up to 72 h where the greatest increment (98%) was observed in the larvae exposed to SP 6 compared to the control group ($P < 0.05$) (Fig. 4-A).

On the other hand, CAT also presented modifications in its activity in larvae exposed to reservoir water. At 24 h, an increase of 70% was observed in SP 6, 91% at 48 h in SP 2 and 99% at 72 h in SP 6 (Fig. 4-B). However, at 96 h of exposure, in SP 2, 3, 4, 5 and 6 CAT activity decreased below 100% with respect to the control group ($P < 0.05$) (Fig. 4-B).

With respect to the GPx enzyme, changes in activity were also observed in *C. carpio* larvae. These effects were observed up to 72 h of exposure, where a 60% increase was observed in all sampling points compared to the controls (Fig. 4-C). After 96 h of exposure, the increase in enzyme activity continued at SP 2, 3 and 6 of 50, 52 and 58%, respectively ($P < 0.05$) (Fig. 4-C).

3.3. AChE activity and gene expression in C. carpio larvae

The activity of the enzyme AChE showed changes after 96 h of exposure to water from the Villa Victoria dam in *Cyprinus carpio* larvae. An increased activity was observed in SP 2, 3, 4, and 5 ($P < 0.05$) with respect to the control group (Fig. 5-A). Likewise, an overexpression compared to the control in the *ache* gene was observed in the larvae of *C. carpio* exposed to the water of the Villa Victoria dam in SP 2 with 4.3%, SP 4 with 6.5%, SP 5 with15.3% and SP 3 with 100% (Fig. 5-B).

3.4. Embryotoxicity and teratogenic index

The embryotoxicity was evaluated through the GMS after 96 h of exposure to the water of the Villa Victoria dam. The GMS showed that the *C. carpio* embryos presented values lower than the score of 15 compared to the control groups. The lowest values that represent a delay in morphological develoment in the *C. carpio* embryos were those exposed to the SP 3 with a GMS of 3, SP 6 with 7, SP 4 and 5 with a GMS

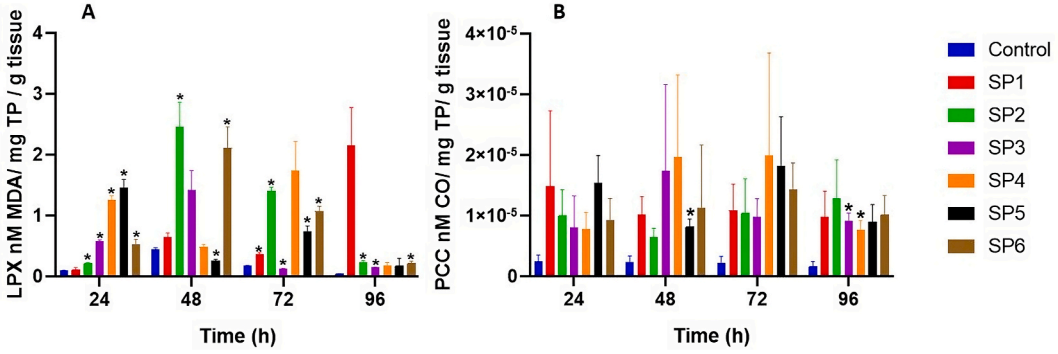

Fig. 3. Oxidative damage in *Cyprinus carpio* larvae by exposure to water from the Villa Victoria dam for 24, 48, 72 and 96 h. A show the degree of lipoperoxidation, and B the carbonyl protein content. Data are expressed as mean and SD with two-way ANOVA, and Dunnett's post hoc test for multiple comparisons. The asterisk (*) represents a $P < 0.05$ with respect to the control group.

