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Long-term exposure to environmentally relevant concentrations of ibuprofen and aluminum alters oxidative stress status on *Danio rerio*

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ABSTRACT

Despite the ubiquitous presence of multiple pollutants in aqueous environments have been extensively demonstrated, the ecological impact of chemical cocktails has not been studied in depth. In recent years, environmental studies have mainly focused on the risk assessment of individual chemical substances neglecting the effects of complex mixtures even though it has been demonstrated that combined effects exerted by pollutants might represent a greater hazard to the biocenosis. The current study evaluates the effects on the oxidative stress status induced by individual forms and binary mixtures of ibuprofen (IBU) and aluminum (Al) on brain, gills, liver and gut tissues of *Danio rerio* after long-term exposure to environmentally relevant concentrations (0.1–11 μg L^{-1} and 0.05 mg L^{-1}- 6 mg L^{-1}, respectively). Lipid peroxidation (LPO), Protein carbonyl content (PCC) and activity of Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX) were evaluated. Moreover, concentrations of both toxicants and the metabolite 2-OH-IBU were quantified on test water and tissues. Results show that ibuprofen (IBU) and aluminum (Al) singly promote the production of radical species and alters the oxidative stress status in all evaluated tissues of zebrafish, nevertheless, higher effects were elicited by mixtures as different interactions take place.

1. Introduction

Chemical pollution of the aquatic environment has become a major matter of concern. Natural and anthropogenic chemicals such as heavy metals and pharmaceutical compounds are released into the water bodies promoting harmful effects on the biocenosis. Moreover, incomplete removal of pollutants with conventional treatments at municipal WWTPs contributes to the constant discharges of toxic compounds into the aquatic system (Gorito et al., 2017; Radović et al., 2015). Previous studies carried out by González-González et al. (2014), Martínez-vieyra et al. (2017) and Pérez-Alvarez et al. (2018) have reported the joint presence of heavy metals and nonsteroidal inflammatory drugs in different water samples at concentrations that exceed the permissible levels for the protection of aquatic life with harmful consequences to the biota.

Ibuprofen (IBU) is a non-steroidal anti-inflammatory drug widely used for pain relief and inflammatory disorders (Jia et al., 2020; Wang et al., 2010). This NSAID acts as an inhibitor of the cyclooxygenase system, involved in the transformation of arachidonic acid into prostaglandins (PGs) (Santos et al., 2010). The wide occurrence of pharmaceutical ingredients in the aquatic environment is the result of irrational human and veterinarian consumption, relative persistence, bioavailability, and metabolic excretion as well as the lack of effective removal treatments at the WWTPs (Khetan and Collins, 2007; Vasquez et al., 2014). Therefore, IBU and its transformation products have been frequently detected in natural and drinking waters as well as wastewater samples at levels ranging up to μg L^{-1} (Table 1) (González-Alonso et al., 2017; Madikizela and Chimuka, 2017).

Abbreviations: Al, aluminum; BSA, bovine serum albumin; CAT, catalase; CYP450, cytochrome P450; GPX, glutathione peroxidase; IBU, ibuprofen; LPO, lipid peroxidation; MDA, malondialdehyde; NSAIDs, no steroidal anti-inflammatory drugs; OE, oxidative stress; PCPP's, pharmaceutical and personal care products; PCC, protein carbonyl content; PGs, prostaglandins; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; TBA, Thiobarbituric acid; TCA, trichloroacetic acid; TP, transformation products; WWTPs, wastewater treatment plants.

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Several studies have shown that IBU is capable of induce variations in biochemical biomarkers, behavioral alterations and morphological changes in various aquatic species at trace levels (Gómez-Oliván et al., 2014b; Grzesiuk et al., 2020; Gutiérrez-Noya et al., 2020; Han et al., 2010; Jeffries et al., 2015; Xia et al., 2017). Besides, it has been demonstrated that some inhibitors of the eicosanoids biosynthesis, can impaired the cellular and humoral immune response as well as the mainteinance of the oxidative homeostasis in different vertebrates and invertebrates (Büyükgüzel et al., 2007, 2010).

Aluminum (Al) is considered ubiquitous in the Earth's crust (García et al., 2010; Schroniltgen et al., 2007). This metal is considered nonessential for the biota, its bioavailability and therefore its toxicity are strongly affected by physicochemical parameters of the aquatic environment including pH, total hardness, dissolved organic carbon (DOC) and temperature (DeForest et al., 2018; Santore et al., 2018; Trenfield et al., 2012). Aluminum can reach levels ranging up to mg L^{-1} in various aquatic systems (Table 2) (González-González et al., 2014; Li et al., 2016; Reutova et al., 2018).

Diverse studies have shown that exposure to high concentrations of Al can trigger a wide range of toxicity mechanisms in different animal models (Martínez-vieyra et al., 2017; Mathiyazahan et al., 2015; Ward et al., 2006). In aquatic organisms environmental exposure to Al has been related to embryolethality and teratogenic effects (Kovrižnych et al., 2013; Monaco et al., 2017), neurotoxicity (Monaco et al., 2017), geno- cytotoxicity and congenital malformations (Gómez-Oliván et al., 2017; Quiroga-Santos et al., 2021), morphological and behavioral alterations (Grassie et al., 2013; Griffitt et al., 2008), iono and osmo regulatory disturbances (Kumar and Gill, 2014) and oxidative stress (González-González et al., 2014; Razo-Estrada et al., 2013).

The quantification of molecular biomarkers such as oxidative damage to lipids, proteins and DNA as well as harmful effects on antioxidant systems has been extensively used to determine the effects of ROS in main biomolecules of organisms exposed to environmental toxicants (Valavanidis et al., 2006). Sustained input of active pharmaceutical ingredients and metallic compounds into the aquatic environment may influence non-target organisms including fish (Ginebreda et al., 2010; González-González et al., 2014).

Danio rerio is widely used as a suitable animal model for toxicological studies (Scholz et al., 2008) due to its sensitiveness to toxicants, small size, fecundity, ease of maintenance under laboratory conditions and complete genome sequence (Hill et al., 2005; Kari et al., 2007; Spitsbergen and Kent, 2003).

Different reports have been made about toxicity of IBU and Al in aquatic animals, nevertheless pollutants never occur alone, they take part of complex mixtures that may affect non-target organisms (Quiroga-Santos et al., 2021). The Ecotoxicological Risk Assessment requires the analysis of the interactions between compounds that can be found in effluents of various sources (Laquaz et al., 2018). The interactions describe the joined effect of chemicals as stronger (synergistic, potentiation effect) or weaker (inhibition, antagonistic response) based on the addition or additive dose/concentration response (Scientific Committee on Consumer Safety (SCCS), 2005). Thus, interactions may vary based on various characteristics such as physicochemical features (including bioavailability and environmental persistence), frequency, timing, dose levels, duration of exposure, and the biological target (Kortenkamp et al., 2019). In view of the great number of different combinations of substances to which humans and other species are exposed, it is important to stablish filters to allow a focus on mixtures potentially harmful to the aquatic biota. On this matter, various studies point out the harmful effects of IBU and Al singly and as part of effluents contaninng PPCP's and heavy metals in aquatic species, nonetheless far less is known about the specific potential interactions induced by mixtures Al and IBU, although they have been detected in combination in environmental samples (González-González et al., 2014).

Therefore, the present study focuses on the evaluation of oxidative stress promoted by IBU, Al singly and binary mixtures on brain, gills, liver and gut of zebrafish *Danio rerio* after long-term exposure at 7, 14, 21 and 28 days.

Table 1
Worldwide occurrence of IBU and its main metabolite 2-hydroxy-Ibuprofen.

Pollutant	Country	Water sample concentration (μg L^{-1})						References
		Drinking water (min.–max.)	Surface waters (min.–max.)	Groundwater (min.–max.)	WWTPs influent (min.–max.)	WWTPs effluent (min.–max.)	Hospital effluent (min.–max.)	
IBU	China	n.q.	n.q.	0.019–0.058	n.q.	n.q.	n.q.	(Peng et al., 2014)
	Czech Republic	n.q.	n.q- 3.2	n.q.	n.q.	n.q.	n.q.	(Marsik et al., 2017)
	Iran	0.022–0.047	0.022–0.037	n.q.	0.2–1.05	0.03–0.04	n.q.	(Eslami et al., 2015)
	Mexico	n.q.	3.78–5.12	n.q.	n.q.	n.q.	n.q.	(Martínez-vieyra et al., 2017)
	Mexico	n.q.	n.q.	n.q.	n.q.	n.q.	n.q-0.62	(Pérez-Alvarez et al., 2018)
	Mexico	n.q.	n.q.	n.q.	n.q.	n.q.	n.q-0.72	(Luja et al., 2019)
	Norway	n.q.	0.001–0.0092	n.q.	n.q.	n.q.	n.q.	(Reinholds et al., 2017)
	South Africa	n.q.	0.153–0.312	n.q.	n.q.	n.q.	n.q.	(Archer et al., 2017)
	South Africa	n.q.	4.8–11	n.q.	28–72	5.1–21	n.q.	(Madikizela and Chimuka, 2017)
	Spain	n.q.	0.16–9.89	n.q.	n.q.	n.q.	n.q.	(Ginebreda et al., 2010)
	Sweden	n.q.	n.q.	n.q.	15.67–22.82	n.q–0.52	n.q.	(Larsson et al., 2014)
	United States	n.q- 5.85	n.q.	n.q.	19.5–25.8	3.23–11.7	n.q.	(Loraine and Pettigrove, 2006)
2-OH-IBU	Canada	n.q.	0.33–0.713	n.q.	n.q.	n.q.	n.q.	(Zojaji et al., 2019)
	Spain	n.q.	1.46–3.0	n.q.	1.21–93.98	0.39–5.87	n.q.	(Ferrando-Climent et al., 2012)
	Spain	n.q.	n.q.	n.q.	2.68–4.92	n.q.	n.q.	(Malvar et al., 2019)
	Sweden	n.q.	n.q.	n.q.	28.59–35.36	0.44–1.45	n.q.	(Larsson et al., 2014)

n.q = not quantified.

Table 2
Worldwide occurrence of Aluminum in different water samples.

