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Combined effects of glyphosate and chemical hypoxia in zebrafish: A new toxicological point of view

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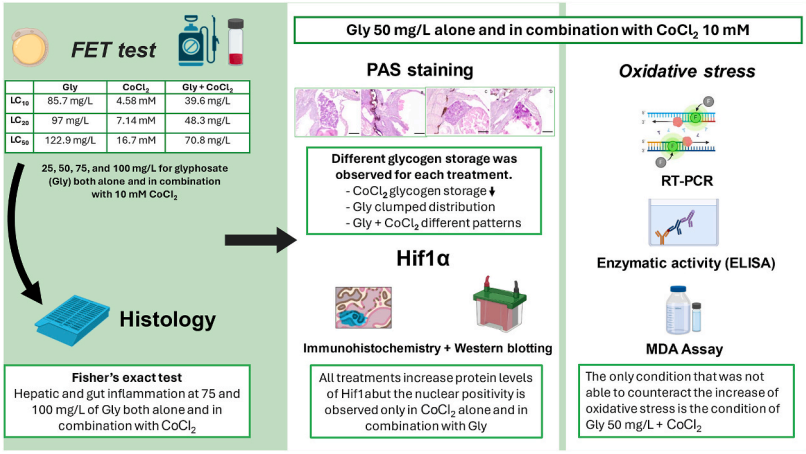
HIGHLIGHTS

- Combined effects of hypoxia-induced by CoCl2 and glyphosate in zebrafish early life stage are studied.
- Results confirmed the toxicity of glyphosate in zebrafish's early stages.
- The phenotypical alterations are supported by histopathological lesions.
- Glyphosate in hypoxia condition is not able to counteract the oxidative stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Glyphosate (Gly), a systemic and non-selective post-emergence herbicide used worldwide, has emerged as a pollutant. However, its toxic effects are debated by regulatory authorities. In addition, in the aquatic environment, often the presence of pollutants is associated with a hypoxia condition that could change their toxicological effects. We used zebrafish embryos to evaluate the toxic effects of Gly and its mechanisms in a hypoxic condition chemically induced by cobalt chloride ($CoCl_2$). We found that Gly induced toxicity in a time and concentration-dependent manner. The toxicity of Gly was determined at 96 h post fertilization as a lethal concentration (LC), and LC_{10}, LC_{20}, and LC_{50} values were 85.7, 97, and 122.9 mg/L, respectively. When Gly was combined with $CoCl_2$ the toxicological endpoints were lower than values referred to the Gly alone indicating the

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worse effects of chemical hypoxia on Gly toxicity. Histological observations were performed at 25, 50, 75, and 100 mg/L for Gly both alone and in combination with 10 mM $CoCl_2$. Fisher's exact test showed significant differences in the presence of hepatic and gut inflammation at 75 and 100 mg/L of Gly both alone and in combination with $CoCl_2$. To deeply investigate the effects of hypoxia on Gly toxicity we decided to test the lowest dose of Gly, 50 mg/L, alone or in combination with $CoCl_2$ 10 mM on liver glycogen storage and oxidative stress. Again the results obtained indicate the worse effects of chemical hypoxia on Gly toxicity. Thus Gly toxicity could be reconsidered in light of the damage it causes to the liver and intestines and its effect in combination with factors that induce chemical hypoxia.

1. Introduction

Glyphosate (*N-(*phosphonomethyl) glycine) is a systemic and non-selective post-emergence foliar herbicide. Gly is now known as the most widely used herbicide in the world (O Duke and B Powles, 2008) and acts through the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).

This enzyme is critical in the shikimate pathway. Such a pathway is highly important in plants as it leads to the development of three aromatic amino acids: tyrosine, phenylalanine and tryptophan. The EPSPS role is to catalyse the condensation reaction between shikimate-3-phosphate (S3P) and the phosphoenolpyruvate (PEP) resulting in 5-enolpyruvylshikimate-3-phosphate (EPSP). The Gly bonds to the EPSPS catalytic site, thus competing with the PEP. As a consequence, it creates a ternary complex glyphosate-EPSPS-S3P, which blocks the metabolic pathway and results in a shikimate stack inside the cells (Duke, 2021).

Because of its extensive use in agriculture, traces of this herbicide are nowadays found in soil, water, and air (Novotny, 2022; Petrovici et al., 2020; Stara et al., 2021, 2022; Tresnakova et al., 2023) as well as in food (Zoller et al., 2018), becoming a growing concern for human health. Indeed, although the shikimate pathway is not found in humans, it is typical in bacteria, including those in the human gut microbiota (Mesnage et al., 2021). In addition, glyphosate and glyphosate-based herbicides can also be toxic to aquatic organisms (Azadikhah et al., 2023; Tresnakova et al., 2023; Yalsuyi et al., 2021). Uren Webster et al. (2014) demonstrated that both pure glyphosate and the glyphosate-based herbicide Roundup® negatively affected the reproductive cycle of zebrafish (*Danio rerio*). It increased the mortality and the premature hatching rate in the early life stage, and it led to changes in *cyp19a1* and *esr1* gene expression in the ovary, as well as *hsd3b2*, *sod1,* and *cat* in testicles (Uren et al., 2014). In the case of goldfish (*Carassius auratus*), Lushchak et al. (2009) observed a reduction in activity for some antioxidant enzymes - glutathione reductase (GR), glutathione S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PD) and superoxide dismutase (SOD) - as a consequence of exposure of fishes to Roundup® concentration between 2.5 and 20.0 mg/L (Lushchak et al., 2009). Ecotoxicology studies based on water organisms as experimental models are particularly useful, as glyphosate-based herbicides and their metabolites are often found in rivers and other water ecosystems (Gasnier et al., 2009) as a result of rains and land erosion (Meftaul et al., 2020). A distinctive feature of some of these water environments, particularly those highly polluted, is the low water oxygen concentration (Sappal et al., 2015). This leads to the development of hypoxic environments which represents a stress factor for the residing organisms (Fulford et al., 2024).

Hypoxia is a condition in which the oxygen concentration in tissues is lower than the physiological one (Filice et al., 2024; Span and Bussink, 2015). Cells can detect hypoxia thanks to Hypoxia-Inducible Factors (HIFs), HIF-1, HIF-2, and HIF-3 (Fitzpatrick, 2019; Fratantonio et al., 2018; Koh and Powis, 2012). HIFs consist of two subunits; the α subunit which reacts to changes in oxygen concentrations, and the β subunit which is constitutively expressed and does not react to such changes (Lee et al., 2009). HIF-1 is perhaps one of the most widely used markers to detect hypoxic conditions. HIF-1α and HIF-1β bond together to produce the HIF-1 heterodimer. In normoxia conditions, HIF-1α is brought to ubiquitination mediated by Von-Hippel-Lindau complex (pVHL). The HIF-1-VHL interaction is made possible by Prolyl Hidroxylase Domain Proteins (PHDs), which promote the hydroxylation of two proline residues on Oxygen-dependent degradation (ODD) domain of HIF-1α subunit. The hydroxylation reaction catalysed by PHDs is also linked to the α-ketoglutarate oxidative decarboxylation to succinate and CO_2. However, the PHDs require oxygen (O_2), iron (Fe^{2+}) and α-ketoglutarate to fulfil their functions. Therefore, hypoxic conditions inhibit PHDs; as a consequence they cannot catalyse hydroxylation of the two proline residues within the ODD domain. This allows the HIF-1α- HIF-1β bond. The bonded subunits, i.e. the HIF-1 heterodimer, create a transcriptionally active complex that supports the expression of hypoxia response elements (HRE) (Lee et al., 2009).

