Single and Joint Acute Toxicity Effects of Glyphosate Isopropyl Amine Salt (GIS) and Atrazine (ATZ) on Juvenile Zebrafish (*Danio rerio*)

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Abstract The widespread use of herbicides such as glyphosate isopropyl amine salt (GIS) and atrazine (ATZ) poses significant risks to aquatic ecosystems. This study investigated the single and joint acute toxicity of a 1 ∶ 1 GIS-ATZ mixture on zebrafish (*Danio rerio*). Acute tests determined 96-h LC_{50} values of 123.41 mg/L for GIS and 103.95 mg/L for ATZ. In the joint toxicity test, these values decreased to 60.96 and 50.88 mg/L, respectively. The Additive Index (AI) analysis revealed a consistent synergistic interaction between the herbicides at all exposure intervals. These findings underscore the enhanced ecological threat of herbicide mixtures and highlight the necessity of considering joint effects in environmental risk assessments.

Key words Acute toxicity; Joint toxicity; Glyphosate isopropyl amine salt; Atrazine; Zebrafish (*Danio rerio*)

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Herbicides, a major class of pesticides with over 400 types used globally, are extensively applied in agriculture and urban environments to control unwanted vegetation, but their widespread use poses serious ecological risks[1]. These chemicals commonly enter aquatic ecosystems through agricultural runoff, leaching, spray drift, and wastewater discharge, where they persist and accumulate, leading to contamination of surface and groundwater resources[2–3]. Among them, GIS and ATZ are the most widely used broad-spectrum systemic herbicides, approved for use on hundreds of crops in over 190 countries[4]. Due to their persistence and mobility, they represent a significant threat to aquatic life, exhibiting both acute and sub lethal toxic effects, including impaired growth, reproduction, and behavioral alterations in fish species such as zebrafish (*Danio rerio*), a standard OECD and ISO test organism for Eco toxicological studies[5–8]. While individual toxicity studies on these compounds exist, limited attention has been given to their combined effects, which may produce synergistic interactions and increase ecological risks. Therefore, in this study, the joint toxicity of a 1 ∶ 1 binary mixture of glyphosate isopropyl amine salt and atrazine on zebrafish was investigated using the Additive Index (AI) approach, aiming to enhance understanding of herbicide interactions and support the formulation of effective water quality standards and pollution control strategies[9–11].

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Materials and Method

Test organism and acclimation

The present study used juvenile health zebrafish with an average weight of (0.5 +0.1) g, length 2.55 +0.3 cm and $n = 100$. The fish were acclimatized for 2 weeks in the laboratory and healthy fish were selected for the test. Feeding was stopped for 24 h before the experiment and a semi-static environment was maintained during the test. No dead fish were observed during the acclimation period.

Test water

Test water was de-chlorinated for 1 week and the temperature was maintained at (31.49 +0.10) ℃ DO 5.4 –5.58 mg/L, and pH 7.78 –7.93, respectively.

Preliminary test

The fish were exposed to test concentrations of 169.00, 151.00, 134.00, and 120.00 mg/L for glyphosate isopropyl amine salt and 119.02, 113.67, 108.55, and 103.67 mg/L for atrazine during the preliminary test. Zebrafish responses were observed for 24 and 48 h to determine the appropriate concentration range for the test.

Acute toxicity tests

After obtaining results from the pre-tests, actual concentrations were established for glyphosate isopropyl amine salt and atrazine. Seven doses with identical intervals determined logarithmically from the preliminary test range zebrafish were added to 5 L glass aquariums, with each concentration triplicated in a 2 L dichlorinated tap water under dynamic test conditions, such as aerated and refreshed daily, resulting in fresh, oxygen-rich water with fish species ($n = 10$) in each eight group including the control. After acclamation at 122.9, 124.61, 126.34, 128.1, 129.88, 131.69, 133.52 mg/L and 101.97, 104.35, 106.78, 109.26, 111.81, 114.41, 117.08 mg/L, respectively, observations for each concentration was done. Dead fish were quickly removed and assessed at 24, 48, 72, and 96 h. Each group was replicated 3 times.