Country	Water sample Al concentration (mg L^{-1})				References
	Drinking water (min.-max.)	Surface water (min.–max.)	Groundwater (min.–max.)	Hospital effluent (min.–max.)	
Iran	n.q	0.0004–0.059	n.q	n.q	(Akbari et al., 2018)
Iran	n.q	n.q	0.005–3.3	n.q	(Shakerkhatibi et al., 2019)
Malaysia	0.11–0.12	n.q	n.q	n.q	(Dzulfakar et al., 2011)
Mexico	n.q	0.06–62.60	n.q	n.q	(Torres Guzmán et al., 2010)
Mexico	n.q	120–239	n.q	n.q	(García et al., 2010)
Mexico	n.q	6.04–24.44	n.q	n.q	(Martínez-vieyra et al., 2017)
Pakistan	n.q	1.01–4.27	n.q	n.q	(Kazi et al., 2009)
Peru	n.q	n.q	0.05–0.7	n.q	(de Meyer et al., 2017)
United States	n.q- 0.112	n.q	n.q	n.q	(Krewski et al., 2007)

n.q = not quantified.

2. Materials and methods

2.1. Chemicals

Aluminum stock solutions (1 g L^{-1}) were prepared with anhydrous aluminum chloride ($AlCl_3$) ReagentPlus® CAS Number 7446-70-0 > 99.9% purity in deionized water, pH 6.0 ± 0.3 and 3 h aging. Stock solutions were not filtered prior to exposure (EPA, 2017). For control, Al and mixtures, pH were adjusted to 6.0 ± 0.3 with 0.1 M HCl according to Cardwell et al. (2018).

IBU stock solutions (1 g L^{-1}) were prepared with Ibuprofen (≥98%) CAS number 15687-27-1. Stock solutions were prepared with deionized water. For control and IBU test tanks, water was maintained with the same characteristics of acclimation tanks.

Unless otherwise indicated, reagents were provided by Sigma-Aldrich, St Louis.

2.2. Analytical quantifications

2.2.1. LC- MS/MS ibuprofen quantification

Analysis was performed following the methods reported by Islas-Flores et al. (2014) and Pérez-Alvarez et al. (2018) with slight modifications. Stock solutions of Ibuprofen (≥98%) CAS number 15687-27-1 and 2-hydroxy-ibuprofen (2-OH-IBU) (≥98%) CAS number 51146-55-5 (Fluka, Sigma Aldrich, Toluca, MX) were prepared by dissolving the compounds in ultrapure water at a concentration of 1000 μg L^{-1}. Chromatographic separation was performed by using an Agilent 1290 Infinity II LC system and an LC column RRHD Eclipse Plus C18 (2.1 × 50 mm, particle size 1.8 μm) both from Agilent Technologies (Palo Alto, CA, USA). Compounds were eluted with a mobile phase consisting of acetonitrile ACN and ammonium acetate 0.1% (60:40 v/v). Flow rate, run time, and injection volume were 0.3 mL min^{-1}, 1.8 min and 2 μL respectively. For MS/MS analysis an Agilent Triple Quadrupole 6340 mass spectrometer (Palo Alto, CA, USA) equipped with electrospray ionization (ESI) in PI mode was used. Triplicates were performed.

2.2.2. Water

Water samples were collected from test tanks on days 0, 7, 14, 21 and 28. Samples (5 mL) were acidified (1 M HCl) and compounds were extracted with a mixture of hexane and ethyl acetate (1:1 v/v). Extraction samples were centrifuged at 1850 ×*g* for 15 min. Organic layers were extracted, evaporated under a nitrogen stream and reconstituted with ACN (30%) and ammonium acetate (70%). Samples were placed in glass vials for further analysis.

2.2.3. Tissues

Approximately 0.2 g of fresh tissue was acidified (1 M HCl) and homogenized with 5.0 mL of extraction solvent (hexane/ethyl acetate 1:1). Tissue extracts were centrifuged at 2500 ×*g* for 10 min. The process was repeated until upper organic layers combined, fully evaporated, and reconstituted with ACN (30%) and ammonium acetate (70%). Samples were placed in glass vials for further analysis.

2.2.4. AAS aluminum quantification

Total Al quantification was performed following methodologies proposed by Eaton et al. (1995) and Cardwell et al. (2018) with slight modifications. Al determination was performed by using an atomic absorption spectrophotometer Varian AA1475 (Melbourne, Australia) at a wavelength of 248.2 nm. A type curve was prepared from a stock solution of aluminum standard for AAS (1000 ppm) (Sigma-Aldrich, Toluca México) and results were interpolated. Percentages of Al recovery ranged between 97 and 98%. Triplicates were performed.

2.2.5. Water

Unfiltered water samples were collected from test tanks on days 0, 7, 14, 21 and 28. Concentrated metal grade HNO_3 (5 mL) was added to 500 μL of samples and digested for 60 min at 15-lb pressure and 120 °C. Next, samples were diluted with Ultrex II ultrapure water (J.T. Baker™) and immediately preserved at 4 °C before analysis.

2.2.6. Tissues

Tissue samples (0.2 g) were collected from specimens on days 0, 7, 14, 21 and 28. Samples were digested with concentrated metal grade HNO_3 (10 mL) for 60 min at 15-lb pressure and 120 °C. Samples were filtered, diluted with ultrapure water, and preserved at 4 °C before analysis.

2.3. Maintenance of specimens of D. rerio

Wild type three-month- old zebrafish specimens (0.89 ± 0.3 g in weight and 3.52 ± 0.05 cm in length) were acquired from a local fish farmer following the requirements cited in the Test Guideline No. 203 Fish, Acute Toxicity Testing (OECD, 2019). Fish were kept in 120-L vessels for 15 days prior the experiments with dechlorinated tap water at 26 ± 2 °C, pH ranging between 7.2 and 7.6, oxygen saturation above 60% and natural photoperiods of 12 h dark and 12 h light. Specimens were fed with commercial flake food two times a day according to methodologies followed by Ferdin and Halili (2017), Senger et al. (2011) and recommendations cited in Test Guideline No. 203 Fish, Acute Toxicity Testing (OECD, 2019).

2.3.1. Toxicity tests

Static-renewal toxicity tests consisted of 25-L glass vessels under laboratory conditions. Water parameters remained constant: hardness of 120 ± 20 mg L^{-1} $CaCO_3$ according to Griffitt et al. (2008), pH ranging between 6.0 and 6.3 for test tanks containing Al and 7.2 for tanks containing IBU singly (Cardwell et al., 2018; OECD, 2013), oxygen saturation above 60% and constant temperature of 26 ± 2 °C (OECD, 2009). Specimens were fed twice a day (Ferdin and Halili, 2017) and waters-ingly was renewed every 48 h with normal cleaning. Two control tests were performed.

The experiment was conducted on seven groups for each exposure

time (7,14,21 and 28 days), a total of twenty-five individuals were used for each assay. Environmentally relevant (nominal) concentrations were used (González-González et al., 2014; Madikizela and Chimuka, 2017; Mathias et al., 2018)

Group- 1: Control group
Group- 2: *D. rerio* exposed to 0.05 mg L^{-1} of Al
Group- 3: *D. rerio* exposed to 6 mg L^{-1} of Al
Group- 4: *D. rerio* exposed to 0.1 μg L^{-1} of IBU
Group- 5: *D. rerio* exposed to 11 μg L^{-1} of IBU
Group- 6: *D. rerio* exposed to mixture 1 (M1) 0.1 μg L^{-1} of IBU + 0.05 mg L^{-1} of Al
Group- 7: *D. rerio* exposed to mixture 2 (M2) 11 μg L^{-1} of IBU + 6 mg L^{-1} of Al

2.4. Assays of oxidative stress induced by individual compounds and mixtures

After exposure, specimens were anesthetized on ice to death and brain, gills, liver, and gut tissues were removed. Organs were suspended in tubes containing buffer solution (pH 7.4), homogenized, centrifuge at 12,500 rpm at a temperature of −4 ± 0.5 °C for 15 min and stored at −79 ± 2 °C. Each sample consisted of combined entire tissues from three fishes.

2.4.1. Lipid peroxidation LPO

This determination was performed according to the TBA reactive substances method (TBARS) as described by Buege and Aust (1978). The malondialdehyde (MDA) content was measured at a wavelength of 535 nm against a blank. Triplicates were performed. The content of MDA was calculated with the MEC molar extinction coefficient = 1.56 × 10^{-5} mM^{-1} cm^{-1} and dilution factors. Values were expressed as millimolar of MDA mg^{-1} of protein tissue.

2.4.2. Protein carbonyl content PCC

Was determined using the method proposed by Levine et al. (1994) with modifications (Burcham, 2007; Parvez and Raisuddin, 2005). The content of carbonyls content was read at a wavelength of 366 nm and results were expressed as nM of reactive carbonyls formed (C=O) mg^{-1} of protein tissue. The molar extinction coefficient = 21,000 M mc^{-1} and dilution factors were also used.

2.4.3. Superoxide dismutase activity

Was determined according to the pyrogallol method proposed by Marklund and Marklund (1974) and modified by Li (2012). The activity was measure spectrophotometrically at 420 nm at intervals of 30 s during 5 min against a reaction blank. Activity values were expressed as IU SOD mg^{-1} of protein tissue. Triplicates were performed.

2.4.4. Catalase

The activity of catalase was performed using the methodology proposed by Radi et al. (1991). Samples and blanks were read at 240 nm after 5 s and 60 s respectively. The MEC of H_2O_2 = 0.043 mM^{-1} cm^{-1} was used to determine the enzymatic activity. Results were expressed as uM H_2O_2 mg^{-1} of protein tissue. Triplicates were performed.

2.4.5. Glutathione peroxidase

The enzymatic activity was determined as specified by Gonzler (1984) with modifications performed by García-Medina et al. (2013). The activity of GPX was measure spectrophotometrically determined at 340 nm against a reaction blank, after intervals of 5 s and 60 s respectively. The MEC of NADPH = 6.2 mM^{-1} cm^{-1} was used to determine the antioxidant activity. Values were expressed as mM NADPH mg^{-1} of protein. Triplicates were performed.