So far, research has focused on the effects of Gly on water organisms, highlighting its neurotoxic and immunotoxic action, but also its ability in nurturing the production of reactive oxygen species (ROS). The latter leads to oxidative damage, as well as to the interference of Gly with the production of antioxidants (Lushchak et al., 2009; Uren et al., 2014 Barathinivas et al., 2022). At the same time, other research focused on the effects of hypoxia showing e.g. that the modulation of HIF-α is closely connected to the development and the increased severity of cancers or inflammatory diseases (Elks et al., 2015).

However, little is still known about the combined effect of Gly and hypoxic conditions on the embryonal and larval forms found in water ecosystems. In zebrafish the adverse outcome pathway of the Gly developmental toxicity and its underlying mechanisms remain still unclear. Therefore, this study aims to assess the biochemical, histological and molecular changes induced by glyphosate-based herbicide in the early life stages of zebrafish and also to evaluate the modifications associated with a hypoxic condition, chemically induced by cobalt chloride ($CoCl_2$). To analyze the concentration of chemicals in both individual and combined samples obtained after 96 h of treatment, we utilized Inductively Coupled Plasma Mass Spectrometry (ICP-MS) which is widely recognized for its accuracy and sensitivity in quantifying trace element concentrations across various matrices and application fields. In fact, the evaluation of Gly toxicity combined with an hypoxic condition is a crucial aspect reflecting the real exposure in a natural environment where different chemicals or conditions can interact in complex ways, potentially resulting in effects that are synergistic, antagonistic, or additive. Concerning the study design, the choice of embryonal form was suggested by their susceptibility to stress factors, while the Gly was selected because of its wide use as herbicide in pre and post-emergence and for its documented presence in water bodies. A commercial formulation was employed to test the Gly toxicity. The evaluation of the toxicity of complex commercial formulations is highly relevant as these formulations reflect the real-world applications in agricultural fields and the forms that are administered to living organisms.

2. Materials and method

2.1. Chemicals

Gliphogan Top CL PFnPE was produced by Bayer Agriculture BVBA (B-2040 Antwerp Belgio). Cobalt (II) chloride hexahydrate ($CoCl_2$; CAS

number 69098-14-2, Pharmaceutical Secondary Standard, C8661, >98%), 3,4-dichloroaniline (CAS number 95-76-1, 437778, >98%) and 10% neutral-buffered formalin were purchased from Bio-Optica (Milano, Italy). Dilution water (DW) was prepared following the OECD TG 203, Annex 2 (OECD, 1992). Nitric acid (HNO3, Suprapur™, 65 %, Merck Life Science S.r.l., 1.00441), Cobalt standard (for AAS, TraceCERT®, Supelco, Merck Life Science S.r.l., 05202), phosphorus standard (for AAS, TraceCERT®, Supelco, Merck Life Science S.r.l., 51474) and Rhodium Standard TraceCERT® solution (1 g/L in hydrochloric acid, Supelco, Merck Life Science S.r.l., 04736) were used for ICP-MS measurements.

2.2. Zebrafish maintenance and fish embryo acute toxicity (FET) tests

Zebrafish early-life stages used in the experiments were obtained from the University of Teramo facility. Adult wild-type AB strain zebrafish were bred in a recirculating water system with a light/dark cycle (14 h of light/10 h of dark) and were kept in 3.5 L ZebTec tanks (Tecniplast S.p.a., Buguggiate, Italy). The afternoon before the spawning, groups of females and males of adult zebrafish were placed in breeding tanks and the eggs were collected the following morning and rinsed by DW. After the macroscopic selection (the unfertilized eggs were eliminated) the eggs were used for the FET tests. The tests were performed according to OECD n. 236 (OECD, 2013). To find out the ideal concentration for hypoxic injury Gly and $CoCl_2$ were tested respectively at 25, 50, 75, 100, and 125 mg/L and 1, 5, 10, 20 mM. The dose-response curves showed a dose-dependent embryotoxicity for both chemicals. The 10 mM $CoCl_2$ concentration was chosen considering both, the survival rate derived from FETs and the data presented by several authors (Kajimura et al., 2006; Yu et al., 2012). These authors demonstrated as $CoCl_2$ 10 mM induces hypoxia stabilizing HIF1α, determining the increase in IGFBP-1 dependent on it, and also causing an alteration of hypoxia-induced vascularization.

The following concentrations were tested: 50 mg/L Gly +10 mM $CoCl_2$, 75 mg/L Gly +10 mM $CoCl_2$, 100 mg/L Gly +10 mM $CoCl_2$, 125 mg/L Gly +10 mM $CoCl_2$. The concentrations employed in FET represented the nominal concentration of the active principle contained in the formulation (error less than 10%). At the end of FET tests, larvae were collected for histological analyses. Selected embryos were placed individually with 2 mL of test solution in each well of 24-well plates within 3 h post fertilization (hpf). Twenty embryos for treatment were exposed to the four concentrations of Gly +10 mM $CoCl_2$, and the working solutions were freshly prepared every 24 h. Negative control (DW) and positive control (4% 3,4 dichloroaniline) were also tested. Three replicates were performed both for single substances and for mixtures. The exposure solutions were replaced every day and the dead embryos were removed daily. Embryos were exposed for 96 h in the incubator at 26 ± 1 °C and photoperiod (14 h light/10 dark) conditions. Zebrafish early-life stages were daily observed up to 96 h with the inverted optical microscope (CKX 41, Olympus, Japan) considering four lethal alterations: coagulation of fertilized eggs, absence of heartbeat lack of somite formation, lack of detachment of the tail from the yolk sac and lack of somite formation. At the end of the exposure period, lethal concentrations 10, 20, and 50 ($LC_{10,\ 20,\ 50}$) and some sublethal effects were evaluated. To deeply investigate the effects of hypoxia on Gly toxicity we decided to test the lowest dose of Gly, 50 mg/L, alone or in combination with $CoCl_2$ 10 mM, in which no macroscopic damage was observed. Thus, the RT-PCR, western blotting, enzymatic activity, TBARS, chemical, and immunohistochemistry analyses were performed only on 50 mg/L Gly +10 mM $CoCl_2$, 10 mM $CoCl_2$, and 50 mg/L Gly.

