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Embryotoxicity Produced by the Mixture of Aluminum, Metformin and Penicillin on Common Carp (*Cyprinus carpio*): a Study of Interactions

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Abstract Penicillin, metformin, and aluminum are commonly used substances and their presence in the environment has increased due to their widespread use. In water bodies, the combined presence of xenobiotics leads to additive, synergistic, or antagonistic interactions that significantly modify the toxic response, being more evident in the early stages of development of individuals. These interactions can be evaluated through biomarkers such as the activity and expression of antioxidant enzymes and the production of congenital malformations in exposed organisms. However, little is known about the effects that mixtures of drugs such as penicillin, metformin, and metals such as aluminum can produce on aquatic species that are constantly exposed to these xenobiotics such as common carp. The objective of this study was to determine the toxicity and type of interaction produced on *Cyprinus carpio* embryos exposed to these pollutants isolated and in mixtures. Here we show that the mixture of these pollutants produces antagonism and synergism at high and low concentrations respectively. In addition, toxicity results show that in embryos subacutely exposed (NOAEL) to the mixture of contaminants, the activity of antioxidant enzymes and their gene expression (PCR-RT) is increased, embryonic development is modified, and

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teratogenesis occurs. As can be observed, the mixture of the contaminants influenced the toxic response, evidencing the importance of continuing to study interactions; since this is the way, they are usually found in contaminated bodies of water.

Highlights 1. Exposure of Cyprinus carpio embryos to the mixture of metformin, penicillin and aluminum at high concentrations (LC50) produces an antagonistic type of interaction.

2. Exposure of Cyprinus carpio embryos to the mixture of metformin, penicillin and aluminum at sublethal concentrations (NOAEL) produces a synergistic type interaction.

3. Antioxidant enzyme activity of Cyprinus carpio embryos exposed to NOAEL-equivalent concentrations of metformin, penicillin and aluminum is modified by simultaneous exposure to the toxicants.

4. Gene expression of antioxidant enzymes in Cyprinus carpio embryos exposed to NOAEL-equivalent concentrations of metformin, penicillin and aluminum is increased by simultaneous exposure to the toxicants during the first hours of development.

5. The embryonic developmental score of Cyprinus carpio is decreased by simultaneous exposure to metformin, penicillin and aluminum.

Keywords Interactions · Embryonic development · Teratogenesis · Oxidative stress · Gene expression · Metals · Emerging pollutants · *Cyprinus carpio*

1 Introduction

Metals and emerging contaminants are two relevant groups of xenobiotics at the environmental level and their presence in water bodies is a potential indicator of human development; pharmaceuticals are included in the group of emerging contaminants and are considered anthropogenic markers of social activity, while metals are more related to industrial activity (Andreu et al., 2016; Pérez-Alvarez et al., 2018; Pérez-Coyotl et al., 2017).

Aluminum (AL) is the most abundant metal in the earth's crust. Its mechanisms of action are still being studied, although its toxicity is characterized by the generation of reactive oxygen species (ROS), lipoperoxidation, DNA cleavage, and a decrease in the concentration of antioxidant enzymes. It can form complexes with sulfhydryl groups, damaging various biomolecules. Additionally, it has been discovered that this metal can bind to phenylalanine and tyrosine hydroxylases, which are involved in the synthesis of catecholamines (García-Medina et al., 2010; Kovacic & Somanathan, 2006). Some findings that have emerged from the study of aluminum toxicity in hydrobionts are behavioral alterations, increased lipoperoxidation, altered activity of antioxidant enzymes, genotoxicity, cytotoxicity, congenital malformations, endocrine disruption, teratogenic effects, and impairment of embryonic and larval development (Capriello et al., 2021; Fernández-Dávila et al., 2012; García-Medina et al., 2011; García-Medina et al., 2013; Gómez-Oliván et al., 2017; Gabriel-Correia et al., 2021; Quiroga-Santos et al., 2021; Sánchez-Aceves et al., 2021; Silva-Pinheiro et al., 2021). Likewise, García-Medina et al. (2022) demonstrated that Al nanoparticles produced alterations in the activity of antioxidant enzymes and increased levels of lipoperoxidation, hydroperoxides, and oxidized proteins in common carp juveniles, and that the metal is accumulated in tissues of the fish. In the same sense, Ferrandino et al. (2022) found that adults of *Danio rerio* exposed to the metal showed an increase in the thickness of the endomysium and resorbed myofibrils, and an increase of myotomes' size in organisms exposed between 10 and 15 days to aluminum. In individuals exposed for less time, they found an activation of anaerobic metabolism and the increased activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione-S-transferases.

Despite the reported results, this metal continues to be used in large quantities and continues to be found around the world in concentrations that could affect the quality of life of those organisms that are exposed. In Brazil, concentrations of 1.5 mgL^{-1} of aluminum were detected in mine tailings (Gimenes et al., 2020), 0.32–1.88 mgL^{-1} in samples of reserves and rivers in the same country (Gabriel-Correia et al., 2021), and concentrations of 0.35–0.378 mgL^{-1} have been detected in rivers in Poland (Senze et al., 2021).

Penicillins belong to the group of beta-lactam antibiotics used in treatments of bacterial infections and constitute about 50% of the antimicrobial agents currently in use (Banti et al., 2020). Havelkova et al. (2016) studied the acute toxicity of the antibiotics penicillin, vancomycin, and tetracycline. The former was the least toxic to *Daphnia magna*, *Pseudokirchneriella*,

and *Vibrio fischeri* by evaluating motility inhibition (1496.9 mgL^{-1}), growth inhibition (7114.3 mgL^{-1}), and luminescence inhibition (not obtained below 100 mgL^{-1}) respectively. Bownik et al. (2019) reported that *Daphnia magna* exposed to 11.79, 117.9, and 1179 mgL^{-1} of penicillin G (PG) reduced its swimming activity and velocity as well as its turning angle at all concentrations.

In recent years, the use of antibiotics in humans and veterinary medicine has increased, reaching an annual consumption of 1.05 to 2×105 tons, and its use in livestock is expected to increase by 67% by 2030 as a result of the growth of intensive agriculture, so the consequent contamination due to their presence in water bodies has also increased considerably (Cheng et al., 2020). One of the most frequently used groups of antibiotics are penicillins (39% of the total in 2015), with consumption ranging from 36 to 71% in European countries (Kovalakova et al., 2020) and even higher in developing countries, so their presence in water bodies is to be expected. In Australia, PG concentrations of 250 ngL^{-1} were reported (Watkinson et al., 2009); in the Beiyun River in Beijin, concentrations of up to 449 ngL^{-1} were detected considering it the antibiotic with the highest environmental impact among those studied by Ma et al. (2017); in Iran, there are reports of 300 ngL^{-1} of this drug in Urmia Lake (Komijani et al., 2021) and in Mexico concentrations ranging from 0.04 to 295 ngL^{-1} were detected in the Madin Dam, located in the State of Mexico (Pérez-Cóyotl et al., 2019).

Metformin (MTF) is considered the first-line oral therapy against diabetes mellitus (Elizalde-Velázquez & Gómez-Oliván, 2020; Zhang et al., 2021). Thus, in 2013, in the UK, 83.6% of people with type two diabetes mellitus were using it as the main hypoglycemic, while in the USA its use increased to 60% in 2005 and 77% in 2016 (Montvida et al., 2018; Triggle et al., 2022). Although the presence of this drug is ubiquitous in the aquatic environment, little is known about the toxicological effects it may exert on organisms that are constantly exposed. However, Niemuth et al. (2015) have described endocrine-disrupting effects in *Pimephales promelas* upon exposure to environmentally relevant concentrations (40 μgL^{-1}) from an early stage to adulthood and observed intersexuality in male fish, as well as reduction in total size and fecundity. Ussery et al. (2018) evaluated exposure of *Oryzias latipes* during early life stages to environmentally relevant concentrations and identified alterations in the metabolome, gene expression, and growth of the organisms. Godoy et al. (2018) reported malformations (scoliosis and abnormal pigmentation) in *Danio rerio* embryos exposed to 1100 mgL^{-1} metformin. For their part, Lee et al. (2019) demonstrated that MTF caused endocrine disruption and oxidative stress in *Oryzias latipes*, since transcription levels of ERa and CYP19a were increased in males, and gene expression of ERb1 and VTG2 was decreased in female fish. In the same sense, Dandan et al. (2022) showed by comet assay and PAPD analysis that MTF is genotoxic to *Dugesia japonica*. Likewise, Elizalde-Velázquez et al. (2022) found that MTF is neurotoxic to *Danio rerio*, through significant inhibition AChE activity and increment in oxidative damage and apoptosis in the brain of fish. They also reported a significantly upregulated expression of Nrf1, Nrf2, BAX, p53, BACE1, APP, and PSEN1, and downregulated CASP3 and CASP9 in the same organism. Feng et al. (2022) mentioned that the production of ROS at the mitochondrial level is related to the effect of this drug, since it can activate and inhibit the activity of the mitochondrial respiratory chain, although the mechanism is still not entirely clear.

