RESEARCH

Insights into the combined toxic impacts of phoxim and deltamethrin on the embryo-larval stage of zebrafish (*Danio rerio*)

Lu Lv[1], Zhongwen Gao[2], Liangang Mao[3], Xinju Liu[1], Qiang Wang[1], Weifeng Shen[1*] and Yanhua Wang[1*]

Abstract

Background: Pesticides are usually applied as mixtures, and their joint impacts can generate substantial toxicity to organisms. Although exposures to chemical pesticide mixtures make up most occurrences of pesticide exposures, minimal concern has been given to their combined toxicity and interplays to date. In the present study, endpoints of multiple levels were determined to examine the combined toxic impacts of phoxim and deltamethrin on zebrafish (*Danio rerio*).

Results: Our study showed that the LC_{50} values of phoxim obtained over a 96-h exposure period for *D. rerio* during different life stages ranged from 0.24 (0.12–0.33) to 3.39 (2.58–4.86) µM, and those of deltamethrin ranged from 0.0041 (0.0031–0.0060) to 2.97 (1.56–4.69) µM. Combinations of phoxim and deltamethrin displayed synergistic effects on zebrafish embryos. The activities of T-SOD, Cu/Zn-SOD, POD, and CarE varied dramatically under most administrations of phoxim, deltamethrin, and phoxim + deltamethrin combinations relative to the baseline value. Nine genes, namely, *Mn-sod*, *Cu/Zn-sod*, *cas3*, *dio1*, *tsh*, *ERa*, *vtg1*, *cyp17*, and *crh*, related to antioxidation, cell apoptosis, immunity, and the endocrine system were altered to a greater degree under the mixture administration compared with the individual administrations.

Conclusions: In summary, our current data offered a detailed insight into the combined toxic impacts of pesticide mixtures at various endpoints and over a wide range of concentrations. The results emphasized the necessity to consider the administration mixtures during the ecological risk assessment of pesticides.

Keywords: Combined toxicity, Aquatic vertebrates, Biomarker, Synergetic impact

Background

In agricultural production, pesticides are often applied to control pests and diseases in various plants [1, 2]. However, most pesticides do not remain attached to crops and are eventually released into aquatic environments through runoff, spray drift, and other pathways, resulting in extensive residue accumulation [3]. Additionally, pesticides that contaminate surface water usually contain a mixture of substances rather than an individual compound, and many types of crops are generally intermingled in agricultural areas [4]. This results in detrimental effects on ecosystems and threatens environmental organisms [5]. Present regulatory procedures for prioritization and environmental quality standards mainly focus on individual compounds [6, 7]. However, a mixture of pesticides may usually pose a greater risk than each

*Correspondence: weifsh@126.com; wangyanh79@hotmail.com

[1] State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Institute of Agro-Product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, Zhejiang, China
Full list of author information is available at the end of the article

Lv *et al. Environmental Sciences Europe*   (2022) 34:90

Page 2 of 16

constituent alone [8]. Consequently, compliance with environmental quality standards for individual pesticides may not sufficiently protect aquatic organisms against toxic impacts from exposure to pesticide mixtures.

The pollution of water ecosystems by pesticides may adversely affect non-target organisms, such as fish [9]. As a valuable model in aquatic ecotoxicology, zebrafish (*Danio rerio*) has been used in different research fields, including aquatic ecotoxicology [10, 11]. Among its several practical merits, including relatively easy maintenance and culturing, its embryos are transparent, and most organ systems can be fully developed within 96 h post-fertilization (hpf) [12]. The experiment on zebrafish embryos following the 3R (Replacement, Reduction, and Refinement) principles of animal research under acute exposure has replaced the mandatory adult fish acute test for the toxicity assessment of wastewater in some European countries [13, 22]. Additionally, exposure to low concentrations of pesticides during the early life stages of *D. rerio* can negatively affect their biology and the characteristics formed during later development stages [12]. However, previous studies on pesticides have mainly focused on their individual toxicity to zebrafish embryos, whereas the possible toxicities caused by pesticide mixtures have seldom been investigated [14–16].