2.3. Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Cobalt chloride and glyphosate were determined by Thermofisher iCAP™ TQe ICP-Triple Quadruple instrument. For ICP-MS analysis, samples were filtered through a 0.2 μm recycled cellulose syringe filters and diluted by a factor of 2000× in 100 mL polymethylpentene (PMP) volumetric flasks with 1% v/v of suprapur HNO_3 in ultrapure deionized water (18.2 MΩ cm resistivity at 25 °C), provided by a purification system Milli Q (Millipore, Germany). All the samples were prepared in duplicate and were analyzed by performing three instrumental repetitions.

Cobalt Standard TraceCERT® (1 g/L in 2% nitric acid, nominal concentration), phosphorus standard TraceCERT® (1 g/L in H2O, nominal concentration) were diluted and employed for the instrument calibration. Standard solutions were prepared using 1% nitric acid (HNO3) in ultrapure deionized water. Matrix effects and instrumentation drift were monitored using a 10 μg/L Rhodium standard TraceCERT® solution as an Internal Standard for all the analyzed solutions. Both the DW prepared according to the OECD TG 203, the non-treated larvae water (NK) and the treated larvae water (10 mM $CoCl_2$, Gly 50 mg/L and 50 mg/L Gly + 10 mM $CoCl_2$) were analyzed.

2.4. Histological analyses

At 96 hpf survived larvae were collected for histological analyses. Ten embryos for each treatment were considered for histological evaluation. Briefly, they were fixed in 10% neutral-buffered formalin for 2 h and processed for routine histological evaluation through 2 h-long consecutive passages in increasing ethanol and xylol, and finally embedded in paraffin. 5 μm thickness serial sections were stained with hematoxylin and eosin and PAS Stain (periodic acid shift) for highlighting hepatic glycogen in magenta red, and then visualized by light microscope (Leika DMRE). A qualitative analysis of the histopathological features has been performed for all the groups. Furthermore, the presence (0 = absent; 1 = present) of the following markers, representative of toxic damage and inflammation, have been evaluated semi-quantitatively in 100 mg/L Gly-treated larvae both in normal and hypoxic conditions: hepatic vacuolization, enlarged hepatic nuclei, hepatic hyperemia, hepatic inflammation, gut inflammation, and turbid bulge. The controls were represented by non-treated larvae (NK).

2.5. Immunohistochemical analysis

HIF1α immunoexpression has been evaluated in 5 larvae serial sections for each group through immunohistochemistry (IHC) using a specific primary antibody (Anti-HIF1α 1:300 dilution, NB100-134). Briefly, sections were treated with citrate buffer solution 0.01 M pH 6.0 in a microwave at 720W for 15 min for antigen retrieval; unspecific binding sites were blocked with 5% bovine serum albumin and 5% normal goat serum for 20 min each. Labeling was detected using an ImmPRESS HRP Universal (horse anti-mouse/rabbit IgG) PLUS polymer kit (Vector Laboratories, CA, USA) with 0.1% hydrogen peroxide in a 3,3′-diaminobenzidine solution (Millipore Sigma, St. Louis, MO, USA) as chromogen. Sections were counterstained with Mayer hematoxylin (Merck, Darmstadt, Germany). A qualitative evaluation of protein localization was performed.

2.6. Protein extraction and western blotting

Total protein extracts were obtained by homogenization of pools of 30 96-dpf larvae in ice-cold RIPA buffer (ThermoFisher, 89901) and a complete EDTA-free protease inhibitor cocktail (ThermoFisher, 78444). Protein concentration was measured using the BCA Protein Assay kit (Thermo Fischer Scientific, 23225) and 30 μg of protein was run on Bolt™ 4–12% Bis-Tris Plus Gels (ThermoFisher, NW04120BOX). Once the electrophoretic run was completed, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad). Nonspecific binding sites were blocked for 1 h at room temperature (RT) with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20 (TBS-T). The membranes were incubated overnight at 4 °C with primary antibodies: anti-HIF1α (1:1000; Novus Bio techne, NB100-134) and anti-βActin (1:1000;

GeneTex, GTX124501) appropriately diluted in blocking solution. Anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000; Jackson ImmunoResearch, AB_2313567) was incubated for 1 h at room temperature, and protein bands were detected by chemiluminescence according to the manufacturer's instructions and acquired by the imaging Alliance 7 (UVITEC Limited, Cambridge, UK). The relative densities of immunoreactive bands were determined using ImageJ.

2.7. mRNA isolation and Real-Time PCR

Three biological replicates were collected for developmental gene expression analysis. In each replicate, 30 zebrafish larvae were homogenized with 200 μL of Trizol™ reagent (Invitrogen, CA, USA). RNA concentration and nucleic acid purity were determined by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA). cDNA was synthesized using 5× All-In-One RT MasterMix (Applied Biological Materials Inc., BC, Canada) according to the manufacturer's instructions. Real-time PCRs were performed with the CFX Opus 96 Real-Time PCR System (Biorad, CA, USA) using Sso Advanced™ Universal Probes Supermix (Biorad, CA, USA) and gene-specific probes (Biorad, CA, USA) for Superoxide dismutase 1 (sod1) (qDreCIP0036226), Glutathione S-transferase M (gst) (qDreCEP0038218) and for Catalase (cat) (qDreCEP0043368). The PCR amplification procedure was as follows: 50 °C × 2 min, 95 °C × 10 min, then 40 cycles of 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C. Results were normalized using β-actin (qDreCEP0045468) as the reference gene. We used the $2^{-\Delta\Delta Ct}$ method to calculate expression levels.

2.8. Enzymatic activity (ELISA)

ELISA kits were purchased from Elabscience Biotechnology and used to determine enzyme activity levels according to the manufacturer's instruction method. Three biological replicates per condition (10 mg corresponding to a pool of 50 96 dpf larvae). The antioxidant enzymes analyzed are the following: Glutathione-S-Transferase (GST) (E-BC-K800-M), Total Superoxide Dismutase (T-SOD) (E-BC-K020-M), and Catalase (CAT) (E -BC-K031-M). Their activity was estimated by colorimetric method using an Infinite® 2000 PRO microplate reader.

2.9. TBARS (MDA) assay kit

The malondialdehyde (MDA) assay was performed using the lipid peroxidation analysis kit (MDA). Three biological replicates per condition obtained by homogenizing a 10 mg pool of 96 dpf larvae in ice-cold lysis buffer were used. MDA levels were measured according to the manufacturer's instructions (MAK085, Sigma-Aldrich).

2.10. Ethics statement

The zebrafish developmental stage at the end of the exposure did not fall into the regulatory frameworks dealing with animal experimentation, and all the experiments complied with "Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes" and with the Italian law "D. Lgs n. 26 4 marzo 2014 Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici".

2.11. Statistical analysis

Statistical analysis of FET test results was performed using ToxRat software version 3.3 (ToxRat Solutions GmbH, Germany).