This drug has been reported in surface water from 1 to 49 ngL^{-1} in the USA (Tisler & Zwiener, 2018), 167.7 to 250.1 ngL^{-1} in wastewater in Germany (Oertel et al., 2018), and 0.05 to 11,694 ngL^{-1} in the Madin Dam, located in the State of Mexico (Pérez-Cóyotl et al., 2019), while concentrations ranging from 1360 to 1480 ngL^{-1} (Luja-Mondragón et al., 2019) and 1290 to 1330 ngL^{-1} (Pérez-Álvarez et al., 2018) have been found in hospital effluents in Mexico.

The information gathered here shows that aluminum, penicillin, and metformin are xenobiotics that are ubiquitous in nature and, in particular, in water bodies around the world, generating toxic effects on hydrobionts. However, although the above pollutants have been found mixed with each other and with several other substances in water bodies, little is known about the effects that they could have together in the early stages of the organisms that inhabit the environment, and recently obtained experimental data suggests that it would be negligent to ignore the risks of mixtures (Schmidt et al., 2016; Drzymala and Kalka, 2020). Thus, considering that aluminum, penicillin, and metformin in isolation have been shown to be

agents that can generate oxidative stress in aquatic organisms, it is possible that they produce embryotoxicity, teratogenesis, and alteration in the gene expression of antioxidant enzymes and that this response can be modified when these xenobiotics are in mixture, producing interactions.

The aim of this work was to evaluate the toxic effects of AL, PG, MTF, and the mixture of them (Mx) on *Cyprinus carpio* embryos using equitoxic concentrations of LC_{50} and NOAEL. This organism is one of the most widely distributed freshwater fish in the world and of great economic importance (Vilizzi & Copp, 2017), whose embryonic development facilitates the observation of the effects that the previously mentioned substances may exert on them.

2 Methodology

2.1 Test Substances

For exposure to AL, the salt $Al_2(SO_4)_3$ were used. MTF and PG stock solutions (1 g L^{-1}) were prepared with metformin hydrochloride (CAS number 1115–70-4) and penicillin G sodium salt (CAS number 69–57-8) in egg water (40 g "Instant Ocean ®" Sea Salt added to 1L distilled water. From this solution, 1.5-mL stock salts added to 1L distilled water = 60 µg/mL final concentration). Unless otherwise indicated, reagents were provided by Sigma-Aldrich.

2.2 Quantification of Exposure Concentrations of Test Toxicants

Analysis was performed following methodologies proposed by Merck © (2022) and Porta et al. (2008) with slight modifications. Stock solutions of PG and MTF were prepared by dissolving the compounds in egg water at a concentration of 1000 ppm and results were interpolated. PG chromatographic separation was performed by using an HPLC–UV method and an Ascentis® C18 column, 15 cm × 4.6 mm, 5 µm. The antibiotic was eluted with a mobile phase consisting of ammonium acetate 10 mM (acetic acid pH 4.5) and acetonitrile (75:25). Flow rate, run time, and injection volume were 1 mL min^{-1}, 3 min, and 10 µL respectively. MTF was eluted with a mobile phase consisting of a mixture of phosphate buffer 0.02 M (pH 7.0) and acetonitrile (50:50) at flow rate of 1.0 mL min^{-1}. Wavelength was set at 236 nm. AL determination was performed following the methods reported by the Mexican Standard for Analysis NMX-AA-051SCFI-2016: Determination of metals by atomic absorption. Graphite furnace method (DOF, 2016). A type curve was prepared from a stock solution of aluminum standard (1000 ppm) in egg water and results were interpolated.

2.3 Collection of Common Carp Embryos

Cyprinus carpio embryos were collected at the Centro Carpícola de Tiacaque (State of Mexico). The fish producers placed the reproductive organisms in ponds of approximately 15 × 7 m, induced the females with chorionic gonadotropin and placed branches of casuarina (*Casuarina equisetifolia*) where the females oviposited and the males fertilized the oocytes. The embryos were then detached one by one from the branches and observed under the stereomicroscope to select those that had developed for approximately 2 h and were in the blastula stage (approximately 128 cells). Once the viability of the embryos was confirmed, they were weighed on an OBI® digital scale to place the appropriate weight in the corresponding exposure systems and were transferred to the aquatic toxicology laboratory of the IPN Zacatenco.

2.4 Experimental Design

Once the embryos were obtained, they were placed in their corresponding test systems, which can be divided into two stages, i.e., acute toxicity and sublethal toxicity evaluation, each of which is described below.

2.4.1 Acute Toxicity Assay

Embryos destined for the determination of LC_{50}, NOAEL, and the type of interactions were individually placed in 24-well plates with 3 mL of the substance to be tested for 96 h. After this time, the lethality produced was recorded and the results were entered into the Compusyn software (Chou & Martin, 2005). To determine the LC_{50} (Median Lethal Concentration) and NOAEL (Non Observed Adversed Effect Level) of each substance, plates with 24 wells were used in which 3 mL of each substance and one embryo per

Table 1 Ranges of concentrations used to determine LC_{50} and NOAEL of the individual substances (0.5 to 5 mgL^{-1} aluminum; 250 to 1000 mgL^{-1} metformin and 50 to 1000 mgL^{-1} penicillin)

	AL μgL^{-1}	PG μgL^{-1}	MTF μgL^{-1}
1	0.5	50	50
2	1	100	100
3	1.5	150	150
4	2	200	200
5	2.5	250	250
6	3	300	300
7	3.5	350	350
8	4	400	400
9	4.5	450	450
10	5	500	500

well was placed. In total, 8 embryos with 3 replicates were exposed to 10 different concentrations of the individual contaminants in intervals from lowest to highest (Table 1). They were maintained at 27° and there was no turnover in the system. At 96 h, the number of dead embryos was determined and with this record the concentration–response curves were elaborated and LC_{50} and NOAEL were obtained by means of Compusyn software. The characteristics considered to recognize an embryo as dead were coagulation, lack of blood circulation, and absence of heartbeat. With these parameters, the design of binary and tertiary mixtures was carried out, maintaining equitoxic concentrations in them, i.e., those in which there was an equivalent effect in the three pollutants studied.

Evaluation of Interactions Once the NOAEL and LC_{50} were determined for each pollutant, the binary and tertiary mixtures were carried out using the following fractions 0.125, 0.25, 0.5, 1, and 2 of the mentioned parameters maintaining in them the corresponding equitoxic concentrations (Table 2). In 24-well plates, 3 mL of each substance (single, binary mixtures, and tertiary mixtures) and one embryo per well were placed. Forty-eight embryos were used for each of the mixtures to be evaluated and death was considered the end point at 96 h. The observed results were entered into Compusyn software (Chou & Martin, 2005) to determine the type of interaction in the mixtures and to obtain the respective graph, in which $CI < 1, = 1, \quad 1$ indicate synergy, addition, and antagonism, respectively.

