Joint Toxicity of Fluxapyroxad and Pyraclostrobin on Zebrafish and Their Mitochondrial Targeting Mechanisms

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Abstract [**Objectives**] This study was conducted to investigate the joint toxicity of fungicides on aquatic ecosystems. [**Methods**] Using zebrafish as a model organism, an LC-MS/MS simultaneous detection method was established for fluxapyroxad and pyraclostrobin (with detection limits at ng/L level), and their acute toxicity, joint toxicity and toxic mechanisms were systematically evaluated. [**Results**] The toxicity of pyraclostrobin (96 h-LC_{50} = 0.052 mg/L) to zebrafish was approximately 25.8 times higher than that of fluxapyroxad (96 h-LC_{50} = 1.34 mg/L). Joint toxicity evaluation using the fixed-ratio ray design revealed that six of the seven mixture ratios exhibited additive effects (AI = 0.62 - 1.47), while the 8 ∶ 1 ratio showed antagonism (AI = 2.14). The analysis of toxicity mechanisms indicated that both fungicides induced oxidative stress, lipid peroxidation, and cellular damage through inhibition of mitochondrial complex III and II, respectively, with pyraclostrobin inducing more pronounced hepatic MDA elevation (2.56-fold) and antioxidant enzyme inhibition. Ecological risk assessment demonstrated that fluxapyroxad posed moderate risk (RQ = 0.16 - 0.90), while pyraclostrobin posed moderate to high risk (RQ = 0.56 - 3.56), and crustaceans faced the highest risk. [**Conclusions**] This study elucidated the mechanism underlying toxicity differences due to distinct mitochondrial targets, providing a scientific basis for fungicide management.

Key words Fluxapyroxad; Pyraclostrobin; Zebrafish; Joint toxicity; Mitochondrion; Oxidative stress

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Fluxapyroxad and pyraclostrobin are widely used fungicides that belong to mitochondrial respiratory chain inhibitors. They target complex II (succinate dehydrogenase) and complex III (cytochrome bc1 complex), respectively[1]. Due to their complementary mechanisms of action and significant efficacy, they are often formulated together to delay the development of resistance, leading to their frequent co-detection in aquatic environments. Current studies indicate that pyraclostrobin exhibits strong acute toxicity to aquatic organisms (with 96 h-LC_{50} values for fish typically at the μg/L level)[2], while fluxapyroxad is relatively less toxic. However, the toxic effects of their combined exposure and the relationship with the differences in mitochondrial targets remain unclear. Traditional mixture toxicity assessment models, mostly based on concentration addition or independent action assumptions, overlook biochemical interactions rooted in shared target sites[3]. These models struggle to explain the complex combined effects of fungicide mixtures in which fungicides act on the mitochondrial respiratory chain but target different complexes. In this study, zebrafish was used as a model organism to establish an LC-MS/MS synchronous detection method. The fixed-ratio ray design was adopted to systematically evaluate the joint toxicity under different mixture ratios, and multidimensional biochemical indicators, including oxidative stress, liver function, and neurotoxicity, were analyzed to reveal the differential toxicity mechanisms of the two fungicides based on mitochondrial complex specificity. The study provides a scientific basis for the differential regulation of mitochondrial inhibitor fungicides and the protection of aquatic ecosystems.

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Materials and Methods

Reagents and materials

Fluxapyroxad standard (CAS: 907204-31-3, purity 98.5%) and pyraclostrobin standard (CAS: 175013-18-0, purity 99.2%) were purchased from Dr. Ehrenstorfer GmbH (Germany). Methanol and acetonitrile (HPLC grade) were purchased from Merck KGaA (Germany). Mass spectrometry-grade formic acid (purity ≥98%) was obtained from Sigma - Aldrich (USA). Analytically-pure dimethyl sulfoxide (DMSO, purity≥99.5%) was used as the carrier solvent, with its final concentration in test solutions controlled below 0.01%. Experimental water was prepared using a Milli-Q ultrapure water system (resistivity≥18.2 MΩ · cm).

The test water was dechlorinated tap water subjected to activated carbon filtration and UV sterilization, followed by standing and aeration for over 24 h. Water quality parameters were as follows: pH 7.0 - 7.8, dissolved oxygen≥6.0 mg/L, total hardness 50 - 100 mg/L (as $CaCO_3$), conductivity 400 - 600 μS/cm, ammonia nitrogen < 0.1 mg/L, and nitrite nitrogen < 0.02 mg/L.