A mixed formulation of organophosphate insecticide phoxim (PHO) and pyrethroid insecticide deltamethrin (DEL) is commonly used all over the world to reduce insect resistance and improve control efficiency and/or economic concerns [17]. Therefore, their residues are likely to coexist in the same aquatic environment [18]. Despite the coexistence of PHO and DEL in water samples, their ecological and health risk evaluations have been primarily carried out based on the toxicological impacts of single compounds [19–21]. The interaction between PHO and DEL has not yet been examined, and their combined toxicity at low doses and the underlying toxicity pathways remain largely unknown. This study investigated the acute toxicity, enzymatic activities, and gene expressions in embryonic zebrafish co-exposed to PHO and DEL. A comprehensive evaluation of PHO and DEL at various endpoints offered noteworthy insights into the overall toxicity triggered by their combinations in zebrafish and the potential mechanism.

Materials and methods
Ethical note
All rearing and exposure procedures were conducted according to the Experimental Animal Management Law of China and authorized by the Independent Animal Ethics Committee of the Zhejiang Academy of Agricultural Sciences.

Pesticides and reagents
PHO (purity of 97%) was obtained from Lianyungang Liben Agrochemical Group (Lianyungang, China). DEL (purity of 98%) was donated by Nanjing Red Sun Chemical Co., Ltd. (Nanjing, China). To prepare the stock solutions, pesticides were dissolved in *N*, *N*-dimethylformamide (DMF) and 10% Tween-80 (Wt: Vol) and then preserved at 4 ℃. These stock solutions were then further diluted to the required concentrations using reconstituted water [22]. Reagents of analytical grade were used in this study.

Experimental animals
Adult zebrafish (AB strain) were used as breeding stocks and purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The organisms were reared in a flow-through system using dechlorinated and aerated water (27 ± 1 ℃, 14-h light:10-h dark) and fed twice with a commercial fish diet (Tetramin) ad libitum daily [7, 25]. For propagation, sexually mature fish at a female/male ratio of 1:2 were separated by isolation boards in spawning containers with a net bottom (Esen Corp, Beijing, China) overnight. Spawning was induced by the light stimulation at seven o'clock in the following morning, and the isolation boards were removed. Embryos were collected within 30 min of light exposure and rinsed with reconstituted water. Viable embryos with normal developmental blastula were transferred into crystallizing dishes (bottom diameter of 10 cm) incorporating Hank's solution.

Detections of single and combined toxicity
Toxicity detection of single pesticides
The acute toxicities of single chemicals to zebrafish at various life stages (embryonic, larval, juvenile, and adult stages) were examined according to OECD guidelines [23, 24]. Embryos at 3 hpf and larvae at 72 h post-hatching were selected for embryo and larva experiments, respectively. Juvenile zebrafish of 1 month old and adult zebrafish of 3 months old were subjected to food deprivation for 24 h before the toxicity test. At least 4 concentrations with a geometrical ratio that caused 10–90% mortality according to the results from pre-trails were assayed for every pesticide. Each concentration and control were tested in triplicate. The optimal dose of chemicals and water quality were kept by exchanging the exposure solutions every 12 h. The temperature and light cycle remained the same as those in the culture environment during exposure. Mortality was assessed after exposure for 96 h. Detailed information on the test procedure

during the multiple life stages is provided in the Additional file 1.

Combined toxicity detection

The combined toxicity of PHO and DEL was evaluated using zebrafish embryos. The toxicities of the single chemicals were linearly compared with their combined toxicity. To investigate the combined impacts of PHO and DEL, the zebrafish were subjected to serial dilutions of each chemical with a fixed equitoxic ratio according to the obtained single LC_{50} values. The sum doses of the mixture were systematically changed, while all of the above-mentioned factors were kept constant to construct the dose–response correlation. All tests were conducted three times for each dose. The other test programs of the equitoxic test were similar to the acute embryo toxicity tests for single chemicals.