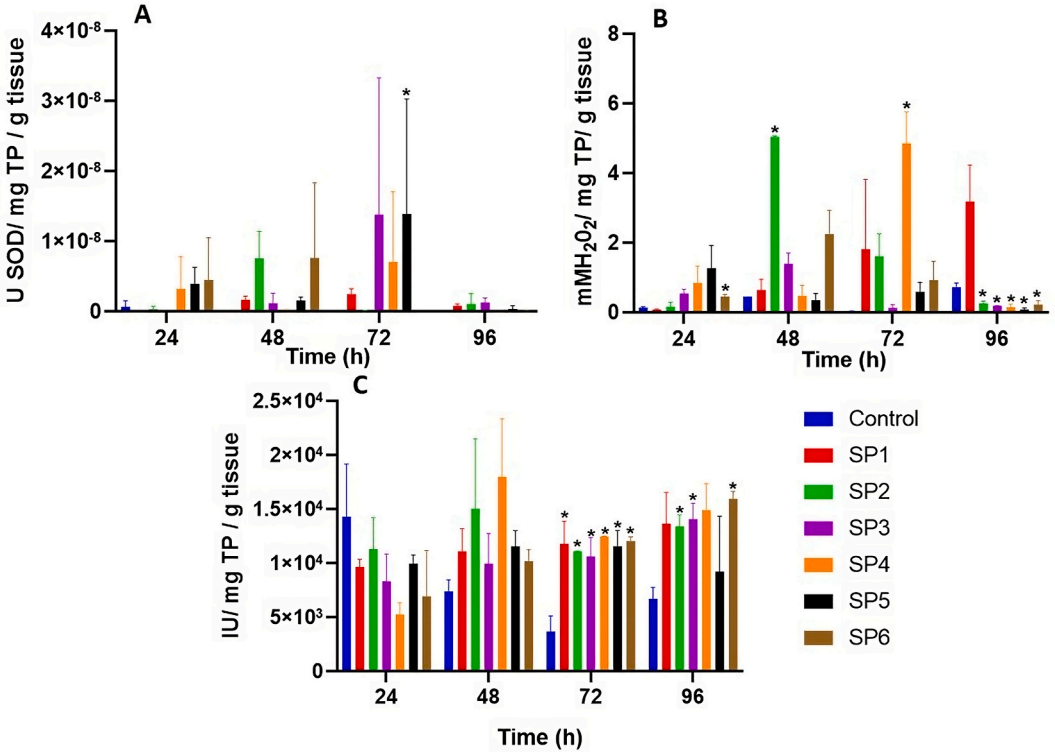

Fig. 4. Antioxidant enzyme activity (24, 48, 72 and 96 h) and acetylcholinesterase (96 h) of *Cyprinus carpio* larvae exposed to water from Villa Victoria dam. A shows superoxide dismutase activity, B catalase, C glutathione peroxidase and D acetylcholinesterase activity. Data are expressed as mean and SD with two-way ANOVA, and Dunnett's post hoc Dunnett's test for multiple comparisons. The asterisk (*) represents a $P < 0.05$ with respect to the control group.

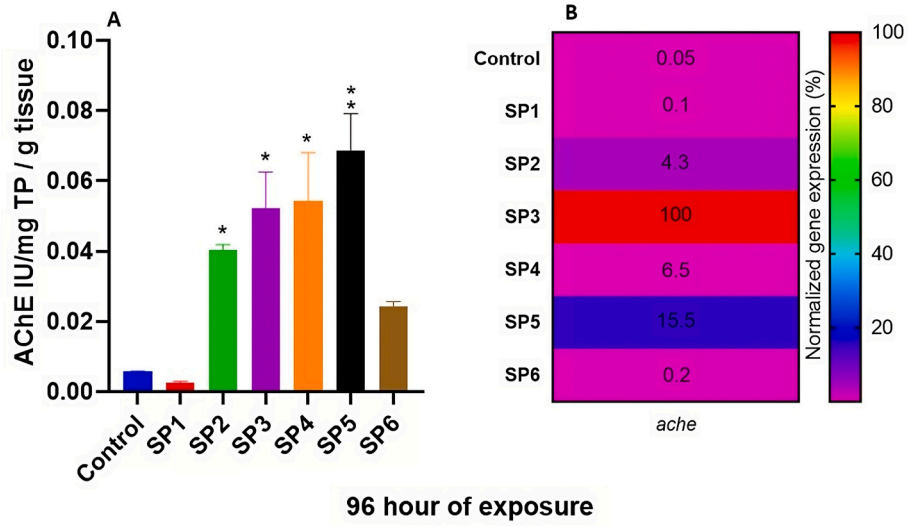

Fig. 5. AChE activity (A) and relative gene expression levels (B) in *Cyprinus carpio* larvae exposed to the Villa Victoria water at 96 h. Values are presented as percent and normalized respect whit the control group. The asterisk (*) represents a $P < 0.05$, asterisk (**) represents a $P < 0.002$.

of 8 (each one) and finally the SP 2 with a GMS of 9 (Table 3).

On the other hand, the teratogenic index in *Cyprinus carpio* embryos was calculated. The teratogenic index was less than 1 at all sampling points wich means that the probability of producing teratogenic effects is low. However, the closed values were those corresponding to SP 1 with a value of 0.94, SP 2 with 0.88 and SP 3 with 0.71, which indicates that, if the water from the Villa Victoria dam does not induce teratogenic effects on the embryos of this species, eventually these high values could increase in a short time (Table 3). Finally, teratogenic effects were recorded in *Cyprinus carpio* embryos. The prevalent abnormalities observed at 24, 48, 72, and 96 h were in the SP1, SP2 and SP3 reflexing head malformation, tail malformation, fin malformation, pericardial edema, and scoliosis (Fig. 6).

