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Health impact assessment after *Danio rerio* long-term exposure to environmentally relevant concentrations of metformin and guanylurea

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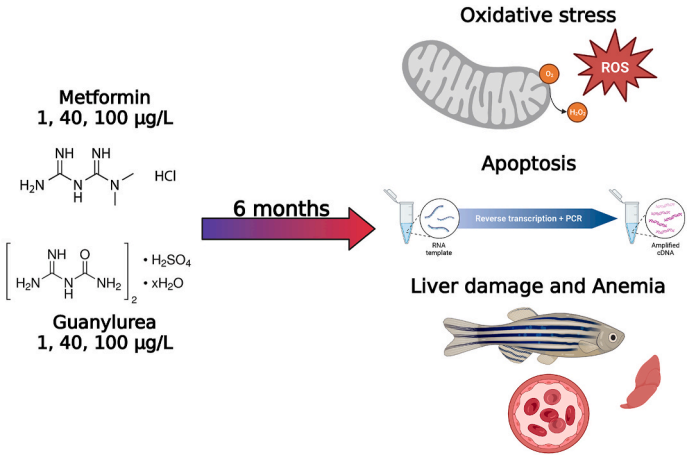
HIGHLIGHTS

- Guanylurea prompts a more toxic response than metformin.
- Both compounds render oxidative damage in the liver, gills, gut, and brain.
- Liver dysfunction was observed in fish exposed to both pollutants.
- Anemia was a consequence of chronic exposure to metformin and guanylurea.

GRAPHICAL ABSTRACT



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ABSTRACT

The antidiabetic drug metformin (MET) and its metabolite guanylurea (GUA) have been frequently and ubiquitously detected in surface water. Consequently, there has been a consistent rise in studying the toxicity of MET and GUA in fish over the past decade. Nonetheless, it is noteworthy that no study has assessed the harmful effects both compounds might trigger on fish blood and organs after chronic exposure. Taking into consideration the data above, our research strived to accomplish two primary objectives: Firstly, to assess the effect of comparable concentrations of MET and GUA (1, 40, 100 µg/L) on the liver, gills, gut, and brain of *Danio rerio* after six months of flow-through exposure. Secondly, to compare the outcomes to identify which compound prompts more significant oxidative stress and apoptosis in organs and blood parameter alterations. Herein, findings indicate that both compounds induced oxidative damage and increased the expression of genes associated with apoptosis (*bax,*

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bcl2, p53, and *casp3*). Chronic exposure to MET and GUA also generated fluctuations in glucose, creatinine, phosphorus, liver enzymes, red and white blood count, hemoglobin, and hematocrit levels. The observed biochemical changes indicate that MET and GUA are responsible for inducing hepatic damage in fish, whereas hematological alterations suggest that both compounds cause anemia. Considering GUA altered to a more considerable extent the values of all endpoints compared to the control group, it is suggested transformation product GUA is more toxic than MET. Moreover, based on the above evidence, it can be inferred that a six-month exposure to MET and GUA can impair REDOX status and generate apoptosis in fish, adversely affecting their essential organs' functioning.

1. Introduction

Metformin (MET), a medication classified as a biguanide, is considered the choice drug for type two diabetes (Foretz et al., 2019). However, recent research has indicated that metformin possesses other beneficial properties, including antioxidant and anti-aging effects, which have substantially increased worldwide usage. For instance, metformin has been employed to manage other medical conditions such as polycystic ovary syndrome, cancer, dementia, cardiovascular disease, and COVID-19 (Poznyak et al., 2022; Bailey and Gwilt, 2022; Zhu et al., 2022; Top et al., 2022). Consequent to those above, there has been a substantial surge in the consumption of MET over the last ten years, leading to its widespread input into wastewater treatment plants (WWTPs), where bioreactors transform it into guanylurea (GUA) (Trautwein et al., 2014; Briones et al., 2018; Briones and Sarmah, 2019; Tisler and Zwiener, 2019; He et al., 2022; Martinez-Vaz et al., 2022; Li et al., 2023). To date, various studies have informed the presence of MET and GUA in the sewages of WWTPs at concentrations oscillating from 0.0075 to 82.7 μg/L and <0.0283–810 μg/L, respectively (Meador et al., 2016; Tisler and Zwiener, 2018; Elizalde-Velázquez and Gómez-Oliván, 2020). Therefore, conventional wastewater treatment techniques cannot eliminate these compounds, resulting in MET (0.0002–33.6 μg/L) and GUA (0.001–222 μg/L) presence and persistence in rivers, lakes, and oceans (Kosma et al., 2015; de Solla et al., 2016; Elliott et al., 2017; Ali et al., 2017; Tao et al., 2018; Posselt et al., 2018; Tisler and Zwiener, 2018; Caldwell et al., 2019; Moreover, Meador et al., 2017 revealed that staghorn sculpin and juvenile Chinook salmon from the estuaries of Puget Sound reached metformin concentrations above the reporting limits, posing a real threat to the health and fitness of aquatic species.

Oxidative damage, due to the rising levels of oxygen radicals, has been one of the most reported effects on fish after metformin and guanylurea exposure (Lee et al., 2019; Elizalde-Velázquez et al., 2021a, 2021b). For instance, Lee et al., 2019 indicated that under a two-generational exposure condition, F0 male fish presented oxidative stress after metformin (40, 120, and 360 μg/L) exposure. Another harmful effect recently studied is the behavioral and neurotoxic effect of the parent compound and transformation product in fish (Phillips et al., 2021; Ussery et al., 2021; Elizalde-Velázquez et al., 2022a, 2022b). Our prior report, for instance, demonstrated anxiety behavior in *Danio rerio* was triggered by an apoptotic response and an acetylcholinesterase inhibition in the brain result of long-term exposure to metformin (1, 20, 40 μg/L) and guanylurea (25, 50, 200 μg/L) (Elizalde-Velázquez et al., 2022a, 2022b). Toxic consequences of these compounds have also been informed in the early-life stages of fish (Ussery et al., 2019; Cano-Viveros et al., 2022; Elizalde-Velázquez et al., 2022c; Ussery et al., 2019 showed that guanylurea in concentrations oscillating from 1 ng/L to 100 ng/L considerably reduced the Japanese medaka body size and weight.

Data concerning metformin and guanylurea toxicity in fish have steadily increased in the last decade. Nonetheless, there is still a knowledge gap about the harmful effects both compounds may prompt in fish blood and organs after chronic exposure. Bearing in mind this information, our research aimed to 1) assess the impact similar concentrations of metformin and guanylurea would have in the liver, gills, gut, and brain of *Danio rerio* adults and 2) compare the outcomes to point out which compound is more toxic. Contemplating our previous findings, we hypothesize that guanylurea would trigger a more severe response in all organs than metformin.

2. Method

2.1. Chemical reagents

Sigma-Aldrich (St. Louis, MO) provided all reagents, including *N*-guanylurea sulfate salt hydrate (CAS: 207,300-86-5). Toronto Research Chemicals (Toronto, ON) was the source of metformin hydrochloride (CAS: 1115-70-4).

2.2. Danio rerio upkeeping and exposure

Before exposure, five-month-old *Danio rerio* adults (AB strain, 3.4 ± 0.3 cm, and 459 ± 17 mg) were harbored in 100 L water tanks. Freshly cultivated *Artemia nauplii* from our laboratory were provided as feed to the fish on a thrice-daily basis. The temperature (27 °C ± 1 °C) and illumination length (14 h) were maintained constant and monitored. Additionally, to ensure the maintenance and exposure of zebrafish in all aquaria met the required water quality parameters, regular measurements of pH, dissolved oxygen levels, nitrate, nitrite, and un-ionized ammonia were conducted every other day. The mean values of these parameters are presented in Table 1. 420 *Danio rerio* adults per experiment were used to carry out all tests described in the following sections. Employed organisms were allocated to seven tanks containing either metformin (1, 40, and 100 μg/L), guanylurea (1, 40, and 100 μg/L), or ultrapure water. The selected concentrations are deemed environmentally relevant (Briones et al., 2016; Tao et al., 2018; Ambrosio-Albuquerque et al., 2021) and were chosen because they have been proven to induce toxic effects in *Danio rerio* embryos and adults (Elizalde-Velázquez et al., 2021a, 2021b, 2022a, 2021b,b). Exposure lasted six months, and during this time, water was renewed every week from all tanks, taking care to maintain constant nominal concentrations of metformin and guanylurea. The temperature (27 °C ± 1 °C) and illumination length (14 h) were also monitored during exposure. This identical procedure was conducted thrice with different organisms, as three independent experiments were performed for this research.

2.3. Blood and organs gathering

From 60 fish exposed per concentration, excluding the control group, a mean of 45 *Danio rerio* adults (SD ± 3) prevailed after six months of exposure. The control group exhibited an average mortality rate of 3 (SD

Table 1
Water quality parameters measured in aquaria.

Parameter	Value
pH	7.38 ± 0.11
Dissolved Oxygen	9.5 ± 0.2 mg/L
Nitrate	2900 ± 300 μ g/L
Nitrite	28 ± 6 μg/L
Un-ionized ammonia	15 ± 2 μg/L

Values are expressed as mean ± standard deviation.

of ±1) following six months of exposure. Fish from each concentration were euthanized by immersion in eugenol 1%. This method was utilized as previous findings indicate eugenol minimizes stress stimulus to euthanasia in *Danio rerio* and allows the collection of considerable volumes of blood postmortem (Davis et al., 2015). Immediately after euthanasia, a transverse incision was made over the caudal fin to gather the blood from the dorsal aorta. Briefly, a 0.1–0.3 cm incision was accomplished by inserting the sharp ends of dissecting scissors (Hergom Medical, Mexico), and the blood pouring out from it was rapidly gathered with a micropipette (Science Med, Finland). Approximately 40–50 μL of blood gushed out from each incision, which was then compiled into BD micro containers (DIKYSA, Mexico). After finishing blood collection, the gut, liver, gills, and brain were extracted and allocated into Eppendorf tubes (Costar, USA) with 1 mL of PBS (pH 7.4). Three Eppendorf tubes were collected for each organ across all treatments, including the control group. Each tube contained an average of 15 organs with a standard deviation of ±1. Two tubes per organ were used to evaluate oxidative stress and gene expression biomarkers. Additionally, the third tube from each organ was employed to measure the quantities of MET and GUA in the organs. Fish from the three independent experiments underwent the same blood gathering, euthanasia, and dissection procedure. Moreover, the distribution and usage of organs in each experiment were the exact as described above.