Detections of biochemical and molecular parameters
Sample preparation

The doses of 1/400, 1/100, and 1/25 of 96-h LC_{50} value for each chemical to the embryos were deemed as the low, moderate, and high doses, respectively. Accordingly, low, moderate, and high doses in the mixture exposure of PHO + DEL (MIT) were the combinations of both PHO and DEL at the corresponding doses. Approximately 300 embryos at 3 hpf were placed into an 800 mL beaker containing 800 mL pesticide test solution as a replicate. Each experiment was repeated three times for each dose. After exposure for 96 h, the treated fish were collected. The collected specimens were preserved at − 80 ℃ for further experimentation.

Tests of biochemical parameters

About 200 fish from each beaker were placed in 50 mM potassium phosphate buffer (pH 7.0) consisting of 0.5 mM EDTA and then subjected to homogenization (1: 20, *w/v*) adopting an electric homogenizer. The tissue homogenate was centrifuged at 12,000 rpm for 30 min at 4 ℃, and the supernatant was applied to analyze biochemical indexes.

The parameters, including oxidative stress, apoptotic enzymes, detoxification enzymes, vitellogenin, and thyroid hormones, were detected using the available commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to previously established protocols [25]. Protein concentrations were examined using the Bradford method [26], and bovine serum albumin (BSA) was adopted to construct the standard curve. Details on the biochemical indices tested are found in the Additional file 1: Table S1.

Gene expression analysis

About 30 fish from each beaker were harvested and homogenized. Total RNA was extracted from the tissue homogenates using TransZol Up Plus RNA Kit (TransGen Biotech Ltd., China). Gene expressions were examined by quantitative real-time PCR (qRT-PCR) using previously established protocols [25]. Briefly, total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China). In addition, *β*-actin was taken as the internal reference gene. The primer sequences are listed in Additional file 1: Table S2. The relative expressions of the target genes were calculated using the $2^{-\triangle\triangle Ct}$ method [27].

Chemical analysis

In order to determine the actual concentrations of PHO, DEL, and their mixtures, the water samples were monitored at the start (0 h) and before water renewal (12 h) during the experimental period. The water samples were filtered using a 13 mm filter (0.25 μm). The samples containing PHO and/or DEL were analyzed using liquid chromatography-tandem mass spectrometry (SHIMADZU, LCMS-8050) performed on an ACE C18 column (2.1 mm × 100 mm, 1.7 μm) with a mobile phase composed of an aqueous solution (5 mmol/L ammonium formate, A) and methanol (B). The separation was conducted using a gradient elution program as follows: 0–0.5 min, 95% A; 0.5–2.0 min, 5% A; 2.0–5.0 min, 5% A; 5.0–5.1 min, 95% A; 5.1–7.0 min, 95% A. Mass spectrometric detection was conducted under positive electrospray ionization (ESI) with a multiple reaction monitoring (MRM) mode. For PHO, the precursor ion was *m/z* 299.0, and the product ions were *m/z* 77.1 for quantization and *m/z* 129.1 for confirmation, with collision energies of 26 and 10 eV, respectively. For DEL, the precursor ion was *m/z* 523.0, and the product ions were *m/z* 281.0 for quantization and *m/z* 506.0 for confirmation, with collision energies of 16 and 11 eV, respectively. The analysis results revealed that the deviations of the nominal and the actual concentrations of PHO, DEL, and their mixtures were less than 20%. Accordingly, the nominal concentration was used as the actual concentration in this study.