2.4.6. Protein content

This analysis was performed following the method proposed by Bradford (1976) using the Coomassie brilliant blue g-250 and BSA as a standard. Absorption was spectrophotometrically determined at 595 nm against a reaction blank and results were expressed as mg^{-1} protein wet tissue. A BSA standard curve was prepared to interpolate the results. Triplicates were performed.

2.5. Statistical analysis

All results were assessed by one-way ANOVA to test differences among treatments (controls, separately compounds and mixtures) and differences between means were evaluated with a Tukey HSD multiple comparison test ($P < 0.05$) by using the SPSS v10 program (SPSS, Chicago IL). Analysis of correlations between concentrations of IBU and Al present in tissues and biomarkers evaluated was performed by using the Pearson's correlation test (SPSS, Chicago IL).

3. Results

3.1. Quantitation of IBU and Al in water samples and tissues of zebrafish (D. rerio)

Ibuprofen and metabolite 2-OH-IBU concentrations in different matrices are presented in Table 3. The mean measured concentrations of IBU in test waters after each exposure time in both separately and binary mixtures ranged between 60 and 81% (0.1 μg L^{-1}) and 57–78% (11 μg L^{-1}) the nominal concentration. Concentrations of the hydroxylated metabolite 2-OH-IBU in test water remain relatively constant throughout the test as a constant renewal of medium was performed. Increasing concentrations of IBU in tissues were observed over the time. Tissues with the highest uptake of IBU were gills and liver. Lower uptake values were found in the brain and gut.

Analytical quantitation of Al in different matrices is presented in Table 4. The mean measured concentrations of aluminum in test waters after each exposure time in both individual forms and binary mixtures ranged between 57 and 81% (0.05 mg L^{-1}) and 74–84% (6 mg L^{-1}) the nominal concentration. As observed, sustained renewal of medium every 48-h maintained relatively constant concentrations of Al in test waters throughout exposure times. In general, results indicate that concentrations of Al increased over the time in all evaluated tissues. Tissues with the highest uptake of Al were gills and gut while the lowest uptake was observed in liver and brain.

3.2. Biomarkers of cellular oxidation and antioxidant activity in tissues of zebrafish (D. rerio)

3.2.1. LPO

LPO results for separately compounds and binary mixtures are shown in Fig. 1. In general MDA concentration was significantly increased in all tissues and times of exposure for Mixture 2 concerning the non-exposed group ($P < 0.05$). Similar behavior was shown by M1 mainly in brain and gills at different times of exposure. Also, the individual form of Al at 6 mg L^{-1} showed significant increases in gills, liver and gut. Significantly higher values concerning the non-exposed group ($P < 0.05$) were observed for IBU (11 μg L^{-1}) in liver after 7, 14 and 28 d. On the other hand, MDA values of the lowest concentrations of both toxicants did not significantly change. In general, significant differences within separately compounds and both mixtures were found at all times of exposure in all evaluated tissues.

3.2.2. PCC

PCC results for compounds alone and binary mixtures are shown in Fig. 2. Carbonyl concentration was significantly increased in all evaluated tissues and times of exposure for specimens exposed to M2. Similar behavior was shown by M1 in all tissues at different times concerning the non-exposed group ($P < 0.05$). Also, the highest concentration of Al showed increased values in all tissues at different times of exposure. On

Table 3
Concentrations of IBU and 2-OH-IBU in water and tissues of *D. rerio* exposed to binary mixtures for 28 d.

Exposure concentration	Exposure time (days)	IBU in water system (μg L^{-1})	2-OH-IBU in water system (μg L^{-1})	IBU in brain (ng g^{-1})	IBU in gills (ng g^{-1})	IBU in liver (ng g^{-1})	IBU in gut (ng g^{-1})
0.1 μg L^{-1}	Control	ND	ND	ND	ND	ND	ND
	0	0.1025 ± 0.001	ND	ND	ND	ND	ND
	7	0.0831 ± 0.001	0.0134 ± 0.0002	ND	0.021 ± 0.002	0.012 ± 0.003	0.031 ± 0.002
	14	0.0677 ± 0.001	0.0209 ± 0.0002	0.0035 ± 0.0006	0.043 ± 0.003	0.025 ± 0.002	0.048 ± 0.006
	21	0.0784 ± 0.006	0.0107 ± 0.0001	0.0043 ± 0.0004	0.065 ± 0.001	0.048 ± 0.001	0.038 ± 0.002
	28	0.0729 ± 0.001	0.0148 ± 0.0002	0.0027 ± 0.0007	0.076 ± 0.004	0.063 ± 0.002	0.043 ± 0.003
11 μg L^{-1}	Control	ND	ND	ND	ND	ND	ND
	0	11.09 ± 0.3	ND	ND	ND	ND	ND
	7	7.75 ± 0.2	0.272 ± 0.002	2.52 ± 0.5	23.8 ± 0.7	24.9 ± 0.3	19.0 ± 0.5
	14	6.34 ± 0.1	0.326 ± 0.001	3.70 ± 0.8	47.7 ± 0.7	39.8 ± 0.2	23.1 ± 0.4
	21	7.66 ± 0.4	0.201 ± 0.002	5.31 ± 0.4	71.6 ± 0.9	44.8 ± 0.2	18.8 ± 0.3
	28	7.12 ± 0.5	0.215 ± 0.003	4.14 ± 0.9	55.4 ± 0.3	49.7 ± 0.5	26.3 ± 0.4
Mixture 1	Control	ND	ND	ND	ND	ND	ND
	0	0.1057 ± 0.003	ND	ND	ND	ND	ND
	7	0.0783 ± 0.003*	0.0106 ± 0.0005*	0.0029 ± 0.0002*	0.047 ± 0.006*	0.027 ± 0.003*	0.039 ± 0.005
	14	0.0706 ± 0.001	ND*	0.0052 ± 0.0006	0.066 ± 0.007*	0.035 ± 0.002*	0.055 ± 0.004
	21	0.0636 ± 0.004*	0.0103 ± 0.0003	0.0067 ± 0.0003*	0.059 ± 0.009	0.053 ± 0.002	0.044 ± 0.003
	28	0.0647 ± 0.006*	0.0134 ± 0.0001	0.0089 ± 0.0007*	0.067 ± 0.003	0.057 ± 0.005	0.117 ± 0.004*
Mixture 2	Control	ND	ND	ND	ND	ND	ND
	0	11.04 ± 0.46	ND	ND	ND	ND	ND
	7	7.04 ± 0.67	0.214 ± 0.02	1.54 ± 0.5*	23.47 ± 0.6	25.78 ± 0.3	20.39 ± 0.5
	14	6.64 ± 0.62	0.304 ± 0.07	2.42 ± 0.8*	35.66 ± 0.4*	42.75 ± 0.2	25.98 ± 0.4
	21	8.67 ± 0.14*	0.203 ± 0.09	4.05 ± 0.3*	34.87 ± 0.9*	51.33 ± 0.2*	34.94 ± 0.3*
	28	6.88 ± 0.22*	0.289 ± 0.08*	5.68 ± 0.7*	67.3 ± 0.3*	58.87 ± 0.5*	39.45 ± 0.4*

Mean values of three replicates ± SE. ND = Non-detected. *Significantly different from individual exposure systems ($P < 0.05$), ANOVA, and Tukey HSD.

the other hand, carbonyls were increased after exposure to the lowest concentration of Al in liver and gills at all times of exposure and 28 d, respectively. The highest concentration of IBU showed statistically significant differences concerning the non-exposed group ($P < 0.05$) in gills at 7 d and liver after 28 d of exposure. In general, significant differences within separately compounds and mixtures were found at all times of exposure in all evaluated tissues.

3.2.3. SOD

The values of antioxidant activity of SOD enzyme are shown in Fig. 3. For specimens exposed to individual forms, no differences with statistical significance when compared to the non-exposed group ($P < 0.05$) occurred for IBU or Al at the lowest concentrations. SOD activity significantly increased for mixture M2 in all tissues at different times of exposure in comparison to the non-exposed group ($P < 0.05$). In liver, the highest concentration of IBU showed an increased antioxidant activity at 7, 14 and 28 d as well as gut at 14 d. Likewise, statistically increased values of enzymatic activity occurred for M1 in gills, liver, and gut at different times of exposure. Significant differences within separately compounds and mixtures were found mainly in gills, liver and gut.

3.2.4. CAT

The antioxidant activity of CAT enzyme is shown in Fig. 4. For specimens exposed to separately forms at the lowest concentrations no significant differences regarding the non-exposed group ($P < 0.05$) occurred. Significant increased values were observed for M1 and M2 in all tissues at different times of exposure. Enzymatic activity significantly incremented concerning the non-exposed group ($P < 0.05$) in gills, liver, and gut for Al (6 mg L^{-1}) at different times of exposure. The highest concentration of IBU showed a statistically significant increased at 21 d in brain. In general, significant differences within compounds alone and both mixtures were observed after all times of exposure in all evaluated tissues.

3.2.5. GPX

The values of antioxidant activity of GPX are shown in Fig. 5. Significant differences of activity concerning the non-exposed specimens ($P < 0.05$) occurred for M2 in all evaluated tissues at various times of exposure. Enzymatic activity for M1 also increased in brain, gills and liver. Likewise, Al (6 mg L^{-1}) induced the activity in all evaluated tissues, regarding the non-exposed group ($P < 0.05$). IBU (11 μg L^{-1}) increased the activity in gills and liver at 21 and 28 d. The lowest concentrations of IBU and Al, significantly increased the GPX activity in gills and gut at 21 and 7 d, respectively concerning the non-exposed group ($P < 0.05$). Differences with statistical significance were found between individual forms and the binary mixtures at different times and tissues.

3.3. Pearson correlation

Table 5 (Supplemental data) shows correlation coefficients between concentrations of compounds in tissues of *D. rerio* exposed to separately forms as well as binary mixtures and values of each evaluated biomarker of oxidative stress. High degree of correlation (˃0.8) between variables is shown in bold letters. As observed, concentrations in tissues of specimens exposed to binary mixtures and values of biomarkers of cellular oxidation and antioxidant enzymes are highly correlated.

3.4. Additive interactions

Table 6 shows real values interaction and calculated additivity of IBU and Al tested in *D. rerio*.

