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Sub-acute toxicity of the herbicide glufosinate-ammonium exposure in adult red swamp crayfish (*Procambarus clarkii*)☆

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ABSTRACT

Glufosinateammonium (GLA) is one of the most widely used agricultural herbicides. It is frequently detected in surface waters near farmland and may pose a risk to non-target aquatic species. This study aimed to explore the toxicity of subacute GLA exposure in crayfish. Adult red swamp crayfish were exposed to GLA (0, 1, 10, and 100 mg/L) for 21 days. Bioaccumulation, oxidative stress, nonspecific immunity, and the expression of genes encoding xenobiotic detoxification-related enzymes were examined. The results showed GLA accumulation and hepatopancreatic histopathological changes (dilation of hepatic tubules and vacuolation of hepatocytes) in the exposed crayfish. GLA exposure induced ROS production, inhibited glutathione expression, and catalase activity in the crayfish hepatopancreas, as well as inhibited immunoenzyme expression (acid phosphatase, alkaline phosphatase, and lysozyme) in the hemolymph. In addition, the total hemocyte number decreased, and the proportion of hemocyte subsets changed significantly. Superoxide dismutase first increased and then decreased with increasing GLA dosage. GLA promoted the expression of biotransformation enzymes (*cypb5*, *gst*) in the hepatopancreas. Our results suggest that subacute GLA exposure caused structural damage to the hepatopancreatic tissue and decreased antioxidant capacity and non-specific immunity in crayfish. These findings provide insight into the toxicity of herbicides on non-target organisms.

1. Introduction

Glufosinateammonium (GLA), a chiral member of the organophosphorus family (Peltzer et al., 2013), is widely used around the world as a broad-spectrum herbicide (Calas et al., 2008). Glufosinate is comprises of L- and D-stereoisomers, and the commercial product is a racemic mixture with ammonium salt (Zhang et al., 2014). Because of its high aqueous solubility (>500 g/L), foliar sprays and surface runoff allow GLA to contaminate surrounding freshwater bodies (Meng et al., 2022). GLA has a long half-life in natural water, so it is ubiquitous in surface waters near farmland(Jia et al., 2019). By contrast, it is rarely detected in soils due to rapid degradation by soil microorganisms (Pelosi et al., 2022). In northern Italy, the annual average concentration of GLA in the river Musoncello (0.72 μg/L) and Teva (0.42 μg/L) exceeded the upper tolerable limit for Europe in river water (0.1 μg/L) for pesticides (Masiol et al., 2018). Similarly, the maximum observed GLA concentrations in China's agricultural surface waters sampled in summer and autumn was 13.15 μg/L (Geng et al., 2021). Most field investigations have demonstrated that average surface water GLA concentrations are lower than the observed concentrations of glyphosate (Masiol et al., 2018; Geng et al., 2021). However, compared with glyphosate, the concentration of GLA (0.63 μg/L) was higher in water samples collected from banana gardens in Hainan Province, China (He et al., 2019). The measured environmental concentrations for glufosinate in the aquatic system are usually far lower than the expected environmental concentration (EEC) of this compound (1 mg/L) in Canada (Faber et al., 1998). Given the increased utilization of GLA due to the global ban on paraquat and glyphosate, the contamination of surface water by GLA residues and the

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risk to aquatic life has raised significant concerns.

Although GLA is considered a relatively safe product when used properly (Ebert et al., 1990; Wibawa et al., 2009; Takano and Dayan, 2020), significant toxic effects on non-target organisms have been established. GLA is an irreversible glutamine synthetase (GS) inhibitor, and its herbicide activity depends on the subsequent ammonium accumulation and oxidative burst of reactive oxygen species (ROS) (Takano and Dayan, 2020). However, GS is a ubiquitously conserved enzyme that catalyzes the transformation of glutamate to glutamine in the vertebrate nervous system (Bak et al., 2006). Inhibition of GS activity leads to the accumulation of glutamate, the major excitatory brain neurotransmitter, which causes neurotoxicity (Lantz et al., 2014). Despite the lack of clarity surrounding the mechanisms of GLA toxicity in humans, acute high-dose exposure to GLA has been reported to cause convulsions, memory loss, and hippocampal pathology (Mao et al., 2012; Park et al., 2013). After three weekly intraperitoneal GLA injections, increased GS activity and mild learning impairment were found in mice (Calas et al., 2008). By contrast, decreased GS activity was reported in Wistar rats after feeding a diet with GLA for 28 days (Hack et al., 1994). Different durations of exposure, concentration or routes and types of exposure lead to different GS responses. Male reproductive and developmental toxicity have been reported in mammals exposed to GLA (Ma et al., 2021), perhaps due to sperm histone modification (Ma et al., 2022). Aquatic animals exposed to GLA (2.5 mg/L) exhibit significant morphological abnormalities during early development, suggesting GLA is teratogenic in amphibians (Boccioni et al., 2022). Tadpoles exposed to GLA might suffer oxidative stress, hormonal disturbance (T4), and DNA damage. In zebrafish, for example, GLA causes spinal deformities, yolk sac edemas, and embryo mortality (Xiong et al., 2019). In reptiles, GLA induces hepatotoxicity and reproductive toxicity in male lizards via oxidative damage and disruption of the hypothalamic–pituitary–gonadal axis (Zhang et al., 2019a).

