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Review

Effects of Glyphosate or Glyphosate-Based Herbicide during the Zebrafish Life Cycle: A Review Addressing the Mechanisms of Toxicity

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Abstract: Herbicides with glyphosate (GLY) as an active ingredient (a.i.) are increasingly used, and GLY is currently the most used herbicide in the world. Consequently, its residues have often been found in aquatic ecosystems. Investigating how this substance affects aquatic species is a priority in ecotoxicology research, especially in fish, as they can absorb and concentrate toxins. In this sense, a critical review was performed, synthesizing data from the peer-reviewed bibliography, reporting on the toxicity of exposure to pure GLY and glyphosate-based herbicides (GBHs), using zebrafish as an animal model. The concentrations of this herbicide that induced toxic effects are highly variable, with some exceeding the limits determined by regulatory agencies. Globally, relevant toxic effects have been reported in zebrafish, namely, teratogenic effects incompatible with life, which translates directly into an increase in reported zebrafish mortality. Neurotoxicity, genotoxicity, changes in energy metabolism and oxidative stress, and immune and hormonal system dysfunction with an impact on fish reproduction were also described. In conclusion, both GLY and GBHs may induce damage to zebrafish, compromising their survival, reproduction, and maintenance. These results may be valid and applied to other fish species and aquatic ecosystems.

Keywords: environmental pollutants; glyphosate; glyphosate-based herbicides; toxicity; zebrafish

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1. Introduction

1.1. Glyphosate and Glyphosate-Based Herbicides Characterization

Glyphosate (GLY, N-phosphonomethylglycine, CAS Number: 1071-83-6) is a substance derived from the amino acid glycine, that was synthesized in 1950 by Henri Martin while working for a Swiss pharmaceutical company called Cilag [1]. It is a molecule classified as an organophosphate compound, specifically a phosphonic acid resulting from the formal oxidative coupling of the methyl group of methyl phosphonic acid with the amino group of glycine [2]. It is an analogue of the natural amino acid glycine with a basic amino group and a strongly ionized phosphate group. GLY is a polar and amphoteric molecule developed to be used for metal chelation. Twenty years after its discovery, the organic chemist John Franz found that GLY was highly effective as an herbicide [3]. Therefore, this compound was registered by the company Monsanto (St. Louis, MO, USA) in the US Environmental Protection Agency (EPA), under the brand name Roundup®, for non-selective weed control [4].

After the emergence of glyphosate-resistant crops, mainly corn, soybeans, canola, and sugar beet, its use in agriculture has increased exponentially, becoming the main herbicide worldwide [5], used in over 140 countries, in 2014 [6]. Initial assessments of GLY toxicity assumed a low concern and a limited risk for vertebrate animals because its stated mechanism of action targeted the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), absent in animals [3]. In addition, relevant regulatory agencies such as the European Food Safety Authority (EFSA), EPA, and the US National Cancer Institute have stated that there is no evidence of potential interaction of GLY with endocrine pathways or a carcinogenic effect in vertebrate animals [1,7]. However, experimental data revealed a wide range of adverse effects in non-target species such as rats, fish, amphibians, and invertebrates [8,9]. Among the reported effects, it is worth mentioning the teratogenic effects [10], oxidative stress [11], immunotoxicity [12], reproductive toxicity [13], cardiotoxicity [14], and neurotoxicity [15]. Despite these findings, its toxicological classification is not yet fully clarified, and it is important to acknowledge the deleterious implications that may result from GLY exposure, in invertebrate and vertebrate species.

1.2. Glyphosate and Glyphosate-Based Herbicides in the Environment

Herbicide formulations are not commercialized as single molecules but in mixtures. In glyphosate-based herbicides (GBHs), GLY is designated as the active ingredient (a.i.), while the other compounds are referred to as 'inert' [16]. Due to their wide use in varied sectors of agriculture and urban environments, GBHs have a diffuse distribution in the environment [17]. Indeed, the manufacture and use of GBHs have increased significantly due to the end of patent protection and the launch of glyphosate-tolerant genetically modified crops [3,18]. As a consequence, several studies stated that trace levels of GLY can be found in water and food, as well as in human serum, breast milk, and urine (Figure 1) [18–20]. This may be due to the resistance of GLY to degradation, since according to EPA data, the GLY molecule is relatively stable to decomposition [1,21]. Its main degradation pathway is by soil microbial action, where it is metabolized by two pathways: by a GLY oxidoreductase that generates aminomethylphosphonic acid (AMPA) and glyoxylate [22], via conversion to glycine. In addition, factors such as ultraviolet radiation, oxidation of peroxides, and mineral oxidation induce an alternative pathway of environmental degradation of GLY [22]. Environmental characteristics such as temperature, sunlight, and soil type can also influence the distribution of GLY in water, which may explain the high variability of GLY concentrations across regions in the world [8]. Thus, in soil, the GLY half-life can range from 2 to 197 days, and in water from a few to 91 days [23].

Environmentally relevant concentrations of GLY are well determined, and, in water, can range from 0.0001 to 105 μg mL^{-1} [19,24–26]. The higher concentrations were found in countries that are large herbicide-dependent agricultural producers [19]. It is noteworthy that the higher concentrations were found during the rainy season when GLY is usually transported from terrestrial ecosystems to surface waters by leaching [26]. The maximum allowable GLY concentration on the surface of freshwater varies according to each country's legislation. For example, in the USA, the EPA established a maximum allowed value of 0.7 μg mL^{-1} [27], while most of the European countries imposed more restrictive values, with a value below 0.0005 μg mL^{-1}, and even a trend towards banning GLY use. Nevertheless, most of the concentrations found in the environment are above established limits [19,28].

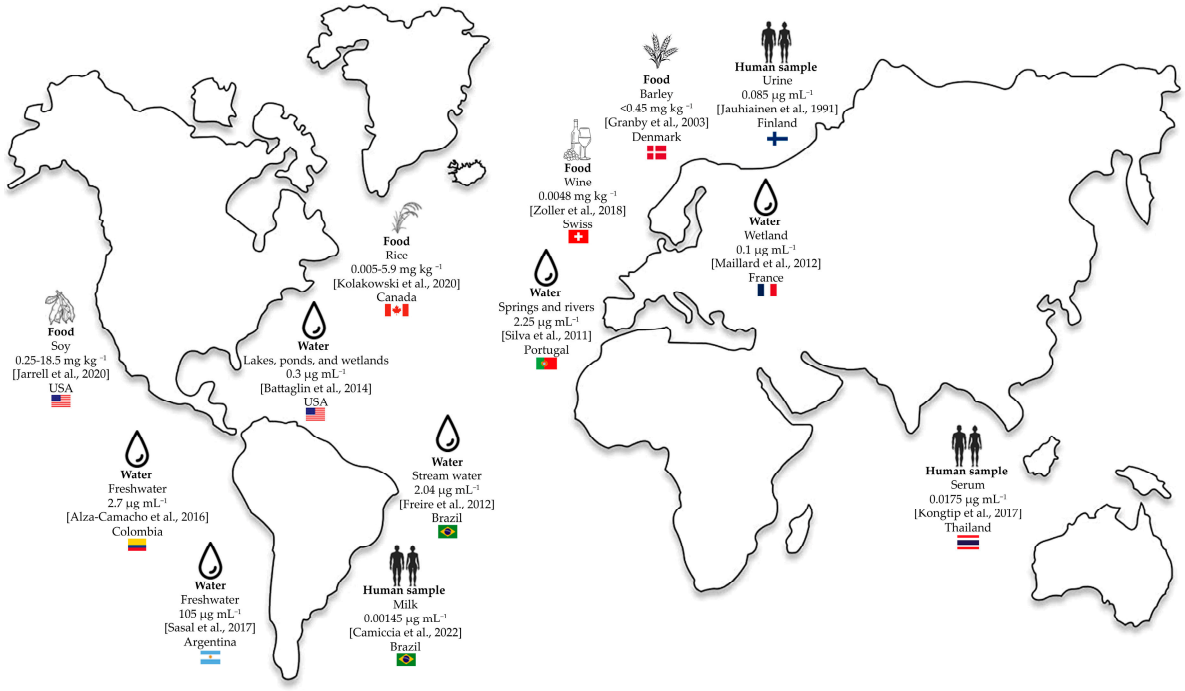

Figure 1. Residual concentrations of glyphosate in the aquatic environment, food, and humans (1991 to 2022). Adapted from [18,19]. References (water [26,29–33], food [13,34–36], and humans [37–39]).

2. Toxicological Effects of GLY/GBHs on Zebrafish

Globally known as zebrafish, *Danio rerio* belongs to the Cyprinidae family and has become an established aquatic vertebrate model widely used in ecotoxicology research to assess the toxicity of environmental pollutants [40,41]. This species has several inherent advantages, including a fast life cycle, high fecundity, external embryogenesis, small size, low maintenance cost, and ability to respond to a wide range of environmental toxicants [42–44]. Embryos show optical transparency through the chorion during the early stages of life, facilitating the observation of morphological processes as well as physical movements [45]. Furthermore, it has 70% homology with the human genome (which increases to 82% when considering genes related to human diseases) [46]. There are several zebrafish transgenic lines, which facilitate the development of ecotoxicological studies with greater specificity [47]. Guidelines have been developed by the Organization for Economic Co-operation and Development (OECD) for the use of zebrafish as a model species in ecological risk management [48]. In particular, the early life stages of zebrafish, defined by the European legislation (Directive 2010/63/EU) as the first 5 days post fertilization (dpf), have become an optimal model choice under 3R policies: replacement, reduction, and refinement [49,50].

For this critical review, publications from the NCBI PubMed and Web of Science databases, between January 2011 and March 2023, that were queried with the keywords "glyphosate, toxicity, zebrafish", were used and synthesized. A literature review was conducted, considering the studies that evaluated toxicity in zebrafish by exposure to pure glyphosate (GLY) and glyphosate-based herbicides (GBHs). Possible information was extracted from the products used by the authors in their respective studies where the GLY concentrations assessed varied from 0.00005 to 1690 µg mL^{-1} and GBH concentrations ranged from 0.0048 to 350 µg a.i. mL^{-1}. Effects resulting from exposure to GLY and GBHs in zebrafish were described, reporting teratogenic effects, cardiotoxicity, neurotoxicity, behavioral changes, oxidative stress, genotoxicity, apoptosis, endocrine disruption, reproduction impairment, energy metabolism disruption, and immunotoxicity (Figure 2). The scope of the studies included, and the effects evaluated, make this a complete and comprehensive review of the effects of this herbicide on the zebrafish lifecycle.

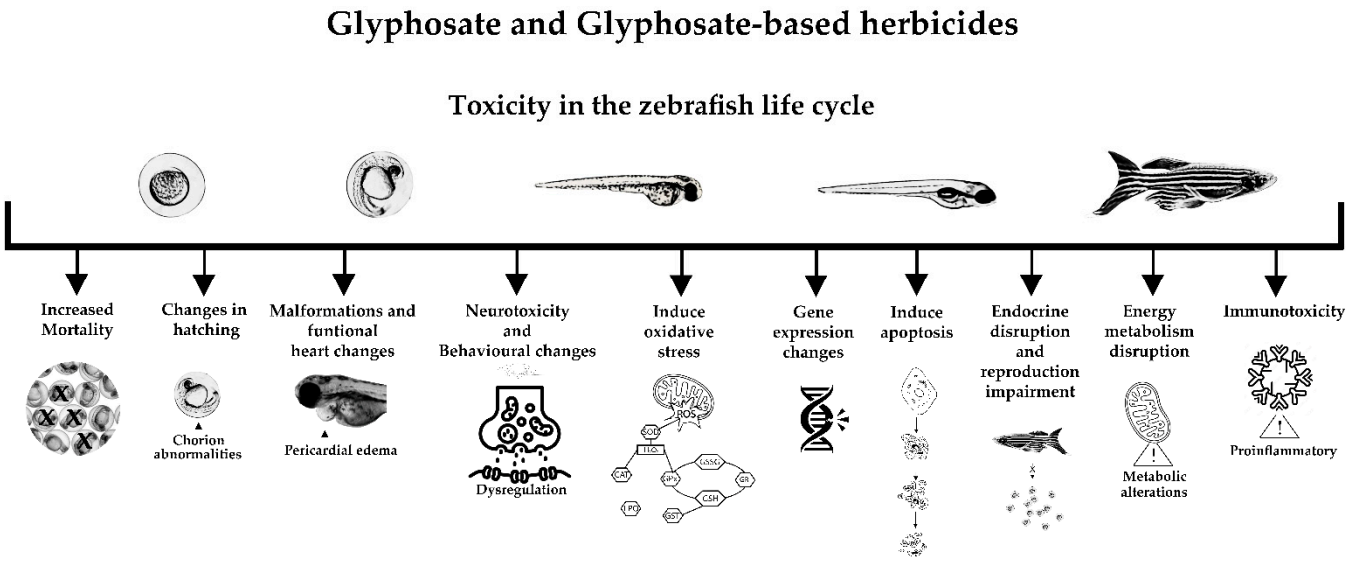

Figure 2. Effects resulting from exposure to GLY and GBHs in zebrafish.

