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Temperature-dependent effects of neonicotinoids on the embryonic development of zebrafish (*Danio rerio*)

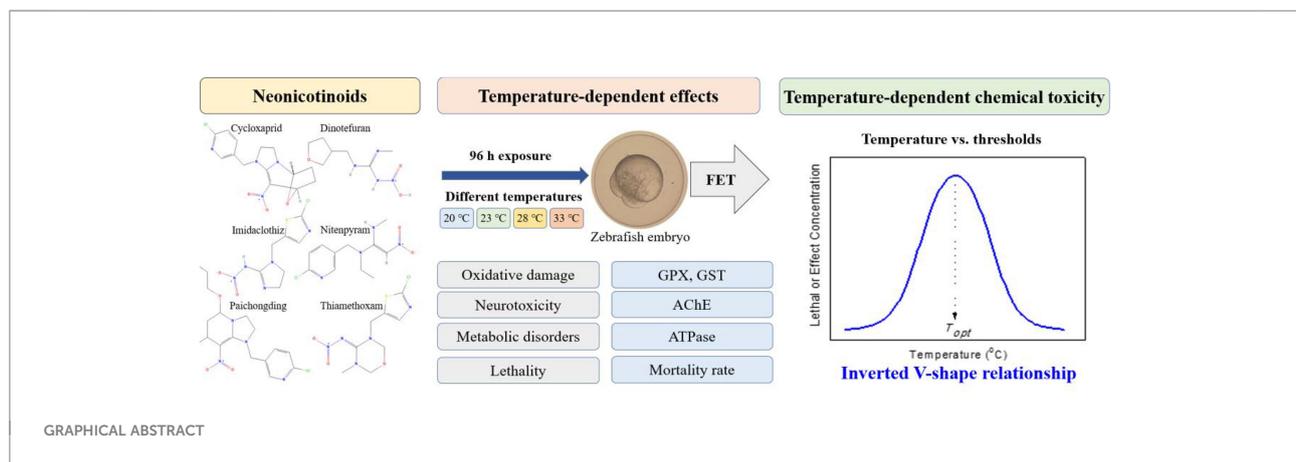
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The agricultural use of neonicotinoids is increasing worldwide and poses a threat to non-target organisms. The existing toxicity data of neonicotinoids that is mainly focused on widely used neonicotinoids ignores the influence of environmental factors, like temperature, related to climate changes, etc. To fill this data gap, the present study assessed the temperature-dependent toxicity of six neonicotinoids at four temperatures. Briefly, a fish embryo toxicity test was performed at the following temperatures—20, 23, 28, and 33°C—on zebrafish embryos to evaluate the lethal and sublethal effects of these toxicants. At 28°C, the lethal toxicity (LC₅₀) values for these toxicants were cycloxaprid—3.36 mg/L, nitenpyram—7.08 mg/L, paichongding—17.2 mg/L, imidaclothiz—738.6 mg/L, dinotefuran—2,096 mg/L, and thiamethoxam—4,293 mg/L, respectively. Among the sublethal effects, the enzymatic activities changed significantly in neonicotinoid treatments, which revealed oxidative stress, metabolic disorders, and neurotoxicity. Particularly, acetylcholinesterase inhibition and glutathione S-transferase activation showed a significant dose–response relationship. However, cycloxaprid, nitenpyram, and paichongding were found to be more potent compared with imidaclothiz and thiamethoxam. The influence of temperature on these neonicotinoids demonstrated an inverted V-shaped relationship, in which toxicity decreased with the increase of temperature and then increased with the increase of temperature after exceeding the optimum temperature. This study provides a reference for the multiscale effects and potential mechanisms of neonicotinoids. Temperature-dependent toxicity is of great significance for future toxicity testing and risk assessment of chemicals in the face of global climate changes.

KEYWORDS

test alternatives, fish embryo toxicity test, insecticides, temperature-dependent chemical toxicity, ecological risk assessment



Highlights

- The temperature-dependent lethal and sublethal effects of neonicotinoids were investigated by a fish embryo toxicity test.
- Multi-biomarkers including AChE, GPX, GST, and ATPase were examined.
- Temperature-dependent thresholds for multi-endpoint were determined.
- The temperature-dependent chemical toxicity for neonicotinoids followed an inverted V-shaped relationship.
- Our results can support data dossier development, toxicity testing, and ecological risk assessment.

1 Introduction

The extensive use of neonicotinoid insecticides and their massive residues in aquatic environments are a serious threat to non-target organisms. To date, neonicotinoid toxicity testing for aquatic species has been limited to the most commonly used categories in major markets (e.g., imidacloprid and thiamethoxam) and some aquatic invertebrates like *Chironomus riparius*, *Mysidopsis bahia*, and *Daphnia* (Hayasaka et al., 2013; Anderson et al., 2015; Miles et al., 2017). Several studies reported that neonicotinoid insecticides could induce acute lethal toxicity to fish species like *Oryzias latipes*, *Lepomis macrochirus*, *Oncorhynchus mykiss*, and *Danio rerio* (Ohi et al., 2004; Ocampo and Sagun, 2007; Clasen et al., 2011; Gibbons et al., 2014). Some studies demonstrated that neonicotinoids can induce sublethal toxicity, e.g., reduced body length and weight and inhibition in the growth and development of *Pimephales promelas* (DeCant and Barrett, 2010), and can influence the development of gonads and the reproductive system in

Oreochromis niloticus (Ocampo and Sagun, 2007; Lauan et al., 2013) as well as immunotoxicity in *O. latipes* (Sánchez-Bayo and Goka, 2005). Moreover, inhibition in the activity of acetylcholinesterase (AChE) and the development of the nervous system in brain tissues, resulting in neurotoxicity in *O. mykiss* and *Gobiocypris rarus*, was reported by Topal et al. (2017); Tian et al. (2018), and Ma et al. (2019). Oxidative stress, DNA damage, inhibition in protein synthesis, and disruption of amino acid metabolism and tricarboxylic acid cycle, leading to energy metabolism imbalance in *D. rerio*, were reported by Yan et al. (2015); Yan et al. (2016); Zhang and Zhao (2017). However, the toxicity data of neonicotinoids is scarce, especially for some newly developed neonicotinoids including nitenpyram, cycloxaprid, paichongding, and imidacloprid, and the sublethal effects of these neonicotinoids on non-target fishes (e.g., *D. rerio*) are largely unknown.