3.5. Behavioral analysis

Changes in the basal locomotor activity (BLA) and time spend in the periphery of the wall (thigmotaxis) were observed in *Cyprinus carpio* larvae after exposure to water from the Villa Victoria dam at the different sampling points. After 24 h of exposure, an increase in BLA was

Table 3
General Morphology Score (GMS) and teratogenic index of *Cyprinus carpio* embryo exposed to the six sampling points of the Villa Victoria dam.

Sampling points of the Villa Victoria Dam	GMS	TI
Control	15	0
PM1	12	0.94
PM2	9	0.88
PM3	3	0.71
PM4	8	0.69
PM5	8	0.42
PM6	7	0.63

observed in all SPs compared to the control group ($P < 0.001$) as well as SP 4 compared to SP 2 ($P < 0.002$) and 6 ($P < 0.001$) (Fig. 7-A). At this same exposure time, the larvae exposed to SP 1, 2, 3, 4 and 5 spent less time on the periphery of the well wall compared to the control group ($P < 0.001$) and SP 6 ($P < 0.002$) (Fig. 7-E). After 48 h, the distance traveled continued to increase only in the larvae of SP 2, 3, 4 and 5 compared to the control group ($P < 0.05$) and SP 6 compared to SP 2 ($P < 0.05$) and 5 ($P < 0.002$) (Fig. 7-B). Likewise, larvae exposed to SP 3 and 4 had a shorter time in the periphery compared to the control group and SP 6 ($P < 0.05$) (Fig. 7-F).

At 72 h, the larvae of SP 2, 4 and 5 continued to increase their distance traveled in the BLA compared to the control group ($P < 0.05$) and SP 6 with the larvae of SP 1 and 4 ($P < 0.05$). A difference was also observed in the time spent outside the periphery between the larvae of SP 2 and 4 with the control group and those of SP 6 ($P < 0.05$). Finally, after 96 h of exposure, the distance traveled in the BLA increased again in the larvae of SP 1, 2, 3, 4 and 5 compared to controls ($P < 0.05$) and the larvae of SP 6 compared to those of SP 2 ($P < 0.001$), 3 and 5 ($P < 0.05$) (Fig. 7-D). Differences were also observed in the time spent outside the periphery of the well in the larvae of SP 1, 2, 3, 4 and 5 compared to the control group ($P < 0.002$) as well as the larvae of SP 2 and 4 with the SP 6 ($P < 0.002$) (Fig. 7-H).

3.6. Correlation study

Fig. 8a shows the chord graph where it is observed that there are interconnections between tigmotaxis with the activity of the enzymes AChE, SOD, CAT, as well as with the expression of ache and the levels of oxidized proteins. In the case of BLA there is a relationship with the activity of antioxidant enzymes SOD, CAT and GPX, as well as with PCC levels. In the case of AChE activity a significant interconnection is seen with PCCs and the activity of CAT, SOD and to a lesser extent with GPx. For ache expression, a direct relationship is observed with AChE enzyme activity, as well as with CAT, SOD and GPx enzyme activity, in addition to PCC and LPX levels. Fig. 8b presents the matrix of Spearman correlation coefficients between oxidative stress, neurotoxicity and behavioral biomarkers at 96 h, in this it is observed that for tigmotaxis there is a high significant negative correlation between AChE levels ($p < 0.001$), oxidized proteins ($p < 0.01$) and SOD activity ($p < 0.05$), as well as a reduction of ache gene expression ($p = 0.061$). While increasing BLA increased the levels of GPx ($p < 0.01$), LPX and PCC ($p < 0.05$). In the case of AChE levels, it can be observed that the expression of its gene is increased ($p = 0.0506$), while increasing AChE reduces CAT levels ($p = 0.056$), the same behavior is observed with the expression levels of the ache gene with this antioxidant enzyme ($p = 0.053$).

4. Discussion

In this study, it was observed that water from the Villa Victoria reservoir caused an increase in lipid and protein oxidation in *C. carpio* larvae up to 96 h of exposure. Increased oxidation in these biomolecules is closely related to ROS that may be generated from contaminants present in the Villa Victoria reservoir. Pérez-Coyotl et al. (2019) and Gobi et al. (2018) observed an increase in lipoperoxidation and protein oxidation upon exposure of *C. carpio* embryos to water from the Madín reservoir and *Oreochromis mossambicus* exposed to selenium, respectively. When the generation of ROS increases, antioxidant enzymes are more activated to recover the redox balance and promote the health of the organism. Metals as aluminum, iron and selenium leads to the generation of redox-active metabolites, which may result in the generation of intracellular damage by calcium release. This triggers the activation of protein-breaking enzymes and nitric oxide synthase, as well as the production of free radicals, eventually causing oxidative stress (Gobi et al., 2018; Capriello et al., 2021; Rosales-Pérez et al., 2022). In Mexico,