Additive values of environmental concentrations of IBU and Al were compared with two binary mixtures. In general, for LPO and PCC, real values of M1 and M2 were statistically higher ($P < 0.05$) than calculated minimal and maximal additive interactions values. SOD values were found to be variable for binary mixtures with respect to both additive interactions. In general, CAT and GPX values were found to be significantly lower ($P < 0.05$) for binary mixtures at different tissues and times of exposure concerning the calculated additive interactions (minimal and maximal).

Table 4
Concentrations of Al present in water and tissues of *D. rerio* exposed to binary mixtures for 28 d.

Exposure concentration	Exposure time (days)	Total Al in water system (mg L^{-1})	Al in brain (μg g^{-1})	Al in gills (μg g^{-1})	Al in liver (μg g^{-1})	Al in gut (μg g^{-1})
0.05 mg L^{-1}	Control	ND	ND	ND	ND	ND
	0	0.0531 ± 0.005	ND	ND	ND	ND
	7	0.0342 ± 0.003	0.013 ± 0.003	0.068 ± 0.002	0.049 ± 0.003	0.043 ± 0.002
	14	0.0364 ± 0.004	0.024 ± 0.006	0.098 ± 0.003	0.091 ± 0.002	0.035 ± 0.006
	21	0.0401 ± 0.004	0.015 ± 0.004	0.125 ± 0.01	0.086 ± 0.001	0.052 ± 0.002
	28	0.0324 ± 0.004	0.036 ± 0.007	0.156 ± 0.04	0.126 ± 0.02	0.068 ± 0.003
6 mg L^{-1}	Control	ND	ND	ND	ND	ND
	0	6.032 ± 0.2	ND	ND	ND	ND
	7	5.104 ± 0.3	1.09 ± 0.05	3.89 ± 0.06	3.16 ± 0.03	2.71 ± 0.05
	14	5.213 ± 0.5	1.98 ± 0.08	7.78 ± 0.07	6.33 ± 0.02	7.32 ± 0.04
	21	4.805 ± 0.03	1.60 ± 0.04	16.65 ± 0.02	6.64 ± 0.02	10.48 ± 0.03
	28	4.465 ± 0.02	2.38 ± 0.08	13.68 ± 0.03	7.43 ± 0.05	13.67 ± 0.04
Mixture 1	Control	ND	ND	ND	ND	ND
	0	0.0524 ± 0.002	ND	ND	ND	ND
	7	0.0323 ± 0.003	0.012 ± 0.005	0.055 ± 0.006*	0.027 ± 0.003*	0.054 ± 0.005
	14	0.0362 ± 0.003	0.028 ± 0.008	0.092 ± 0.007	0.035 ± 0.002*	0.091 ± 0.004*
	21	0.0424 ± 0.002	0.039 ± 0.003*	0.152 ± 0.009*	0.098 ± 0.002*	0.131 ± 0.003*
	28	0.0401 ± 0.006*	0.027 ± 0.007*	0.168 ± 0.003*	0.142 ± 0.005*	0.169 ± 0.004*
Mixture 2	Control	ND	ND	ND	ND	ND
	0	6.075 ± 0.4	ND	ND	ND	ND
	7	5.617 ± 0.6	1.47 ± 0.05	3.28 ± 0.06	2.61 ± 0.03*	3.85 ± 0.05*
	14	4.603 ± 0.6*	1.14 ± 0.08*	8.09 ± 0.04	5.98 ± 0.02	5.28 ± 0.04*
	21	5.702 ± 0.1*	2.28 ± 0.03*	11.88 ± 0.09*	5.58 ± 0.02*	10.09 ± 0.03
	28	4.703 ± 0.2	3.56 ± 0.07*	10.03 ± 0.03*	8.64 ± 0.05*	16.92 ± 0.04*

Mean values of three replicates ± SE. ND = Non-detected. *Significantly different from individual exposure systems ($P < 0.05$), ANOVA, and Tukey HDS.

4. Discussion

Exposure to pharmaceutical compounds and metals promotes the overproduction of radical species and induces harmful effects such as oxidative damage and depletion of antioxidant defenses in aquatic species (Abdalla et al., 2019; Sivakumar et al., 2012). In Mexico, regulatory authorities have established maximum concentrations permitted of Al for drinking water (0.02 mg L^{-1}) and aquatic life protection (0.05–0.1 mg L^{-1}) (SEDUE, 1989), nevertheless the presence of pharmaceuticals in aquatic ecosystems remains unregulated. On this subject, the present study aims to evaluate whether long-term exposure to individual forms and binary mixtures of Ibuprofen and Al is capable of stimulate toxicity mechanisms involved in the oxidative damage to target molecules in zebrafish *D. rerio*.

IBU is a propionic acid derivate (Montes et al., 2016) known to intervene in the synthesis of prostaglandins (PGE_2) in humans and animals (Manku et al., 2019; Wagner et al., 2019). IBU undergoes abiotic and biotic reactions due to environmental conditions and diverse cytochrome P450 (CYP) enzymes (Gagné et al., 2006; Islas-Flores et al., 2017). As in mammals, similar IBU biotic transformation products and metabolic pathways have been found in fish species. According to Gomez et al. (2011), 2-hydroxy-ibuprofen (2-OH-IBU) was the major metabolite found in different organs of *Oncorhynchus mykiss*. Also, Jones et al. (2012) observed that *Danio rerio* larvae were capable of metabolize IBU to hydroxy-ibuprofen (OH-IBU) suggesting that the CYP450 isoforms found in mammals are also present in zebrafish tissues. As noted in Table 3, IBU parent compound and metabolite 2-OH-IBU were both detected in water samples at different concentrations. As stated in previous studies, the presence of this hydroxylated metabolite in test waters indicates that *D. rerio* is capable of metabolize IBU to similar products than mammals and other fish species.

Uptake and bioconcentration of pharmaceutical compounds depend on physicochemical properties like lipophilicity (K_{ow}) and half-life in biological systems (distribution, metabolism and excretion) (Bhandari and Venables, 2011). Ibuprofen hydrophobic character (Log $K_{ow} = 3.97$) indicates that bioconcentration in fish tissues is possible (Ferrando-Climent et al., 2012). As observed in our study, IBU concentration increased over the time in evaluated tissues mainly gills and liver while decreasing in water systems. Similar results were observed by Islas-Flores et al. (2014) who noted that IBU may bioconcentrate as concentration increased in various tissues of common carp exposed to IBU after an acute test. Contrarily, Nallani et al. (2011) noted that even though IBU concentrations fluctuated in tissues of different teleost fish, BCF levels (0.08–1.4) suggested that IBU may not bioconcentrate to levels higher than nominal exposure concentrations.

Aluminum is considered no essential for the biota, its bioavailability is strongly affected by physicochemical parameters of the aquatic environment (Santore et al., 2018; Trenfield et al., 2012). Toxicity of Al is strongly affected by solubility and speciation, parameters directly correlated with water pH changes (DeForest et al., 2018). In our study, the test water pH = 6.0–6.3 was considered as it represents the lowest pH range of a many natural waters found in Europe, USA and Mexico (Cardwell et al., 2018; González-González et al., 2014).Moreover, bioavailability of metals increases when pH is lower than 6.5 as more soluble forms are found (Coz et al., 2004). Al concentrations increased over the time, with higher uptake found in gills and gut (Table 4). Similar to this study, teleost fish *Cirrhinus mrigala*, *Geophagus brasiliensis*, *O. mossambicus* and *M. salmoides* presented higher concentrations of Al in gills and gut tissues suggesting that this metal is capable of bioaccumulate after long-term exposure (Oberholster et al., 2012; Sivakumar et al., 2012; Voigt et al., 2015). Bioconcentration leads to the generation of long-term toxic effects due to increases in the internal tissue concentrations (Nallani et al., 2011).

Lipid peroxidation is an oxidative phenomenon by which polyunsaturated fatty acids, react with ROS and RNS to form hydroperoxides, MDA and 4-hydroxy-2-nonenal as final products (Gentile et al., 2017; Guéraud et al., 2010; Wilhelm Filho et al., 2005; Żur et al., 2018). As can be seen in Fig. 1, in general, higher values of MDA were observed in specimens exposed to binary mixtures than those exposed to separately forms, being liver and gut the most damaged tissues. On the other hand, inactivation of proteins due to oxidative damage has been reported (Wong et al., 2008). Yao and Rahman (2011) mentioned that protein carbonylation which is the generation of reactive carbonyl groups such as aldehydes, ketones and methylglyoxal, among others