Temperature variations by climatic changes have profound implications on the chemical toxicity to individual organisms by influencing their physical and chemical properties (e.g., thermal dynamics, solubility, aggregation, ion dissolution, precipitation, partition coefficient, diffusion rates, chemical bonding, reaction rate, chemical speciation, and bioavailability) and biological reactions including growth and development, physiology and biochemistry, energy metabolism, chemical uptake, detoxification, and biological elimination (Heugens et al., 2003; Brocchi et al., 2013; Bourgeault et al., 2013; Wang et al., 2019). However, the toxicity of pesticides such as neonicotinoids under different temperature exposure scenarios is scarcely explored. The toxicity data and risk thresholds obtained from laboratory standard toxicity testing ignore the influence of temperature variations on neonicotinoid toxicity. These factors could potentially bring large uncertainty into the derivation of appropriate water quality guidelines or the risk assessment of chemicals (Zhou et al., 2014). Keeping in view the increasingly global climatic changes and the emergence of a wide variety of pollutants like neonicotinoids in the environment with relatively poor safety information, it is necessary to investigate the

influence of temperature changes on the multi-endpoint toxicity induced by neonicotinoids to provide safety information and theoretical basis for a comprehensive ecological risk assessment (ERA) of chemicals.

The data based on some traditional individual-level endpoints (e.g., mortality, growth, development, behavior, and morphology) or considering a single toxic effect as endpoint may not provide sufficient protection for aquatic organisms exposed to low doses of neonicotinoids over long time periods (Wang et al., 2021) because neonicotinoids at low concentrations may not directly cause mortality at the individual level but could potentially cause some chronic or sublethal effects at the cellular/molecular level (e.g., biochemistry, enzymes, and hormones). Some previous studies reported that neonicotinoids can induce oxidative stress (Priya and Maruthi, 2013), followed by changes in cells/tissues/organs like the liver and spleen (Nicolas and Ocampo, 2006) and gonads (Lauan et al., 2013), ultimately leading to developmental toxicity, immunotoxicity, and thus mortality (Morrissey et al., 2015), whereas the mode of action (MOA) and the relationship among these effects (i.e., adverse outcome pathway—AOP) induced by neonicotinoids are unclear. Meanwhile, the selection of test endpoints is another key factor for toxicity identification and evaluation (TIE) and the ERA of pollutants. However, the traditional toxicity test using adult fish is time-consuming and resource-wasting and violates the 3R principles (reduction, refinement, and replacement of animal testing). The embryonic life stage could supplement enough to cover these deficiencies because an embryo is the simplest multicellular individual developed from a single cell, which not only complies with the 3R principles but also could allow a convenient study of its toxic effects from cell/tissue level to the ontogenetic level. Therefore, the fish embryo toxicity test (FET) may be a rapid and effective alternative test method to examine multiscale effects from the cellular/molecular level, including neurotoxicity, energy metabolism, and oxidative stress, to the individual level like hatching rate, mortality, and potential MOA of neonicotinoids (OECD, 2013; Su et al., 2021).

In this study, FET was performed with the exposure of neonicotinoids (nitenpyram, cycloxaprid, paichongding, imidaclothiz, dinotefuran, and thiamethoxam) at different temperature levels (20, 23, 28, and 33°C) to investigate the temperature-dependent effects of neonicotinoids on the development of zebrafish embryo. Firstly, this study provides a reference for the toxic effects of neonicotinoids and their potential mechanisms on zebrafish embryos as well as the theoretical basis for the construction of AOPs of the chemicals. Secondly, the determination of temperature-dependent thresholds [e.g., LC/EC_{10/50}, no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC)] at diverse endpoints can enrich the toxicity database for TIE and the ERA of neonicotinoids. Finally, the influences of temperature on neonicotinoid

toxicity to the embryonic development of zebrafish were systematically investigated by analyzing the relationship between the temperature and toxicity thresholds of each test endpoint. It could provide vital information/guidelines for the construction of predictive models based on temperature-dependent chemical toxicity as well as the derivation of temperature-dependent or region-specific risk thresholds for neonicotinoids.

2 Materials and methods

2.1 Zebrafish maintenance and embryo collection

Adult wild-type (AB) zebrafish (*D. rerio*) were obtained from China Zebrafish Resource Center (Wuhan, China). All fish were reared in a recirculation system at $28 \pm 0.5^\circ\text{C}$ under 14:10-h light/dark photoperiod and 54–324-lx light intensity following the standard zebrafish breeding protocols. Meanwhile, NaHCO₃ and NaCl were added in reverse osmosis-filtered water to maintain the pH and conductivity at 7.5 ± 0.5 and 500–800 $\mu\text{S}/\text{cm}$, respectively. The adult zebrafish were fed twice a day with live brine shrimps. The male and female fishes were kept separately at each designated temperature conditions for more than 2 weeks before they were determined to spawn. On the night before spawning, the paired adult fishes were placed in spawning boxes with a male/female ratio of 1:1 and separated by a partition overnight. The partition was removed earlier the next day, and spawning was induced at the beginning of the light cycle. Within 0.5 h of spawning, embryos were collected, rinsed with standard diluent (Appendix I) thrice, and transferred to the embryo medium before use. Fertilized and healthy embryos were selected for toxicity testing under the stereomicroscope SZX 7 (Olympus, Tokyo, Japan) following Kimmel et al. (1995).

2.2 Chemicals and reagents

Cycloxaprid (CAS number: 1203791416), dinotefuran (165252700), imidaclothiz (105843365), nitenpyram (150824478), paichongding (948994169), and thiamethoxam (153719234) with 98% purity were purchased from HaoRong Biological Technology Co., Ltd. (Wuhan, China). Stock solutions were prepared by dissolving the neonicotinoids in standard diluent (Appendix I) prior to the commencement of an exposure experiment to ensure the proper dissolution of the substance. The tested concentrations were obtained by further diluting the stocks with standard diluents (Appendix I). To prevent photodegradation, both solutions (test and stock) were prepared in glass bottles wrapped in aluminum foil and stored in a refrigerator at 4°C until each experiment is finished.

2.3 Standard acute lethal toxicity test

Standard acute lethal toxicity tests were conducted following OECD TG 236 (OECD, 2013). The highest tested concentration should preferably result in 100% lethality, and the lowest concentration tested should preferably give no observable effect. Range-finding tests were conducted with concentrations ranging from 0.1 to 10,000 mg/L (10 embryos per concentration) before the definitive test to allow the selection of an appropriate concentration range. After 96 h of exposure, the approximate concentration range of the embryonic acute lethal toxicity of each neonicotinoid could be preliminarily determined to set the formal test concentration gradient.