Experimental animals

Wild-type zebrafish were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences, with an equal ratio of males and females. The fish had a body length of (3.0 ± 0.5) cm and a body weight of (0.35 ± 0.08) g, and were 3 - 4 months

old. The test fish were acclimated under laboratory conditions for more than two weeks, with a water temperature of (26 ± 1) ℃, a photoperiod of 14 h light/10 h darkness, and a light intensity of 300 – 500 lux. During the acclimation period, the fish were fed commercial zebrafish feed twice daily, and the mortality rate was <5%. Prior to the experiment, the fish were fasted for 24 h, and healthy individuals exhibiting active swimming, no physical damage and uniform size were selected for the toxicity tests.

Pesticide residue analysis method

Pesticide concentrations were determined by ultra-high performance liquid chromatography-triple quadrupole tandem mass spectrometry (ACQUITY UPLC I-Class/Xevo TQ-S micro, Waters Corporation). Water samples were pretreated using liquid-liquid extraction. In specific, 1.0 ml of water sample was precisely transferred, 1.0 ml of acetonitrile was added for extraction. The sample was vortexed for 3 min, and centrifuged at 4 ℃ and 12 000 rpm for 15 min, and 0.8 ml of the supernatant was taken and diluted to 1.5 ml with ultrapure water. The solution was filtered through a 0.22 μm PTFE membrane before analysis.

Chromatographic separation was performed using an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with a column temperature of 35 ℃ and an injection volume of 5 μl. The mobile phase A used was an aqueous solution containing 0.1% formic acid and 5 mmol/L ammonium acetate, while mobile phase B was acetonitrile. The gradient elution program was as follows: 0 – 0.5 min, 5% B, 0.5 – 3.0 min, 5% – 95% B, 3.0 – 5.0 min, 95% B, 5.0 – 5.1 min, 95% – 5% B, and 5.1 – 7.0 min, 5% B. The flow rate was set at 0.4 ml/min.

MS detection was carried out using an electrospray ionization (ESI) source in positive ion mode with multiple reaction monitoring (MRM). Fluxapyroxad was detected at a retention time of 3.85 min, using the transition from precursor ion m/z 398.1 to the product ion m/z 342.1 for quantification and m/z 262.1 for qualification. Pyraclostrobin was detected at a retention time of 4.12 min, using the transition from precursor ion m/z 388.1 to the product ion m/z 194.0 for quantification and 163.0 for qualification. The detection limits of the method were 0.003 μg/L for fluxapyroxad and 0.002 μg/L for pyraclostrobin.

Acute toxicity test

The acute toxicity test for individual pesticides was conducted according to the OECD Test Guideline 203, with a test duration of 96 h. Based on preliminary test results, fluxapyroxad was tested at six concentration gradients: 0.3, 0.6, 1.0, 1.5, 2.0, and 2.5 mg/L. Pyraclostrobin was tested at six concentration gradients: 0.015, 0.030, 0.050, 0.070, 0.090, and 0.120 mg/L. A blank control group and a carrier control group (containing 0.01% DMSO) were also included, and four replicates were set for each treatment group.

The exposure test was conducted using a semi-static method in 2 L borosilicate glass beakers, with 10 test fish placed in each container and a test solution volume of 1.5 L. The test solution was renewed every 24 h, during which the number of deaths was recorded and dead fish were removed. During the test period, survival status and behavioral performance were observed every 8 h, and symptoms of poisoning such as swimming behavior, respiratory frequency, body color changes, and imbalance were recorded. The criterion for death was defined as the loss of swimming ability and no response to external stimuli.

Joint toxicity assessment

The joint toxicity test was designed using the fixed-ratio ray method, with seven mixture ratios of fluxapyroxad to pyraclostrobin set at 1∶1, 1∶2, 1∶4, 2∶1, 4∶1, 8∶1, and 1∶8. For each ratio, 5 – 6 concentration gradients were established. The experimental methodology was consistent with that of the acute toxicity test of single pesticide.

The joint effect was evaluated using the additive index method: $AI = EC_{50_m}/(EC_{50_\alpha}/EC_{50_{m\alpha}} + EC_{50_\beta}/EC_{50_{m\beta}})$, where EC_{50_m} is the median effect concentration of the mixture, EC_{50_α} and EC_{50_β} are the EC_{50} values of individual compounds A and B, respectively, and $EC_{50_{m\alpha}}$ and $EC_{50_{m\beta}}$ are the concentrations of compounds A and B in the mixture, respectively. The joint effect is classified as synergism with $AI < 0.5$, antagonism with $AI > 1.5$, and additive with $0.5 \leqslant AI \leqslant 1.5$.

