Science of the Total Environment 806 (2022) 150541

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Teratogenic effects induced by paracetamol, ciprofloxacin, and their mixture on *Danio rerio* embryos: Oxidative stress implications

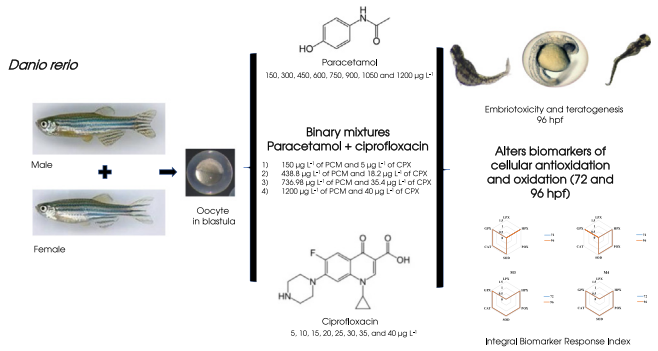
Jonathan Ricardo Rosas-Ramírez [a,1], José Manuel Orozco-Hernández [a,1], Gustavo Axel Elizalde-Velázquez [a], Demetrio Raldúa [b], Hariz Islas-Flores [a], Leobardo Manuel Gómez-Oliván [a,*]

[a] Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón Intersección Paseo Tollocan s/n, Col. Residencial Colón, 50120 Toluca, Estado de México, Mexico
[b] Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain

HIGHLIGHTS

- Developmental abnormalities and oxidative stress were evaluated in zebrafish.
- Paracetamol, ciprofloxacin and their mixtures can alter fish embryogenesis.
- Paracetamol, ciprofloxacin and their mixture induced oxidative damage in fish embryos.
- Concentrations of evaluated drugs and their mixtures pose a threat to aquatic species.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 July 2021
Received in revised form 26 August 2021
Accepted 19 September 2021
Available online 25 September 2021

Editor: Henner Hollert

Keywords:
Embryotoxicity
Teratogenesis
Antioxidant defense
Cellular oxidation
Zebrafish embryos

ABSTRACT

Even though the toxic effects of paracetamol (PCM) and ciprofloxacin (CPX) have been deeply studied in the last decades, the impact of the PCM-CPX mixture may induce in aquatic organisms is poorly known. Thus, the objective of this work was to investigate the teratogenic effects and oxidative stress that PCM, CPX, and their mixture induce in *Danio rerio* embryos. Moreover, we aimed to determine whether the PCM-CPX mixture induces more severe effects on the embryos than the individual drugs. For this purpose, zebrafish embryos (4 hpf) were exposed to environmentally relevant concentrations of PCM, CPX, and their mixture until 96 hpf. In addition, at 72 hpf and 96 hpf, we also evaluated the oxidative stress biomarkers (superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation, and hydroperoxides and carbonyl content) in the embryos. Our results demonstrated that PCM, CPX, and their mixture reduced the survival rate of embryos by up to 75%. In addition, both drugs, induced morphological alterations in the embryos, causing their death. The most observed malformations were: scoliosis, craniofacial malformations, hypopigmentation, growth retardation, pericardial edema. Concerning oxidative stress, our integrated biomarkers response (IBR) analysis demonstrated that PCM, CPX, and their mixture induce oxidative damage on the embryos. In conclusion, PCM, CPX, and their mixture can alter zebrafish embryonic development via an oxidative stress response.

1. Introduction

The consumption of analgesics and antipyretics, as well as non-steroidal anti-inflammatory drugs (NSAIDs), is increasing because

* Corresponding author at: Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico.
E-mail address: lmgomezo@uaemex.mx (L.M. Gómez-Oliván).
[1] JRRR and JMOH. contributed equally to this work.

https://doi.org/10.1016/j.scitotenv.2021.150541
0048-9697/

these drugs are sold without prescription. Among these, the best known and most consumed NSAID worldwide is paracetamol (PCM). This drug is the most commonly used for severe pain and osteoarthritis, for gastric and duodenal ulcers, gastritis, and hiatal hernia, as an alternative treatment in case of salicylates allergies and patients with hemophilia or receiving anticoagulants (Lau et al., 2016). According to Acetaminophen Global Market Report, 2018, worldwide sales of PCM in 2019 summed a total of 740 million USD. Nonetheless, it is forecasted that by 2023 its sales will rise to 780 million USD.

As a result of its high usage and partial degradation (14–75%), huge amounts of PCM are spilled out every day into the aquatic environment (Gutiérrez-Noya et al., 2021). Recent studies have reported the occurrence of this drug in worldwide water bodies. Table 1 shows the worldwide occurrence of PCM in aquatic environments. As can be seen from this table, the highest concentration of PCM found in WWTP effluent and surface water were 3, 000 μg L^{-1} and 30.4 μg L^{-1}, respectively.

Paracetamol is bio-transformed by cytochrome P450 (CYP450) in the liver via CYP2E1, CYP1A2, and CYP3A4 subfamilies causing the formation of a very unstable metabolite with a high affinity for thiol groups *N*-acetyl-p-benzoquinone-imine (NAPQI). *N*-acetyl-p-benzoquinone-imine can cause structural modifications in cellular proteins and therefore damage to deoxyribonucleic acid (DNA), ribonucleic acid (RNA), as well as oxidative stress (Moriarty and Carroll, 2016; Xu et al., 2008). Choi et al., 2018 for instance, showed that PCM (10 & 30 μg L^{-1}) induced histological damage to the gill, structural damage to the kidney, and decrease glycogen levels in the liver of *Oncorhynchus mykiss*, after 4 weeks of exposure. Moreover, Liu et al., 2019 showed that PCM altered the expression of Nrf1, a transcription factor related to the anti-oxidant system, in *Daphnia magna*. Similarly, Gutiérrez-Noya et al., 2021 pointed out that oxidative stress PCM-induced teratogenesis on embryos of *Cyprinus carpio*. Thus, adverse effects induced by PCM are closely related to the increased production of ROS during its biotransformation process.

Antibiotics are also an important group of drugs whose worldwide consumption has been increasing (Israel et al., 2021). This group includes the fluoroquinolones, used in human and veterinary medicine for the treatment of multiple bacterial infections (Ávila et al., 2019). Ciprofloxacin is a broad-spectrum fluoroquinolone commonly utilized for the treatment of diseases such as pharyngitis, sinusitis, and earaches, as well as airway diseases such as pneumonia and bronchitis (Yi et al., 2017).

Table 1
Worldwide occurrence of paracetamol and ciprofloxacin.

Country	Concentration (μg L^{-1})	Matrix	Reference
Paracetamol			
Brazil	30.4	Surface water	Campanha et al., 2015
Canada	57.5	WWTP effluent	Ba et al., 2014
Canada	90.2	WWTP effluent	Ba et al., 2014
China	150	WWTP effluent	Wu et al., 2012
Colombia	39.25	WWTP effluent	Botero-Coy et al., 2018
Costa Rica	13.21	Surface water	Spongberg et al., 2011
EE. UU.	1000	WWTP effluent	Wilcox et al., 2009
Italy	246	WWTP effluent	Verlicchi et al., 2012
Japan	1.7	WWTP effluent	Okuda et al., 2008
Mexico	3000	WWTP effluent	SanJuan-Reyes et al., 2015
Spain	246	WWTP effluent	Gómez et al., 2007
UK	211.3	WWTP effluent	Kasprzyk-Hordern et al., 2009
Ciprofloxacin			
Argentina	7.7	Surface water	Teglia et al., 2019
China	5.9	Surface water	Wei et al., 2012
EE.UU.	1.3	WWTP effluent	He et al., 2015
India	5528	Surface water	Gothwal and Shashidhar, 2017
Pakistan	341	WWTP effluent	Riaz et al., 2017
Portugal	38.6	Hospital effluent	Santos et al., 2013
South Korea	8.7	WWTP effluent	Sim et al., 2011
Vietnam	0.25	Surface water	Andrieu et al., 2015

Due to the abuse in its consumption and its poor biodegradability, CPX has been increasingly detected in wastewater (Li et al., 2011). The three major sources of CPX entering wastewater are industry, hospital, and household. Table 1 shows the worldwide occurrence of CPX in aquatic environments. Until now, the highest concentrations of this drug found in wastewater treatment plants (WWTPs effluents), hospital effluents, and surface water were 341 μg L^{-1}, 38.6 μg L^{-1}, and 5528 μg L^{-1}, respectively.

As same as PCM, it has also been demonstrated that CPX can generate oxidative stress and cell death by stimulating the production of reactive oxygen species (ROS), affecting lipids, DNA, and other cellular components. (Becerra and Albesa, 2002). Zivna et al., 2016 for instance, reported that the exposure of *C. carpio* embryos to CPX (1000 μg L^{-1} and 3000 μg L^{-1}) led to the death of 32% of the embryos. Moreover, Gomes et al., 2017 reported that CPX induced oxidative stress in *Lemna minor L* by impairing normal electron flow in the respiratory electron transport chain.

Since the mechanism of toxicity by both drugs is oxidative stress, and the latter has been associated with teratogenic effects in several aquatic species. The purpose of this study was to assess the teratogenic effects and oxidative stress that PCM, CPX, and their mixtures have in *Danio rerio* embryos. Furthermore, we also carried out an integrated biomarkers (IBR) analysis to better understand the influence that oxidative stress biomarkers (lipid peroxidation, hydroperoxides content, protein carbonyl content, superoxide dismutase, catalase, and glutathione peroxidase) has on the embryos of this freshwater fish.

2. Material and methods

2.1. Reagents

Paracetamol (*N*-acetyl-4-aminophenol, CAS number: 103–90-2, linear formula CH3CONHC6H4OH, analytical purity of 99%) and ciprofloxacin (1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid, CAS number 85721-33-1 linear formula C17H18FN3O3, analytical purity > 98%) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents referred to in the methodology were purchased from the same company, unless reference is made to another company.

2.2. Acclimatization and maintenance of broodstock fish

Wild-type zebrafish (0.9 ± 0.5 g in weight and 3.5 ± 0.5 cm in length) were purchased from a commercial supplier (Aquanimals MX) following the requirements requested in the OECD guidelines. None fish showed visible signs of disease and stress, nor apparent malformations. For their acclimatization, fish were kept in 120 L tanks, at a ratio of one fish per two liters of water (12 h of light by 12 h of darkness). Along the experiment water from each tank was monitored and maintained under the following conditions: T: 26 ± 2 °C, pH: 7.2–7.6, and oxygen saturation: 85%. Moreover, water was changed daily, maintaining the above-mentioned characteristics. Breeding fish were fed with commercial flakes (TetraMin Tropical Flakes®) [48% protein, 8% fat, and 2% fiber], twice a day (Test Guideline No. 203 Fish) and supplemented with *Artemia sp* once a day, 15 days before egg production (Elizalde-Velázquez et al., 2021a, 2021b).

2.3. Egg production

D. rerio eggs were obtained by natural fertilization (Elizalde-Velázquez et al., 2021a, 2021b). Briefly, eight males and four females (with visible gravidity) of 4–4.5 cm in length were placed in breeding tanks. Each tank contained 12 L of tap water previously aerated, dechlorinated, and reconstituted with commercial salts Instant Ocean® (9 mg L^{-1}). In addition, all tanks contained a breeding trap (4 × 4 mm mesh). Early next day, eggs were collected rinsed with ultrapure water, and bleached according to the methods of Westerfield, 2007

and Varga, 2011. Fertilized oocytes were classified under a stereoscopic microscope (Zeiss Stemi 305 at 10× of magnification) according to the protocol of Kimmel et al., 1995. Middle blastula stage oocytes (equivalent to 2.5 hpf) were selected and used for subsequent experiments.

2.4. Embryolethality test

Paracetamol (150, 300, 450, 600, 750, 900, 1050, and 1200 μg L^{-1}) and CPX 5, 10, 15, 20, 25, 30, 35, and 40 μg L^{-1}) concentrations were selected considering their occurrence in surface water, WWTP effluents, and hospital effluents (Table 1). In addition, four binary mixtures (M) were tested:

Mixture 1 (M1): corresponding to the lowest concentrations of both drugs (150 μg L^{-1} of PCM and 5 μg L^{-1} of CPX).

Mixture 2 (M2): corresponding to the highest concentrations of both drugs (1200 μg L^{-1} of PCM and 40 μg L^{-1} of CPX).

Mixture 3 (M3): corresponding to the effective concentration 50 of malformations (EC_{50}) obtained for each drug in isolation (438.8 μg L^{-1} of PCM and 18.2 μg L^{-1} of CPX).

Mixture 4 (M4): corresponding to the lethal concentration 50 for embryos (LC_{50}) obtained for each drug in isolation (736.98 μg L^{-1} of PCM and 35.4 μg L^{-1} of CPX).

All concentrations were prepared from the standards mentioned in Section 2.1. Moreover, these were prepared from the stock solution in dark volumetric flasks to avoid photodegradation.