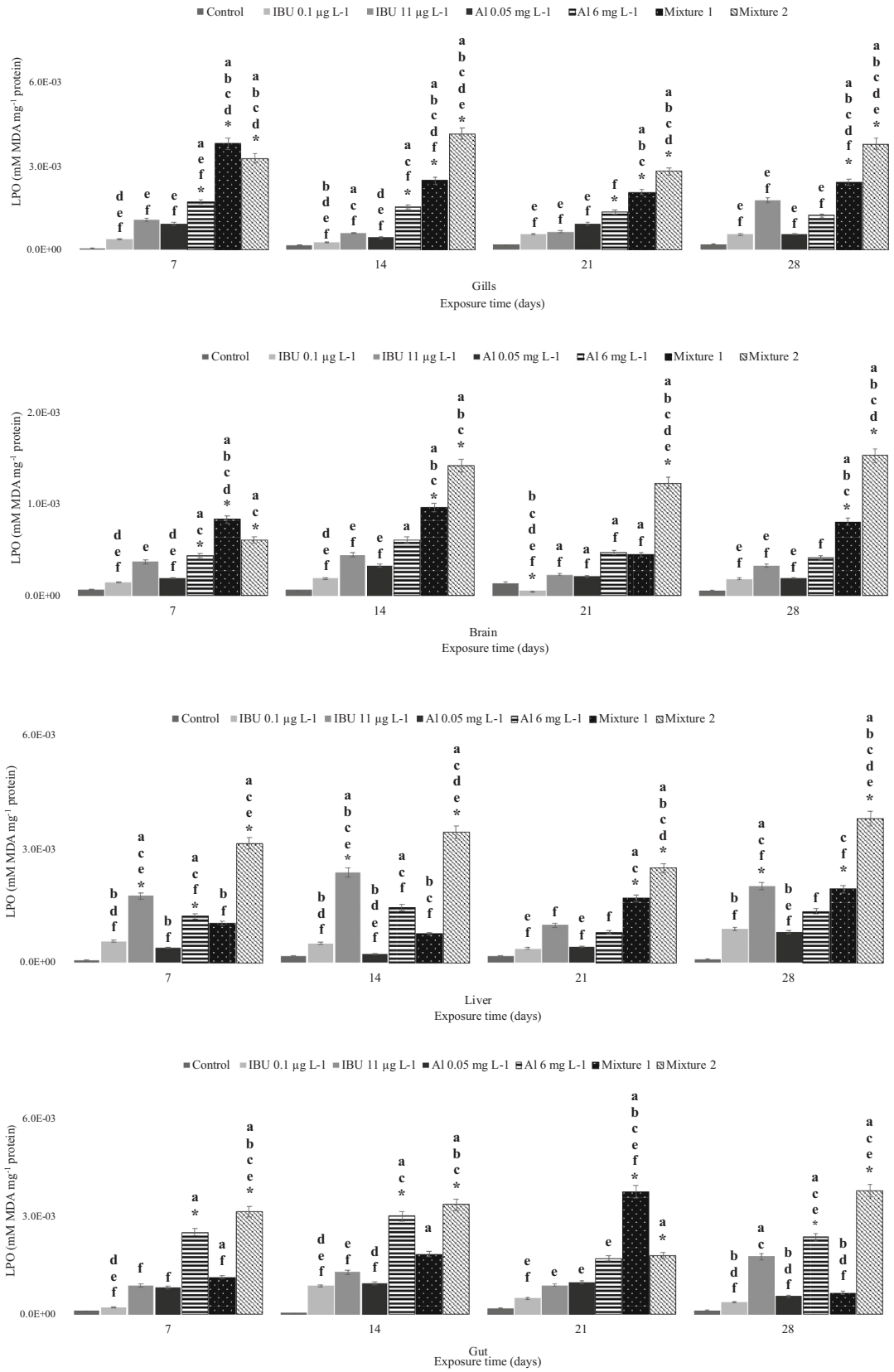

Fig. 1. LPO determination in brain, gills, liver and gut tissues of *Danio rerio* after exposure to IBU, Al and binary mixtures at 7, 14, 21 and 28 days. * Statistically different values regarding the non-exposed group ($P < 0.05$). Letters show statistically different values in comparison to specimens exposed to [a] ibuprofen 0.1 µg L^{-1}, [b] ibuprofen 11 µg L^{-1}, [c] aluminum 0.05 mg L^{-1}, [d] aluminum 6 mg L^{-1}, [e] mixture 1 (M1), [f] mixture 2 (M2). ANOVA and Tukey HSD Test.

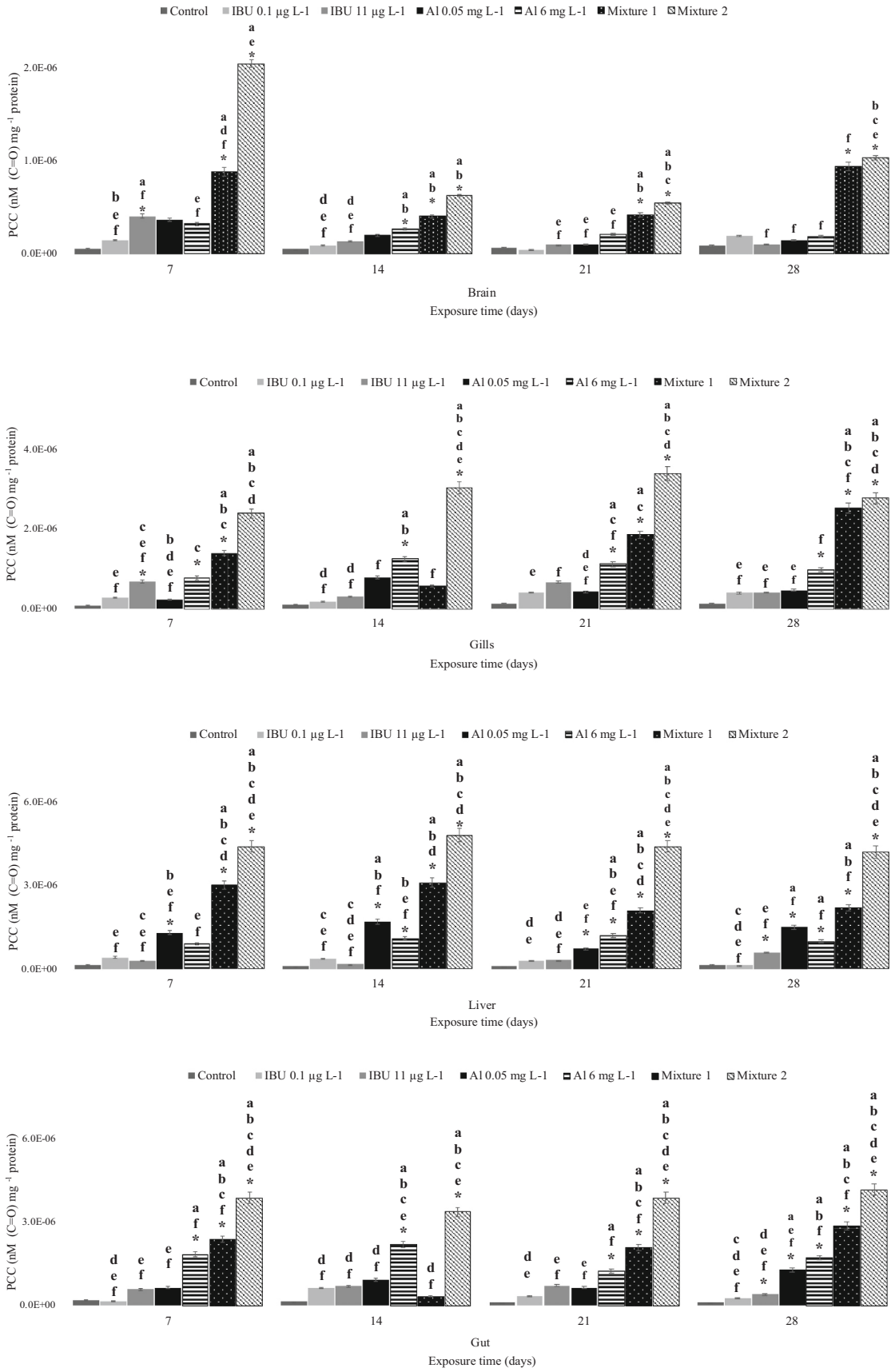

Fig. 2. PCC determination in brain, gills, liver and gut of *Danio rerio* after exposure to IBU, Al and binary mixtures at 7, 14, 21 and 28 days. * Statistically different values regarding the non-exposed group ($P < 0.05$). Letters show statistically different values in comparison to specimens exposed to ^{a} ibuprofen 0.1 μg L^{-1}, ^{b} ibuprofen 11 μg L^{-1}, ^{c} aluminum 0.05 mg L^{-1}, ^{d} aluminum 6 mg L^{-1}, ^{e} mixture 1 (M1), ^{f} mixture 2 (M2). ANOVA and Tukey HSD Test.