2.1. Lethality and Mortality Caused by Exposure to GLY or GBHs in Zebrafish

Survival evaluation is usually the first approach to assess the environmental safety and toxicity of chemicals [51]. These data generally vary according to the life stage of the fish, body size, physicochemical parameters of the water, absorption rate, detoxification mechanisms, and the differences in the composition of the tested formulations [52]. The effects of exposure to GLY and GBHs on the mortality and lethality of zebrafish are shown in Table 1. Studies have shown that there is a proportional relationship between herbicide concentration and mortality. The median lethal concentration reported in zebrafish embryos/larvae after exposure to GLY was LC50 48 h = 66.04 μg mL^{-1}. A mortality of 100% was observed after exposure to a concentration of 400 μg mL^{-1} for 24 h and a concentration of 200 μg mL^{-1} for 48 h. Mortality also increased after exposure to concentrations above 50 μg mL^{-1} for 96 h [53]. In another study, at 6 hpf, all embryos treated with 600 μg mL^{-1} GLY died, and the concentration of 400 μg mL^{-1} showed increased mortality after 96 hpf [54]. High mortality rates were observed after exposures ranging from 84.54 to 1690 μg mL^{-1} at 96 hpf [55]. GLY increased mortality was reported in embryos at 24 hpf (>90 μg mL^{-1}) and after 48 hpf (>60 μg mL^{-1}) [56]. Even at lower concentrations, GLY was able to exert high mortality rates when compared to the control group, as demonstrated by [57] with tested concentrations of >0.05 μg mL^{-1}. Other studies showed increased mortality at >0.01 μg mL^{-1} (74 hpf) [58], and at 0.8 μg mL^{-1} after 7 days [59]. Exposures to GLY (10 μg mL^{-1}) for 21 d in adult zebrafish induced an increase in mortality in embryos after 3 hpf [60]. Another cross-generational study also found an increase in embryonic mortality caused by exposure to GLY along with increased water temperature [61].

Regarding the mortality induced by exposure to GBHs, the embryos/larvae were more sensitive to Roundup® UltraMax (Monsanto, St. Louis, MO, USA) LC50 72 h = 8.53 μg a.i. mL^{-1}, with an increase in mortality at concentrations of 8.5 and 15 μg a.i. mL^{-1} at 72 h [62]. Roundup Original (LC50 96 h = 13.56 μg a.i. mL^{-1}) was followed by AKB 480 (LC50 96 h = 36.17 μg a.i. mL^{-1}) [63], Roundup® (Monsanto, St. Louis, MO, USA) (LC50 96 hpf = 58.3 μg a.i. mL^{-1}) [64] and Atanor (LC50 96 h = 102 μg a.i. mL^{-1}) [65]. Lethal effects were observed at the highest concentration tested of Atanor 48 (133 μg a.i. mL^{-1}) at 24 and 48 h [66]. Results shared by [64] showed an increase in mortality at Roundup® concentrations exceeding 11.7 μg a.i. mL^{-1} at 96 hpf. Exposures to Roundup GC (10 μg a.i. mL^{-1}) for 21 d in adult zebrafish induced an increase in mortality in embryos after 3 hpf [60]. An increase in mortality was also observed at the concentration of 0.0048 μg a.i. mL^{-1} of Roundup® in zebrafish larvae [67]. The larvae offspring of the exposed animals also showed an increase in mortality (7 dpf) [68]. A study that did not mention the commercial

name of the GBH used observed an increase in mortality at a concentration greater than 1 µg a.i. mL^{-1} (96 h) [69]. Regarding the exposure to GBHs in adults, a LC50 96 h of 53.75 µg mL^{-1} was obtained with Scout® Herbicide [70], and a LC50 96 h of 42.61 µg mL^{-1} with the commercial formulation Roundup WG® [51]. Evidence shows that commercial products have a toxicity profile that is different from that of GLY. As they are complex mixtures formed by several ingredients that are considered inert, clarification of its potential toxic effects should be guaranteed [71].

Table 1. Lethality and mortality caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**µg mL^{-1}**			
[53]	GLY Cat#45521	10 to 400	24, 48, 72 and 96 h	Embryos/Larvae	-LC_{50} 66.04 ± 4.6 µg mL^{-1} (48 h). -100% mortality 400 µg mL^{-1} (24 h). -100% mortality 200 µg mL^{-1} (48 h). -Mortality increased (>50 µg mL^{-1}).
[54]	GLY 99.8% purity	0.01 to 600	0.75 to 96 hpf	Embryos/Larvae Wild-type AB strain	-Increased mortality 400 and 600 µg mL^{-1} was observed.
[55]	GLY 96% purity CAS#1071-83-6	1.69 to 1690	2 to 96 hpf	Embryo/Larvae	-High mortality was observed in exposures >84.54 µg mL^{-1}.
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-Increased mortality at 24 hpf (>90 µg mL^{-1}). -Increased mortality at 48 hpf (>60 µg mL^{-1}).
[57]	GLY ≥ 99% purity	0.005 to 50	0.75 to 120 hpf	Embryos/Larvae	-Mortality increased at >48 hpf (>0.05 µg mL^{-1}).
[58]	GLY 96% purity CAS#1071-83-6	0.001 to 0.7	2 to 74 hpf	Embryos/Larvae Wild-type TU	-Mortality increased at >0.01 µg mL^{-1}.
[59]	GLY 99% purity CAS#1071-83-6	0.8	7 d	Larvae (8 days post-fertilization (dpf))	-Mortality increased.
[60]	GLY	0.01 to 10	21 d (Adult) and maintained across generations	Embryos/Larvae WIK strain	-Increase in mortality in embryos at 3 hpf.
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Intergenerational exposure caused an increase mortality, together with the effect of increasing temperature.
GBHs in Embryos/Larvae		**µg a.i. mL^{-1}**			
[62]	Roundup® UltraMax (Bayer, Portugal) 35.5 wt% of glyphosate	2 to 15	72 h	Embryos/Larvae Wild-type AB strain	-LC_{50} 72 h = 8.53 µg a.i. mL^{-1}. -Mortality increased at concentrations of 8.5 and 15 µg a.i. mL^{-1} at 72 h.
[63]	-AKB 480 (Kelldrin Industrial) (480 g a.i. L^{-1} or 360 g glyphosate acid equivalent (a.e.) L^{-1} of formulation). -Roundup Original (Monsanto, Brazil) (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation).	2.4 to 240	24, 48, 72 and 96 h	Embryo/Larvae	-AKB LC_{50} at 24 h: 64.17; at 48 h: 37.30; at 72 h: 36.85; at 96 h: 36.17 µg a.i. mL^{-1}. -Roundup LC_{50} at 24 h: 36.52; at 48 h: 23.33; at 72 h: 14.4; at 96 h: 13.56 µg a.i. mL^{-1}.
[69]	GBH 360 mg a.i. L^{-1}	1 to 100	4 to 96 h	Embryo Larvae AB strain	-Increased mortality >1 µg a.i. mL^{-1}.
[64]	Roundup® (Monsanto, St. Louis, MO USA)	3.5 to 350	96 hpf	Embryo/Larvae	-LC_{50} 96 h: 58.3 µg a.i. mL^{-1} -An increase in mortality was observed. (>11.7 µg a.i. mL^{-1}).
[65]	Atanor 48 (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation).	2.2 to 133	24, 48, 72 and 96 h	Embryo/Larvae	-LC_{50} 96 h: 102 µg a.i. mL^{-1}.

Table 1. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
[66]	Atanor 48, (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation)	0.6 to 133	24, 48, 72 and 96 h	Embryo/Larvae	-Lethal effects were observed at the highest concentration tested (133 µg a.i. mL^{-1}) at 24 and 48 h.
[60]	Roundup GC (containing 120 g L^{-1} glyphosate acid, UK)	0.01 to 10	21 d (Adult) and maintained across generations	Embryos/Larvae WIK strain	-Increase in mortality in their embryos at 3 hpf.
[67]	Roundup®	0.0048	3 to 120 hpf and maintained up to 7 dpf	Embryo/Larvae Wild-type	-Decreased survival was observed.
[68]	Roundup®	0.0048	3 to 120 hpf and maintained across generations	Embryos/Larvae Wild-type	-Intergenerational exposure caused an increase in larval mortality (7 d).
GBHs in adult		**µg a.i. mL^{-1}**			
[51]	Roundup WG® (Monsanto, Brazil)	0.065 to 6.5	15 d	Adult females	-LC_{50} 96 h: 42.61 µg a.i. mL^{-1}.
[70]	Scout® (Monsanto, Brazil)	0.065 to 10	7 d	Adult Wild-type	-LC_{50} 96 h: 53.75 µg a.i. mL^{-1}.

2.2. Effects on the Hatching Caused by Exposure to GLY or GBHs in Zebrafish

Hatching is a relevant transition event for fish, when the chorion is ruptured and the larvae start to swim freely, normally occurring between 48 and 72 h post fertilization (hpf) [72]. Hatching success is a parameter used in the assessment of acute toxicity during zebrafish development under the action of exogenous chemicals. Changes in hatching can occur as a delay, acceleration, or inhibition [42]. The timing of hatching has been recorded in several studies investigating the acute toxicity of GLY or GBHs (Table 2). Studies testing GLY have been contradictory, with some reporting premature hatching and others reporting a delay. After exposure to GLY, embryos hatched prematurely at 48 hpf, in concentration of 90 µg mL^{-1}. However, hatch rates decreased after 72 hpf (>90 µg mL^{-1}), which could be attributed to developmental delay or death of unhatched embryos caused by high GLY concentrations [56]. A decrease in chorion elasticity and an increase in hatching rate was observed after exposure to 400 µg mL^{-1} (72 hpf) [54]. An increase in hatching was also observed at concentrations of >5 µg mL^{-1} (72 hpf) [57], which is in agreement with another study that also demonstrated premature hatching at concentrations >7 µg mL^{-1} [73]. Exposures to GLY (10 µg mL^{-1}) for 21 d in adult zebrafish induced an increase in hatching in their embryos after 54 hpf [60]. On the contrary, other studies using GLY reported a delay in hatching rate at concentrations of >0.01 µg mL^{-1} (72 hpf) [58], >50 µg mL^{-1} (48 h) [53], and >42.27 µg mL^{-1} [55]. Delayed hatching also occurred in embryos where their parents were exposed to GLY along with increased water temperature [61].