The lethality tests were based on FET (Fish Embryo Acute Toxicity) analysis. Briefly, 144 eggs at the mid-blastula stage (2.5 hpf) were selected and randomly distributed into 24-well plates (1 embryo per well). Plates were incubated in bioclimatic chambers at 28 ± 1 °C, with light-dark cycles of 8:14 h. All systems were static with daily medium renewal. The temperature and humidity of the incubation chamber were monitored until the end of the test. The number of living, dead (coagulated or absence of heartbeat), and malformed embryos was quantified at 12, 24, 48, 72, and 96 hpf. The data obtained were used to calculate the embryo LC_{50} and EC_{50} using a maximum likelihood linear regression test, and their respective 95% confidence intervals were calculated ($p < 0.05$). This analysis was performed with US-EPA software ver. 1.5, according to the Trimmed Spearman-Karber method (Hamilton et al., 1977). The LC_{50}/EC_{50} ratio was used to determine the teratogenic index (TI). If the value of TI is >1, substance should be considered teratogenic, but if this is <1, substance should be categorized as embryo-lethal (Weigt et al., 2011).

2.5. Evaluation of morphology scoring and embryonic developmental alterations

Systems cited in Section 2.4 were used for this purpose. The main malformations induced by PCM, CPX, and their mixture were recorded based on the scoring scale established by Kimmel et al. (1995) and Hermsen et al. (2011). Briefly, an experimental embryo is compared to the reference embryo and receives points for each developmental hallmark dependent on its stage of development. All deviations, for instance, incomplete detachment of the tail, will result in a lower point score which corresponds to a certain extent of developmental retardation. The tail formation, somite formation, eye development, embryo movement, heartbeat, blood circulation, head and body pigmentation, tail pigmentation, presence of mouth protrusion, and hatching were all the developmental hallmarks evaluated.

Malformations and other teratogenic effects were separately recorded as present or absent. Along the experiment, we evaluated the following alterations: delay in the hatching process, hypopigmentation, hemorrhaging in the head, hemorrhaging in the tail, hemorrhaging in the yolk, miscellaneous; severe malformations, modified chord structure, malformation of the head, malformation of the heart, malformation of the tail, pericardial edema, scoliosis, yolk deformation, and yolk edema. The total malformations were expressed as the percentage of embryos with at least one malformation in comparison to the control. The alterations to embryonic development presented by exposure to the drugs and their mixtures were recorded at each exposure time, and with these data, the concentration-response curves for PCM, CPX, and their binary mixtures were constructed.

2.6. Evaluation of oxidative stress in zebrafish embryos

40 systems, each with one gram of zebrafish embryos (corresponding to 1600 embryos), were distributed to aquaria of 4 L. Each system was spiked with the environmentally relevant concentrations of PCM, CPX, and binary mixtures. During the exposure period, temperature (27 ± 1 °C) and light/dark cycles (14:10 h) were kept constant in all systems. At 72 hpf and 96 hpf, 800 embryos, respectively, were randomly selected and homogenized using 1 mL of phosphate buffer solution (pH 7.4) at 12,500 rpm. The exposure times were selected considering that at this time, the larvae had already hatched and their antioxidant enzyme system was already functioning. For the evaluation of oxidative stress the following biomarkers of cellular oxidation were determined: lipid peroxidation level (LPX) (Buege and Aust, 1978), hydroperoxide content (HPC) (Jiang et al., 1992), and protein carbonylation content (PCC) (Levine et al., 1994). In addition to the antioxidant enzymes superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (CAT) (Radi et al., 1991) and glutathione peroxidase (GPX) (Günzler and Flohé, 1985, as modified by Stephenson, 2000). All results were harmonized considering the protein contents determined by the method of Bradford (1976). The experiments were replicated in triplicate.

2.7. Integrated biomarker response index (IBR)

The integrated biomarker response index was employed to analyze the influence of oxidative stress biomarkers at each tested environmentally relevant concentration of PCM, CPX, and their respective binary mixtures (Sanchez et al., 2013). For IBR determination, the biomarkers of each tested drug and mixtures (Xi) were compared with the biomarkers of the control group (Xo). The ratio of Xi to Xo was log-transformed (Yi) to reduce variance. Subsequently, Yi values were standardized by applying the formula $Zi = (Yi-\mu)/s$ and using the mean (μ) and standard deviation (s) of Yi. Then, the biomarker deviation index (A) was calculated by the difference between Z_i and Z_0. Finally, the values of A were depicted in star plots representing the integrated responses of biomarkers. In addition, the absolute value of A of each biomarker was summed to obtain IBR.

2.8. Traceability and test validity criteria

To ensure traceability of the results, batches of eggs were only used whether the fertilization rate was greater than 90% and whether the control group did not show more than 2% of alterations in their development in all exposure times.

2.9. Ethical approval

This research protocol was evaluated and approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM) to ensure that the experiment was conducted in accordance with the institutional standards and guidelines for animal care (approval ID: UAEM.CEI.DCTF.REC.078.2020). The provisions referred to in the official Mexican standard on breeding, care,

and use of laboratory animals (NOM-062-ZOO- 1999) were considered.

2.10. Data analysis

The LC_{50} and EC_{50} were calculated by probit analysis with maximum likelihood linear regression (EPA Analysis Program v1.5). The teratogenic index (TI) was obtained applying the following eq. TI = (LC_{50}/EC_{50}).

A Student's *t*-test (one-tailed) was performed to identify statistically significant differences between treatment and controls groups ($p < 0.05$, IBM SPSS Statistics 22 software). Statistics were done based on affected embryos (embryos with lethal or teratogenic effects). At first, the scores for each concentration of PCM, CPX, and binary mixtures had to be registered, then for the control were gathered and finally, a comparison was completed using the *t*-test.

Oxidative stress biomarkers data was examined using a two-way analysis of variance (ANOVA), considering time as factor A and concentration as factor B. Variations between the means were examined with the Student-Newman-Keuls method, using SigmaPlot 12.3 software. All oxidative stress biomarkers passed the normality test.

3. Results

3.1. Embryolethality test

Paracetamol, ciprofloxacin, and the binary mixtures showed concentration-dependent behavior in the number of dead and malformed embryos. LC_{50} of PCM was 736.9 μg L^{-1} with 95% confidence intervals (647.2–853.9 μg L^{-1}). Meanwhile, the LC_{50} value of CPX was 35.4 μg L^{-1} (29.2–47.9 μg L^{-1}). The results of the mixture of the two drugs indicated that the M4 mixture had the highest embryolethal index by causing the death of 75% of the embryos. EC_{50} of PCM and CPX were 438.8 μg L^{-1} and 18.2 μg L^{-1} with 95% confidence intervals of 302.6–569.5 μg L^{-1} and 16.1–20.4 μg L^{-1}, respectively. The teratogenic indexes were 1.6 for PCM and 1.9 for CPX, which according to the criteria of Weigt et al., 2011, both drugs should be classified as teratogenic. M4 mixture had the highest number of malformed embryos (Table 2).

Fig. 1 shows the proportions of live, dead, and malformed embryos for each concentration of PCM, CPX, and their binary mixtures. In all cases, the number of live embryos decreased with increasing drug concentration. Ciprofloxacin had lower death rates. However, the PCM-CPX mixtures showed the highest rates of deaths and malformations compared to the individual drugs. The M4 mixture showed lower numbers of live embryos.

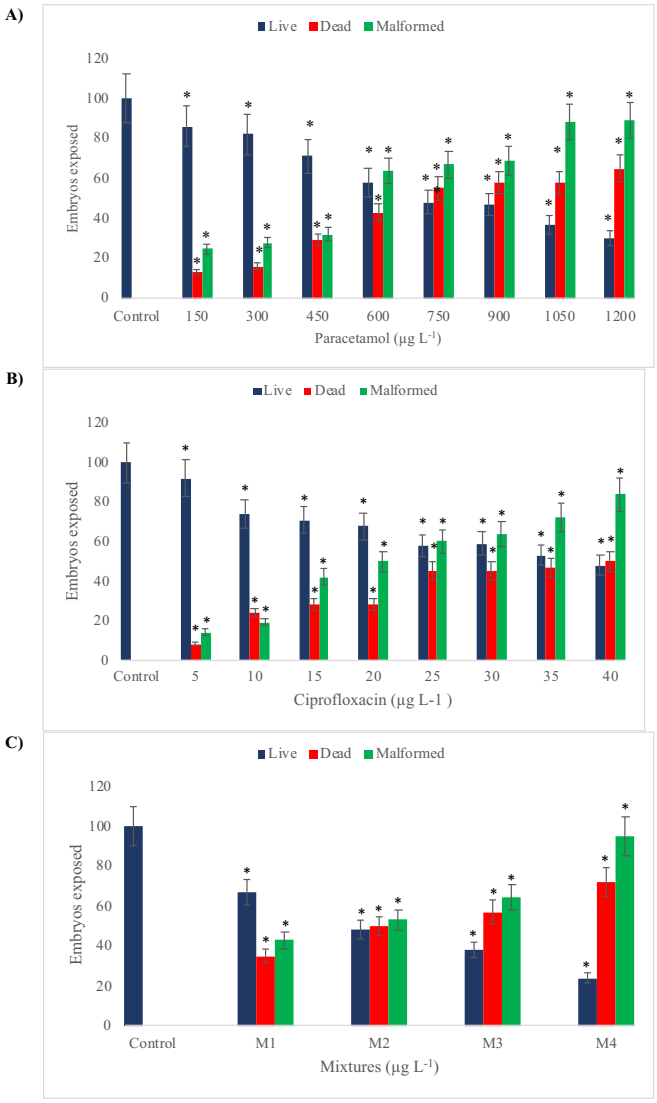


Fig. 1. Effects on survival and malformations embryos of *D. rerio* exposed to **A)** PCM, **B)** CPX and **C)** Binary mixtures of PCM-CPX. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; two-way ANOVA($p < 0.05$).

Table 2
Mortality and malformations rates by environmentally relevant concentrations of PCM, CPX and their binary mixtures on *D. rerio* embryos.

	Paracetamol				Ciprofloxacin				Mixtures			
	Live	Dead	Malformed		Live	Dead	Malformed	PCM-CPX	Live	Dead	Malformed
μg L^{-1}	%	%	%	μg L^{-1}	%	%	%		%	%	%
Control	100.0	0.0	0.0	Control	100	0.0	0.0	Control	100	0.0	0.0
150	86.1	13.9	25.0	5	91.7	8.3	15.3	M1	65.3	34.7	43.1
300	83.3	16.7	29.2	10	75.0	25.0	20.8	M2	48.6	51.4	54.2
450	70.8	29.2	33.3	15	70.8	29.2	43.1	M3	38.9	61.1	68.1
600	56.9	43.1	63.9	20	69.4	30.6	52.8	M4	25.0	75.0	93.1
750	45.8	54.2	68.1	25	55.6	44.4	58.3				
900	43.1	56.9	75.0	30	55.6	44.4	65.3				
1050	38.9	61.1	87.5	35	51.4	48.6	76.4				
1200	31.9	68.1	88.9	40	45.8	54.2	83.3				
LC_{50} = 736.9				**LC_{50}** = 35.4							
CI = [647.2–853.9]				CI = [29.2–47.9]							
EC_{50} = 438.8				**EC_{50}** = 18.2							
CI = [302.6–569.5]				CI = [16.1–20.4]							
TI = 1.6				**TI** = 1.9							

3.2. Alterations in the development of D. rerio embryos by PCM, CPX and their binary mixture

Fig. 2 shows the number of embryos that presented malformations generated by exposure to different concentrations of PCM, CPX, and their mixture. Paracetamol induced nine different malformations. Delayed hatching and body hypopigmentation were the most frequent malformations in embryos exposed to each of the PCM concentrations evaluated. Exposure to different concentrations of CPX generated 16 different malformations. Craniofacial and yolk sac malformations were the most frequent in the exposed embryos. The PCM-CPX mixtures showed the highest number of different malformations. Delayed hatching and pericardial edema occurred most frequently in the embryos exposed to the binary mixture. The M4 mixture generated the highest number of embryos with malformations. The M1 mixture presented the lowest number of malformed embryos.