Most studies focus on GLA toxicity in humans and vertebrates, and few invertebrate studies have been reported. Among the few investigations in arthropods, GLA had a significant, short-term effect on the predatory activity of *Pardosa agrestis spiders* (Niedobová et al., 2019), and it caused larval and nymph death in *Orius strigicollis Poppius* and *Harmonia axyridis* (Pallas) via direct larvicidal and nymphicidal action (Ahn et al., 2001). To fully understand the toxic effects of GLA, it is necessary to test more aquatic species. The red swamp crayfish (*Procambarus clarkii*) has become one of China's most important cultured aquatic animals due to its high survival rate and reproductive performance, rich nutrition, and delicious taste (Tan et al., 2017). It is an attractive freshwater model organism for toxicology studies because of its wide distribution, high fecundity, and tendency to accumulate pollutants in water (Brittle et al., 2016; Velisek et al., 2013). Due to the cost and time, herbicides remain the preferred weed management method in integrated rice–aquatic animal systems (Edwards and Hannah, 2014). Promoting this farming system and using GLA has made contamination of agricultural fields and nearby water sources inevitable (Liu et al., 2020). Crayfish are raised in rice fields and trenches in rice–crayfish systems (Cao et al., 2017; Lin et al., 2021) and are directly exposed to GLA residues in the surrounding environment, but there have been no studies of the potential toxicological effects of GLA on crayfish.

This study aimed to determine whether subacute exposure to GLA would induce toxicity in adult red swamp crayfish and to characterize the role of oxidative stress and the nonspecific immunity response in toxicity. A range of concentrations based on the measured 96-h median lethal concentration (LC50) were applied over a 21-day exposure (0, 1, 10, and 100 mg/L). The results of this study will provide an important foundation for evaluating the ecotoxicology and risk of GLA.

2. Materials and methods

2.1. Crayfish maintenance

Red swamp crayfish (weight 26.82 ± 5.24 g, length 90.42 ± 7.97 mm) were purchased from an aquaculture farm in Wuhan, China. All crayfish were temporarily placed in glass aquariums for two weeks to acclimate before the experiment. Each tank was filled with aerated and dechlorinated tap water, which was maintained at pH 7.75–8.12, total ammonia–nitrogen 0.052–0.067 mg/L, and 22.8°C–24.5 °C. To maintain water quality, all exposure solutions were renewed daily. Each tank was equipped with a polyvinyl chloride pipe (7.5 cm × 25 cm) for shelter. The crayfish were fed twice a day using commercial feed.

2.2. Glufosinate ammonium exposure

An acute toxicity assay was performed to determine the 96-h median lethal concentration (LC_{50}) of GLA. After two weeks of maintenance, 180 crayfish were randomly selected and divided into 18 chambers. The concentrations for acute exposure were 0, 250, 500, 1000, 1500, and 2000 mg/L. Each treatment group comprised three parallel chambers of 10 crayfish. Dead crayfish were counted and removed every 12 h.

The gradient concentration of 21-day toxicity test was set according to the 96-h LC_{50} obtained from the acute toxicity. 120 animals in twelve chambers with a water control and three nominal GLA concentrations (1, 10, and 100 mg/L), corresponding to 1/1000, 1/100, and 1/10 of the 96-h LC_{50}. Three replicates of 10 animals were assigned at each concentration of GLA and control. Crayfish were maintained in the same conditions as they were during acclimatization.

After 21 days, three crayfish were selected randomly from each tank for hemolymph collection, assessments of GLA and histological analysis. Another three crayfish were used for biochemical analysis, and the remaining crayfish were used for gene expression. Crayfish in molting cycles did not undergo subsequent experiments. To ensure an adequate sample size and eliminate individual differences, the tissues of three random crayfish in each tank were combined into one sample for quantification of GLA and biochemical analysis.

2.3. Quantification of GLA in crayfish hepatopancreas

The hepatopancreas samples were centrifuged, and the supernatant was collected for analysis by liquid-mass spectrometry (LC-MS). Samples (1 g) were combined with 200 μL of internal standard (1 mg/kg) in a 50-mL centrifuge tube, and then mixed with 10 mL of 5% borate and 5 mL of methylene chloride. The supernatant was extracted by ultrasound and purified by column centrifugation with an EclipsePlus C18 column at an average velocity of 0.4 mL/min.

2.4. Histological analysis

Three hepatopancreas samples were randomly selected from each tank, then fixed in paraformaldehyde, dehydrated in ethanol, embedded in paraffin wax, sectioned (5 μm), and stained with hematoxylin and eosin. The sections were observed by light microscopy and reviewed by at least two independent, blinded graders (Nikon H600L Microscope, Japan). Histologic injury was quantified as described elsewhere (Table S1) (Corbett et al., 2015).

2.5. Oxidative stress

Commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to measure indicators of oxidative stress (SOD, CAT, MDA, and GSH) in the hepatopancreas. Total protein was measured by Bradford assay (absorbance at 595 nm) against bovine serum albumin as a reference. SOD, CAT, MDA, and GSH were normalized to the total protein content. ROS concentrations in the hepatopancreas were

determined using a kit (Jiancheng Bioengineering Institute, Nanjing, China). Detailed protocols are provided in the Supporting Information (S1).

