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The Relationship Between Embryotoxicity and Oxidative Stress Produced by Aluminum, Iron, Mercury, and Their Mixture on *Cyprinus carpio*

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Abstract Aluminum, mercury, and iron have been found in high concentrations in various freshwater bodies around the world and have been shown to be very harmful contaminants to hydrobionts, causing metabolic dysfunction and damage at various levels. However, studies on mixtures of these pollutants are scarce, particularly in younger or developing organisms, which are more sensitive to damage. Therefore, the objective of this work was to evaluate the toxicity of Al, Fe, and Hg, in isolation and in mixture, on

Highlights
1. Common carps embryos exposed to concentrations equivalent to the maximum permissible limit for the protection of aquatic life of Al and Hg exhibit modifications to the activity of antioxidant enzymes, but only Al produces oxidative damage.
2. Al and Hg produce modifications to common carp embryonic development at concentrations equivalent to the maximum permissible limit for the protection of aquatic life.
3. Fe at concentrations equivalent to the maximum permissible limit for the protection of aquatic life, produces neither oxidative stress nor modifications to the embryonic development of *C. carpio*.
4. The toxicity of Fe, Al and Hg is modified by the interaction of the three toxicants

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common carp embryos exposed to the maximum permissible limits described in the Mexican regulations for the protection of aquatic life, correlating the biomarkers of oxidative stress with the effects on embryonic development. For this purpose, the *Cyprinus carpio* embryos were exposed to iron (0.1 mg L^{-1}), mercury (0.00001 mg L^{-1}), aluminum (0.05 mg L^{-1}), and their mixture, throughout their development and until hatching. The activity of the antioxidant enzymes (SOD, CAT, and GPx), the degree of lipoperoxidation, and the content of hydroperoxides and total proteins, as well as the morphological development of the embryos were evaluated at 12 h, 24 h, 48 h, 72 h, and 96 h of exposure. The results showed that the metals under study are toxic to *C. carpio* embryos and that their interaction modifies the toxic response. Thus, iron generates alterations in the activity of antioxidant enzymes and modification in embryonic development to a lesser extent than aluminum and the mixture of metals, while mercury exerts its toxicity on embryos by mechanisms other than oxidative stress, but the modification to the activity of antioxidant enzymes may contribute to the changes observed in embryonic development. The changes in the response of biomarkers of embryotoxicity and oxidative stress suggest that the combination of metals produces an antagonism-type interaction, so this study provides a precedent for future research to determine the type of interaction that a mixture of contaminants generates in developing aquatic organisms.

Keywords Interactions · Metals · Oxidative stress · Embryotoxicity · *Cyprinus carpio*

1 Introduction

The amount of pollutants such as aromatic hydrocarbons, pesticides, pharmaceuticals, personal care products, nanoparticles, and metals, among others, has increased rapidly in different water bodies around the world in recent years (Ali et al., 2019; Fent et al., 2006; Schmidt et al., 2016; Zhou et al., 2020) and can have detrimental effects on the health of hydrobionts (Lushchak, 2016), particularly those in early stages of development.

Among the most common metals in freshwater bodies are aluminum (Al), iron (Fe), and mercury (Hg). Aluminum is the third most abundant element in the Earth's crust after oxygen and silicon, and previous studies have shown that this metal has the capacity to generate adverse effects in humans and aquatic organisms (Barabasz et al., 2002; Roy & Bhadra, 2014; Slaninova et al., 2014) with the nervous, hematopoietic, and skeletal systems being the most sensitive to this metal (Exley, 2004; García-Medina et al., 2010). Iron is the metal most exploited for its industrial uses, in addition to being an essential metal, which, in small amounts, fulfills biological functions in animals and plants; however, in higher concentrations, it can cause damage in exposed organisms, derived from the precipitation of excess iron in organs and by the effect of oxidative stress (Slaninova et al., 2014). On the other hand, mercury is a metal that, in small concentrations, can exert toxic effects on the biotic elements of an ecosystem. Hg can occur in the environment in three forms: elemental (Hg^0), organometallic (Me-Hg), and inorganic (Hg^{2+}), the latter being the most frequent in water bodies, followed by Me-Hg, which is highly liposoluble, resulting in its bioaccumulation in aquatic species (Carrasco et al., 2011; Chang et al., 2017; Driscoll et al., 2013). Hg is known to have genotoxic, nephrotoxic, cardiotoxic, neurotoxic, and immunotoxic potential, being a public health and environmental problem (Lubick, 2010; Fernandes-Azevedo et al., 2012; Chang et al., 2017; Bridges & Zalups, 2017; Choi et al., 2017).

These metals are frequently found in water bodies. In the Ologe Lagoon in Lagos, Nigeria, Fe concentrations in water and sediments were found with maximum values in the dry season of 0.080 mg L^{-1} and 0.5 mg L^{-1}, respectively (Kumolu-Johnson et al., 2010). In another study, Fe concentrations were determined from an urban runoff in industrial, residential, and roadside areas in South Korea, finding maximum concentrations of 34 mg L^{-1}, 2 mg L^{-1}, and 7 mg L^{-1}, respectively (Lee & Han, 2012). Ravenscroft et al. (2018) determined the iron concentration in drinking water consumed by school children, with a median of 0.0157 mg L^{-1}, while Aba et al. (2019) found concentrations close to 1 mg L^{-1} in deep well water in Sulawesi Province, Indonesia.

In the case of Al, Guibaud and Gauthier (2003) found concentrations ranging from 0.008 to 0.275 mg L^{-1} in four rivers belonging to the Limousin region of France. In another study conducted in

groundwater, Al present in 871 samples from Sistan and Baluchistan provinces, Iran, was quantified and values ranging from 0.01 to 0.059 mg L^{-1} were found. Recently, the content of this metal was determined in water samples from the Mogi Guaçu River in Brazil, obtaining concentrations of 0.1 to 1 mg L^{-1} (Silva Pinheiro et al., 2020).

Hg is a pollutant metal whose presence in water bodies has been reported at very low concentrations; however, these are sufficient to produce toxic effects in aquatic organisms. This metal was quantified in several water samples from the Sacramento River basin in California, where concentrations ranging from 30 to 105 ng L^{-1} of total Hg were obtained (Domagalski, 1998) Bosch et al. (2009) quantified Hg concentrations between 0.5 and 15.1 μg g^{-1} in sediments obtained from the Ebro River in Spain. Subsequently, in a study conducted in Lake Zapotlán in the state of Jalisco, Mexico, total Hg concentrations of between 0.5 and 240.1 ng L^{-1} were determined in samples from this water body (Malczyk & Branfireun, 2015). Recently, environmental contamination caused by an abandoned chloralkali plant in southeastern China was characterized, where concentrations of dissolved Hg in groundwater and total Hg in surface water, as well as in soil samples in the area of the factory, were quantified. The results showed that dissolved Hg ranged from 0.003 and 3600 μg L^{-1} in groundwater samples, 8.4 to 1940 ng L^{-1} total Hg for surface water, and 0.09 to 1.3 mg kg^{-1} for soil samples (Song et al. 2018). At the Madín dam in the state of Mexico, Al, Fe, and Hg were found in surface water samples at concentrations ranging from 6.04 to 24.44, 1.42 to 4.28, and less than 0.001 mg L^{-1}, respectively (Pérez-Coyotl et al., 2019; Valerio-García et al., 2017), which exceed the maximum permissible limits established in the legislation for the protection of aquatic life (DOF, 1989).

Previous research demonstrates the occurrence of various metals in water bodies, forming complex mixtures that can modify the toxic response of exposed organisms unlike if they were exposed individually, due to interactions between different environmental contaminants (Aba et al., 2019; Jaishankar et al., 2014; Kumolu-Johnson et al., 2010; Ravenscroft et al., 2018). An interaction is defined as the simultaneous or consecutive exposure of two or more toxicants and may result in additive, synergistic, potentiating, or antagonistic effects (Feng et al., 2018; Hernández et al., 2017). The study of metal interactions is a key to the understanding of the physicochemical, toxicokinetic, and toxicodynamic mechanisms that produce them and the toxic effects that may occur in aquatic organisms (Sadeq & Beckerman, 2020). Additionally, age is another factor that modifies the toxic response, where individuals in early stages of development, such as the embryonic stage, are more susceptible to the combined effects of contaminants (Pérez-Coyotl et al., 2017).

Fe, Al, and Hg have been shown to be toxic to various aquatic species, sharing oxidative stress as a mechanism of toxicity. Oxidative stress is defined as the imbalance between the generation of reactive oxygen species (ROS) and the antioxidant capacity of the organism (Valavanidis et al., 2006). The chemical coordination characteristics and oxidation–reduction properties of metals make them function as pro-oxidant agents, which favor the production of ROS in cells (such as superoxide anion, hydroxyl radical, or nitric oxide), which bind to biomolecules, such as proteins, lipids, and nucleic acids, generating toxic effects at the functional and structural level triggering cell death (Jaishankar et al., 2014). This mechanism of toxic action can cause depletion of antioxidant molecules essential for cellular processes, such as glutathione, destabilization of the cell membrane, denaturation of proteins, and damage to genetic material, triggering processes of cytotoxicity, mutagenicity, genotoxicity, reproductive disorders, embryotoxicity, and teratogenesis (Ercal et al., 2001; Pérez-Coyotl et al., 2017, 2019).

Previous studies show that these metals exert toxic effects on developing organisms individually through oxidative stress; Al toxicity has been demonstrated on several species of developing organisms such as *Palaemonetes pugio* (Rayburn & Aladdin, 2003), *Rhinella arenarum* (Herkovits et al., 2015), *Xenopus laevis*, and *Danio rerio*, producing malformations and increased lethality associated with altered expression of genes involved in the development and maturation of the digestive, circulatory, and nervous systems (Ismail et al., 2019). On the other hand, Li et al. (2009) demonstrated that increasing concentrations of Fe nanoparticles increased the degree of lipoperoxidation (LPx) and reduced superoxide dismutase (SOD) activity in

Oryzias latipes embryos. It is important to highlight that studies concerning Fe toxicity in early stages of aquatic organisms are scarce; however, it is well established that Fe has the ability to induce oxidative stress in different organs of adults of *Oryzias latipes* (Chen et al., 2013; Li et al., 2009), *Labeo rohita* (Singh et al., 2019), and *Cyprinus carpio* (Slaninova et al., 2014). For its part, Hg in both organic and inorganic forms has been associated with modifications in the expression patterns of transport genes and transcriptional regulation associated with oxidative stress, as well as fluctuations in the activity of antioxidant enzymes in zebrafish (*Danio rerio*) embryos and larvae (Ho et al., 2013; Zhang et al., 2016), while chronic exposure to Hg^{2+} generates reduced growth and metamorphosis rate, as well as thyroid and liver dysfunction due to oxidative stress in *Bufo gargarizans* tadpoles (Shi et al., 2018).

In general, the combination of biomarkers of oxidative stress and early morphological development constitute a battery of tests that is very useful to determine the effects of environmental pollutants, since ROS play a fundamental role in normal embryonic development by shaping the organs and systems of individuals, which will be compromised when exposed to toxicants that produce oxidative damage to biomolecules and modify the activity of antioxidant defense systems (Pérez-Coyotl et al., 2019).