According to the results of the preliminary experiment, five test concentrations were set between 0% and 100% lethal concentration, and standard diluent was used as the blank control group. The embryos were immersed in the test solutions before cleavage of the blastodisc commences or, at the latest, by the 16-cell stage. Thus, embryos at 1 h of post-fertilization (1 hpf) were randomly selected and individually exposed to the neonicotinoids in 24-well plates, and the acute toxicity test was terminated after 96 h of exposure (static exposure with no renewal over the period of 96 h). Each well on the plate was refilled with 2 ml of freshly prepared test solutions and one embryo (24 embryos per plate). The test was performed under four temperatures (20, 23, 28, and 33°C) and under 14:10-h light/dark photoperiod. Each neonicotinoid exposure concentration at each temperature was set as one treatment, and each treatment was performed in triplicates (three 24-well plates for each treatment and 72 embryos per treatment). Coagulation of embryos, malformation, and lack of heartbeat were used to determine the lethality. Additionally, hatching was recorded in the treatment and control groups every day starting from 48 h. The observations of embryos hatching and mortality under a microscope were recorded every 24 h until the end of the test. Finally, the mortality rate was computed to generate the log (dose) mortality curves, and the 96-h embryonic 10%/50% lethal concentration ($LC_{10/50}$) values under different temperatures were calculated.

2.4 Standard sublethal FET test

Following the OECD TG 236 and based on the acute toxicity test results, the exposure concentrations of neonicotinoids for the sublethal toxicity test were set as 1/2 \times , 1/4 \times , 1/8 \times , 1/16 \times , and 1/32 \times 96-h LC_{50} . Standard diluent without chemicals was used as the blank control group. The 1-hpf embryos were randomly selected for exposure to different neonicotinoid test solutions under four temperatures (20, 23, 28, and 33°C) and 14:10-h light/dark photoperiod. Each treatment was performed in triplicates (24 embryos per replicate). The homogenate of the embryo tissue was prepared after exposure for 96 h. Firstly, 24 embryos were rinsed

in cold normal saline (pH 7.4, 0.6% sodium chloride solution), dried with filter paper, and then weighed at 0.2 g. The weighed embryos (0.2 g) were added into 1.8 ml 0.6% NaCl solution by ratio of weight (g) to volume (ml) = 1:9 in a 5-ml glass homogenizer (also called tissue grinding tube, which consisted of a grinding rod with an outer surface of ground sand and a glass tube with an inner surface of ground sand) for homogenizing. The homogenizing tube was placed on ice and fully ground for a dozen times up to 6–8 min to make 10% homogenate (the embryonic tissue was crushed and ground by manually pushing and pulling and by using a rotating rod). Finally, the prepared 10% homogenate was centrifuged at 4°C, 2,500 rpm for 15 min, and the supernatant was taken for determination. The embryo homogenate of each test group was used to detect the different endpoints, including the following: (1) oxidative stress: GPX and GST activity, (2) metabolic disorders/ATPase activity, and (3) neurotoxicity/AChE activity.

Total protein content and enzyme activity were determined by various kits, including Bradford Protein Content Assay Kit (Beyotime), Total GPX Activity Detection Kit (Beyotime), GST Activity Detection Kit (Solarbio), ATPase Activity Detection Kit (Nanjing Jiancheng), and AChE Activity Detection Kit (Solarbio). The enzyme activity in each sample was expressed as the enzyme activity in protein per unit mass (U/mg protein). The contents of the above-mentioned enzyme activities were detected by a TECAN microplate analyzer.

2.5 Statistical analysis

Experimental data was analyzed using GraphPad Prism (version 5.0, San Diego, CA, USA), and the results were expressed as mean \pm standard deviation (SD). One-way ANOVA was performed to compare the significant difference between the control and the neonicotinoid treatment groups as well as to determine the LOEC and NOEC. Two-way ANOVA was computed to test the significance of temperature and exposure concentration and their interactions on the changes of indicators. To determine $LC_{10/50}$ (10%/50% lethal concentration) and $EC_{10/50}$ (10%/50% effective concentration), the data were fitted to a sigmoidal (log) dose–response curve (variable slope) with a four-parameter logistic equation. All analyses were conducted in SPSS (version 23, Chicago, IL, USA), and the significance level α was set at 0.05.

3 Results and discussion

3.1 Acute lethal toxicity of neonicotinoids to zebrafish embryos

Based on the preliminary experimental results, the five exposure concentrations for each neonicotinoid were set as

follows: cyclozaprid—0.1, 1, 10, 50, and 100 mg/L; dinotefuran—1, 10, 100, 1,000, and 10,000 mg/L; imidaclothiz—0.1, 1, 10, 50, and 100 mg/L; nitenpyram—0.1, 1, 10, 100, and 1,000 mg/L; paichongding—0.1, 1, 10, 50, and 100 mg/L; and thiamethoxam—1,000, 2,000, 3,000, 4,000, and 5,000 mg/L, respectively. After 96 h of exposure under four temperatures (20, 23, 28, and 33°C), the mortality rate of zebrafish embryos was calculated (Supplementary Table S1). The dose–response curves at four temperatures for the six neonicotinoids were plotted and demonstrated in Supplementary Figure S5. The 96-h LC₅₀ of the six neonicotinoids under different temperatures was calculated and listed in Table 1—for example, at 28°C, the LC₅₀ of cyclozaprid, dinotefuran, imidaclothiz, nitenpyram, paichongding, and thiamethoxam was 3.36, 2,096, 738.6, 7.08, 17.2, and 4,293 mg/L, respectively. The acute lethal toxicity of these neonicotinoids to zebrafish embryos is presented in the following order: cyclozaprid > nitenpyram > paichongding > imidaclothiz > dinotefuran > thiamethoxam. However, a similar toxicity trend was also found at three other temperatures (20, 23, and 33°C).

3.2 Sublethal effects of neonicotinoids to zebrafish embryos

According to the LC₅₀ values of the neonicotinoids, five sublethal concentrations for each neonicotinoid were set as follows: cyclozaprid—0.125, 0.25, 0.5, 1, and 2 mg/L; imidaclothiz—6.25, 12.5, 25, 50, and 100 mg/L; nitenpyram—0.125, 0.25, 0.5, 1, and 2 mg/L; paichongding—0.625, 1.25, 2.5, 5, and 10 mg/L; and thiamethoxam—62.5, 125, 250, 500, and 1,000 mg/L, respectively. After 96 h of exposure, the enzyme activities in all neonicotinoid treatment groups and in the control group under different temperatures were measured (Supplementary Tables S2–S5).