2.4. Oxidative stress evaluation

The organs obtained during dissection were homogenized using a rotor-stator homogenizer (IKA, Germany) at a velocity of 10,000 rpm for 20. The resulting homogenate was divided into two Eppendorf tubes as follows. Tube 1 contained 300 μL from the homogenate with an equal volume of a trichloroacetic acid (TCA) solution (20%). Tube 2, on the other hand, consisted of 700 μL of the homogenate alone. All Eppendorf were stored at −20 °C until their intended use. Tube 2 was centrifuged at 12,500 rpm for 15 min at four-degree Celsius, and the resulting supernatant was used to measure the levels of superoxide dismutase (SOD) and catalase (CAT). Meanwhile, tube 1 was centrifuged at 11,495 rpm for 15 min at 4 °C, and the resulting supernatant was used to ascertain lipid peroxidation (LPX) levels and hydroperoxides (HPC). Moreover, The precipitate obtained from this centrifugation was used to determine protein carbonyls (PCC). All methods employed were colorimetric and followed step by step as each protocol indicated (Table 2). Results were normalized against total proteins (TP). Samples from each organ and concentration were assessed three times. Moreover, organs from the three independent experiments were evaluated as previously indicated.

2.5. Gene expression assessment

After dissection of the fish, the brains were placed in an Eppendorf tube free of RNases that contained RNALater (QIAGEN) to prevent nucleotide degradation. The ratio of RNALater added to each Eppendorf was 10 μL:1 mg of tissue, as indicated manufacturer's manual. All Eppendorf were stored at −20 °C until RNA isolation. RNA isolation was carried out in the molecular biology room, which has a laminar flow hood (Bioevopeak, China) that works with positive pressure to prevent the infiltration of environmental contaminants. The samples were thawed in an ice bath, and each tissue was treated with RNeasy Mini Kit from QIAGEN. Agarose gel electrophoresis (1%) was used to evaluate the quality of RNA, while its purity was determined by spectrophotometry (NanoDrop, 2000/2000c Thermo Scientific, USA) through 260/280 and 260/230 ratios. The RNA obtained was kept at −20 °C until use. The RNA samples were thawed in an ice bath, and 2.0 μL of 7x gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit QIAGEN), 10.0 μL of the RNA template, and 2.0 μL of RNase-free water (QuantiTect Reverse Transcription Kit QIAGEN) were placed in an Eppendorf tube free of RNases. These tubes were incubated for 2 min at 42 °C and immediately placed on ice to subsequently add 1.0 μL Quantiscript reverse transcriptase (QuantiTect Reverse Transcription Kit QIAGEN), 4.0 μL Quantiscript RT Buffer 5× (QuantiTect Reverse Transcription Kit QIAGEN), 1.0 μL RT Primer Mix (QuantiTect Reverse Transcription Kit QIAGEN). All above was mixed and incubated at 42 °C for 15 s and then at 93 °C for 3 min for reverse transcription inactivation. After the reverse transcription process, the quality of the resulting cDNA was evaluated using agarose gel electrophoresis (1%). The purity of the cDNA was also analyzed through spectrophotometry using the NanoDrop 2000/2000c Thermo Scientific instrument, with the 260/280 and 260/230 ratios utilized as indicators of purity. The qPCR reaction was performed by adding 25 μL of the 2× QuantiTect SYBR Green PCR (QIAGEN), 1.0 μL of each primer, 4.0 μL of cDNA, and 19 μL of RNase-free water. The genes evaluated are shown in Table 3. All reagents were mixed, and the qPCR was run using these conditions: 94 °C for 10 min, followed by 35 cycles of denaturation 94 °C for 15 s, primer annealing for 30 s (annealing temperature in Table 3), and extension 72 °C for 30 s. The Gene Q Rotor (QIAGEN) was employed as the qPCR equipment. The β-actin gene was utilized to normalize the expression of target genes. The $2^{-\Delta\ \Delta Cq}$ method (Pfaffl, 2001; Pfaffl et al., 2002; Schmittgen and Livak, 2008) was applied to calculate mRNA expression changes. Following the completion of the qPCR cycles, a dissociation curve was executed with these conditions: 10 s at 95 °C, 10 s at 65 °C, and 10 s at 95 °C with 0.2 °C increments to distinguish between specific and non-specific products. Data were obtained at each increment of the melting curve. qPCR reactions for all organs and concentrations were carried out in triplicate, and data from all three experiments were gathered to perform the statistical analyses. As a negative control, samples lacking temper were used, with RNase-free water being used instead.

Table 2
Colorimetric methods employed.

Biomarker	Wavelength	Protocol
SOD	480 nm	Misra and Fridovich (1972)
CAT	240 nm	Radi et al. (1991)
LPX	535 nm	Buege and Aust (1978)
HPC	560 nm	Jiang et al. (1992)
PCC	366 nm	Levine et al. (1994)
TP	595 nm	Bradford,1976

SOD: superoxide dismutase; CAT: catalase, LPX: lipid peroxidation, HPC: hydroperoxides, PCC: protein carbonyls; TP: total proteins.

2.6. Biochemical and hematological analysis

Red-capped microtubes were centrifuged for 10 min at 2500 rpm, and the serum was extracted with a micropipette. The serum was then used to quantify alkaline phosphatase (ALP), alanine aminotransferase (ALT), albumin (ALB), blood urea nitrogen (BUN), creatinine (CRE), globulin (GLOB), glucose (GLU), phosphorus (Phos), total protein (TP), and total bilirubin (TBIL). Analysis was done using the VetScan VS2 (Abaxis, USA). In addition, blood stored in purple-cap microtubes was directly analyzed employing HEMA-V6190 (Bioevopeak, China). The parameters evaluated were mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) red blood cell count (RBC), hematocrit (HTC) hemoglobin (Hb), white blood cell count (WBC), percentage of basophils (BAS), eosinophils (EOS), lymphocytes (LYM), monocytes (MXD), neutrophils (NEU). Blood from the three independent experiments was analyzed as indicated above (n = 3 per concentration).

2.7. Chemicals quantification

Metformin and guanylurea were quantified in all organs and water samples. A 10 mL water sample from each system was collected every week before the renewal of the medium. All water samples were stored at −20 °C until their quantification. Organs were processed according to the technique reported by Łabuzek et al. (2010). Briefly, the

Table 3
QPCR genes evaluated.

Gene	Forward primer	Reverse primer	Accession numbers	Tm	Annealing Temp (°C)	Product Size	Primer Efficiency	Source
β-actin	AAGTGCGACGTGGACA	GTTTAGGTTGGTCGTTCGTTTGA	NM_131031.2	55.69/ 59.69	55	226	94	Gonzalez et al. (2005)
nrf1	TTT GGT TCC CGA TGA AGA CG	TGA TTA GCG TGA GAC TGA GC	JX867114.1	58.2/ 57.43	55	264	97.8	Sant et al. (2017)
nrf2	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	BC045852	53.63/ 56.53	51	184	102.4	Sant et al. (2017)
bax	GGC TAT TTC AAC CAG GGT TCC	TGC GAA TCA CCA ATG CTG T	AF231015	58.9/ 58.05	55	197	106.7	Soares et al., 2018
casp3	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT	NM_131877.3	55.55/ 52.69	53	129	109.1	Félix et al. (2018)
p53	GCA GCG ATG AGG AGA TCT TT	GGG CTC AGA TGA TTC ACG AT	NM_001328588.1	57.76/ 57.46	53	176	101.3	Lei et al. (2017)
bcl2	TCACTCGTTCAGACCCTC	ACGCTTTCCACGCACAT	NM_001030253.2	55.56/ 57.46	53	235	99.8	Kim et al. (2018)

Nrf1: Nuclear factor erythroid 2-related factor 1, *nrf2*: Nuclear factor erythroid 2-related factor 2, *bax*: BCL2 associated X, apoptosis regulator, *casp3*: Caspase 3, *p53*: tumor protein p53, *bcl2*: BCL2 apoptosis regulator.

homogenate was deproteinized (MeCN, MeOH, and internal standard), filtered (10 μm), and evaporated to dryness (N2). Next, the samples were mixed with 100 μL of the mobile phase and centrifuged for 15 min at 16, 000 g. The eluent A in the mobile phase was composed of 2 mM $MeCOO^-$, while the mobile phase B consisted of 100% acetonitrile. Then, 50 μL of supernatant was injected into the autosampler. An Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS with a Turbo V Ion spray source was used to analyze samples. Separation was achieved