2.6. Immune-related enzyme analysis

Hemolymph samples were extracted and fixed with anticoagulant solution (1:1), then centrifuged (3000×*g*, 15 min, 4 °C), and the supernatants were assayed for enzyme activity. Serum activities of AKP (alkaline phosphatase), LZM (lysozyme), and ACP (acid phosphatase) were measured with commercial kits (Jiancheng Bioengineering Institute, China). Detailed methods for measuring enzyme activity are described elsewhere and in the Supporting Information (S2).

2.7. Hemocyte analysis

Hemocytes were stained with Wright–Giemsa in 100-μL hemolymph samples mixed with anticoagulant (1:1). The total hemocyte count was determined using a hemocytometer and microscopy. The differential hemocyte count was determined by counting the various hemocyte subtypes using differential interference contrast microscopy: (1) small hyalinocytes without cytoplasmic granules; (2) larger semigranulocytes with low-density cytoplasmic granules; (3) granulocytes with high-density cytoplasmic granules (Dolar et al., 2021).

2.8. Quantitative real-time polymerase chain reaction (q-PCR)assay

Hepatopancreas samples from each treatment group were extracted and stored at −80 °C. Total RNA was extracted, purified, and quantified, followed by first-strand cDNA synthesis (Takara, Dalian, China). Real-time PCR was performed with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) using primers designed with Primer 3 (http://bioinfo.ut.ee) and dehydrogenase (gapdh) as the internal control (Table S2). Relative expression was determined using the $2-\Delta\Delta Ct$ method and expressed in terms of fold-change (Livak and Schmittgen, 2001).

2.9. Statistical analysis

All the data were displayed as mean ± standard error (SEM). Statistical analysis was performed using Statistic Package for Social Science 22.0 (SPSS, Chicago, IL, USA). A one-way analysis of variance (ANOVA) followed by Tukey's test was used. Statistical significance was indicated as $*P < 0.05$ and $**P < 0.01$.

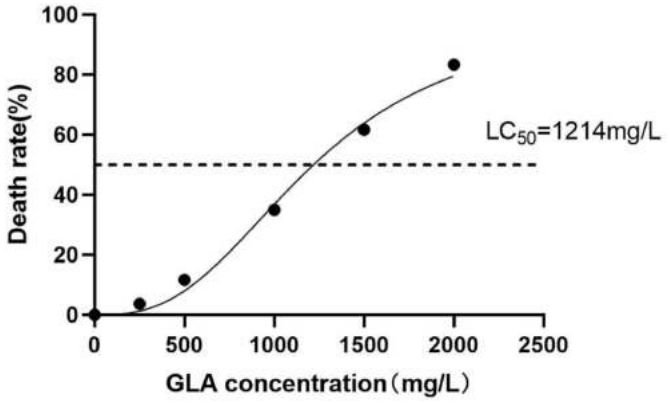

Fig. 1. The fitted sigmoidal dose-response curve of GLA in crayfish.

3. Results

3.1. Acute exposure test

The 96-h LC50 value of GLA in crayfish was 1214 mg/L (Fig. 1). The mortality rate of the exposed crayfish showed a curve-fitting correlation with GLA dose. No mortality occurred in the control group, whereas 85% of crayfish died after exposure to 2000 mg/L GLA.

3.2. Concentration of GLA in the crayfish hepatopancreas

GLA concentrations of 1, 10, and 100 mg/L were associated with crayfish hepatopancreas accumulations of 0.814 ± 0.193, 5.525 ± 0.694, and 80.022 ± 5.832 mg/kg wet mass (ww). No GLA was observed in the organs of the control group (See Fig. 2).

3.3. Histological evaluation

As shown in Figs. 3 and 1S, a normal structure with compactly arranged epithelial cells was observed in the control group. Hepatic tubule lumens were expanded in the 1 mg/L group (Fig. 3b). 10 mg/L GLA-induced hepatocyte vacuolation and increased the interstitial width (Fig. 3c). Degeneration of the hepatic tubule lumens and hepatocyte membrane lysis were observed in the 100 mg/L treatment group (Fig. 3d). The quantitative evaluation showed that 10 mg/L and 100 mg/L GLA caused significant histologic injury to the microscopic structures of the crayfish hepatopancreas ($P < 0.01$) (Fig. 3B).

3.4. Effect on oxidative stress

ROS induction was significantly induced in the 10 and 100 mg/L groups versus the control group, and SOD activity was significantly induced. CAT activity and GSH content were reduced in the 100 mg/L group, but MDA content was unaffected by GLA. Positive correlations were found between the concentrations of ROS and GSH and the level of GLA in the hepatopancreas, and there was a negative correlation with CAT activity (Fig. 4). Thus, GLA significantly inhibits CAT and GSH activity, but there was no significant correlation between GLA concentration and SOD or MDA activity (Table S3).