3.2.1 AChE activity

The tested neonicotinoids at sublethal concentrations (1/32–1/2 LC₅₀) showed no significant effect on the survival of zebrafish embryos. However, the AChE activity followed a

dose–effect relationship of being significantly decreased by increasing the neonicotinoid concentrations, which indicated that the neonicotinoids are potentially neurotoxic to zebrafish embryos (Supplementary Figure S1). Sigmoidal dose–response curves (variable slope) were fitted with a four-parameter logistic equation to obtain EC₅₀ (Supplementary Figures S6A–E). One-way ANOVA was also performed to determine the NOEC/LOEC of neonicotinoids inhibiting the AChE activity of zebrafish embryos at four temperatures (Table 2). At 28°C, the EC₅₀ of cyclozaprid, imidaclothiz, nitenpyram, paichongding, and thiamethoxam AChE activity was 0.998 (0.786, 1.244), 52.55 (29.74, 189.8), 0.957 (0.546, 2.184), 0.695 (0.019, 1.442), and 24.33 (17.09, 32.7) mg/L, whereas at 33°C the EC₅₀ of these neonicotinoids was 0.693 (0.595, 0.806), 6.57 (2.10, 11.15), 0.494 (0.416, 0.587), 0.503 (0.137, 0.824), and 128.0 (96.21, 163.2) mg/L, respectively. However, it is worth mentioning that the inhibitory effect of cyclozaprid, nitenpyram, and paichongding on the AChE activity of zebrafish embryo was significantly greater than those of imidaclothiz and thiamethoxam.

As nicotinic acetylcholine receptor (nAChR) agonists, neonicotinoids selectively bind to the nAChR and compete with the insect's acetylcholine, thus blocking the normal transmission between neurons and thereby causing paralysis and the ultimately death of the organism (Mitchell et al., 2017). AChE is a multi-molecule glycoprotein that hydrolyzes acetylcholine and can terminate the transmission of acetylcholine in synapses. It exists in nerve tissue, liver, muscle, red blood cell, or spleen in animals and plays an important role in nerve conduction and functional regulation of the neurotransmitter. Changes in the activity of AChE are sensitive toxicological indicators for pesticide pollution in an aquatic environment, which can be further used as molecular biomarkers for toxicity evaluation and risk assessment as well as in the environmental monitoring of pesticides in water (Lionetto et al., 2013; Gravato et al., 2021). Neonicotinoids particularly inhibit the AChE activity, resulting in the accumulation of acetylcholine which causes inhibition in the normal functioning of the central nervous system (Lu et al., 2018).

TABLE 1 The embryonic 96-h 10% and 50% lethal concentration (LC_{10/50}, mg/L) of six neonicotinoids under different temperature conditions [curves were fitted to sigmoidal (log) dose–response curve (variable slope) with a four-parameter logistic equation in GraphPad Prism (version 5.0, San Diego, CA, USA)].

Neonicotinoids	20°C			23°C			28°C			33°C		
	R ²	LC ₁₀	LC ₅₀	R ²	LC ₁₀	LC ₅₀	R ²	LC ₁₀	LC ₅₀	R ²	LC ₁₀	LC ₅₀
Cyclozaprid	0.361	3.294	5.910	0.570	1.302	12.94	0.999	1.105	3.362	0.997	1.203	3.328
Dinotefuran	0.816	1,350	11,141	0.775	1,733	14,897	0.977	1,157	2,096	0.973	566.2	2,096
Imidaclothiz	0.525	100.2	892.8	0.496	197.3	2,398	0.521	72.62	738.6	0.543	37.87	253.8
Nitenpyram	0.857	12.59	18.75	0.971	43.57	109.2	0.984	4.944	7.079	0.969	4.332	6.407
Paichongding	0.751	181.9	400	0.797	107.5	507.7	0.993	10.36	17.23	0.963	0.8682	2.113
Thiamethoxam	0.880	2,190	12,205	0.792	4,111	19,910	0.909	2,976	4,293	0.999	1,817	2,182

TABLE 2 Toxicity threshold of AChE inhibition and GST activation (EC_{50} , NOEC, LOEC, mg/L) of five neonicotinoids under different temperature conditions.

Neonicotinoids	Temperature	Toxicity threshold of AChE inhibition (mg/L)				Toxicity threshold of GST activation (mg/L)			
		R^2	EC_{50}	NOEC	LOEC	R^2	EC_{50}	NOEC	LOEC
Cycloxaprid	20°C	0.904	0.762 (0.629, 0.932)	0.125	0.25	0.942	0.319 (0.198, 0.54)	ND	0.125
	23°C	0.893	0.851 (0.682, 1.086)	0.25	0.5	0.936	1.032 (0.451, 3.623)	ND	0.125
	28°C	0.949	0.998 (0.785, 1.244)	0.25	0.5	0.935	0.962 (0.541, 1.992)	0.125	0.25
	33°C	0.993	0.693 (0.594, 0.806)	ND	0.125	0.848	0.028 (0.002, 0.063)	0.25	0.5
Imidaclothiz	20°C	0.916	22.03 (17.92, 27.02)	ND	6.25	0.824	41.42 (21.58, 138.3)	6.25	12.5
	23°C	0.655	12.92 (5.5, 23.44)	ND	6.25	0.677	120.4 (43.1, 246.8)	6.25	12.5
	28°C	0.414	52.55 (29.74, 189.8)	25	50	0.834	97.45 (19.66, 146.6)	6.25	12.5
	33°C	0.924	6.566 (2.096, 11.15)	6.25	12.5	0.674	17.91 (9.866, 94.74)	25	50
Nitenpyram	20°C	0.834	0.729 (0.586, 0.915)	0.125	0.25	0.468	0.216 (0.061, 0.652)	ND	0.125
	23°C	0.904	0.919 (0.687, 1.261)	0.125	0.25	0.978	0.314 (0.162, 0.585)	0.125	0.25
	28°C	0.595	0.957 (0.746, 2.184)	ND	0.125	0.766	0.978 (0.536, 1.87)	0.5	1
	33°C	0.949	0.494 (0.416, 0.587)	ND	0.125	0.903	0.181 (0.089, 0.336)	0.125	0.25
Paichongding	20°C	0.926	0.963 (0.023, 2.053)	ND	0.625	0.817	2.867 (1.905, 12.92)	1.25	2.5
	23°C	0.802	3.125 (1.009, 7.260)	ND	0.625	0.886	14.17 (11.73, 38.74)	0.625	1.25
	28°C	0.866	0.695 (0.019, 3.442)	ND	0.625	0.609	2.3 (1.842, 4.08)	1.25	2.5
	33°C	0.976	0.503 (0.137, 0.824)	0.625	1.25	0.845	1.299 (0.483, 4.689)	0.625	1.25
Thiamethoxam	20°C	0.926	122.3 (99.52, 150.8)	ND	62.5	0.997	214.7 (205.3, 223.9)	ND	62.5
	23°C	0.798	359.4 (255.6, 512.9)	ND	62.5	0.852	274.8 (230.5, 324.6)	62.5	125
	28°C	0.993	128.0 (96.21, 163.2)	ND	62.5	0.436	2,886 (1,440, 3,775)	ND	62.5
	33°C	0.918	24.33 (17.09, 32.70)	ND	62.5	0.983	130.9 (119.6, 145.9)	62.5	125

The curves were fitted to sigmoidal (log) dose–response curve (variable slope) with a four-parameter logistic equation in GraphPad Prism (version 5.0, San Diego, CA, USA). ND, no available data.