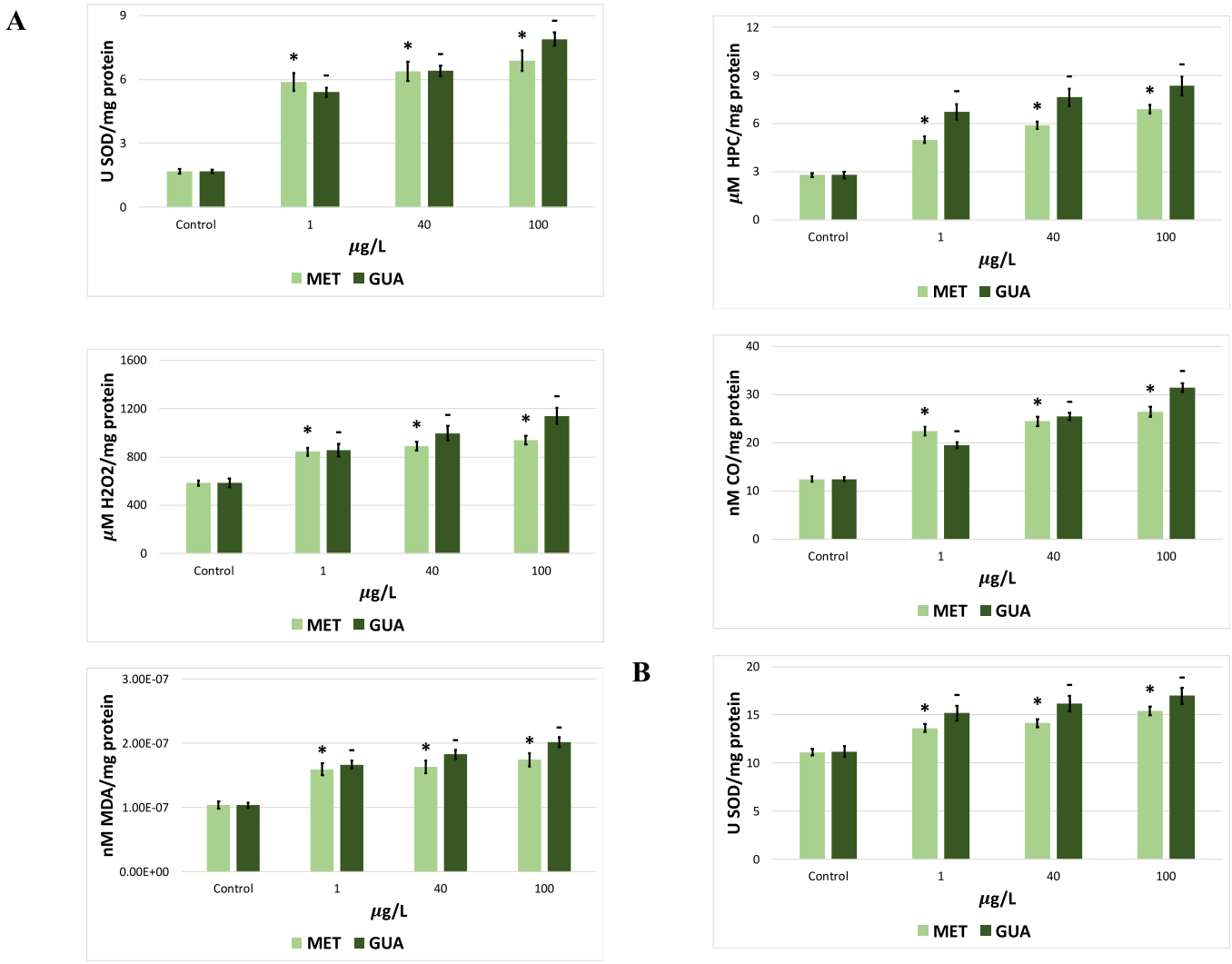

Fig. 1. Metformin- and guanylurea-induced oxidative stress (a: SOD: superoxide dismutase, b: CAT: catalase, c: LPX: lipid peroxidation, d: HPC: hydroperoxides, e: PCC: protein carbonyls) in *Danio rerio* liver (**A**), gut (**B**), brain (**C**), and gills (**D**). The significant differences between the treatments and the control group are depicted with an *. Data represent mean ± standard deviation of three independent experiments ($p < 0.001$).

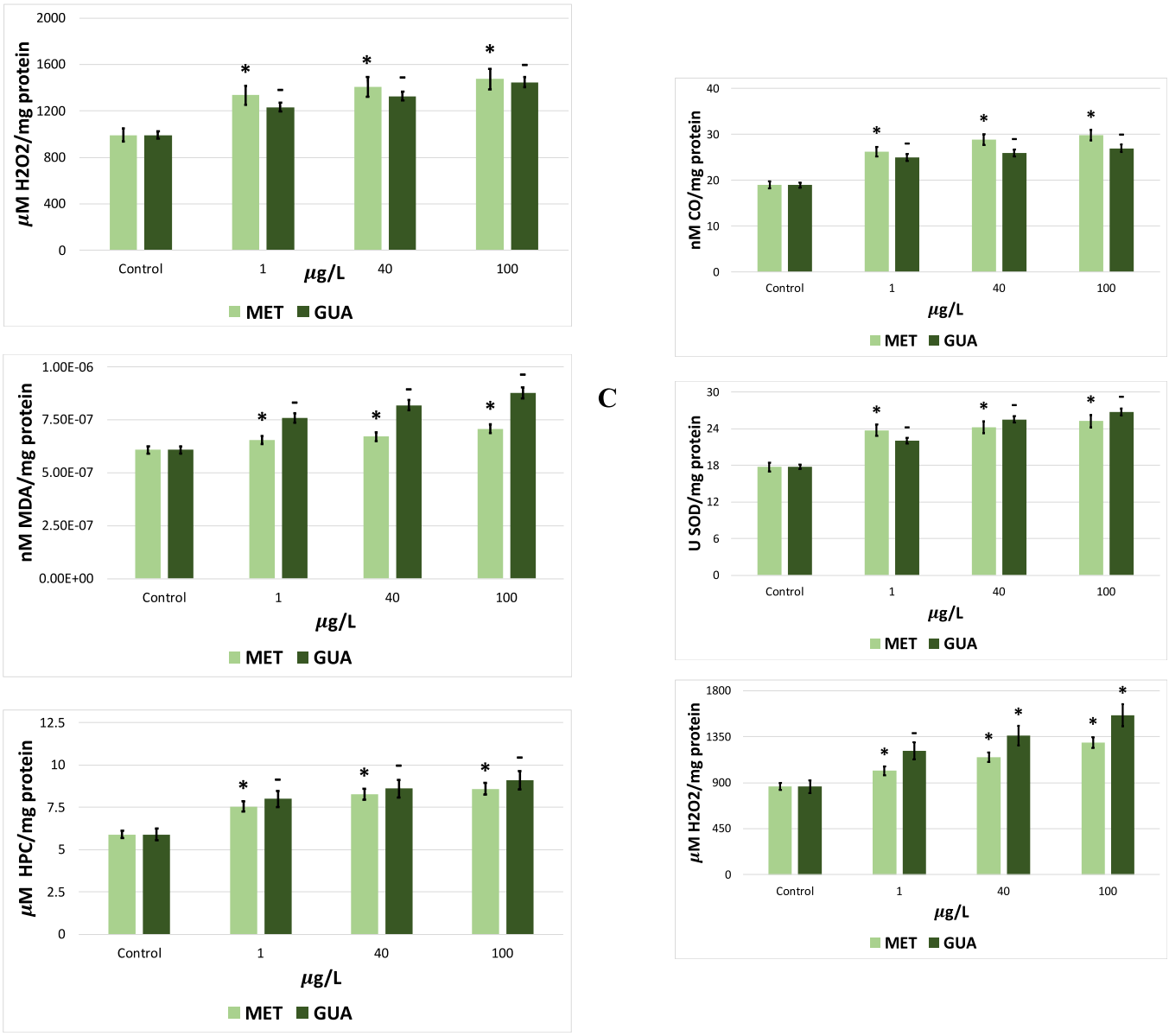

Fig. 1. (*continued*).

using an Xbridge C18 column (50 mm × 3.0 mm, particle size 3.5 µm) and Xbridge Phenyl column (150 mm × 2.1 mm, particle size 3.5 µm) for metformin and guanylurea, respectively. The samples were eluted at a flow of 100 µL/min.

2.8. Statistical analysis

Homoscedasticity and normality were confirmed by Bartlett tests and Shapiro-Wilk, respectively. Differences between means were ascertained by the Tukey test, considering a $p < 0.05$. Dissimilarities between treatment groups were verified with a one-way ANOVA test ($\alpha = 0.05$) (Sigma Plot 12.3). Outcomes from all trials were represented as the mean ± standard deviation (SD). Two Pearson correlation analyses, with a significance level set at $p < 0.05$, were conducted to assess 1) the strength of the relationship between outcomes from oxidative stress and gene expression and 2) the magnitude of the association between the results obtained from the biochemical and hematological results (R software). Moreover, the resulting correlations were presented through various network correlations and two correlograms.