Sublethal toxicity test and biochemical indicator determination

Based on the acute toxicity test results, 1/2, 1/5, and 1/10 of the 96 h-LC_{50} values were selected as sublethal exposure concentrations. The exposure concentrations for fluxapyroxad were 0.67, 0.268, and 0.134 mg/L, while those for pyraclostrobin were 0.026, 0.010 4, and 0.005 2 mg/L. Meanwhile, a blank control group and a carrier solvent control group were established. Four replicates were set for each treatment group, and 15 test fish were put in each container. A semi-static exposure method was implemented for 21 d, with the test solution renewed every 24 h. Sample collections were conducted at 7, 14, and 21 d of exposure, respectively.

During sample collection, eight fish were randomly selected from each treatment group and anesthetized with MS-222 (100 mg/L) before rapid dissection. Liver and gill tissues were used for oxidative stress indicator measurements, while brain tissue was used for acetylcholinesterase activity determination. Tissue samples were quickly frozen in liquid nitrogen and stored at −80 ℃.

Tissue homogenate preparation: Precisely a 50 mg sample of tissue was weighed, and 450 μl of pre-cooled phosphate buffer (0.1 mol/L, pH 7.4) was added. Homogenization was performed under an ice bath condition for 2 min, followed by centrifugation at 4 ℃ and 3 000 rpm for 15 min. The supernatant was collected for enzyme activity assays. Protein concentration was determined using the Bradford method.

Biochemical indicator assays: Superoxide dismutase (SOD) activity was measured using the WST-1 method. Catalase (CAT) activity was determined based on the absorbance change of H_2O_2 at 240 nm. Glutathione peroxidase (GSH-Px) activity was assessed

using the DTNB colorimetric method. Malondialdehyde (MDA) content was quantified by the thiobarbituric acid method. Liver function indicators included alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Acetylcholinesterase (AChE) activity was determined using the Ellman colorimetric method.

Ecological risk assessment

A deterministic risk assessment method based on the Risk Quotient (RQ) was adopted: RQ = PEC/PNEC, where PEC represents the predicted environmental concentration, and PNEC denotes the predicted no-effect concentration. Environmental exposure concentration data were sourced from measured values reported in the literature and predictions generated by the TOP-rice model. The PNEC value was derived from chronic toxicity data of the most sensitive species, and calculated using an assessment factor (AF) of 10. Risk levels were categorized as follows: low risk (RQ < 0.1), moderate risk (0.1 ≤ RQ < 1), and high risk (RQ ≥ 1).

Data analysis

Probit analysis was performed on acute toxicity data using the EPA Probit Analysis Program 1.5 software to calculate the median lethal concentration (LC_{50}) and its 95% confidence interval. Continuous data were expressed as mean ± standard deviation ($\bar{x} \pm SD$). Comparisons among multiple groups were performed using one-way analysis of variance (One-way ANOVA). When the homogeneity of variance assumption was met, the LSD multiple comparison test was conducted. All statistical analyses were conducted using SPSS 26.0 software, with the significance level set at $\alpha = 0.05$.

Results and Analysis

Validation of the pesticide residue analysis method

The established LC-MS/MS method demonstrated excellent linearity within the range of 0.01 – 10.0 μg/L ($r^2 > 0.998$). The limits of detection (LOD) for fluxapyroxad and pyraclostrobin were 0.003 and 0.002 μg/L, respectively, achieving detection sensitivity at the ng/L level. Precision validation showed that the repeatability RSD ranged from 2.5% to 4.2%, and the intermediate precision RSD ranged from 4.8% to 6.1% (Table 1). The recovery rates were in the range of 88.3% – 96.8% with *RSD* < 5%, meeting the requirements for residue analysis. The average retention rates of the two pesticides in water over 96 h were 90.16% and 89.37%, respectively, and the concentrations showed a significant decreasing trend over time ($P < 0.001$).