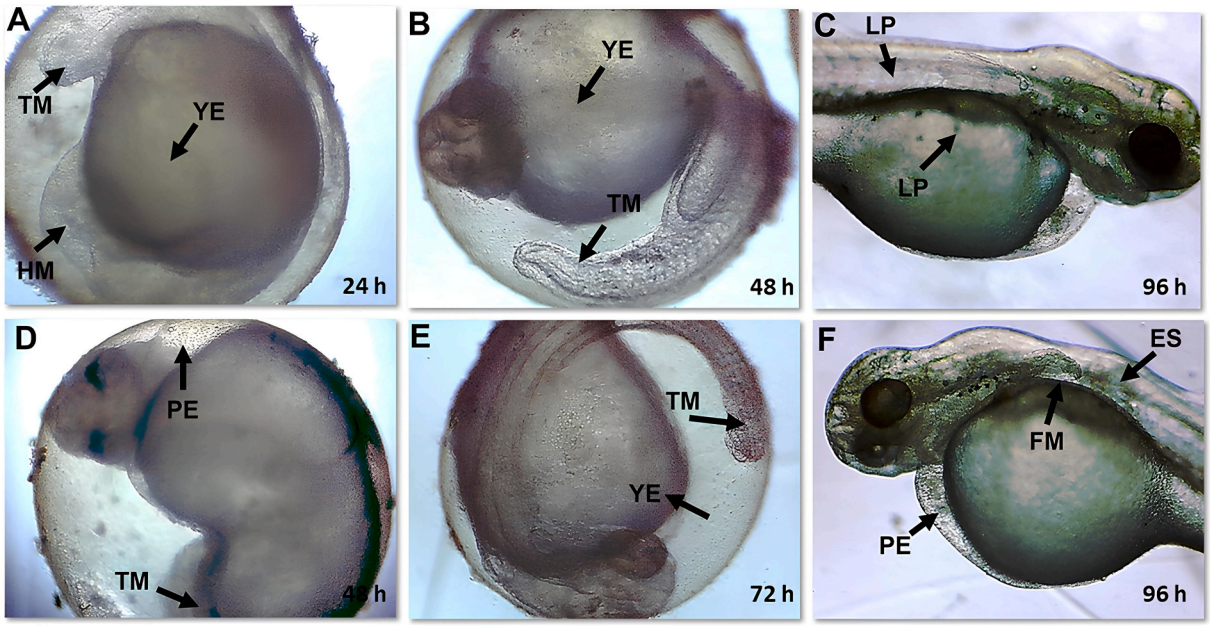

Fig. 6. Teratogenic effects of *Cyprinus carpio* embryos exposed to the Villa Victoria dam water. In A shows tail malformation (TM), head malformation (HM) and yolk edema (YE). In B yolk edema (YE) and tail malformation (TM). C shows low pigmentation (LP). D pericardic edema (PE) and tail malformation (TM). In E tail malformation (TM) and yolk edema (YE) and F shows pericardic edema (PE), fin malformation (FM) and escoliosis (ES).