3. Results

3.1. Oxidative damage

Both metformin and guanylurea raised the levels of all oxidative stress biomarkers in all organs after six months of exposure. All concentrations from both compounds were significantly different from the control group. In the liver (Fig. 1A), metformin exhibited a more significant increase in SOD (a) and PCC (e) than guanylurea at 1.0 µg/L. However, at 40 µg/L, guanylurea displayed a more increased boost in CAT (b), LPX (c), and HPC (d) levels than the parent compound. Moreover, the transformation product at the highest concentration always showed a considerable difference in all oxidative stress biomarkers compared to metformin. In the gut (Fig. 1B), guanylurea exposure caused a more remarkable rise in SOD (a) and LPX (c) than metformin at all concentrations. Nonetheless, metformin showed a more significant increase in CAT (b) and PCC (e) than guanylurea. In the brain (Fig. 1C), guanylurea exposure resulted in a considerably higher increase in CAT (b), LPX (c), and HPC (d) levels than metformin at all concentrations. Metformin significantly increased SOD (a) levels at the lowest

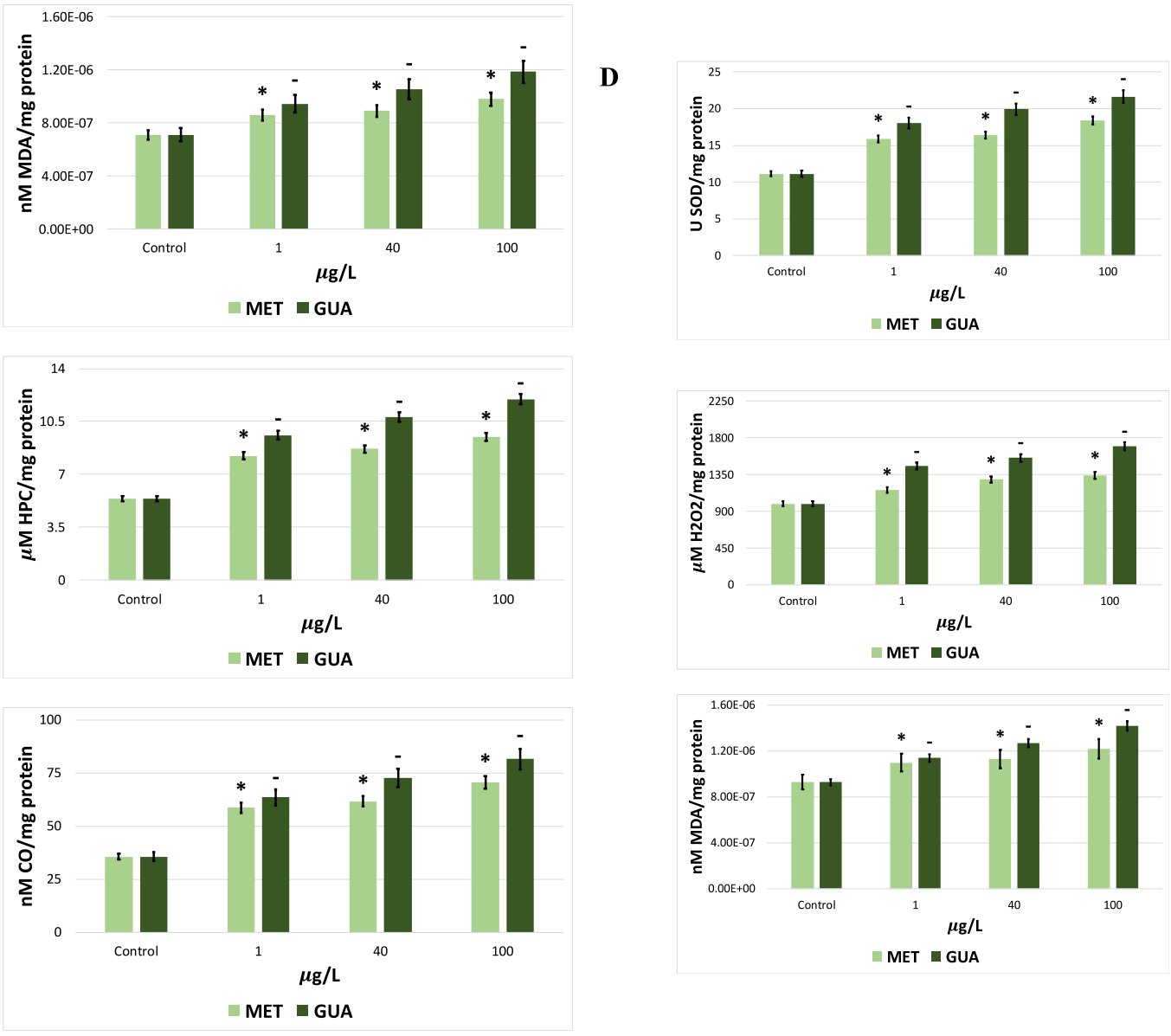

Fig. 1. (*continued*).

concentration compared to guanylurea. Nevertheless, as the concentration increased, the transformation product raised much more levels of SOD than metformin. A similar situation was observed with the PCC biomarker. Finally, in the gills (Fig. 1D), guanylurea showed a more significant response than metformin in all biomarkers and all concentrations, except for LPX (c) at the lowest concentration.

3.2. Gene expression damage

Guanylurea remarkably augmented the gene expression of *nrf1, nrf2, casp3, p53, bax*, and *blc2* in all organs and at all concentrations compared to the control group (Fig. 2A-D). In contrast, metformin only modified the response of all genes at the middle and highest concentrations compared to the control group. In the liver, guanylurea upregulated all genes at all concentrations, except *nrf2* at 1.0 µg/L, significantly compared to the parent compound. Apoptosis genes *p53, bax*, and *bcl2* suffered a more remarkable change than *nrf1, nrf2,* and *casp3* in the liver after chronic exposure to both chemicals. *Casp3* was down-regulated by metformin at 40 and 100 µg/L, but no significant differences were observed. Except for *nrf1* and *blc2* at 40 g/L, the expression of all genes in the gut was more upregulated by the transformation product than by metformin. As in the liver, *p53* and *bax* expression increased much more than the other genes. The expression of all genes in the brain, similar to the liver and gut, was significantly upregulated by guanylurea compared to metformin. However, no considerable change in the expression of *nrf2* was observed between the middle concentration of both compounds in the brain. *Nrf2* was the most upregulated gene in the brain after metformin treatment, as *casp3* and *p53* following guanylurea exposure. In the gills, *nrf1* and *p53* exhibited the most significant up-regulation in fish treated with metformin, while *nrf2, p53*, and *bax* demonstrated a more substantial response in organisms treated with guanylurea. No significant fold difference was observed in the expression of *nrf2* between the lowest concentrations of both compounds.

3.3. Biochemical and hematological damage

Biochemical values from fish of the control group are closely related to reference ones reported in the literature. The parent compound and transformation product widely altered the expected values of all

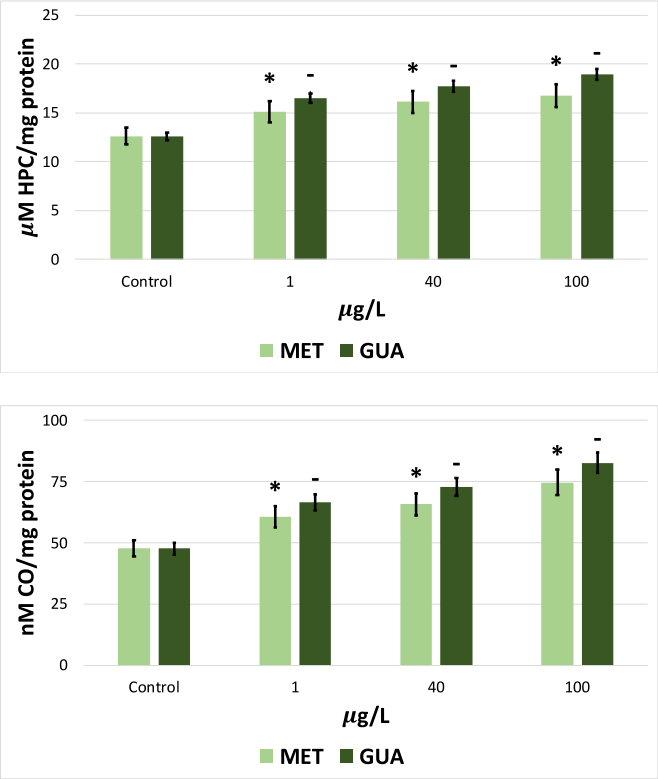

Fig. 1. (*continued*).

biochemical biomarkers in exposed organisms (Table 4). Glucose levels from all treatments were significantly different from those reported in the control group. Unlike metformin, the transformation product increased the blood glucose levels concentration-dependent. BUN values were only substantially different from the control group at the highest concentration of guanylurea. Metformin kept the levels of BUN steady through the treatments, while guanylurea increased them. Both compounds, at all concentrations, remarkably reduced the levels of CRE compared to the control group. As in BUN, CRE values were maintained invariant through the metformin treatments, but guanylurea reduced them at the middle and highest concentrations. TBIL did not suffer any significant change after fish exposure to any of the treatments. TP values were significantly reduced at the lowest concentration of guanylurea and metformin and the middle one of the latter. Metformin and guanylurea increased in a concentration-dependent manner the blood levels of ALT. However, significant differences were not observed at the lowest concentration of metformin compared to the control group. Blood ALP values from all treatments were significantly raised regarding the control group. ALP levels augmented concentration-dependent in both compounds, except at 40 μg/L of metformin. ALB was decreased concentration-dependent, but only significant differences regarding the control group were found at 40 and 100 μg/L of guanylurea. All treatments remarkably lowered the levels of GLOB compared to the control group. Phos levels in the blood decreased concentration-dependent after metformin exposure, but they remained significantly below the values of the control group.

Hematological parameters from the control group are inside the ones reported in other research, while the ones from treatments were broadly altered (Table 5). RBC count significantly decreased in fish after exposure to all treatments. The decrease was concentration-dependent, showing a higher impact in organisms exposed to the transformation product. Hb and HCT values were also remarkably reduced in all fish treated with metformin and guanylurea compared to the control group. Metformin at the lowest and middle concentration augmented the MCV compared to the control group, but no significant changes were observed at the highest concentration. Guanylurea, on the other hand, decreased the MCV in fish; however, substantial differences regarding the control group were only observed at 40 and 100 μg/L. The parent compound did not modify the MCHC values of fish, but guanylurea did, generating the most remarkable decrease at the highest concentration. MXD percentage was substantially decreased in fish exposed to both treatments, but most values remained inside reference values. No changes were observed in NEU, EOS, and BAS rates with any treatments.