2.4.2 *Sublethal Toxicity Assay*

Evaluation of Antioxidant Enzyme Activity Five groups with 5 g of embryos each were organized in 1-L fish tanks. The first four groups were exposed to aluminum, penicillin, metformin, and the mixture at NOAEL concentrations of 0.074, 45.8, and 0.01 mgL^{-1} respectively, dissolved in egg water (60 μgL^{-1} of Instan Ocean salts), and the fifth group was the control, which was only egg water. After 12, 24, 48, 72, and 96 h of exposure to the abovementioned pollutants, the embryos were homogenized in 1.5 mL of phosphate buffer (PBS, pH 7.4) and centrifuged at 15 $000 \times g$ and 4 °C for 15 min to obtain the supernatant to subsequently evaluate the antioxidant activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), as well as the total protein content which was used to express the results of all biomarkers evaluated. Samples were stored at − 70 °C until their measures. The exposure concentrations were measured in egg water (Table 3).

1. **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 milligram of protein per gram of tissue.
2. **Determination of the antioxidant activity of catalase**
   CAT enzyme activity was determined by the method of Radi et al. (1991). To 100 µL of supernatant were added 900 µL 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. The absorbance was determined at 240 nm at 0 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.
3. **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 minutes 1, 2 and 3. The concentration of GPx was calculated using the fol-

Table 2 Experimental design of the concentrations used for the binary and tertiary mixtures of AL, PG, and MTF maintaining the equitoxicity and considering the LC_{50} and NOAEL

CL_{50}	AL mgL^{-1}	PG mgL^{-1}	MTF mgL^{-1}	NOAEL	AL mgL^{-1}	PG mgL^{-1}	MTF mgL^{-1}
1/8 CL_{50}	0.36	280.42		$^{1/8}$ NOAEL	0.00925	0.00125	
¼ CL_{50}	0.725	560.82		¼ NOAEL	0.0185	0.0025	
½ CL_{50}	1.45	1,121.67		½ NOAEL	0.037	0.005	
1 CL_{50}	2.9	2,243.33		**1 NOAEL**	0.074	0.01	
2 CL_{50}	5.8	4,486.66		2 NOAEL	0.148	0.02	
1/8 CL_{50}	0.36		78.82	$^{1/8}$ NOAEL	0.00925		5.725
¼ CL_{50}	0.725		157.63	¼ NOAEL	0.0185		11.45
½ CL_{50}	1.45		315.26	½ NOAEL	0.037		22.9
1 CL_{50}	2.9		630.52	**1 NOAEL**	0.074		45.8
2 CL_{50}	5.8		1,261.04	2 NOAEL	0.148		91.6
1/8 CL_{50}		280.42	78.82	$^{1/8}$ NOAEL		0.00125	5.725
¼ CL_{50}		560.82	157.63	¼ NOAEL		0.0025	11.45
½ CL_{50}		1,121.67	315.26	½ NOAEL		0.005	22.9
1 CL_{50}		2,243.33	630.52	**1 NOAEL**		0.01	45.8
2 CL_{50}		4,486.66	1,261.04	2 NOAEL		0.02	91.6
1/8 CL_{50}	0.36	280.42	78.82	$^{1/8}$ NOAEL	0.00925	0.00125	5.725
¼ CL_{50}	0.725	560.82	157.63	¼ NOAEL	0.0185	0.0025	11.45
½ CL_{50}	1.45	1,121.67	315.26	½ NOAEL	0.037	0.005	22.9
1 CL_{50}	2.9	2,243.33	630.52	**1 NOAEL**	0.074	0.01	45.8
2 CL_{50}	5.8	4,486.66	1,261.04	2 NOAEL	0.148	0.02	91.6

Table 3 Exposure concentration and quantification of Al, metformin, and penicillin in egg water

Substance	Exposure concentration mg L^{-1}	Quantification in egg water mg L^{-1}
Aluminum NOAEL	0.074	0.076
Aluminum CL_{50}	2.89	3.0
Penicillin NOAEL	0.01	0.01
Penicillin CL_{50}	2243.33	2226.09
Metformin NOAEL	45.8	41.21
Metformin CL_{50}	630.52	600.0

lowing formula: $U/L = 8412 \times \Delta A$ 340 nm/min. Results are expressed as IU of GPx per milligram per gram of tissue.

4. **Determination of total protein content**

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

Antioxidant Enzyme Gene Expression by PCR To evaluate gene expression, 5 groups of 3 g embryos and 3 replicates were used. They were exposed to NOAEL concentrations of AL (0.074 mgL^{-1}), MTF (45.8 mgL^{-1}), and PG (0.01 mgL^{-1}), the mixture of the 3 and a control group. One hundred milligrams was taken from each group at 12, 24, 48, 48, 72, and 96 h and placed in RNAlater® stabilizing reagent for subsequent storage at −20 °C following the protocol instructions. RNA was extracted with the help of the RNeasy® kit followed by reverse transcription to obtain cDNA with the QuantiTect® kit, and aliquots of each completed reverse transcription reaction were added to the PCR-Real-Time mix of the QuantiTect® SYBR® kit; all kits used belong to the QIAGEN brand. The cycler (Rotor-Gene Q from QIAGEN) was programmed according to the protocol. The concentration of RNA and DNA was measured with a Thermo scientific NanodropTM 2000 spectrophotometer. The primers used are shown in Table 4.

Evaluation of Embryonic Development The evaluation of morphological development was performed on ELISA plates, in which a 4-h post-fertilization embryo

Table 4 Gene sequence of primers used for gene expression of antioxidant enzymes and the reference gene

Gene	Sequence (5′-3′)
fe α	Forward: CACGTCGACTCCGGAAAGT Reverse: ATGGTGATACCACGCTCACG
sod	Forward: AGAAGATGTCAGCTGCCACA Reverse: ATGCATGCTCCCAGACATCT
cat	Forward: AGCCAAAGTGTTCGAGCATGT Reverse: TCACCAGCCACAGTGGAAAA
gpx	Forward: TGCAACCAGTTCGGACATCA Reverse: GAAGCCATTTCCAGGACGGA

at blastula stage showing a round and transparent chorion without signs of coagulation was placed in each of the wells. Each of the five test groups was composed of 24 embryos and exposed to 1 mL of the solutions corresponding to the NOAEL concentrations and a control group. The morphological development of each embryo was evaluated at 12, 24, 24, 48, 48, 72, and 96 h of exposure, using an adaptation of the general morphological scoring system (GMS) proposed by 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 traits assessed with which it complies. The first score was assigned at 12 h, using an Optika XDS-2 inverted microscope, 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 whose development was described by Kimmel et al. (1995).

Evaluation of Teratogenicity The embryos were observed at 96 h under an inverted microscope and the number of times that a given malformation was visible in each of the exposed embryos and in the control group was recorded. The eleven malformations evaluated were the following: pericardial edema, yolk sac edema, eye edema, head malformation, saccule/otolith malformation, tail malformation, heart malformation, notochord modification, scoliosis, rachischisis, and yolk sac deformation. The experiment was considered valid when < 10% of control embryos showed coagulation (Hermsen et al., 2011).

2.5 Statistical Analysis

For the determination of LC_{50}, NOAEL, and interaction type, Compusyn software was used. Data normality and homoscedasticity were verified by Shapiro–Wilk and Bartlett tests, respectively. Antioxidant enzyme activity was analyzed by two-factor ANOVA and post hoc Tukey's. For embryonic development, two-way RM ANOVA with Holm-Sidak as post hoc was used. The statistical package used was SigmaPlot 12.0. The averages of the gene expression results were normalized in Prism 9.3.1, as well as the heat map performance.

Table 5 LC_{50} and NOAEL values for individual substances

	CL_{50} mgL^{-1}	NOAEL mgL^{-1}
Aluminum	2.89	0.074
Penicillin	2243.33	0.01
Metformin	630.52	45.8

3 Results and Discussion

3.1 Quantification of AL, PG and MTF in Egg Water

Concentrations of the three contaminants are shown in Table 3. The quantified values show slight differences with respect to the concentrations considered for the exposure of embryos with AL, while for PG and MTF the differences reach 17 mg L^{-1}.