Regarding GBHs, AKB caused premature hatching of embryos with EC50 48 h of 8.30 µg a.i. mL^{-1} and Roundup Original caused premature hatching of EC50 48 h of 11.05 µg a.i. mL^{-1} [63]. Exposures to Roundup GC (10 µg a.i. mL^{-1}) for 21 d in adult zebrafish induced an increase in hatching in embryos at 54 hpf [60], although other GBH caused a delaying effect on hatching rate (72 h) at 100 µg a.i. mL^{-1} [69]. Another study also reported a decrease in the percentage of hatched embryos under GBH exposure (Roundup® UltraMax) at a concentration of 8.5 µg a.i. mL^{-1} [62]. Based on these studies, divergences were observed in hatch rate induced by GLY and GBH according to the concentration and type of herbicide used. In addition, these results may be correlated with possible morphological and neuronal changes in embryos, which limited the mobility that precedes their hatching [73].

Table 2. Effects on hatch rate caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[54]	GLY 99.8% purity	0.01 to 600	0.75 to 96 hpf	Embryos/Larvae Wild-type AB strain	-The elasticity of the chorion decreased and there was an increase in the hatching rate at 400 μg mL^{-1} (72 h).
[57]	GLY ≥ 99% purity	0.005 to 50	0.75 to 120 hpf	Embryos/Larvae	-Early hatching increased at concentrations of >5 μg mL^{-1} (72 hpf).
[73]	GLY ≥ 99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf	Embryos/Larvae Wild-type AB strain	-Premature hatching was observed (>7 μg mL^{-1}) (52; 56; 60 hpf).
[55]	GLY 96% purity CAS#1071-83-6	1.69 to 1690	2 to 96 hpf	Embryo/Larvae	-Delay in the hatching, >42.27 μg mL^{-1}.
[58]	GLY 96% purity CAS#1071-83-6	0.001 to 0.7	2 to 74 hpf	Embryos/Larvae Wild-type TU	-Hatching decreased at 0.01, 0.1, and 0.7 μg mL^{-1} (74 hpf).
[53]	GLY Cat#45521	10 to 400	24, 48, 72 and 96 h	Embryos/Larvae	-Reduced hatching was observed (48 h) (>50 μg mL^{-1}).
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-Premature hatching at 48 hpf (90 μg mL^{-1}). -Reduced hatching at 72 hpf (>90 μg mL^{-1}).
[60]	GLY	0.01 to 10	21 d (Adult) and maintained across generations	Embryos/Larvae WIK strain	-Increase in hatching in embryos at 54 hpf (10 μg mL^{-1}).
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Delayed hatching in embryos at 72 h.
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[63]	-AKB 480 (Kelldrin Industrial) (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation). -Roundup Original (Monsanto, Brazil) (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation).	2.4 to 240	24, 48, 72 and 96 h	Embryo/Larvae	-AKB caused premature hatching of embryos. EC50/48 h: 8.30 μg a.i. mL^{-1}. -Roundup caused premature hatching of embryos. EC50/48 h, 11.05 μg a.i. mL^{-1}.
[69]	GBH 360 mg a.i. L^{-1}	1 to 100	4 to 96 h	Embryo Larvae AB strain	-Delaying effect on hatching rate (72 h) at 100 μg a.i. mL^{-1}.
[62]	Roundup® UltraMax (Bayer, Portugal) 35.5 wt% of glyphosate	2 to 15	72 h	Embryos/Larvae Wild-type AB strain	-Decrease in the percentage of hatched embryos at concentration of 8.5 μg a.i. mL^{-1}.
[60]	Roundup GC (containing 120 g L^{-1} glyphosate acid, U.K)	0.01 to 10	21 d (Adult) and maintained across generations	Embryos/Larvae WIK strain	-Increase in hatching in embryos at 54 hpf (10 μg a.i. mL^{-1}).

2.3. Malformations Caused by Exposure to GLY or GBHs in Zebrafish

Development is an extremely sensitive process, and chemical exposures can threaten the normal progression of embryo organogenesis [74]. Common morphological abnormalities observed during zebrafish development include body morphology, eye and head size and shape, body curvature, tail formation, pigmentation, swim bladder inflation, and edema and malformation in the pericardial sac and yolk sac [75]. Zebrafish embryos/larvae exhibit a diverse range of morphological alterations after treatment with GLY or GBHs (Table 3). Regarding GLY exposures, a study reported a reduction in head and eye size at 24 hpf to a concentration of 50 μg mL^{-1} [76]. Ocular distance reduction also occurred at 0.5 μg mL^{-1} after 96 h [77]. Decreased body length, and eye and head size, have also been reported at concentrations greater than 10 μg mL^{-1} after 96 hpf [54]. Other studies have also reported morphological abnormalities, which are concentration- and time-dependent,

such as decreased body size, pericardial edema, swim bladder deficiency, spinal curvature, and yolk sac edema [53,55–58,61,73]. Embryos/larvae exposed to 10 and 50 μg mL^{-1} of GLY, up to 96 h, and reared for 10 months to adulthood, were assessed for skeletal alterations, showing spinal deformities [78]. Regarding malformations caused by GBH exposure in zebrafish embryos/larvae, Roundup® at a concentration of 50 μg a.i. mL^{-1} induced a reduction in the head and eye size in embryos (24 hpf) [76]. After 72 h of Roundup® UltraMax (8.5 μg a.i. mL^{-1}), malformations were reported, such as pericardial and yolk sac edema, body, head and tail malformations, spinal curvature, decreased body length and eye diameter, and reduced length of the brain vesicles (forebrain, midbrain, and hindbrain) [62]. After 96 h, a decrease in body length was observed due to exposure to Roundup® (>0.01 μg a.i. mL^{-1}) [77]. Roundup® also caused the inability of the larvae to inflate the swim bladder (>11.7 μg a.i. mL^{-1}) [64]. GBH caused pericardial edema, yolk sac edema, curvature of the spine, and body malformation (>1 μg a.i. mL^{-1}) [69]. Malformations in the development of zebrafish caused by exposure to glyphosate may be closely related to energy dysfunctions and disruptions in lipid metabolism, leading to smaller fish. Malformations may also be associated with high levels of cellular apoptosis caused by increased production of ROS [64,69].

Table 3. Malformations caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[76]	GLY	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Head and eye reduction.
[77]	GLY	0.01 to 0.5	96 h	Larvae (3 d)	-Reduction in ocular distance (0.5 μg mL^{-1}).
[54]	GLY 99.8% purity	0.01 to 600	0.75 to 96 hpf	Embryos/Larvae Wild-type AB strain	-Delay in the epibolic process and decrease in body length, eye area and head were observed at concentrations greater than 10 μg mL^{-1}.
[57]	GLY ≥ 99% purity	0.005 to 50	0.75 to 120 hpf	Embryos/Larvae	-Pericardial and yolk sac edema, hematoma, and late development, were found. (>0.005 μg mL^{-1}).
[53]	GLY Cat#45521	10 to 400	24, 48, 72 and 96 h	Embryos/Larvae	-Pericardial edema, yolk sac edema and body malformations (100 μg mL^{-1}).
[73]	GLY ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf	Embryos/Larvae Wild-type AB strain	Malformations and decreased body size (>7 μg mL^{-1}).
[55]	GLY 96% purity CAS#1071-83-6	1.69 to 1690	2 to 96 hpf	Embryo/Larvae	-Reduced eye size, cardiac or yolk sac edemas, shortening of the tail and tail and spine malformations (>1.69 μg mL^{-1}).
[58]	GLY 96% purity CAS#1071-83-6	0.001 to 0.7	2 to 74 hpf	Embryos/Larvae Wild-type TU	-Body malformation, pericardial edema, swim bladder deficiency, spinal curvature, and yolk sac edema at >0.01 μg mL^{-1} (74 hpf).
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-Spine curvature, pericardial edema, shortened body length, and curved tail.
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Effect of increasing temperature, causes body malformation, pericardial edema, curved body axis, and yolk sac edema.
[78]	Glyphosate 99% purity CAS#1071-83-6	1 to 50	96 h (bred for 10 months to adulthood)	Embryo/Larvae	-Spinal deformities in adults observed at 10 and 50 μg mL^{-1}.

Table 3. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[76]	Roundup®	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Head and eye reduction was observed.
[62]	Roundup® UltraMax (Bayer, Portugal) 35.5 wt% of glyphosate	2 to 15	72 h	Embryos/Larvae Wild-type AB strain	-Pericardial and yolk sac edema, body, head and tail malformations and spinal curvature (8.5 μg a.i. mL^{-1}). -Decrease in the body length and eye diameter (8.5 μg a.i. mL^{-1}). -Reduced length of the brain vesicles (forebrain, midbrain and hindbrain) (8.5 μg a.i. mL^{-1}).
[77]	Roundup® (Monsanto, St. Louis, MO, USA)	0.01 to 0.5	96 h	Larvae (3 d)	-Decreased body length (>0.01 μg a.i. mL^{-1}).
[69]	GBH 360 mg a.i. L^{-1}	1 to 100	4 to 96 h	Embryo/Larvae AB strain	-Pericardial edema, yolk sac edema, curvature of the spine and body malformation (>1 μg a.i. mL^{-1}).
[64]	Roundup® (Monsanto, St. Louis, MO, USA)	3.5 to 350	96 hpf	Embryo/Larvae	-Incapacity to inflate the swim bladder was observed (>11.7 μg a.i. mL^{-1}).

2.4. Cardiotoxicity Caused by Exposure to GLY or GBHs in Zebrafish

Zebrafish is an excellent model to study cardiotoxicity due to the easy visualization of the heart during its development. Although the zebrafish heart contains two chambers, an atrium and a ventricle, constituted by myocardium and endocardium, it shares histological and structural similarities with that of mammalians [56,79]. Assessment of cardiotoxicity can be performed through different parameters, but one of the most used is the heart rate [53]. Additionally, congenital cardiac abnormalities can occur due to exposure to potentially teratogenic agents during the early stages of zebrafish life [80]. Studies concerning the cardiotoxicity of glyphosate are controversial, reporting either an increase or a decrease in heart rate (Table 4) [53]. Studies using zebrafish embryos exposed to GLY have reported concentration-dependent changes in heart rate. Exposure to concentrations of >0.01 μg mL^{-1} (50/74 hpf) not only induced an increase in heart rate, but also cardiac malformations, cardiomyocyte apoptosis, and a decrease in the activity of Na+/K+-ATPase and Ca^{2+}-ATPase, important enzymes for the maintenance of normal physiological functions of the heart [58]. On the other hand, some studies have reported a decrease in heart rate. As demonstrated by [79], GLY at a concentration of 50 μg mL^{-1} at 48 hpf induced a decrease in heart rate, and structural heart and vascular abnormalities. Another study found a decrease in heart rate related to increased concentrations of GLY [55]. GLY (50 and 100 μg mL^{-1}) caused a decrease in heart rate (48 and 72 h) and reduced nitric oxide generation in the heart (50 μg mL^{-1}) (72 h) [53]. A decrease in heart rate was also observed under the exposure to concentrations of 0.1; 1 μg mL^{-1} (48 h) [81], 10 μg mL^{-1} (48 h), 10; 50 μg mL^{-1} (72 h) [78] and 7; 35 μg mL^{-1} (48; 72; 96 and 120 hpf) [73]. Embryos exposed to GLY exhibited cardiac malformations, including enlarged chambers, thinned ventricular walls, and rhythm disturbances. In addition, defective intersegmental vasculature occurred, indicating impaired angiogenesis [56]. A cross-generational study also showed that GLY (1 and 5 μg mL^{-1}) had the ability to alter the cardiac function of embryos [61]. Regarding the effects of GBH, a study reported that Roundup® UltraMax caused a reduction in heart rate in zebrafish larvae at concentrations of 5 and 8.5 μg a.i. mL^{-1} [62]. In fact, studies indicate that an alteration in heart rate may be related to structural abnormalities caused by exposure to glyphosate, with impairment of cardiac pumping [58,79]. In this sense, there is a need for studies on cardiac development after exposure to both GLY and GBHs to elucidate these effects.