Cyprinus carpio Linnaeus, 1758 is an organism commonly used in ecotoxicological studies, due to its ecological and economic importance as it is found in 80% of water bodies and is a product for human consumption, occupying eighth place in world fish production (Nava-Alvarez et al. 2014). Additionally, their embryonic development is particularly rapid, with a semitransparent chorion that allows easy observation of the appearance of structural features over time until hatching and monitoring of developmental delay and teratogenicity (Beker van Woudenberg et al., 2014; Hermsen et al., 2011; Pérez-Coyotl et al., 2019).

The objective of this work was to evaluate the toxicity of Al, Fe, and Hg, in isolation and in mixture, on common carp (*Cyprinus carpio*) embryos exposed to the maximum permissible limits described in the Mexican regulations for the protection of aquatic life, correlating the biomarkers of oxidative stress with the effects on embryonic development.

2 Material and Methods

2.1 Test Substances

For exposure to Fe, Al, and Hg, the salts $FeSO_4$, $Al_2(SO_4)_3$, and $HgCl_2$ (Sigma-Aldrich) were used. The nominal concentrations tested were as follows: Fe = 0.1 mg L^{-1}, Al = 0.05 mg L^{-1}, and Hg = 0.00001 mg L^{-1}.

2.2 Procurement of Cyprinus carpio Embryos

Embryos were obtained by natural fertilization carried out at the Centro Carpícola Tiacaque (state of Mexico). Four adult females and two adult males were placed in fertilization tanks. Oviposition and fertilization of eggs were monitored. Embryos were selected at 6 hpf and observed under a stereomicroscope to confirm fertilization, embryonic stage (gastrulation period), and viability (Hermsen et al., 2011). The embryos were then exposed to the different metals and the mixture to investigate possible oxidative stress and embryotoxicity.

2.3 Determination of Antioxidant Defenses and Oxidative Stress Parameters

Five groups with 7 g of embryos each were organized in 1-L fish tanks. The first three groups were exposed to aluminum, mercury, and iron at concentrations of 0.05 mg L^{-1}, 0.00001 mg L^{-1}, and 0.1 mg L^{-1}, respectively, and dissolved in egg water (60 µg L^{-1} of Instant Ocean salts), corresponding to the concentrations of the maximum permissible limit for the protection of aquatic life (DOF, 1989); the fourth group was exposed to the mixture of the three metals at the aforementioned concentrations, and the fifth group was the control, which was only egg water. After 12 h, 24 h, 24 h, 48 h, 48 h, 72 h, and 96 h of exposure to the abovementioned metals, the embryos were homogenized in 1.5 mL of phosphate-buffered solution (PBS), pH 7.4, to subsequently evaluate oxidative stress. The homogenate was divided into two parts: one was used to determine the degree of LPx and hydroperoxide content, and the other was centrifuged at 15,000 × *g* and 4 °C for 15 min to obtain the supernatant. This was used to evaluate the antioxidant activity of SOD, catalase (CAT), and glutathione peroxidase (GPx), as well as the carbonyl protein

content. Both fractions were stored at − 70 °C. Total protein content was used to express the results of all biomarkers evaluated.

2.4 Determination of Superoxide Dismutase Antioxidant Activity

SOD enzyme activity was determined using the Ransod kit (Randox). Readings were carried out on an Elx800 reader (BioTek) at 490 nm and interpreted with a calibration curve included in the kit. Data are expressed as U SOD per mg of protein per gram of tissue.

2.5 Determination of the Antioxidant Activity of Catalase

CAT enzyme activity was determined by the method of Radi et al. (1991). Nine hundred microliters of isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, and 5 mM KH_2PO_4; Sigma) and 200 µL of 20 mM H_2O_2 were added to 100 µL of the supernatant. The absorbance was determined at 240 nm at 0 s and 60 s, and the CAT activity per minute was calculated using the CME of H_2O_2 (0.043 mM^{-1} cm^{-1}). Results are expressed as millimoles of H_2O_2 per milligram of protein per gram of tissue.

2.6 Determination of the Antioxidant Activity of Glutathione Peroxidase

GPx enzyme activity was determined using the Ransel kit (Randox). The absorbance was determined at 340 nm at 1 min, 2 min, and 3 min. The concentration of GPx was calculated using the following formula: U $L^{-1} = 8412 \times \Delta A$ 340 nm min^{-1}. Results are expressed as IU of GPx per mg per gram of tissue.

2.7 Determination of the Degree of Lipoperoxidation

The degree of LPx was determined using the method of Buege and Aust (1978). Two hundred microliters of the homogenate was added to 1 mL of the reaction solution composed of 15% TCA, HCl, and TBA. The samples were brought to boiling for 15 min and, upon removal, were allowed to cool, and then the precipitate was removed by centrifugation at $2350 \times g$ for 10 min. The absorbance was determined at 535 nm with the reaction solution as blank. The results are expressed as nM of malondialdehyde per milligram of protein per gram of tissue.

2.8 Determination of Hydroperoxide Content

The hydroperoxide (HPX) content was determined by the method of Nourooz-Zadeh et al. (1994). Four hundred microliters of 15% trichloroacetic acid was added to 400 µL of the homogenate. After 15 min, it was centrifuged for 10 min at $850 \times g$. Three hundred microliters of the supernatant was taken, and 700 µL of the reaction solution (xylenol orange tetrasodium and butylated hydroxytoluene (Sigma), dissolved in 90% methanol and mixed with ferrous sulfate and H_2SO_4) was added. It was kept in incubation for 1 h. The absorbance was determined at 560 nm. Results are expressed as micromoles of cumene per milligram of protein per gram of tissue.

2.9 Determination of Carbonyl Protein Content

The oxidized protein (POX) content was determined by the method of Levine et al. (1994), modified as follows: 300 µL of 10 mM dinitrophenylhydrazine in HCl (2 M, Sigma) was added to 200 µL of the supernatant, prior to incubation away from light for 1 h. Then, 1 mL of trichloroacetic acid was added and rested for 15 min before centrifugation for 10 min at 10,000 rpm. The precipitate was washed three times with ethanol:ethyl acetate (1:1, Baker), dissolved in 1 mL of guanidine (6 M, sigma) in formic acid, and incubated at 37 °C for 15 min. The absorbance was read at 366 nm, and the results were expressed as nmol of reactive carbonyls (C = O) formed per mg protein, based on their EMF of 21,000 M^{-1} cm^{-1}.

2.10 Determination of Total Protein Content

The total protein content was determined by the method of Bradford (1976); 7.5 µL of deionized H_2O and 250 µL of Bradford reagent were added to 2.5 µL of the supernatant and left for incubation out of the light for 5 min. Samples were read at 595 nm in ELISA (BioTek).

2.11 Embryotoxicity Evaluation

Embryos for morphological development evaluation were arranged in 20 wells with three replicates

for each group on ELISA plates containing 300 µL of the metal solutions and a control group (egg water), maintained at a temperature of 24 ± 1 °C, and observed under an inverted microscope (Optika XDS-2) at 12 h, 24 h, 48 h, 48 h, 72 h, and 96 h. The tests were carried out in a static system without renewal. The evaluation was performed using the general morphological scoring (GMS) system adapted by Kimmel et al. (1995) and Hermsen et al. (2011), which is based on the presence or absence of specific features at defined times. Each embryo scores points for the developmental features assessed with which it complies. The first score was assigned at 12 h, and the last score was assigned at 96 h, corresponding to the time of hatching. The final score of the exposed embryos was compared with the final score of the control group. Dead embryos were not considered.

2.12 Statistical Analysis

The values obtained for each biomarker were subjected to a bifactorial analysis of variance, considering time as factor A and metals as factor B (two-way analysis of variance (ANOVA), $p < 0.05$). Differences between means were examined with the Tukey method. The results of the overall morphological score for the evaluation of embryonic development were subjected to a repeated measures (RM) ANOVA, followed by a Tukey post hoc test. All analyses were performed in the SigmaPlot 12.3 statistical package.

For the association between oxidative stress biomarkers and embryotoxicity, the development of each embryo was classified as a binary result (yes/no) based on the morphological score and the relationship of the embryotoxic effect was established with each of the groups tested with the control embryos; in the case of the mixture, the frequency of embryos with/without modifications in embryonic development was also compared with respect to the embryos exposed to the metals individually. First, a bivariate analysis was performed to establish the association between exposure to the metals and the mixture with the effect on the modification of embryo development, in order to subsequently perform the multivariate logistic regression analysis to evaluate the effect of several factors such as LPx, HPX, and POX concentration, as well as SOD, CAT, and GPx activities on embryotoxicity rates for each group evaluated. Logistic regression was also used to calculate the odds ratio (OR) and their 95% confidence interval (CI) using SPPS program, version 18.

3 Results

The results obtained in this work show certain enzymatic activity patterns in the control group, where SOD (Fig. 1) and GPx (Fig. 2) follow a decreasing tendency from 12 to 72 h post fertilization. Before hatching and after this event, there was a slight increase in the activity of this enzyme. In contrast, CAT (Fig. 3) reduced its activity after hatching of individuals (96 hpf).

Embryos exposed to Fe concentration (0.1 mg L^{-1}) present an enzymatic activity of SOD, CAT, and GPx, similar to that of the control group (Figs. 1, 2, and 3, respectively). On the other hand, embryos that were exposed to a concentration of 0.00001 mg L^{-1} of inorganic Hg showed statistically significant differences in SOD and GPx activities with respect to control group ($p < 0.05$), with 85% reduction in both cases and at all exposure times (Figs. 1 and 2, respectively). CAT activity did not show significant differences throughout the experiment; however, an increase in enzyme activity was observed at 12 h and 24 h (42% and 44%, respectively), followed by a 68% reduction in activity at 72 h, just before hatching of the individuals.

Organisms exposed to 0.5 mg L^{-1} of Al showed a significant decrease ($p < 0.05$) in SOD enzyme activity at 12, 24, and 48 hpf (70%, 72%, and 63%, respectively; Fig. 1) and then had a similar behavior to the control group until the end of the experiment. For the CAT enzyme, no significant differences were observed with the control group; however, a tendency to increase the activity of this enzyme was observed at 72 (94%) and 96 (186%) hpf (Fig. 2). For the GPx enzyme, an increase in activity was observed at 24, 48, and 72 hpf (37%, 73%, and 46%, respectively), with significance ($p < 0.05$) only at 48 hpf (Fig. 3).