Regarding the histological data, to better identify the effect of Gly and the role of hypoxia, Fisher's exact test was used to evaluate the statistical differences in the frequencies of over-mentioned toxic and inflammatory markers in 100 mg/L Gly-treated larvae both in normal and hypoxic conditions with respect to the controls. For gene expression, western blotting, protein activity analysis, and lipid peroxidation dosage we used ANOVAs. Significant effects of treatment were further investigated with Tukey's post hoc tests.

GraphPad Prism 8 software (GraphPad Software) was used to compare statistical differences between exposed and control groups for gene expression data. Differences were considered statistically significant for all statistical analyses if their *p*-values were 0.05 or lower.

3. Results

3.1. ICP-MS analysis

The solution treated with $CoCl_2$ revealed the presence of 7.805 mM of cobalt, with reference to the nominal concentration of 10 mM, while in the Gly sample with nominal concentration of 50 mg/L (equivalent to 9.1 mg/L of phosphorous) ICP-MS measured 12.0 mg/L of phosphorous. In the 50 mg/L Gly +10 mM $CoCl_2$ solution the Co amount was 7.666 mM, while phosporous was 8.4 mg/L (Table 1).

3.2. Fish embryo acute toxicity (FET) tests

In accordance with the OECD guidelines n° 236, the acceptance criteria of the FET tests were respected as the mortality of the negative control group was less than 10%, while the mortality of the positive control group was 60%.

The toxicity of Gly was determined at 96 hpf as a lethal concentration, and LC_{10}, LC_{20}, and LC_{50} values were 85.7, 97, and 122.9 mg/L, respectively (Table 2). Late coagulation was the only lethal endpoint at the highest concentration of Gly (125 mg/L): specifically, 61.7 % of embryos were coagulated after 24 hpf. At 96 hpf the number of coagulated embryos increased to 73.3 %. Furthermore, zebrafish larvae treated with Gly developed sublethal alterations. From the concentration of 25 mg/L at 48 hpf, macroscopically the larvae showed pericardial edema, impaired blood flow, and blood stasis (Fig. 1S). The number of survived embryos developing sublethal alterations increased in a concentration-dependent manner and in a time-dependent manner (from 72 hpf to 96 hpf).

Concerning FET tests performed on $CoCl_2$, at 96 hpf the LC_{10}, LC_{20}, and LC_{50} were respectively 4.58 mM, 7.14 mM, and 16.7 mM (Table 2). Also in this case the main lethal alteration observed was the coagulation of the embryo that appeared in the first 24 hpf. In fact at the highest concentration (20 mM) at 24 hpf the percentage of coagulated embryos was 33 %. At 48 hpf the main sub-lethal alteration was blood stasis in all tested concentrations, while reduction of blood circulation and pericardial edema started to be observed from 72 hpf. Furthermore, at 72 hpf, hatching was significantly delayed in zebrafish embryos exposed to

Table 1
ICP-MS results: nominal concentrations versus measured concentrations for elemental Cobalt and Phosphorous at 96 hpf.

Sample	Cobalt Nominal concentration (mM)	Cobalt (mM)	Phosphorous Nominal concentration (mg/L)	Phosphorous (mg/L)
DW	–	3.9E-05 (±2E-07)	–	<LOD
NK	–	3.2E-05 (±1.0E-05)	–	9.0E-03 (±2.0E-03)
$CoCl_2$	10	7.805 (±0.111)	–	<LOD
Gly	–	7.0E-04 (±2.0E-04)	9.1[a]	12.0 (±0.8)
$CoCl_2$ + Gly	10	7.666 (±0.062)	9.1[a]	8.4 (±0.4)

[a] Equivalent to 50 mg/L of Gly.

Table 2
Toxicological parameters.

	CL_{10}	CL_{20}	CL_{50}
Gly	85.7 mg/L	97 mg/L	122.9 mg/L
$CoCl_2$	4.58 mM	7.14 mM	16.7 mM
Gly + $CoCl_2$	**39.6 mg/L**	**48.3 mg/L**	**70.8 mg/L**

$CoCl_2$ at concentrations of 10 mM (90.6 %) and 20 mM (92.5 %). At the highest concentrations of Gly (100 mg/L and 125 mg/L), the percentage of not hatched larvae decreased at 55.6 % and 56.3 %, respectively, arriving at 44.7 % in the 100 mg/L Gly + 10 mM.

Finally, as regards the FET tests toxicological endpoints for the Gly and $CoCl_2$ combination, as shown in Table 2, the toxicological endpoints were lower than values referred to the Gly.

In the mixture too, the coagulation of embryos was the main observed lethal effect. In fact, at 24 hpf 8.3% of embryos at 50 mg/L +10 mM were coagulated, 6.7% at 75 mg/L+ 10 mM, 20% at 100 mg/L Gly +10 mM, and 8.3% at 125 mg/L Gly +10 mM.

In the case of hypoxia and Gly combination too the sub-lethal alterations related to the cardiovascular system were observed above all. The main sub-lethal effects grossly observed were, in fact: pericardial edema, yolk sac edema and blood stasis visible above all at the highest concentrations as early as 48 hpf.

3.3. Histopathological effect of treatments

Histological observation has been performed for each Gly concentration (25, 50, 75, 100 mg/L) both alone and in combination with 10 mM $CoCl_2$.

An initial qualitative analysis of the histopathological features highlighted the presence of the following histopathological patterns only in higher concentrations-treated groups (Gly 75 and 100 mg/L) both alone and combined with 10 mM $CoCl_2$.

The larvae showed major lesions in the liver and in the intestine. In the liver were found derangement of hepatic cords, loss of cellular and nuclear contour, loss of cell-to-cell contact, increased cell volume, variable intense cytoplasmic vacuolization, decreased numbers of nuclei, nuclear atrophy or hypertrophy of the nuclei, cytoplasmic and nuclear degeneration, cell disruption, hyperemia and rare infiltration of leucocytes (Fig. 2A–D).

In the same groups, the intestine showed alterations referable to dilatation of the villous vessels, very mild detachment of the epithelial