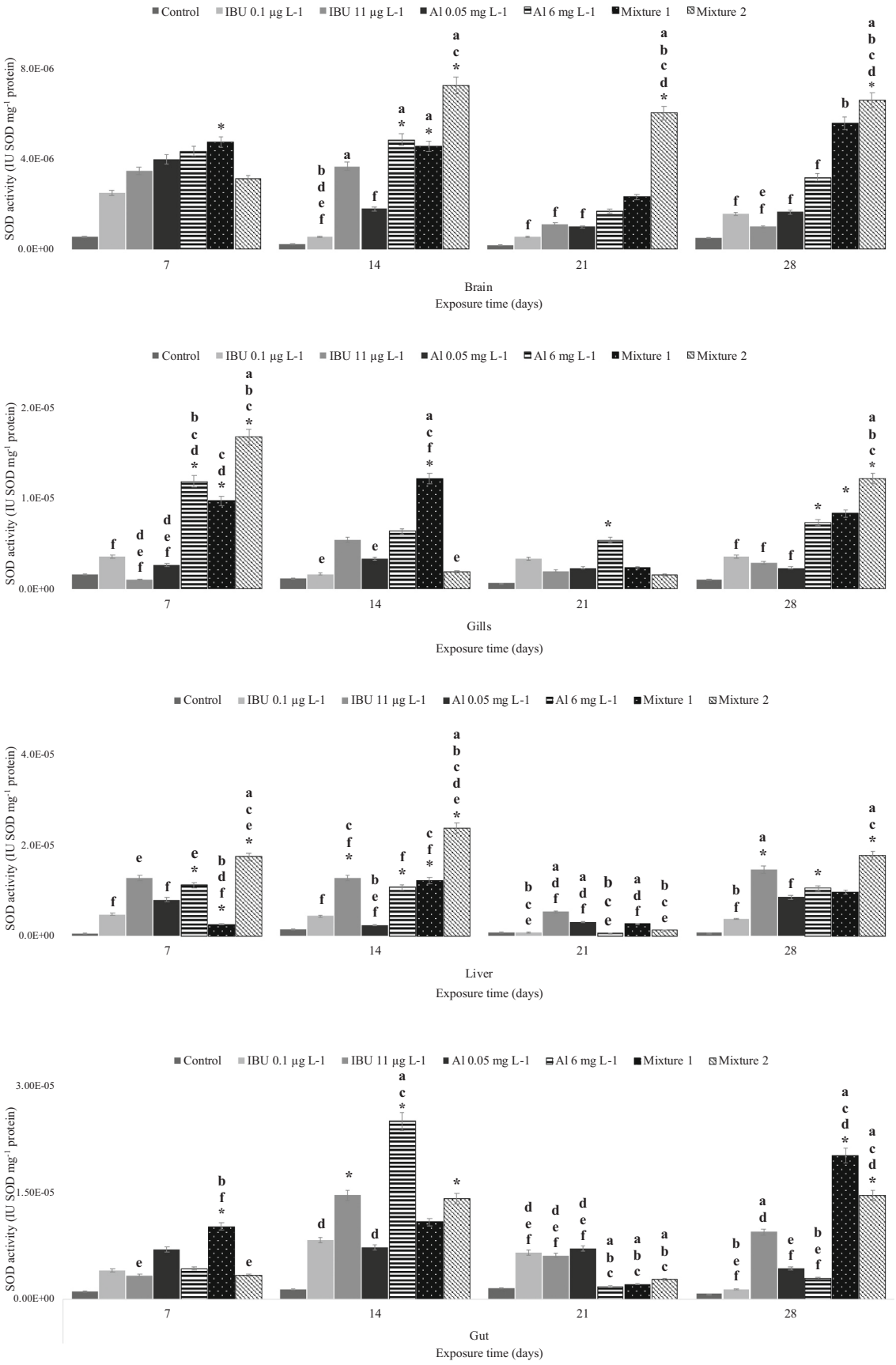

Fig. 3. Superoxide dismutase (SOD) enzymatic activity in brain, gills, liver and gut of *Danio rerio* after exposure to IBU, Al and binary mixtures at 7, 14, 21 and 28 days. * Statistically different values with respect to non-exposed group ($P < 0.05$). Letters show statistically different values in comparison to specimens exposed to [a] ibuprofen 0.1 µg L^{-1}, [b] ibuprofen 11 µg L^{-1}, [c] aluminum 0.05 mg L^{-1}, [d] aluminum 6 mg L^{-1}, [e] mixture 1 (M1), [f] mixture 2 (M2). ANOVA and Tukey HSD Test.

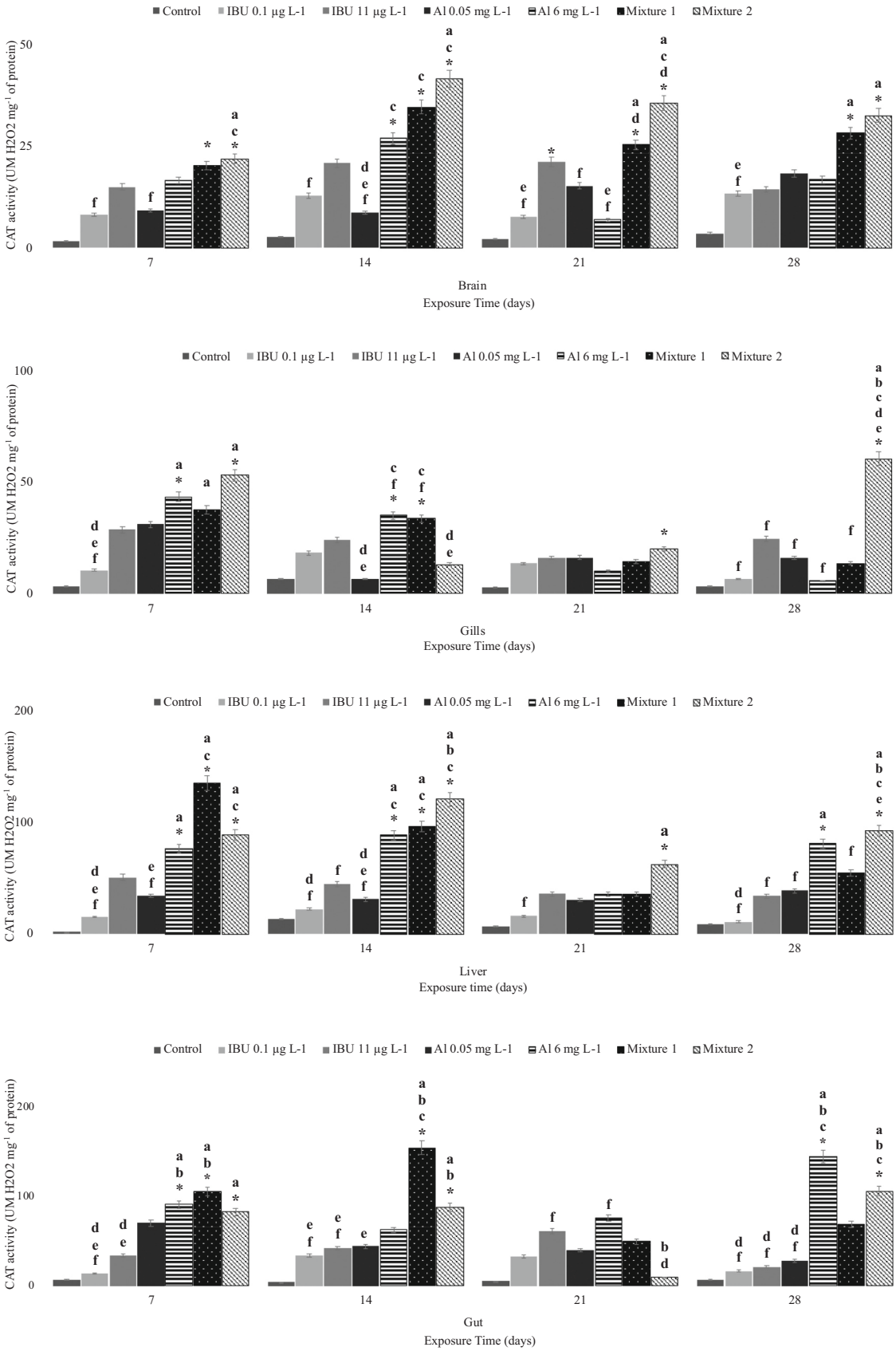

Fig. 4. Catalase (CAT) enzymatic activity in brain, gills, liver and gut of *Danio rerio* after exposure to concentrations of IBU, Al and binary mixtures at 7, 14, 21 and 28 days. * Statistically different values with respect to non-exposed group ($P < 0.05$). Letters show statistically different values in comparison to specimens exposed to [a] ibuprofen 0.1 µg L^{-1}, [b] ibuprofen 11 µg L^{-1}, [c] aluminum 0.05 mg L^{-1}, [d] aluminum 6 mg L^{-1}, [e] mixture 1 (M1), [f] mixture 2 (M2). ANOVA and Tukey HSD Test.

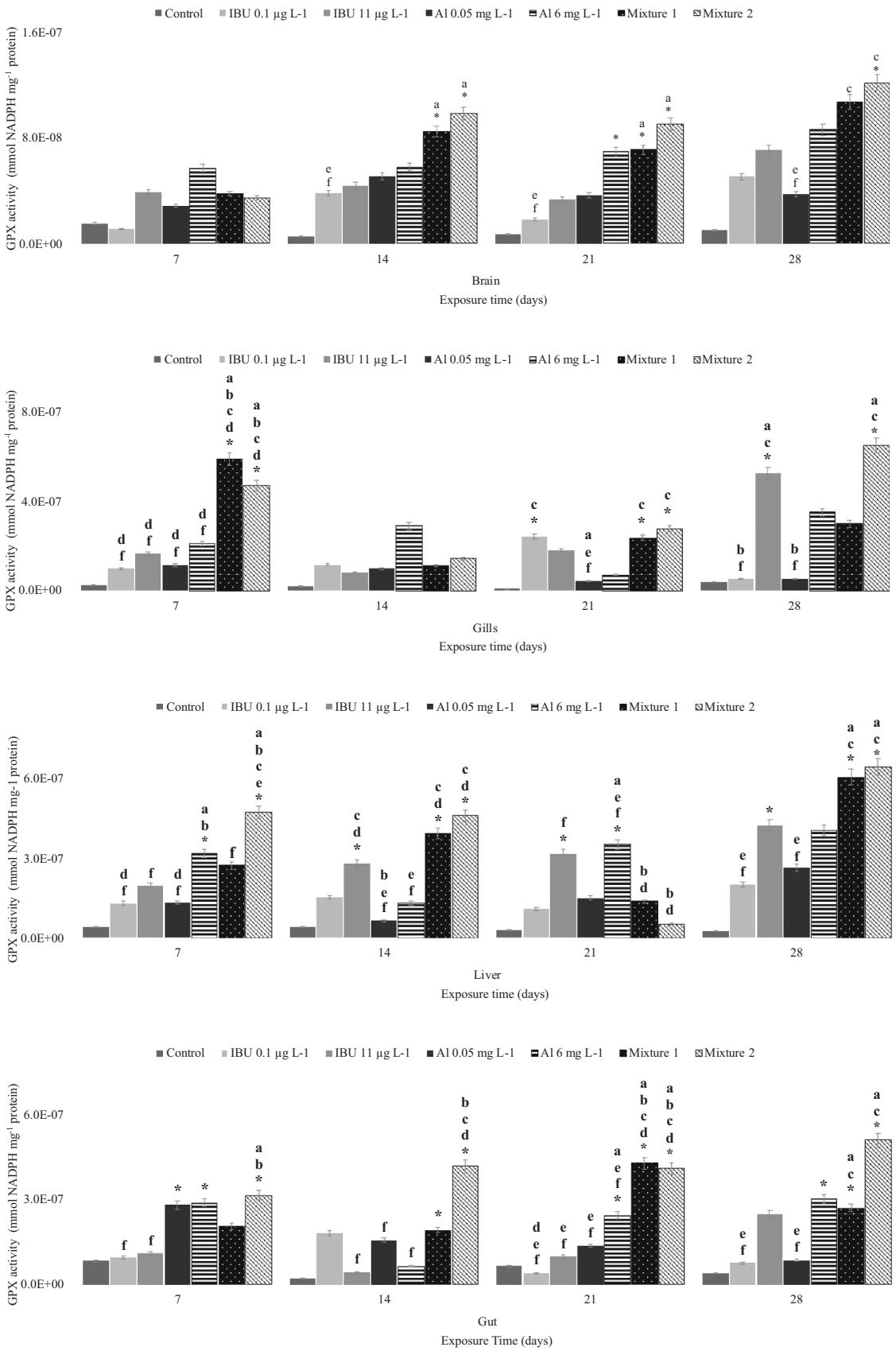

Fig. 5. Glutathione peroxidase (GPX) enzymatic activity in brain, gills, liver and gut of *Danio rerio* after exposure to concentrations of IBU, Al and binary mixtures at 7, 14, 21 and 28 days. * Statistically different values with respect to non-exposed group ($P < 0.05$). Letters show statistically different values in comparison to specimens exposed to [a] ibuprofen 0.1 µg L^{-1}, [b] ibuprofen 11 µg L^{-1}, [c] aluminum 0.05 mg L^{-1}, [d] aluminum 6 mg L^{-1}, [e] mixture 1 (M1), [f] mixture 2 (M2). ANOVA and Tukey HSD Test.