Finally, in embryos exposed to the mixture of the three metals, it was observed that SOD activity (Fig. 1) was drastically reduced at all times evaluated, with statistical differences at 12, 24, and 48 hpf (89%, 86%, and 82%, respectively). In the case of CAT (Fig. 2), it was observed that the activity was similar to that of the control group at 12, 24, and 48 hpf

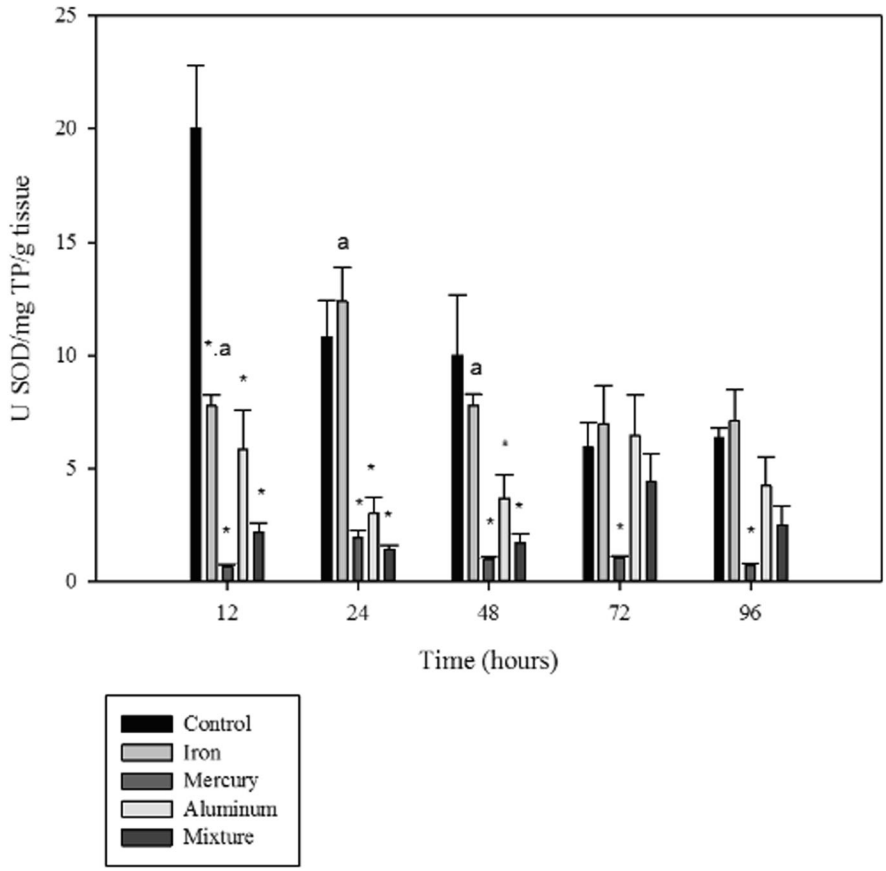


Fig. 1 Superoxide dismutase (SOD) in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. [a]Significantly different ($p < 0.05$) from the mixture group. Two-way ANOVA and Tukey test

and, subsequently, there were increases at 72 and 96 hpf (250% and 160%, respectively). The GPx enzyme (Fig. 3) follows an ascending pattern where at 12 and 24 hpf, the activity was lower than that of the control group; at 48 h, it became equal, and from 72 hpf onwards, there was an increase (102%) in activity which was significant ($p < 0.05$) with respect to the control.

The results about oxidative damage are shown in Figs. 4, 5, and 6, where the findings related to the level of LPx and the content of reactive carbonyls and hydroperoxides, respectively, are observed.

It is important to note that a reduction in the level of LPx (Fig. 4) was observed in all exposed groups at 12 hpf with respect to the control group; however, the embryos exposed to Al and the mixture showed a tendency to increase from 24 hpf (136% and 57%, respectively) onwards, reaching the maximum point at 96 hpf (150% and 334%, respectively), with significant differences between the mixture and the control ($p < 0.01$) and the groups exposed to Al, Hg, and Fe individually ($p < 0.01$). Embryos exposed in isolation to iron showed similar behavior to the control, while those exposed to mercury remained downward throughout the exposure time.

With regard to carbonyl proteins (Fig. 5), it was observed that embryos exposed to iron had a similar behavior to the control group, those exposed to mercury showed a decrease, while in the group exposed to aluminum, there was an increase from 12 h (8% with respect to the control group) with a maximum value at 72 h (139% with respect to the control group). In the organisms exposed to the mixture of metals, there was a slight increase at 24 h (21% with respect to the control group) followed by a decrease at 48 h (20%) and again an increase at 72 h which was maintained until 96 h (147% and 97%, respectively).

Regarding the hydroperoxide content (Fig. 6), the embryos exposed to iron showed a decrease at 12 h and 24 h (38% and 35%) with respect to the control group, having an increase at 48 h, 72 h, and 96 h (60%, 142%, and 670%, respectively), being

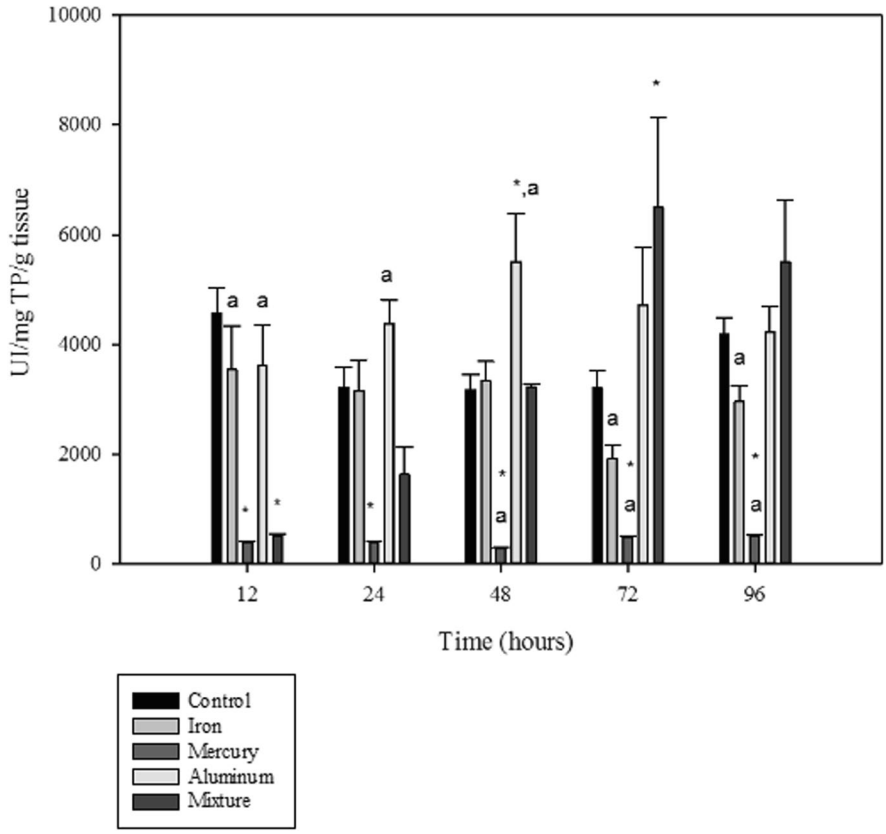


Fig. 2 Glutathione peroxidase (GPx) activity in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. [a]Significantly different ($p < 0.05$) from the mixture group. Two-way ANOVA and Tukey test

statistically significant at 72 and 96 hpf ($p < 0.05$). In the group exposed to mercury, hydroperoxides remained decreased during the whole exposure time and the group exposed to aluminum showed an increase after 48 h (12%), at 72 h (266%), and finally at 96 h (496%). The mixture showed an increase at 72 h (265% with respect to the control group) and at 96 h (623%).

The evaluation of the general morphological development (Fig. 7) was uniform at 12 h in all groups, indicating that there were no significant changes between the groups of embryos with respect to the control after exposure to the contaminants. At later times, the group of embryos exposed to mercury was the one that presented the lowest score, that is to say, the greatest damage, and this was maintained until 96 h. The group exposed to iron showed a significant decrease with respect to the control at 24 h and 48 h, as did the groups exposed to aluminum and the mixture of metals, with the difference that the latter two maintained their significant decrease at 96 h.

Table 1 shows the correlation analysis of the biomarkers of embryotoxicity between the different treatments; it was observed that the embryos exposed to the three metals, individually and in mixture, present a higher relative risk (OR > 1) of presenting adverse effects with respect to the control ($p < 0.001$), while the embryos exposed to the metals individually have a lower relative risk of embryotoxicity (OR < 1) with respect to the individuals exposed to the mixture.

The effect of exposure to individual metals and their mixture on modification of embryonic development shows that the OR increases significantly in 152% ($p < 0.001$) for Al, 128% ($p < 0.001$) for Fe, 178% ($p < 0.001$) for Hg, and 121% ($p < 0.001$) for the mixture. When comparing the effect of the mixture of metals on embryotoxicity with respect to the exposure of individual metals, it was observed that the OR decreased by 12% for Al, 3% for Fe, and 20% for Hg.

Table 2 presents the results of the multivariate logistic regression analysis, which establishes the effect of the levels of oxidative damage (LPx, HPX, and POX) and the activity of antioxidant enzymes

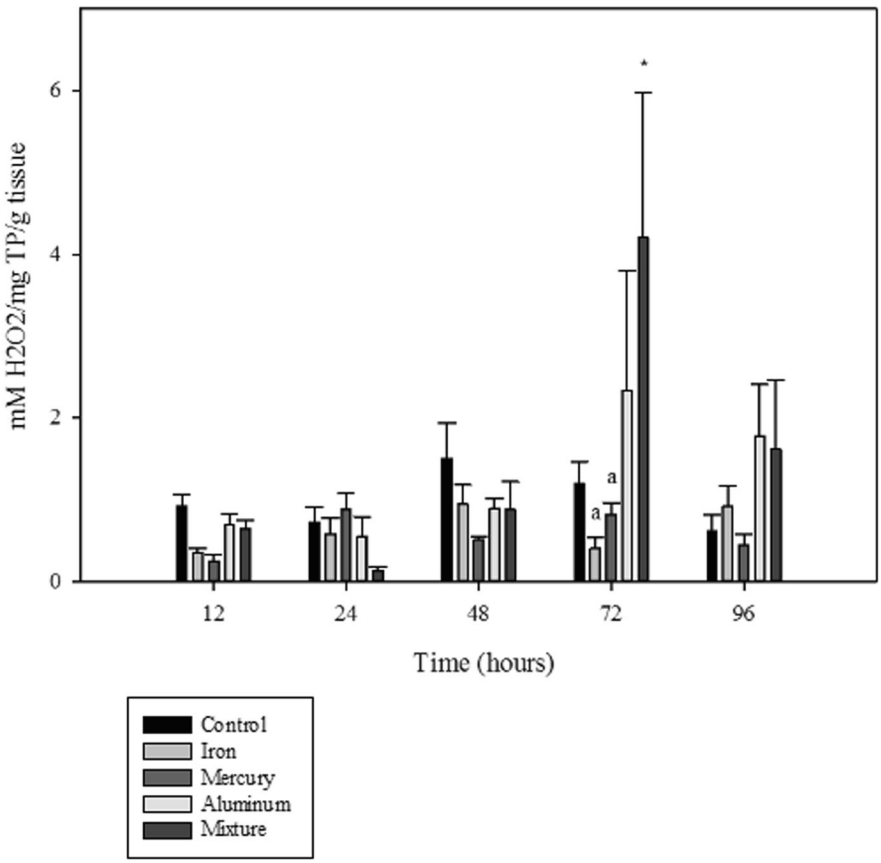


Fig. 3 Catalase (CAT) activity in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. Two-way ANOVA and Tukey test

(SOD, CAT, and GPX) on the OR of the association between the frequency of embryos that presented changes in embryonic development and exposure to metals separately or to their mixture. The effect of SOD and CAT enzyme activities on the association between aluminum exposure and embryonic developmental modification shows 43% and 44% reductions in the OR, while GPx and LPx activities show an increased risk of 0.1% and 409%, respectively. For Fe-exposed embryos, antioxidant enzyme activity and HPX content increased the risk of embryonic developmental impairment following metal exposure, being 0.1% for SOD, 45% for CAT, 180% for GPx, and 86% for HPX. For embryos exposed to Hg, two oxidative stress biomarkers showed an effect on the association between embryotoxic damage with metal, increasing the OR up to 699% for SOD, while for GPx, the risk was reduced by 0.4%. Finally, in the mixture, the SOD activity presented in embryos has a protective effect for the association between embryonic development and exposure to the mixture (43%), not so with LPx levels since the effect on the OR increased, presenting an increased risk of 44%.