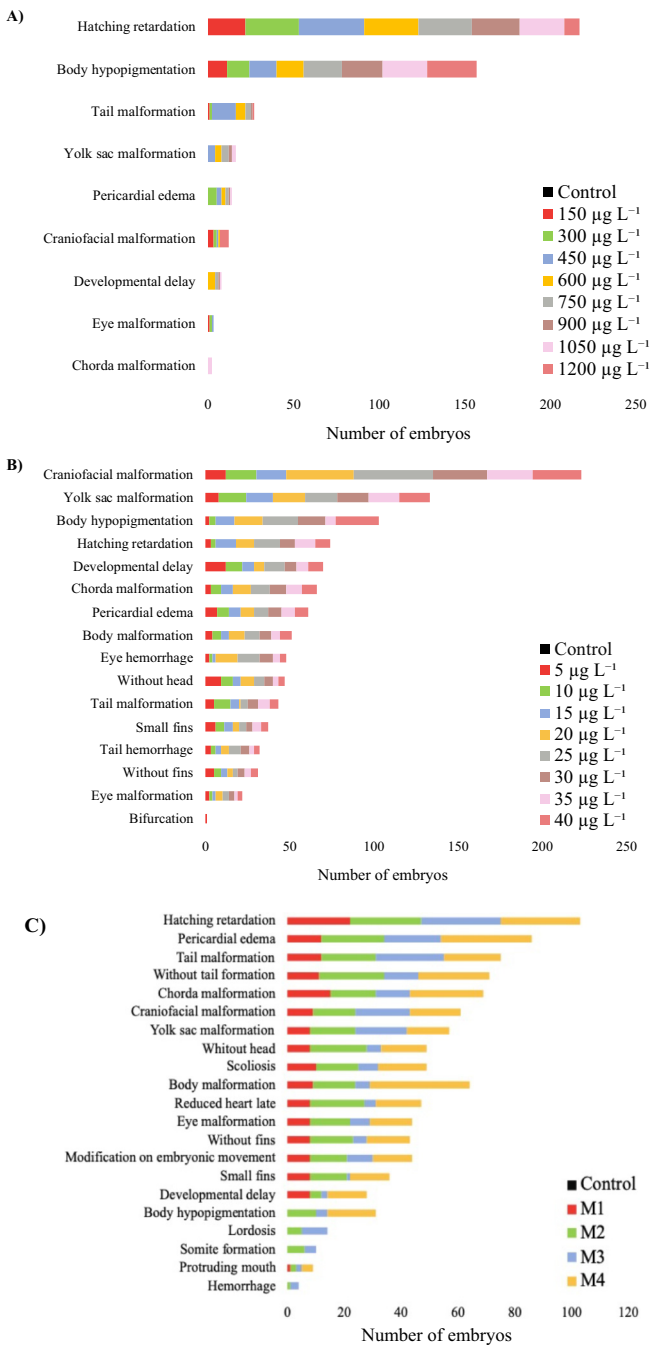


Fig. 2. Main malformations induced by **A)** PCM, **B)** CPX and **C)** Mixtures PCM-CPX in *D. rerio* embryos.

3.3. Embryonic development score at different times and concentrations of PCM, CPX and their binary mixtures

Fig. 3 shows the effect-concentration curves of PCM, CPX and the four binary mixtures PCM-CPX tested about the normal development of zebrafish embryos according to the score of Hermsen et al., 2011. In all cases, a decrease in the score is shown with respect to the control organisms, due to the different morphological alterations presented. Concentrations from 300 to 1200 µg L^{-1} of PCM showed statistically significant differences in relation to the control group ($p < 0.05$). In

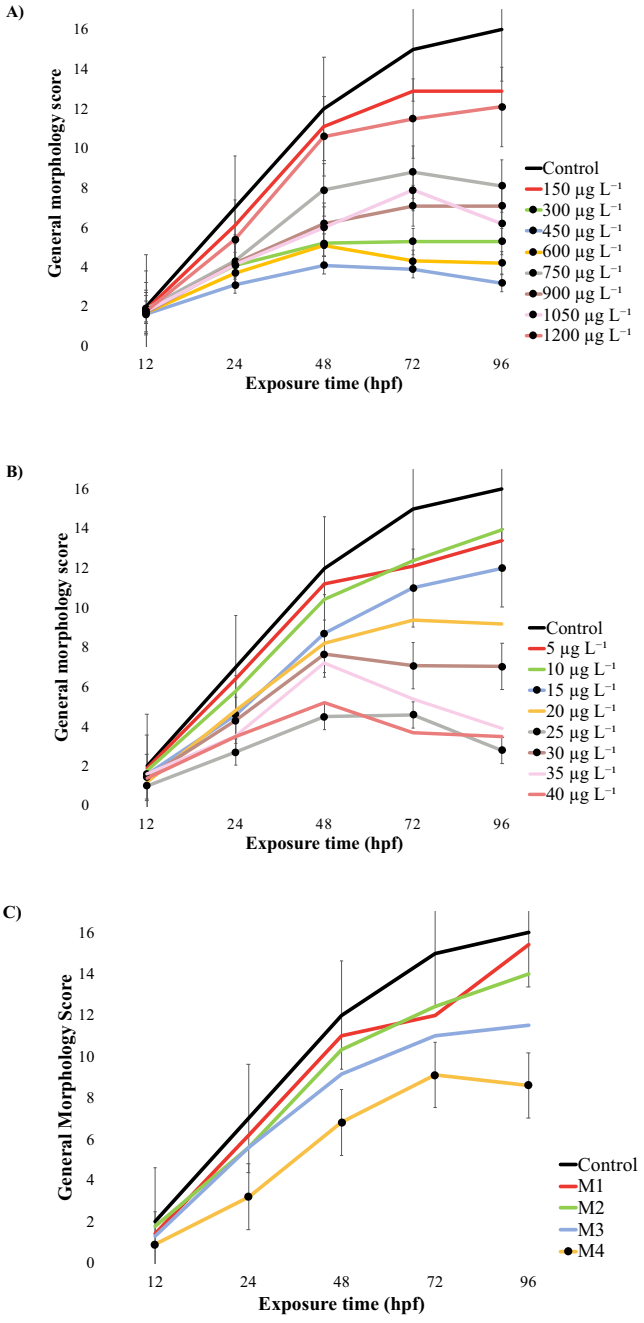


Fig. 3. Concentration-response curves of PCM, CPX and their binary mixtures in *D. rerio* embryos. All concentrations of PCM, CPX and their mixtures were significantly different from the control group ($p < 0.05$). The differences are not indicated so that the figure can be easily appreciated (Student's *t*-test).

the case of CPX, the concentration that showed the greatest influence on embryo development was 25 μg L^{-1}. Meanwhile, the concentrations from 15 μg L^{-1} showed significant differences with respect to the control group ($p < 0.05$). In the case of binary mixtures of PCM-CPX, M4 was the only mixture that showed significant differences with respect to the control group.

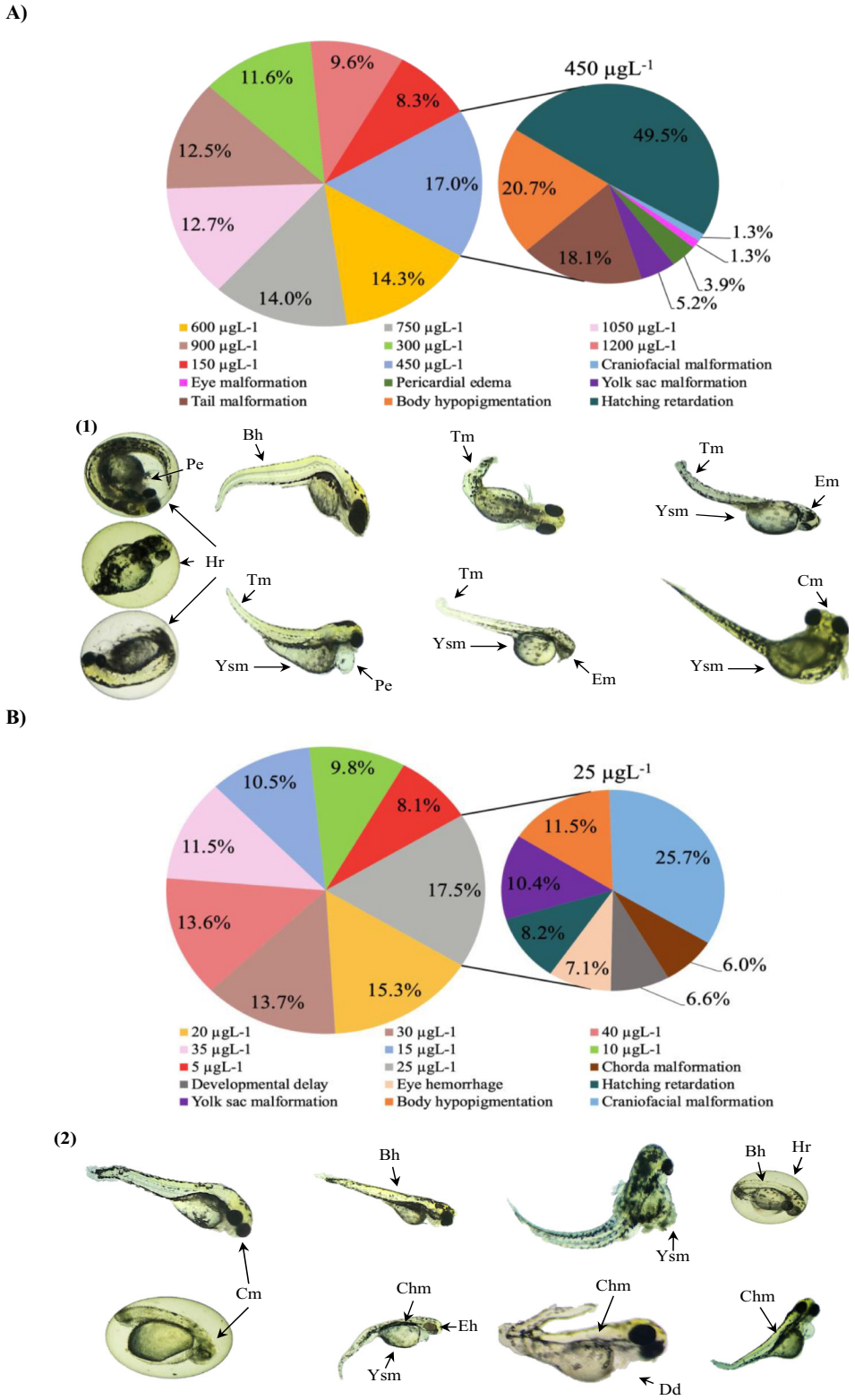


Fig. 4. Proportion of the number of malformations by concentration and main malformations in *D. rerio* embryos at the critical concentration of **A)** PCM, **B)** CPX and **C)** Mixtures PCM-CPX. *Malformations of less than 5.0% in **B)** and **C)** are not presented.
Representation of the main teratogenic effects at 96 hpf at the critical concentration of **(1)** paracetamol, **(2)** ciprofloxacin and **(3)** M4 mixture. **(4)** Control organisms at 96 hpf.
Chm = Chorda malformation; **Dd** = Developmental delay; **Eh** = Eye hemorrhage; **Hr** = Hatching retardation; **Bh** = Body hypopigmentation; **Tm** = Tail malformation; **Ysm** = Yolk sac malformation; **Pe** = Pericardial edema; **Em** = Eye malformation; **Cm** = Craniofacial malformation; **Bm** = Body malformation; **Wt** = Without tail formation; **Tm** = Tail malformation.

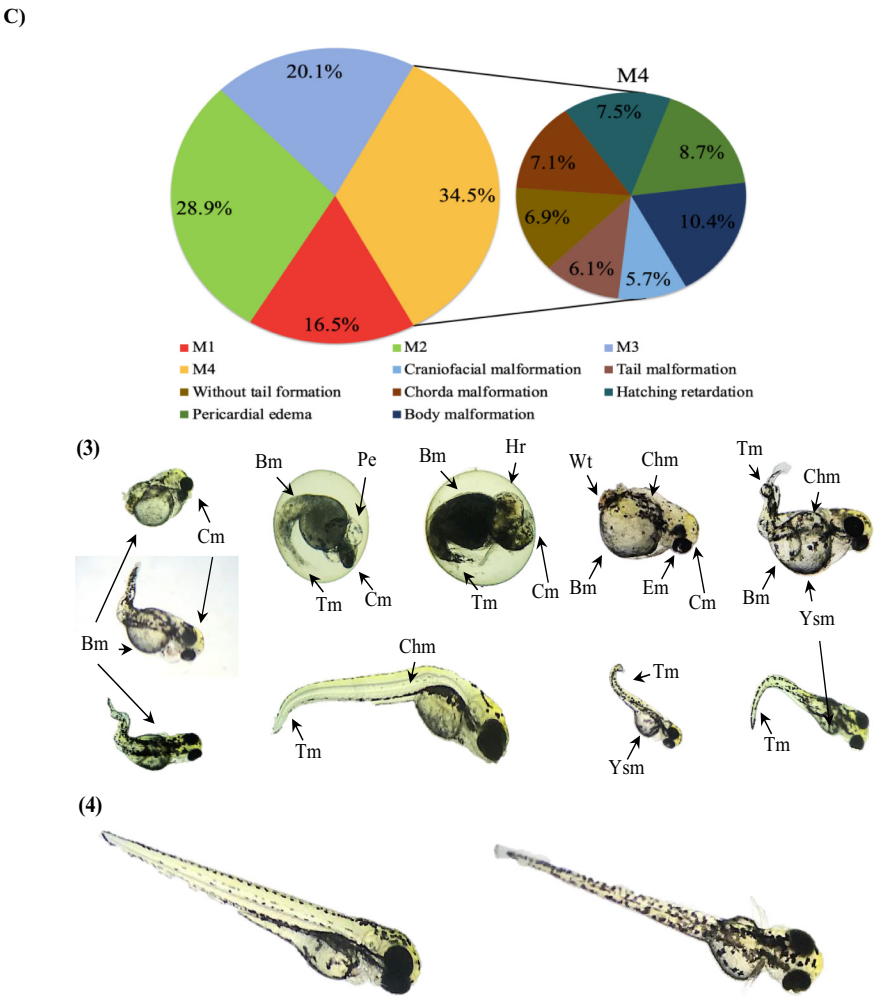


Fig. 4 (*continued*).