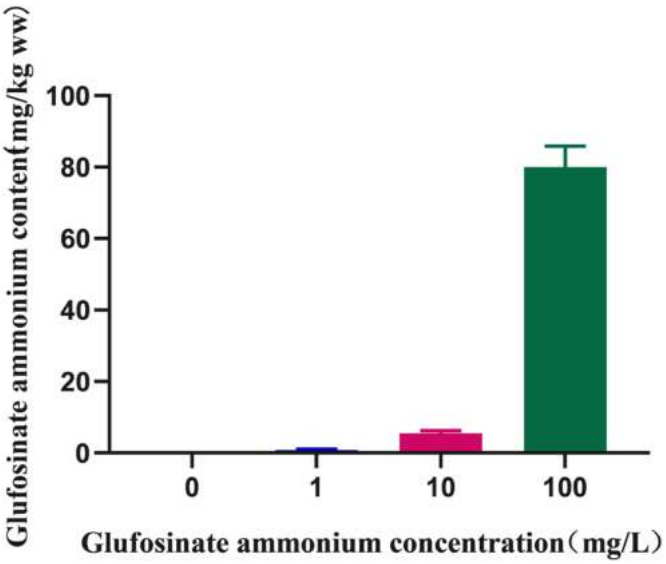

Fig. 2. Average GLA levels in the crayfish hepatopancreas after 21 days of treatment. Values represent means ± SEM (n = 3).

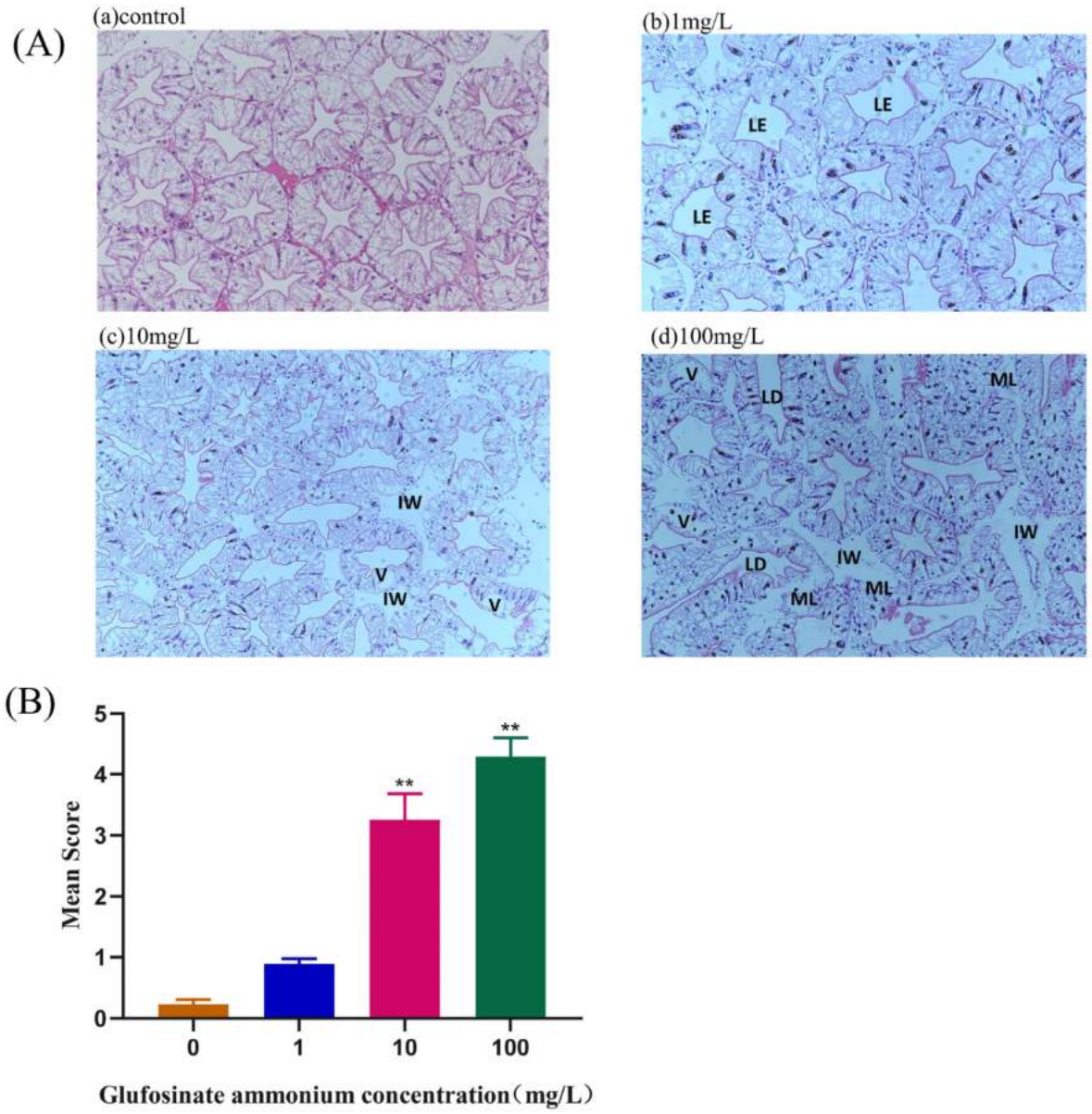

Fig. 3. Histological changes and injury scores of crayfish hepatopancreas. A: Representative images (40 ×) of the (a) control, (b) 1 mg/L, (c) 10 mg/L, and (d) 100 mg/L groups. B: Histologic injury scores. LE, hepatic tubule lumen expansion. IW, hepatopancreatic interstitial widening. V, hepatocyte vacuolation. LD, hepatic tubule lumen degeneration. ML, hepatocyte membrane lysis. Values represent mean ± SEM (n = 9). Significant differences between the treatment and control are indicated as *P < 0.05 and **P < 0.01.