3.4. MET and GUA quantification

Metformin and guanylurea concentrations in all control group organs remained below the quantification limit. However, all organs of fish from treatment groups other than the control showed a significant increase in the concentration of both compounds (Table 6). Overall, guanylurea was more absorbed in all organs than metformin. Absorption of both compounds was higher in the gut, followed by the gills, liver, and brain (Table 7). Regarding the water samples, the concentrations of MET and GUA in the control group were below the limit of quantification (LOQ). In contrast, the measured concentrations of both compounds in the other treatment groups decreased, but not by more than 20% compared to the control group.

3.5. Pearson correlations

In network correlation analysis, the nodes represent the variables, and the edges represent the relationships between them. Furthermore, colors indicate the strength and type of relation. Therefore, the correlation gets more positive as nodes get closer and the green color is more intense. For example, in the liver (Fig. 3A), all variables but LPX, PCC, and *nrf1* were closely related after MET and GUA exposure. Moreover, *casp3* was negatively associated with other gene expression and oxidative stress biomarkers. All correlations were positive in the gut (Fig. 3B); however, *nrf2*, LPX, SOD, and PCC did not show a close relationship with other variables. Regarding variable correlation in the brain (Fig. 3C), it

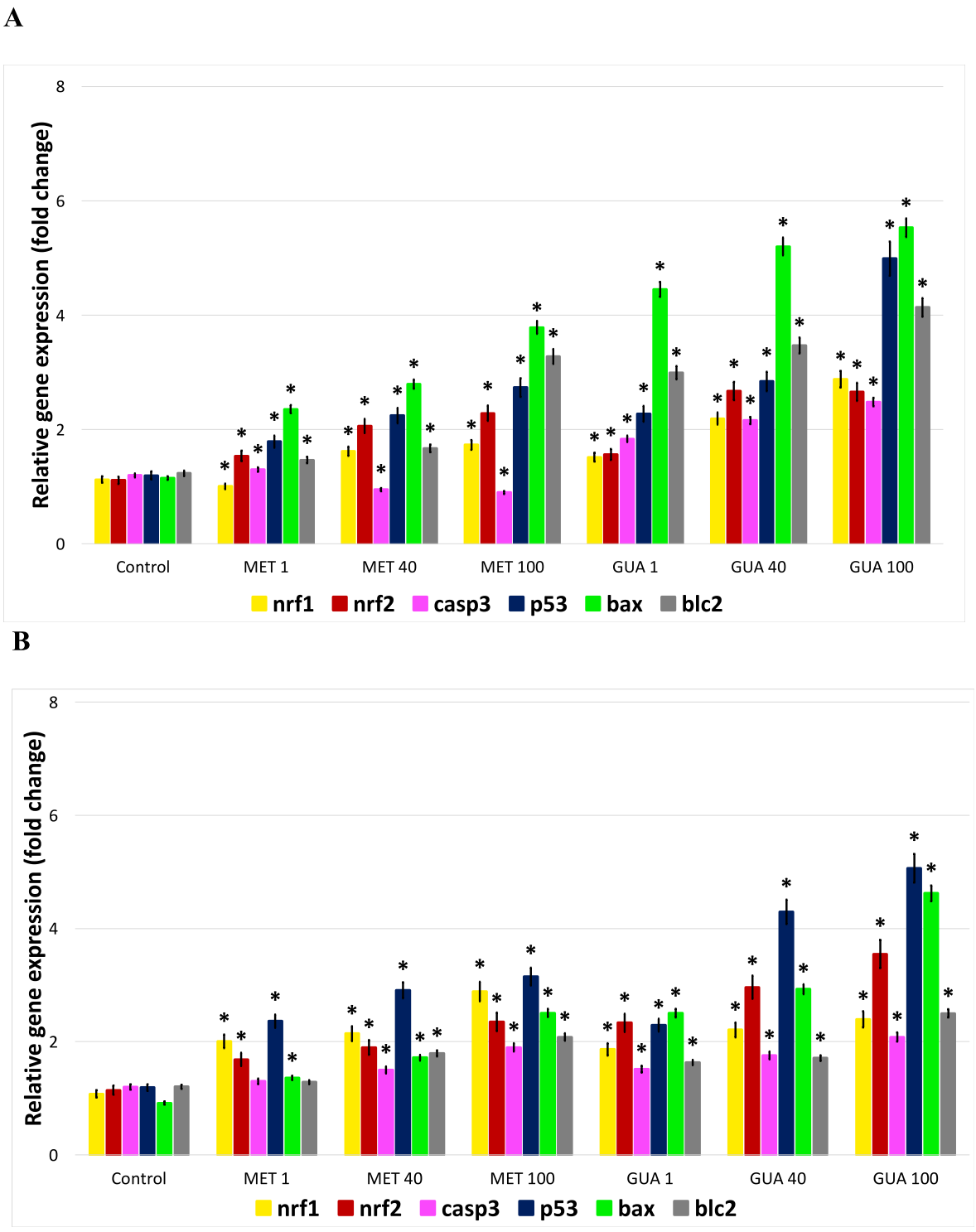

Fig. 2. Gene expression changes in the liver (**A**), gut (**B**), brain (**C**), and gills (**D**) of *Danio rerio* after metformin (MET) and guanylurea (GUA) exposure. The significant differences between the treatments and the control group are depicted with an *. Data represent mean ± standard deviation of three independent experiments ($p < 0.001$). *Nrf1*: Nuclear factor erythroid 2-related factor 1, *nrf2*: Nuclear factor erythroid 2-related factor 2, *bax*: BCL2 associated X, apoptosis regulator, *casp3*: Caspase 3, *p53*: tumor protein p53, *bcl2*: BCL2 apoptosis regulator.

was observed that SOD, *nrf2*, and *bax*, respectively, did not show a close relation to the rest of the variables. Furthermore, *casp3* was negatively correlated with all other variables after MET exposure, as in the liver. LPX, *nrf2*, SOD, and PCC neither showed a closely correlated with the rest of the variables in the gills (Fig. 3D), as in other organs. Correlograms follow the same color trend as the network correlation; thus, as the intensity of the red color augments, the strength of the correlation also increases. Fig. 4A and B showed glucose, TP, ALT, ALP, and BUN are positively related to ALB, RBC, CRE, and GLOB.

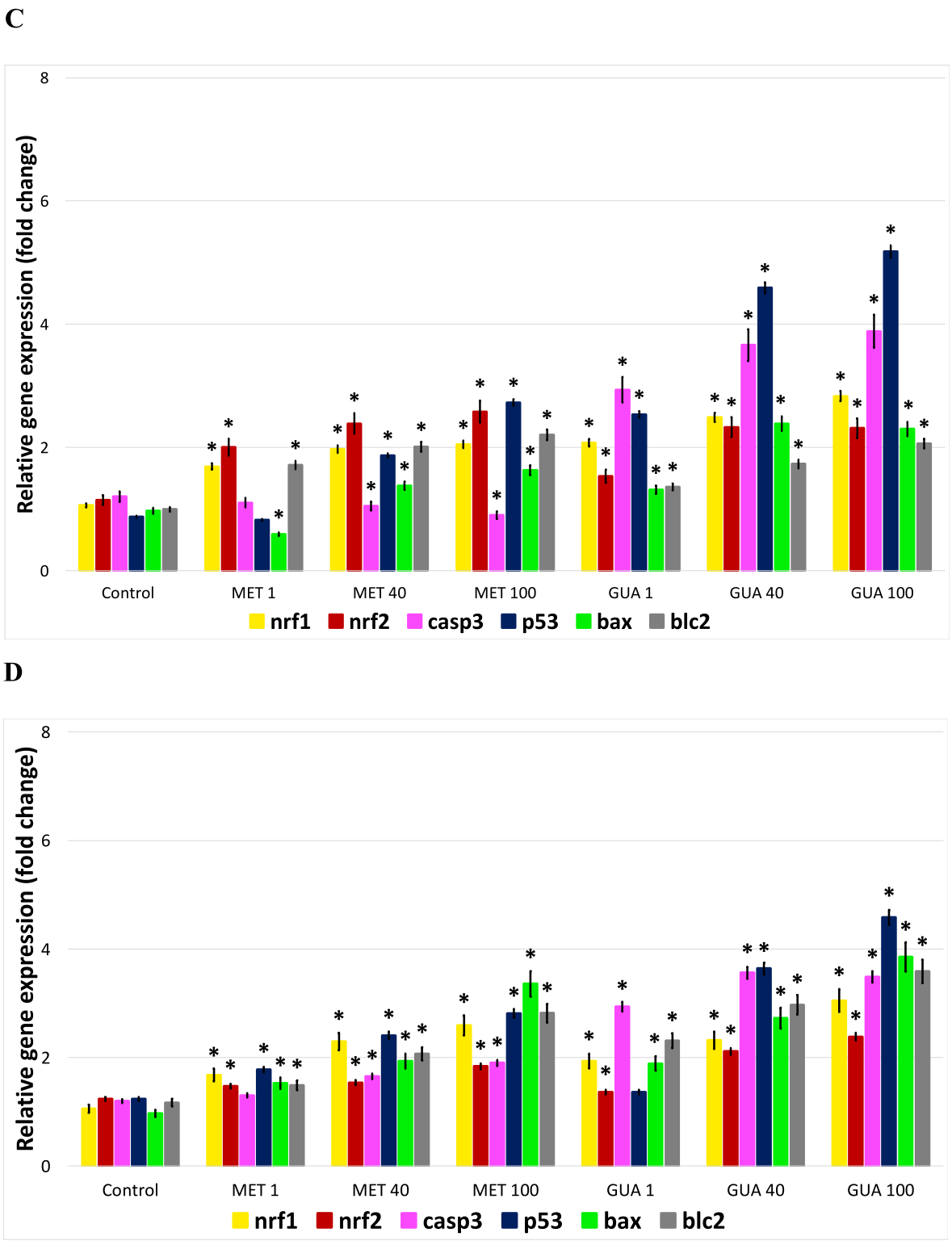

Fig. 2. (*continued*).