3.4. Teratogenic effects induced by PCM, CPX and their binary mixtures

Fig. 4 shows the total proportion malformations that occurred at each concentration of PCM, CPX and their mixtures. In the PCM case, the highest proportion of malformations occurred at 450 μg L^{-1}, and delayed hatching was the most frequent malformation associated with this concentration. The highest proportion of malformations by CPX occurred at the 25 μg L^{-1} and craniofacial malformation was the most frequent malformation associated with this concentration. Regarding the mixtures, M4 had the highest proportion of malformations and body malformation was the most frequent malformation in this mixture.

3.5. Biomarkers of cellular oxidation

Fig. 5 shows the results of cellular oxidation. Lipid peroxidation results are shown in Fig. 5A. Statistically significant increases were found with respect to the control group ($p < 0.05$) in a concentration-dependent manner. For PCM, increases were observed at 72 and 96 hpf respectively with respect to the control at concentrations of 150 μg L^{-1} (1.09 and 0.84 times), 300 μg L^{-1} (2.17 and 1.65 times), 450 μg L^{-1} (3.24 and 2.45 times), 600 μg L^{-1} (4.32 and 3.26 times), 750 μg L^{-1} (5.39 and 4.07 times), 900 μg L^{-1} (6.47 and 4.87 times), 1050 μg L^{-1} (7.54 and 5.68 times) and 1200 μg L^{-1} (8.62 and 6.49 times). For CPX, the same trend was observed, the increases in times with respect to the control at 72 and 96 hpf were 5 μg L^{-1} (0.108 and 0.334), 10 μg L^{-1} (0.213 and 0.616), 15 μg L^{-1} (0.319 and 0.880), 20 μg L^{-1} (0.425 and 1.44), 25 μg L^{-1} (0.549 and 1.40), 30 μg L^{-1} (0.636 and 1.65), 35 μg L^{-1} (0.745 and 1.97) and 40 μg L^{-1} (0.849 and 2.21), respectively. Finally, for the binary mixtures (PCM-CPX), there was a significant difference with respect to the control group at 72 and 96 hpf: M2 (3.0 and 3.74), M3 (5.05 and 6.31), M4 (8.11 and 10.14). Protein carbonyl content results are shown in Fig. 5B. Significant increases with respect to the control group ($p < 0.05$) were found at 72 and 96 hpf of 1.02 and 1.23; 1.93 and 2.35; 2.83 and 3.46; 3.73 and 4.57; 4.62 and 5.68; 5.52 and 6.78; 6.42 and 7.89; and 7.31 and 8.99 times, respectively for 150, 300, 450, 600, 750, 900, 1050 and 1200 μg L^{-1} of PCM. For CPX of 1.02 and 1.24; 1.93 and 2.34; 2.83 and 3.45; 3.73 and 4.56; 4.62 and 5.64; 5.49 and 6.74; 6.38 and 7.84; and 7.26 and 8.92 times respectively at 5, 10, 15, 20, 25, 30, 35 and 40 μg L^{-1}. Finally, for the binary mixtures, there was a difference for M2 (3.16 and 2.77 times), M3 (5.24 and 4.58 times), and M4 (8.34 and 7.28 times). Hydroperoxides content results are shown in Fig. 5C. For PCM, increases were observed at 72 and 96 hpf respectively with respect to the control at concentrations of 150 μg L^{-1} (1.63 and 1.16 times), 300 μg L^{-1} (3.16 and 2.20 times), 450 μg L^{-1} (4.70 and 3.24 times), 600 μg L^{-1} (6.23 and 4.29 times), 750 μg L^{-1} (7.76 and 5.33 times), 900 μg L^{-1} (9.30 and 6.37 times), 1050 μg L^{-1} (9.79 and 7.41 times) and 1200 μg L^{-1} (9.87 and 8.45 times). For CPX, the same trend was observed, the increases in times with respect to the control at 72 and 96 hpf were 5 μg L^{-1} (1.65 and 1.20), 10 μg L^{-1} (3.20 and 2.22), 15 μg L^{-1} (4.74 and 3.26), 20 μg L^{-1} (6.29 and 4.31), 25 μg L^{-1} (7.81 and 5.37), 30 μg L^{-1} (9.34 and 6.41), 35 μg L^{-1} (9.42 and 7.45) and 40 μg L^{-1}

(9.70 and 8.49), respectively. Finally, for the binary mixtures (PCM-CPX) there was a significant difference with respect to the control group at 72 and 96 hpf respectively of M2 (3.16 and 3.10), M3 (5.28 and 5.16), M4 (8.42 and 8.22).

3.6. Antioxidant defense biomarkers

Fig. 6 shows the results of antioxidant activity. Superoxide dismutase activity results are shown in Fig. 6A. Statistically significant increases

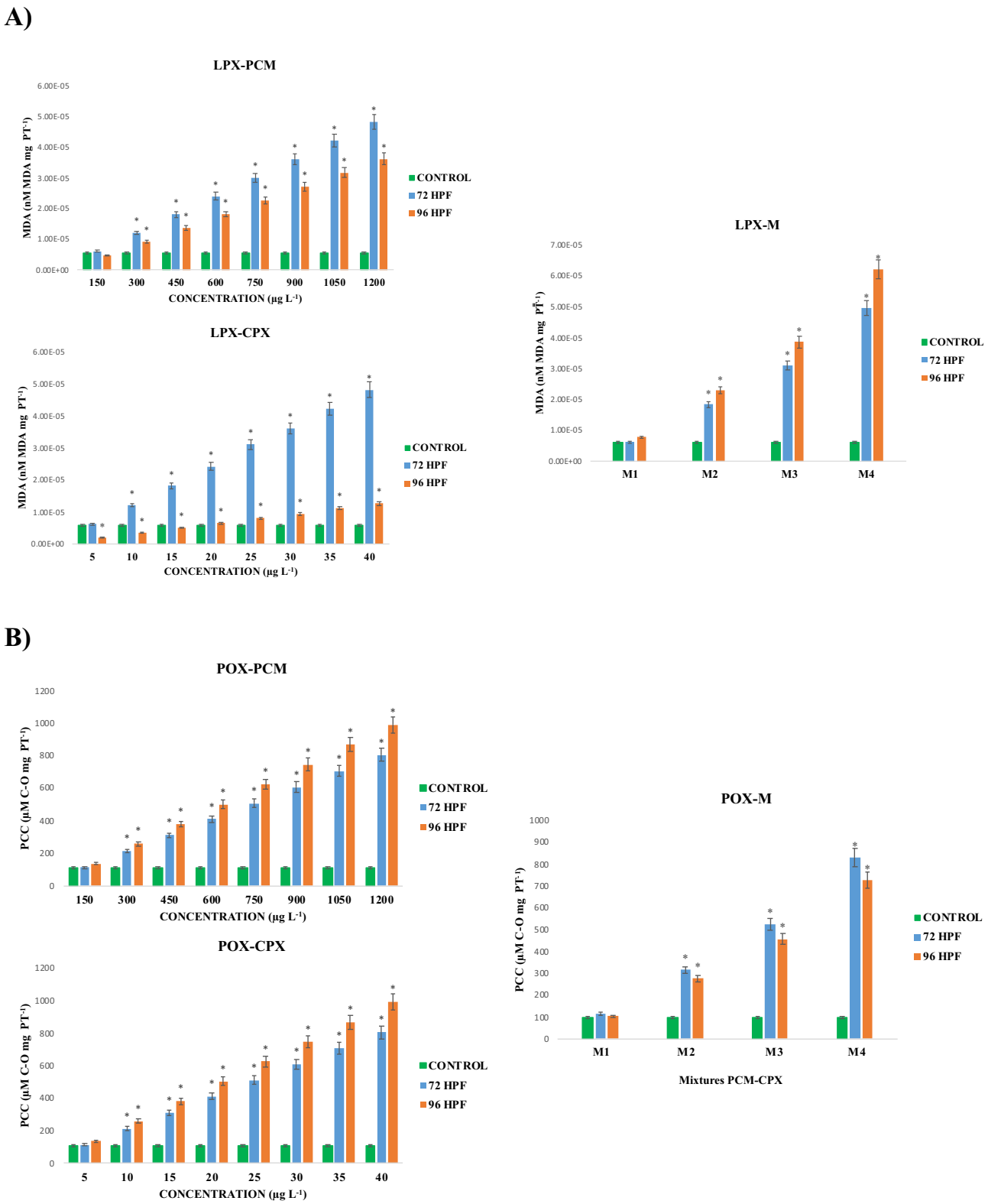


Fig. 5. A) Lipid peroxidation (LPX), **B)** Protein carbonyl content (PCC) and **C)** Hydroperoxide content (HPC) in embryos of zebrafish *D. rerio* exposed to eight different concentration of PCM, CPX and their binary mixtures at 72 and 96 hpf. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; two-way ANOVA($p < 0.05$).

C)

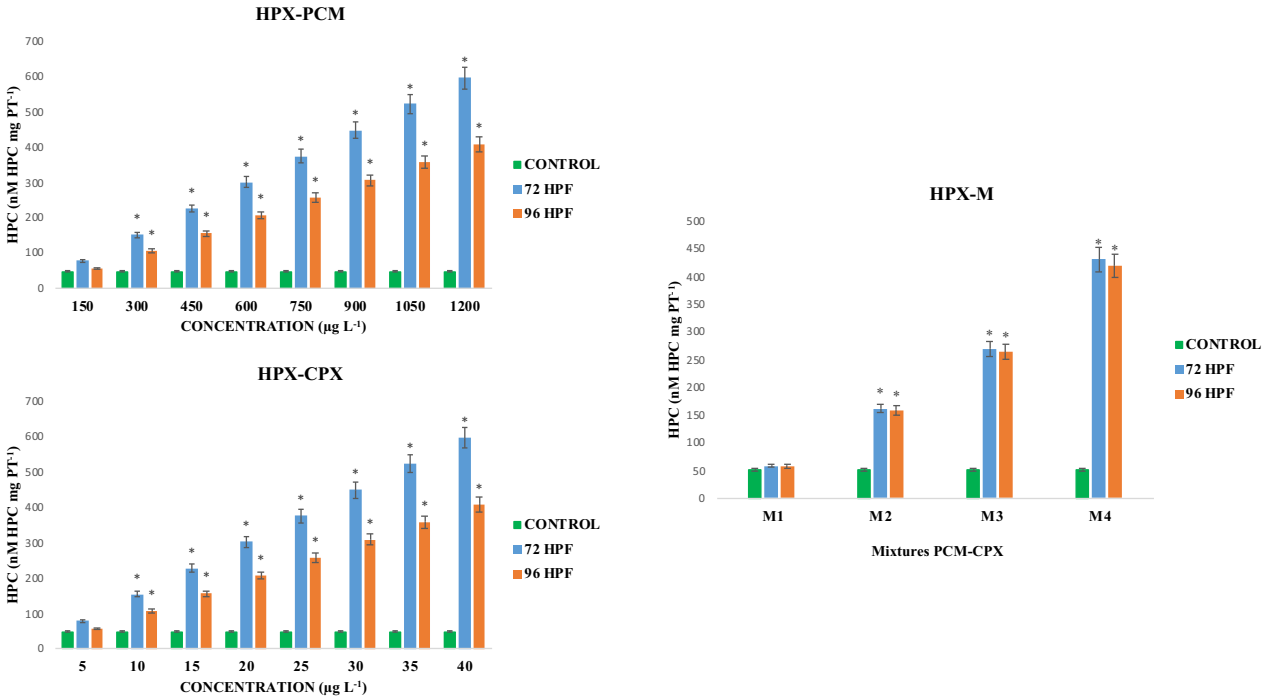


Fig. 5 (*continued*).