Table 1 Summary of verification results of analysis methods

Compound	Linearity range//μg/L	r^2	LOD//μg/L	LOQ//μg/L	Repeatability *RSD*//%	Intermediate precision *RSD*//%	Recovery//%
Fluxapyroxad	0.01 – 10.0	0.998 7	0.003	0.010	2.8 – 4.2	5.3 – 6.1	88.3 – 94.7
Pyraclostrobi	0.01 – 10.0	0.999 2	0.002	0.008	2.5 – 3.9	4.8 – 5.7	91.2 – 96.8

Acute toxicity of individual pesticides

The 96 h-LC_{50} for fluxapyroxad was 1.34 mg/L (95% CI: 1.13 – 1.59 mg/L), while that for pyraclostrobin was 0.052 mg/L (95% CI: 0.044 – 0.061 mg/L). The toxicity of pyraclostrobin was approximately 25.8 times that of fluxapyroxad (Table 2). The dose-response relationships for both pesticides were well-fitted ($r^2 > 0.982$, $P > 0.05$). Analysis of median survival time showed that the LT_{50} for pyraclostrobin (32.4 h) was significantly shorter than that for fluxapyroxad (48.6 h). Behavioral observation revealed that both pesticides induced neurotoxic symptoms, but the symptoms appeared earlier with pyraclostrobin (6 – 22 h vs. 12 – 36 h) and were accompanied by significant convulsions and respiratory distress.

Table 2 Acute toxicity parameters of fluxapyroxad and pyraclostrobin to zebrafish

Compound	Duration of exposure//h	LC_{50}//mg/L	95% confidence interval//mg/L	Slope	r^2	*P* value
Fluxapyroxad	24	2.180	1.840 – 2.580	1.64	0.982	0.147
	48	1.760	1.490 – 2.080	1.78	0.987	0.194
	72	1.520	1.280 – 1.800	1.92	0.990	0.213
	96	1.340	1.130 – 1.590	2.03	0.993	0.226
Pyraclostrobin	24	0.078	0.066 – 0.092	2.14	0.985	0.138
	48	0.065	0.055 – 0.077	2.28	0.991	0.156
	72	0.058	0.049 – 0.068	2.35	0.994	0.168
	96	0.052	0.044 – 0.061	2.41	0.996	0.179

Joint toxicity effect

Evaluation of joint toxicity at seven mixture ratios showed that six of them had additive index (AI) values ranging from 0.62 to 1.47, indicating additive effects (Table 3). The mixture ratio of 8 ∶ 1 had an AI value of 2.14 (>1.5), demonstrating significant antagonism. The mixture ratio of 8 ∶ 1 exhibited an AI value of 2.14 (>1.5), indicating significant antagonism. The LC_{50} values differed significantly among the different mixture ratios ($F = 156.82$, $P < 0.001$), with the 1 ∶ 8 ratio showing the highest toxicity ($LC_{50} = 0.058$ mg/L) and the 8 ∶ 1 ratio the lowest ($LC_{50} = 0.624$ mg/L). Analysis of median survival time revealed that the 1 ∶ 8 ratio had the shortest LT_{50} (28.7 h), while the 8 ∶ 1 ratio had the longest (52.3 h). The Kaplan – Meier survival curve showed significant differences (Log-rank $\chi^2 = 68.42$, $P < 0.001$).

Table 3 Joint toxicity evaluation of fluxapyroxad and pyraclostrobin

Mixture ratio *	LC_{50} // mg/L	95% confidence interval // mg/L	Slope	r^2	Additive index (AI)	Toxic unit (TU)	Joint effect
1 ∶ 01	0.142	0.124 – 0.163	2.18	0.992	0.92	1.08	Additive
1 ∶ 02	0.089	0.077 – 0.103	2.31	0.994	0.84	1.04	Additive
1 ∶ 04	0.067	0.058 – 0.077	2.26	0.993	0.78	0.96	Additive
2 ∶ 01	0.234	0.204 – 0.268	2.08	0.99	1.13	1.19	Additive
4 ∶ 01	0.397	0.346 – 0.456	1.95	0.988	1.47	1.35	Additive
8 ∶ 01	0.624	0.544 – 0.716	1.87	0.991	2.14	0.67	Antagonistic
1 ∶ 08	0.058	0.051 – 0.066	2.44	0.995	0.62	1.43	Additive

The mixture ratio represents the molar ratio of fluxapyroxad to pyraclostrobin.

Lipid peroxidation and oxidative stress

Exposure to both pesticides significantly induced an increase in MDA content, showing dose-effect and time-effect relationships (Table 4). After 21 d of exposure, the hepatic MDA content in the high-concentration groups of fluxapyroxad and pyraclostrobin was 2.21 and 2.56 times that of the control group, respectively ($P<0.01$), while in gill tissue, it was 2.28 and 2.63 times, respectively. Two-way ANOVA indicated that both pesticide concentration ($F=98.47$, $P<0.001$) and exposure time ($F=76.23$, $P<0.001$) had highly significant effects on MDA content, with a significant interaction between the two factors ($F=12.84$, $P<0.001$). Pyraclostrobin exhibited a slightly stronger ability to induce lipid peroxidation than fluxapyroxad ($P<0.05$).