3.5. Total and differential count of hemocyte

Compared to the control group, total hemocyte counts were decreased by 50% and 66% in the 10 and 100 mg/L groups, respectively (P < 0.01) (Fig. 5A). All exposed groups had a greater proportion of granulocytes versus the control, and the proportion of hyalinocytes and semi-granulocytes was significantly lower in the 10 and 100 mg/L groups versus the control (Fig. 5B).

3.6. Effects on non-specific immune enzymes

The effects of GLA on nonspecific immune enzyme expression are shown in Fig. 6. ACP activity was significantly inhibited in the 100 mg/L group versus the control. AKP and LZM activities were significantly inhibited in the 10 and 100 mg/L GLA groups versus the control. Spearman correlation analysis showed that ACP, AKP, and LZM activity were inversely correlated with GLA concentration in the hepatopancreas (Table S4).

3.7. Transcript expression

The transcript expression of metabolic enzyme-related genes is shown in Fig. 7. The results suggested that GLA significantly induced expression of gst, and 100 mg/L GLA significantly induced expression of cypb5.

4. Discussion

The impact of pesticides on aquatic creatures has drawn increasing attention (Caglayan et al., 2019; Caglayan et al., 2020). GSA is highly water-soluble and one of the most common pesticides. It can enter natural water bodies through groundwater infiltration and surface runoff. The 96-h LC_{50} provides data on the acute toxicity of GLA and a reference for sublethal exposure concentrations. In this study, the 96-h

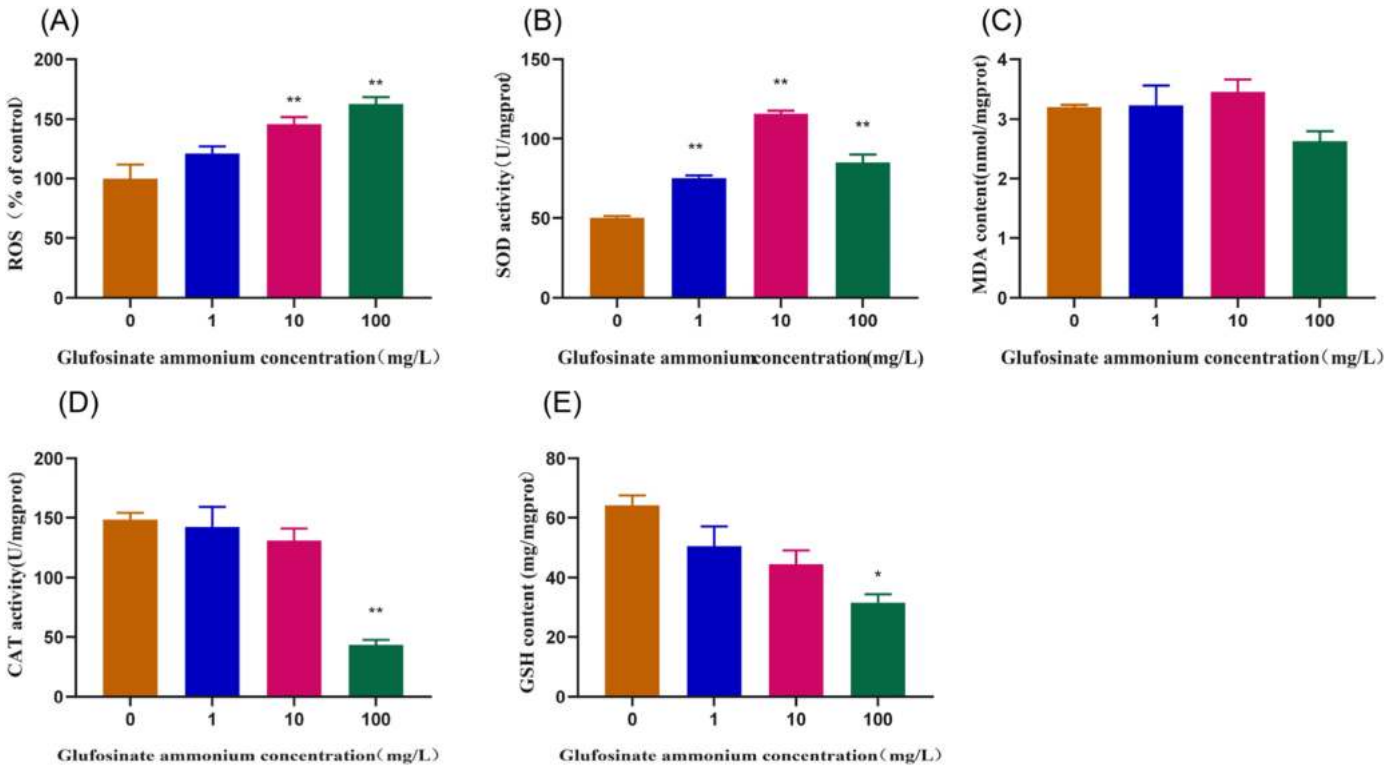

Fig. 4. ROS (A), MDA (C), and GSH (E) content and SOD (B) and CAT (D) activity in crayfish hepatopancreas. Values represent mean ± SEM (n = 3). Significant differences between treatment and control groups are indicated by *P < 0.05 and **P < 0.01.