According to pioneer studies, neonicotinoids can cause neurotoxic effects (*e.g.*, motor behavior) to invertebrates and aquatic vertebrates by inhibiting the AChE activity, *e.g.*, exposure of imidacloprid resulted in decreased AChE activity in *Chironomus riparius* (Azevedo-Pereira et al., 2011), *Eisenia foetida* (Wang et al., 2015), and *Caenorhabditis elegans* (Meyer and Williams, 2014) as well as in the brain of *D. rerio* (Guerra et al., 2021), *O. mykiss* (Topal et al., 2017), and *Rhamdia quelen* (Marins et al., 2021). In the muscle of *Astyanax altiparanae* (Almeida et al., 2021), sulfoxaflor exposure can also significantly decrease AChE enzyme activity in the brain and muscle tissues of zebrafish (Piner Benli and Çelik, 2021). In this study, the inhibitory effect on AChE activity of zebrafish embryos indirectly reflects the neurotoxic effect of neonicotinoids on zebrafish, which can be used as a quantitative indicator to evaluate the sublethal toxicity of neonicotinoid pesticides (Sheets et al., 2015).

3.2.2 ATPase activity

Compared with the control group, under four temperatures (20, 23, 28, and 33°C), the ATPase activity of zebrafish embryos was slightly increased in the low concentration ($p > 0.05$) and significantly decreased ($p < 0.05$) in the high concentration of the six neonicotinoid treatment groups (Supplementary Figure S2)—for example, at 33°C, the ATPase activity was decreased in 1 mg/L and increased in 0.5 mg/L, whereas the enzyme activity was increased at other temperatures in 0.125 and 0.25 cycloxaprid treatments (Supplementary Figure S2A). At 20 and 33°C, the enzyme activity was decreased in 6.25 mg/L and decreased at two other temperatures in 12.5- and 25-mg/L treatments of imidaclothiz (Supplementary Figure S2B). Furthermore, at all four temperatures, the ATPase activity was activated by nitenpyram exposure in 0.125 and 0.25 mg/L and decreased in the 0.5-, 1-, and 2-mg/L treatment groups (Supplementary Figure S2C). Meanwhile, at 23°C, the ATPase

activity was promoted in 0.625-mg/L treatment and inhibited by 0.625-, 1.25-, and 2.5-mg/L paichongding treatments at three other temperatures (Supplementary Figure S2D). However, at 20 and 28°C, the ATPase activity was enhanced in 0.625 mg/L, followed by a decrease at 250 and 500 mg/L, and inhibited at 23 and 33°C by 0.625- and 1.25-mg/L treatments of thiamethoxam (Supplementary Figure S2E).

ATPase (divided into Na^+K^+ -ATPase and $\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase), a protease in the membrane of a cell or an organelle, can regulate the membrane potential and maintain the intracellular ion gradients which are essential for signal transduction, excitability, conductivity, and release of neurotransmitters in nerve cells (Ji et al., 2009). Na^+K^+ -ATPase is a sodium pump on the cell membrane, which can transport Na^+ and K^+ to maintain the normal physiological function of cells. When Na^+K^+ -ATPase activity is inhibited, the intracellular and extracellular Na^+ concentration will be imbalanced, and excessive intracellular Na^+ can inhibit the uptake of excitatory amino acids in the synaptic membrane (Davis and Wedemeyer, 1971; Farley et al., 1984; Agrahari and Gopal, 2008), whereas $\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase is a calcium pump on the membrane, which can hydrolyze the ATP to maintain a low intracellular Ca^{2+} concentration which may help in maintaining cell homeostasis. When the activity of $\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase inhibits, the activity of a large number of intracellular enzymes will be reduced, and this will cause an increase of free radical production, ultimately leading to peroxidation damage of the membrane, destruction of the mitochondria, and cell death (Al-Rajhi, 1990; Blasiak, 1995; Li et al., 2006). The inhibition in energy conversion—such as inhibition in the activity of mitochondrial enzyme—may be the key component of neonicotinoid insecticide mechanism (Kumar et al., 2018; Li et al., 2022; Sevçiler and Atlı, 2022; Veedu et al., 2022). The results of our study indicated that the high-concentration exposure of neonicotinoids inhibited the ATPase activity in zebrafish embryos, which may cause the functional disturbance of the membrane ion pump (e.g., sodium pump and calcium pump). Because the dysfunction of a membrane ion pump leads to a massive outflow of K^+ and Mg^{2+} , there will be a massive influx of Na^+ , Ca^{2+} , and H_2O into the cell, resulting in the retention of water and sodium, energy metabolism disorders, and mitochondrial dysfunction, ultimately leading to nerve cell damage and dysfunction of the central nervous system (Suvetha et al., 2010; Serafini et al., 2019; Oyovwi et al., 2021). However, at present, the mechanism which demonstrates how neonicotinoid induces the inhibition of ATPase activity is not clear. It could be either the direct inhibition of ATPase after neonicotinoid pesticides enter into the cell or by indirectly inhibiting the activity of ATPase by structural and functional disorders of the biomembrane through oxidative/peroxidation damage, or both possibilities could have happened (Chauhan et al., 2002; Akbar et al., 2016; Kumar et al., 2018).