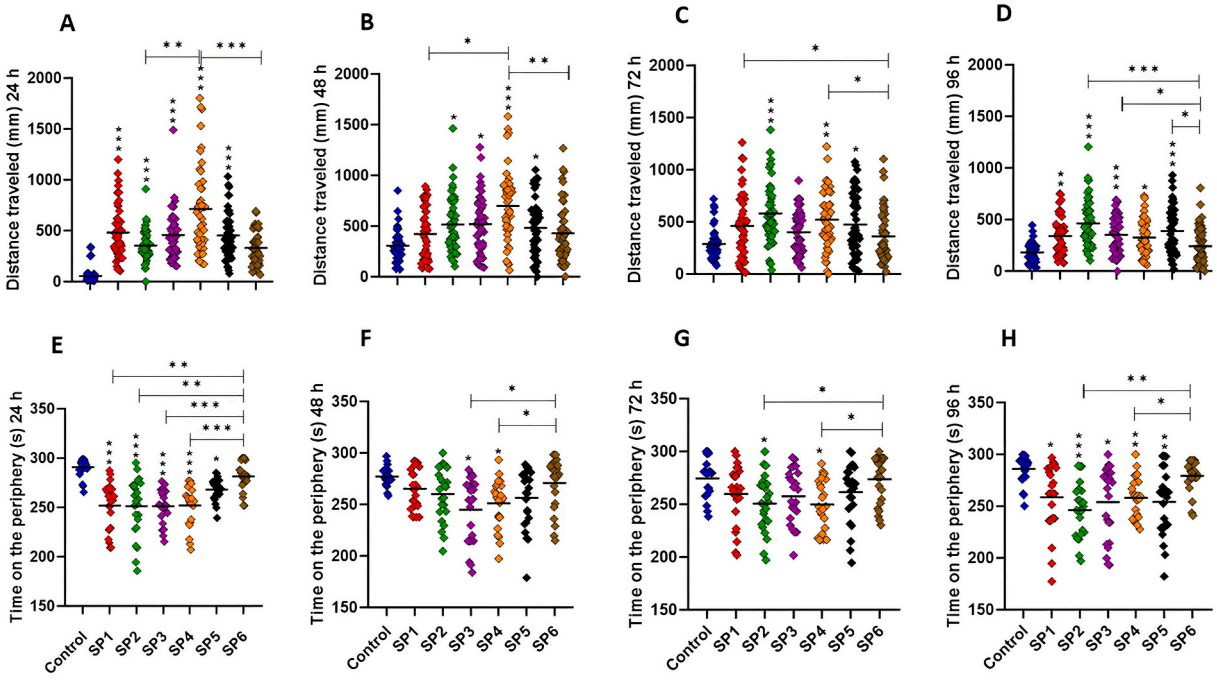

Fig. 7. Basal locomotor activity and time in the periphery (thigmotaxis) of *Cyprinus carpio* larvae exposed to water from the Villa Victoria dam at 24, 48, 72 and 96 h. Data are expressed as the median with one-way ANOVA, Kruskal-Wallis nonparametric tests and Dunnett's post hoc test for multiple comparisons. The asterisk (*) represents a $P < 0.05$, asterisk (**) represents a $P < 0.002$ and (***) represents a $P < 0.0001$.

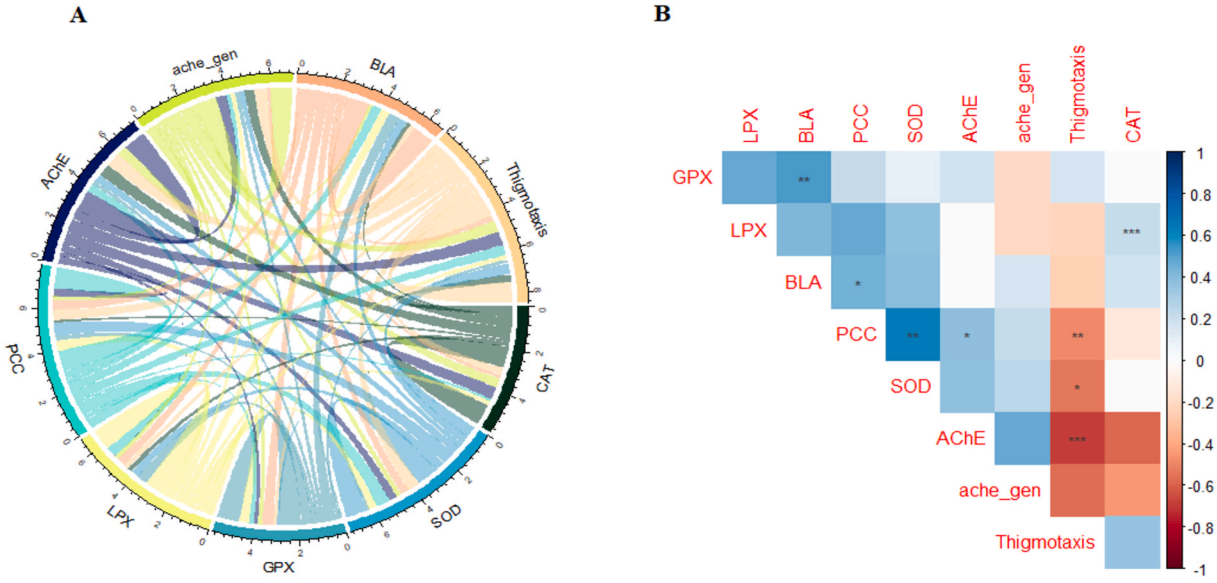

Fig. 8. Interconnections by chord diagram (A) and Sperman correlations (B) between the biomarkers studied in *Cyprinus carpio* larvae exposed to the Villa Victoria water at 96 h.

water bodies and storage reservoirs such as Conchos in Chihuahua, Atoyac in Puebla, the Lerma River Basin and Madin Dam in the State of Mexico, among others; present contaminants such as metals, drugs, PCB's and pesticides that promote the production of ROS and modify the chemical and molecular structure of proteins, enzymes and genetic material, important for cell differentiation and organogenesis in fish embryos and larvae (Blahová et al., 2013; Velki et al., 2017; Cano-Viveros et al., 2022).

To evaluate oxidative stress produced by pollutants, enzymatic biomarkers have been used in aquatic organisms, such as the enzyme SOD, which forms the first line of antioxidant defense together with CAT and GPx (Anifowoshe et al., 2022; Cano-Viveros et al., 2022; Gopi et al., 2022). The activity of these enzymes has been modified in *Cyprinus carpio* embryos exposed to drugs, metals, PCB's and pesticides in water from Madín reservoir as reported by Pérez-Coyotl et al. (2019). Anifowoshe et al. (2022) and Gopi et al. (2022) reported similar results in *Tilapia zillii* exposed to reservoirs and rivers from Ilorin, Nigeria and in *Oreochromis niloticus* to sub-lethal copper concentrations. Metals and pesticides have been reported in the water of Villa Victoria dam (Table 1), which could explain the increased CAT and GPx activity in *C. carpio* larvae in SP 3, 4 and 5, which are livestock and agricultural areas evaluated in this study, and therefore with a higher load of this

type of pollutants.