4. Discussion

Reactive oxygen species (ROS) are highly reactive molecules that arise from metabolic processes and immune responses. These molecules function as secondary messengers, regulating various signaling cascades crucial for cellular activities such as function, proliferation, differentiation, and apoptosis (Jakubczyk et al., 2020). Nevertheless, an imbalance characterized by excessive ROS production or a decline in antioxidant defenses can lead to a condition known as oxidative stress. External factors, including exposure to environmental pollutants, radiation, and certain drugs, can lead to the generation and overproduction of ROS (Xie et al., 2019). Herein, the outcomes indicated that chronic exposure to MET and GUA disrupted the equilibrium between ROS and antioxidant defense mechanisms in fish, leading to oxidative stress responses in the liver, gut, brain, and gills. Nonetheless, it is worth mentioning that GUA induced significantly greater levels of oxidative

Table 4
Biochemical biomarkers.

Biomarkers	Control	Metformin			Guanylurea			Units	Reference values	Reference
		1 μg/L	40 μg/L	100 μg/L	1 μg/L	40 μg/L	100 μg/L			
Glucose	80.22 ± 2.51	63.47 ± 1.32*	49.62 ± 1.56*	57.34 ± 2.21*	70.93 ± 3.90*	98.51 ± 3.64*	128.24 ± 4.77*	mg/dL	62–91	Murtha et al. (2003)
BUN	2.87 ± 0.24	2.02 ± 0.13	1.97 ± 0.19	2.02 ± 0.19	2.08 ± 0.26	3.77 ± 0.65*	5.63 ± 0.96*	mg/dL	3–4	
CRE	0.49 ± 0.02	0.20 ± 0.03*	0.20 ± 0.02*	0.19 ± 0.01*	0.21 ± 0.02*	0.09* ± 0.02	0.10 ± 0.01*	mg/dL	.5–.9	
TBIL	0.50 ± 0.03	0.39 ± 0.02	0.40 ± 0.04	0.39 ± 0.02	0.39 ± 0.03	0.39 ± 0.03	0.39 ± 0.02	mg/dL	.2–.6	
TP	4.23 ± 0.70	3.22 ± 0.66*	3.43 ± 0.45*	3.53 ± 0.57*	3.29 ± 0.48*	3.50 ± 0.62*	3.62 ± 0.71*	g/dL	4.4–5.8	
ALT	345.44 ± 5.83	358.04 ± 8.50	375.32 ± 9.32*	493.30 ± 7.86*	400.93 ± 4.79*	497.78 ± 5.14*	509.71 ± 8.27*	U/L	343–410	
ALP	4.10 ± 0.74	7.08 ± 0.99*	7.19 ± 0.91*	11.06 ± 1.92*	8.05 ± 0.95*	11.24 ± 1.49*	12.86 ± 1.37*	U/L	0–10	
ALB	2.69 ± 0.30	2.72 ± 0.36	2.84 ± 0.29	2.22 ± 0.40	2.58 ± 0.25	2.10 ± 0.34*	1.46 ± 0.23*	g/dL	2.7–3.3	
GLOB	1.57 ± 0.18	0.57 ± 0.03*	0.57 ± 0.04*	0.65 ± 0.03*	0.59 ± 0.03*	0.30 ± 0.02*	0.30 ± 0.04*	g/dL	1.3–2.8	
Phos	22.79 ± 2.44	9.86 ± 0.98*	11.66 ± 1.52*	11.31 ± 1.36*	16.63 ± 2.65*	16.44 ± 2.11*	11.30 ± 1.34*	g/dL	20.3–24.3	

Data represent mean ± standard deviation of three independent experiments. * denote significant difference compared to the control group. BUN: blood urea nitrogen, CRE: creatinine, TBIL: total bilirubin, TP: total protein, ALT: alanine aminotransferase, ALP: alkaline phosphatase, ALB: albumin, GLOB: globulin, Phos: phosphorus.

Table 5
Hematological biomarkers.

Biomarkers	Control	Metformin			Guanylurea			Units	Reference values	Reference
		1 μg/L	40 μg/L	100 μg/L	1 μg/L	40 μg/L	100 μg/L			
RBC	3.04 ± 0.70	2.45 ± 0.41*	1.80 ± 0.25*	1.02 ± 0.20*	1.16 ± 0.23*	0.9 ± 0.13*	0.69 ± 0.09*	$X10^6$/mL	2.9–3.2	Elizalde-Velázquez et al. (2023)
Hb	7.37 ± 0.91	3.53 ± 0.71*	3.18 ± 0.66*	3.57 ± 0.63	3.45 ± 0.74*	3.26 ± 0.69*	3.8 ± 0.72*	g/dL	4.75–7.7	
HTC	32.27 ± 3.88	0.54 ± 0.02*	0.32 ± 0.02*	0.30 ± 0.02*	0.40 ± 0.03*	0.30 ± 0.02*	0.43 ± 0.03*	%	25–36	
MCV	131.00 ± 3.14	236.89 ± 5.04*	238.52 ± 4.87*	234.91 ± 2.9*	126.26 ± 2.71	105.08 ± 2.67*	104.07 ± 3.22*	fL	119–252	
MCHC	225.63 ± 5.91	221.83 ± 5.74	235.88 ± 4.45	212.02 ± 5.28	179.31 ± 2.15*	199.79 ± 2.90*	173.12 ± 2.94*	%	183–276	
WBC	23.82 ± 1.48	25.54 ± 1.24	22.37 ± 1.33	24.89 ± 1.42	24.12 ± 2.45	25.28 ± 1.69	25.65 ± 1.62	mL	13.6–31.7	
LYM	72.90 ± 3.83	78.21 ± 2.48	81.96 ± 2.23	91.45 ± 3.11*	73.18 ± 2.31	85.15 ± 3.17*	92.63 ± 3.29*	%	71–92	Murtha et al. (2003)
MXD	13.72 ± 0.96	11.96 ± 0.32*	10.94 ± 0.44*	10.04 ± 0.19*	11.09 ± 1.41*	10.81 ± 0.22*	9.13 ± 0.15*	%	5–15	
NEU	12.03 ± 1.56	11.93 ± 0.73	11.99 ± 0.64	11.99 ± 0.74	11.95 ± 0.57	11.93 ± 0.76	11.01 ± 0.29	%	2–18	
EOS	0	0	0	0	0	0	0	%	0–2	
BAS	0	0	0	0	0	0	0	%	0–2	

Data represent mean ± standard deviation of three independent experiments. * denote significant difference compared to the control group. RBC: red blood cell count, Hb: hemoglobin, HTC: hematocrit, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, WBC: white blood cell count, LYM: lymphocytes, MXD: monocytes, NEU: neutrophils, EOS: eosinophils, BAS: basophils.

damage in all organs except for the gut, where the parent compound displayed a similar effect to GUA. The data presented do not resemble those documented by Lin et al. (2020). Lin et al. conducted an experiment wherein adult *Danio rerio* were subjected to different concentrations of MET (0.1, 1, 10, 100, and 1000 μg/L) to ascertain its accumulation in the liver, gut, and gills, as well as to evaluate the MET-induced DNA methylation in tissues. The authors reported that the liver was the most vulnerable to MET toxicity after 30 days of exposure. Nonetheless, the author's outcomes also indicated that the bioaccumulation potential of MET was more significant in the gastrointestinal tract than in other organs, which is consistent with our results (Table 6). Besides producing a more substantial disruption of fish REDOX status, GUA-affected organs suffered a different degree of oxidative damage. Specifically, the gills were the most damaged organ, followed by the brain, liver, and gut. In agreement with previous findings, Barbieri et al. (2022) indicated MET (50, 100, 1000, 10,000 μg/L) prompted lamellar fusion, telangiectasia hyperplasia, and disappearance of micro ridges in Astyanax lacustris after 90 days of exposure. In addition, they suggested that the influence of metformin on the ultra-structures of the gill may be attributed to its established cellular mechanism of action, specifically the inhibition of Complex I within the mitochondrial respiratory chain. The above data is remarkable because Complex I inhibition of mitochondria is closely related to MET-induced oxidative stress (Lee et al., 2019; Elizalde-Velázquez et al., 2022a) and because our previous findings have shown that GUA may exert its toxic effects using the same pathway as the parent compound (Ussery et al., 2021; Elizalde-Velázquez et al., 2022b). Hence, inhibition of complex I may also lead to oxidative damage observed in *Danio rerio* gills after GUA exposure.

In addition to inducing oxidative damage, MET and GUA also caused

Table 6
Metformin and guanylurea concentrations in water samples.