3.2 Acute toxicity Assay

The mean lethal concentrations (LC_{50}) obtained show that the exposure of *Cyprinus carpio* embryos to different concentrations of the studied pollutants places AL as the most toxic substance, followed by MTF and finally PG (Table 5). These results coincide with similar studies in which the responses of other organisms were evaluated in acute exposures of AL as in embryos of *Coregonus albula* (Duis & Oberemm, 2001), *Macrobrachium lanchesteri*, and *Nais elinguis* (Shuhaimi-Othman et al., 2011) of PG as *Daphnia magna* and *Nitzschia fonticola* (Zoetis, 2015) and MTF as blue mojarra (ABC laboratories Inc., 1994) and rats, in whom mortality was observed with the administration of more than 900 mg/kg/day (Quaile et al., 2010). In the case of penicillin, even for mice with experimental diet (heat-treated beef) containing high levels of PG, the LD_{50} was 933.04 $mgkg^{-1}$ (Cui et al., 2018). Its high LC_{50} and LD_{50} rank it

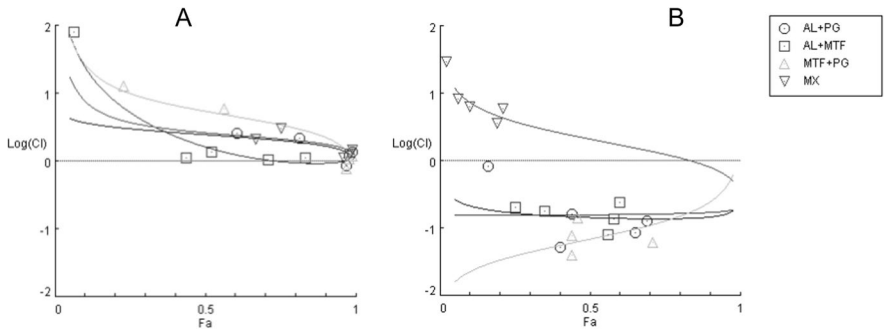

Fig. 1 Combination index (Fa-IC) in logarithm, of concentrations corresponding to (**A**) LC_{50} and (**B**) NOAEL for 3 binary mixtures between (AL), penicillin (PG), and metfomine (MTF) and a tertiary mixture (MX). CI values are plotted using CompuSyn software as a function of mortality (Fa) of *Cyprinus carpio* embryos exposed to the mixtures. CI < 1, = 1 and > 1 indicate synergism, addition and antagonism, respectively

as one of the safest antibiotics for humans and animals, although it is very common to cause allergy, which affects 1–10% of the population. However, it is important to consider the effects at the sublethal level that can lead to important medium- and long-term effects that can even compromise the life of the exposed organisms and the health of the ecosystem.

The relevance of conducting studies using (sublethal) NOAEL concentrations lies in observing the effects that contaminants can exert on organisms that are constantly exposed to them, detecting if it is possible that they cause sublethal toxicity as has been published in different studies: AL induces DNA damage in human peripheral blood lymphocytes (Lankoff et al., 2006), as well as genotoxicity and cytotoxicity in *Cyprinus carpio* lymphocytes (Garcia-Medina et al., 2011); PG caused reduction in abundance and antibiotic resistance in free-living nematodes (Nasri et al., 2015), oxidative stress in *Gallera mellonella* (Büyükgüzel & Kalender, 2007), and modification in root architecture and secondary metabolite metabolism in *Arabidopsis thaliana* (Gudiño et al., 2018) while MTF caused immobilization and growth inhibition in *Daphnia magna*, *Desmodesmus subspicatus*, and *Lemna minor* (Cleuvers, 2003); acts as an endocrine disruptor in *Pimephales promelas* (Niemuth & Klaper, 2015); and inhibits growth and development of *Daucus carota* (Eggen et al., 2011).

It is widely known that AL is considered a neurotoxic agent implicated in behavioral, neuropathological, and neurochemical changes that are associated with cognitive impairments (Monteiro-Fernandes et al., 2021). However, the mechanisms of toxicity and the concentrations that reach to be safe for different organisms need to be further studied.

PG has been shown to generate changes in the composition of the microbiota when administered in early stages of development in humans and mice, which affects the immune system making them vulnerable to various conditions (Daniluk et al., 2017; Volkova et al., 2021), while several studies in MTF have shown that this drug can trigger the expression of various genes, particularly those responsible for endocrine hormone pathways (Ambrosio-Albuquerque et al., 2021). The interaction between these three pollutants present in water bodies remains uninvestigated, so it is interesting to evaluate the effects and changes they may cause in the toxic response.

3.2.1 Determination of Interaction Types in Mixtures

In Fig. 1A, B on the abscissa axis is the fraction affected, i.e., the fraction of embryos killed by the substances to which they were exposed and on the ordinate axis, the value of the combination index in logarithm (this function allows to condense the data for appreciation), which indicates the type of interaction present. CI less than 1, equal to 1, and greater than 1 indicate synergy, addition, and antagonism, respectively.

Graph 1A represents the response of embryos exposed to LC_{50}. It is observed that the mixture of aluminum and metformin initiates with a marked antagonism and subsequently remains in addition, while the other three mixtures have a concentration-dependent behavior since there was antagonism at low concentrations and a tendency to synergism at high concentrations.

According to the article published in 2021 by Wang et al., the factors that influence the type of interaction in the mixtures will determine the complexity of the combined mechanism of toxicity, which are mainly caused by changes in the structure and function of the cell membrane, interference with physiological and biological activities, competition in the sites of action, and formation of complexes or chelates. In this study, it was observed that the toxicity of binary mixtures of aminoglycoside antibiotics was modified after the addition of copper resulting in different effects (decrease in the intensity of synergism, increase in antagonism, or disappearance of both) possibly due to the formation of chelates between antibiotics and the heavy metal. Multiple widely used antibiotics such as tetracyclines and quinolones contain in their structure carboxylic groups, carbonyls, and/or piperazines that act as possible electron donors for coordinating metals. These antibiotics acting as ligands can bind to a variety of metals forming complexes (Zhang et al., 2012).

In the present study, three mixtures have the presence of PG. This drug is known to have the ability to form conjugates with some metals. Ketikidis et al. (2020) reported that this binding increases the antimicrobial activity in humans, and in turn could be causing the antagonism in the mixtures. It has also been reported that one of the degradation metabolites of this drug (penicillamine) is used in pharmacology as a chelator in the treatment of some diseases, so it could be chelating with AL. These results are similar to those obtained by Zhou et al. (2021) where the mixture of cadmium and the antibiotic sulfamethazine presented antagonistic and also synergistic effects in the growth inhibition of *Brassica chinensis* when exposed to high concentrations (1 and 10 mg kg^{-1}).

The complexes formed appear to have different behaviors in physiological activity when compared to antibiotics independently, since a complex may be more active than the free antibiotic, or increase the affinity of the antibiotic to the target RNA, while the drug alone would have a weak binding to the target. Some results have revealed that complexes could be the active species, not to mention that high concentrations of metals can reduce the biological activities of antibiotics (Zhang et al., 2012).

Regarding the predominant antagonism of the mixtures, the study by Liao et al. (2021) showed that the bioaccumulation of the lead-cadmium mixture in *Danio rerio* decreased significantly versus the individual bioaccumulation of each metal, which can be explained because both divalent cations share similar uptake systems such as competition for Ca + channels in the cell membrane. The inhibition/decrease of effects as a consequence of sharing sites or mechanisms of action has been documented by Ribeiro et al. (2021) where the mixture between selenium and mercury presented antagonistic effects in *Oncorhynchus mykiss*; in *Cyprinus carpio*, the mixture between copper and zinc produced a decrease in the consumption of these metals (Castaldo et al., 2021).

Figure 1B corresponds to NOAEL concentrations; the antagonistic effect of the tertiary mixture could be explained by the complex possibly formed between aluminum and penicillin as described above. In addition, there are reports in which antioxidant activity of MTF has been found at low concentrations. In a 2010 study, Hou et al. suggest that this may be carried out by increased expression of thioredoxin, a protein that acts as an antioxidant by decreasing intracellular ROS concentrations in human aortic endothelial cells. Godoy et al. (2018) found that the mixture of MTF and ranitidine produced synergism in the immobilization of *Daphnia similes*, and in two other binary mixtures that also included metformin, mild to moderate antagonism occurred. In that study, the determination of the type of interaction was also carried out by Compusyn software.