Table 4 Effects of fluxapyroxad and pyraclostrobin exposure on MDA content in zebrafish tissues

Compound	Concentration mg/L	Hepatic MDA content // nmol/mg prot			Gill MDA content // nmol/mg prot		
		7 d	14 d	21 d	7 d	14 d	21 d
Control group	0	2.43 ± 0.32	2.51 ± 0.29	2.47 ± 0.31	1.86 ± 0.25	1.89 ± 0.26	1.88 ± 0.24
Fluxapyroxad	0.134	2.67 ± 0.38	2.89 ± 0.42 *	3.24 ± 0.48 *	2.08 ± 0.31	2.32 ± 0.34 *	2.58 ± 0.39 *
	0.268	2.91 ± 0.43 *	3.45 ± 0.52 * *	4.12 ± 0.61 * *	2.34 ± 0.37 *	2.78 ± 0.43 * *	3.34 ± 0.51 * *
	0.67	3.42 ± 0.54 * *	4.28 ± 0.67 * *	5.47 ± 0.82 * *	2.71 ± 0.42 * *	3.45 ± 0.58 * *	4.29 ± 0.67 * *
Pyraclostrobin	0.005 2	2.74 ± 0.41	3.18 ± 0.48 *	3.68 ± 0.56 *	2.15 ± 0.33	2.47 ± 0.38 *	2.84 ± 0.44 *
	0.010 4	3.16 ± 0.52 *	3.89 ± 0.64 * *	4.76 ± 0.73 * *	2.53 ± 0.41 *	3.12 ± 0.49 * *	3.78 ± 0.59 * *
	0.026	3.87 ± 0.68 * *	4.94 ± 0.81 * *	6.32 ± 0.97 * *	2.98 ± 0.51 * *	3.95 ± 0.67 * *	4.94 ± 0.78 * *

Data are expressed as mean ± standard deviation ($n=8$); * $P<0.05$, * * $P<0.01$, compared with the control group.

Response of the antioxidant enzyme system

The activities of SOD, CAT, and GSH-Px all exhibited a biphasic response pattern of "increasing first and then decreasing" (Table 5). At 7 d of exposure, SOD activity in the high-concentration groups increased by 27.9% – 31.5% ($P<0.01$). After 21 d of exposure, it decreased by 20.8% – 24.4% ($P<0.01$). CAT and GSH-Px activities showed similar trends, decreasing by 23.3% – 31.3% and 32.0% – 38.1%, respectively, at 21 d ($P<0.01$). ANOVA for repeated measurement indicated that the time factor had a highly significant effect on the activities of all three antioxidant enzymes ($P<0.001$), and there was a significant interaction between time and concentration ($P<0.01$). Pyraclostrobin exhibited a slightly stronger inhibitory effect on the antioxidant enzyme system than fluxapyroxad.

Table 5 Effects of fluxapyroxad and pyraclostrobin exposure on hepatic antioxidant enzyme activities in zebrafish

Compound	Concentration mg/L	SOD activity // U/mg prot			CAT activity // μmol/(min · mg prot)			GSH-Px activity // μmol/(min · mg prot)		
		7 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
Control group	0	44.82 ± 5.73	44.56 ± 5.29	45.14 ± 6.02	15.38 ± 2.09	15.72 ± 1.98	15.54 ± 2.18	8.69 ± 1.14	8.87 ± 1.18	8.76 ± 1.12
Fluxapyroxad	0.67	57.34 ± 7.62 * *	46.28 ± 6.18	35.74 ± 4.87 * *	20.84 ± 2.89 * *	17.94 ± 2.46 *	11.92 ± 1.63 * *	11.18 ± 1.54 * *	6.82 ± 0.94 * *	5.96 ± 0.81 * *
Pyraclostrobin	0.026	58.92 ± 7.89 * *	47.83 ± 6.35	34.12 ± 4.53 * *	21.47 ± 3.02 * *	18.56 ± 2.54 *	10.67 ± 1.48 * *	11.73 ± 1.69 * *	6.59 ± 0.89 * *	5.42 ± 0.74 * *

Data are expressed as mean ± standard deviation ($n=8$); * $P<0.05$, * * $P<0.01$, compared with the control group.