3.2.3 Antioxidant enzymes

Oxidative stress refers to oxidative damage caused by the accumulation of reactive oxygen species (ROS) and reactive nitrogen species when there is an imbalance between the generation and the elimination of oxygen-free radicals in the body or cells, resulting in the damage of lipids, amino acids/proteins, nucleic acids, and chromosomes and, ultimately, cell death (Droge, 2002; Deng et al., 2021). Antioxidant enzymes, including superoxide dismutase, catalase, peroxidase, GPX, and GST, are important indexes to detect oxidative stress (Lacourse et al., 2009). GPX can remove oxygen metabolites and maintain the activity of glutathione (GSH). GST is a dual-function enzyme acting as an antioxidant and phase II detoxification enzyme. The combination of GSH with various toxic substances and their metabolites by catalyzing them into hydrophobic substances which are excreted in the form of urine or bile is a vital mechanism of GSH. This mechanism helps GSH to achieve the detoxification effect of degrading and discharging various toxic substances in the body. Meanwhile, GST can also repair macromolecular proteins, DNA, and other substances, remove lipid peroxides, and inhibit lipid peroxidation, which can protect the body from damage by exogenous stimulation (Ullah et al., 2022). Thus, GPX and GST are commonly known as biochemical indicators or biomarkers for evaluating the oxidative damage of environmental pollutants (Pan et al., 2022; Ullah et al., 2022).

In this study, at four temperatures (20, 23, 28, and 33°C), the GPX activity was promoted or not affected by the lower concentrations of the six neonicotinoids compared with the higher concentrations which cause the inhibition in GPX activity (Supplementary Figure S3)—for example, at 20, 23, and 28°C, the GPX activity was enhanced in 0.125-, 0.25-, and 0.5-mg/L concentrations and inhibited in 1- and 2-mg/L concentrations of cyclozaprid (Supplementary Figure S3A); at all four temperatures, enzyme activity was inhibited in 6.25-, 12.5-, and 25-mg/L imidaclothiz concentrations (Supplementary Figure S3B); at 20 and 33°C, enzyme activity increased in 0.125-mg/L concentration and decreased in 0.5- and 1-mg/L concentrations of nitenpyram (Supplementary Figure S3C); at 20°C, the enzyme activity was promoted in 0.625-, 1.25-, and 2.5-mg/L paichongding concentrations and inhibited in 10-mg/L concentration (Supplementary Figure S3D); and at 20, 23, and 28°C, enzyme activity was activated in 62.5- and 125-mg/L concentrations and inhibited in 250- and 500-mg/L concentrations of thiamethoxam (Supplementary Figure S3E).

These findings demonstrated that the inhibition of GPX activity by cyclozaprid and nitenpyram is much stronger than by the other neonicotinoids (especially imidaclothiz and thiamethoxam). The overall effect of neonicotinoids demonstrated a strong dose-response relationship between GPX activity and neonicotinoid concentrations. At low-concentration exposures of the six neonicotinoids, the GPX

activity was generally promoted, whereas at higher concentrations the enzyme activity was inhibited. One possible reason could be the excessive production of ROS after neonicotinoids entered the embryos (Shukla et al., 2017). GPX can participate in controlling ROS homeostasis by regulating the intracellular H₂O₂ levels, antioxidant enzyme activities, and gene transcription levels and by maintaining the GSH/GSSG balance (Zhang et al., 2020). In turn, GPX activities can be regulated by ROS level. An increased concentration of intracellular ROS caused by external stimulus (neonicotinoid exposure) can increase the expression level of the *gpx* gene and thus promote GPX activities (Li et al., 2015), whereas with increased pesticide concentration and continuous action on cells, excessive ROS cannot be eliminated by the GPX enzyme, leading to the accumulation of ROS, which will oxidize the amino acid residues of the GPX enzyme protein to produce a carbonyl group and change its secondary structure (reducing its helicity) and thus reducing the enzyme activity (Lin et al., 2021). Meanwhile, the excessive ROS can damage lipid, DNA, protein, and other biological macromolecules as well as the structure and functions of membranes, which can directly or indirectly affect the synthesis of the GPX enzyme (Tian et al., 2020; Benchaabane et al., 2022).

The findings of our study demonstrated that the GST activity was activated by neonicotinoids in a dose-dependent manner (Supplementary Figure S4). It can be noticed that, at 20°C, the GST activity was increased by 0.125 mg/L cycloxaprid, 12.5 mg/L imidaclothiz, 1 mg/L nitenpyram, 2.5 mg/L paichongding, and 62.5 mg/L thiamethoxam, while at 33°C the enzyme activity was promoted by 0.5 mg/L cycloxaprid, 50 mg/L imidaclothiz, 0.25 mg/L nitenpyram, 1.25 mg/L paichongding, and 125 mg/L thiamethoxam. It should be noted that the fluctuations in GST activity may be caused by oxidative stress and the detoxification is induced by pollutant stimulation because the activity of GST could be activated to catalyze the binding of GSH with lipid peroxides or other harmful metabolites produced by oxidative damage and exert antioxidant and detoxification effects (Qi et al., 2013; Benchaabane et al., 2022).

Dose–response curve and one-way ANOVA were employed to obtain the EC_{10/50} and the LOEC/NOEC of neonicotinoids, which revealed that the GST activity was promoted in zebrafish embryos at different temperatures (Table 2 and Supplementary Figures S6F–J). At 28°C, the EC₅₀ of GST activation induced by cycloxaprid, imidaclothiz, nitenpyram, paichongding, and thiamethoxam was 0.9619 (0.5409, 1.992), 17.91 (9.866, 94.74), 0.9780 (0.5363, 1.870), 2.300 (1.842, 14.08), and 2,886 (1,440, 9,775) mg/L, respectively, whereas the oxidative damage by cycloxaprid and nitenpyram to zebrafish embryo was stronger compared that of with thiamethoxam. Previous studies have shown that antioxidant and detoxification enzymes are crucial in maintaining cell homeostasis and can be used as a biochemical

marker to evaluate the potential toxicity of the contaminants—inducing oxidative stress in aquatic organisms, especially in fish (Santana et al., 2018; Gallego-Rios et al., 2021; Solomando et al., 2022). In this study, the changes of antioxidant enzyme (GPX) and detoxification enzyme (GST) revealed that the neonicotinoids induce oxidative damage to zebrafish embryo. These responses can be used as molecular biomarkers to evaluate the oxidative stress and toxicity mechanisms of neonicotinoids or other pollutants.

Overall, the neonicotinoids potentially caused neurotoxicity and CNS dysfunction by AChE inhibition. Furthermore, neonicotinoid toxicity can cause the peroxidation of biological macromolecules including lipid, protein, and DNA by inducing oxidative damage. This phenomenon can destroy the structure and function of biological membranes, including those of the mitochondrial membrane with the potential inhibition of respiratory enzyme activity, ultimately leading to cellular metabolic disorders (Meiliana and Wijaya, 2012). This implies that neurotoxicity, oxidative damage, and metabolic disorders may be the key components of MOA of neonicotinoid insecticides. These biomarkers—including AChE, GST, GPX, and ATPase activity at the molecular/cellular level—can be utilized as signal indicators for monitoring pesticide contamination in aquatic environments at the early stages.