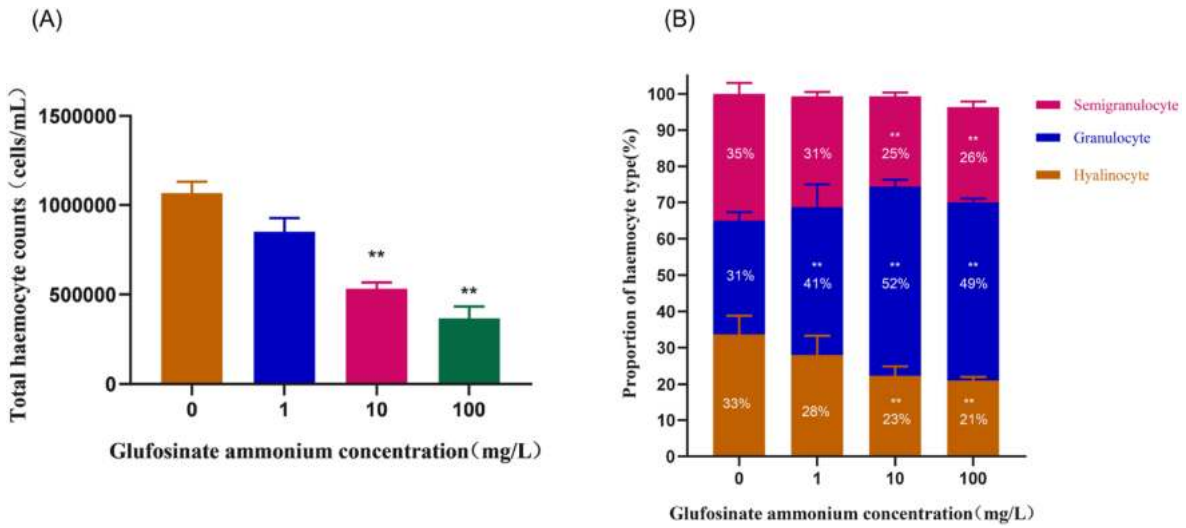

Fig. 5. Total hemocyte count (A) and differential hemocyte count (B). Values represent mean ± SEM (n = 3). Significant differences between treatment and control groups are indicated by *P < 0.05 and **P < 0.01.

LC_{50} of GLA in crayfish was 1214.57 mg/L, compared to 8.7 mg/L in *Oryzias dancena* (Kang et al., 2014), suggesting that the crayfish is less sensitive to GLA than fish. Similar discrepancies in species susceptibility were found in studies of glyphosate, another widely used organophosphorus pesticide (Folmar et al., 1979; Banaee et al., 2020). Although GLA is considered safe at the recommended dose (Takano and Dayan, 2020), sublethal adverse effects caused by GLA are a concern due to the exponential increase in the use of GLA over the past decade. The exposure concentration in most toxicology studies (Zhang et al., 2019a; Boccioni et al., 2022) is much higher than the levels found in environmental samples (Masiol et al., 2018; Geng et al., 2021) and provides the basis for acute LC_{50} data and the EEC for GLA in Canada (Faber et al., 1998). Accordingly, we chose GLA exposure concentrations based on the measured 96-h LC_{50}. Though this exposure level exceeds observed environmental concentrations, the findings can provide mechanistic information useful for hazard identification and be considered important for the general screening of GLA toxicity. GLA induces acute developmental immunotoxicity at an environmentally relevant concentration (10 μg/L) in zebrafish embryos (Xiong et al., 2019), suggesting that glufosinate safety in nontarget aquatic organisms is far from proven, and that more studies are required. The great diversity of arthropods and their widespread distribution routinely expose them to

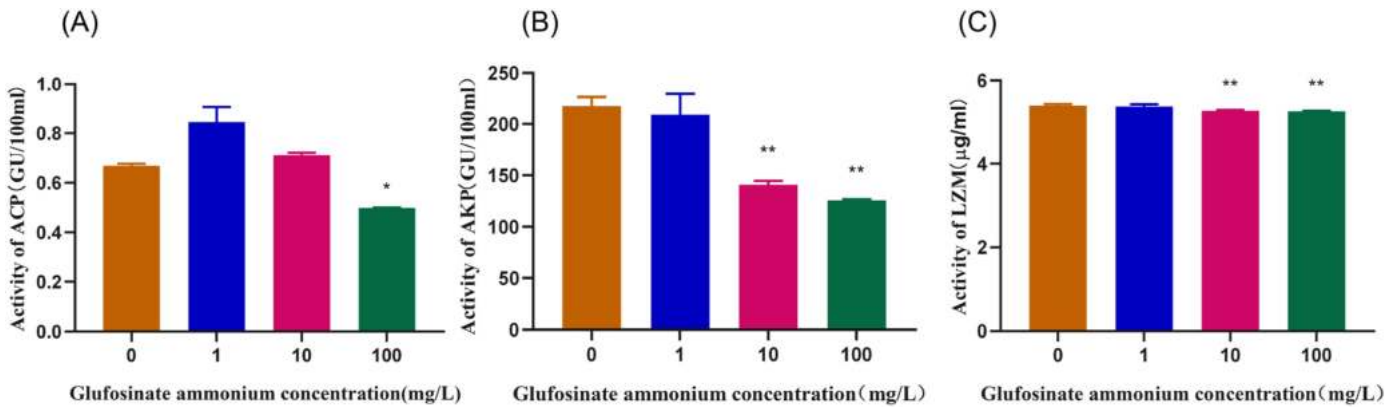

Fig. 6. Activities of ACP (A), AKP (B), and LZM (C) in crayfish hemolymph. Values represent mean ± SEM (n = 3). Significant differences between treatment and control groups are indicated by *P < 0.05 and **P < 0.01.