In the study by Long et al. (2016), inhibition in the growth of *Escherichia coli* exposed to individual and pooled antibiotics was evaluated. They found antagonism-type effects, between tetracyclines and penicillin V, in addition, between sulfonamides and some β-lactams, and synergism between sulfonamides. They attribute the additive effect to the fact that the substances have different targets and the same mechanism of action, while the antagonism could be due to differences in the binding capacity to the target protein. Therefore, they suggest that the toxic effects of complex mixtures do not depend solely on the pharmacological mechanism of action, and that the chemical structure of the substances and the binding energy to the target proteins are factors that should be taken into account to determine the type of interaction present in the mixtures studied. Additionally, regarding the addition effect, Chen et al. (2014) suggest that the mixture presents this effect when the individual components bind to the same target and show a similar binding pattern.

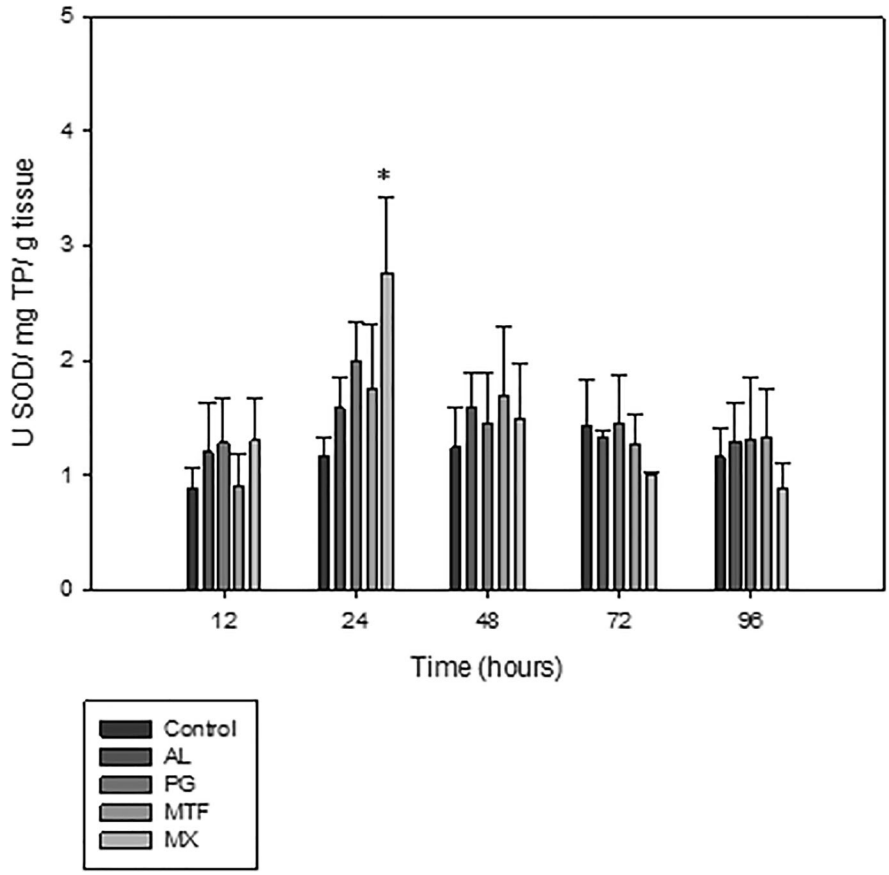

Fig. 2 Antioxidant activity of the enzyme superoxide dismutase. Embryos exposed to aluminum, penicillin, metformin and mixture of them. The results are expressed as the mean ± SE. Bifactor ANOVA ($F_{4,50}=0.565$, $p=0.689$ for groups factor, $F_{4,50}=2.951$, $p=0.029$ for time factor and $F_{16,50}=0.685$, $p=0.794$ for interaction time and groups), post hoc Tukey $^{*}p=0.039$ with respect to the control

3.3 Sublethal Toxicity Assay

3.3.1 Antioxidant Enzyme Activity

Excess ROS generation and oxidative stress are mechanisms of toxicity shared by prooxidants such as AL, PG, and MTF. Organisms have developed systems of protection, prevention, and repair against these events. The first line of defense of the antioxidant enzyme system is formed by SOD, CAT, and GPx, and the evaluation of their activities gives us clues to the behavior and possible mechanism of toxicity of the pollutants evaluated.

Superoxide Dismutase The three pollutants and the mixture of them generated changes in the enzymatic activity of SOD (Fig. 2); however, the mechanisms by which they could exert and interact with the molecules that cause the observed changes are different. The greatest increase with respect to the control group occurred in embryos exposed to the mixture of contaminants at 24 h at 134%, followed by PG (70%) and MTF (49%) at the same time. On the other hand, the decrease in enzyme activity compared to the control was mainly observed at 72 h in MX (30%), MTF (12%), and AL (7%) and at 96 h only in MX (23%).

The increase in the first hours could be due to the increase in ROS. In vertebrate embryos, SOD has been found to be involved in neural tube development during the initial hours of development (Dhage et al., 2017), which could explain the antioxidant activity observed in the early exposure times, while the decrease at 72 h is related to the increase in respiratory rate (derived from the proximity of hatching), leading to a demand for ATP and consequent decrease in ROS (Brooks, 2005).

AL is a non-essential metal and potentially toxic to organisms that are constantly exposed to it. One of its best known mechanisms of toxicity is the displacement of intracellular iron, which facilitates the generation of free radicals such as superoxide, hydroperoxides, and hydroxyls through the Fenton

and Haber–Weiss reaction. Additionally, aluminum has the ability to bind to the SOD enzyme optimizing oxidation reactions in different biological systems (Monteiro-Fernandes et al., 2021).

The increase of SOD in the first exposure times, as well as the subsequent progressive decrease was also reported by Capriello et al. (2021), who exposed *Danio rerio* larvae to concentrations of 50, 100, and 200 µM $AlCl_3$ for 72 h, in that study additionally GPx had a concentration-dependent behavior and CAT did not show significant changes. Benavides et al. (2016) exposed *Carassius auratus* to different concentrations of Al_2O_3 and ZnO for 21 days, observing an increase in the antioxidant activity of SOD and CAT enzymes and then their decrease in longer exposures, indicating that vitamins C and E have the ability to decrease the toxicity of ZnO nanoparticles. Canli and Canli (2020) reported the ability to scavenge aluminum nanoparticles 14 days after exposure in *Oreochromis auratus* despite the decrease in SOD and CAT enzyme activity. They also point out the importance of taking into account that metals can be detoxified by different molecules such as metallothioneins (Canli & Canli, 2020).

Similar enzyme behavior to that of the embryos exposed to the penicillin antibiotic in this study has been observed in *Daphnia magna* after the first hours of exposure to triclosan where an increase in SOD and a subsequent decrease after 48 h was observed (Peng et al., 2013).

The MTF drug impacts cellular energy balance by activating AMPK, a suppressor of mitochondrial complex I of the respiratory chain (Niemuth & Klaper, 2015). Lee et al. (2019) studied two generations of *Oryzias latipes* proving that it caused oxidative stress, as MTF increased ROS and decreased glutathione content in male fish, while in females it increased CAT activity. In this study, they indicate that the mentioned increases could be due to MTF reducing the mononucleotide cofactor flavin, altering the flow of electrons and the consequent generation of the superoxide radical. This could explain the increase in the enzymatic activity of embryos exposed to this drug.

Catalase CAT enzymatic activity (Fig. 3) was increased with respect to the control mainly at 48 h of exposure by PG (134%) and MTF (127%) and at 24 h by PG (119%) and MX (101%). The decrease occurred at 12 h by MX (76%) and MTF (65%) and at 72 h by AL (71%). The changes in the activity of this enzyme could be related to conformational changes and alterations in its secondary structure, since it has been shown that substances such as naphthalene and others are able to bind to CAT and generate such variations (Jing et al., 2020).

The increase in CAT activity could be due to the presence of high concentrations of H_2O_2, as described by Wu et al. (2011) in *Oryzias latipes* embryos in the first hours post-fertilization, which gradually increased as development progressed until reaching maximum levels in recently hatched organisms, and conversely, the absence of the substrate could result in a decrease in its activity (Monteiro-Fernandes et al., 2020). Carmo et al. (2018) propose that in prolonged exposures, other antioxidants such as GSH increase, producing the decrease of certain enzymes such as CAT.