Table 4. Cardiotoxicity caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[58]	GLY 96% purity CAS#1071-83-6	0.001 to 0.7	2 to 74 hpf	Embryos/Larvae Wild-type TU	-Increased cardiac function and cardiac malformations, observed at >0.01 μg mL^{-1} (50/74 hpf). -Cardiomyocyte apoptosis was observed at >0.001 μg mL^{-1} (74 hpf). -Decrease in Na+/K+-ATPase and Ca^{2+}-ATPase activities were observed at >0.01 μg mL^{-1} (74 hpf)
[79]	GLY	50	5 to 48 hpf	Embryos Wild-type AB strain and transgenic fli-1 gfp.	-Heart rate decrease. -Structural abnormalities of the heart. -Vasculature alterations.
[55]	GLY 96% purity CAS#1071-83-6	1.69 to 1690	2 to 96 hpf	Embryo/Larvae	-Heart rates showed a concentration-dependent relationship, decreasing with increasing glyphosate concentration.
[53]	GLY Cat#45521	10 to 400	24, 48, 72 and 96 h	Embryos/Larvae	-Reduced heart rate (50 and 100 μg mL^{-1}) (48/72 h). -Reduction in nitric oxide generation in the heart (50 μg mL^{-1}) (72 h).
[81]	GLY 96% purity CAS#1071-83-6	0.01 to 1	96 h	Embryos/Larvae	-Reduced heart rate at 48 h (0.1 and 1 μg mL^{-1}).
[78]	GLY 99% purity CAS#1071-83-6	1 to 50	96 h (bred for 10 months to adulthood)	Embryo/Larvae	-Decreased heart rate at 10 μg mL^{-1} (48 hpf) and 10; 50 μg mL^{-1} (72 hpf).
[73]	GLY ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf.	Embryos/Larvae Wild-type AB strain	-Reduced heart rate (7; 35 μg mL^{-1}) (48, 72, 96, 120 hpf).
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-Cardiac malformations. -Defective intersegmental vasculature (30 and 90 μg mL^{-1}). -Reduced heart rate (90 μg mL^{-1}).
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Decrease blood flow. -Decrease heart rate.
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[62]	Roundup® UltraMax (Bayer, Portugal) 35.5 wt% of glyphosate	2 to 15	72 h	Embryos/Larvae Wild-type AB strain	-Reduced heart rate (5 and 8.5 μg a.i. mL^{-1}).

2.5. Neurotoxicity and Behavioral Changes Caused by Exposure to GLY or GBHs in Zebrafish

Behavioral patterns are considered a functional indicator of neural activity [82]. The behavioral patterns of the zebrafish are encompassed in its ability to adapt to the environment and respond to different conditions, being controlled by genetic, biochemical, and physiological pathways [24]. Sensorimotor alterations can be observed in the embryonic stage of zebrafish since their chorion is transparent. More complex behavioral activities arise in the larvae when they reach a state of free swimming and actively seek food (120 hpf) [83]. The complexity of zebrafish behavioral patterns varies between locomotor responses, social behaviours, and cognitive performance [84]. Thus, exploring the effects of pollutants on fish behavioral patterns can be a tool for neurotoxicological assessment [85]. In addition, it is recognized that GLY can interfere with processes involved in neurotransmission [86]. The effects of exposure to GLY and GBHs on the neurotoxicity and behavioral changes of zebrafish are shown in Table 5.

Table 5. Neurotoxicity and behavioral changes caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[76]	GLY	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Structural changes in the developing brain, such as loss of cerebral ventricles.
[78]	GLY 99% purity CAS#1071-83-6	1 to 50	96 h (bred for 10 months to adulthood)	Embryo/Larvae	-Change in the spontaneous movement pattern, observed at 50 μg mL^{-1} (24 h). -Defects in craniofacial development, observed at 50 μg mL^{-1} (96 h).
[87]	GLY CAS#1071-83-6	5 to 50	96 hpf	Embryo/Larvae Wild-type	-Significant reductions in acetylated α-tubulin levels (50 μg mL^{-1}). -Reduction in polymeric tubulin (10 and 50 μg mL^{-1}).
[54]	GLY 99.8% purity	0.01 to 600	0.75 to 96 hpf	Embryos/Larvae Wild-type AB strain	-Increased locomotor activities (0.01–1 μg mL^{-1}).
[88]	GLY CAS#1071-83-6	0.016 to 1.6	5 hpf to 7 dpf	Embryo/Larvae Wild-type AB/TU strain	-Hyperactivity, but anxiety-like behaviours were absent.
[77]	GLY	0.01 to 0.5	96 h	Larvae (3d)	-Decreased distance traveled (0.5 μg mL^{-1}). -Decreased absolute turning angle (0.01 μg mL^{-1}). -Increased aversive stimulus (>0.01 μg mL^{-1}).
[89]	GLY CAS#071-83-6	0.00005 to 10	1.5 to 120 hpf.	Embryo/Larvae Wild-type AB strain Transgenic lines: Tg(fli1a:GFP)y1Tg Tg(mpeg1:mCherry) Tg(HuC:Tomato)	-Significant decrease in locomotor activity after exposure to 1 μg mL^{-1} or higher. -Midbrain electrophysiological recordings indicated abnormal peak activity and variable at 1 μg mL^{-1}. -Morphological changes of microglia (0.0001 and 1 μg mL^{-1}).
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Increase 5HT4R and GNAT2 expression in the brain. -Increase histopathologic finding in the brain. -Decrease dark/light locomotor activity. -Increase thigmotaxis.
GLY in adult		**μg mL^{-1}**			
[77]	GLY	0.01 to 0.5	96 h	Adult (6–7 months)	-The distance traveled decreased (0.5 μg mL^{-1}). -The average speed decreased (0.5 μg mL^{-1}). -The number of line crossings decreased (0.5 μg mL^{-1}). -There was no memory impairment. -The aggressive stimulus decreased (0.01; 0.065; 0.5 μg mL^{-1}).
[90]	GLY 98% purity CAS#1071-83-6	0.0003 and 0.003	14 d	Adult/ AB-Wild-type	-An impairment of exploratory behaviors and a social anxiety increase were observed. -Was observed an increase in dopamine and serotonin levels, as well as in DOPAC/dopamine and homovanillic acid/dopamine turnover ratios.
[91]	GLY	1 and 5	96 h	Adult Wild-type AB strain	-Increase 5HT4R and GNAT2 expression in the brain. -Increase histopathologic finding in the brain. -Disruption in circadian rhythm. -Anxiety-like behaviors. -Decrease mobility. -Metabolic alterations.

Table 5. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[76]	Roundup®	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Loss of cerebral ventricles was observed.
[77]	Roundup® (Monsanto, St. Louis, MO, USA)	0.01 to 0.5	96 h	Larvae (3d)	-Decrease in the distance traveled (0.065 and 0.5 μg a.i. mL^{-1}). -Decrease in the absolute turning angle (0.01 μg a.i. mL^{-1}). -Increase in time mobile (0.065 and 0.5 μg a.i. mL^{-1}). -Increase in the aversive stimulus (0.065 and 0.5 μg a.i. mL^{-1}).
[92]	Roundup® UltraMax (Bayer, Portugal) 35.5 wt% of glyphosate	1 to 5	2.5 to 75 hpf and maintained up to 144 hpf	Embryos/Larvae Wild-type AB strain	-Changes in avoidance behaviour and decreased distance traveled (5 μg a.i. mL^{-1}). -Increased in AChE activity.
[67]	Roundup®	0.0048	3 to 120 hpf and maintained up to 7 dpf	Embryo/Larvae Wild-type	-Changes in animal behaviour, such as an increase in the number of rotations (hypermobility) and non-response to the aversive stimulus.
[68]	Roundup®	0.0048	3 to 120 hpf and maintained across generations	Embryos/Larvae Wild-type	-Intergenerational exposure caused an increase in AChE activity and changes in behaviour.
GBHs in adult		**μg a.i. mL^{-1}**			
[93]	Roundup Original™, 360 g L^{-1} of N-phosphonomethylglycine, CAS#1071-83-6	0.00659 and 5.2	150 s	Adult (180 d) Wild-type	-Induced aversion in fish. -Induced a decrease in the number of body rotations in the time intervals 30–60, 60–90 and 120–150 s.
[77]	Roundup® (Monsanto, St. Louis, MO, USA)	0.01 to 0.5	96 h	Adult (6–7 months)	-Decrease in the distance traveled (0.065; 0.5 μg a.i. mL^{-1}). -Decrease in the average speed (0.065; 0.5 μg a.i. mL^{-1}). -Decrease in the number of midline crossings (0.065; 0.5 μg a.i. mL^{-1}). -Memory impairment was observed (0.5 μg a.i. mL^{-1}).
[70]	Scout® (Monsanto, Brazil)	0.065 to 10	7 d	Adult Wild-type	-Decrease in aggressive stimulus (0.01; 0.065; 0.5 μg a.i. mL^{-1}). -Behavioural impairments were observed at low concentration (0.065 μg a.i. mL^{-1}).
[94]	Roundup Original® 360 g L^{-1} of glyphosate	1 to 5	96 h	Adult (50:50, male:female)	-Fish moved between zones more often, spending more time in the upper zone and less time in the lower zone (3 and 5 μg a.i. mL^{-1}). -Increase in rotations (3 μg a.i. mL^{-1}).
[95]	Roundup® (Monsanto, St. Louis, MO, USA)	0.0014	30 min	Adult Wild-type of the short-fin phenotype	-Fish have lost the ability to react properly to the simulated predator attack. -Exposed fish stay longer in the central area, which was the preferred area.
[68]	Roundup®	0.0048	3 to 120 hpf and maintained up to 180 dpf	Adult Wild-type	-Hypermobility and antipredator reaction were impaired.
[96]	Roundup formulation (Roundup Pro Scotts Ortho Roundup, USA, containing 41% glyphosate)	0.015 and 0.5	14 d	Adult males of the AB Wild-type zebrafish (age: 8–12 months)	-AChE activity in the brain was suppressed.

In zebrafish embryos, GLY was shown to be able to induce brain toxicity depending on concentration. A study that exposed embryos to GLY for 24 hpf at a concentration of 50 μg mL^{-1} reported alterations in the structure of the developing brain, such as loss

of brain ventricles [76]. At the same concentration and exposure time, changes in the spontaneous movement pattern were reported and after 96 h defects in the craniofacial development of the larvae were described [78]. GLY in larvae also caused significant reductions in acetylated α-tubulin levels (50 μg mL^{-1} at 96 hpf) and a decrease in polymeric tubulin (10 and 50 μg mL^{-1}), which are microtubule-associated proteins that are vital for many developmental processes in the nervous system [87]. Larvae exposed up for 48 hpf to GLY (0.01–1 μg mL^{-1}) and maintained up to 96 hpf showed an increase in locomotor activities [54]. Hyperactivity was also reported in larvae exposed to GLY after 7 dpf [88]. On the other hand, larvae (3 d) exposed to 0.5 μg mL^{-1} of GLY for 96 h evidenced a decrease in their movements, with a reduction in the distance traveled, a decrease in the absolute turning angle (0.01 μg mL^{-1}), and an increase in the aversive stimulus (0.01; 0.065; 0.5 μg mL^{-1}) [77]. In a study carried out in larvae (120 hpf), a significant decrease in locomotor activity was also observed after exposure to >1 μg mL^{-1}, and midbrain electrophysiological recordings indicated an abnormal peak activity and morphological changes of microglia (0.0001 and 1 μg mL^{-1}) [89]. Exposure to GLY (1 and 5 μg mL^{-1}) for 96 h, with a simultaneous increase in water temperature of 0.5 °C, caused an increase in anxiety in adult fish as well as relevant neurological effects in their offspring, in which were observed an increase in histopathologic findings in the brain, an increase in Serotonin type 4 receptor (5HT4R) and G Protein Subunit Alpha Transducin 2 (GNAT2) expression in the brain, a decrease in dark/light locomotor activity, and an increase in thigmotaxis [61].