4 Discussion

Metals enter the environment due to natural processes such as volcanic eruptions, erosion, soil formation processes, or evaporation of water contained in oceans, lakes, or rivers. However, in most cases, metals come from human activities including various branches of industry, energy production, transportation, and municipal waste management. Once metals enter the ecosystem, they become air, soil, and water pollutants (Stankovic et al., 2014; Szyczewski et al., 2009). Metals often exert adverse effects on reproduction and early development of exposed organisms, either directly at low concentrations or indirectly through systemic toxicity at high concentrations, compromising the quantity and quality of gametes, or the survival of embryos (Apostoli & Catalani, 2011).

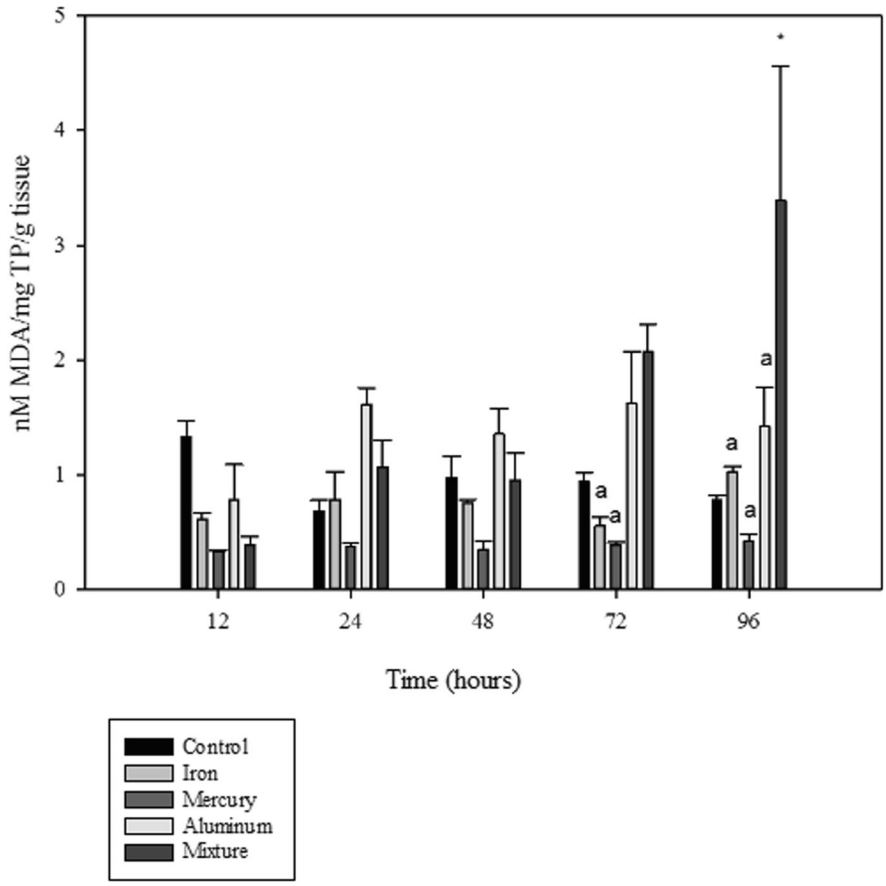


Fig. 4 Lipid peroxidation (LPx) in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. MDA, malondialdehyde. *Significantly different ($p < 0.05$) from the control group. [a]Significantly different ($p < 0.05$) from the mixture group. Two-way ANOVA and Tukey test

In normal embryonic development, ROS act as second messengers to exert cellular responses that regulate cell proliferation, differentiation, and death. However, when there is an excess of ROS, embryo integrity and survival can be compromised (Torres-Osorio et al., 2019). One of the common mechanisms of toxic action of metals is oxidative stress, because they function as pro-oxidant agents, generating ROS, which have the ability to react with the macromolecules of cells, such as proteins, lipids, and nucleic acids (Jaishankar et al., 2014). The toxicity of each metal will depend on its speciation in the environment, its solubility, and its ability to form coordination complexes, so each metal will act in a particular way in contact with cells (Sevcikova et al., 2011).

Embryonic developing organisms are nourished from the contents of the yolk sac, which is mostly composed of polyunsaturated fatty acids that are highly susceptible to oxidation by ROS, so antioxidant enzyme systems are of critical importance for the survival and development of the embryo (Surai, 1999). The removal of ROS occurs by enzymes such as SOD which dismutates superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen, subsequently H_2O_2 is removed by the action of CAT or GPx, forming the first line of defense against oxidative stress (Aceto et al., 1994).

As mentioned above, changes in antioxidant enzyme activity prior to hatching were observed in the unexposed group of embryos. This coincides with previous studies where *Cyprinus carpio* embryos were used to evaluate the toxicity of water from the Madin dam in the state of Mexico, observing similar activity trends over time in the non-exposed groups for these three enzymes (Pérez-Coyotl et al., 2019). Isuev et al. (2008) monitored the enzymatic activity of SOD and GPx in embryos of *Misgurnus fossilis* and *Acipenser gueldenstaedtii*, determining that changes in the activity of antioxidant enzymes during the embryonic stage are associated with the different oxygen conditions of embryonic development of each species; thus, enzyme activity will be dependent on

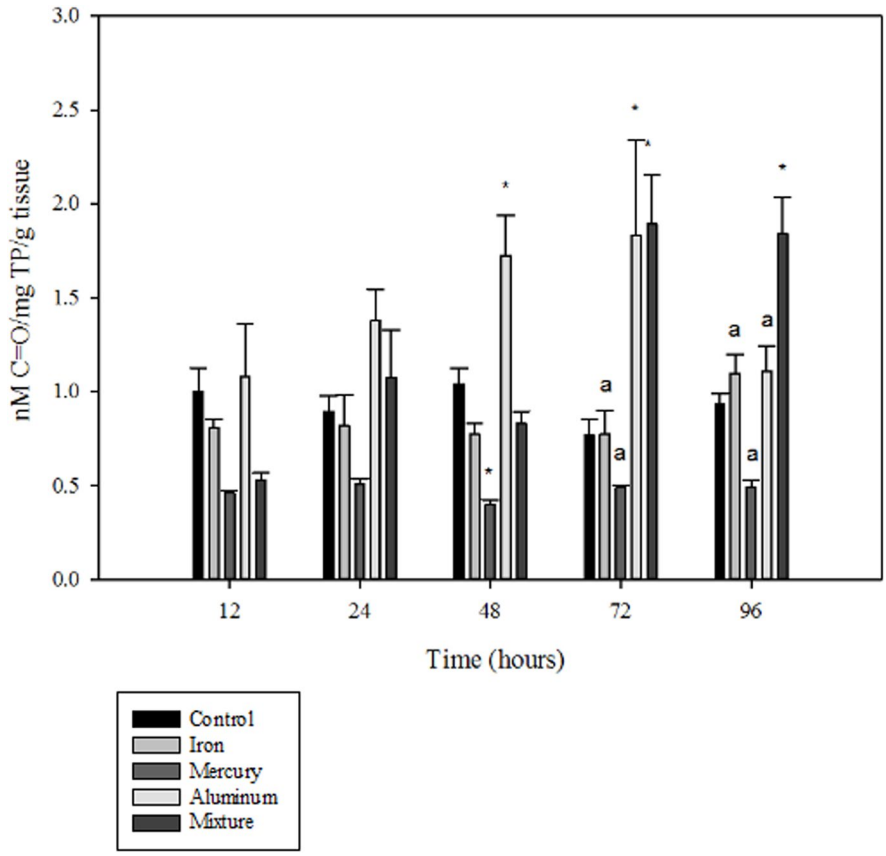


Fig. 5 Protein carbonyl (POX) content in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. [a]Significantly different ($p < 0.05$) from the mixture group. Two-way ANOVA and Tukey test

the oxygen concentrations in the embryo development medium. In a study carried out in *Salmo iridaeus*, it was shown that the activity of the antioxidant enzymes SOD, CAT, and GPx increased in stages prior to embryo hatching; however, this increase was exponential only for SOD and CAT in fry, in contrast to the activity of GPx which is reduced to almost undetectable levels (Aceto et al., 1994). On the other hand, Peters and Livingstone (1996) observed that *Scophthalmus maximus* embryos showed higher SOD and GPx activities than fry, 3 days and 11 days post hatching. This is associated with the fact that in the embryonic stages, oxygen uptake by the organisms increases, and subsequently, the oxygen in the medium decreases, generating a hypoxic environment that favors hatching. In *Oncorhynchus mykiss* species, it was observed that SOD and GPx activity increased from fertilization to 32 days of embryo development, while CAT activity had an opposite behavior, with a reduction in activity to 50% at day 32 (Zengin et al., 2015). This makes it clear that enzyme activity patterns vary between test species throughout embryonic development, and post hatching, depending on the needs of the organism and oxygen availability.