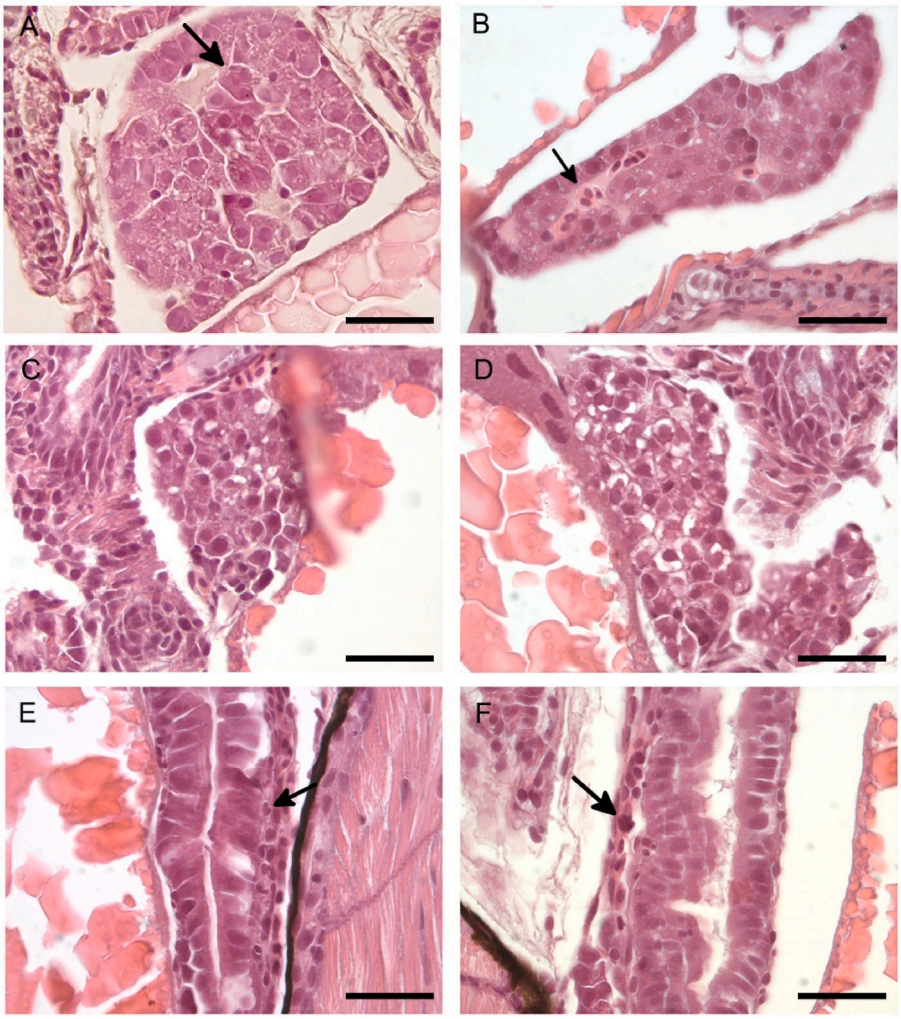

Fig. 1. A: Derangement of hepatic cords, loss of cellular and nuclear contour, and increased cell volume (black arrow) in 100 mg/L Gly treated larvae in chemical hypoxic condition. B: the presence of hyperemia (black arrow) in 75 mg/L Gly treated larvae in chemical hypoxic condition. Both moderate (C) and intense (D) cytoplasmic vacuolization with nuclear atrophy or hypertrophy of the nuclei, cytoplasmic and nuclear degeneration, loose cell-to-cell contact, cell disruption in 75 mg/L (C) and 100 mg/L (D) of Gly alone treated larvae. E-F dilatation of the villous vessels, very mild detachment of the epithelial lining of the apex of the intestinal villous, and displacement of the lamina propria in 75 mg/L (E) and 100 mg/L (F) of Gly chemical hypoxic condition treated larvae. Focal and mild leukocytes (neutrophils and lymphocytes) (black arrow). Scale bar 25 μm (100× immersion oil).

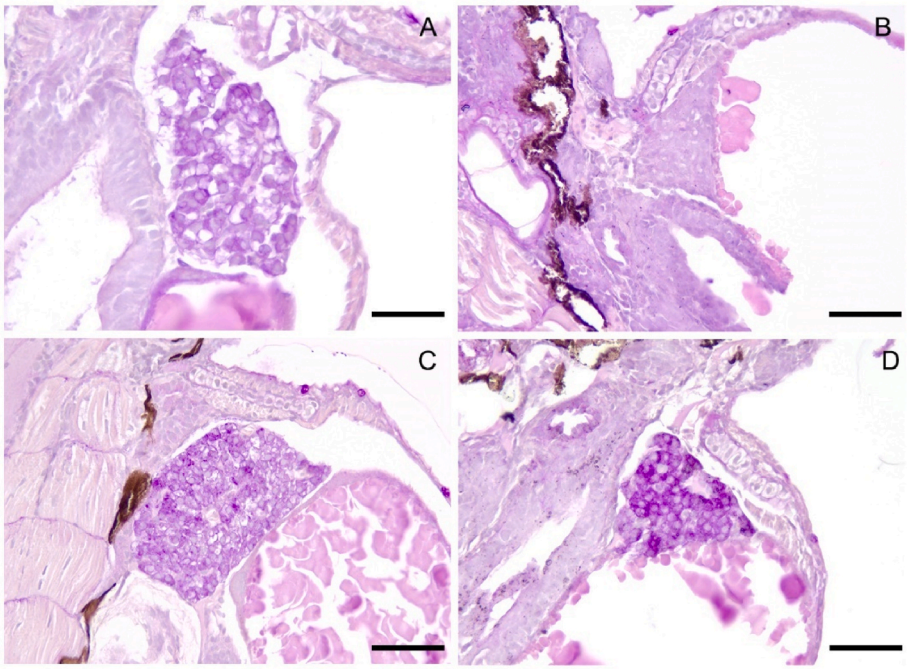

Fig. 2. PAS staining highlighting hepatic glycogen in control larvae (A), hypoxic larvae (B), 50 mg/L Gly treated larvae (C) and 50 mg/L Gly treated larvae in hypoxic condition (D). Scale bar 50 μm (40×).

lining of the apex of the intestinal villous, displacement of the lamina propria, focal and mild leukocyte (neutrophils and lymphocytes) infiltration among the lamina propria. Finally, an expanded gut lumen together with smooth gut lining was observed (Fig. 1E and F).

In the subjects treated with 100 mg/L Gly +10 mM $CoCl_2$ or with 100 mg/L Gly alone, dilatation and cardiac edema were also seen, in addition to the overmentioned histopathological features. In 10 mM $CoCl_2$ alone, only hyperemia has been observed in all the chemical hypoxic larvae, as well as in the tail vessels.

To gain deeper insights into the role of 10 mM $CoCl_2$ in the damage determined by Gly, the presence of the toxic damage and inflammation markers listed in Materials and Methods have been evaluated in 100 mg/L Gly-treated larvae both in normal and chemical hypoxic conditions (10 mM $CoCl_2$).

Fisher's exact test was used to measure the statistical differences among groups. Results showed significant differences in the presence of hepatic inflammation that is observable only in the 100 mg/L Gly treated larvae both alone and in chemical hypoxic conditions (13% of the total cases; $p = 0.003$; Table 3) and it is completely missing in NK and $CoCl_2$-treated larvae. Liver damage in the form of enlarged hepatic nuclei has also been detected in Gly-treated larvae alone and in chemical hypoxic conditions with respect to the NK and the $CoCl_2$-treated larvae which is mostly absent ($p = 0.046$; Table 3).

Furthermore, significant differences were also present in gut inflammation frequencies. It has been observed in Gly-treated larvae without hypoxia (23% of total cases) and with hypoxia (18% of total cases; $p < 0.001$; Table 3), while resulting in predominantly absent in the other groups (Table 3).