were found with respect to the control group ($p < 0.05$) in a concentration-dependent manner. In the case of PCM, increases were observed at 72 and 96 hpf with respect to the control at concentrations of 150 µg L^{-1} (1.16 and 1.04 times), 300 µg L^{-1} (2.33 and 2.07 times), 450 µg L^{-1} (3.49 and 3.10 times), 600 µg L^{-1} (4.64 and 4.13 times), 750 µg L^{-1} (5.80 and 5.15 times), 900 µg L^{-1} (6.96 and 6.18 times), 1050 µg L^{-1} (8.12 and 7.21 times) and 1200 µg L^{-1} (9.27 and 8.23 times). For CPX, the same trend was observed, the increases in times with respect to the control at 72 and 96 hpf were 5 µg L^{-1} (1.21 and 1.05), 10 µg L^{-1} (2.69 and 2.09), 15 µg L^{-1} (3.58 and 3.11), 20 µg L^{-1} (4.70 and 4.15), 25 µg L^{-1} (5.84 and 5.17), 30 µg L^{-1} (7.0 and 6.20), 35 µg L^{-1} (8.14 and 7.22) and 40 µg L^{-1} (9.29 and 8.24), respectively. Finally, for the binary mixtures PCM-CPX there was a significant difference with respect to the control group at 72 and 96 hpf of M2 (3.90 and 3.46), M3 (6.58 and 5.85), M4 (10.57 and 9.38), respectively. Catalase activity results are shown in Fig. 6B. Significant increases with respect to the control group ($p < 0.05$) were found at 72 and 96 hpf of 0.94 and 1.14; 1.86 and 2.24; 2.79 and 3.34; 3.71 and 4.44; 4.64 and 5.54; 5.56 and 6.64; 6.49 and 7.74; and 7.41 and 8.83 times, respectively for 150, 300, 450, 600, 750, 900, 1050 and 1200 µg L^{-1} of PCM. For CPX of 1.25 and 1.14; 2.48 and 2.24; 3.72 and 3.34; 4.95 and 4.44; 6.18 and 5.54; 7.42 and 6.64; 8.65 and 7.73; and 9.88 and 8.83 times respectively at 5, 10, 15, 20, 25, 30, 35 and 40 µg L^{-1}. Finally, for the binary mixtures there was difference for M2 (5.18 and 3.10 times), M3 (8.73 and 4.21 times) and M4 (8.75 and 8.35 times). Glutathione peroxidase activity results are shown in Fig. 6C. For PCM, increases were observed at 72 and 96 hpf with respect to the control at concentrations of: 150 µg L^{-1} (1.46 and 1.05 times), 300 µg L^{-1} (2.92 and 2.09 times), 450 µg L^{-1} (4.38 and 3.14 times), 600 µg L^{-1} (5.84 and 4.18 times), 750 µg L^{-1} (7.30 and 5.22 times), 900 µg L^{-1} (8.75 and 6.26 times), 1050 µg L^{-1} (8.82 and 7.30 times) and 1200 µg L^{-1} (9.14 and 8.34 times), respectively. For CPX, the same trend was observed, the increases in times with respect to the control at 72 and 96 hpf were 5 µg L^{-1} (1.48 and 1.07), 10 µg L^{-1} (3.19 and 2.12), 15 µg L^{-1} (4.51 and 3.16), 20 µg L^{-1} (5.85 and 4.20), 25 µg L^{-1} (7.30 and 5.24), 30 µg L^{-1} (8.77 and 6.30), 35 µg L^{-1} (8.83 and 7.32) and 40 µg L^{-1} (9.19 and 8.65), respectively. Finally, for the binary mixtures PCM-CPX there was a significant difference with respect to the control group at 72 and 96 hpf respectively of M2 (4.38 and 3.14), M3 (7.40 and 5.29), M4 (9.14 and 8.49).

3.7. Integral biomarker response index (IBRv2)

Fig. 7 shows the analysis of integrated biomarkers for PCM, CPX, and their binary mixtures. In the case of PCM (Fig. 7A), our IBR analysis indicated that at low concentrations the antioxidant enzymes got more influence on the embryos than oxidative damage biomarkers. Nonetheless, as concentration increases, oxidative damage biomarkers (LPX and HPC) got more impact on the embryos than antioxidant enzymes. Concerning CPX (Fig. 7B), we can observe that no matter the concentration of this drug, antioxidant enzymes still have more influence over the embryos than oxidative damage biomarkers. Finally, in the case of binary mixtures (PCM-CPX) (Fig. 7C), we can observe that as same as PCM, as concentration increases, oxidative damage biomarkers (HPC and PCC) got more impact on the embryos than antioxidant enzymes.

4. Discussion

In this research work, we evaluated the toxic effects that PCM, CPX, and their mixture induced in *D. rerio* embryos. According to our results, environmentally relevant concentrations of PCM, CPX, and their mixture produced different body malformations in the embryos, affecting their integrity and consequently increasing the mortality rate of embryos. For PCM, previous studies have demonstrated that 100 µg L^{-1} of this drug-induced the death of 50% of *D. rerio* embryos after 96 h of exposure (David and Pancharatna, 2009). Nonetheless, in our study, we found that 1200 µg L^{-1} of PCM induced the dead of 68% of *D. rerio* embryos after 96 h of exposure. These results suggest that either low or high concentrations of PCM may increase the mortality rate of fish

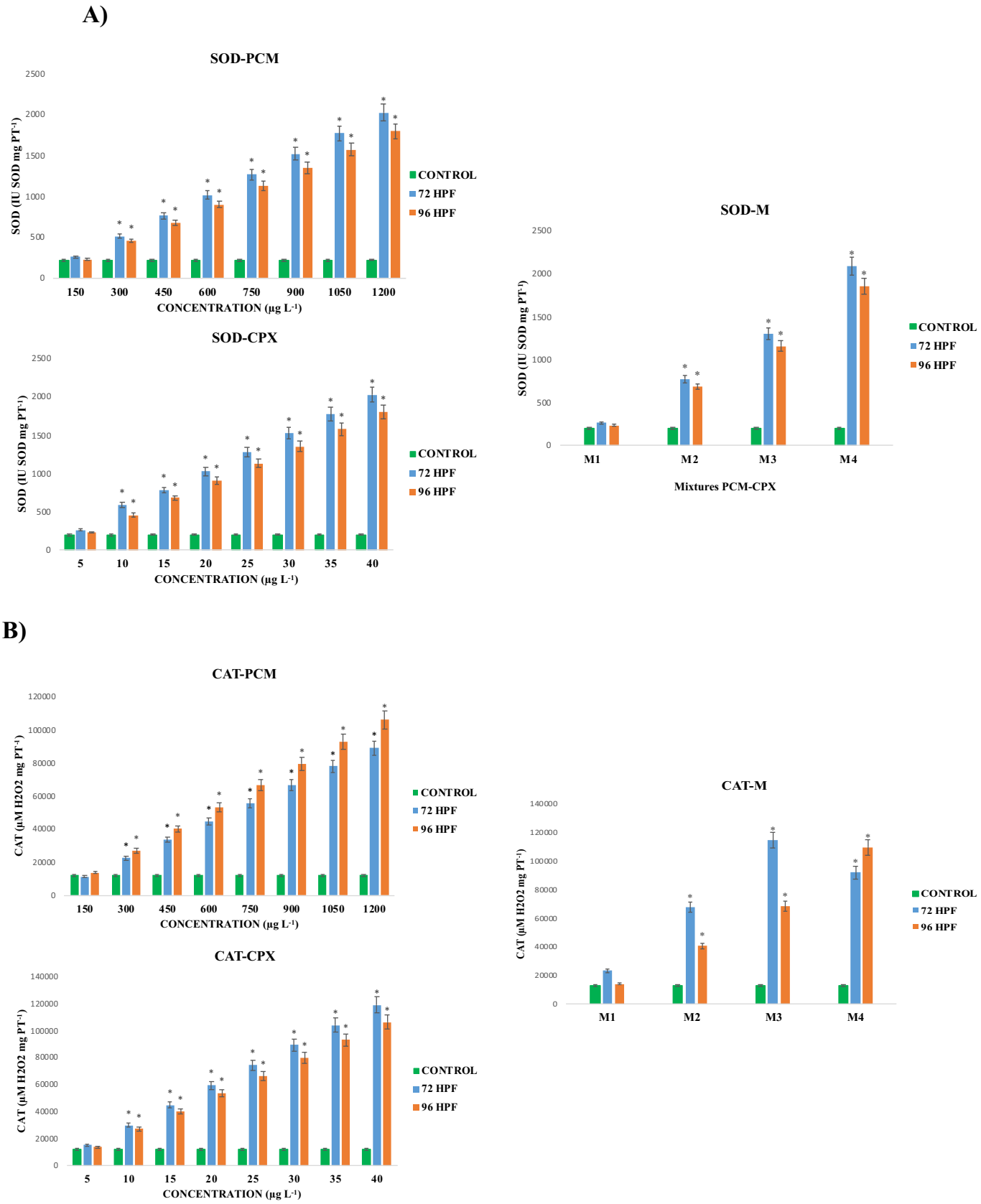


Fig. 6. A) Superoxide dismutase (SOD), **B)** Catalase (CAT) and **C)** Glutathione peroxidase (Gpx) activity in embryos of zebrafish *D. rerio* exposed to eight different concentrations of PCM, CPX and their binary mixtures at 72 and 96 hpf. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; two-way ANOVA *($p < 0.05$)*.

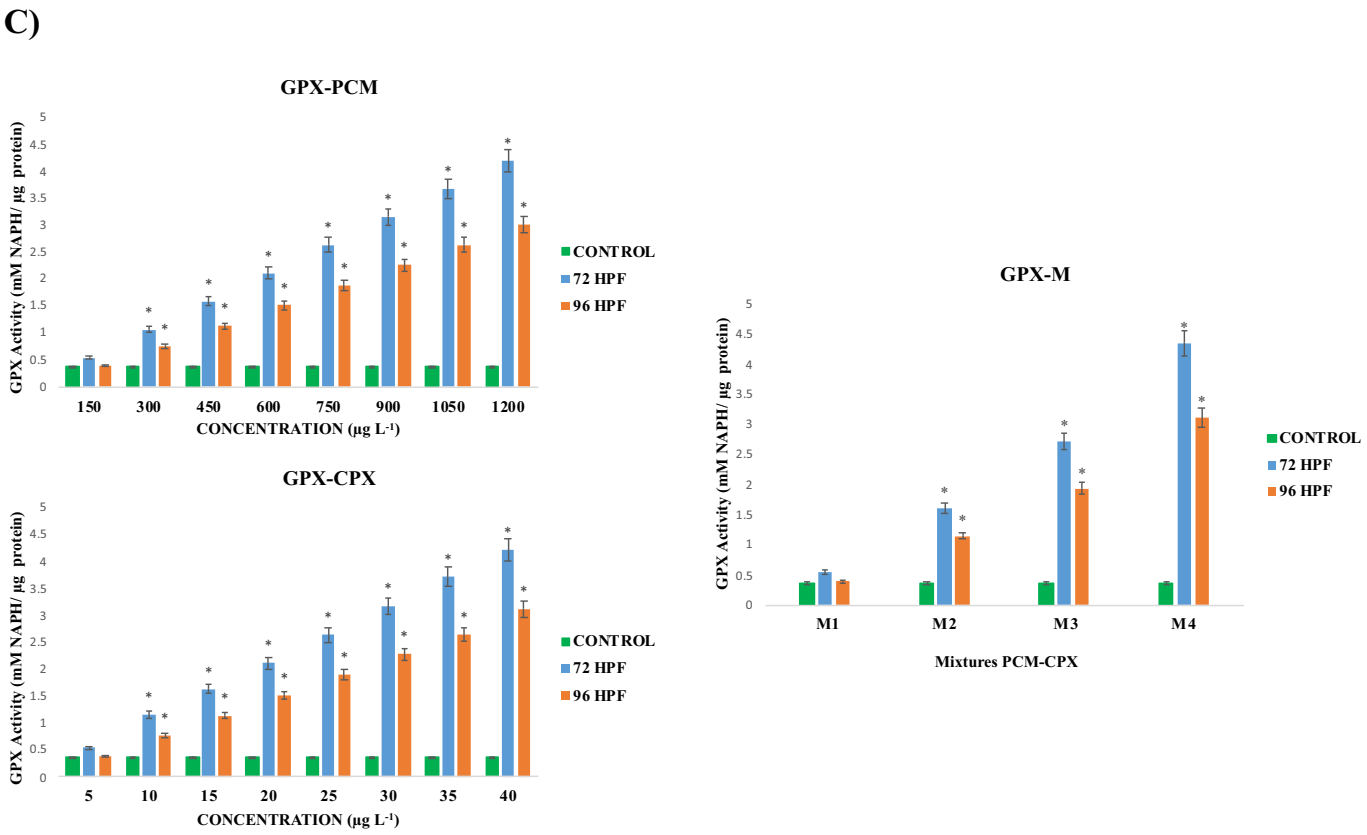


Fig. 6 (*continued*).

and that *D. rerio* embryos are highly sensitive to emerging pollutants such as PCM.

According to Gutiérrez-Noya et al., 2021, LC50 of PCM in *C. carpio* embryos was of 1.29 µg L^{-1}. On the other hand, Cedron et al., 2020 established that LC50 of PCM in embryos was 6.6 mM (997.6 mg L^{-1}). Nevertheless, in this study, LC50 of PCM was 736 µg L^{-1}.