3.3 Temperature-dependent effects of neonicotinoids to zebrafish embryos

3.3.1 Effects of temperature on embryonic development

Temperature can profoundly influence the physiological and ecological performance of organisms such as during embryonic development—including growth and development (Brett, 1979), malformation and death (Kamler et al., 1998), adaptations and acclimation (Burleson and Silva, 2011), resource acquisition and behavior (Van der Have, 2002), metabolic and ventilation rate (Vinagre et al., 2014), and survivorship and fecundity (Frazier et al., 2006; Angilletta et al., 2009; Kingsolver, 2009; Amarasekare and Savage, 2012). In this study, the hatching rate of zebrafish embryos which are maintained as the control group at four temperatures was recorded for 192 h. The hatching curves of zebrafish embryos at different temperatures (20, 23, 28, and 30°C) were plotted and demonstrated in Supplementary Figure S7. The results showed that embryos started hatching at 20 and 23°C within 108 and 84 h and completely hatched after 192 h at both of these temperatures, while at the two other temperatures (28 and 33°C) the embryos started hatching at 36 h and completely hatched after 72 h. These findings clearly indicated that a low temperature has an inhibitory effect on embryo hatching, and a higher temperature can reduce the hatching time of embryos.

Meanwhile, in the control group, the enzyme activities of AChE, ATPase, GPX, and GST were highest at 28°C, decreased at 33°C, and significantly inhibited at low temperatures of 20 and 23°C (Supplementary Tables S2–S5). As demonstrated in Supplementary Figure S7, low temperature inhibited the enzyme activity and high temperature enhanced the enzyme activity. Therefore, it can be assumed that temperatures below or above T_{opt} can inhibit embryonic development.

3.3.2 Temperature-dependent chemical toxicity of neonicotinoids

In this study, a significant interaction was found between various concentrations of the tested neonicotinoids and the embryonic responses (lethal including mortality and sublethal like changes of AChE, ATPase, GPX, and GST activities) of zebrafish at different temperatures. Furthermore, the findings of this study also demonstrated that the interaction between neonicotinoid toxic concentrations and temperature significantly influenced the embryonic mechanisms (Supplementary Table S6).

Nonlinear regression curves were plotted on the toxicity threshold values of lethal (LC_{50}) and sublethal effect (EC_{50}) of neonicotinoids at different temperatures and fitted by Gaussian distribution (Figure 1). Significant variations in LC_{50} and EC_{50} can be noticed at variable temperatures. The effect of temperature on neonicotinoid toxicity showed an inverted V-shaped relationship, where the toxicity decreased with the increase of temperature and then increased with the increase of temperature after exceeding the T_{opt} . The temperature corresponding to the highest amplitude of the Gaussian curve represents the T_{opt} , at which toxicity is the least (toxicity is enhanced when the temperature is above/below the T_{opt}). T_{opt} values were also obtained at 23.5, 22.3, 23.8, 23.6, 21.9, and 22.9°C for cycloxyprid, dinotefuran, imidacloprid, nitenpyram, paichongding, and thiamethoxam, respectively, when considering acute lethal toxicity (Table 3), while for the sublethal effects of AChE inhibition, T_{opt} values were derived at 26.3, 27.3, 25.4, 23.8, and 24.0°C for cycloxyprid, imidacloprid, nitenpyram, paichongding, and thiamethoxam, respectively. Temperature can affect the toxicity of neonicotinoids (e.g., imidacloprid and thiacloprid) on the embryonic level by enhancing the respiratory efficiency or energy metabolism (Osterauer and Köhler, 2008; Scheil and Köhler, 2009). It is generally accepted that elevated temperatures tend to increase the rate of chemical contaminant uptake by increasing the metabolic and ventilation rate of ectotherms, hence boosting the bioaccumulation of chemicals in body tissues (Cherkasov et al., 2006; Tsui and Wang, 2006; Cherkasov et al., 2007; Schiedek et al., 2008). This mechanism ultimately leads to a higher chemical toxicity at higher temperature levels (Cairns et al., 1975; Bourgeault et al., 2013).

It is commonly reported that chemical toxicity increases with increasing temperature (linear model) (Leung et al., 2000; McConnachie and Alexander, 2004; Kwok and Leung, 2005), but a non-linear function (e.g., inverted V-shaped model) tends to describe the relationship between temperature and chemical toxicity more adequately with wider temperature ranges as demonstrated in further comprehensive toxicity studies (Heugens et al., 2003; Li et al., 2006; Bao et al., 2008; Osterauer and Köhler, 2008; Prato et al., 2008; Wong and Leung, 2013). Following the inverted V-shaped model, chemical toxicity to aquatic organisms was lowest at T_{opt} and increased at a temperature below or above T_{opt} . In general, aquatic organisms living in optimal conditions are more tolerant to chemical toxicity than those living in conditions near their thermal tolerance limits (Heugens et al., 2001; Li et al., 2014). Every aquatic organism has a thermal tolerance range (Cairns et al., 1975; Cairns et al., 1978); when aquatic ectotherms (e.g., fish) generally live within an optimum range of temperatures, they have sufficient energy and oxygen supply and strong activities of detoxification enzymes and antioxidant enzymes (Pörtner, 2010), resulting in a high clearance rate of exogenous toxins and low sensitivity to their toxicity (Frederich and Pörtner, 2000). When the temperature exceeds (higher/lower than) the optimal range, its internal energy budget cannot maintain its normal growth state (energy deficit) (Sokolova and Pörtner, 2007), the tolerance of the organism is reduced, and such a thermal stress at temperature extremes may further enhance the toxicity of a chemical (Jusup et al., 2016). Therefore, such an integrative inverted V-shaped response can be typically explained by the temperature-regulated mechanisms (Mu et al., 2018).