Liver function impairment

Exposure to both pesticides significantly increased ALT and AST activities (Table 6). After 21 d of exposure, ALT activity in the high-concentration groups of fluxapyroxad and pyraclostrobin increased by 91.5% and 112.5%, respectively ($P<0.01$), while AST activity increased by 80.0% and 97.3%, respectively ($P<0.01$). The AST/ALT ratio decreased with prolonged exposure time, indicating aggravated hepatocyte damage. Correlation analysisshowed that ALT and AST activities were significantly positively correlated with MDA content ($r=0.742$ and 0.718, respectively; $P<0.001$). Liver function impairment caused by pyraclostrobin was slightly more severe than that caused by fluxapyroxad ($P<0.05$).

Table 6 Effects of fluxapyroxad and pyraclostrobin exposure on liver function enzyme activities in zebrafish

Compound	Concentration mg/L	ALT activity // μmol/(min · mg prot)			AST activity // μmol/(min · mg prot)		
		7 d	14 d	21 d	7 d	14 d	21 d
Control group	0	2.79 ± 0.41	2.87 ± 0.39	2.81 ± 0.38	5.18 ± 0.69	5.34 ± 0.61	5.26 ± 0.68
Fluxapyroxad	0.134	3.06 ± 0.47	3.24 ± 0.48	3.47 ± 0.54 *	5.67 ± 0.81	6.14 ± 0.87 *	6.73 ± 0.96 *
	0.268	3.42 ± 0.53 *	3.78 ± 0.59 * *	4.26 ± 0.67 * *	6.24 ± 0.94 *	6.89 ± 1.04 * *	7.84 ± 1.18 * *
	0.67	3.91 ± 0.62 * *	4.47 ± 0.71 * *	5.38 ± 0.84 * *	7.03 ± 1.12 * *	8.12 ± 1.26 * *	9.47 ± 1.49 * *
Pyraclostrobin	0.005 2	3.18 ± 0.49	3.41 ± 0.52 *	3.79 ± 0.58 * *	5.84 ± 0.84	6.37 ± 0.91 *	7.12 ± 1.02 *
	0.010 4	3.57 ± 0.56 *	4.02 ± 0.63 * *	4.74 ± 0.74 * *	6.48 ± 0.98 *	7.34 ± 1.13 * *	8.56 ± 1.32 * *
	0.026	4.13 ± 0.67 * *	4.89 ± 0.79 * *	5.97 ± 0.94 * *	7.29 ± 1.17 * *	8.74 ± 1.39 * *	10.38 ± 1.64 * *

Data are expressed as mean ± standard deviation (n = 8); * P < 0.05, * * P < 0.01, compared with the control group.

Neurotoxic effects

Both pesticides inhibited AChE activity, though the degree of inhibition was relatively mild (Table 7). After 21 d of exposure, the inhibition rates of AChE in the high-concentration groups of fluxapyroxad and pyraclostrobin were 20.2% and 23.4%, respectively (P < 0.01). AChE activity showed significant negative correlations with both exposure concentration (r = −0.673, P < 0.001) and exposure time (r = −0.548, P < 0.001). Compared with classical cholinesterase inhibitors, the AChE inhibitory effects of both fungicides were relatively weak (maximum inhibitory rate < 25%), which is consistent with their non-cholinesterase targeting mechanisms. AChE activity showed a significant negative correlation with MDA content (r = −0.586, P < 0.001).

Table 7 Effects of fluxapyroxad and pyraclostrobin exposure on AChE activity in brain tissue of zebrafish

Compound	Concentration mg/L	AChE activity // μmol/(min · mg prot)			Inhibitory rate // %		
		7 d	14 d	21 d	7 d	14 d	21 d
Control group	0	24.67 ± 3.18	24.84 ± 3.12	24.73 ± 3.21	–	–	–
Fluxapyroxad	0.134	24.12 ± 3.02	23.78 ± 2.94	23.21 ± 2.87	2.2	4.3	6.1
	0.268	23.45 ± 2.84	22.67 ± 2.73 *	21.84 ± 2.64 *	4.9	8.7	11.7
	0.67	22.34 ± 2.61 *	21.12 ± 2.46 * *	19.73 ± 2.34 * *	9.4	15.0	20.2
Pyraclostrobin	0.005 2	23.89 ± 2.97	23.52 ± 2.87	22.94 ± 2.79	3.2	5.3	7.2
	0.010 4	23.18 ± 2.82	22.31 ± 2.67 *	21.46 ± 2.51 *	6.0	10.2	13.2
	0.026	21.87 ± 2.58 *	20.64 ± 2.41 * *	18.94 ± 2.21 * *	11.3	16.9	23.4

Data are expressed as mean ± standard deviation (n = 8); * P < 0.05, * * P < 0.01, compared with the control group.