In this project, it is assumed that PG concentrations are not sufficient to generate an increase in enzymes that are significant, since studies such as the one conducted by Zhong et al. (2021) found that CAT activity increased significantly only at the highest concentrations when evaluating 3 antibiotics on *Anabaena cylindrica* and *Chlorella pyrenoidosa*.

The decrease in CAT at 96 h compared to earlier times where the increase was constant is explained in *Danio rerio* by changes in the structure of the chorion over time, as its hardness increases and permeability decreases. It has been proven that environmentally relevant concentrations of MTF can cause disturbances in the embryogenesis of *Danio rerio*, and according to Elizalde-Velázquez et al. (2021), the mechanism that causes it is oxidative stress, since they observed an increase in the enzymatic activity of SOD, CAT, and GPx at concentrations ranging from 1 to 100 µg L^{-1}, at 72 and 96 hpf, although the lowest activity corresponded to the highest concentration of the exposures. Additionally, it has been shown that exposure to 6 µM of MTF for prolonged times generates oxidative stress, alters genes related to this mechanism, and increases lipoperoxidation in human breast cancer cells (Marinello et al., 2020). However, in contrast to these studies, it has been observed that the interaction of MTF with mitochondrial function could decrease ROS formation and improve cellular energy metabolism, as well as prevent the release of cell death mediators from mitochondria (Ommati et al., 2021) .

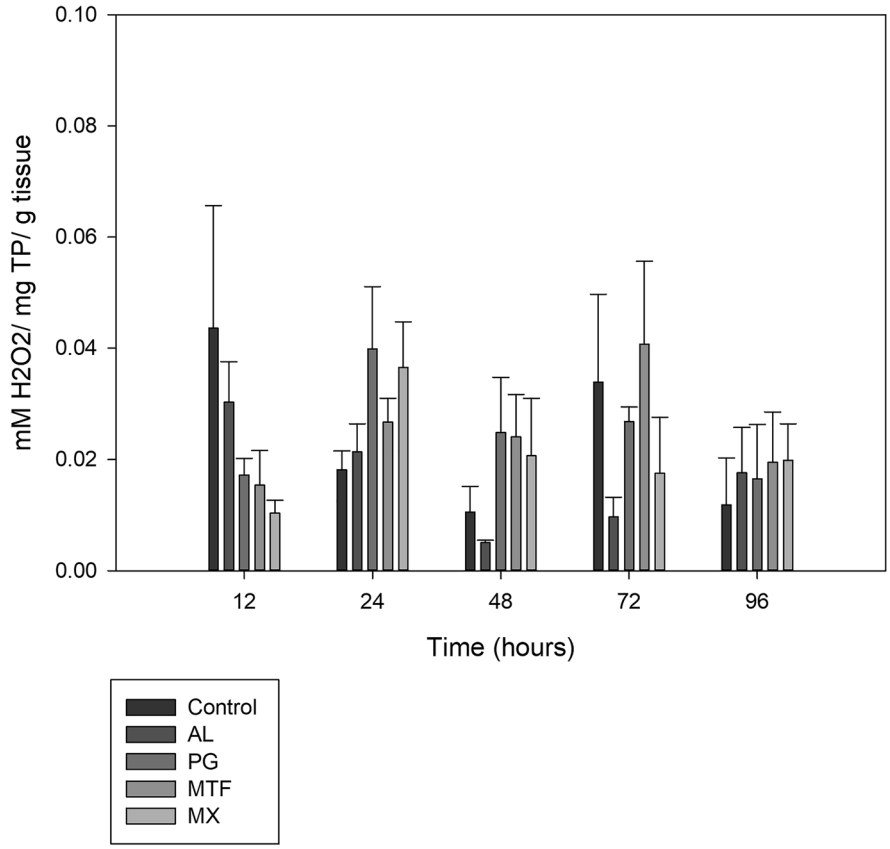

Fig. 3 Antioxidant activity of the enzyme catalase. Embryos exposed to aluminum, penicillin, metformin the mixture of them. The results are expressed as the mean ± SE. ANOVA Two-factor ($F_{16,50} = 1.354$, $p = 0.204$ for interaction time and groups), post hoc Tukey

Sanchez-Aceves et al. (2021) evaluated the effect of ibuprofen, AL, and the binary mixture in *Danio rerio*, observing that the toxic response was affected by the mixture in gill, liver, and intestinal tissue. The enzymatic activities of SOD, CAT, and GPx showed variation in the mixture compared to the individual toxicants, highlighting that the decrease in SOD and CAT activity in mixtures of metals and NSAIDs has been an effect observed in other studies.

Glutathione Peroxidase The GPx enzyme can react with hydrogen peroxide and a wide range of lipid hydroperoxides, including cholesterol derivatives and cholesterol esters. Although it has long been considered solely as an antioxidant enzyme, evidence suggests that it plays an important role in signal transduction, inflammation, and apoptosis (Hermesz & Ferencz, 2009). The most evident increase in GPx activity compared to the control group was in the 24-h MX with 68% and 48-h MTF with 35%, while the main decrease in its activity was in the group exposed to 12-h AL (38%) and 96-h MX (27%) (Fig. 4).

Some GPx isoforms are fundamental in the participation of *Danio rerio* embryonic development because they exhibit a very dynamic localization throughout the early stage and because of their ability to protect the organism during differentiation between muscle cells and ROS. Muscle contractions increase ROS production. These contractions start around 17 hpf, being more coordinated, stronger, and more frequent as development progresses (Mendieta-Serrano et al., 2015), which could influence the increase in enzyme activity in the first hours of exposure, subsequently presenting a tendency to decrease as a result of the effect that contaminants could exert on embryos.

The activity of the GPx enzyme in *Danio rerio* embryos exposed to AL increased in a concentration-dependent manner in the study by Capriello et al. (2021), although it always remained below that recorded in the controls. A possible explanation could be the stabilization by Al(III) of a superoxide radical anion. This may induce the formation of several ROS both by a direct pathway with the formation of the hydroxyl radical and indirectly by influencing the

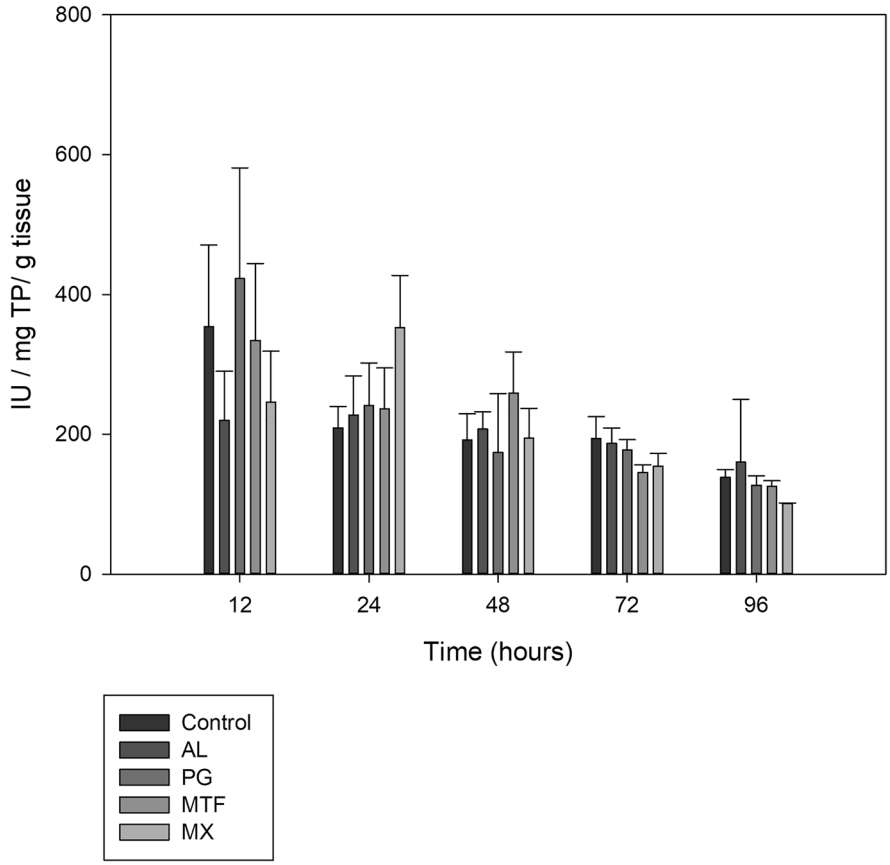

Fig. 4 Antioxidant activity of the enzyme glutathione peroxidase. Embryos exposed to aluminum, penicillin, metformin the mixture of them. The results are expressed as the mean ± SE. ANOVA Two-factor ($F_{16,50} = 0.697$, $p = 0.783$ for interaction time and groups), post hoc Tukey

redox equilibrium in the Fenton reaction. Therefore, increased GPx activity may not respond to a direct increase in ROS, and subsequently intervene to form secondary species. In addition, GPx plays an important role in the protection of neural cells in response to extreme oxidative stress. In this case, its protective action probably contributed to a decrease in cell mortality or an improvement in larval swimming activity, recorded at higher concentrations.