3.4. *Investigation of sublethal dose of glyphosate in liver glycogen storage and oxidative stress*

To deeply investigate the effects of hypoxia on Gly toxicity in liver glycogen storage and oxidative stress we decided to test the lowest dose of Gly, 50 mg/L, alone or in combination with $CoCl_2$ 10 mM. The result of the PAS technique on zebrafish showed that the PAS staining of the liver of $CoCl_2$-treated subjects (Fig. 2B) was either slightly, but always noticeably reduced with respect to the control group, as in Fig. 2. Gly 50 mg/L alone showed staining only very slightly reduced in intensity, but a clumped distribution was observed in some hepatocytes (Fig. 2C), while Gly combined with $CoCl_2$ (Fig. 2D) showed a varied pattern, with areas of hepatocytes in which PAS intensity was weak and others with marked reactivity.

Subsequently, we analyzed the protein levels of Hif1α (Fig. 3). We

Table 3
Hepatic and gut frequency of alteration. Fisher's exact test was used to measure the statistical differences among groups. Gly 100 mg/L; Gly 100 mg/L + $CoCl_2$ 10 mM.

	NK	$CoCl_2$	Gly	Gly + $CoCl_2$	Absence (0) or presence (1)	Fisher's exact test p value
Hepatic vacuolization	10%	18%	10%	5%	**0**	0.208
	15%	8%	15%	20%	**1**	
Hepatic inflammation	25%	25%	13%	13%	**0**	**0.003**
	0%	0%	13%	13%	**1**	
Enalarged hepatic nuclei	23%	23%	15%	10%	**0**	**0.046**
	3%	3%	10%	15%	**1**	
Hepatic hyperemia	10%	8%	13%	5%	**0**	0.689
	15%	18%	13%	20%	**1**	
Gut inflammation	18%	25%	3%	8%	**0**	**<0.001**
	8%	0%	23%	18%	**1**	
Turbid bulge	20%	15%	13%	13%	**0**	0.506
	5%	10%	13%	13%	**1**	

found a stabilization of Hif1 α protein in all conditions (Fig. 3 left). To better investigate the Hif1 α protein we also performed its immunolocalization with particular attention to the liver. Expression of Hif1α at the cytoplasmic level was generally widespread, whereas its evidence at the nuclear level was practically absent in the normal subject's liver. In the 10 mM $CoCl_2$ treatment, several positive hepatic nuclei were evident (Fig. 3B), accounting for around 5% of the total nuclei present. However, this expression was significantly reduced in subjects treated with Gly 50 mg/L (Fig. 3C), exhibiting a similar immunoexpression to NK. Furthermore, in the combination treatment of 10 mM CoCl2 and Gly 50 mg/L (Fig. 3D), positive nuclei were once again visible. Much nuclear positivity of Hif1α in other parts of the fish has been observed in all treated samples, confirming western blotting results.

Regarding the expression levels of the major enzymes involved in the oxidative stress response (Fig. 4A), we saw a different modulation depending on the treatment. In all treatments, we observed a decrease in *cat* expressions while for Gly 50 mg/L treatment we also observed an upregulation of *gst,* a key enzyme involved in xenobiotics detoxification. However, the stress response of Gly 50 mg/L + $CoCl_2$ appeared to be different, although the decreased expression of *cat* remained, the increase in expression of *gst* was no longer observed. Regarding the enzymatic activity (Fig. 4B), we observed an increase in the enzymatic activity only for Gst in $CoCl_2$ and in Gly 50 mg/L alone while for Gly + $CoCl_2$ a decreased Gst activity was observed. To better understand the effects of this enzymatic activity we performed the MDA assay to evaluate the levels of lipid peroxidation products (Fig. 4C). The only condition that was not able to counteract the increase of oxidative stress is the condition of Gly 50 mg/L + $CoCl_2$.

4. Discussion

With the extensive use of Gly in agriculture, traces of this herbicide are nowadays found in soil, water, and air (Krüger et al., 2014) as well as in food (Zoller et al., 2018), becoming a growing concern for human health. The toxicity of Gly has indeed been extensively studied by various authors, and it's not uncommon to find variations in the results due to differences in experimental conditions or the specific substances used (Gaur and Bhargava, 2019; Lanzarin et al., 2019).

In this study, we leveraged the well-established ease of detecting and quantifying elemental phosphorus by ICP-MS/MS to indirectly quantify trace levels of phosphorus-containing organic compounds (like glyphosate). From the ICP-MS analysis of the various samples at 96 hpf, the Co quantification was highly accurate using the ICP-MS technique, regardless of the presence of Gly. In both the $CoCl_2$ and $CoCl_2$+Gly samples, there was a reduction in Co concentration in the solution, with a decrease of 22 % and 23 %, respectively, compared to the nominal value of 10 mM for the initial solution (Table 1). It is noteworthy that the solution in contact with the embryos was freshly prepared and renewed every 24 h. This observed decrease in Co concentration aligns with the possibility of salt internalization in the larvae during the 24-h treatment period. The quantity of Gly in mg/L was converted into mg/L of the element phosphorus alone to ensure consistency of results, as the ICP-MS technique quantifies elemental species rather than compounds. The quantification of phosphorus contained in Gly solution presented promising yet seemingly contradictory results in fact the ICP-MS measurement of phosphorus after 96 h of treatment showed a slight increase compared to the nominal value (approximately 30 %). At this level of analysis, it is challenging to distinguish between the phosphorus from Gly and the phosphorus produced by larval metabolic processes and released into the water. In fact, it is important to consider that at 96hpf the zebrafish liver is perfused with blood and is metabolically functional (Bauer et al., 2021). On the other hand, in the $CoCl_2$+Gly solution, an 8 % decrease is measured, indicating a quantity likely internalized by the larvae. The quantity of phosphorus and Co present in the DW and NK samples is negligible compared to that of the other samples.