Weeks		1	2	3	4	5	6	7	8
Metformin (ng/L)	Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.82 ± 0.07*	0.81 ± 0.08*	0.79 ± 0.06*	0.82 ± 0.09*	0.80 ± 0.07*	0.82 ± 0.08*	0.81 ± 0.09*	0.81 ± 0.09*
	20	16.32 ± 0.95*	17.01 ± 0.87*	16.54 ± 0.95*	16.28 ± 0.91*	16.06 ± 0.89*	16.71 ± 0.99*	15.98 ± 0.84*	16.17 ± 0.65*
	40	33.54 ± 1.11*	33.21 ± 1.02*	32.83 ± 0.97*	33.91 ± 1.00*	32.47 ± 0.93*	33.03 ± 1.05*	32.97 ± 0.91*	33.78 ± 1.06*
Guanylurea (ng/L)	**Control**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.80 ± 0.09*	0.82 ± 0.06*	0.82 ± 0.07*	0.83 ± 0.08*	0.82 ± 0.05*	0.79 ± 0.04*	0.83 ± 0.11*	0.81 ± 0.06*
	20	16.14 ± 0.80*	16.55 ± 0.91*	16.30 ± 0.91*	16.09 ± 0.73*	16.90 ± 0.84*	16.84 ± 0.96*	17.09 ± 0.97*	17.01 ± 0.77*
	40	33.81 ± 0.96*	32.97 ± 1.01*	33.62 ± 0.84*	33.19 ± 0.97*	33.61 ± 0.89*	32.53 ± 1.01*	32.90 ± 0.90*	32.81 ± 1.00*
Weeks		**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Metformin (ng/L)	**Control**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.81 ± 0.07*	0.81 ± 0.07*	0.82 ± 0.05*	0.80 ± 0.09*	0.80 ± 0.08*	0.81 ± 0.09*	0.81 ± 0.06*	0.80 ± 0.07*
	20	17.02 ± 0.79*	16.81 ± 0.92*	16.93 ± 0.88*	16.73 ± 0.63*	16.55 ± 0.71*	16.62 ± 0.84*	17.01 ± 0.92*	16.45 ± 0.89*
	40	32.38 ± 0.81*	33.24 ± 1.00*	32.91 ± 0.89*	32.74 ± 0.93*	33.07 ± 1.01*	32.66 ± 0.91*	31.99 ± 0.69*	33.36 ± 1.12*
Guanylurea (ng/L)	**Control**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.83 ± 0.08*	0.82 ± 0.04*	0.82 ± 0.06*	0.81 ± 0.07*	0.81 ± 0.07*	0.82 ± 0.08*	0.82 ± 0.09*	0.82 ± 0.08*
	20	16.87 ± 0.94*	17.01 ± 1.01*	17.03 ± 0.93*	16.90 ± 0.78*	16.85 ± 0.83*	16.41 ± 0.76*	16.53 ± 0.89*	16.59 ± 0.94*
	40	32.94 ± 0.81*	33.64 ± 1.00*	33.91 ± 0.89*	33.52 ± 0.93*	33.11 ± 1.01*	32.98 ± 0.91*	33.50 ± 0.69*	32.83 ± 1.12*
Weeks		**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Metformin (ng/L)	**Control**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.81 ± 0.09*	0.80 ± 0.09*	0.81 ± 0.08*	0.82 ± 0.07*	0.80 ± 0.03*	0.81 ± 0.08*	0.82 ± 0.09*	0.82 ± 0.08*
	20	15.92 ± 0.60*	16.44 ± 0.88*	16.51 ± 0.94*	16.79 ± 0.81*	17.01 ± 1.00*	16.86 ± 0.69*	16.92 ± 0.91*	16.79 ± 0.79*
	40	33.51 ± 0.99*	33.19 ± 1.04*	32.99 ± 0.96*	32.85 ± 0.97*	32.94 ± 0.87*	32.74 ± 0.90*	32.71 ± 0.74*	33.01 ± 1.00*
Guanylurea (ng/L)	**Control**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	1	0.83 ± 0.09*	0.83 ± 0.07*	0.82 ± 0.08*	0.82 ± 0.09*	0.81 ± 0.08*	0.82 ± 0.07*	0.83 ± 0.08*	0.82 ± 0.09*
	20	17.06 ± 0.87*	17.11 ± 0.95*	16.97 ± 1.05*	16.84 ± 0.86*	16.97 ± 0.91*	16.82 ± 0.84*	16.88 ± 0.93*	16.99 ± 0.89*
	40	33.13 ± 0.90*	33.48 ± 0.76*	32.96 ± 0.85*	32.76 ± 0.69*	33.04 ± 1.00*	32.79 ± 0.89*	33.19 ± 0.92*	32.90 ± 0.93*

Data represent mean ± standard deviation of three independent experiments. LOQ-MET: 1.0 ng/L, LOQ-GUA: 5 ng/L.

Table 7
Metformin and guanylurea concentrations in *Danio rerio* organs.

Organ	Control	Metformin (ng/g)			Guanylurea (ng/g)		
		1	20	40	1	20	40
Liver	<LOQ	19 ± 0.6*	320 ± 3.8*	516 ± 6.7*	25 ± 0.7*	542 ± 3.7*	906 ± 9.9*
Gut	<LOQ	28 ± 1.1*	505 ± 4.6*	871 ± 7.1*	36 ± 0.9*	724 ± 6.1*	1252 ± 10.1*
Brain	<LOQ	15 ± 0.7*	263 ± 5.9*	438 ± 4.4*	22 ± 0.4*	480 ± 7.3*	871 ± 9.4*
Gill	<LOQ	23 ± 0.9*	391 ± 4.6*	649 ± 5.3*	29 ± 1.0*	603 ± 4.8*	1037 ± 9.7*

Data represent mean ± standard deviation of three independent experiments. LOQ-liver-MET: 1.02 ng/g, LOQ-liver-GUA: 1.56 ng/g, LOQ-gills-MET: 5.32 ng/g, LOQ-gills-GUA: 4.32 ng/g, LOQ-brain-MET: 3.52 ng/g, LOQ-brain-GUA: 4.08 ng/g, LOQ-gut-MET: 1.31 ng/g, LOQ-gut-GUA: 2.08 ng/g * denote significant difference compared to the control group.

modifications in the expression of multiple genes associated with regulating antioxidant defense and programmed cell death. These changes were detected in all examined organs, albeit the response was organ-specific and compound-specific. For example, the outcomes indicated that GUA prompted more substantial damage than the parent compound. This was evidenced as MET only exerted significant gene expression changes at the middle and highest concentration, while GUA did it at all systems. Furthermore, the alterations in gene expression induced by GUA were more significant in most organs and genes than those elicited by MET. Concerning each organ's gene expression, outcomes showed bax, bcl2, and p53, in that order, were the genes that suffered the most remarkable change in the liver after chronic exposure to both compounds. Based on the information presented earlier, Lin and colleagues (2021) provided evidence that various concentrations of MET (0.1, 1, 10, 100, and 1000 μg/L) induced up-regulation of *p53* expression, a human tumor suppressor gene that is frequently associated with the process of apoptosis. The upregulation of apoptosis genes was not exclusive to the liver of fish. The *bax, bcl2, p53*, and *casp3* genes were also overexpressed in the other organs, with the most notable response, after the liver, occurring in the gut, followed by the gills and brain. *Nrf1 and nrf2* are two essential transcription factors that help cells respond to various stressors and maintain cellular homeostasis. Herein, *nrf1* and *nrf2* were remarkably up-regulated in all organs after chronic exposure to MET and GUA. The brain was the organ that showed the most substantial change compared to the control group, followed by the gut, liver, and gills. Changes in *nrf1* and *nrf2* are noteworthy, as ROS, specifically H2O2, triggers their increase (Escobar-Huerfano et al., 2022; Colín-García et al., 2022), which agrees with our oxidative stress results.

Similar to what has been observed in the context of gene expression and oxidative stress, the study revealed significant alterations in fish's hematological and biochemical blood parameters following chronic exposure to both the parent compound and its transformation product. Even though MET displayed a similar trend as GUA in most of the blood parameters, it is remarkable 1) GUA increased or reduced the values of haemato-biochemical indices in a more significant way than MET, and 2) there were some indices in which GUA prompted and increase, while MET decreased them. An evident illustration of the latter phenomenon can be observed in the changes in glucose levels. Specifically, the exposure to the antidiabetic drug MET substantially decreased the levels of glucose in fish, whereas GUA led to an increase in its concentration. Oxidative stress can give rise to elevated glucose levels. One of the predominant pathways by which oxidative stress affects glucose homeostasis is via the impairment of insulin function (Hurrle and Hsu, 2017). Insulin facilitates glucose uptake by cells, which can be utilized for energy production or stored for future use. However, oxidative stress can induce insulin resistance, causing cells to become less receptive to insulin's actions (Luc et al., 2019). Consequently, glucose transport into the cells is hindered, accumulating glucose in the bloodstream and causing hyperglycemia. Considering our oxidative stress results, it can be suggested that GUA-induced oxidative damage might be the reason for increasing glucose levels. With regards to kidney function, it was observed that there was no significant change in blood urea nitrogen (BUN) levels across all concentrations of MET, as well as the lowest concentration of GUA. However, the levels of BUN significantly increased with exposure to 40 g/L and 100 g/L of GUA. In addition, chronic exposure to both pollutants resulted in a significant reduction in