Regarding the effects of GLY in adults, a study reported, after exposure of GLY (0.5 μg mL^{-1}) for 96 h, a decrease in the distance covered and in the average speed in relation to the control. At lower concentrations, a decrease in the aggressive stimulus was identified (>0.01 μg mL^{-1}) [77]. Another study showed that exposure to GLY (0.003 μg mL^{-1}) for 14 d caused an impairment of exploratory behaviours and an increase in social anxiety. An increase in dopamine and serotonin levels was also observed, being acknowledged that both monoaminergic neurotransmitters are involved in anxiety disorders [90]. Exposure to GLY (1 and 5 μg mL^{-1}) for 96 h, with a simultaneous increase in water temperature of 0.5 °C, caused increased anxiety in adult fish, alteration in circadian rhythm, and also an increase in histopathological findings and 5-HT4R and GNAT2 immunopositivity in the brain [91].

Considering the neurotoxic effects reported in embryo/larvae exposed to GBHs, in a study conducted with embryos (24 hpf), Roundup® 50 μg a.i. mL^{-1} caused alterations in brain development, with loss of cerebral ventricles [76]. Another study using Roundup® reported changes in the behavior of larvae exposed for 96h, decreasing the distance traveled (0.065 and 0.5 μg a.i. mL^{-1}), and decreasing the absolute turning angle of the larvae (0.01 μg a.i. mL^{-1}). In addition, an increase in the mobile time (0.065; 0.5 μg a.i. mL^{-1}) and an increase in the area of the aversive stimulus (0.065 and 0.5 μg a.i. mL^{-1}) was observed [77]. Larvae exposed from 2.5 to 75 hpf to Roundup® UltraMax (5 μg a.i. mL^{-1}), and maintained up to 144 hpf, showed changes in avoidance behavior and decreased distance traveled [92]. In larvae exposed to Roundup® (0.0048 μg a.i. mL^{-1}), an increase in the activity of the enzyme acetylcholinesterase (AChE) was observed, as well as changes in the behavior, such as an increase in the number of rotations (hypermobility) and non-response to aversive stimulus [67]. In the continuation of this study that evaluated Roundup® exposure between generations reported similar results with an increase in AChE activity and behavioral changes in the larvae [68]. The AChE is responsible for degrading acetylcholine (ACh), an essential neurotransmitter of the central and peripheral nervous system, which establishes the communication between cholinergic neuronal cell synapses and in the neuromuscular junction. The imbalance of AChE activity can lead to constraints in cell signaling and changes in muscle contraction [24].

The exposure of adult zebrafish to GBHs was able to alter the natural behavior of fish. Adults exposed to Roundup Original™ (0.00659 μg a.i. mL^{-1}) for just 15 s showed behavioral changes, with a decrease in speed and in the number of body rotations [93]. Another study in adults exposed for 96 h to Roundup® showed a decrease in the distance

covered and in mean speed (0.065 and 0.5 µg a.i. mL^{-1}), in addition to memory impairment (0.5 µg a.i. mL^{-1}) and a decrease in aggressive stimulus (>0.01 µg a.i. mL^{-1}) [77]. In a study with a different GBH (Scout®), after exposure for 7 d, a reduction in the exploratory activity in adults was observed at a concentration of 0.065 µg mL^{-1} [70]. In adults exposed to Roundup Original® for 96 h, it was found that fish moved less between zones, spending more time in the upper zone and less time in the lower zone (3 and 5 µg mL^{-1}), and there was an increase in the number of rotations (3 µg a.i. mL^{-1}) [94]. Another study, where adults were exposed to Roundup® (0.0014 µg a.i. mL^{-1} for 30 min), showed the loss of ability to react adequately to a simulated predator attack. [95]. Adult zebrafish exposed to Roundup® (0.0048 µg a.i. mL^{-1}) during the entire period of organogenesis showed hypermobility and impairment of the antipredator reaction [68]. Other study reported that Roundup (0.015 and 0.5 µg a.i. mL^{-1}) caused a decrease in AChE activity in fish brain after 14 dpe [96].

Despite being scarce, the information provided indicates that exposure to both GLY and GBHs affects normal neuronal development, and induces neurotoxicity with dysregulation of neuronal transmission, with repercussions on the behavioral patterns of the zebrafish. These adverse effects make zebrafish prone to predation, interfere with the search for food, and affect reproduction, compromising their perpetuation and survival in polluted environments. It is worth mentioning that behavioral changes may also be related to hormonal dysregulation, related to the activation of the hypothalamic–pituitary–inter-renal axis induced by stress conditions [24].

2.6. Oxidative Stress Caused by Exposure to GLY or GBHs in Zebrafish

The generation of reactive oxygen species (ROS) is a mechanism of toxicity of several toxicants, which leads to the induction of cell damage [97]. ROS can initiate the process of oxidative damage to nucleic acids, lipids, and proteins, eventually leading to organelle damage and ultimately cell death [98]. The synthesis of antioxidants, including enzyme and non-enzymatic metabolites, counteract the harmful effects of ROS. However, when this response can no longer compensate for free radicals, oxidative damage occurs [99]. There is evidence that oxidative stress is one of the mechanisms of glyphosate toxicity in animals [100]. In fact, there is evidence that this herbicide may change parameters related to oxidative stress in zebrafish (Table 6), although there are contradictory data.

Studies have shown that, in zebrafish, GLY caused changes in oxidative stress parameters. One study reported an increase in ROS levels (7; 35 µg mL^{-1}) at 72 and 120 hpf, and changes in antioxidant enzyme activity, showing a decrease in superoxide dismutase (SOD) activity (0.7 µg mL^{-1}) (72 hpf), and an increase in Catalase (CAT) activity (7 µg mL^{-1}) (72 hpf), but at a higher concentration of 35 µg mL^{-1}, a decrease in CAT (72 hpf) was observed. These enzymes play a fundamental role in protecting cells from radical attack. An increase in malondialdehyde (MDA) (35 µg mL^{-1}) (120 hpf) was also observed, indicating oxidative damage to lipids. A change in the levels of endoplasmic reticulum stress signaling pathway factors has also been reported (7; 35 µg mL^{-1}) (120 hpf), indicating that massive ROS and lipid oxidative injury may be involved in the toxicity of GLY in larvae, which may cause developmental malformations in zebrafish [73]. Exposure to GLY in adults also caused an increase in ROS levels in their offspring [61].

In adult zebrafish exposed to GLY, a study showed an improved antioxidant response against peroxyl radicals (ACAP) in the gills at a concentration of 5 µg mL^{-1} after 24 h, and in the muscles, an increase in lipid peroxidation (LPO) at a concentration of 10 µg mL^{-1} after 96 h [101]. In another study, after exposure to 0.1 µg mL^{-1}, an increase was observed in the activity of ATP binding cassette subfamily C (ABCC) after 24 h and after 96 h in the gills, liver, intestine, and brain [102]. After 14 d, GLY (0.003 µg mL^{-1}) caused an increase in CAT and SOD activities, induced LPO, and decreased glutathione stores [90]. At a concentration of 3.5 µg mL^{-1}, GLY increased SOD and CAT activity in adult zebrafish exposed for 21 d [103].

Table 6. Effects on oxidative stress caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[73]	Glyphosate ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf	Embryos/Larvae Wild-type AB strain	-Decreased SOD activity (0.7 μg mL^{-1}) (72 hpf). -Increased CAT activity (7 μg mL^{-1}) (72 hpf). -Decreased CAT activity (35 μg mL^{-1}) (72 hpf). -Increase ROS levels (7; 35 μg mL^{-1}) (72/120 hpf). -MDA increase (35 μg mL^{-1}) (120 hpf). -Alteration in the levels of endoplasmic reticulum stress signaling pathway factors (7; 35 μg mL^{-1}) (120 hpf).
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Increase ROS levels.
GLY in Adult		**μg mL^{-1}**			
[101]	GLY	5 and 10	96 h	Adult Male	-Increase in ACAP in gills (24 h) was observed in animals exposed to 5 μg mL^{-1}. -Decreased LPO brain tissue exposed to 10 μg mL^{-1} after 24 h was observed. -Increase in LPO was observed in the muscle after 96 h (10 μg mL^{-1}).
[102]	GLY 98.6% purity	0.1	24 and 96 h	Adult	-ABCC activity in the gills (24 h) was increased. -ABCC activity in gills, liver, gut and brain (96 h) was increased.
[90]	GLY 98% purity CAS#1071-83-6	0.0003 and 0.003	14 d	Adult/AB-Wild-type	-Increase in CAT and SOD activities was observed.
[103]	Glyphosate 99.5% purity CAS#1071-83-6	3.5	7, 14 and 21 d	Adult (6 months) /AB-Wild-type	-Increased SOD and CAT was observed (21 d).
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[104]	Roundup® Flex (Bayer, Portugal) 35.5 wt% of glyphosate	1 to 10	4 h 30 min	Larvae (72 hpf) Wild-type AB strain and Tg(mpxGFP)^{i114}	-Increased ROS levels (10 μg a.i. mL^{-1}).
[69]	GBH 360 mg a.i. L^{-1}	1 to 100	4 to 96 h	Embryo Larvae AB strain	-ROS production was increased especially in the gill. -Activity of carbonic anhydrase was inhibited.
[68]	Roundup®	0.0048	3 to 120 hpf and maintained across generations	Embryos/Larvae Wild-type	-Increased SOD activity. -Inhibition of CAT activity.
GBHs in Adult		**μg a.i. mL^{-1}**			
[105]	Roundup®	5 and 10	24 to 96 h	Adult	-In the gills, was observed an increase in ACAP after 96 h (10 μg a.i. mL^{-1}). -In the liver, was observed a reduction ACAP after 24 h, however there was an increase in ACAP after 48 h (10 μg a.i. mL^{-1}).

Table 6. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
[106]	Roundup Original®	5	96 h	Adult	-MDA levels in the brain and liver increased significantly. -Increased hepatic CAT activity was observed. -Decreased hepatic GPx activity was observed.
[102]	Roundup Transorb® 480 g L^{-1} of glyphosate	0.1	24 and 96 h	Adult	-An increase in ABCC activity was observed in the gills, liver, and intestine (96 h).
[96]	Roundup formulation (Roundup Pro Scotts, USA, containing 41% glyphosate).	0.015 and 0.5	14 d	Adult males of the AB Wild-type zebrafish (age: 8–12 months)	-An increase in tissue levels of RNS was observed (0.5 µg a.i. mL^{-1}). -An increase in tissue levels of TBARS was observed (0.015 and 0.5 µg a.i. mL^{-1}). -Antioxidant capacity in the liver fluctuated, increasing at 0.015 µg a.i. mL^{-1}, and decreasing at 0.5 µg a.i. mL^{-1}. -A decrease in GST activity was observed (0.5 µg a.i. mL^{-1}). -Total GSH concentration was elevated in the liver tissues (0.015 µg a.i. mL^{-1}). -Tissue levels of oxidized glutathione (GSSG) increased in the liver (0.015 and 0.5 µg a.i. mL^{-1}). -GSSG/GSH ratio increased (0.5 µg a.i. mL^{-1}).

Studies have revealed that GBHs can elevate ROS levels. An acute exposure study with 72 hpf larvae exposed for 4h 30min to Roundup® Flex (10 µg a.i. mL^{-1}) reported an increase in ROS levels [104]. Another study also demonstrated an increase in ROS levels, especially in the gills, at concentrations >1 µg a.i. mL^{-1} after 96 h. Under these conditions the inhibition of carbonic anhydrase activity was also reported. This enzyme is involved in several biological processes, such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis, and body fluid generation [69]. Intergenerational exposure to Roundup® was able to alter the larva's first line of antioxidant defense, causing an increase in SOD activity and inhibition of CAT activity [68].