Fe is an essential element for the vital processes of aerobic organisms at low concentrations, since it is part of various metabolic processes such as oxygen transport, nucleic acid synthesis, and cellular respiration and forms part of the catalytic center of certain metalloproteins (Bury & Grosell, 2003). This versatility is due to the two different oxidation states that this metal presents in biological systems: the ferric ion (Fe^{3+}) and the ferrous ion (Fe^{2+}). The toxicity of Fe is dependent on the formation of ROS through the Fenton and Haber–Weiss reactions mediated by the change in the oxidation state from Fe^{2+} to Fe^{3+} (Papanikolaou & Pantopoulos, 2005). Embryos exposed to Fe concentration (1 mg L^{-1}) exhibit enzyme activity for SOD, CAT, and GPx similar to that of the control group. This is probably due to the fact that the Fe concentration used is not sufficient to generate subacute effects that lead to inhibition

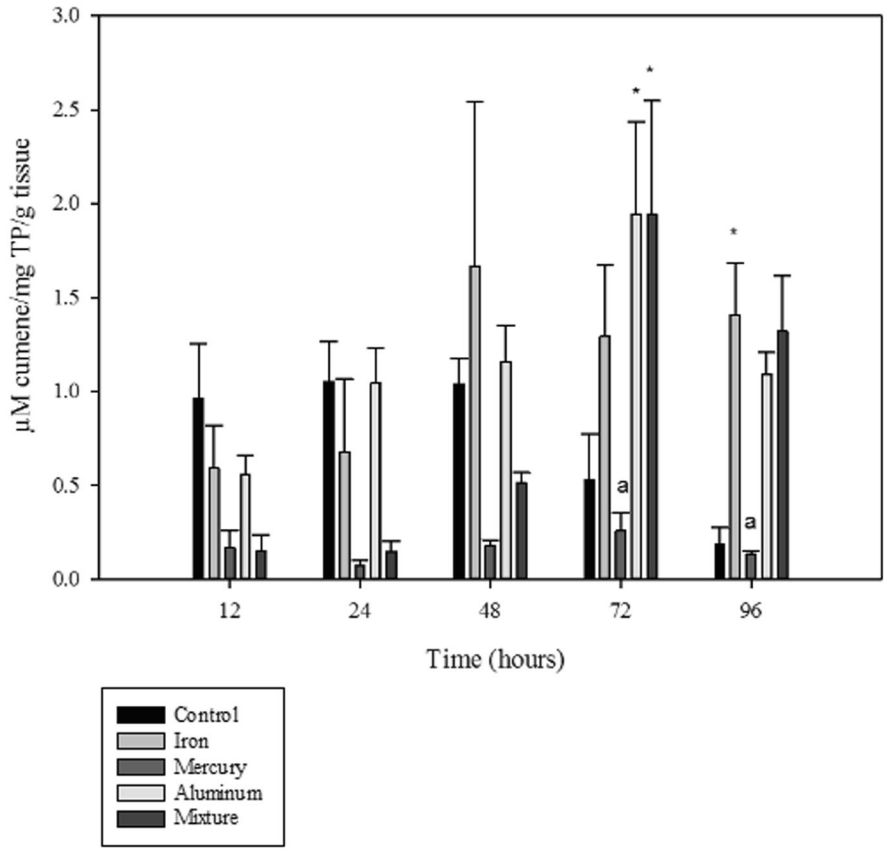


Fig. 6 Hydroperoxide (HPX) content in embryos of *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. [a]Significantly different ($p < 0.05$) from the mixture group. Two-way ANOVA and Tukey test

of enzyme activity. In studies conducted in juvenile *Leporinus friderici* exposed to nominal concentrations of Fe (1 to 30 mg L^{-1}) in its two oxidation states, it was observed that Fe^{3+} is more toxic than Fe^{2+}; however, both ionic forms generated reduction in glutathione concentration, as well as increase in hemoglobin and methemoglobin concentration (Cunha Gemaque et al., 2019). On the other hand, Pereira et al. (2020) determined that concentrations of 1.25 mg L^{-1} of Fe^{3+} ions, although they did not increase mortality, produced a reduction in the frequency of spontaneous contractions, bradycardia, as well as severe teratogenic effects in *Danio rerio* embryos. Regarding the oxidative damage produced, it was observed that the degree of LPx and the concentration of reactive carbonyls did not present differences with respect to the control group; however, in the content of hydroperoxides, an increase in this variable was observed after 48 hpf, being statistically significant at 72 and 96 hpf ($p < 0.05$). Iron also had an influence on embryonic development, since exposure to this metal in isolation increased the risk of embryonic alterations up to two times. When evaluating the effect of oxidative stress biomarkers on this risk, antioxidant enzyme activity and LPx levels were found to contribute significantly to an increase, particularly CAT activity and HPX levels. This may be due to the fact that Fe^{2+} reacts with oxygen in the aqueous medium favoring the production of O_2^- anions which generate H_2O_2 and Fe^{3+}. This H_2O_2 has the capacity to induce the formation of OH^- radicals through the Fenton reaction, increasing the concentration of hydroperoxides in the tissue that will subsequently cause oxidation of biomolecules (Boveris, 1998). This behavior is consistent with that observed in *Mya arenaria* bivalves, in which LPx is not observed in the first days of exposure to concentrations of 50 µM Fe; however, this biomarker shows a significant increase after 7 days of exposure and is attributed to the bioaccumulation of Fe in the tissues of treated organisms (González et al., 2010).

In embryos exposed to Hg, a reduction in antioxidant activity was observed at all times. This reduction of antioxidant activity may be due to the fact that one

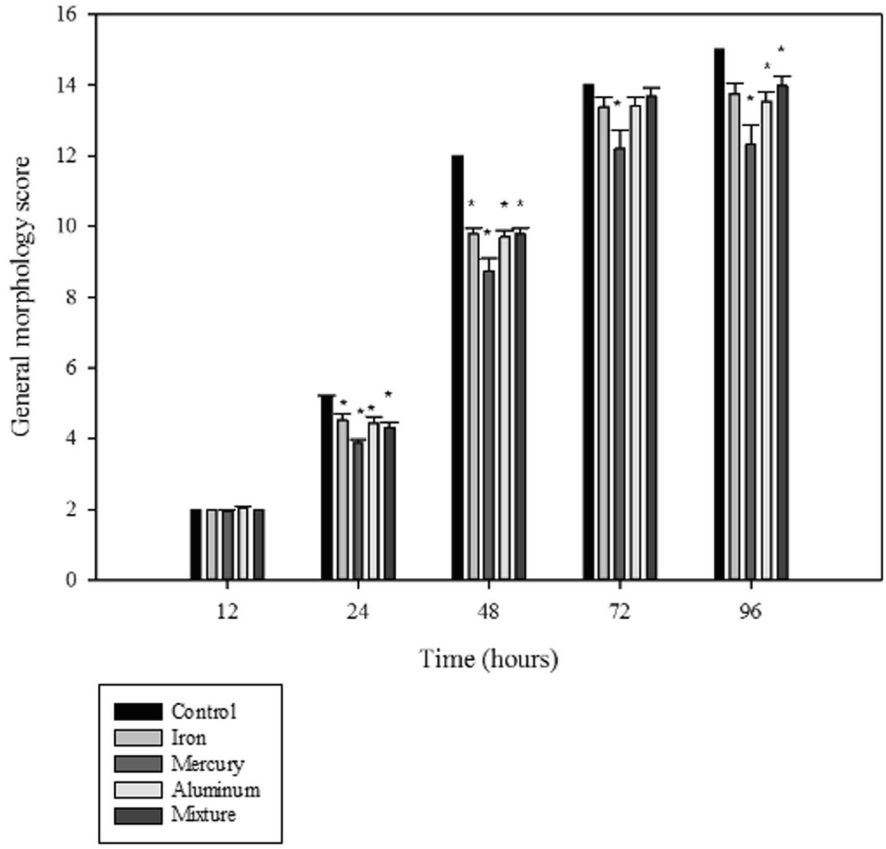


Fig. 7 Morphological development of embryos *C. carpio* exposed for 12 h, 24 h, 48 h, 72 h, and 96 h to Fe, Al, Hg, and the mixture. Values are the mean of three replicates ± SEM for each exposure time. $N = 60$. *Significantly different ($p < 0.05$) from the control group. Repeated measures analysis of variance (RM ANOVA), followed by a Tukey post hoc test

of the mechanisms of toxicity of the inorganic form of Hg is the binding to thiol groups of biomolecules, which are found in low molecular weight antioxidants, such as glutathione, and in proteins, such as antioxidant enzymes, generating substrate depletion and conformational changes that drastically reduce catalytic functionality (Jaishankar et al., 2014; Patrick, 2002). In the results obtained, it is observed that the oxidative damage parameters (LPx, HPX, and POX) decreased with respect to the control without significant differences, except in the concentration of reactive carbonyls where a significant reduction was observed at 48 hpf ($p < 0.05$). Hg has been shown to induce oxidative stress and mitochondria-dependent apoptosis in various tissues (Kim et al., 2020; Pal et al., 2012; Sinha et al., 2013), so the reduction in the levels of oxidized biomolecules may be due to an increase in apoptosis and, therefore, produces alterations to embryonic development. In this sense, when evaluating the association between metal exposure and changes in embryonic development, it was observed that this metal produces a higher risk compared to Al, Fe, or the mixture. This is in agreement with reports from other researchers indicating that Hg can modify mitotic divisions of cells as well as suppress embryogenesis at different stages of development (Perry et al., 1988; Wang et al. 2009; Ismail & Yosuf, 2011). When evaluating the effect of oxidative

Table 1 Bivariate analysis of embryotoxicity and exposure to Al, Fe, and Hg individually and mixture

	p value	Odds ratio (OR)	95% confidence interval	
			Inferior	Superior
Control vs Al	< 0.001	2.525	2.167	2.943
Control vs Fe	< 0.001	2.273	1.976	2.614
Control vs Hg	< 0.001	2.778	2.355	3.277
Control vs mixture	< 0.001	2.212	1.930	2.536
Mixture vs Al	0.205	0.876	0.714	1.076
Mixture vs Fe	0.787	0.973	0.801	1.184
Mixture vs Hg	0.036	0.796	0.643	0.987

Table 2 Relationship between oxidative stress biomarkers and embryonic development of *C. carpio* exposed to Al, Fe, Hg, and their mixture

	Variable	*p* value	Odds ratio (OR)	95% confidence interval
Al	CAT	< 0.001	0.563	0.429–0.738
	SOD	< 0.001	0.556	0.483–0.659
	GPx	0.005	1.001	1.001–1.001
	LPx		5.902	2.598–13.403
	HPX	0.304	–	–
	POX	0.955	–	–
Fe	CAT	< 0.001	18.788	7.330–48.187
	SOD	< 0.001	1.452	1.274–1.654
	GPx	0.001	1.001	1.00–1.001
	LPx	0.227	2.863	0.519–15.779
	HPX	0.007	1.865	1.185–2.935
	POX	0.354	–	–
Hg	CAT	0.557	–	–
	SOD	< 0.001	7.990	3.269–19.527
	GPx	0.006	0.996	0.993–0.999
	LPx	0.400	–	–
	HPX	0.541	–	–
	POX	0.206	–	–
Mixture	CAT	0.075	–	–
	SOD	< 0.001	0.575	0.438–0.754
	GPx	0.438	–	–
	LPx	0.044	1.441	1.010–2.056
	HPX	0.713	–	–
	POX	0.189	–	–

stress biomarkers on the association between Hg exposure and embryonic development, in contrast to the other metals, SOD activity exerts a higher risk, while GPx reduces the risk.