As same as PCM, CPX may induce embryotoxic and teratogenic effects in fish. Zivna et al., 2016 for instance, reported that the exposure of *C. carpio* embryos to 1000 and 3000 µg L^{-1} of this drug led to the death of 32% of the embryos. On the other hand, in our study, we showed that CPX reached an LC50 of 35.4 µg L^{-1}. Thus, it can be suggested that *D. rerio* embryos are more sensitive to CPX than *C. carpio* embryos.

Regarding the mixture PCM-CPX, at mixture 4, we observed an increase in the number of dead and malformed embryos (1200 µg L^{-1} of PCM and 40 µg L^{-1} of CPX), reaching a mortality rate of 75% and a malformation rate of 93.1%. In agreement with our results, Richards et al., 2004 reported that the mixture of CPX (6, 10, 10 µg L^{-1}) with other drugs such as ibuprofen (6 µg L^{-1}) and fluoxetine (10 µg L^{-1}) induced an increase in the mortality rate and malformations of sunfish (*Lepomis gibbosus*).

Our results demonstrated that different concentrations of PCM, CPX, and their mixture PCM-CPX induced different malformations in *D. rerio* embryos. For the particular case PCM, the main malformations observed in the embryos were: craniofacial malformation, eye malformation, pericardial edema, yolk sac malformation, tail malformation, hatching retardation, and body hypopigmentation. From these, the most frequent were hatching retardation and body hypopigmentation. These results are consistent with those reported by David and Pancharatna (2009), who demonstrated that PCM (1–100 µg L^{-1}) induced different malformations (alterations in the hatching process, alterations in the growth and development of embryos, and hypopigmentation) in *D. rerio* embryos.

Hypopigmentation in fish is due to an affectation of the cells responsible for the distribution of the pigment both in the cells of the retina and in the melanophores of the head, body, and yolk sac. According to Cedron et al., 2020, PCM (2.5 mM - 4.9 mM) may affect the synthesis of the pigment cells: melanocytes, iridophores, and xanthophores that are derived from the neural crest (NCC) in *D. rerio*. Regarding other malformations, it is well known that PCM may generate different malformations in fish due to its capacity to produce ROS. Reactive oxygen species produced by PCM are produced through the formation of NAPQI, during the biotransformation process of this drug. Reactive oxygen species include the hydroxyl radical (OH-), superoxide anion (O2-), and hydrogen peroxide (H_2O_2), which modulate different cellular processes invertebrate organisms. Moreover, ROS act as messengers within cells and can induce cell growth, development, or death. Nonetheless, when organisms are exposed to some toxic agents such as PCM, the production of ROS increase, leading to important damage in cells and consequently disrupt the normal development of embryos. Thus, ROS produced by PCM may affect the cells of NCC and then induce several body abnormalities (abnormal spinal development, yolk sac malformation, hypopigmentation, delay of the hatching process) in fish.

Hatching in fish is caused via the movement of the embryo from the first hours of development (17 hpf). These movements arise in the spinal cord and continue until the development of the motor neurons of the muscular nerves (Brustein et al., 2003). Several studies have reported a delay in the hatching process induced by PCM. David and Pancharatna, 2009 for instance, reported that PCM (1, 10, 50 y 100 µg L^{-1}) induced important retardation in the hatching process of *D. rerio*. Furthermore, they pointed out that this retardation in the hatching was extended until 120 hpf. Similarly, Xia et al., 2017, established that ibuprofen, diclofenac, and PCM (5–500 µg L^{-1}) induced a delay in the hatching process in *D. rerio*, as a result of a reduction of the movements of zebrafish during their first hours of development. In

agreement with this data, we also demonstrate that PCM induced an important delay in the hatching process of the embryos (Fig. 2A.)

Concerning CPX, we showed that the main malformations that this drug-induced in *D.rerio* embryos were yolk sac malformation and craniofacial malformation. The yolk sac is a unique compartment in embryos rich in phospholipoproteins and with properties that facilitate the sorption of chemical substances or external agents. Furthermore, this is considered the source of nutrients necessary for the development of the embryo (Fraher et al., 2016). In the literature, it has been reported that CPX can react with phospholipoproteins and form adducts (S—H) modifying its structure (Miyares et al., 2014; He et al., 2013).

Genes, such as soxE, responsible for the normal development of the body, are expressed in the central nervous system (CNS). Sox9b for instance is involved in craniofacial development, while Sox10 is needed for the normal development of pigment cells, spinal ganglia, and sympathetic, enteric, and glial neurons. This is noteworthy, as ROS generated by CPX disrupt functions of the CNS cells and consequently induce different malformations in fish, such as spinal deformities, yolk sac

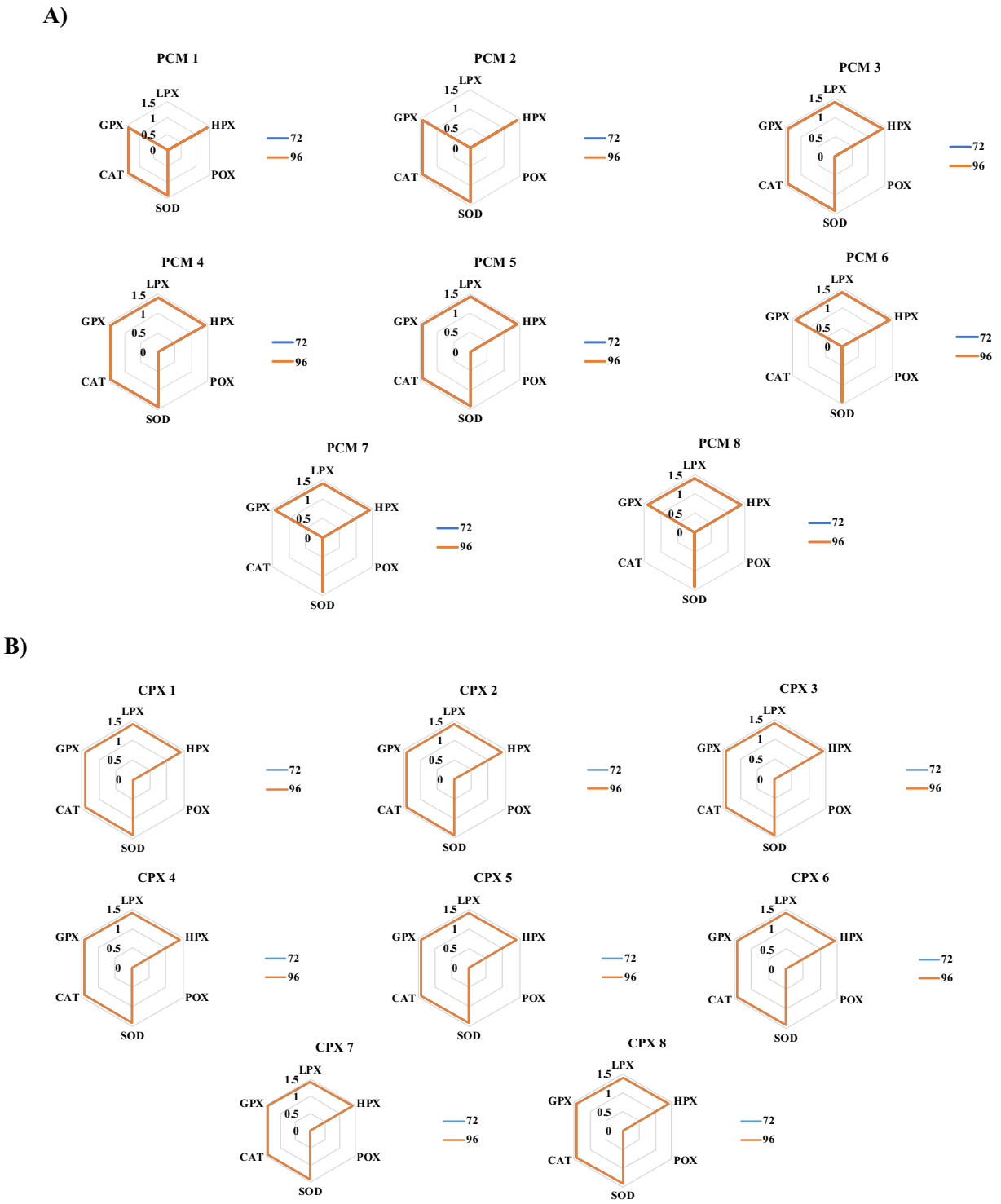


Fig. 7. Integral Biomarker Response Index (IBRv2) analysis performed to evaluate the different biomarkers of oxidative stress mentioned above, for each concentration of **A)** PCM, **B)** CPX and **C)** Binary mixtures of PCM-CPX at 72 and 96 hpf. The higher the IBR value, the greater the damage in the embryo.

C)

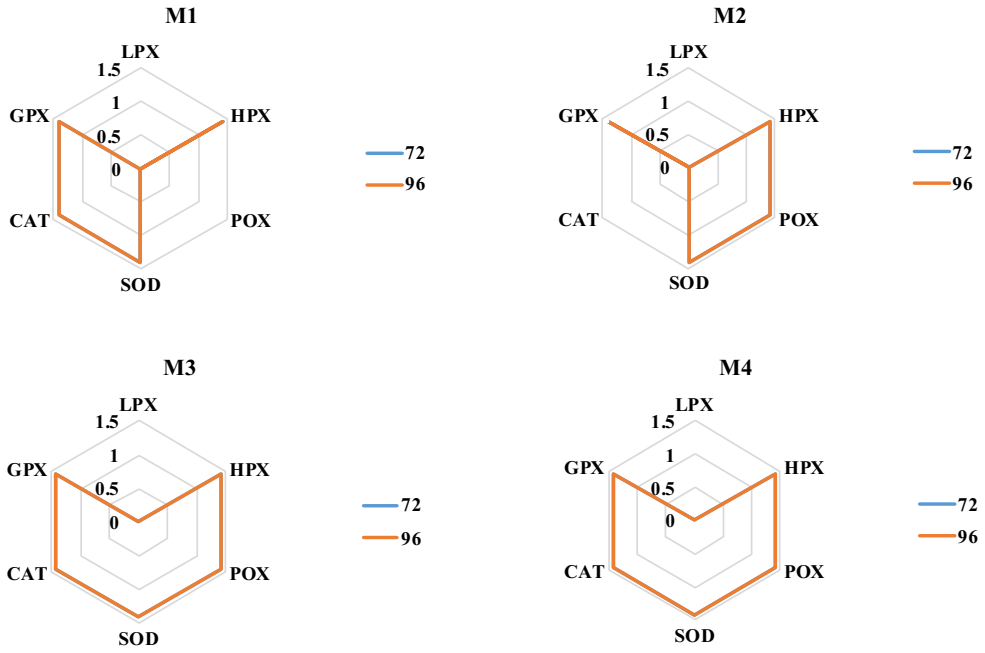


Fig. 7 (*continued*).

malformations, craniofacial malformation, eye malformation, and hypopigmentation. Zivna et al., 2016 for instance, reported that CPX (100 μg L^{-1}–3000 μg L^{-1}) generated different body abnormalities (lordosis, kyphosis, craniofacial malformation, pericardial edema, yolk sac deformation) in *C. carpio* embryos after 6 days of exposure. Similarly, in our study, we found that *D. rerio* embryos exposed to CPX (20 μg L^{-1}-40 μg L^{-1}) presented different malformations. The most frequent malformations found in our study were craniofacial malformation and yolk sac malformation, while the less frequent were hypopigmentation, pericardial edema, and delay of the hatching process.

Paracetamol and ciprofloxacin are simultaneously prescribed and administered simultaneously in the common therapeutic practice, generating a pharmacokinetic interaction between the two drugs (M. M. Issa et al., 2007). Until now, there are no studies that we are aware of that describe the toxic effects of the mixture PCM-CPX in *D. rerio* embryos. Nonetheless, previous studies have established that CPX affects the pharmacokinetics of PCM (Issa et al., 2006, 2007). In this research work, *D. rerio* embryos were exposed to the PCM-CPX mixture at different concentrations (M1 – M4). As we aforementioned, we decided to use this mixture as these drugs are commonly prescribed together (M. M. Issa et al., 2006). Moreover, from an environmental point of view, this is important as the occurrence of drugs in aquatic environments does not manifest itself in a single type, but in a very extensive group of different molecules that are biologically active and are mixed in different concentrations (Graham et al., 2013; Muramatsu et al., 2016).

Paracetamol-Ciprofloxacin mixtures (M1 - M4) got mortality rates of 34.7%, 51.4%, 61.1%, and 75%, respectively. As can be seen, mortality rates increase in a concentration- and time-dependent manner; thus, we can say that the mixture PCM-CPX is more embryotoxic than drugs administered single (Table 1).