Studies with adult zebrafish exposed to GBHs reported that Roundup (10 µg mL^{-1}) caused an increase in ACAP in the gills after 48 and 96 h in the liver [105]. Roundup Original® (5 µg mL^{-1}) had the ability to decrease brain thiol levels, increase brain and liver MDA levels, increase hepatic CAT activity, and decrease GPx activity in liver, after 96 h of exposure [106]. Roundup Transorb® (0.1 µg mL^{-1}) caused an increase in ABCC activity in the gills, liver, and intestine after 96 h of exposure [102]. Another study reported that Roundup at concentrations of 0.015 and 0.5 µg mL^{-1} exposed for 14 d showed an altered antioxidant defense capacity, noting an increase in tissue levels of reactive nitrogen species (RNS) (0.5 µg mL^{-1}), an increase in tissue levels of LPO (0.015 and 0.5 µg mL^{-1}), and a decrease in GST activity (0.5 µg mL^{-1}). The total concentration of GSH (0.015 µg mL^{-1}), as well as the levels of oxidized glutathione (GSSG) (0.015 and 0.5 µg mL^{-1}), were elevated in the liver, and an increase in the GSSG/GSH ratio was also reported (0.5 µg mL^{-1}) [96].

Overall, the evidence suggests that GLY causes oxidative damage in zebrafish, dependent on the concentration and duration of exposure, through ROS and lipid peroxidation. The induction of antioxidant systems during low-level exposures may allow cells to combat oxidative stress effectively and reduce the impact of oxidative damage. To our knowledge, there are very few studies showing changes in these parameters in zebrafish embryos exposed to GLY/GBHs.

2.7. Genotoxicity Caused by Exposure to GLY or GBHs in Zebrafish

The genotoxicity of GLY and GBHs has been studied in different test systems [107]. In contaminated environments, the loss of structural or functional DNA integrity in exposed organisms can result in deleterious effects, with late consequences at the population level. This damage can lead to mutation, which can affect cell form and function, even inducing cell death [108]. Genotoxic effects caused by exposure to GLY and GBHs in zebrafish showed that this herbicide causes negative effects on this species (Table 7).

Zebrafish has 26 206 protein-coding genes [81,109]. A study in zebrafish embryos reported that GLY (>0.01 μg mL^{-1}) had the ability to alter 30 expressed genes, showing that this herbicide causes a transcriptomic response with uncertain consequences in zebrafish development or transgenerational implications for this species [81]. Another study performed with embryos, up to 24 h, reported that GLY at a concentration of 50 μg mL^{-1} caused a decrease in gene expression in the eye, fore, and midbrain regions (*pax2*, *pax6*, *otx2*, and *ephA4*), and loss of retinoic acid expression in the retina was also observed [76]. The same concentration was able to change the expression of the cardiac progenitor gene (myocyte increase factor 2) at 48 hpf [79]. Exposure to GLY also changed the expression of *ntl*, an important gene required for early notochord formation, and of *krox20* as glyphosate concentration increased after 13 hpf (1 to 100 μg mL^{-1}). The gene *krox20* is a zinc-finger transcription factor that is involved in vertebrate hindbrain segmentation [54]. Regarding the genes involved in the coupling of cardiac excitation-contraction, alterations were reported in the gene expression of *cacna1c* (L type calcium channel), *ryr2a* (ryanodine receptor), and *hspb11* (heat shock protein) at concentrations of 50 and 100 μg mL^{-1} at 48 and 72 h [53]. Genotoxic effects were also observed in fish exposed to >1.7 μg mL^{-1}, causing high levels of DNA damage (96 h) [65]. Increased gene expression of *kim1* and *pax2* was observed at 0.01 and 0.1 μg mL^{-1} (72 hpf). These are highly sensitive markers of renal toxicity, with *pax2* being a critical gene for kidney development, and increased kim1 expression indicating acute kidney injury [110]. GLY has been shown to have the ability to cause RNAseq dysregulation of transcriptional families implicated in neuronal physiology, synaptic transmission, and inflammation (0.0001 e 1 μg mL^{-1}) (72 hpf) [89]. At 7 dpf, GLY (0.16 and 1.6 μg mL^{-1}) induced an increase in *sod2* mRNA, suggesting an oxidative stress response. An increase was also observed in expression of *cytochrome c oxidase subunit 4 isoform 1* and *citrate synthase*, genes linked to metabolic capacity and oxidative phosphorylation [88]. GLY exposure (0.7; 7 and 35 μg mL^{-1}) (120 hpf) disrupted gene expression patterns related to the hypothalamic-pituitary-thyroid axis (HPT) (*crh*, *tshβ*, *tr α*, *tr β*, and *t tr*) and growth hormone/insulin-like growth factor axis (GH/IGF) (*gh*, *ghrα*, *ghrβ*, *igf1*, *igf1rα*, and *igf1rβ*), also significantly altering the levels of endoplasmic reticulum stress signaling pathway factors (*perk*, *eif2α*, *gadd34*, *atf4*, *ire1α*, *xbp1*, *atf6*, *hspa5*, and *chop*) [73].

Regarding the effects of GLY in adult zebrafish, a study of exposure to adult males for 96 h at concentrations of 10 μg mL^{-1} reported that DNA functionality was reduced [111]. A change in the expression of AChE gene (*ache*) was also reported; first a decrease in the brain was reported after 24 h, and later, after 96 h, an increase in brain and muscle tissues was observed [101]. Another study (96 h) reported that GLY (0.1 μg mL^{-1}) caused a reduction in *abcc5* gene expression in the gills and in *abcc1* gene expression in the intestine, and an increase in *abcc1* and *abcc4* expression in the brain. These genes are involved in cellular detoxification [102]. GLY (0.0003 and 0.003 μg mL^{-1}) deregulated the expression of genes involved in the dopaminergic system, such as *th1*, *th2*, *comtb*, and *scl6a3*, after exposure for 14 d [90]. After exposure to GLY (3.5 μg mL^{-1}) for 21 d, altered expression of miRNAs was observed (miR-146a, miR-155, miR-16, miR-21, and miR-223), associated with the inflammatory response in the intestine; inhibition of claudin-5 and occludin transcription levels also occurred [103]. After 21 d exposure, the gene involved in the conversion of testosterone to 11-ketotestosterone was downregulated, and endocrine disruption was supported by hormone and gene expression levels, identified as NCBI gene ID 322626 [112]. Exposure for 28 d to GLY (0.7 μg mL^{-1}) increased transcript levels of the genes involved

in the stress response, including *nr3c1* and *hsp70.2*, possibly mediated by glucocorticoid receptors. Decreased transcript levels of genes involved in oxidative stress response *sod1*, *sod2*, and *gpx1* and an increase in the level of cat transcription were observed. The mRNA levels of pro-inflammatory interleukins litaf and *cxcl8b.1* were increased [113].

In embryo/larvae exposed to Roundup® at 50 µg a.i. mL^{-1} (24 hpf) a decrease was observed in the expression of genes in the eye, fore, and midbrain (*pax2*, *pax6*, *otx2* and *ephA4*) and there was a loss of retinoic acid expression in the retina [76]. Atanor 48 genotoxic effects (DNA strand breaks) were observed at all concentrations tested (>2.2 µg a.i. mL^{-1}) in larvae after 96 h [65].

The exposure of adult zebrafish to GBHs demonstrated that Roundup GC (10 µg a.i. mL^{-1}) exposed for 21 d increased the expression of cyp19a1 and esr1 genes in the ovary. The increase in this gene may have resulted from compensatory mechanisms in the ovary to maintain or restore estrogen signaling pathways [60]. Another study showed that Roundup altered genes related to reduction in ROS levels. Reducing the expression of *sod2* and *gstt*π increased expression of the uncoupling protein 1 (ucp1) in the gills at 24 h (10 µg a.i. mL^{-1}), reduced gpx gene expression in the gills (5 µg a.i. mL^{-1}), and increased *gpx* gene expression in liver tissue after 96 h of exposure (10 µg a.i. mL^{-1}) [105]. Another GBH evaluated was Roundup Transorb® for 96 h of exposure at a concentration of 0.1 µg a.i. mL^{-1}. In gills, an increase was observed in the expression of the *abcc2* gene and a decrease in *abcc5* gene expression; in the intestine, there was a reduction in the expression of the *abcc1* gene and an increase in the gene *abcc3*; and in the liver, there was an increase in the expression of the *abcc3* and *abcc5* genes [102]. A 14 d study in adults showed that Roundup caused an increase in double-strand DNA breaks in liver tissues (0.015 and 0.5 µg a.i. mL^{-1}). Attenuated levels of gene mRNA RAD51 in the liver were observed; this gene encodes a protein with a fundamental role in the repair of double-stranded DNA breaks. An increase was observed in the expression of mRNA Nrf2, which is an emerging regulator of cellular resistance to oxidants [96]. Other genetic biomarker is assessment of micronucleus formation during cell division, corresponding to chromosomal fragments not incorporated by the nucleus [114]. A study showed that Roundup Original had the ability to increase micronucleus formation, and cellular and nuclear abnormalities in blood cells, after exposure for 72 h [115].

Table 7. Genotoxicity caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**µg mL^{-1}**			
[81]	GLY 96% purity CAS#1071-83-6	0.01 to 1	96 h	Embryos/Larvae	-Changes in gene expression: Positive regulation: 0.01 µg mL^{-1}: *mllt1b/ralyl/ncoa5/gpr185a.* 0.1 µg mL^{-1}: *mllt1b/ralyl/cxxc5b/fhod1/rhot1b/mier1a.* 1 µg mL^{-1}: *mier1a/egf/cep170ab/thumpd3/asap2a/apba1a/hmgra/armc6/tpd52.* Negative regulation: 0.01 µg mL^{-1}: *rbm14b/rnf34b/vit/pde3a/edem3/wdr59.* 0.1 µg mL^{-1}: *nav1a/robo1/edem3/wdr59.* 1 µg mL^{-1}: *nav1a/ebf1a/eif4g8b/myt1b/picalmb/sorbs1/nav2a/edem3/wdr59.*
[76]	GLY	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Decreased gene expression in the eye, fore and midbrain regions occurred (*pax2*, *pax6*, *otx2* and *ephA4*). -Loss of retinoic acid expression in the retina was observed.
[79]	GLY	50	5 to 48 hpf	Embryos Wild-type AB strain and transgenic fli-1 gfp.	-Alteration of cardiac progenitor gene expression (myocyte-enhancing factor 2).