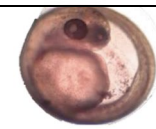
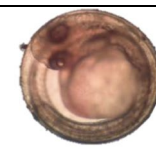
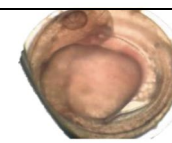
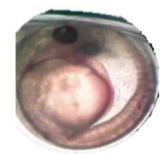
In aquatic organisms, Al has the capacity to replace calcium, producing accumulation of the metal in the gills, affecting gas exchange and respiration, besides interfering with reproductive processes and generating DNA damage by binding to thiol groups of biomolecules (Barabasz et al., 2002; García-Medina et al., 2010; Pereira et al., 2013). Al caused changes in the activity of antioxidant enzymes: a significant reduction in SOD from 12 to 48 h, an increase in GPx at 48 h, and a trend towards an increase in CAT at 72 h and 96 h. On the other hand, oxidative damage in lipids and proteins showed a significant increase between 72 and 96 h. These changes coincide with the study of Capriello et al. (2021a), who found an increase in antioxidant enzymes and lipid and protein oxidation in *Danio rerio* larvae exposed to sublethal concentrations of Al (50 µM, 100 µM, and 200 µM), due to an increased production of free radicals. In contrast, García-Medina et al. (2010) reported decreased SOD and CAT activities with a significant increase in lipoperoxidation levels in Al-exposed carp lymphocytes. The mechanism by which Al produces oxidative stress has been proposed to be through indirect production of ROS via the Fenton reaction (Exley, 2004), stabilization of superoxide ions (Mujika et al., 2014), and direct damage to mitochondria by affecting electron transport in the respiratory chain (Kumar and Gill 2014). On the other hand, alterations in antioxidant activity and increased oxidative damage to proteins and lipids in zebrafish embryos are likely to be related to the changes in embryonic development observed for this metal. In this sense, it was observed that Al caused a decrease in the morphological score, with the risk of effect of this metal on embryonic development being 2.5 times greater than that presented by the control. Likewise, when evaluating the effect of biomarkers of oxidative stress on this risk, it was found that the activity

of antioxidant enzymes and LPx levels modify it. Other authors have reported that, after Al exposure in fish, complex biological processes occur that affect embryonic development and that mechanisms such as oxidative stress, genotoxicity/cytotoxicity, metabolic processes, and neuromuscular transmission are usually related (Capriello et al., 2019, 2021b; Quiroga-Santos et al., 2021).

In the metal mixture, it was observed that SOD decreased its activity at all times of the assay, being significant at 12, 24, and 48 hpf, while GPx and CAT had a reduced activity at 12 hpf, followed by an increase that was significant at 72 hpf. On the other hand, the mixture of the three metals induced an increase in oxidative damage biomarkers after 72 hpf with respect to the control and the individual metals. It was observed that the changes in enzyme activity are very similar in the mixture with respect to the Al-exposed group, while biomarkers of damage show a similar pattern. This is in agreement with other studies where metal mixtures were used. Boukadida et al. (2017) observed that exposure of *Mytilus galloprovincialis* embryos to Cu and Ag individually and in mixture modified the expression and enzyme activity of SOD, CAT, and GST, showing no changes in the degree of LPx with respect to the control; however, these changes were not significant between the mixture groups and exposure to Cu and Ag separately. In the freshwater bivalve *Anodontites trapesialis* exposed to Zn (0.18–5 mg L^{-1}), Mn (0.1–5 mg L^{-1}), and Fe (5 mg L^{-1}) and their mixture, no differences in SOD and GST activity and oxidative damage biomarkers were observed when compared to individual exposure (Oliveira et al., 2018). This behavior can be attributed to the interactions that occur between metals when they are in a mixture. Interactions can be of three types: physicochemical interactions, where the components of the mixture can interfere with each other before entering the organism; toxicokinetic interactions, where the components of the mixture can favor or hinder the processes of absorption, distribution, biotransformation, and elimination of one or more of the toxicants present; toxicodynamic interactions, where exposure to the constituents of the mixture can modify the processes of damage, repair, compensation, or signaling; and toxicodynamic interactions, where exposure to the constituents of the mixture can modify the processes of damage, repair, compensation, or signaling (Sexton & Hattis, 2007). According to the above, the effects of the interactions of the components of a mixture could be additive when the effect is equal to the sum of the individual toxic effects, synergistic when the effect is greater than the sum of the individual effects, and antagonistic when the effects are less than the sum of the individual effects (Hertzberg & Teuschler, 2002). In the case of our study, the results suggest that in the mixture of Al, Hg, and Fe, at least one of them exerts an antagonistic effect. This is reinforced by the bivariate analysis between the exposure groups (Table 1), where a reduction in the OR is observed when comparing exposure to the mixture on the presence of embryotoxicity with respect to exposure to the metals individually. On the other hand, multivariate analysis (Table 2) indicates that SOD activity decreases the risk of the mixture producing modifications in embryonic development and that LPx is associated with an increase in this risk, so that the embryonic toxicity produced by the mixture is related to oxidative stress, as was observed in aluminum, but to a lesser extent than in this metal.

Furthermore, embryotoxicity results show that both the individual metals and the mixture significantly ($p < 0.01$) reduce the general developmental score by delaying hatching as shown in Table 3; however, mercury produces more pronounced effects, as it additionally inhibits pigmentation of the eyes, head, and tail. The low number of hatched embryos is attributed to increased metal penetration through the chorion, inducing functional and structural disturbances, while the delay in hatching could be due to abnormal chorionase function. Chorionase is a zinc-dependent metalloproteinase that is secreted from a gland on the head of the hatching larva. This enzyme is found as a zymogen and requires Zn^{2+} ions for activation and proteolytic effect (Small et al., 2020). The metals used in the exposure systems are transient bivalents, so they can interfere with the action of chorionase by displacing Zn. In addition, it has been reported that Hg has the ability to reduce head size, modify cAMP signaling and cause embryo mortality (Green & Planchart, 2018). This last point seems to be involved in the reduction of pigmentation of embryos exposed to Hg, since intracellular cAMP levels seem to be the main regulator of melanogenesis in mammals, amphibians, and teleosts (Logan et al., 2006). This is congruent with the study of Abbot et al. (2017) where *Danio rerio* embryos

Table 3 Results of embryotoxicity in *Cyprinus carpio* embryos exposed for 96 h to Al, Fe, Hg, and their mixture

Group	Total number of embryos exposed	Total number of deaths	Total number of lives	% mortality	Developmental endpoint	Embryos at 96 hpf
Control	60	0	60	0.0	-	
Aluminium	60	3	57	5	Delayed hatching	
Iron	60	6	54	10	Delayed hatching	
Mercury	60	7	53	11	Delayed hatching, lack of pigmentation in the eyes, body and tail	
Mixture	60	3	57	5	Delayed hatching	

were exposed to increasing concentrations of $HgCl_2$, observing that from 50 μg L^{-1}, there was a reduction of pigmentation in exposed embryos, due to alterations in the expression of genes associated with the differentiation, migration, and survival of chromatophore cells (Kelsh 1996).

In this study, the mixture of metals produces an antagonistic type of interaction, as the deleterious effects on exposed embryos were smaller than the effects of exposure to individual metals. In contrast, Cobbina et al. (2015) demonstrated by multivariate analysis that oxidative damage in the liver and kidney of ICR mice was correlated with exposure to low-dose metal mixtures, and that the damage produced by the metal mixtures was greater than the effects produced by exposure to individual metals. However, there is evidence that, for some metal mixtures, most of the effects observed for binary and ternary combinations of Cd, Cu, and Zn may be primarily antagonistic, followed by synergistic (Vijver et al., 2011). The above is reinforced by the meta-analysis conducted by Norwood et al. (2003), where it is observed that a large part of the studies analyzed present antagonistic (less than additive) or synergistic (more than additive) effects. This demonstrates the importance of conducting studies with mixtures of environmental pollutants, as is the case of metals, since this will allow us to understand the mechanisms by which toxicants exert their combined effects and how organisms respond to them.

5 Conclusions

This study evaluated the effect of exposure to individual or mixed Fe, Al, and Hg at concentrations indicated in Mexican legislation as adequate for the protection of aquatic life, on fertilized embryos of *Cyprinus carpio*. The results show that Fe individually induces an increase in hydroperoxide content at 96 hpf. On the other hand, embryos exposed only to Hg show a significant reduction in antioxidant activity along with a reduction in damage parameters, as well as a reduction in the overall developmental score compared to the control, suggesting a mechanism of damage other than oxidative stress. Exposure to Al individually produces a higher lethality of the study organisms, as well as a reduction in SOD activity in the early exposure times and an increase in SOD activity, as well as CAT and GPx, accompanied by an increase in the concentration of reactive carbonyls, hydroperoxides, and degree of lipoperoxidation with respect to the control for the rest of the study, indicating an oxidative stress process. Finally, exposure to the mixture of metals modifies the activity of antioxidant enzymes and increases damage to biomolecules such as lipids and proteins, with greater damage observed at 96 hpf compared to individual metals, suggesting an additive effect. In the case of embryonic development, it is observed that the mixture of metals produces an antagonistic effect with respect to the effect produced in organisms exposed to individual metals. This study sets a precedent for future research to determine the type of interaction generated by a mixture of contaminants on developing aquatic organisms as well as starting the debate about the structuring of laws that derive not only from experiments with individual substances but also from systems attached to reality (mixtures) to guarantee the quality of aquatic life with greater efficiency.

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Data Availability The data will be made available on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

References

Aba, L., Prasetyo, A., Sitti-Ilmawati, W. O., Ode-Ahmad, L., & Sahidin, L. O. (2019). Reduction of iron and manganese concentration in dug well water by using Moramo beach sand as filter media. *Journal of Physics: Conference Series*, *1153*(9th International Conference on Physics and Its Applications), 012078.

Aceto, A., Amicarelli, F., Sacchetta, P., Dragani, B., Bucciarelli, T., Masciocco, L., et al. (1994). Developmental aspects of detoxifying enzymes in fish (Salmo iridaeus). *Free Radical Research, 21*(5), 285–294. https://doi.org/10.3109/10715769409056581

Ali, H., Khan, E., & Ilahi, I. (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry, 2019*, 14. https://doi.org/10.1155/2019/6730305

Apostoli, P., & Catalani, S. (2011). Metal ions affecting reproduction and development. *Metal Ions in Life Sciences, 8*, 263–303.

Barabasz, W., Albinska, D., Jaskowska, M., & Lipiec, J. (2002). Ecotoxicology of aluminium. *Polish Journal of Environmental Studies*, *11*(3), 199–203. http://www.pjoes.com/Ecotoxicology-of-Aluminium,87442,0,2.html. Accessed 29 June 2020

Beker van Woudenberg, A., Snel, C., Rijkmans, E., De Groot, D., Bouma, M., Hermsen, S., et al. (2014). Zebrafish embryotoxicity test for developmental (neuro)toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. *Reproductive Toxicology, 49*, 101–116. https://doi.org/10.1016/j.reprotox.2014.07.082

Bosch, C., Olivares, A., Faria, M., Navas, J. M., del Olmo, I., Grimalt, J. O., et al. (2009). Identification of water soluble and particle bound compounds causing sublethal toxic effects. A field study on sediments affected by a chloralkali industry. *Aquatic Toxicology*, *94*(1), 16–27. https://doi.org/10.1016/j.aquatox.2009.05.011.

Boukadida, K., Cachot, J., Clérandeaux, C., Gourves, P. Y., & Banni, M. (2017). Early and efficient induction of antioxidant defense system in Mytilus galloprovincialis embryos exposed to metals and heat stress. *Ecotoxicology and Environmental Safety, 138*, 105–112. https://doi.org/10.1016/j.ecoenv.2016.12.021

Boveris, A. (1998). Biochemistry of free radicals: From electrons to tissues. *Medicina (B Aires)*, *58*(4), 350–356. https://pubmed.ncbi.nlm.nih.gov/9816695/. Accessed 3 Sept 2020.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bridges, C. C., & Zalups, R. K. (2017). The aging kidney and the nephrotoxic effects of mercury. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*. Taylor and Francis Inc. https://doi.org/10.1080/10937404.2016.1243501.