Metals such as aluminum, copper and iron can originate free radicals such as hydroxyl ion (OH-), through the Fenton and Haber-Weiss reaction, which can alter proteins, enzymes and genetic material causing modifications in organogenesis and regulatory apoptotic mechanisms in the formation of the gastrula by SOD and CAT enzymes in fish embryos and larvae (Pérez-Coyotl et al., 2019; Cano-Viveros et al., 2022). On the other hand, pesticides such as atrazine and diazinon, widely used and sold in Mexico for agriculture and detected in Villa Victoria reservoir, have been shown to modify SOD and GPx gene expression, as well as delayed organogenesis, slowing of movements and heart alterations in *Danio rerio*, *Cyprinus carpio* embryos and other fish species exposed to these contaminants (Blahová et al., 2013; Pérez-Coyotl et al., 2019; Cano-Viveros et al., 2022).

The knowledge of AChE activity turns out to be an important neurotoxic biomarker in studies of aquatic pollution since this enzyme is modified to various contaminants in water such as organophosphorus pesticides and metals such as cadmium, lead, iron, selenium and aluminum (Rodríguez-Fuentes et al., 2015; Gobi et al., 2018; Marinho et al., 2019; Rosales-Pérez et al., 2022). The primary action of organophosphates involves blocking AChE by adding a phosphate group to the serine residue in its active site. This inhibition leads to higher levels of ACh at nicotinic and muscarinic receptors, causing an accumulation of acetylcholine and overstimulation of cholinergic receptors affecting the central nervous and muscular system (Rodríguez-Fuentez et al., 2015; Bedrossiantz et al., 2023). In this study, modifications in AChE levels were observed in *C. carpio* larvae due to water exposure mainly in points 2, 3, 4 and 5 where there is a relationship between the wasted waters and agriculture that may use organophosphate pesticides like diazinon and dimethoate, that tend to bioaccumulate in the brain. The changes in AChE levels in *C. carpio* larvae can be explained according to what was suggested by Yen et al. (2011) where pesticides such as parathion, diazinon and chlorpyrifos, reported in reservoirs and rivers in northern and southern Mexico, directly inhibit AChE by blocking neuro-muscular receptors, thus causing a decrease in neuromuscular function in fish larvae.

Another mechanism of action produced by metals, such as aluminum and iron, is explained by Senger et al. (2011), where these metals interact with the cholinergic system in the brain as a cholinotoxin by blocking synaptic cleavages and preventing the hydrolysis of ACh to choline and acetate and, consequently, increasing AChE activity. Other researchers have reported that aluminum and malathion can increase AChE activity through allosteric interaction between the cation and the peripheral anionic site of the enzyme (Ramanathan, 2014). In *C. carpio* larvae exposed to water from the Villa Victoria dam, an increase in AChE activity was observed in the different sampling points and this may be due to the fact that, in the reservoir, there are different contaminants such as metals, PCB's and pesticides that could be interacting in parallel in the sites of action and in the toxic response of the larvae, evidencing these variations in concentration, as well as changes in the behavior activity of the larvae evaluated.

The presence of pesticides, metals, and pharmaceuticals products in water can induce modifications in the expression of the acetylcholinesterase gene (*ache*) within fish larvae (Wang et al., 2019; Rosales-Pérez et al., 2022; Bedrossiantz et al., 2023). Research has indicated that organophosphate pesticides impact ACh, given that prooxidant agents influence the regulation of AChE activity and associated gene expression. Experiments carried out in controlled environments have revealed that low concentrations of H2O2 stimulate the expression of the recombinant *ache* gene, while high concentrations not only inhibit it, but also promote the oxidation of key amino acids such as methionine, cysteine and tryptophan, related to its active site (Wang et al., 2019; Capriello et al., 2021; Rosales-Pérez et al., 2022). This gene encodes a crucial enzyme vital for the regulation of cholinergic neurotransmission in the nervous system. Inhibiting AChE can lead to the accumulation of neurotransmitters in the synaptic cleft, causing an excessive stimulation of the nervous system. Exposure to commonly used agricultural pesticides like organophosphates has been proven to diminish AChE activity in fish larvae. Likewise, metals like lead, mercury and aluminum have displayed an ability to affect acetylcholine in these organisms, demonstrating lowered AChE enzyme activity when exposed to high concentrations (Rodríguez-Fuentes et al., 2015; Senger et al., 2011; Rosales-Pérez et al., 2022). Additionally, drugs like nicotine, carbamazepine and NSAIDs, when encountered by fish larvae, have been linked to alterations in AChE activity. Certain studies propose that nicotine might escalate the enzyme's activity in specific tissues. This phenomenon gives rise to diverse health outcomes in fish larvae, such as hindered growth, heightened mortality, neurotoxicity, and modified behavior (Rodríguez-Fuentes et al., 2015; Senger et al., 2011; Rosales-Pérez et al., 2022).