Table 7. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
[58]	GLY 99.8% purity	0.01 to 600	0.75 to 96 hpf	Embryos/Larvae Wild-type AB strain	-Expression of *ntl* (tailless) shortened and *krox20* (also known as *Egr2b*, early growth response 2b) changed as glyphosate concentration increased (1 to 100 μg mL^{-1}) (13 hpf).
[53]	GLY Cat#45521	10 to 400	24, 48, 72 and 96 h	Embryos/Larvae	-Alteration of gene expression of *cacna1c*, *ryr2a*, *hspb11* (50 and 100 μg mL^{-1}) (48/72 h).
[65]	GLY 99% purity CAS#1071-83-6	1.7 to 100	24, 48, 72 and 96 h	Embryo/Larvae	-Genotoxic effects (>1.7 μg mL^{-1}). -High levels of DNA damage.
[110]	GLY Cat#45521	0.01 and 0.1	7 hpf to 8 dpf	Embryo/Larvae AB strain	-Increased expression of *kim1* and *pax2* genes (72 hpf).
[89]	GLY CAS#071-83-6	0.00005 to 10	1.5 to 120 hpf.	Embryo/Larvae Wild-type AB strain Transgenic lines: Tg(fli1a:GFP)y1Tg Tg(mpeg1:mCherry) Tg(HuC:Tomato)	-RNAseq showed dysregulation of transcriptional families implicated in neuronal physiology, synaptic transmission and inflammation (0.0001 and 1 μg mL^{-1}).
[88]	GLY CAS#1071-83-6	0.016 to 1.6	5 hpf to 7 dpf	Embryo/Larvae Wild-type AB/TU strain	-*sod2* mRNA increased. -*Isoform 1 of subunit 4 of cytochrome c oxidase* and *citrate synthase* mRNA increased.
[73]	GLY ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf	Embryos/Larvae Wild-type AB strain	-Abnormal HPT and GH/IGF expression was observed (0.7; 7; 35 μg mL^{-1}) (120 hpf). -Alteration in the levels of endoplasmic reticulum stress signaling pathway factors was observed (7; 35 μg mL^{-1}) (120 hpf).
GLY in Adult		**μg mL^{-1}**			
[111]	GLY	5 and 10	24 and 96 h	Adult	-DNA functionality was reduced (96 h) (10 μg mL^{-1}).
[101]	GLY	5 and 10	96 h	Adult Male	-Gene expression of AChE gene (*ache*) in the brain was reduced after 24 h and was increased in brain and muscle tissues after 96 h.
[102]	GLY 98.6% purity	0.1	24 and 96 h	Adult	-In the gills, was observed a reduction in *abcc5* gene expression (96 h) -In the gut, was observed a reduction in *abcc1* gene expression (96 h) -In the brain, was observed an increase in *abcc1* and *abcc4* gene expression (96 h).
[90]	GLY 98% purity CAS#1071-83-6	0.0003 and 0.003	14 d	Adult/AB-Wild-type	-Expression of genes involved in the dopaminergic system, such as *th1*, *th2*, *comtb* and *scl6a3* were deregulated.
[103]	GLY 99.5% purity CAS#1071-83-6	3.5	7 to 21 d	Adult (6 months) /AB-Wild-type	-Altered expression of miRNAs was observed (miR-146a, miR-155, miR-16, miR-21 and miR-223). -In the intestine, inhibition of *claudin-5* and *occludin* transcription levels occurred.
[112]	GLY 95% purity CAS#1071-83-6	0.5 and 10	21 d	Adult	-Gene involved in the conversion of testosterone to 11-ketotestosterone (ID 322626) was downregulated. -Increased stress response in both sexes was observed, as suggested by *nr3c1* expression. -The transcription level of *hsp70.2* was increased in females but decreased in males.
[113]	Glyphosate 98% purity	0.7	28 d	Adult, AB Wild-type strain	-Decreased transcript levels of genes *sod1*, *sod2*, and *gpx1*, and increase in the level of *cat* transcription. -mRNA levels of the pro-inflammatory interleukins *litaf* and *cxcl8b.1* were increased in females.

Table 7. *Cont.*

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GBHs in Embryos/Larvae		**µg a.i. mL^{-1}**			
[76]	Roundup®	50	5 to 24 hpf	Embryos Wild-type AB strain and transgenic RGYn	-Decrease in the expression of genes in the eye, fore and midbrain (*pax2*, *pax6*, *otx2 and ephA4*). -Loss of retinoic acid expression in the retina.
[63]	Atanor 48 (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation).	2.2 to 133	24, 48, 72 and 96 h	Embryo/Larvae	-Genotoxic effects (DNA strand breaks) (>2.2 µg a.i. mL^{-1}).
GBHs in Adult		**µg a.i. mL^{-1}**			
[60]	Roundup GC (120 g L^{-1} glyphosate acid, Monsanto, UK)	0.01 to 10	21 d	Adult WIK strain	-In the ovary: increased expression of *cyp19a1* and *esr1* genes.
[105]	Roundup®	5 and 10	24 to 96 h	Adult	-Regarding gene expression, a reduction in superoxide dismutase 2 (*sod2*) and glutathione S-transferase (*gstt*π) was observed. -An increase in the expression of protein uncoupling 1 (*ucp1*) was observed in the gills at 24 h (10 µg a.i. mL^{-1}). -There was a reduction in the expression of the glutathione peroxidase gene (*gpx*) in the gills (5 µg a.i. mL^{-1}). -There was an increase in the expression of the glutathione peroxidase (*gpx*) gene in liver tissue after 96 h of exposure. (10 µg a.i. mL^{-1}).
[102]	Roundup Transorb® 480 g L^{-1} of glyphosate	0.1	24 and 96 h	Adult	-In the gills, there was an increase in the expression of the *abcc2* gene and a reduction in the *abcc5* gene (96 h). -In the intestine, there was a reduction in the expression of the *abcc1* gene and an increase in the *abcc3* gene (96 h). -In the liver, there was an increase in the expression of *abcc3* and *abcc5* genes.
[96]	Roundup formulation (Roundup Pro Scotts, USA, containing 41% glyphosate).	0.015 and 0.5	14 d	Adult males AB Wild-type (8–12 months)	-An increase in concentration of double-stranded DNA breaks in the liver tissues was observed (0.015 and 0.5 µg a.i. mL^{-1}). -Attenuated *RAD51* mRNA levels in the liver were observed. -An increase in mRNA Nrf2 expression was observed.
[115]	Roundup Original®	0.001 to 5	72 h	Adult	-Cellular and nuclear abnormalities of blood cells and formation of micronucleus.

Computational toxicology can be used to identify new herbicide exposure targets. An approach to elucidate cellular processes and pathways potentially altered by GLY exposure in zebrafish was used. With the Comparative Toxicogenomics Database (CTD), the interactions between GLY exposure and zebrafish genes/proteins were described [116]. Subsequently, the affected gene was related to its function in cellular processes and respective consequences in the functioning of zebrafish systems (Supplementary Data, Table S1). This analysis opens new doors for a better understanding of this subject, offering new targets for future investigations in zebrafish.

2.8. Effects on Apoptosis Caused by Exposure to GLY or GBHs in Zebrafish

Cell apoptosis is a form of programmed cell death involved in the removal of harmful or invasive cells. Apoptotic pathways play a significant role in fish development [42]. Evidence shows that exposure to GLY and GBHs can trigger apoptosis in zebrafish (Table 8).

GLY (>0.7 μg mL^{-1}) on larvae (120 hpf) caused abnormal expression of apoptosis-related genes (*p53*, *caspase-3*, *-8*, and *-9*) [73]. Apoptotic cells were found in the heart and vasculature of zebrafish exposed to GLY, and transcript levels indicated that upregulation of the *caspase-3*, *caspase-9*, and *bax* genes coincided with increased concentrations, while the *bcl-2* decreased as the concentration increased [56]. In adult zebrafish exposed for 21 d to GLY (3.5 μg mL^{-1}), markers used to assess apoptosis (*bax*, *bcl-2*, and *caspase-9*) were increased [103]. An increase in apoptotic cells has been observed in offspring of adults exposed to GLY (1 and 5 μg mL^{-1}) for 96 h [61]. Regarding GBH, in larvae (96 h) exposed to Atanor 48 (0.6 μg a.i. mL^{-1}) apoptosis was detected [66]. A study using acridine orange (AO) staining revealed fluorescence in the larvae exposed to GBH at concentrations >1 μg a.i. mL^{-1} after 96 h, indicating cellular apoptosis [69]. A study with larvae 72 hpf exposed for 4 h 30 min to Roundup® Flex (10 μg a.i. mL^{-1}) reported increased levels of apoptotic cells [104]. Exposure to Roundup (0.5 μg mL^{-1}) for 14 d in adults also increased expression of the apoptotic executor caspase 3 [96]. The aforementioned studies suggest that both GLY and GBHs cause imbalance in zebrafish anti- and apoptotic systems, although the precise mechanisms require further characterization as the apoptosis process involves complex pathways.

Table 8. Effects on apoptosis caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[73]	GLY ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf.	Embryos/Larvae Wild-type AB strain	-Abnormal expression of apoptosis-related genes (*p53, caspase-3, -8*, and *-9*) 0.7; 7 and 35 μg mL^{-1}) (120 hpf). -Apoptosis increased (>60 μg mL^{-1}).
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-Abnormal expression of: *bax* (>60 μg mL^{-1}), *caspase-3* (>30 μg mL^{-1}), *caspase-9* and *bcl-2* (90 μg mL^{-1}).
[61]	GLY	1 and 5	96 h (Adult) and maintained across generations (120 hpf)	Embryos/Larvae Wild-type AB strain	-Increase apoptotic cell.
GLY in Adult		**μg mL^{-1}**			
[103]	GLY 99.5% purity CAS#1071-83-6	3.5	7, 14 and 21 d	Adult AB-Wild-type (6 months)	-Markers used to assess apoptosis (*bax*, *bcl-2* and *caspase-9*) were increased (21 d).
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[66]	Atanor 48, (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation)	0.6 to 133	24, 48, 72 and 96 h	Embryo/Larvae	-Apoptosis induction (0.6 μg a.i. mL^{-1}).
[69]	GBH 360 mg L^{-1}	1 to 100	4 hpf to 96 h of exposure	Embryo Larvae AB strain	-Triggered cellular apoptosis, >1 μg mL^{-1} after 96 hpf.
[104]	Roundup® Flex (Bayer, Portugal) 35.5 wt% of glyphosate	1 to 10	4h 30 min	Larvae (72 hpf) Wild-type AB strain and Tg(mpxGFP)^{i114}	-Triggered cellular apoptosis (10 μg a.i. mL^{-1}).
GBHs in Adult		**μg a.i. mL^{-1}**			
[96]	Roundup (Scotts, USA), containing 41% glyphosate).	0.015 and 0.5	14 d	Adult males of the AB Wild-type zebrafish (8–12 months)	-The increase in expression of the apoptotic executor *caspase 3* and mRNA *Bax* (0.5 μg a.i. mL^{-1}).

2.9. Endocrine Disruption and Reproduction Impairment Caused by Exposure to GLY or GBHs in Zebrafish

Studies in different animal models exposed to GLY or GBHs showed that the effects on the female reproductive system may be related to endocrine disruption, differentiation of ovarian follicles, and uterus malformation, affecting the fertility of the species exposed [117]. The effects of exposure to GLY and GBHs on hormonal regulation and reproductive system of zebrafish are shown in Table 9.

Table 9. Effects on endocrine disruption caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryo/Larvae		**μg mL^{-1}**			
[78]	GLY 99% purity CAS#1071-83-6	1 to 50	96 h (bred for 10 months to adulthood)	Embryo/Larvae	-Changes in the level of estrogen receptor alpha osteopontin and bone sialoprotein were observed at 5; 10 and 50 μg mL^{-1} (96 h).
[73]	GLY ≥99.5% purity CAS#1071-83-6	0.7 to 35	1 to 120 hpf	Embryos/Larvae Wild-type AB strain	-T3 decrease (35 μg mL^{-1}) (120 h). -T4 increase (0.7; 35 μg mL^{-1}) (120 hpf). -Decreased T3/T4 ratio (35 μg mL^{-1}) (120 hpf).
GLY in Adult		**μg mL^{-1}**			
[118]	GLY	0.065	15 d	Adult females	-Significant increase in oocyte diameter was observed. -Changes in ovarian ultrastructure were observed, with the presence of concentric membranes, appearing as myelinated structures and associated with the outer membranes of mitochondria and with yolk granules.
[60]	GLY	0.01 to 10	21 d	Adult WIK strain	-Evidence of ovarian abnormalities. -Decrease in the gonadosomatic index. -Reduced production of embryos.
[111]	GLY	5 and 10	24 and 96 h	Adult	-Sperm motility and motility period were reduced.
[112]	GLY 95% purity CAS#1071-83-6	0.5 and 10	21 d	Adult	-Reduce androgen and concentrations of 11-ketotestosterone and estrone (0.5 μg mL^{-1}).
GBHs in Adult		**μg a.i. mL^{-1}**			
[60]	Roundup GC (containing 120 g L^{-1} glyphosate acid, UK)	0.01 to 10	21 d	Adult WIK strain	-Evidence of ovarian abnormalities (>0.01 μg a.i. mL^{-1}).
[51]	Roundup WG® (Monsanto, Brazil)	0.065 to 6.5	15 d	Adult females	-An increase in the number of early ovarian follicles, a decrease in late ovarian follicles and a smaller diameter of ovarian follicles were observed in fish exposed to 0.065 and 6.5 μg a.i. mL^{-1}. -A reduction in the thickness of the yolk envelope and an increase in the content of yolk protein in the ovarian follicle were observed at the two highest concentrations.
[112]	Rodeo® (DOW-Agrosciences, USA)	0.5 and 10	21 d	Adult	-A decrease in total androgen concentration was observed at 0.5 μg a.i. mL^{-1}. -Decreased concentrations of 11-ketotestosterone and estrone were observed. (0.5 μg a.i. mL^{-1}).