Buege, J. A., & Aust, S. D. (1978). Biomembranes - Part C: Biological oxidations. *Methods in Enzymology, 52*, 302–310.

Bury, N., & Grosell, M. (2003). Iron acquisition by teleost fish. In *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology* (Vol. 135, pp. 97–105). Elsevier Inc. https://doi.org/10.1016/S1532-0456(03)00021-8

Capriello, T., Monteiro, S., Félix, L., Dozinetti, A., Aliperti, V., & Ferrandino, I. (2021a). Apoptosis, oxidative stress and genotoxicity in developing zebrafish after aluminium exposure. *Aquatic Toxicology, 23*(2021), 105872. https://doi.org/10.1016/j.aquatox.2021.105872

Capriello, T., Grimaldi, M. C., Cofone, R., D'Aniello, S., & Ferrandino, I. (2019). Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish. *Chemosphere, 222*, 243–249. https://doi.org/10.1016/j.chemosphere.2019.01.140

Capriello, T., Monteiro, S. M., Félix, L. M., Donizetti, A., Aliperti, V., & Ferrandino, I. (2021b). Apoptosis, oxidative stress and genotoxicity in developing zebrafish after aluminium exposure. *Aquatic Toxicology, 236*, 105872. https://doi.org/10.1016/j.aquatox.2021.105872

Carrasco, L., Barata, C., García-Berthou, E., Tobias, A., Bayona, J. M., & Díez, S. (2011). Patterns of mercury and methylmercury bioaccumulation in fish species downstream of a long-term mercury-contaminated site in the lower Ebro River (NE Spain). *Chemosphere, 84*(11), 1642–1649. https://doi.org/10.1016/j.chemosphere.2011.05.022

Chang, Y., Lee, W. Y., Lin, Y. J., & Hsu, T. (2017). Mercury (II) impairs nucleotide excision repair (NER) in zebrafish (Danio rerio) embryos by targeting primarily at the stage of DNA incision. *Aquatic Toxicology, 192*, 97–104. https://doi.org/10.1016/j.aquatox.2017.09.001

Chen, P. J., Wu, W. L., & Wu, K. C. W. (2013). The zerovalent iron nanoparticle causes higher developmental toxicity than its oxidation products in early life stages of medaka fish. *Water Research, 47*(12), 3899–3909. https://doi.org/10.1016/j.watres.2012.12.043

Choi, J., Bae, S., Lim, H., Lim, J. A., Lee, Y. H., Ha, M., & Kwon, H. J. (2017). Mercury exposure in association with decrease of liver function in adults: A longitudinal study. *Journal of Preventive Medicine and Public Health, 50*(6), 377–385. https://doi.org/10.3961/jpmph.17.099

Cobbina, S. J., Chen, Y., Zhou, Z., Wu, X., Zhao, T., Zhang, Z., et al. (2015). Toxicity assessment due to sub-chronic exposure to individual and mixtures of four toxic heavy metals. *Journal of Hazardous Materials, 294*, 109–120. https://doi.org/10.1016/j.jhazmat.2015.03.057

Cunha Gemaque, T., Pereira da Costa, D., Vaz Pereira, L., & Miranda Filho, K. C. (2019). Evaluation of iron toxicity in the tropical fish Leporinus friderici. *Biomed J Sci & Tech Res*, *18*(2), 13436–13441. https://doi.org/10.26717/BJSTR.2019.18.003127.

de Oliveira, L. F., Santos, C., Risso, W. E., & dos Reis Martinez, C. B. (2018). Triple-mixture of Zn, Mn, and Fe increases bioaccumulation and causes oxidative stress in freshwater neotropical fish. *Environmental Toxicology and Chemistry, 37*(6), 1749–1756. https://doi.org/10.1002/etc.4133

DOF. (1989). ACUERDO por el que se establecen los Criterios Ecológicos de Calidad del Agua. México: Diario Oficia de la Federación. dof.gob.mx/index.php?year=1989&month=12&day=13

Domagalski, J. (1998). Occurrence and transport of total mercury and methyl mercury in the Sacramento River Basin, California. *Journal of Geochemical Exploration*, *64*(1–3–3 pt 1), 277–291. https://doi.org/10.1016/S0375-6742(98)00038-7

Driscoll, C. T., Mason, R. P., Man Chan, H., Jacob, D. J., & Pirrone, N. (2013). Mercury as a global pollutant: Sources, pathways, and effects terms of use. *Environmental Science and Technology, 47*, 4983. https://doi.org/10.1021/es305071v

Ercal, N., Gurer-Orhan, H., & Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: Mechanisms involved in metal-induced oxidative damage. *Current Topics in Medicinal Chemistry, 1*(6), 529–539. https://doi.org/10.2174/1568026013394831

Exley, C. (2004). The pro-oxidant activity of aluminum. *Free Radical Biology and Medicine, 36*(3), 380–387. https://doi.org/10.1016/j.freeradbiomed.2003.11.017

Feng, J., Gao, Y., Ji, Y., & Zhu, L. (2018). Quantifying the interactions among metal mixtures in toxicodynamic process with generalized linear model. *Journal of Hazardous Materials, 345*, 97–106. https://doi.org/10.1016/j.jhazmat.2017.11.013

Fent, K., Weston, A. A., & Caminada, D. (2006, February 10). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology*. Elsevier. https://doi.org/10.1016/j.aquatox.2005.09.009

Fernandes-Azevedo, B., Barros-Furieri, L., Maciel-Peçanha, F., Wiggers, G. A., Frizera-Vassallo, P., Ronacher-Simões, M., et al. (2012). Toxic effects of mercury on the cardiovascular and central nervous systems. *Journal of Biomedicine & Biotechnology, 2012*, 11. https://doi.org/10.1155/2012/949048

García-Medina, S., Razo-Estrada, A. C., Gómez-Oliván, L. M., Amaya-Chávez, A., Madrigal-Bujaidar, E., & Galar-Martínez, M. (2010). Aluminum-induced oxidative stress in lymphocytes of common carp (Cyprinus carpio). *Fish Physiology and Biochemistry, 36*(4), 875–882. https://doi.org/10.1007/s10695-009-9363-1

González, P. M., Abele, D., & Puntarulo, S. (2010). Exposure to excess dissolved iron in vivo affects oxidative status in the bivalve Mya arenaria. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 152*(2), 167–174. https://doi.org/10.1016/j.cbpc.2010.04.006

Green, A. J., & Planchart, A. (2018). The neurological toxicity of heavy metals: A fish perspective. *Comparative Biochemistry and Physiology Part - c: Toxicology and Pharmacology, 208*, 12–19. https://doi.org/10.1016/j.cbpc.2017.11.008

Guibaud, G., & Gauthier, C. (2003). Study of aluminium concentration and speciation of surface water in four catchments in the Limousin region (France). In *Journal of*

Inorganic Biochemistry (Vol. 97, pp. 16–25). Elsevier Inc. https://doi.org/10.1016/S0162-0134(03)00254-X

Herkovits, J., Castañaga, L. A., D'Eramo, J. L., & Jourani, V. P. (2015). Living organisms influence on environmental conditions: PH modulation by amphibian embryos versus aluminum toxicity. *Chemosphere, 139*, 210–215. https://doi.org/10.1016/j.chemosphere.2015.05.025

Hermsen, S. A. B., van den Brandhof, E. J., van der Ven, L. T. M., & Piersma, A. H. (2011). Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies. *Toxicology in Vitro, 25*(3), 745–753. https://doi.org/10.1016/j.tiv.2011.01.005

Hernández, A. F., Gil, F., & Lacasaña, M. (2017, October 1). Toxicological interactions of pesticide mixtures: An update. *Archives of Toxicology*. Springer Verlag. https://doi.org/10.1007/s00204-017-2043-5

Hertzberg, R. C., & Teuschler, L. K. (2002, December 1). Evaluating quantitative formulas for dose-response assessment of chemical mixtures. *Environmental Health Perspectives*. Public Health Services, US Dept of Health and Human Services. https://doi.org/10.1289/ehp.02110s6965

Ho, N. Y., Yang, L., Legradi, J., Armant, O., Takamiya, M., Rastegar, S., & Strähle, U. (2013). Gene responses in the central nervous system of zebrafish embryos exposed to the neurotoxicant methyl mercury. *Environmental Science and Technology, 47*(7), 3316–3325. https://doi.org/10.1021/es3050967

Ismail, A., & Yusof, S. (2011). Effect of mercury and cadmium on early life stages of Java medaka (Oryzias javanicus): A potential tropical test fish. *Marine Pollution Bulletin, 63*(5–12), 347–349. https://doi.org/10.1016/j.marpolbul.2011.02.014

Ismail, T., Lee, H. K., Kim, C., Kim, Y., Lee, H., Kim, J. H., et al. (2019). Comparative analysis of the developmental toxicity in Xenopus laevis and Danio rerio induced by Al_2O_3 nanoparticle exposure. *Environmental Toxicology and Chemistry, 38*(12), 2672–2681. https://doi.org/10.1002/etc.4584

Isuev, A. R., Ismailova, S. I., & Ozernyuk, N. D. (2008). Comparative study of superoxide dismutase and glutathione peroxidase activity in embryos and skeletal muscles of adult fish. *Biology Bulletin, 35*(5), 494–498. https://doi.org/10.1134/S1062359008050105

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology*. Slovak Toxicology Society. https://doi.org/10.2478/intox-2014-0009

Kim, M. J., Kim, C. H., An, M. J., Shin, G. S., Lee, H. M., Kim, J. Y., et al. (2020). Exposure to mercury induced early apoptotic signals in human placental BeWo cells through alteration of cell cycle regulation. *Molecular and Cellular Toxicology, 16*(4), 419–429. https://doi.org/10.1007/s13273-020-00098-2

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics, 203*(3), 253–310. https://doi.org/10.1002/aja.1002030302

Kumar, V., & Gill, K. D. (2014). Oxidative stress and mitochondrial dysfunction in aluminium neurotoxicity and its amelioration: A review. *Neurotoxicology*. https://doi.org/10.1016/j.neuro.2014.02.004

Kumolu-Johnson, C. A., Ndimele, P. E., Akintola, S. L., & Jibuike, C. C. (2010). Copper, zinc and iron concentrations in water, sediment and Cynothrissa mento (Regan 1917) from Ologe Lagoon, Lagos, Nigeria: A preliminary survey. *African Journal of Aquatic Science, 35*(1), 87–94. https://doi.org/10.2989/16085914.2010.466588

Lee, J. Y., & Han, M. (2012). Iron removal using adsorptive filtration treatment for low iron concentration water from urban industrial region. *International Journal of Urban Sciences, 16*(1), 115–121. https://doi.org/10.1080/12265934.2012.662587