Regarding toxicological effects, the mortality of zebrafish embryos was concentration-dependent as observed by Liu et al. (2022). Zebrafish larvae exposed to Gly at the lower concentrations developed mainly

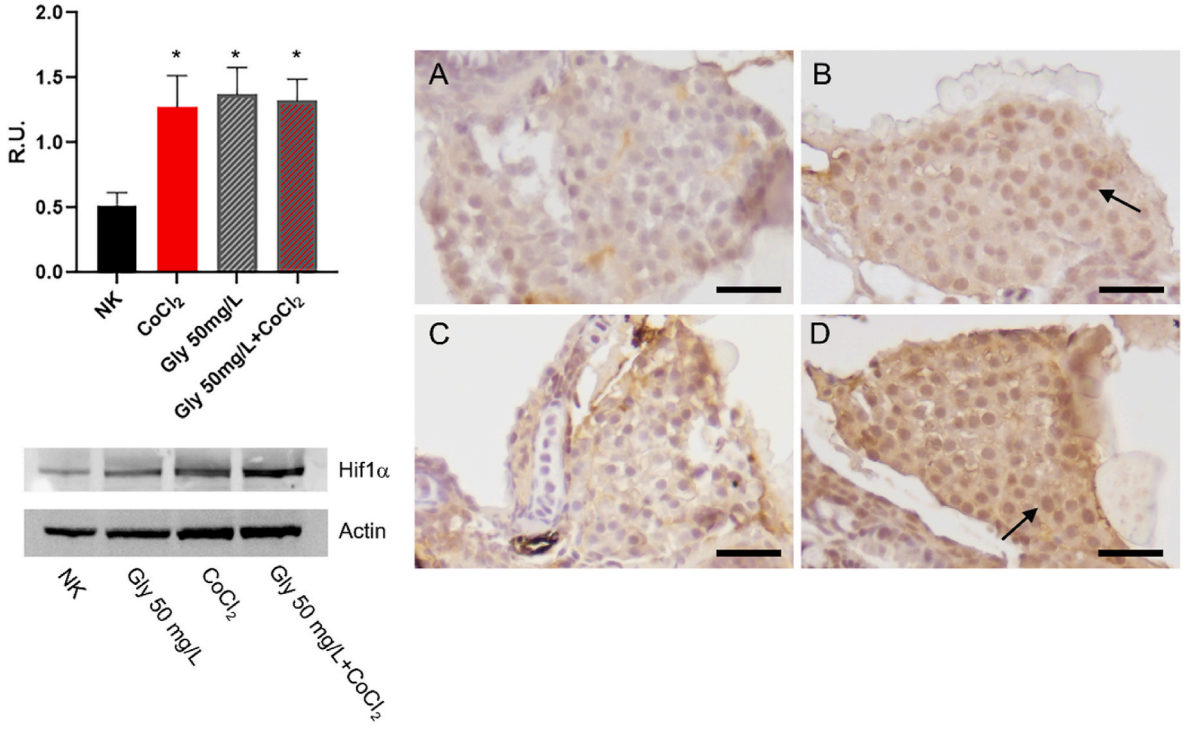

Fig. 3. Western blotting analysis for Hif1α in control (NK) and treated larvae (left), in the bottom histogram represents the relative densitometric analysis for Hif1α, bellow a representative blotting is shown. Data are mean ± SE of three different experiments. *p < 0.05 vs NK values.
On the right, immunolocalization of Hif1α in the liver of control larvae (A), hypoxic larvae (B), 50 mg/L Gly treated larvae (C) and 50 mg/L Gly treated larvae in hypoxic condition (D). Arrows indicate an example of positive nuclei among others. Scale bar 25 μm, (80×).

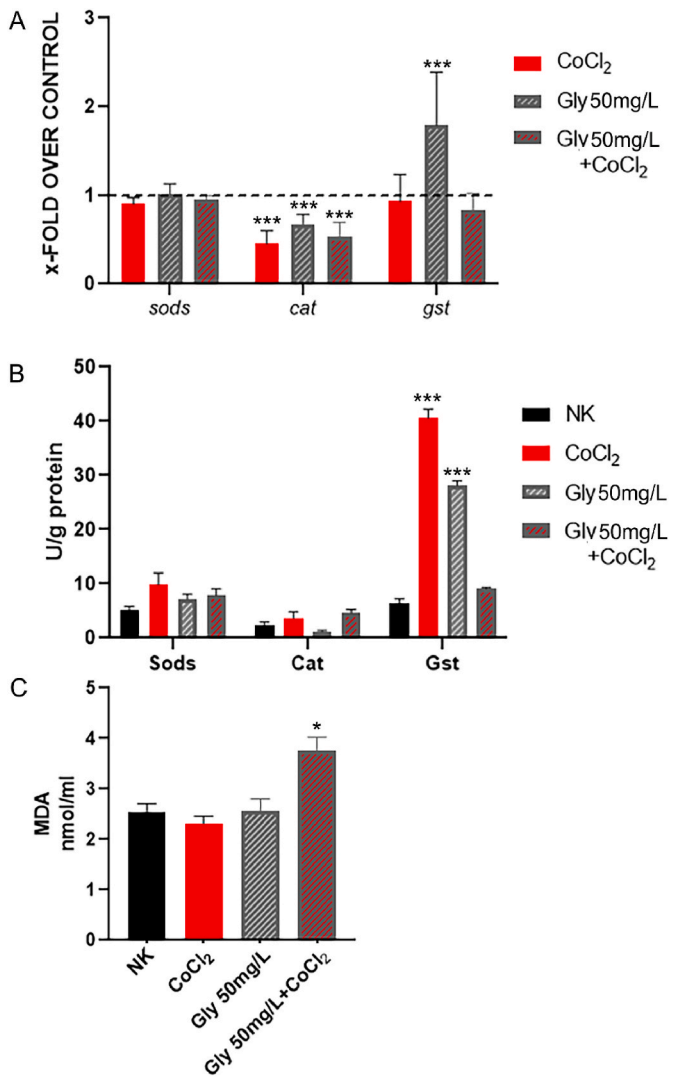

Fig. 4. Impaired antioxidant enzymatic defense. A) histogram shows the expression levels of sod, cat, and gst. Data are mean ± SE of three different experiments. ***$p < 0.0005$ vs NK values. B) histogram shows the enzymatic activity of Sods, Cat, and Gst. Data are mean ± SE of three different experiments. ***$p < 0.0005$ vs NK values. C) Histogram shows the TBARS levels, which are a direct measure of lipid peroxidation. Data are mean ± SE of three different experiments. *$p < 0.05$ vs NK values.

some sublethal alterations referring to the cardiovascular system including edema of the yolk sac, pericardial edema, impaired blood flow, and blood stasis. These alterations were also reported by Sulukan et al. (2017).

Considering the hypoxia condition, no significant variations in morphology or mortality were reported at the lowest concentrations (1 mM and 5 mM) while, at the highest concentration (20 mM), 67% of larvae resulted dead at 96 hpf. The most interesting effects were reported in larvae treated with 10 mM $CoCl_2$ (20 % of mortality), where sub-lethal alterations, including yolk sac edema, scoliosis/lordosis, pericardial edema, and delayed hatching rate, were observed. Similar results were shown by Han et al. (2021).

The results of our study showed that in the combination Gly + $CoCl_2$ the potency of Gly is increased (LC_{50} 70.8 mg/L) with respect to the larvae treated only with Gly or $CoCl_2$ (Table 2). In this case, these two stressors (xenobiotic and hypoxia) synergistically interacted in zebrafish larvae suggesting that, in an environmental context, they may present a particular concern for the fish population. Our conclusion is supported by previous studies done on other species of fish exposed to hypoxia and pesticides in combination, which showed that in this context the toxic effects of chemicals were exacerbated (McBryan et al., 2013; Sula et al., 2020).