In embryos exposed to GLY, changes in estrogen receptor alpha osteopontin and bone sialoprotein levels were observed at concentrations of 5, 10, and 50 μg mL^{-1} after 96 h, and these alterations may be interconnected with the reported malformations [78]. GLY also induced changes in thyroid hormone levels: a decrease in T3 (35 μg mL^{-1}) (120 hpf), an increase in T4 (0.7; 35 μg mL^{-1}) (120 hpf), and a decrease in the T3/T4 (35 μg mL^{-1}) (120 hpf) [73]. The balance of thyroid hormones is important during the transition of zebrafish embryos into larvae. Changes in these hormones may be related to malformations in zebrafish [73].

In adult zebrafish, after exposure to GLY for 15 d at 0.065 μg mL^{-1}, a significant increase was observed in oocyte diameter, as well as alterations in the ovarian ultrastructure, with the presence of concentric membranes appearing as myelinated structures associated with the outer membranes of mitochondria and veal granules [118]. Another study also reported evidence of ovarian abnormalities at 21 d (10 μg mL^{-1}), showing a reduced production of embryos and a decrease in the gonadosomatic index [60]. In adult males, GLY at a concentration of 5 and 10 μg mL^{-1} (96 h) altered sperm motility and reduced the motility period [111]. GLY also showed the ability to reduce androgen and concentrations of 11-ketotestosterone and estrone at 21 d (0.5 μg mL^{-1}) [112].

Regarding the toxic effects on reproduction and hormone regulation of GBHs, there is evidence that Roundup GC causes ovarian abnormalities in adult females at 21 d (>0.01 μg a.i. mL^{-1}) [60]. After 15 d, Roundup WG® (0.65 and 6.5 μg mL^{-1}) also caused abnormalities in the number of ovarian follicles, a decrease in late ovarian follicles, and a smaller diameter of ovarian follicles of exposed adult females. A reduction in the thickness of the yolk envelope and an increase in the content of yolk protein in the ovarian follicle was also observed, in addition to structural changes in the ovarian follicular component [51]. In a study of adults exposed to Rodeo® (0.5 μg mL^{-1}) for 21 d, a decrease in total androgen concentration and decreased concentrations of 11-ketotestosterone and estrone were observed [112].

2.10. Energy Metabolism Disruption Caused by Exposure to GLY or GBHs in Zebrafish

Energy homeostasis is critical for aquatic organisms' development. Disruption of this balance can lead to energy deficits in zebrafish embryos, endangering their survival. In this process, mitochondria play a preponderant role with complex functions in the cell, including the production of energy and the maintenance of redox balance to the regulation of apoptosis. These organelles are also important in the innate immune system and amino acid metabolism [42,70]. Several works show that metabolites related to energy metabolism pathways were impacted in zebrafish exposed to GLY and GBHs (Table 10). For example, in embryos exposed to GLY (0.1 μg mL^{-1}) there were changes in mitochondrial bioenergetics, showing damaged mitochondria (31 hpf) [110]. ATP levels of zebrafish larvae (72 hpf) were reduced after exposure to GLY (>30 μg mL^{-1}), suggesting that the toxicity may be related to apoptosis [56]. In another study, it was shown that GLY (0.8 μg mL^{-1}) after 7 d caused a decrease in total protein content and lower carbohydrate levels [59]. Studies with adult zebrafish exposed to GLY (10 μg mL^{-1}) after 96 h also showed that the herbicide altered mitochondrial functionality [111]. GLY (0.7 μg mL^{-1}) after 28 d in adult females affected purine metabolism, decreasing levels of AMP and GMP. Decreased UMP levels were also reported in the pyrimidine metabolism pathway. In male zebrafish, a decreased aminoadipic acid in the lysine degradation pathway shows adverse effects on fish liver metabolism [113]. Exposure to GLY (1 and 5 μg mL^{-1}) for 96 h with an increase in water temperature of 0.5 °C caused metabolic alterations in the pathways of riboflavin, purine, nicotinate, nicotinamide, tryptophan, vitamin B6, pyrimidine, valine leucine, phenylalanine, and glutathione [91].

Table 10. Effects on energy metabolism disruption caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Embryos/Larvae		**μg mL^{-1}**			
[110]	GLY Cat#45521	0.01 and 0.1	7 hpf to 8 dpf	Embryo/Larvae AB strain	-Alteration of mitochondrial bioenergetics occurred (31 hpf).
[56]	GLY 96% purity CAS#1071-83-6	30 to 120	72 hpf	Embryos/Larvae Wild-type, Tg(myl7:eGFP) and Tg(Flk:eGFP)	-ATP levels reduced (>30 μg mL^{-1}).
[59]	GLY 99% purity CAS#1071-83-6	0.8	7 d	Larvae (8 dpf)	-Decrease in total protein content and lower carbohydrate levels.
GLY in adult		μg mL^{-1}			
[111]	GLY	5 and 10	24 and 96 h	Adult	-Altered mitochondrial functionality (10 μg mL^{-1}).
[113]	GLY 98% purity	0.7	28 d	Adult AB Wild-type strain	-In females, exposure affected purine metabolism, decreasing AMP, GMP and inosinic acid levels, and increasing uric acid levels. -Decreased UMP levels in the pyrimidine metabolism pathway were observed. -In males, exposure decreased aminoadipic acid in the lysine degradation pathway.
[91]	GLY	1 and 5	96 h	Adult Wild-type AB strain	-Metabolic alterations.
GBHs in Embryos/Larvae		**μg a.i. mL^{-1}**			
[64]	Roundup® (Monsanto, St. Louis, MO, USA)	3.5 to 350	96 hpf	Embryo/Larvae	-Decreased hexokinase activity (>11.7 μg a.i. mL^{-1}).
[66]	Atanor 48, (480 g a.i. L^{-1} or 360 g a.e. L^{-1} of formulation)	0.6 to 133	24, 48, 72 and 96 h	Embryo/Larvae	-Concentration-dependent decrease in mitochondrial potential of cells (6.6 and 66,6 μg a.i. mL^{-1}).
GBHs in adult		**μg a.i. mL^{-1}**			
[70]	Scout® (Monsanto, Brazil)	0.065 to 10	7 d	Adult Wild-type	-Induced a reduction in cell viability (0.065 and 1 μg a.i. mL^{-1}). -Inhibition of complex I and IV activity was detected at 0.065 and 1 μg a.i. mL^{-1}. -Mitochondrial hyperpolarization was observed at >0.065 μg a.i. mL^{-1}.
[96]	Roundup formulation (Roundup Pro Scotts, USA, containing 41% glyphosate).	0.015 and 0.5	14 d	Adult males AB Wild-type (8–12 months)	-A significant increase in plasma LDH activity was observed.

Studies performed with GBH exposure showed that Roundup® after 96 h in larvae decreased hexokinase activity. This erythrocyte enzyme plays a role in the glycolytic pathway and catalyzes the initial step in glucose utilization. Therefore, it is required for both glycolysis and pentose derivation and produces glucose-6-phosphate (>11.7 μg a.i. mL^{-1}) [64]. Another GBH (Atanor 48: 6.6 and 66.6 μg a.i. mL^{-1}) showed the ability to decrease the mitochondrial potential of cells in larvae (96 h) [66]. In adults exposed to Scout® for 7 d (0.065 and 1 μg a.i. mL^{-1}), a reduction was observed in cell viability, inhibition of complex I and IV activity, and mitochondrial hyperpolarization at >0.065 μg a.i. mL^{-1} [70]. In adults exposed to Roundup 0.015 and 0.5 μg a.i. mL^{-1} for 14 d, a significant increase was observed in activity of plasma LDH, an enzyme that participates in the process of transforming glucose into energy [96].

2.11. Immunotoxicity Caused by Exposure to GLY or GBHs in Zebrafish

Relatively little is known about the effect of GLY and GBHs on the immune system of fish, but in other species it has been found that this herbicide can cause immunotoxicity [12]. A few studies have explored this issue in zebrafish, as shown in Table 11. Adults exposed to GLY for 14 and 21 d (3.5 μg mL^{-1}) showed alterations in intestinal levels of the enzyme diamine oxidase, which is related to histamine degradation. Increased levels of intestinal IL-1β and IL-8 (14; 21 d) and decreased levels of IL-10 and TGF-β (21 d) were observed, suggesting that inflammation occurred in the intestine. These are crucial anti-inflammatory cytokines that can inhibit the production of proinflammatory cytokines [103]. In adult zebrafish, GLY and Rodeo® caused alterations in immune system pathways. GLY enriched cytokine–cytokine receptor interaction in the trunk kidney, while Rodeo® enriched cell-adhesion molecules, extracellular matrix–receptor interaction, and focal adhesions [112]. In adults, exposure to GBH Roundup for 14 d had the ability to raise immunoglobulin (IgM) levels in the blood (0.5 μg mL^{-1}) [96].

Table 11. Immunotoxicity caused by exposure to GLY or GBHs in zebrafish.

Reference	Product	Exposure Concentrations	Exposure Period	Strain	Main Effects
GLY in Adult		**μg mL^{-1}**			
[103]	GLY 99.5% purity CAS#1071-83-6	3.5	7, 14 and 21 d	Adult AB-Wild-type (6 months)	-Diamine oxidase increased 7 d and reduced at 21 d. -Increased levels of intestinal IL-1β and IL-8 were observed (14; 21 d). -Decreased levels of IL-10 and TGF-β (21d) were observed.
[112]	GLY 95% purity CAS#1071-83-6	0.5 and 10	21 d	Adult	-Enriched cytokine–cytokine receptor interaction in the trunk kidney (10 μg mL^{-1}).
GBHs in adult		**μg a.i. mL^{-1}**			
[112]	Rodeo® (DOW-Agrosciences, USA)	0.5 and 10	21 d	Adult	-Enriched cell-adhesion molecules, extracellular matrix–receptor interaction, and focal adhesions (10 μg a.i. mL^{-1}).
[96]	Roundup formulation (Roundup Pro Scotts, USA, containing 41% glyphosate)	0.015 and 0.5	14 d	Adult males AB Wild-type (8–12 months)	-Elevation of IgM levels were observed (0.5 μg a.i. mL^{-1}).

3. Conclusions and Proposals for Future Research

Studies show that both GLY and GBHs can be considered toxic for the development of zebrafish. The mechanisms associated with this toxicity were explored through exposure experiments designed to discern morphological defects, developmental delays, behavioral responses, and molecular signatures. In the embryonic and larval stages, increased mortality, changes in hatching, and organic malformations were reported. In both embryo/larvae and adult zebrafish, an increase was observed in lethality, behavioral constraints, oxidative stress and apoptosis induction, mechanisms of genotoxicity, and endocrine and energy metabolism disruption. Additionally, in adult zebrafish, reproductive impairment and interference in the immune system with the induction of a pro-inflammatory state have been reported. These lethal and sub-lethal effects, which disrupt predation, feeding, locomotion, reproduction, and survival, can translate into the population decline of aquatic species. Thus, awareness of the potential effects of these herbicides on aquatic ecosystems must be promoted, so that measures can be taken to mitigate them.

However, there are disagreements regarding the effects triggered by exposure to GLY, particularly concerning its commercial formulations. Accordingly, it is crucial to assess the individual and combined toxicity of excipients in commercial GLY formulations, which are complex mixtures with limited safety information. In addition, the molecular mechanisms

underlying the effects of GBHs in the early stages of development remain elusive and further studies are needed. It is also necessary to examine the simultaneous use of different pesticides in agriculture, an important topic for ecotoxicological studies.

Overall, it was noted that most studies evaluate GLY and GBHs in concentrations higher than those typically found in aquatic systems. Thus, future research should prioritize environmentally relevant concentrations to enhance our understanding of the actual effects of GLY and GBHs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15122276/s1.

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