Ecological risk assessment

Based on aquatic toxicity data, the geometric means of acute toxicity of fluxapyroxad for fish, crustaceans, and algae were 1.34, 0.96, and 2.78 mg/L, respectively, while those of pyraclostrobin were 0.052, 0.038, and 0.142 mg/L, respectively (Table 8). Crustaceans showed the highest sensitivity to both fungicides. Based on chronic toxicity data for crustaceans, the PNEC values for fluxapyroxad and pyraclostrobin were 4.10 and 0.50 μg/L, respectively. Risk quotient assessment (Table 9) indicated that fluxapyroxad posed a moderate risk (RQ = 0.16 − 0.90), while pyraclostrobin posed moderate to high risks (RQ = 0.56 − 3.56). In intensively used areas, the ecological risk of pyraclostrobin was significantly higher than that of fluxapyroxad. Species-specific assessments revealed that crustaceans faced the highest risk.

Table 8 Summary of toxicity data of fluxapyroxad and pyraclostrobin to aquatic organisms

Compound	Biological group	Geometric mean of acute toxicity // mg/L	Geometric mean of chronic toxicity // mg/L	Data size (n)
Fluxapyroxad	Fish	1.340	0.058	15
	Crustaceans	0.960	0.041	12
	Algae	2.780	0.089	8
Pyraclostrobin	Fish	0.052	0.014	15
	Crustaceans	0.038	0.009	12
	Algae	0.142	0.029	8

Table 9 Ecological risk assessment results under different exposure scenarios

Compound	Exposure scenario	PEC // μg/L	PNEC // μg/L	RQ	Risk level
Fluxapyroxad	Typical use	0.65 − 1.85	4.1	0.16 − 0.45	Moderate risk
	Intensive use	1.75 − 3.70	4.1	0.43 − 0.90	Moderate risk
	Extreme exposure	6.80 − 14.80	4.1	1.66 − 3.61	High risk
Pyraclostrobin	Typical use	0.28 − 0.89	0.5	0.56 − 1.78	Moderate-high risk
	Intensive use	0.84 − 1.78	0.5	1.68 − 3.56	High risk
	Extreme exposure	2.80 − 5.60	0.5	5.60 − 11.20	High risk

Discussion

The results of this study showed that the toxicity of pyraclostrobin to zebrafish was approximately 25.8 times that of fluxapyroxad. Such a significant difference is closely related to their specific inhibition on different complexes in the mitochondrial respiratory chain. Fluxapyroxad targets complex II (succinate dehydrogenase), while pyraclostrobin targets complex III (cytochrome bc1 complex). The critical role of complex III in proton pumping and electron transport means that its inhibition leads to more extensive downstream effects. In contrast, complex II inhibitors such as fluxapyroxad, while also affecting mitochondrial function, exhibit relatively milder toxic effects[4-5]. Zhang *et al.* [6] reported a 96 h-LC_{50} of 56 μg/L for pyraclostrobin, which is highly consistent with the results of this study (52 μg/L), while the toxicity value for fluxapyroxad was significantly higher. This toxicity classification based on differences in mitochondrial targets provides a molecular mechanism foundation for the differential risk assessment of fungicides.

In this study, the joint toxicity at seven mixture ratios was systematically evaluated using the fixed-ratio ray design, and it was found that most ratios (6/7) exhibited additive effects (AI = 0.62 – 1.47), with only the high fluxapyroxad ratio (8∶1) showing antagonism (AI = 2.14). This result aligns with the mixture toxicity review by Cedergreen[7], which indicates that compounds acting on different mitochondrial sites typically exhibit additive or slightly antagonistic effects rather than synergism. Kumar *et al.* [8] also observed a similar pattern of additive effects in their study on the joint toxicity of azoxystrobin and pyraclostrobin. Furthermore, the antagonism observed at the 8∶1 mixture ratio may be related to the positions of complex II and complex III inhibitors in the electron transport chain. When the concentration of the complex II inhibitor is significantly higher than that of the complex III inhibitor, electrons may partially bypass the inhibition through alternative pathways via complex I, thereby mitigating the toxic effects of complex III inhibition[9]. Yang *et al.* [10] compared the mitochondrial toxicity of three streptomycin fungicides and found that the toxic effects of mixed exposures at different ratios exhibited concentration-dependent and ratio-dependent characteristics. The results of this study indicate that in real-world environments where both fungicides coexist, their joint toxicity can generally be predicted using the concentration addition model, but the influence of mixture ratio on toxicity must be taken into account.