The mixture of contaminants such as PG and MTF and metals such as AL, iron, and mercury, among other substances present in water samples from Madin Dam, was used to determine the antioxidant activity of SOD, CAT, and GPx in *Cyprinus carpio*. The results showed that SOD increased in almost all exposed groups during the first hours of exposure, CAT increased in almost all groups and exposure times, while GPx presented a similar behavior to the control group up to 72 h. Despite the increase in enzyme activity, oxidative damage in embryos was inevitable. It is highlighted that most of the pollutants present in the dam water are prooxidant agents capable of interacting with each other and causing serious damage to the exposed organisms (Pérez-Coyotl et al., 2019).

3.4 Gene Expression of Antioxidant Enzymes

3.4.1 Superoxide Dismutase

Gene expression of *sod* in embryos exposed to MX was increased mainly at 12 and 24 h (Fig. 5a, b), followed by AL at 48 and 72 h (Fig. 5c, d). This could be due to the fact that SOD synthesis occurs mainly in the first half of development and is available to exert its activity in the last hours before embryos hatch, which has been reported in another work from our laboratory (Cano-Viveros et al., 2021) where an increase in antioxidant enzyme activity is observed at 72 and 96 hpf. However, Yang et al. (2021) reported variations in antioxidant enzyme gene expression in *Danio rerio* embryos exposed to binary mixtures of mycotoxins

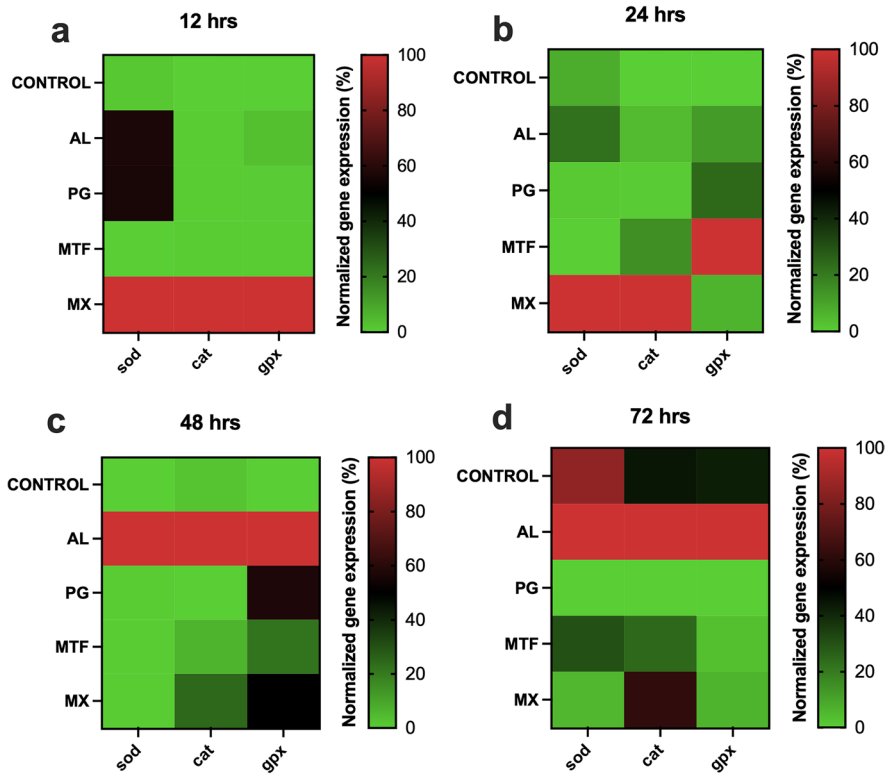

Fig. 5 Relative gene expression levels of superoxide dismutase, catalase and glutathione in *Cyprinus carpio* embryos exposed to aluminum, penicillin, metformin and the mixture of them at **a** 12 h, **b** 24 h, **c** 48 h, and **d** 72 h. Values are presented as mean ± EE

and since the *sod* mRNA expression pattern was not consistent with changes in its enzyme activity, they suggest that the regulation of this enzyme is at the post-transcriptional level.

Daphnia magna exposed to 5 and 10 mg of the fungicide boscalid reduced sod gene expression and attribute this to boscalid affecting nuclear factor erythroid signaling pathways, inhibiting the expression of genes related to the antioxidant system (Aksakal, 2020). According to Wang et al. (2019), MTF may alter the signaling pathway involving AMPK, also related to oxidative stress response.

3.4.2 Catalase

cat is overexpressed at 12, 24, and slightly 72 h by MX, while at 48 and 72 h it is due to exposure to AL. The increase in the first hours could be due to the removal of excess hydrogen peroxide. In addition to this, it is also considered the possibility that gene expression is carried out in the first hours so that in the last times of embryonic development, the enzymes are available and exert their antioxidant activity.

Gaaied et al. (2019) observed that the gene expression level of *cat* showed an opposite trend to the corresponding enzyme activity as at 48 h in this study with the exception of AL. Gaaied attributes this to the increased antioxidant activity as it causes a decrease in *cat* gene expression. While the increase in enzymes such as glutathione reductase in that study and cat at 24 h in the present study compared to high corresponding gene expression could be due to possible compensation in low levels of antioxidant defense.

3.4.3 Glutathione Peroxidase

The overexpression of the enzyme *gpx* occurs at 12 and 24 h for MX and MTF (Fig. 5a, b) respectively and at 48 and 72 h (Fig. 5c, d) for AL. This enzyme in addition to sharing substrate with CAT (hydrogen peroxide) can reduce hydroperoxidation of lipids and other hydroperoxides. On the other hand, Zheng et al. (2016) observed that the gene expression levels of the antioxidant enzymes studied did not reflect their corresponding activities and consider that the mismatch between antioxidant gene expression and enzyme activities may be