The main isoforms of CYP450 responsible for PCM metabolism are CYP2E1, CYP1A2, and CYP3A4 (Nelson, 1995). This is important as CPX is a potent competitive inhibitor of CYP1A2 (Herrlin et al., 2000), so it could cause an increase in the concentration of non-metabolized PCM and NAPQI, and consequently induced an increase of the mortality and malformation rates (Dai and Cederbaum, 1995; Pasăre et al., 1979). In humans, it has been shown that PCM and CPX can cross the placenta and cause mutagenic effects when these are administered in high doses (Black and Hill, 2003; Briggs et al., 2011). Moreover, it has been also reported that PCM is capable of entering the chorion in the phase before embryonic hatching, and consequently increasing its potential to produce malformations in organisms (Kim et al., 2004). Here, we demonstrated that the mixture PCM-CPX generated different malformations in *D. rerio* embryos, being body malformation the most common. Body malformation could lead to other malformations (pericardial edema, delay in hatching process, and/or tail malformation, Fig. 4C) as from the beginning of embryonic development the functioning of organs, systems, and biotransformation pathways could be affected by the pharmacokinetic interaction of PCM and CPX.

Oxidative stress is an intracellular process that occurs by an imbalance between antioxidant enzymes and oxidant species. This process is closely related to the biotransformation process of PCM and CPX. Thus, once PCM is bio transformed, several ROS are generated, which are capable to induce damage in biomolecules (Gómez-Oliván et al., 2012). Similarly, in the case of CPX, ROS are generated in hepatic microsomes, after the metabolism of this drug (Wagai and Tawara, 1992; Gürbay et al., 2001). This is remarkable, as, in our study, we observed that PCM (150–1200 μg L^{-1}) and CPX (10–40 μg L^{-1}) alone increased the production of malondialdehyde (MDA) at 72 hpf, while the mixture PCM-CPX increased the production of MDA at 96 hpf (Fig. 5A).

During embryonic development, the relative rate of oxygen in metabolism fluctuates, causing changes in the redox state of the cell (Hansen et al., 2018). During early organogenesis, these changes could have a much more devastating effect on embryos for two reasons. Firstly, because alterations in the reducing environment may result in a decrease or cessation of proliferation or promote apoptosis (Timme-Laragy et al., 2018). Secondly, because antioxidant defenses during early organogenesis are not as mature as later stages of development, and may allow for longer periods of oxidative imbalance and alter redox signaling (Hansen, 2006).

Antioxidant enzymes are vital to protect cells from damage caused by oxidative stress. Catalase, for instance, decomposes intramolecular H2O2 into O2 and H2O, while GPX catalyzes the reduction of peroxides

(hydroperoxides and lipid peroxides) in alcohol. An increase in CAT activity may be the result of high levels of H2O2, generated during PCM biotransformation (Nunes et al., 2014; Ramos et al., 2014; Nogueira et al., 2019). According to our results, PCM (300–1200 μg L^{-1}), CPX (10–40 μg L^{-1}) and the mixture PCM-CPX (M2-M4) increased the activity of CAT in *D. rerio* embryos at 72 and 96 hpf (Fig. 6B). Similarly, Zivna et al. (2016) revealed that CPX at environmentally relevant concentrations (1 μg L^{-1}, 100 μg L^{-1}, 500 μg L^{-1}, 1000 μg L^{-1}, 3000 μg L^{-1}) induced an increase in the activity of CAT and GPX in *C. carpio* embryos.

One of the main objectives of ROS is the reversible oxidation of cysteines, leading to the formation of disulfide bonds that modulate the conformation and activity of proteins. The primary product of NOX activity is O2, which is immediately and largely transformed into H2O by an enzyme called superoxide dismutase (SOD) (Bedard and Krause, 2007; Brandes et al., 2014). In our study, we demonstrated that SOD activity increased in *D. rerio* embryos, after exposure to PCM (300–1200 μg L^{-1}), CPX (10–40 μg L^{-1}), and the mixture PCM-CPX (M2-M4) (Fig. 6A). In agreement with our results, Guiloski et al. (2017) and Gürbay et al. (2001) found that antioxidant enzymes, SOD and GPX, increased their activity in male fish *Rhamdia quelen* after exposure to PCM. Together these results suggest that both PCM and CPX, as well as their mixture, increase ROS production and the activity of antioxidant enzyme SOD.

The oxidation of reduced glutathione to glutathione disulfide is catalyzed by GPX. Reduced glutathione is very important for the proper progress of the reactions catalyzed by GPX and glutathione-S-transferase (GST). Glutathione reductase is an enzyme that promotes the conversion of glutathione disulfide to reduced glutathione (Di Giulio and Hinton, 2008). Glutathione-S-transferase is a group of biotransformation enzymes that catalyze the conjugation of xenobiotics and endogenous substances with glutathione, during the biotransformation reactions of phase II. Furthermore, these enzymes also protect cells against damage from oxidative stress and the toxic effects of a wide range of chemicals (Chambers, 1987; Di Giulio and Hinton, 2008). Since we found a higher GPX activity in all treatment groups (PCM, CPX, and their mixture), we suggest that these results are due to the gradual collapse of the GST enzymatic pathway. In agreement with our results, Zivna et al. (2016) found that environmentally relevant concentrations of CPX increased the activity of GPX in *C. carpio* embryos. Similarly, Nogueira et al. (2019) also reported that the exposure of *D. rerio* embryos to CPX produced an important increase in the activity of GPX.

Our IBR analysis showed that PCM, CPX and their mixture, may induce an important damage in *D. rerio* embryos, as a result of an important increase of oxidative damage biomarkers (LPX and HPC). Furthermore, it confirms that despite the antioxidant activity of SOD, CAT, GPX, oxidative damage biomarkers still having an important influence over the embryos. This may be explained due to the increased production of ROS via the biotransformation of PCM and CPX.

5. Conclusions

This study provided information on the embryotoxic, teratogenic, and oxidative stress effects of two pharmaceutical products, PCM, CPX, as well as their mixtures PCM-CPX. Paracetamol, ciprofloxacin, and their mixtures induced different morphological alterations in embryos, affecting the integrity of the organisms, and consequently leading to their death. Furthermore, we also demonstrated that the teratogenic and embryotoxic effects that both drugs induced in *D. rerio* embryos have resulted from the oxidative stress response generated by each drug. Therefore, it can be concluded that the presence of these drugs can be harmful to aquatic species. Overall, our results, contribute to better understand the toxic effects of pharmaceutical products, individually and in a mixture.

CRediT authorship contribution statement

Jonathan Ricardo Rosas-Ramírez and José Manuel Orozco-Hernández performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván, Jonathan Ricardo Rosas-Ramírez and José Manuel Orozco-Hernández were involved in the conception.

Leobardo Manuel Gómez-Oliván, Gustavo Elizalde-Velázquez and Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from Demetrio Raldúa.

Declaration of competing interest

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

Acknowledgements

This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Project 300727).

References

Andrieu, M., Rico, A., Phu, T.M., Phuong, N.T., Van den Brink, P.J., 2015. Ecological risk assessment of the antibiotic enrofloxacin applied to Pangasius catfish farms in the Mekong Delta, Vietnam. Chemosphere 119, 407–414.

Ávila, F., Luppi, M., Gaete, P., Rivas, A., Silva, F., Olivares, R., 2019. Changes in prescriptions and antibiotic consumption after the implementation of recommendations for use: experience in a university hospital. Rev. Chil. Infectol. 36, 253–264. https://doi.org/10.4067/S0716-10182019000300253.

Ba, S., Haroune, L., Cruz-Morató, C., Jacquet, C., Touahar, I.E., Bellenger, J.P., Cabana, H., 2014. Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. Sci. Total Environ. 487, 748–755.

Becerra, M., Albesa, I., 2002. Oxidative stress induced by ciprofloxacin in Staphylococcus aureus. Biochem. Biophys. Res. Commun. 297, 1003–1007. https://doi.org/10.1016/S0006-291X(02)02331-8.

Bedard, K., Krause, K.H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. https://doi.org/10.1152/physrev.00044.2005.

Black, R.A., Hill, D.A., 2003. Over-the-counter medications in pregnancy - American Family Physician. Am. Fam. Physician 67 (12), 2517–2524. https://www.aafp.org/afp/2003/0615/p2517.html.

Botero-Coy, A.M., Martínez-Pachón, D., Boix, C., Rincón, R.J., Castillo, N., Arias-Marín, L.P., Hernández, F., 2018. An investigation into the occurrence and removal of pharmaceuticals in colombian wastewater. Sci. Total Environ. 642, 842–853.

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

Brandes, R.P., Weissmann, N., Schröder, K., 2014. Nox family NADPH oxidases: molecular mechanisms of activation. Free Radic. Biol. Med. 76, 208–226. https://doi.org/10.1016/J.FREERADBIOMED.2014.07.046.

Briggs, G.G., Freeman, R.K., Yaffe, S.J., 2011. Drugs in Pregnancy and Lactation : A Reference Guide to Fetal and Neonatal Risk. Wolters Kluwer Health/Lippincott Williams & Wilkins.

Brustein, E., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R., Drapeau, P., 2003. Steps during the development of the zebrafish locomotor network. J. Physiol.-Paris 97 (1), 77–86.

Buege, J.A., Aust, S.D., 1978. [30] microsomal lipid peroxidation. Methods Enzymol. 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Campanha, M.B., Awan, A.T., de Sousa, D.N., Grosseli, G.M., Mozeto, A.A., Fadini, P.S., 2015. A 3-year study on occurrence of emerging contaminants in an urban stream of São Paulo State of Southeast Brazil. Environ. Sci. Pollut. Res. 22 (10), 7936–7947.

Cedron, V.P., Weiner, A.M.J., Vera, M., Sanchez, L., 2020. Acetaminophen affects the survivor, pigmentation and development of craniofacial structures in zebrafish (Danio rerio) embryos. Biochem. Pharmacol. 174, 113816. https://doi.org/10.1016/j.bcp.2020.113816.

Chambers, F.L., 1987. A textbook of modern toxicology. Trends Pharmacol. Sci. 8 (10), 408. https://doi.org/10.1016/0165-6147(87)90110-6.

Choi, E., Alsop, D., Wilson, J.Y., 2018. The effects of chronic acetaminophen exposure on the kidney, gill and liver in rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 198, 20–29.

Dai, Y., Cederbaum, A.I., 1995. Cytotoxicity of acetaminophen in human cytochrome P4502E1-transfected HepG2 cells. J. Pharmacol. Exp. Ther. 273 (3).

David, A., Pancharatna, K., 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio. J. Appl. Toxicol. 29, 597–602. https://doi.org/10.1002/jat.1446.

Di Giulio, R.T., Hinton, D.E., 2008. The toxicology of fishes. CRC Press.

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., García-Medina, S., Islas-Flores, H., Hernández-Navarro, M.D., Galar-Martínez, M., 2021a. Antidiabetic drug metformin disrupts the embryogenesis in zebrafish through an oxidative stress mechanism. Chemosphere 285, 131213. https://doi.org/10.1016/j.chemosphere.2021.131213.

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., Islas-Flores, H., Hernández-Navarro, M.D., García-Medina, S., Galar-Martínez, M., 2021b. Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of Danio rerio. Sci. Total Environ. 799, 149432. https://doi.org/10.1016/j.scitotenv.2021.149432.

Fraher, D., Sanigorski, A., Mellett, N.A., Meikle, P.J., Sinclair, A.J., Gibert, Y., 2016. Zebrafish embryonic lipidomic analysis reveals that the yolk cell is metabolically active in processing lipid. Cell Rep. 14 (6), 1317–1329.

Gomes, M.P., Gonçalves, C.A., de Brito, J.C.M., Souza, A.M., da Silva Cruz, F.V., Bicalho, E.M., et al., 2017. Ciprofloxacin induces oxidative stress in duckweed (Lemna minor L.): implications for energy metabolism and antibiotic-uptake ability. J. Hazard. Mater. 328, 140–149.

Gómez, M.J., Bueno, M.M., Lacorte, S., Fernández-Alba, A.R., Agüera, A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. Chemosphere 66 (6), 993–1002.

Gómez-Oliván, L.M., Neri-Cruz, N., Galar-Martínez, M., Vieyra-Reyes, P., García-Medina, S., Razo-Estrada, C., Dublán-García, O., Corral-Avitia, A.Y., 2012. Assessing the oxidative stress induced by paracetamol spiked in artificial sediment on hyalella azteca. Water Air Soil Pollut. 223, 5097–5104. https://doi.org/10.1007/s11270-012-1261-y.

Gothwal, R., Shashidhar, 2017. Occurrence of high levels of fluoroquinolones in aquatic environment due to effluent discharges from bulk drug manufacturers. J. Hazard. Toxic Radioact. Waste 21, 05016003. https://doi.org/10.1061/(ASCE)HZ.2153-5515.0000346.

Graham, G.G., Davies, M.J., Day, R.O., Mohamudally, A., Scott, K.F., 2013. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. Inflammopharmacology. https://doi.org/10.1007/s10787-013-0172-x.