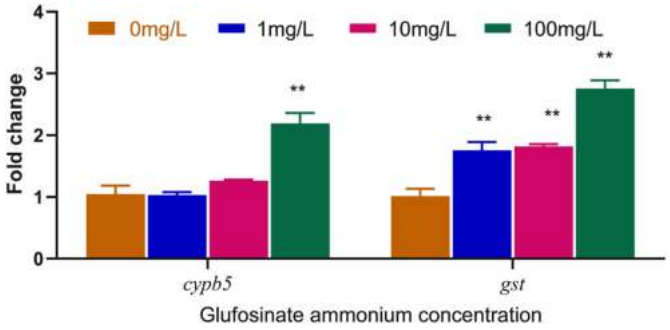

Fig. 7. Relative transcript expression level of cypb5 and gst in the crayfish hepatopancreas. Values represent mean ± SEM (n = 3). Significant differences between treatment and control groups are indicated by *P < 0.05 and **P < 0.01.

various levels of pollutants, providing the rationale for using them as biological models in ecotoxicological studies. Crayfish are a rational surrogate for aquatic invertebrate species because of their prominent role in the physical and biological modification of the ecosystems they inhabit (Edwards et al., 2009). However, the effect of GLA exposure on crayfish is poorly understood despite its direct contact with GLA in surface water and sediments. In this study, GLA was detected in the hepatopancreas of exposed crayfish, showing bioaccumulation under experimental conditions. We also observed changes in oxidative stress parameters accompanied by varied degrees of hepatic tissue damage.

In the present study, we did not measure the actual concentrations in exposure solutions. It was reported that GLA was quite stable in water samples, and less than 22% of the applied substance degraded after 100 days (Jia et al., 2019). Consistently, the mean concentration of GLA in the 10 mg/L exposure solutions was 9.05 mg/L using LC-MS at 8 days post-exposure (Xu et al., 2022). In our study, the solution was refreshed every 48 h with carbon-filtered water containing the corresponding concentration of GLA, and thus the concentrations of GLA in exposure solutions were comparable to nominal concentrations. The estimated GLA log Kow (−3.49) showed that this compound had a low bioaccumulation potential. Similarly, the estimated bioconcentration factor (BCF) values in our study were less than 1 mg/L (results not shown), suggesting the limited ability of GLA to accumulate in the hepatopancreas of crayfish. Although there is no need to pay excessive attention to bioaccumulation of GLA, the detection of GLA in the hepatopancreas indicated that the hepatotoxicity was worthy of further study.

The hepatopancreas plays a critical role in digestion, absorption, excretion, and immune functions in crustaceans (Rőszer, 2014; Yang et al., 2015). As a crucial organ in alleviating environmental stress, the hepatopancreas is engaged in accumulating and detoxifying chemical contaminants. (Zhang et al., 2019b). It is well-known that histopathological investigations can contribute to understanding pathophysiological processes under environmental stress (Sula et al., 2020). Our study showed that GLA exposure causes histological damage to the hepatopancreas of crayfish, making this a useful model indicator of water pollution (Wolf and Wheeler, 2018). The crayfish hepatopancreas comprises several blind tubes (Loizzi, 1971). We observed clear expansion of the hepatic tubules in the exposed groups, and the basement membrane of the inner lumen wall was gradually expanded. Consistent with our results, injuries such as hepatopancreas vacuolization, luminal dilation, and eosinophil deposition have also been observed in crayfish exposed to ammonia (Lin et al., 2023). The hepatopancreas of BPA-treated crayfish also shows comparable pathological characteristics, such as an increase in the number of lipid droplets in hepatocytes (Zhang et al., 2020). Quantitative analysis using the histologic injury scoring system showed a dose-dependent negative effect of GLA on morphology. It should be noted that these histopathological changes of the hepatopancreas will not be evident in the natural aquatic environment due to the low concentrations of GLA.

The oxidative stress and antioxidant capacity observed in the crayfish hepatopancreas supported the hypothesis that GLA-induced hepatoxicity occurs in aquatic organisms. One of the most common mechanisms of injury is oxidative stress (Tang et al., 2019). Pollutant exposure may increase or inhibit antioxidant enzyme activity in animals (Oruç and Uner, 2000). Excessive ROS generated by toxic substances have a significant effect on hepatopancreas damage and could directly indicate the degree of cellular oxidative stress (Chen et al., 2020). ROS may increase protein and lipids and lead to the peroxidation of membrane lipids (Mercan et al., 2013). One of the products of lipid peroxidation is MDA, which is another indicator of the severity of cellular damage (Frijhoff et al., 2015). To combat oxidative stress, organisms express various antioxidant enzymes and antioxidants (Sen et al., 2010). CAT is a biomarker of oxidative stress, converting hydrogen peroxide into water and oxygen (Zeinab et al., 2016). Our study showed that ROS and MDA content increased after exposure to GLA, while CAT activity was reduced. The inability of the antioxidant defense system to properly clear excess ROS accumulation may account for these observations (Adrees et al., 2015) and result in damage to the antioxidant system. This same result has been observed with other pesticides, as CAT activity in zebrafish liver significantly declined after 24-h exposure to imidacloprid (Vieira et al., 2018). SOD is an oxidative stress biomarker that affects peroxide radicals and is a precursor of intracellular ROS (Yi et al., 2008). SOD activity in the crayfish hepatopancreas increased in the 1 mg/L and 10 mg/L groups and decreased in the 100 mg/L group versus the control. This observation may be attributable to an increase in superoxide anion free radicals, which would induce SOD activity, followed