4 Conclusion

In this study, the multi-scale toxic effects of neonicotinoids on zebrafish embryos under exposure at different temperatures were investigated by using standard FET test. The neonicotinoids significantly inhibited the AChE activity but significantly promoted the GST activity, while GPX activity and ATPase activity were enhanced in the low-concentration treatment group and inhibited by high concentrations of neonicotinoids. Dinotefuran, imidacloprid, and thiamethoxam were less toxic than cycloxyprid, nitenpyram, and paichongding. Neurotoxicity, oxidative stress responses such as changes of GPX and GST activities, and metabolic dysfunction indicators such as ATPase activities may be the key endpoints of neonicotinoid toxicity as revealed from our study. These multi-biomarkers can be used for monitoring the early contamination or long-term exposure of trace contaminants in water environments. Temperature-dependent toxicity thresholds (e.g., LC/EC_{50} ,

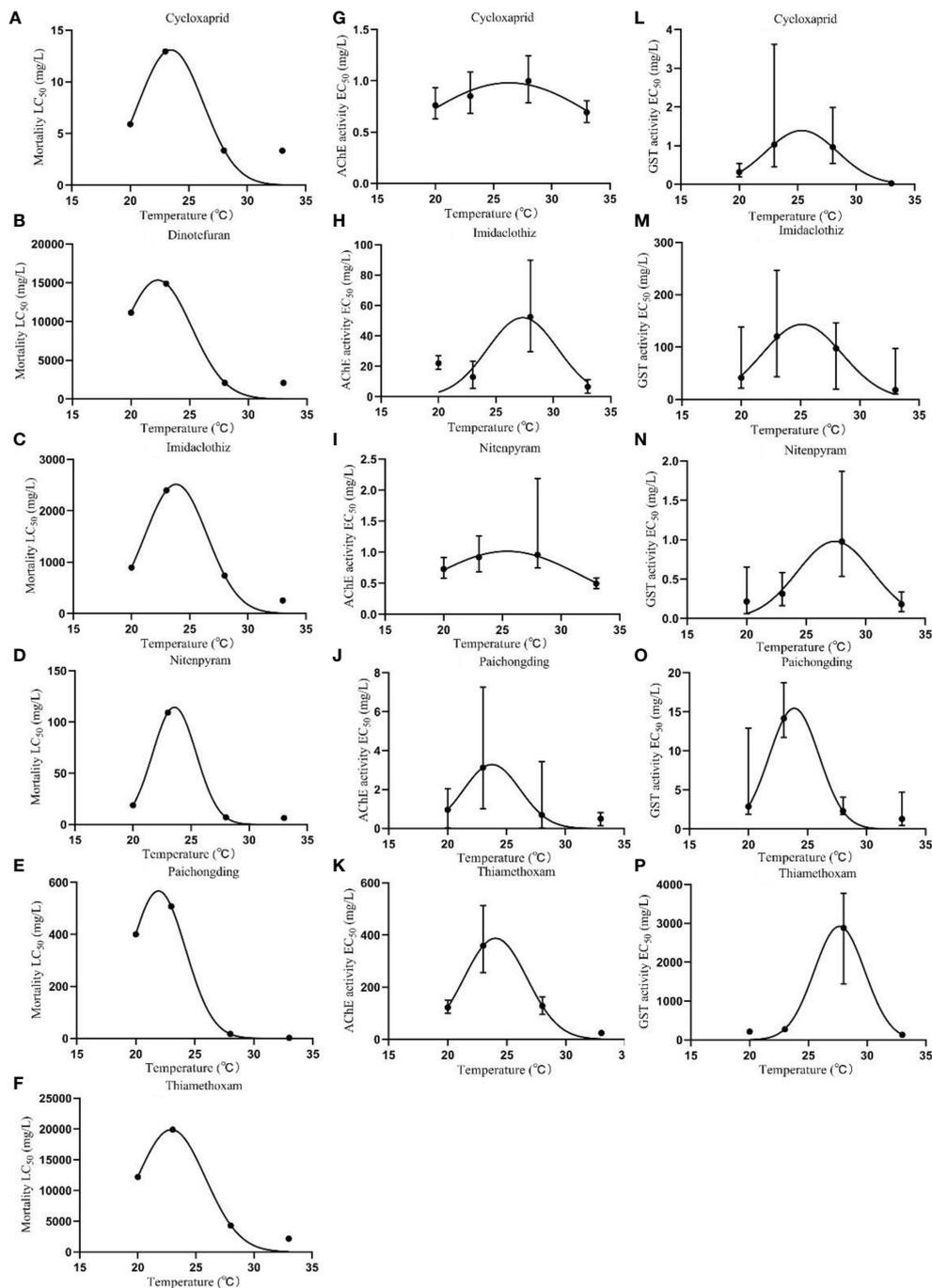


FIGURE 1

Temperature-dependent chemical toxicity of neonicotinoids to zebrafish embryos. (A–F) Effect of temperature on the lethal toxicity (LC_{50}). (G–K) Effect of temperature on the toxicity threshold (EC_{50}) of AChE inhibitory effect. (L–P) Effect of temperature on the toxicity threshold (EC_{50}) of glutathione S-transferase activation.

LOEC, and NOEC) at multi-endpoints were determined by lethal and sublethal toxicity tests to fill the data gaps on neonicotinoids. The influence of temperature on neonicotinoid toxicity to zebrafish embryos represented an inverted V-shaped relationship where toxicity increased with increasing and

decreasing temperature from T_{opt} . Therefore, the influence of environmental parameters, especially temperature, should be considered in future toxicity testing and risk assessment of contaminants of emerging concern, including neonicotinoids, and such an inverted V-shaped model can support the

TABLE 3 Effect of temperature on the lethal and sublethal toxicity of neonicotinoids (R^2 represents the goodness of nonlinear regression curves fitted by Gaussian distribution in GraphPad Prism (version 5.0, San Diego, CA, USA).

Neonicotinoids	Lethal effect (LC ₅₀)		Effect of AChE inhibition (EC ₅₀)		Effect of GST promotion (EC ₅₀)	
	R^2	T_{opt} (°C)	R^2	T_{opt} (°C)	R^2	T_{opt} (°C)
Cyloxaprid	0.824	23.5	0.897	26.3	0.998	25.3
Dinotefuran	0.966	22.3	ND	ND	ND	ND
Imidaclothiz	0.976	23.8	0.668	27.3	0.985	25.1
Nitenpyram	0.994	23.6	0.989	25.4	0.935	27.4
Paichongding	0.999	21.9	0.944	23.8	0.985	23.9
Thiamethoxam	0.977	22.9	0.991	24.0	0.992	27.7

The optimum temperature (T_{opt}) refers to the temperature corresponding to the highest amplitude of the Gaussian curve, at which point the toxicity threshold is the highest, meaning the least toxicity.
ND, no available data.

prediction of temperature-dependent chemical toxicity to aquatic organisms and the enactment of appropriate water quality guidelines for protecting aquatic ecosystems under different thermal scenarios.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee in Shantou University (Approval Number: 20221205).

Author contributions

YYLW: investigation, methodology, and writing—original draft. Y-EC: investigation and writing—review and editing. SK, JY, PL, and WL: writing—review and editing. YW: investigation, supervision, and writing—review and editing. ZW: conceptualization, supervision, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1101737/full#supplementary-material>

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