Joint toxicity test

The single acute toxicity testing of the two herbicides provided the basis for determining the joint toxicity. The concentrations for glyphosate isopropyl amine salt and atrazine were 61.45, 62.31, 63.17, 64.05, 64.94, 65.85, 66.76 mg/L and 51.30, 52.01, 52.73, 53.47, 54.21, 54.97, 55.73 mg/L, respectively. Interactions between the chemicals and zebrafish were evaluated using the Additive Index (AI)[12]. The mortalities and behavioral changes of exposed zebrafish were well observed at the 24 h time interval for 96.

Results and Analysis

Single acute toxicity of glyphosate isopropyl amine salt and atrazine

The acute toxicity tests revealed that both GIS and ATZ exhibited strong, time- and dose-dependent toxic effects on zebrafish, with mortality rates increasing significantly with higher concentrations and longer exposure durations. The calculated LC_{50} values for glyphosate were 131.07, 126.27, and 123.41 mg/L at 24, 48, and 96 h, respectively, while those for atrazine were 115.48, 111.85, and 103.95 mg/L, indicating higher toxicity of atrazine. Statistical analysis showed a significant positive correlation ($P < 0.05$) between concentration and mortality across all exposure periods. Also, zebrafish exhibited loss of activity, spiral movement and loss of equilibrium after exposure to the GIS & ATZ. Detailed information is displayed in Table 1 and Table 2.

Joint toxicity effects of glyphosate isopropyl amine salt and atrazine to juvenile zebrafish (*Danio rerio*)

The joint toxicity of glyphosate isopropylamine salt (GIS) and atrazine (ATZ) on zebrafish demonstrated a clear concentration and time-dependent increase in mortality. Mortality escalated with higher concentrations and longer exposure, reaching 100% at the highest concentrations by 96 h. Acute toxicity analysis revealed the 96-h LC_{50} values for the herbicides in the mixture were 60.96 mg/L for GIS and 50.88 mg/L for ATZ. These values are substantially lower than their individual LC_{50s}. The interaction was quantitatively assessed using the Additive Index (AI), which yielded positive values (0.026 at 24 h, 0.004 at 48 h, 0.017 at 96 h). Furthermore, the biological activity (S) values were consistently close to 1 across all time intervals. Based on these AI and S values, the joint effect of GIS and ATZ was concluded to be synergistic. In addition, zebrafish exhibited loss of activity, spiral movement and loss of equilibrium after exposure to the mixture of GIS & ATZ. The exhaustive information for joint toxicity effects of GIS and ATZ on zebrafish is shown in Table 3 and Table 4.

Table 1 Acute toxicity of glyphosate isopropyl amine salt to juvenile zebrafish (*Danio rerio*)

Concentrations // mg/L	Death rate // %		
	24 h	48 h	96 h
Control	0	0	0
	17	30	50
124.61	23	43	63
126.34	24	53	75
128.10	27	57	83
129.88	30	67	98
131.69	50	77	100
133.52	77	87	100
Regression equation	$Y = 40.125x - 79.965$	$Y = 42.563x - 84.437$	$Y = 78.695x - 159.58$
Correlation coefficient (R)	0.893 532 *	0.990 707 *	0.951 104 *
LC_{50}	131.071 35	126.265 6	123.414 3
95% Confidence limit	128.600 7 – 133.584 7	124.022 7 – 128.549	21.997 8 – 124.847

Table 2 Acute toxicity of atrazine to juvenile zebrafish (*Danio rerio*)

Concentrations // mg/L	Death rate // %			
	24 h	48 h	72 h	96 h
Control	0	0	0	0
101.97	7	10	30	37
104.35	20	30	40	53
106.78	23	40	53	63
109.26	27	47	64	89
111.81	30	53	67	100
114.41	49	57	80	100
117.08	56	60	87	100
Regression equation	$y = 24.112x - 44.731$	$y = 22.576x - 41.25$	$y = 25.826x - 47.322$	$y = 49.306x - 94.441$
Correlation coefficient	0.956 *	0.914 *	0.992 *	0.964 *
LC_{50}	115.478	111.850	106.156	103.947
95% Confidence limit	111.882 – 119.190	243.121 – 207.431	103.066 – 109.338	101.841 – 106.097