The oxidative stress response patterns observed in this study are consistent with typical pathways of mitochondrial dysfunction. After 21 d of exposure to both fungicides, hepatic MDA content increased by 2.21 (fluxapyroxad) and 2.56 times (pyraclostrobin), respectively, while antioxidant enzyme activities exhibited a biphasic response of increasing first and decreasing then. Mao *et al.* [11] also observed a similar oxidative stress pattern in their developmental toxicity study of pyraclostrobin, suggesting it as a primary mechanism of toxicity resulting from mitochondrial respiratory chain inhibition. Zhai *et al.* [12], through RNA-Seq analysis, found that pyraclostrobin significantly affected the transcription of genes related to oxidative phosphorylation, cardiac muscle contraction, and nervous system development, confirming the complete toxic pathway of mitochondrial dysfunction → ROS generation → oxidative stress → cellular damage.

The biphasic response of antioxidant enzyme activities reflects the transition of the organism from compensatory adaptation to decompensatory damage. The initial increase in enzyme activity represents a defensive response of the organism to oxidative stress, while the sustained decline in activity following long-term exposure indicates the collapse of the antioxidant system[13-14]. The significant elevation of liver function enzymes (ALT and AST) observed in this study aligns with the findings of Wang *et al.* [15], who reported that pyraclostrobin caused histological damage and energy deficiency in adult zebrafish through multiple exposure pathways, with the gills being the primary target organ. Correlation analysis revealed a significant positive relationship between ALT/AST activity and MDA content ($r > 0.7$), confirming that oxidative stress is a key driver of liver injury.

The risk assessment in this study revealed that fluxapyroxad posed a moderate risk (RQ = 0.16 – 0.45) under typical usage scenarios, while pyraclostrobin presented moderate to high risks (RQ = 0.56 – 1.78), with higher risks in intensively used areas (RQ = 1.68 – 3.56). This finding is consistent with the ecological risk review of fungicides by Zubrod *et al.* [16], which highlighted that streptomycin fungicides pose significant threats to aquatic organisms, particularly crustaceans and fish. The present study identified crustaceans as facing the highest risk, attributed to their high sensitivity to mitochondrial inhibitors.

Although this study systematically elucidated the toxicity differences and joint effect of the two fungicides, certain limitations remain. First, the study primarily focused on adult zebrafish and short-term exposure effects, while the developmental toxicity and transgenerational effects of the fungicides on early life stages have not been fully assessed. Second, the toxicity mechanisms investigated were primarily focused on oxidative stress and liver function impairment, while other potential mechanisms such as endocrine disruption and immunotoxicity were not thoroughly explored. Third, although this study highlighted the importance of differences in mitochondrial targets, the molecular mechanisms underlying the differential downstream signaling pathways resulting from complex II and complex III inhibition require further investigation.

Future research should focus on five directions. First, it is necessary to conduct multigenerational exposure studies to evaluate the transgenerational toxicity and population-level effects of fungicides. Second, omics technologies (transcriptomics, metabolomics) can be integrated to comprehensively analyze the molecular networks of toxic effects. Third, Adverse outcome pathway (AOP)-based predictive models can be established to achieve quantitative correlations from molecular initiating events to community-level effects. Fourth, multi-species sensitivity comparisons should be conducted to identify key vulnerable species in ecosystems. Fifth, the impact of climate change (temperature, pH) on fungicide toxicity should be investigated to provide a basis for risk assessment under future environmental conditions. These studies will offer more comprehensive scientific support for the scientific management of mitochondrial inhibitor fungicides and the

protection of aquatic ecosystems.

Conclusions

In this study, an LC-MS/MS synchronous detection method was established for fluxapyroxad and pyraclostrobin (with LOD reaching ng/L level), and the toxic effects of these two fungicides on zebrafish were systematically evaluated. The results showed that the toxicity of pyraclostrobin (96 h-LC_{50} = 0.052 mg/L) was approximately 25.8 times that of fluxapyroxad (1.34 mg/L). This significant difference stems from their specific inhibition of mitochondrial complex III and complex II, respectively. The joint toxicity assessment revealed that most mixture ratios exhibited additive effects (AI = 0.62 – 1.47), while a high fluxapyroxad ratio showed antagonism. The analysis of toxicity mechanisms demonstrated that both fungicides induced oxidative stress and cellular damage through mitochondrial dysfunction, with pyraclostrobin exhibiting stronger effects. Ecological risk assessment indicated that fluxapyroxad posed a moderate risk, while pyraclostrobin posed moderate to high risks, with crustaceans being the most sensitive species. This study provides a scientific basis for the differentiated management of mitochondrial inhibitor fungicides and the protection of aquatic ecosystems.

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