Regarding the sublethal effects, the Gly and $CoCl_2$ combination showed no particular or new morphological alterations but the main effects affected the cardiovascular system and were observed at the highest concentrations. This data provides evidence for a link between Gly exposure and cardiotoxicity, that remains in all our treatments at phenotypical and histological levels and should be investigated in further studies.

Regarding the hatching rate at 72 hpf, both treatments, the hypoxic condition and Gly exposure, determined a significant delay in the zebrafish larvae hatching with an increase of not hatched larvae dose-dependent. In the Gly and $CoCl_2$ combination, this trend is inverted, and the number of hatched larvae was higher than 50 % in all tested concentrations. Delayed hatching in our experiments is in agreement with the studies of Sulukan et al. (2017) and Gaur and Bhargava (2019) showing the potency of Gly to modulate hatching, inducing developmental toxicity. When the Gly is combined with the hypoxia this delay decreases significantly.

The histological lesions observed in the liver and in the gut resulted in be correlated with Gly toxicity. In the intestine, a typical feature of injury caused by noxious compounds is inflammation in the lamina propria and luminal dilation, according to our observation. (Borges et al., 2019; Fleming et al., 2010).

Indeed, according to Borges et al., (2019), the most common alterations observed in the hepatocytes and intestine of zebrafish exposed to toxic substances include cytoplasmic vacuolization. This phenomenon is often caused by a decrease in glycogen stores and lipid accumulation, as a consequence of the action of toxic agents (Borges et al., 2018).

Changes in nuclei morphology, vacuolization, and atrophy are frequently observed in hepatocytes when functional alterations occur, and these changes can precede pyknosis and cellular degeneration. Conversely, hypertrophy of nuclei indicates intense metabolic activity in hepatocytes which may result from exposure to toxic agents. Caspers (1984) and, according to Chen et al. (2018) observations, sustain that nuclear enlargement may be a potential indicator of hepatic lipo-metabolic disorder as observed upon naphthalene exposure.

Bawa et al. (2017) report Gly-induced liver alterations in *Cyprinus carpio* similar to those we observed, and the article includes an interesting compendium review on the effects of Gly or similar toxicants in fish liver.

Analyzing PAS staining results, $CoCl_2$ reduces hepatic glycogen, and the lack of liver glycogen increases fat accumulation, and the development of liver insulin resistance as demonstrated by Irimia et al. (2017). Conversely, in the Gly-treated groups, both in normoxia and hypoxia an accumulation of glycogen was observed such as after the administration of different toxicants (Yancheva et al., 2019). This phenotype may be due to the production of hepatocyte-induced by the generation of ROS caused by exposure to Gly that activates the oxidative stress response transcription factor (NFR2), these growth factors can stimulate the accumulation of lipids and glycogen (Hong et al., 2020; Dos Santos et al., 2023). In contrast, in Pieractus Mesopotamicus PAS- positive hepatocyte staining of the fish exposed to Gly decreased as the Gly concentration increased in water (Shiogiri et al., 2012). This can explain the clumped distribution in our cases where it is likely that Gly alone may also induce a reduction in glycogen because of its toxic effect on the cell. In the combination of Gly and $CoCl_2$ the variegated pattern could be an expression of the glycolytic action of hypoxia, counteracted in several areas by the Gly effects.

The histochemical morphological data on PAS staining are due to a careful qualitative assessment unquantifiable because the liver is an organ that is greatly affected by many functional activities, so even the concentration of PAS-positive material may not be constant in different subjects (Bawa et al., 2017; Vali et al., 2022).

Paralleled to these results, we observed an increase in Hif1α protein

in all treatment conditions, indicating a possible involvement of this transcription factor in Gly toxicity. Although Hif1α is predominantly involved in activating glycolytic pathways that justified the decrease of glycogen in the hypoxia group, it also drives fatty acid uptake and suppression of fatty acid oxidation, as indicated by the different vacuolization observed in Gly-treated groups both in normoxia and hypoxia conditions (Tang et al., 2022).

Since Hif1α is implicated in oxidative stress balance we decided to investigate this pathological status in all conditions. All treatment conditions altered the gene expression of some key enzymes (Reuter et al., 2010).

Regarding the mRNA expression, we saw a different modulation of *cat* and *gst*. This first set of data allowed us to understand that all treatments were responding to oxidative stress, thus we decided to investigate also the enzymatic activity. As expected, the picture of enzymatic activity is also modulated by the treatments, indicating a response to oxidative stress. Moreover, in hypoxia and Gly alone, we observed an increase in the enzymatic activity of Gst indicating a strong oxidative stress response. In hypoxia, Gly decreased Gst activity with respect to Gly in normoxia. To better understand the effects of this enzymatic activity we performed the TBARs assay to evaluate the levels of lipid peroxidation products, which is one of the effects of ROS production. The only condition that was not able to counteract the increase of oxidative stress is the condition of Gly in hypoxia suggesting a worsening effect of chemical hypoxia on Gly toxicity, in fact, as demonstrated in the FET test, the LC_{50} was lower. At 96 hpf with hatching and thus with the increase of oxygen level, this condition results to be the one with the greatest tissue damage as suggested by histochemistry and TBARs assay.

5. Conclusion

Gly is currently approved in the EU until December 15, 2033 (COMMISSION IMPLEMENTING REGULATION EU, 2660) even if its use is not authorized in the pre-harvest phase. Furthermore, the European Commission is involved to complete the evaluation of the dossiers relating to the approval of active substances that are potential alternatives to glyphosate.

The results of the present study confirmed the toxicity of Gly in zebrafish's early life stages and an increase of its toxicity when the Gly is in co-presence of moderate hypoxia condition. Furthermore, this synergism produces biochemical and physiological changes as well as histopathological alterations.

Considering that hypoxia, or low dissolved oxygen, is a widespread water quality problem affecting freshwater, marine, and estuarine ecosystems around the world and that by using agricultural products (Fulford et al., 2024; Sula et al., 2020), may develop hypoxia more frequently and for a longer duration the co-occurrence of hypoxia and Gly could become extremely common in natural environments and pose problems for aquatic population.

Moreover, even its use in agriculture has been proclaimed safe because humans and other animals do not have the target EPSPS enzyme, however, increasing numbers of studies have demonstrated risks to humans and animals because the shikimate metabolic pathway is present in many microbes, and several studies demonstrate that more than one-half of human microbiome are intrinsically sensitive to Gly.

CRediT authorship contribution statement

Annamaria Iannetta: Writing – original draft, Methodology, Formal analysis. **Silvana Zugaro:** Methodology, Formal analysis. **Marcella Massimini:** Formal analysis, Data curation. **William Gentile:** Investigation. **Tommaso Silvestrini:** Methodology. **Giulia Fioravanti:** Methodology, Formal analysis. **Martina Foschi:** Formal analysis. **Monia Perugini:** Writing – review & editing, Supervision, Conceptualization. **Elisabetta Benedetti:** Writing – review & editing, Supervision, Conceptualization. **Leonardo Della Salda:** Supervision.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143484.

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