Table 3 Death rate of juvenile zebrafish under joint effects of glyphosate isopropyl amine salt and atrazine

Glyphosate isopropyl amine salt mg/L	Death rate // %			
	Atrazine // mg/L	24 h	48 h	96 h
Control	0	0	0	0
61.45	51.30	13	14	59
62.305	52.01	22	25	77
63.17	52.73	24	27	85
64.05	53.47	31	33	94
64.94	54.21	40	43	100
65.845	54.97	52	54	100
66.76	55.73	66	69	100

Table 4 Joint toxicity effects of glyphosate isopropyl amine salt and atrazine to juvenile zebrafish

Pesticide	Parameter	24 h	48 h	96 h
Glyphosate isopropyl amine salt	Regression equation	$y = 39.826x - 67.362$	$y = 39.945x - 67.501$	$y = 71.528x - 122.68$
	Correlation coefficient	0.987 2 *	0.983 0 *	0.995 0 *
	LC_{50}	65.61	65.32	60.96
	95% Confidence limit	64.36 - 66.88	64.081 - 66.58	60.10 - 61.82
Atrazine	Regression equation	$y = 39.818x - 64.225$	$y = 39.934x - 64.35$	$y = 71.57x - 117.14$
	Correlation coefficient	0.987 0 *	0.983 0 *	0.995 1 *
	LC_{50}	54.77	53.53	50.88
	95% Confidence limit	53.73 - 55.83	53.50 - 55.58	50.17 - 51.61
Joint Effect	S	0.974 853	0.995 892	0.983 429
	AI	0.025 796	0.004 108	0.016 85
	Conclusion	Synergistic	Synergistic	Synergistic

Discussion

This study quantified significant, dose-dependent acute toxicity for glyphosate isopropylamine salt (GIS; 96-h LC_{50} = 123.41 mg/L) and atrazine (ATZ; 96-h LC_{50} = 103.95 mg/L) in zebrafish, establishing ATZ as the more potent toxicant. Exposure to both herbicides induced marked physiological and behavioral alterations, including reduced swimming activity, loss of equilibrium, and spiral movements, indicative of severe neurotoxicity. The pivotal finding, however, is the unequivocal synergistic interaction in their 1 : 1 mixture. The 96-h LC_{50} values were nearly halved (GIS: 60.96 mg/L; ATZ: 50.88 mg/L), and consistent positive Additive Index (AI) values confirmed this synergism[16]. This suggests the co-exposure exacerbates the neurotoxic effects, potentially through toxicokinetic interactions that increase internal concentrations[5,17] and/or toxicodynamic interactions at shared neurological targets such as sodium channels and GABA receptors[18]. The observed behavioral deficits strongly support this enhanced toxicodynamic disruption. These results demonstrate that environmental co-contamination poses a greater-than-additive risk, underscoring the imperative to incorporate mixture toxicity into regulatory Eco toxicological risk assessments[10-11, 19].

Conclusions

Exposure to agrochemicals such as glyphosate isopropyl amine salt and atrazine presents serious ecological risks, heightening the vulnerability of aquatic organisms and threatening ecosystem stability. Ensuring the protection of non-target species requires comprehensive, evidence-based risk assessments and consistent ecological monitoring.

In pursuit of sustainable development, chemical formulation and application should prioritize reduced environmental persistence and minimized health hazards. Addressing the challenges of combined chemical toxicity demands collaborative efforts among manufacturers, regulators, and environmental scientists. Through stricter pre-market testing, innovation of biodegradable alternatives, and the adoption of integrated chemical management frameworks, the cumulative toxic effects of agrochemicals can be mitigated. Such coordinated actions are critical to preserving the integrity and resilience of aquatic ecosystems.

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lodging during the initial pod-setting stage. For fields where plant height exceeds 35 cm and shows a tendency toward vigorous growth, chemical growth regulation is implemented. This involves spraying 1 050-1 500 g/hm^2 of 5% uniconazole WP mixed with 750 kg/hm^2 of water[12], while avoiding over-application, missed spots, or rainfall shortly after spraying. To reduce costs, growth regulation can be integrated with disease and pest control measures.

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