Table 6
Biomarkers values of calculated additive interactions among IBU and Al alone and the actual values obtained after exposure to mixtures of both pollutants.

Biomarker	Time (d)	Tissue	Minimal additive interaction	Maximal additive interaction	Actual value M1	Actual value M2	Biomarker	Time (d)	Tissue	Minimal additive interaction	Maximal additive interaction	Actual value M1	Actual value M2
LPX (mM MDA mg^{-1} protein)	7	Brain	0.00034	0.00081	0.00084[a]	0.00061[a]	PCC (nM of reactive carbonyls (C=O) mg-1 protein)	7	Brain	5.0E-07	8.4E-04	8.8E-07[a,b]	2.0E-06[a,b]
		Gills	0.00132	0.00106	0.00380[a,b]	0.00328[a,b]			Gills	5.2E-07	5.3E-06	1.4E-06[a]	2.4E-06[a]
		Liver	0.00235	0.00070	0.00105[a,b]	0.00315[a,b]			Liver	1.7E-06	8.4E-04	2.9E-06[a,b]	4.4E-06[a,b]
		Gut	0.00105	0.00075	0.00115[b]	0.00317[a,b]			Gut	8.2E-07	3.9E-06	2.4E-06[a]	3.9E-06[a]
	14	Brain	0.00052	0.00270	0.00097[a,b]	0.00142[a,b]		14	Brain	2.8E-07	1.2E-06	4.0E-07[a,b]	6.2E-06[a,b]
		Gills	0.00068	0.00281	0.00248[a]	0.00416[a,b]			Gills	4.2E-07	6.1E-06	5.7E-07[a,b]	8.9E-06[a,b]
		Liver	0.00075	0.00213	0.00077[b]	0.00345[a,b]			Liver	2.1E-06	5.1E-06	3.1E-06[a]	7.8E-06[b]
		Gut	0.00184	0.00198	0.00185	0.00337[a,b]			Gut	1.6E-06	3.4E-06	3.3E-07[a,b]	3.4E-06
	21	Brain	0.00026	0.00070	0.00046[b]	0.00123[a,b]		21	Brain	1.4E-07	2.4E-06	4.2E-07[a,b]	5.1E-06[b]
		Gills	0.00148	0.00213	0.00207[a]	0.00281[a,b]			Gills	8.3E-07	5.5E-06	1.8E-06[a,b]	3.4E-06[a]
		Liver	0.00080	0.00181	0.00170[a]	0.00250[a,b]			Liver	1.0E-06	6.5E-06	2.1E-06[a,b]	1.0E-05[a,b]
		Gut	0.00148	0.00263	0.00378[a,b]	0.00181[b]			Gut	1.0E-06	3.9E-06	2.1E-06[a]	3.9E-06
	28	Brain	0.00037	0.00075	0.00081[a]	0.00154[a,b]		28	Brain	3.3E-07	3.5E-06	9.4E-07[a,b]	7.9E-06[a,b]
		Gills	0.00111	0.00198	0.00242[a,b]	0.00379[a,b]			Gills	8.7E-07	4.9E-06	2.5E-06	2.7E-06[a]
		Liver	0.00171	0.00337	0.00195[b]	0.00381[a]			Liver	1.6E-06	7.1E-06	2.2E-06[a,b]	1.0E-05[b]
		Gut	0.00092	0.00416	0.00066[a,b]	0.00381[a]			Gut	1.5E-06	4.2E-06	2.9E-06[a]	2.3E-05[b]
SOD activity (IU SOD mg^{-1} protein)	7	Brain	5.9E-06	7.8E-06	1.3E-06[a,b]	4.2E-06[a]	CAT activity (umol H2O2 mg^{-1} protein)	7	Brain	23.08	46.27	9.21[a,b]	16.59[a,b]
		Gills	1.6E-06	1.3E-05	4.6E-06[a,b]	3.8E-06[a,b]			Gills	39.16	52.54	28.13[a,b]	43.51
		Liver	1.3E-05	2.4E-05	6.6E-06[a,b]	1.5E-05			Liver	66.44	71.60	34.28[a,b]	76.49
		Gut	5.8E-06	7.6E-06	5.0E-06[a]	1.2E-05[a,b]			Gut	47.92	79.00	70.55[a]	90.83[a,b]
	14	Brain	6.1E-06	8.5E-06	4.5E-06[a,b]	3.8E-06[a]		14	Brain	33.66	55.92	8.59[a,b]	26.92[b]
		Gills	7.9E-06	1.2E-05	4.4E-06[a,b]	4.9E-06[a,b]			Gills	42.55	68.47	6.69[a,b]	35.14[b]
		Liver	1.5E-05	2.4E-05	1.1E-05	9.3E-06			Liver	67.53	150.96	31.20[a,b]	88.65[a,b]
		Gut	1.7E-05	4.0E-05	9.1E-06[a,b]	1.9E-05[b]			Gut	76.88	120.89	44.20[a,b]	62.45[b]
	21	Brain	3.6E-06	2.8E-06	3.7E-06	1.7E-06		21	Brain	28.83	22.89	15.27	6.86[a,b]
		Gills	4.5E-06	7.4E-06	3.8E-06	5.0E-06[b]			Gills	29.47	20.43	16.30	10.13[a,b]
		Liver	7.8E-06	5.9E-06	1.1E-05[a,b]	1.9E-05[a,b]			Liver	52.10	73.42	30.88[b]	35.93[a,b]
		Gut	8.6E-06	7.9E-06	6.7E-06[a]	3.7E-06[a]			Gut	93.99	98.83	40.14[a,b]	75.73[a]
	28	Brain	3.5E-06	4.2E-06	2.1E-06[a]	1.6E-06		28	Brain	27.67	41.70	18.37[b]	16.78[b]
		Gills	5.3E-06	1.0E-05	6.0E-06[b]	6.2E-06[b]			Gills	31.03	21.71	16.01	5.66[a,b]
		Liver	1.7E-05	2.5E-05	1.3E-05	1.5E-05			Liver	45.28	117.37	38.97[b]	81.01[a]
		Gut	1.2E-05	1.2E-05	8.3E-06[a,b]	4.7E-06[a,b]			Gut	38.29	174.98	28.19[b]	144.67[a,b]
GPX activity (mmol NADPH mg-1 protein)	7	Brain	5.0E-08	9.6E-08	7.3E-09[a,b]	1.9E-08[a,b]							
		Gills	2.7E-07	3.8E-07	1.2E-07	4.2E-07							
		Liver	3.3E-07	5.1E-07	1.6E-07[a]	3.0E-07							
		Gut	2.1E-07	4.0E-07	4.9E-07[a]	5.5E-07[a]							
	14	Brain	8.2E-08	1.0E-07	2.1E-08[a,b]	7.7E-08[b]							
		Gills	2.0E-07	3.7E-07	8.5E-08[a,b]	1.7E-07[b]							
		Liver	4.3E-07	4.1E-07	1.6E-07[a]	3.4E-07							
		Gut	2.3E-07	1.1E-07	4.6E-08[a,b]	1.5E-07							
	21	Brain	5.2E-08	1.0E-07	4.3E-08[b]								

(*continued on next page*)

Table 6 (*continued*)

Biomarker	Time (d)	Tissue	Minimal additive interaction	Maximal additive interaction	Actual value M1	Actual value M2	Biomarker	Time (d)	Tissue	Minimal additive interaction	Maximal additive interaction	Actual value M1	Actual value M2
						2.9E-08[a, b]							
		Gills	4.3E-07	2.5E-07	7.7E-08[a]	3.2E-08[a, b]							
		Liver	4.2E-07	6.7E-07	1.9E-07[b]	6.1E-08[a, b]							
		Gut	1.4E-07	3.4E-07	6.5E-08[a, b]	2.2E-07							
	28	Brain	1.2E-07	1.6E-07	1.1E-07	8.8E-08							
		Gills	5.8E-07	8.8E-07	2.6E-07[b]	5.6E-07[b]							
		Liver	6.2E-07	8.3E-07	2.4E-07[b]	3.2E-07[a, b]							
		Gut	3.3E-07	5.5E-07	4.9E-07[a]	4.2E-07							

[a] Statistically different to minimal additive values.
[b] Statistically different to maximal additive values. (Statistical test:Tukey HSD $P < 0.05$).

through the action of highly reactive hydroxyl radical molecules that oxidize the amino acid residues or the protein backbone, can be considered a major endpoint of oxidative stress as it causes irreversible modifications to the structure and loss of function of various proteins (Dalle-Donne et al., 2003; Fritz and Petersen, 2011; Stadtman and Levine, 2003). In general, specimens exposed to both mixtures showed the highest values of reactive carbonyls, therefore oxidative damage to proteins in all evaluated tissues was demonstrated. The liver is the key organ that participates in the storage, distribution, biotransformation, and detoxification of xenobiotics, therefore it is highly vulnerable to harmful effects induced by pollutants (Sivakumar et al., 2012). Metabolites like 2-OH-IBU may induce oxidative damage to biomolecules due to the formation of reactive species via redox cycling (Barata et al., 2005; Boelsterli, 2003). Also, molecules such as *p*-benzoquinones and IBU-derived glucuronides formed in the hepatocytes as biotransformation by-products, can interact directly with lipids present in the cell membrane as they possess a strong electrophilic behavior (Bakr and Rahaman, 2019; Brozinski et al., 2013; Oviedo-Gómez et al., 2010; Wilhelm et al., 2009). Our results are in accordance with Ogueji et al. (2017) who concluded that LPO values in liver tissue significantly increased after exposure to IBU on *Clarias gariepinus*. On the other hand, this organ which is considered a primary site of metal-binding proteins, may show high concentrations of heavy metals including Al (Sivakumar et al., 2012). Al is capable of promote the Fenton reaction as it alters the metabolism of metals by increasing their intracellular concentration (Ruipérez et al., 2012; Yousef, 2004). Similar to our study, Jolly et al. (2014) found that sub-chronic exposure to Al (100 μg L^{-1}) significantly increased lipid peroxidation products in the liver of *Rutilus* L. Also, Kryndushkin et al. (2017) demonstrated that protein carbonylation can be induced by metal-catalyzed oxidation, supporting the results obtained in this study.