Levine, R. L., Williams, J. A., Stadtman, E. P., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology*, *233*(C), 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9

Li, H., Zhou, Q., Wu, Y., Fu, J., Wang, T., & Jiang, G. (2009). Effects of waterborne nano-iron on medaka (Oryzias latipes): Antioxidant enzymatic activity, lipid peroxidation and histopathology. *Ecotoxicology and Environmental Safety, 72*(3), 684–692. https://doi.org/10.1016/j.ecoenv.2008.09.027

Logan, D. W., Burn, S. F., & Jackson, I. J. (2006). Regulation of pigmentation in zebrafish melanophores. *Pigment Cell Research, 19*(3), 206–213. https://doi.org/10.1111/j.1600-0749.2006.00307.x

Lubick, N. (2010). Mercury alters immune system response in artisanal gold miners. *Environmental health perspectives*. National Institute of Environmental Health Sciences. https://doi.org/10.1289/ehp.118-a243

Lushchak, V. I. (2016, April 1). Contaminant-induced oxidative stress in fish: A mechanistic approach. *Fish Physiology and Biochemistry*. Springer Netherlands. https://doi.org/10.1007/s10695-015-0171-5

Malczyk, E. A., & Branfireun, B. A. (2015). Mercury in sediment, water, and fish in a managed tropical wetland-lake ecosystem. *Science of the Total Environment, 524–525*, 260–268. https://doi.org/10.1016/j.scitotenv.2015.04.015

Mujika, J. I., Rezabal, E., Mercero, J. M., Ruipérez, F., Costa, D., Ugalde, J. M., & Lopez, X. (2014). Aluminium in biological environments: A computational approach. *Computational and Structural Biotechnology Journal*. https://doi.org/10.5936/csbj.201403002

Norwood, W. P., Borgmann, U., Dixon, D. G., & Wallace, A. (2003). Effects of metal mixtures on aquatic biota: A review of observations and methods. *Human and Ecological Risk Assessment*. Taylor and Francis Ltd. https://doi.org/10.1080/713610010

Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., & Wolff, S. P. (1994). Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Analytical Biochemistry, 220*(2), 403–409. https://doi.org/10.1006/abio.1994.1357

Pal, P. B., Pal, S., Das, J., & Sil, P. C. (2012). Modulation of mercury-induced mitochondria-dependent apoptosis by glycine in hepatocytes. *Amino Acids, 42*(5), 1669–1683. https://doi.org/10.1007/s00726-011-0869-3

Papanikolaou, G., & Pantopoulos, K. (2005, January 15). Iron metabolism and toxicity. *Toxicology and Applied*

Pharmacology. Academic Press. https://doi.org/10.1016/j.taap.2004.06.021

Patrick, L. (2002). Mercury toxicity and antioxidants: Part I: Role of glutathione and alpha-lipoic acid in the treatment of mercury toxicity. *Alternative Medicine Review, 7*(6), 456–471.

Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., & Adam-Guillermin, C. (2013). Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. *Mutation Research, 750*, 19–26. https://doi.org/10.1016/j.mrgentox.2012.07.007

Pérez-Coyotl, I., Galar-Martínez, M., García-Medina, S., Gómez-Oliván, L. M., Gasca-Pérez, E., Martínez-Galero, E., et al. (2019). Polluted water from an urban reservoir (Madín dam, México) induces toxicity and oxidative stress in Cyprinus carpio embryos. *Environmental Pollution, 510–521*. https://doi.org/10.1016/j.envpol.2019.04.095

Pérez-Coyotl, I., Martínez-Vieyra, C., Galar-Martínez, M., Gómez-Oliván, L. M., García-Medina, S., Islas-Flores, H., et al. (2017). DNA damage and cytotoxicity induced on common carp by pollutants in water from an urban reservoir. Madín reservoir, a case study. *Chemosphere, 185*, 789–797. https://doi.org/10.1016/j.chemosphere.2017.07.072

Perry, D., Weis, J., & Weis, P. (1988). Cytogenetic effects of methylmercury in embryos of the killifish Fundulus Heteroclitus. *Arch Environ Contam Toxicol, 17*(5), 569–574. https://doi.org/10.1007/BF01055824

Quiroga-Santos, E. H., Galar-Martínez, M., García-Medina, S., Gasca-Pérez, E., Cano-Viveros, S., Ruíz-Lara, K., et al. (2021). Geno-cytotoxicity and congenital malformations produced by relevant environmental concentrations of aluminum, diclofenac and their mixture on Cyprinus carpio. An interactions study. *Environmental Toxicology and Pharmacology*, *82*, 103555. https://doi.org/10.1016/j.etap.2020.103555

Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D., & Freeman, B. A. (1991). Detection of catalase in rat heart mitochondria. *Journal of Biological Chemistry, 266*(32), 22028–22034.

Ravenscroft, J., Roy, A., Queirolo, E. I., Mañay, N., Martínez, G., Peregalli, F., & Kordas, K. (2018). Drinking water lead, iron and zinc concentrations as predictors of blood lead levels and urinary lead excretion in school children from Montevideo, Uruguay. *Chemosphere, 212*, 694–704. https://doi.org/10.1016/j.chemosphere.2018.07.154

Rayburn, J. R., & Aladdin, R. K. (2003). Developmental toxicity of copper, chromium, and aluminum using the shrimp embryo teratogenesis assay: Palaemonid with artificial seawater. *Bulletin of Environmental Contamination and Toxicology, 71*(3), 481–488. https://doi.org/10.1007/s00128-003-8741-0

Roy, B., & Bhadra, S. (2014). Effects of toxic levels of aluminium on seedling parameters of rice under hydroponic culture. *Rice Science, 21*(4), 217–223. https://doi.org/10.1016/S1672-6308(13)60182-1

Sadeq, S. A., & Beckerman, A. P. (2020). Evaluating additive versus interactive effects of copper and cadmium on Daphnia pulex life history. *Environmental Science and Pollution Research, 27*(2), 2015–2026. https://doi.org/10.1007/s11356-019-06622-9

Schmidt, S., Busch, W., Altenburger, R., & Küster, E. (2016). Mixture toxicity of water contaminants-effect analysis using the zebrafish embryo assay (Danio rerio). *Chemosphere, 152*, 503–512. https://doi.org/10.1016/j.chemosphere.2016.03.006

Sevcikova, M., Modrá, H., Slaninova, A., & Svobodova, Z. (2011). Metals as a cause of oxidative stress in fish: A review. *Veterinarni Medicina, 56*(11), 537–546.

Sexton, K., & Hattis, D. (2007). Assessing cumulative health risks from exposure to environmental mixtures - Three fundamental questions. *Environmental Health Perspectives, 115*(5), 825–832. https://doi.org/10.1289/ehp.9333

Shi, Q., Sun, N., Kou, H., Wang, H., & Zhao, H. (2018). Chronic effects of mercury on Bufo gargarizans larvae: Thyroid disruption, liver damage, oxidative stress and lipid metabolism disorder. *Ecotoxicology and Environmental Safety, 164*, 500–509. https://doi.org/10.1016/j.ecoenv.2018.08.058

Silva Pinheiro, J. P., Bertacini de Assis, C., Sanches, E. A., & Moreira, R. G. (2020). Aluminum, at an environmental concentration, associated with acidic pH and high water temperature, causes impairment of sperm quality in the freshwater teleost Astyanax altiparanae (Teleostei: Characidae). *Environmental Pollution, 262*, 114252. https://doi.org/10.1016/j.envpol.2020.114252

Singh, M., Barman, A. S., Devi, A. L., Devi, A. G., & Pandey, P. K. (2019). Iron mediated hematological, oxidative and histological alterations in freshwater fish Labeo rohita. *Ecotoxicology and Environmental Safety, 170*, 87–97. https://doi.org/10.1016/j.ecoenv.2018.11.129

Sinha, K., Das, J., Pal, P. B., & Sil, P. C. (2013). Oxidative stress: The mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Archives of Toxicology*. https://doi.org/10.1007/s00204-013-1034-4

Slaninova, A., Machova, J., & Svobodova, Z. (2014). *Fish kill caused by aluminium and iron contamination in a natural pond used for fish rearing: A case report*. *Veterinarni Medicina* (Vol. 59).

Small, C. D., el-Khoury, M., Deslongchamps, G., Benfey, T. J., & Crawford, B. D. (2020). Matrix metalloproteinase 13 activity is required for normal and hypoxia-induced precocious hatching in zebrafish embryos. *Journal of Developmental Biology*, *8*(1). https://doi.org/10.3390/JDB8010003

Stankovic, S., Kalaba, P., & Stankovic, A. R. (2014, March 9). Biota as toxic metal indicators. *Environmental Chemistry Letters*. Springer International Publishing. https://doi.org/10.1007/s10311-013-0430-6

Surai, P. F. (1999). Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. *British Poultry Science, 40*, 397–405. https://doi.org/10.1080/00071669987511

Szyczewski, P., Siepak, J., Niedzielski, P., & Sobczyński, T. (2009). Research on heavy metals in Poland. *Polish Journal of Environmental Studies*, *18*(5).

Torres-Osorio, V., Urrego, R., Julián Echeverri-Zuluaga, J., & López-Herrera, A. (2019). Oxidative stress and antioxidant use during in vitro mammal embryo production.

Review. *Revista Mexicana de Ciencias Pecuarias*, *10*(2), 433–459. https://doi.org/10.22319/rmcp.v10i2.4652

Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety, 64*(2), 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013

Valerio-García, R. C., Carbajal-Hernández, A. L., Martínez-Ruíz, E. B., Jarquín-Díaz, V. H., Haro-Pérez, C., & Martínez-Jerónimo, F. (2017). Exposure to silver nanoparticles produces oxidative stress and affects macromolecular and metabolic biomarkers in the goodeid fish Chapalichthys pardalis. *Science of the Total Environment, 583*, 308–318. https://doi.org/10.1016/j.scitotenv.2017.01.070

Vijver, M. G., Elliott, E. G., Peijnenburg, W. J. G. M., & de Snoo, G. R. (2011). Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. *Environmental Toxicology and Chemistry, 30*(6), 1482–1487. https://doi.org/10.1002/etc.499

Zengin, H., Yilmaz, Ö., Demir, E., & Gökce, Z. (2015). Antioxidant enzymatic defences during embryogenesis of rainbow trout Oncorhynchus mykiss (Walbaum 1792). *Turkish Journal of Fisheries and Aquatic Sciences, 15*, 437–446. https://doi.org/10.4194/1303-2712-v15_2_30

Zhang, Q. F., Li, Y. W., Liu, Z. H., & Chen, Q. L. (2016). Exposure to mercuric chloride induces developmental damage, oxidative stress and immunotoxicity in zebrafish embryos-larvae. *Aquatic Toxicology, 181*, 76–85. https://doi.org/10.1016/j.aquatox.2016.10.029

Zhou, Q., Yang, N., Li, Y., Ren, B., Ding, X., Bian, H., & Yao, X. (2020). Total concentrations and sources of heavy metal pollution in global river and lake water bodies from 1972 to 2017. *Global Ecology and Conservation, 22*, e00925. https://doi.org/10.1016/j.gecco.2020.e00925