Contaminants in water can modify the activity of antioxidant enzymes, which are closely related to embryonic development, embryotoxic and teratogenic effects in fish. Pérez-Coyotl et al. (2019), and Cano-Viveros et al. (2022) observed a decrease in GMS in *C. carpio* embryos exposed to water from the Madín reservoir and concentrations of 0.074 mg/L of aluminum, 45.8 mg/L of metformin and 0.01 mg/L of penicillin G compared to control groups, respectively. The presence of pesticides, as well as aluminum, metformin and other emerging contaminants, has been found in the Villa Victoria dam (Table 1), and the presence of these compounds could be related to the decrease in MSG observed in *C. carpio* embryos exposed to water from the Villa Victoria dam in this study. Metals like iron and aluminum can interfere with critical signaling pathways involved in embryonic development. They can disrupt the signaling cascades that regulate cell proliferation, migration, and differentiation, leading to abnormal morphological development. Metals have been shown to interfere with signaling pathways like Notch, Wnt, and Sonic hedgehog (Shh), which are essential for proper embryonic morphogenesis as transmembrane signaling receptors for the development of swim bladder, central nervous system and induction and formation of the ectoderm and mesoderm (Kiyooka et al., 2020; Zhang et al., 2022).

Regarding the teratogenic index, the water from the Villa Victoria dam presented an index less than 1 at all sampling points, however some teratogenic alterations were recorded in *C. carpio* embryos. Luja-Mondragón et al. (2019), Pérez-Coyotl et al. (2019) and Vasamsetti et al. (2020) reported teratogenic indices of 1.46, 0.98 and 1.52 in hospital effluents, water of the Madín dam and environment concentrations of pesticides in *C. carpio* and *D. rerio* embryos, as well as embryonic alterations, observed in our study, such as pericardial edema, edema of the yolk sac, fin deformation, tail malformation, scoliosis and delayed hatching. Although the index in this study was less than 1, there is a prevalence of teratogenic effects in *C. carpio* embryos due to exposure to Villa Victoria water. It is important to consider that given the conditions of the reservoir it is possible that over time the contaminants and their mixture may increase, generating more prevalent teratogenic effects in the fish in the reservoir that may put the survival of the organisms at risk and reduce the population by not being able to swim adequately to carry out their biological functions.

In studies related to behavioral changes in fish, one of the pollutants directly related to the central nervous system and its effects on locomotor swimming activity are pesticides. Velki et al. (2017), Faria et al. (2021) and Bedrossiantz et al. (2023), found an increased and decrease in 24 h on the basal locomotor activity in *Danio rerio* larvae by exposure to organophosphorus pesticides at concentrations of 2 mg/L diuron and diazinon, 17 μg/L of fenitrothion and 66 ng/L of carbaryl. Bedrossiantz et al. (2023) and Ricarte et al. (2023) relate hermetic changes in BLA after the exposure to pollutants, with the strongest effects at the lower concentrations. The hyperactivity found in *Cyprinus carpio* larvae in this study it is an ecological relevant effect, as it not only implies a waste or energy but may also attract the attention of its predators (Bedrossiantz et al., 2023; Burgess and Granato, 2007; Calabrese and Agathokleous, 2022; Ricarte et al., 2023).

Thigmotaxis is frequently employed as a tool to assess anxiety and fear in fish after they've been exposed to toxic substances, as indicated by (Hamilton et al., 2021). When fish larvae increase the time spent in the center of the experimental arena it means a decrease in thigmotaxis (negative thigmotaxis), which suggests an anxiogenic effects of substances (Hamilton et al., 2021; Hutton et al., 2023; Li et al., 2019). In other studies, Li et al. (2019) and Hutton et al. (2023) observed negative thigmotaxis (less time spend in the peripheric area) in *D. rerio* larvae exposed to 10 and 100 ng/L of pyrethroids and 1 and 10 μg/L deltamethrin, pesticides that are widely used to control insect pests in crops. Pesticides act directly on neurotransmitters such as ACh, dopamine and GABA, modifying gene expression or inhibiting signal transmission over chemical synapse and ion channels, affecting the behavior in fish larvae (Cao et al., 2019; Rodríguez-Fuentes et al., 2015; Yen et al., 2011). In Villa Victoria, in SP 3, 4 and 5, potato, corn and wheat cultivation activities are conducted as well as wasted water in SP 2, that can contain contaminants as pesticides, drugs and metals that are reported to change the basal locomotor activity and thigmotaxis. The results of this work indicate an increase in the distance traveled in the BLA as well as negative thigmotaxis in *C. carpio* larvae. These effects reflect periods of hyperactivity and anxiety-modulation that could be related to the presence of insecticides, herbicides and their mixtures that could be present in the Villa Victoria dam.