by the conversion of superoxide anion free radicals to H_2O_2 with increasing GLA concentration, oxidizing cysteine and reducing SOD activity (Oruç and Uner, 2000). Through the conjugation process and GPx-mediated reduction, GSH protects against the harmful effects of pesticides (Backos et al., 2012). In this study, GSH content decreased with increasing GLA concentration, perhaps due to GSH induction of GPx (Temiz et al., 2021). It is also possible that long-term GLA exposure leads to H_2O_2 accumulation, which induces lipid peroxidation and decreases GSH content. These results demonstrate that GLA exposure influences oxidative stress levels in crayfish, but the mechanism by which each antioxidant enzyme is regulated remains to be studied.

The hemolymph system of crustaceans acts as a fundamental membrane for pathogen protection and elimination, and blood lymphocytes are both the immune factor and the provider of the humoral immune factor (Yang et al., 2021). Hemocytes perform various functions, including cell-to-cell communication, phagocytosis, and recognition (Qin et al., 2019). The number of hemocytes is correlated with immunity and is an early indicator of infection in aquaculture conditions (Ellis et al., 2011). According to a previous study, crayfish hemocytes fall into three main categories: HC, SGC, and GC (Ding et al., 2012; Du et al., 2012). In this study, a negative correlation was observed between the exposure concentration and total hemocyte count. A reduction in crustacean hemocyte count is generally considered the result of immobilization in the gills (Johansson et al., 2000). In this study, the most significant change in hemocytes was detected in HC, with a significant increase in GC versus a decrease in HC and SGC. Metabolic processes alter granule formation, leading to an increase in the GC content of *Rhynocoris kumarii* exposed to insecticides (George and Ambrose, 2010) and *Porcellio scaber* exposed to environmentally relevant concentrations of polyester fibers and crumb rubber (Dolar et al., 2021). The hydrolase ACP and the multifunctional enzyme AKP are important in nonspecific immunity (Matozzo et al., 2011). LZM is a basic protein that can kill and remove bacteria in the blood and maintain physiological homeostasis (Muta and Iwanagaz, 1996). In this experiment, AKP and LZM decreased in a dose-dependent manner, with a significant difference between the control and the 10 mg/L and 100 mg/L groups. However, ACP activity decreased in the 1 mg/L and 10 mg/L treatment groups, perhaps because low-concentration GLA exposure induced metabolism and inhibited ACP activity at high concentrations to maintain a low level of physiological activity to adapt to the external environment. Reductions in ACP and AKP were also detected in ammonia-exposed crayfish and glyphosate-exposed *Eriocheir sinensis* (Hong et al., 2017; Lin et al., 2023).

Pollutant biotransformation by crustaceans involves xenobiotic-metabolizing phase I and phase II enzymes. Cytochrome P450s (CYP450s) are a family of enzymes responsible for phase I biotransformation of endogenous and exogenous compounds. Some crustaceans metabolize xenobiotics due to a well-developed CYP450 enzyme system (Martin-Diaz et al., 2008). CYPB5 is the most important transport protein in drug metabolism and is important in metabolizing xenobiotics. Intermediate metabolites are tightly bound by endogenous substances (e.g., GST, UST) via the phase II enzymes to improve the polarity of organic matter and promote excretion. In crustaceans, GST is a common phase II metabolic enzyme used as a biomarker to assess environmental contamination (Martín-Díaz et al., 2007). However, less is known about these pathways in GLA detoxification. Upregulation of both *cypb5* and *gst* in the hepatopancreas of crayfish provided support for the important roles of these genes in GLAdetoxification.

5. Conclusion

This study demonstrated that exposure to GLA for 21 days might cause the accumulation of GLA in the hepatopancreas as well as histopathological injuries and oxidative stress. Inhibition of enzymes associated with nonspecific immunity and upregulation of xenobiotic detoxification-related genes were observed. Our findings provide a novel demonstration of the impact of GLA on non-target species and remind us that the specific mechanism and potential toxicity of prolonged GLA exposure remain worthy of investigation.

CRediT authorship contribution statement

Yang Zhang: Investigation, Data curation, Writing – original draft. **Yao Dang:** Methodology, Data curation, Writing – original draft. **Fucheng Pei:** Investigation, Data curation. **Yongchao Yuan:** Investigation, Data curation. **Junfa Yuan:** Investigation, Data curation. **Zemao Gu:** Conceptualization, Methodology. **Jianghua Wang:** Supervision, Methodology, Conceptualization, Writing – original draft.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122605.

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