Guiloski, I.C., Ribas, J.L.C., Piancini, L.D.S., Dagostim, A.C., Cirio, S.M., Fávaro, L.F., Boschen, S.L., Cestari, M.M., da Cunha, C., Silva de Assis, H.C., 2017. Paracetamol causes endocrine disruption and hepatotoxicity in male fish Rhamdia quelen after subchronic exposure. Environ. Toxicol. Pharmacol. 53, 111–120. https://doi.org/10.1016/J.ETAP.2017.05.005.

Günzler, W.A., Flohé, L., 1985. Glutathione peroxidase. Handbook Methods for Oxygen Radical Research. CRC Press, pp. 285–290. https://doi.org/10.1201/9781351072922.

Gürbay, A., Gonthier, B., Daveloose, D., Favier, A., Hincal, F., 2001. Microsomal metabolism of ciprofloxacin generates free radicals. Free Radic. Biol. Med. 30, 1118–1121. https://doi.org/10.1016/S0891-5849(01)00508-1.

Gutiérrez-Noya, V.M., Gómez-Oliván, L.M., Islas-Flores, H., Galar-Martínez, M., García-Medina, S., Ramírez-Montero, M.del C., 2021. Survival and malformations rates, oxidative status in early life stages of Cyprinus carpio due to exposure to environmentally realistic concentrations of paracetamol. Sci. Total Environ. 768, 144585. https://doi.org/10.1016/J.SCITOTENV.2020.144585.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed spearman-karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714–719. https://doi.org/10.1021/es60130a004.

Hansen, J.M., 2006. Oxidative stress as a mechanism of teratogenesis. Birth Defects Res. C Embryo Today Rev. 78 (4), 293–307.

Hansen, J.M., Jacob, B.R., Piorczynski, T.B., 2018. Oxidative stress during development: chemical-induced teratogenesis. Curr. Opin. Toxicol. 7, 110–115.

He, J.-H., Guo, S.-Y., Zhu, F., Zhu, J.-J., Chen, Y.-X., Huang, C.-J., Gao, J.-M., Dong, Q.-X., Xuan, Y.-X., Li, C.-Q., 2013. A zebrafish phenotypic assay for assessing drug-induced hepatotoxicity. J. Pharmacol. Toxicol. Methods 67 (1), 25–32. https://doi.org/10.1016/J.VASCN.2012.10.003.

He, K., Soares, A.D., Adejumo, H., McDiarmid, M., Squibb, K., Blaney, L., 2015. Detection of a wide variety of human and veterinary fluoroquinolone antibiotics in municipal wastewater and wastewater-impacted surface water. J. Pharm. Biomed. Anal. 106, 136–143.

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. Toxicol. in Vitro 25, 745–753. https://doi.org/10.1016/j.tiv.2011.01.005.

Herrlin, K., Segerdahl, M., Gustafsson, L.L., Kalso, E., 2000. Methadone, ciprofloxacin, and adverse drug reactions. Lancet 356, 2069–2070. https://doi.org/10.1016/S0140-6736(00)03409-7.

Israel, O., Miguel, Á., Ruelas, C., 2021. Pharmaceuticals Market, Consumption Trends and Disease Incidence Are Not Driving the Pharmaceutical Research on Water and Wastewater.

Issa, M.M., Nejem, R.M., El-Abadla, N.S., 2006. Oral ciprofloxacin affects the pharmacokinetics of paracetamol in saliva. Clin. Drug Investig. 26, 223–226. https://doi.org/10.2165/00044011-200626040-00007.

Issa, M.M., Nejem, R.M., El-Abadla, N.S., El-Naby, M.K., Roshdy, A.A., Kheiralla, Z.A., 2007. Effects of paracetamol on the pharmacokinetics of ciprofloxacin in plasma using a microbiological assay. Clin. Drug Investig. 27, 463–467. https://doi.org/10.2165/00044011-200727070-00003.

Jiang, Z.Y., Hunt, J.V., Wolff, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. 202, 384–389. https://doi.org/10.1016/0003-2697(92)90122-N.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43 (2), 363–380.

Kim, D.H., Sun, Y., Yun, S., Kim, B., Hwang, C.N., Lee, S.H., Nelson, B.J., 2004. Mechanical property characterization of the zebra!sh embryo chorion. Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings , pp. 5061–5064. https://doi.org/10.1109/iembs.2004.1404399.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. https://doi.org/10.1002/aja.1002030302.

Lau, S.M., McGuire, T.M., Van Driel, M.L., 2016. Consumer concerns about paracetamol: a retrospective analysis of a medicines call centre. BMJ Open 6, e010860. https://doi.org/10.1136/bmjopen-2015-010860.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. [37] carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9.

Li, Z., Hong, H., Liao, L., Ackley, C.J., Schulz, L.A., MacDonald, R.A., et al., 2011. A mechanistic study of ciprofloxacin removal by kaolinite. Colloids Surf. B: Biointerfaces 88 (1), 339–344.

Liu, S., Ding, R., Nie, X., 2019. Assessment of oxidative stress of paracetamol to Daphnia magna via determination of Nrf1 and genes related to antioxidant system. Aquat. Toxicol. 211, 73–80.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175. https://doi.org/10.1016/S0021-9258(19)45228-9.

Miyares, R.L., de Rezende, V.B., Farber, S.A., 2014. Zebrafish yolk lipid processing: a tractable tool for the study of vertebrate lipid transport and metabolism. Dis. Model. Mech. 7 (7), 915–927 https://doi.org/10.1242/dmm.015800.

Moriarty, C., Carroll, W., 2016. Paracetamol: pharmacology, prescribing and controversies. Arch. Dis. Child. Educ. Pract. Ed. 101, 331–334. https://doi.org/10.1136/archdischild-2014-307287.

Muramatsu, S., Shiraishi, S., Miyano, K., Sudo, Y., Toda, A., Mogi, M., Hara, M., Yokoyama, A., Kawasaki, Y., Taniguchi, M., Uezono, Y., 2016. Metabolism of AM404 from acetaminophen at human therapeutic dosages in the rat brain. Anesthesiol. Pain Med. 6, e32873. https://doi.org/10.5812/aapm.32873.

Nelson, S.D., 1995. Mechanisms of the formation and disposition of reactive metabolites that can cause acute liver injury. Drug Metab. Rev. 27 (1–2), 147–177 https://doi.org/10.3109/03602539509029821.

Nogueira, A.F., Pinto, G., Correia, B., Nunes, B., 2019. Embryonic development, locomotor behavior, biochemical, and epigenetic effects of the pharmaceutical drugs paracetamol and ciprofloxacin in larvae and embryos of Danio rerio when exposed to environmental realistic levels of both drugs. Environ. Toxicol. 34, 1177–1190. https://doi.org/10.1002/tox.22819.

Nunes, B., Antunes, S.C., Santos, J., Martins, L., Castro, B.B., 2014. Toxic potential of paracetamol to freshwater organisms: a headache to environmental regulators? Ecotoxicol. Environ. Saf. 107, 178–185. https://doi.org/10.1016/j.ecoenv.2014.05.027.

Okuda, T., Kobayashi, Y., Nagao, R., Yamashita, N., Tanaka, H., Tanaka, S., Houwa, I., 2008. Removal efficiency of 66 pharmaceuticals during wastewater treatment process in Japan. Water Sci. Technol. 57 (1), 65–71.

Pasăre, G., Pasăre, V., Eftimescu, N., Ivan, M., Milici, A., 1979. Combination of Antibiotics with Analgesic and Antipyretic Substances. Zentralblatt Für Bakteriologie, Parasitenkunde, Infektionskrankheiten Und Hygiene. Zweite Naturwissenschaftliche Abteilung: Mikrobiologie Der Landwirtschaft, Der Technologie Und Des Umweltschutzes 134 (7), 640–647 https://doi.org/10.1016/s0323-6056(79)80116-0.

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. J. Biol. Chem. 266, 22028–22034.

Ramos, A.S., Correia, A.T., Antunes, S.C., Gonçalves, F., Nunes, B., 2014. Effect of acetaminophen exposure in Oncorhynchus mykiss gills and liver: detoxification mechanisms, oxidative defence system and peroxidative damage. Environ. Toxicol. Pharmacol. 37 (3), 1221–1228 https://doi.org/10.1016/J.ETAP.2014.04.005.

Riaz, L., Mahmood, T., Kamal, A., Shafqat, M., Rashid, A., 2017. Industrial release of fluoroquinolones (FQs) in the waste water bodies with their associated ecological risk in Pakistan. Environ. Toxicol. Pharmacol. 52, 14–20.

Sanchez, W., Burgeot, T., Porcher, J.-M., 2013. A novel "integrated biomarker response" calculation based on reference deviation concept. Environ. Sci. Pollut. Res. 20, 2721–2725. https://doi.org/10.1007/s11356-012-1359-1.

SanJuan-Reyes, N., Gómez-Oliván, L.M., Galar-Martínez, M., García-Medina, S., Islas-Flores, H., González-González, E.D., Jiménez-Vargas, J.M., 2015. NSAID-manufacturing plant effluent induces geno-and cytotoxicity in common carp (Cyprinus carpio). Sci. Total Environ. 530, 1–10.

Santos, L.H., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., Montenegro, M.C.B., 2013. Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. Sci. Total Environ. 461, 302–316.

Sim, W.J., Lee, J.W., Lee, E.S., Shin, S.K., Hwang, S.R., Oh, J.E., 2011. Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. Chemosphere 82 (2), 179–186.

Spongberg, A.L., Witter, J.D., Acuña, J., Vargas, J., Murillo, M., Umaña, G., Perez, G., 2011. Reconnaissance of selected PPCP compounds in Costa Rican surface waters. Water Res. 45 (20), 6709–6717.

Stephenson, M., 2000. Development and validation of the Stephenson Multigroup Acculturation Scale (SMAS). Psychol. Assess. 12, 77–88. https://doi.org/10.1037/1040-3590.12.1.77.

Teglia, C.M., Perez, F.A., Michlig, N., Repetti, M.R., Goicoechea, H.C., Culzoni, M.J., 2019. Occurrence, distribution, and ecological risk of fluoroquinolones in rivers and wastewaters. Environ. Toxicol. Chem. 38 (10), 2305–2313.

Timme-Laragy, A.R., Hahn, M.E., Hansen, J.M., Rastogi, A., Roy, M.A., 2018. Redox stress and signaling during vertebrate embryonic development: regulation and responses. Seminars in Cell & Developmental Biology. vol. 80. Academic Press, pp. 17–28.

Varga, Z.M., 2011. Aquaculture and husbandry at the zebrafish international resource center. Methods Cell Biol. 104, 453–478.

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci. Total Environ. 430, 109–118.
Wagai, N., Tawara, K., 1992. Possible direct role of reactive oxygens in the cause of cutaneous phototoxicity induced by five quinolones in mice. Arch. Toxicol. 66, 392–397. https://doi.org/10.1007/BF02035128.
Wei, R., Ge, F., Chen, M., Wang, R., 2012. Occurrence of ciprofloxacin, enrofloxacin, and florfenicol in animal wastewater and water resources. J. Environ. Qual. 41 (5), 1481–1486.
Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology 281, 25–36. https://doi.org/10.1016/j.tox.2011.01.004.
Westerfield, M., 2007. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish Danio ("Brachydanio Rerio"). University of Oregon.
Wilcox, J.D., Bahr, J.M., Hedman, C.J., Hemming, J.D., Barman, M.A., Bradbury, K.R., 2009. Removal of organic wastewater contaminants in septic systems using advanced treatment technologies. J. Environ. Qual. 38 (1), 149–156.
Wu, S., Zhang, L., Chen, J., 2012. Paracetamol in the environment and its degradation by microorganisms. Appl. Microbiol. Biotechnol. 96 (4), 875–884.
Xia, L., Zheng, L., Zhou, J.L., 2017. Effects of ibuprofen, diclofenac and paracetamol on hatch and motor behavior in developing zebrafish (Danio rerio). Chemosphere 182, 416–425. https://doi.org/10.1016/j.chemosphere.2017.05.054.
Xu, J.J., Hendriks, B.S., Zhao, J., de Graaf, D., 2008. Multiple effects of acetaminophen and p38 inhibitors: towards pathway toxicology. FEBS Lett. https://doi.org/10.1016/j.febslet.2008.01.063.
Yi, K., Wang, D., Li, X., Chen, H., Sun, J., An, H., et al., 2017. Effect of ciprofloxacin on biological nitrogen and phosphorus removal from wastewater. Sci. Total Environ. 605, 368–375.
Zivna, D., Plhalova, L., Chromcova, L., Blahova, J., Prokes, M., Skoric, M., Marsalek, P., Praskova, E., Stepanova, S., Svobodova, Z., 2016. The effects of ciprofloxacin on early life stages of common carp (Cyprinus carpio). Environ. Toxicol. Chem. 35, 1733–1740. https://doi.org/10.1002/etc.3317.