On the other hand, metals such as iron and aluminum have been shown to have neurotoxic effects by progressively interacting with the lipid membrane composition and changing the molecular structure of neurotransmitters responsible for movement, orientation, and locomotion in fish. Capriello et al. (2021) and Senger et al. (2011) detected an increase and decrease, respectively, in distance traveled, thigmotaxis and hiperactivity of adult *Danio rerio* by exposure to 11 mg/L of aluminum and cadmium for 15 days. Metals such as aluminum are widely used as an adjuvant in the coagulation process for water treatment, in addition to having neurodegenerative capacity in aquatic organisms causing diseases related to the central nervous system such as Parkinson's and Alzheimer's (Cano-Viveros et al., 2022; Capriello et al., 2019, 2021; Fan et al., 2021; García-Medina et al., 2010; Senger et al., 2011). In Villa Victoria Dam, concentrations above 0.05 mg/L of aluminum were reported in the sample points (Table 1), so the increase in the basal locomotor activity and thigmotaxis changes could also be related to the presence of this metal in the Villa Victoria dam water.

Clandestine wastewater discharges are an important source of drug contamination in water bodies, which have been reported in the Villa Victoria dam. NSAIDs interact by inhibiting cyclooxygenases which catalyze the synthesis of prostaglandins, and these are necessary in fish embryo and larvae development in muscular moved processes (Gutiérrez-Noya et al., 2020; Qiang et al., 2016; Zhou et al., 2019). On the other hand, benzodiazepines, and anticonvulsants such as carbamazepine modify enzymatic mechanisms such as GABA at the synapse, delimiting embryo movements in chorion rupture or swimming activities in larvae (Cao et al., 2019; Gebauer et al., 2011; Pohl et al., 2019; Qiang et al., 2016).

Although the behavioral biomarker is sensitive and assesses individual-level effects, the observed behavioral effects may be related to physicochemical variations in water, environment, and climate, as this biomarker is integral. However, in this study it was observed that SP related to agriculture, livestock and wastewater discharge also presented changes in oxidative damage, activity of antioxidant enzymes as well as AChE and its gene expression, suggesting a relationship between the sensitivity of specific biomarkers accompanied by the behavioral biomarker. For this reason, the presence of drugs, as well as metals, pesticides, PCBs and their mixtures, in addition to changes in the physicochemical dynamics of the water, could be modifying the behavior that was observed in *C. carpio* larvae exposed to water from this reservoir.

5. Conclusions

The results obtained in this study show that water from the Villa Victoria dam caused oxidative damage, modification in the enzymatic activity of superoxide dismutase, catalase and glutathione peroxidase, as well as increase in gene expression and activity of ACh esterase. Embryotoxicity, teratogenic effects and changes in the basal locomotor activity and thigmotaxis of *Cyprinus carpio* larvae was also observed. These reported changes could be associated with the presence of drugs, PCB's, metals and pesticides in the dam which, in turn, can compromise biological and ecological activities in fish such as development, swimming, recognition, orientation, depredators scape and food capture, as well as neurotoxic effects related to stress and anxiety. It is important to implement programs to restore the reservoir, community work, talks on the consequences of pollution, and investment by decision-makers to repair and renovate the treatment plants. Finally, the Villa Victoria dam belongs to the Cutzamala system and is one of the main water sources for the Metropolitan Zone of the Valley of Mexico, where more than 20 million people live, so paying attention and acting on its prevention is essential to mitigate the increase of contamination of this reservoir, since it can eventually pose a risk to the life of aquatic organisms and human health.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All international and national standards and/or institutional guidelines for the care and use of animals were followed. The study protocol was approved by the Research Ethics Committee of the National School of Biological Sciences, IPN.

Declaration section

All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors".

CRediT authorship contribution statement

Misael Hernández Díaz: Writing – original draft, Methodology, Investigation, Formal analysis. **Marcela Galar Martínez:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sandra García Medina:** Validation, Methodology, Data curation. **Alejandra Cortés López:** Methodology. **Karina Ruiz Lara:** Investigation, Formal analysis. **Selene Cano Viveros:** Methodology. **Alba Lucero García Medina:** Methodology, Formal analysis. **Ricardo Pérez-Pastén Borja:** Methodology. **Karina Elisa Rosales Pérez:** Methodology. **Leobardo Manuel Gómez Oliván:** Validation, Methodology. **Demetrio Raldúa:** Pérez, Data curation. **Juliette Bedrossiantz:** Data curation.

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.

Data availability

Data will be made available on request.

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