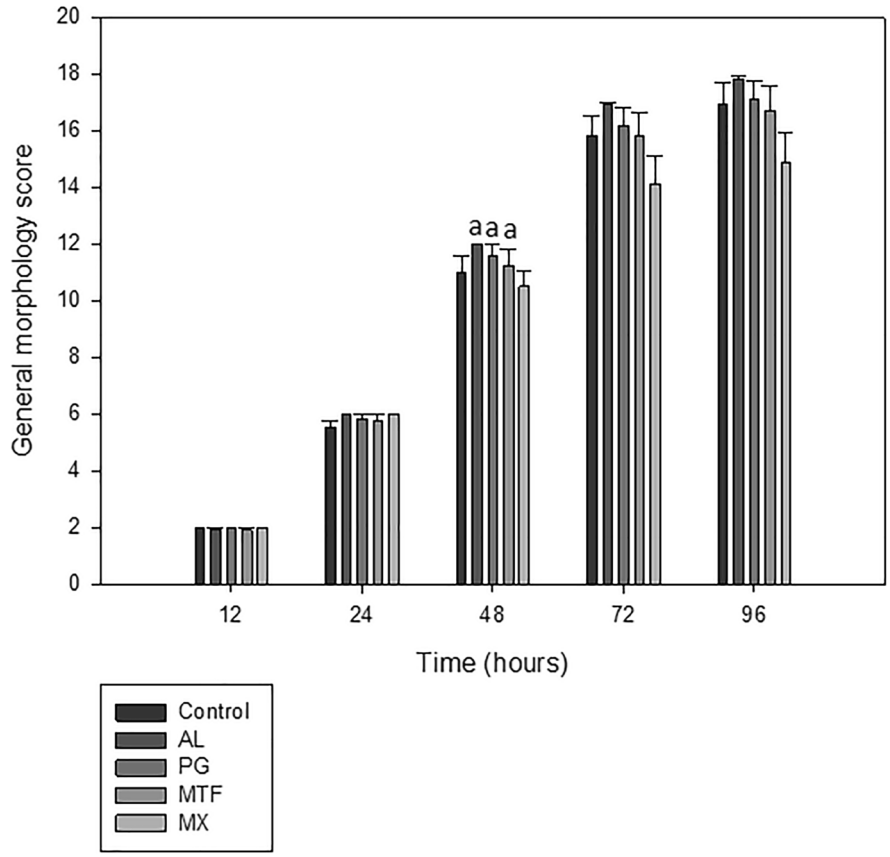

Fig. 6 Morphological development score in *Cyprinus carpio* embryos. Each value represents the mean of the organisms ± SE. *$p < 0.05$ significant difference with respect to the control group and a with respect to the mixture of the three pollutants at concentrations corresponding to the NOAEL. Two-way RM ANOVA ($F_{4,332} = 1.963$, $p = 0.107$ for groups factor, $F_{4,332} = 8528.770$, $p < 0.001$for time factor and $F_{16,332} = 2.598$, $p < 0.001$for interaction time and groups), post hoc Holm-Sidak

due to several reasons. One of them is that the change of a specific isoenzyme at the mRNA level is not always reflected at the enzyme level due to the presence of multiple copies of genes in fish; therefore, the enzyme activity detected could be equal to the total enzyme activity of the different isoenzymes, but the level of mRNA transcription would be limited to a subtype of the antioxidant gene encoding a single isoenzyme, gpx1 in this case. Another reason they consider is the likelihood of enzyme modulation at post-translational levels, as indicated by Pillet et al. (2019) in *Cyprinus carpio* exposed to metal mixtures, showing impact on gill antioxidant enzymes CAT and GPx only after 3 to 7 days of exposure, although relative gene expression of CAT and glutathione reductase was indeed triggered, suggesting responses at the transcriptional level.

3.5 Embryonic Development and Teratogenesis

In Fig. 6, it can be observed that during the first hours of exposure, the behavior of all groups was similar compared to the control. At 48 h, the only significant differences were observed for AL, MTF, and PG (increase of 9%, 5%, and 2% respectively) compared to the mixture group, while the mixture decreased by 4.5% with respect to the control group. At 72 and 96 h, the GMS of the embryos exposed to the mixture of the three contaminants presented a notorious decrease with respect to the control, which corresponded to 17 and 12%, respectively. All the embryos evaluated survived until hatching, although this was delayed in the mixture group. Delayed hatching of *Danio rerio* embryos (Aksakal & Ciltas, 2018) usually occurs due to inhibition of the enzyme coriolysin, general delay in embryonic development, or inability of embryos to break the chorion; similar reasons could account for the delays in embryo hatching in this study.

Wang et al. (2020) analyzed *Oryzias melastigma* embryos exposed to copper and found that the metal was mainly found in the chorion and only a small amount was able to penetrate the chorion and accumulate in the embryo, so the concentration of AL to

Table 6 Teratogenic effects on *Cyprinus carpio* exposed to aluminum, penicillin, metformin, and the mixture at NOAEL concentrations ($n = 24$)

	Teratogenic effects scored	Control	AL	PG	MTF	MX
1	Pericardial edema	0	1	1	0	0
2	Yolk sac edema	0	0	0	0	2
3	Eye edema	0	0	0	0	0
4	Malformation of the head	0	0	0	0	0
5	Malformation of sacculi/otoliths	0	0	0	0	0
6	Malformation of tail	0	2	1	2	3
7	Malformation of heart	0	1	0	0	0
8	Modified chorda structure	0	0	0	0	0
9	Scoliosis	0	0	0	0	0
10	Rachischisis	0	0	0	0	0
11	Yolk deformation	0	4	2	2	6
	% Lethality	0	8.33	16.66	4.16	4.16

which *Cyprinus carpio* embryos were exposed might not be sufficient to generate decrease in GMS.

In the study by Carpiello et al. (2021), it is presumed that lead and AL exposure may be associated with cardiovascular malformations. It is known that AL cannot initiate peroxidation, but it does have the ability to attack cellular components including the lipid membrane, which could be causing the pericardial edema observed in *Cyprinus carpio* embryos.

In another work with metals, Liu et al. (2021) exposed *Oryzias melastigma* to nickel, presenting affections in cardiac development, decrease in hatching rate, increase in malformations, decrease in body length of newly hatched larvae, and finally, greater sensitivity in embryos than in larvae. They argue that exposure to metals can affect the process of water accumulation in fertilized egg tissues and that metal ions enter the egg to change the structure of the chorion and affect membrane permeability. The concentrations used in that study (1.18–65.8 mg L^{-1}) at which the main effects occurred are higher than those of the present work (0.074 mg L^{-1}), which may be one of the reasons why there was no significant difference between the exposed groups of the same.

Table 6 shows that the malformation generated was mainly in the yolk sac and the most affected group was the one exposed to the mixture of contaminants. This could be due to the fact that NOAEL concentrations are too low to generate strong teratogenic effects. Quiroga-Santos et al. (2021) reported that the concentration at which aluminum produces teratogenesis in *Cyprinus carpio* embryos is 24.45 mg L^{-1}. In *Fathead minnows* (Parrott et al., 2021) exposed to different concentrations of MTF, no significant changes in growth, maturation, reproduction, and hatching were observed, while in Japanese medaka (Lee et al., 2019) there was also no effect on fecundity, hatching rate, and larval abnormalities, in a bigenerational study. Nevertheless, Luja-Mondragón et al. (2019) studied embryotoxicity and teratogenesis in *Cyprinus carpio* exposed to hospital effluent in which PG and MTF, among other contaminants, were detected. They reported malformations such as scoliosis, alterations of the notochord, oral hyperplasia, and pericardial edema, as well as a decrease in GMS due to alterations in the development of embryos exposed to different concentrations of hospital effluent when compared to the control group.

In summary, it is important to note that the results of the interaction study show that the tertiary mixture at high concentrations (LC_{50}) produces an antagonistic effect, while at concentrations equivalent to the NOAEL they generate a synergistic effect. However, these determinations were made considering the death of the organisms as the end point. Thus, these divergences should be studied in greater detail, given that at the environmental level, organisms are regularly exposed to sub-lethal but continuous concentrations of the xenobiotic and understanding the toxic response under these circumstances is fundamental.

On the other hand, the subacute study shows that the antioxidant enzyme activity of SOD, CAT, and GPX does not correlate with the increases or decreases observed in the gene expression of these same enzymes at all times. Thus, for example, while gene expression in the group exposed to the mixture

increases at 12 h, the corresponding enzyme activity increases only to a lesser extent in the enzyme activity of CAT. These results show the need to continue studying the correlation between the activity and expression of these enzymes, which are also determinant in the morphological changes of the embryos.

Finally, in this work, all experiments were completed until the end of the embryonic stage, which lasts 96 h; however, other chronic studies of up to months of exposure have shown that at low concentrations, MTF is capable of generating morphological and behavioral damage that makes the organisms susceptible to predators, so it is important to consider continuing the study of interactions in larval stages using other types of biomarkers.

4 Conclusion

In conclusion, the study demonstrates that the interactions present in the contaminant mixtures influenced the toxic response of exposed *Cyprinus carpio* embryos compared to the individual substances. In sublethal studies, the three contaminants and the mixture generated changes in antioxidant enzyme activity, as well as in the expression of the corresponding genes. Alterations in embryonic development were observed mainly in the group exposed to the mixture at late exposure times, while the main malformation observed was in the yolk sac. These results suggest that exposure to mixtures of contaminants could be a potentially dangerous factor for organisms that are constantly exposed, so the mechanisms and effects of the interactions should be carefully studied.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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