Aquatic organisms are exposed to xenobiotics accumulation by both, aqueous and dietary pathways (EPA, 2017). The intestinal tract represents the main contact with the dietary environment by forming the largest adsorption surface area of the body and an important barrier between internal and external environments (Salim and Söderholm, 2011; Vignal et al., 2016). Increased values of LPO and PCC in gut of *D. rerio* observed in our study are in accordance with Goldsmith et al. (2013) who noted that NSAIDs were capable of induce mitochondrial and endoplasmic reticulum stress by ceasing cell stress response in the intestinal tissue of zebrafish specimens. Metal-induced oxidative stress in gut tissues has been previously studied. Zebrafish exhibited oxidative effects on gut tissue after exposure to 5 mg L^{-1} of ZnO nanoparticles due to the increased formation of $OH^{\bullet}$ (Xiong et al., 2011). Copper particles have also been related with the induction of oxidative stress in gut of grass carp *Ctenopharyngodon idella* (Jiang et al., 2016).

In teleost, gills represent the primary site of contact with the surrounding environment, hence they play a significant role in gas exchange, cellular and systemic osmotic and ionic regulation mechanisms as well as enzymatic activity including the activity of the COX system (Choe et al., 2006; Evans et al., 2005). As previously noted, gills showed the highest uptake of both, IBU and Al. Similar to our study, Gonzalez-Rey et al. (2014) found that non-steroidal inflammatory drugs along with copper were capable of promote higher lipid peroxidation in gills of *Mytilus galloprovincialis*. Furthermore, Wei and Yang (2015) demonstrated that metals are able to induce the production of reactive carbonyls in gills of freshwater crayfish.

In contrast, the brain is a particularly vulnerable tissue to radical species due to its high demand for oxygen, iron-rich constitution, restricted antioxidant defenses and its constitution highly rich in lipids (Dehay and Bezard, 2011; Friedman, 2011; Nunes et al., 2017). In our study, the brain presented the lowest values of MDA. Similar to our study, Jamil et al. (2016) concluded that IBU was able to decrease the expression of genes associated with neuropathology induced by $AlCl_3$ in a murine model. Moreover, Islas-Flores et al. (2014) noted that the brain presented no significant differences in biomarkers of oxidative stress after acute exposure to IBU in *C. carpio*. These results may be explained as brain presented the lowest uptake of both IBU and Al. Even though Al is able to cross the blood-brain barrier (Richetti et al., 2011) by binding to the transferrin transport protein (Supriadi et al., 2019), IBU cannot easily cross due to its physicochemical properties (Brown et al., 2007). ROS- altered proteins have been related to the Alzheimer's disease (AD) in mammals (Dalle-Donne et al., 2003). Diverse evidence shows that Al can be considered an etiological factor in AD (Mustafa, 2020), therefore it is possible to infer that carbonylation of proteins induced by aluminum may be a mechanism that participates in the development of different neurodegenerative disorders.

In general, LPO and PCC actual values of M1 and M2 (Table 6) showed to be higher than those additive calculated values of separately compounds in all tissues, therefore it is possible to hypothesize that potentiation interactions occur among IBU and Al. Pérez-Alvarez et al. (2018) noted that effluents containing metals and NSAIDs were able to increase MDA and PCC values in larvae of *X. laevis* and L. *catesbeianus*.

The sustained production of radical species is considered a normal phenomenon of metabolic processes in cells (Urso and Clarkson, 2003). As mammals, teleost fish possess an antioxidant defense system known to neutralize the endogenous generation of ROS (Gómez-Oliván et al., 2014a; Venditti et al., 2013).

As previously stated, SOD activity (Fig. 2) was found to be increased mainly in those specimens exposed to binary mixtures, nevertheless, this behavior was not consistent over the time. Moreover, enzymatic antioxidant activity was higher in gills and liver. Our results were consistent with previous studies; González-González et al. (2014) observed significant increases in superoxide dismutase activity in tissues of common

carp after acute exposure to an effluent containing NSAIDs and heavy metals. This behavior may be explained as the superoxide anion radical is formed during normal processes of cellular oxidative phosphorylation and in the case of NSAIDs, it is considered a reactive by-product of the oxidative metabolism that takes place in mitochondria (Islas-Flores et al., 2013). Valko et al. (2005) suggest that significantly high SOD activity may compensate for the oxidative effects induced by heavy metals as an adaptive response of the organism to counteract cellular damage. Our significantly increased SOD activity values are consistent with the results of Razo-Estrada et al. (2013) who concluded that exposure to 0.05 up to 220 mg L^{-1} of Al was capable of increase SOD antioxidant activity in liver of *C. carpio*. The lack of SOD response also observed in tissues where enzymatic activity values did no show significant differences could be due to an undermined capability of *D. rerio* to respond throughout an adaptive behavior caused by irreversible damage exerted into the antioxidant enzyme.

The values of catalase (Fig. 3) showed variability in enzymatic activity. Higher CAT values were found in liver and gut. The upregulation of CAT and other antioxidant enzymes is related to the presence of LPO products such as hydroperoxides and MDA (Khan et al., 2013). Increased values of CAT activity after exposure to IBU observed in this study are consistent to Parolini et al. (2011) who found moderate increases in CAT activity on *Dreissena polymorpha* after exposure to 1–35 nM of IBU as well as a notable activation of GPX and Razo-Estrada et al. (2013) who reported similar results and conclude that significantly high values of CAT activity were induced after exposure to low doses of Al in liver of *C. carpio*.

GPX antioxidant activity (Fig. 4) values increased in both maximum concentrations of the compounds and binary mixtures in gills and liver. Statistically increased values of GPX activity were also found after exposure to IBU (Gutiérrez-Noya et al., 2020; Parolini et al., 2011) and Al (González-González et al., 2014) in different fish tissues. As seen in the activity of SOD and CAT, no significant differences were also detected in the brain, gills and gut at different exposure times for IBU and Al alone and binary mixtures. This behavior may be explained by the effect on Al-induced oxidative stress as SOD, CAT and GPX can be considered possible target molecules of Al toxicity as these enzymes depend on various trace metals like to properly function (Kumar and Gill, 2009) or by depleting concentrations of antioxidant glutathione that acts as a metal chelator agent and substrate of scavenging enzymes (Hossain et al., 2012).

According to Mujika et al. (2011), this metal can bind with superoxide anion resulting in an aluminum superoxide anion complex, which seems to have a stronger pro-oxidant character than superoxide on its own when promoting the formation of radical species in biological systems. This behavior might partly explain that even though increasing values of superoxide dismutase and glutathione peroxidase were found mainly for binary mixtures in certain tissues at specific times of exposure in the present study, significantly increased values of lipid peroxidation were also observed. Hasan et al. (2018) detected that Al ions were capable of interact (ion-protein/protein-protein) affecting the normal biological function of target proteins including antioxidant enzymes CAT and GPX with the subsequent loss of catalytic function.

As observed in Table 6 antioxidant activities of SOD, CAT and GPX showed variation for binary mixtures when compared to additive interaction of IBU and Al alone at both minimum and maximum concentrations in all evaluated tissues at different exposure times. Mainly significant decreases in all enzymatic antioxidant activities concerning the additive calculated values were observed. According to Gonzalez-Rey et al. (2014), joint-action of metals like copper with non-steroidal anti-inflammatory drugs might produce antagonistic interactions in comparison to individual compounds by promoting significant decreases in SOD and CAT activities as observed in the present study. Additionally, Büyükgüzel et al. (2010) demonstrated that eicosanoid biosynthesis inhibitors are capable of impaired basal levels of antioxidant enzymes and increased oxidative response, as PGs are considered necessary in the maintenance of physiological homeostasis of MDA and PCC, and antioxidant activities as well as cellular and humoral immune defense.

In accordance to our study, Luja et al. (2019), Pérez-Coyotl et al. (2019) and more recently Tenorio-Chávez et al. (2020) found that domestic and hospital effluents containing different NSAIDs and heavy metals were capable to induce lethality and severe malformations in oocytes of *C. carpio* and *D. rerio* infering that the toxic effects were related, at least in part, to the induction of oxidative stress. Moreover, Quiroga-Santos et al. (2021) concluded that a mixture of environmental concentrations of diclofenac and Aluminum induced genotoxic damage and apoptosis in *C. carpio* juveniles and the toxic response was modified by potential interactions between them.

Finally, correlations between concentrations of both toxicants in evaluated tissues and biomarkers of OE (Table 5, Supplementary data) were studied. On that subject it can be seen that OS biomarkers are strongly associated with concentrations of IBU and Al in all evaluated tissues mainly for those specimens exposed to binary mixtures.

5. Conclusions

General toxicity pathways for many environmental pollutants are mainly mediated through the rise of intracellular reactive oxygen species and the depletion of antioxidant defense with the consequent oxidative stress and oxidation of cellular biomolecules. Long-term exposure to environmentally relevant concentrations of IBU and Al may constitute a potential hazard to *D. rerio*. Pollutants in the aquatic ecosystem have the potential to highly increase the formation of radical species such as ROS and RSN and simultaneously excessive levels of free radicals might alter the metabolism of diverse xenobiotics, with major consequences to aquatic organisms exposed to complex mixtures (Regoli and Giuliani, 2014). Induced effects are greater on specimens exposed to binary mixtures than those exposed to separately forms of evaluated toxicants, and it is possible to hypothesize that potentiation interactions occur between IBU and Al in cell oxidation biomarkers, while in specific cases antagonistic interactions take place in enzymatic activity when compared to the additive interactions. This study aimed to investigate the potential environmental harmful effects that can be generated when compounds with different nature and behavior interact in aquatic systems.

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Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that may have influenced the work reported in this document.

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