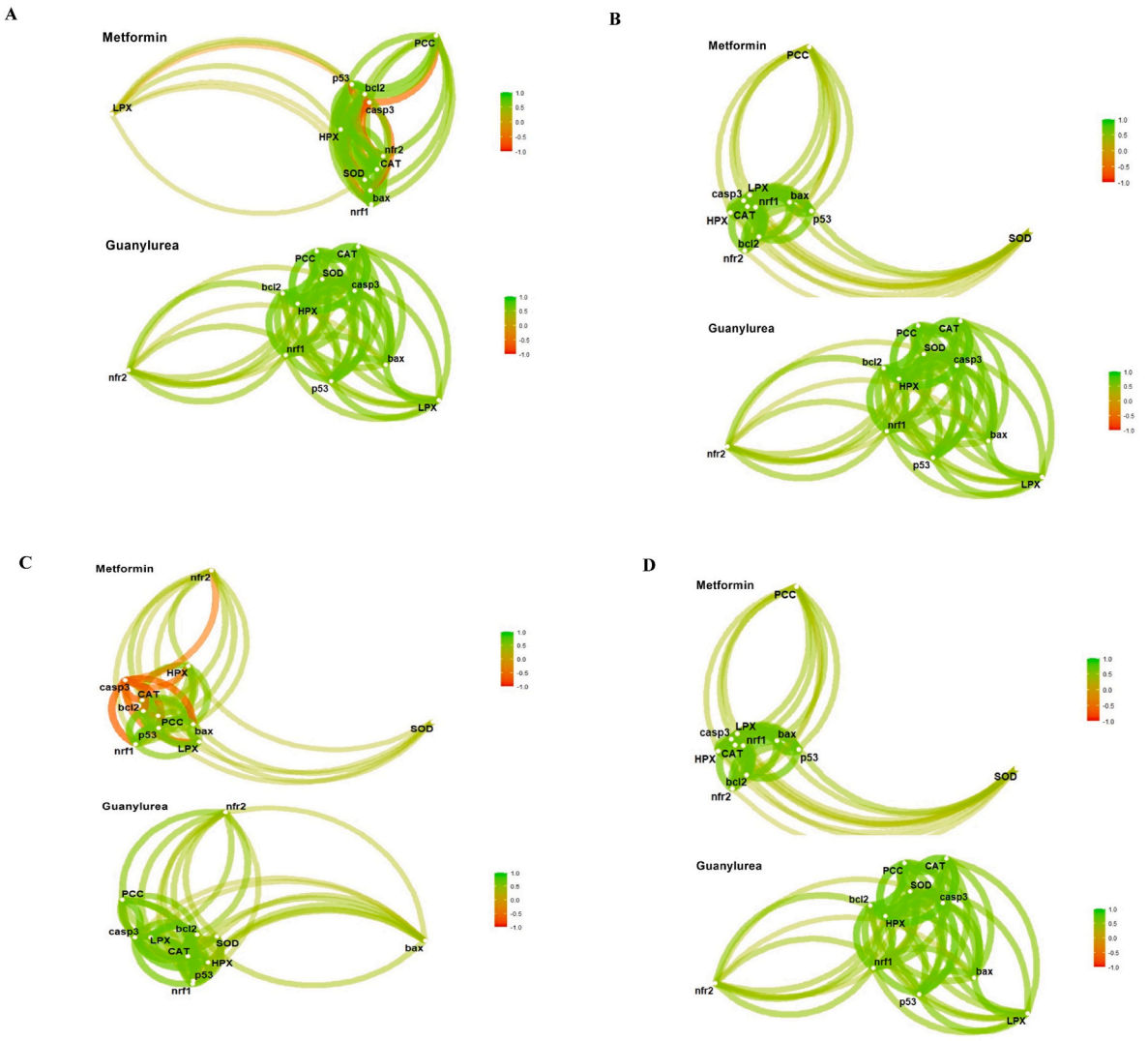

Fig. 3. Network correlation of oxidative stress and gene expression outcomes in **A**: liver, **B**: gut, **C**: brain, and **D**: gills.

creatinine (CRE) levels. BUN and CRE are commonly used blood tests to evaluate kidney function. BUN measures the amount of nitrogen in the blood from urea, a byproduct of liver metabolism, during protein breakdown (Tomizawa et al., 2015). Creatinine is a byproduct produced during muscle metabolism and eliminated from the body through renal excretion. Reduced creatinine levels can be seen in decreased muscle mass or severe malnutrition cases (Keller, 2019). Recent research has identified a relationship between exposure to MET and vitamin B12 deficiency. Low vitamin B12 levels can result in malnutrition, mainly when the deficit is left untreated for a prolonged period (Infante et al., 2021). The current evidence suggests that MET exposure may lead to decreased creatinine levels in fish due to the malnutrition it triggers in organism. However, muscular dystrophy (Kang et al., 2022) and liver dysfunction (Hartleb and Gutkowski, 2012) due to MET chronic exposure are not discarded as likely explanations for low CRE levels in fish. Although hepatotoxicity is not considered a significant side effect of metformin, several cases of metformin-induced liver injury have been reported in the last decade (de la Poza Gómez and Boixeda de Miquel, 2008; Cone et al., 2010; Olivera-Gonzalez et al., 2010; Miralles-Linares et al., 2012; Volarevic et al., 2015; Zheng, 2016; Elyigit et al., 2016; Wang et al., 2022). Moreover, one recent study indicated chronic exposure to MET (390–14,423 ng/L) disrupted molecular and biochemical processes related to *Danio rerio* metabolism and concomitantly produced changes in the hepatosomatic index (HSI) (Barros et al., 2022). Alterations in the HSI can serve as an indicator of hepatic malfunction. In agreement with the above data, our outcomes demonstrated levels of ALT and ALP, two liver enzymes that play a role in protein metabolism, significantly increased in fish exposed to both compounds. Meanwhile, TP and GLOB, two blood markers often used to assess liver function and diagnose liver disease, were significantly reduced in fish of all systems. Together, these outcomes indicated MET and GUA might render liver damage in fish. In addition to liver damage, MET may also trigger hemolytic and megaloblastic anemia, as numerous studies have indicated (Packer et al., 2008; Kirkiz et al., 2014; Gholoobi et a., 2015; Zhang et al., 2016; Ruggiero et al., 2016; Abdel-Moneim et al., 2019; Wang et al., 2022; Bell, 2022). Concordantly with the above data, this research showed the parent compound and the transformation product substantially reduced RBC, Hb, and HTC levels in fish blood. Furthermore, MET exposure remarkably augmented levels of MCV, while GUA decreased it. Even though it has not been fully clarified the mechanism by which MET prompts anemia, it is suggested by the scientific community that B12 and dehydrogenase deficiency might be responsible for it.

5. Conclusion

The findings of this study are the first evidence MET and GUA may prompt anemia and induce hepatic damage in fish after chronic

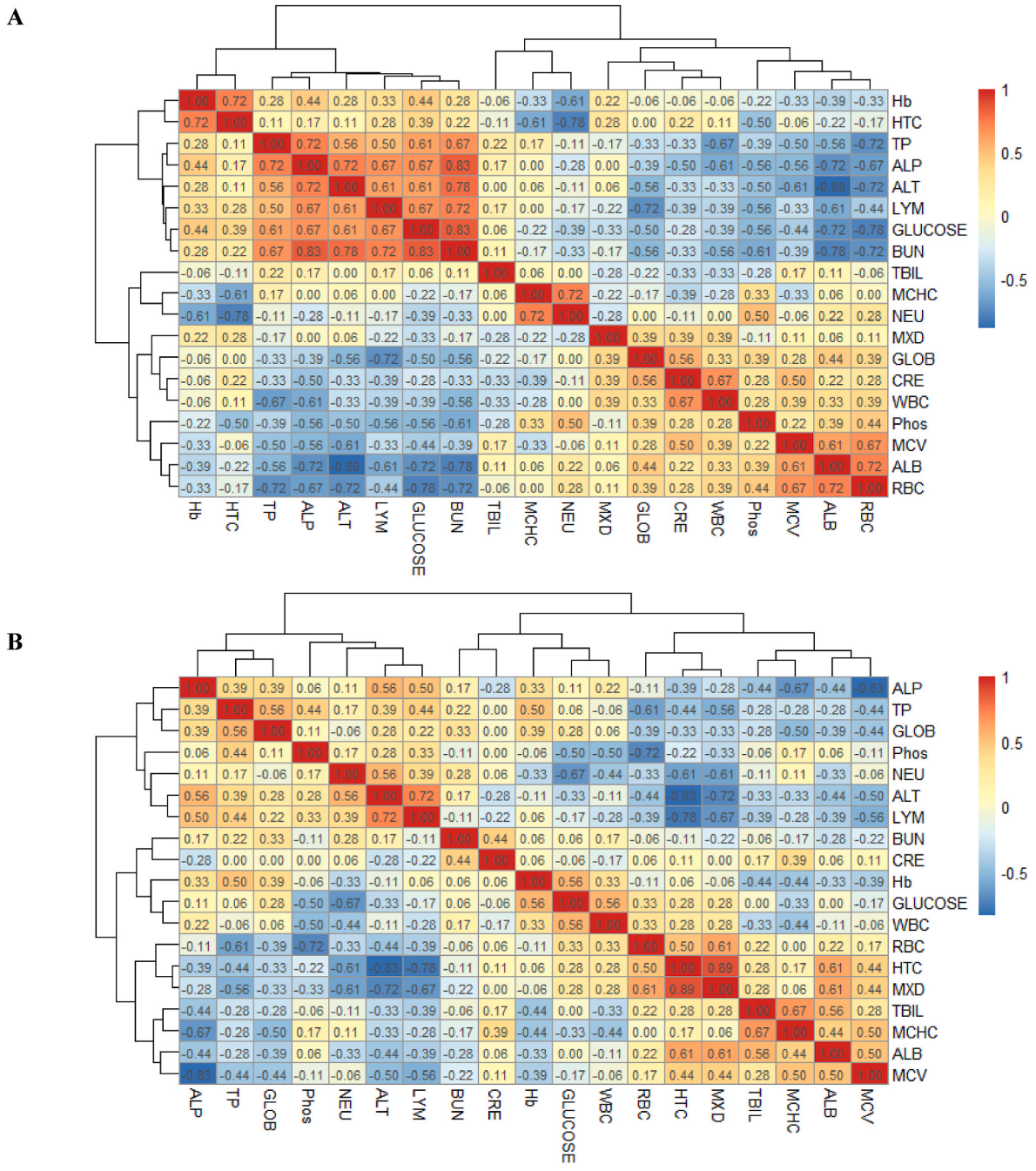

Fig. 4. Correlograms of biochemical and hematological results from GUA (**A**) and MET (**B**).

exposure. Furthermore, our results validate the notion that MET and GUA pose a significant peril to aquatic organisms, as their existing levels within water systems can impair fish health. Even though both compounds generated a similar toxic response, our results show that GUA altered the values of all endpoints to a more considerable extent than its parent compound. Thus, it can be concluded GUA is more toxic than its parent compound, MET.

Credit Author Statement

Gustavo Axel Elizalde-Velázquez performed all the exposure experiments.Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception, Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez, were involved in the design and interpretation of the data and the writing of the manuscript with input from Selene Elizabeth Herrera-Vázquez and Sandra García-Medina.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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