Statistical analysis

The acute toxicities of pesticides to *D. rerio* were analyzed by adopting a probit analysis described by Chi [28]. The interplay pattern of the chemical combinations was assessed according to an additive index (AI) method [29, 30]. Details about the AI method are provided in the Additional file 1. The one-way ANOVA

Lv *et al. Environmental Sciences Europe*   (2022) 34:90

Page 4 of 16

statistical divergence was determined using a Dunnett's post-hoc test conducted with SPSS software (SPSS version 18.0, USA).

Results

Single pesticide toxicity determinations

Results from the single pesticide toxicity tests showed that the toxic selectivity of various chemicals to zebrafish varied greatly, and each chemical showed conspicuous toxicities to various life periods of *D. rerio*. PHO showed a narrower toxic range in different developmental periods of zebrafish, with the 96-h LC_{50} values ranging from 0.24 (0.12–0.33) to 3.39 (2.58–4.86) µM. In contrast, DEL elicited a broader toxic scope to the tested fish, with the 96-h LC_{50} values ranging from 0.0041 (0.0031–0.0060) to 2.97 (1.56–4.69) µM (Table 1). Overall, the embryonic stage was the most tolerant, while the adult period was the most sensitive to the 2 pesticides.

Mixture toxicity determinations

The combination of PHO and DEL resulted in synergistic effects, and the corresponding AI values after exposure for 24, 48, 72, and 96 h were 2.29, 2.50, 3.41, and 4.24, respectively. Additionally, the AI values for the combination were increased with the increase in exposure time, indicating that the combined toxicity was positively associated with the exposure period (Table 2).

Biochemical index determinations

Evaluation of oxidative stress

The MDA level was noticeably enhanced under all the administrations (apart from the high dose of PHO and MIT administrations) relative to the baseline value. In contrast, a conspicuous decrease was found under the low and moderate doses of MIT administration relative to the corresponding PHO administration. The MDA level was also conspicuously reduced under the moderate dose of MIT administration relative to the corresponding DEL administration (Fig. 1A). The CAT activity was conspicuously elevated under all PHO and DEL treatments (apart from the moderate PHO and low DEL treatments) relative to the baseline value. Additionally, a dramatic enhancement was also observed under the low dose of MIT treatment relative to the baseline value and corresponding DEL treatment (Fig. 1B). T-SOD activity was dramatically raised under all the treatments relative to the baseline value. Conversely, an obvious decrease was recorded under the moderate dose of MIT treatment relative to the corresponding PHO treatment (Fig. 1C). Similar to the MDA level, the Cu/Zn-SOD activity was distinctly induced under all PHO and DEL treatments (apart from the high dose of PHO treatment) relative to the baseline value. Additionally, a distinct up-regulation was also observed under the low dose of MIT treatment relative to the baseline value. Contrastingly, its activity was conspicuously inhibited under the moderate dose of MIT treatment relative to the baseline value, as well as corresponding PHO or DEL treatments (Fig. 1D).

Table 1 Acute toxicity of phoxim and deltamethrin to various life stages of zebrafish

Pesticide	LC_{50} (95% FL)[a] µM			
	Embryo	**Larvae**	**Juvenile**	**Adult**
Phoxim	1.44 (0.91–2.08)	0.64 (0.46–0.84)	3.39 (2.58–4.86)	0.24 (0.12–0.33)
Deltamethrin	2.97 (1.56–4.69)	0.26 (0.19–0.35)	0.0041 (0.0031–0.0060)	0.065 (0.030–0.091)

[a] FL fiducial limit

Table 2 Mixture toxicities of phoxim and deltamethrin to zebrafish embryos

Exposure time (h)	LC_{50} (95% FL)[a] µM		LC_{50} (95% FL)[b] µM		AI[c] value
	Phoxim	**Deltamethrin**	**Phoxim**	**Deltamethrin**	
24	4.63 (3.55–5.73)	15.01 (9.94–24.23)	0.70 (0.57–0.87)	2.28 (1.84–2.83)	2.29
48	3.05 (2.45–3.86)	12.85 (8.69–17.93)	0.44 (0.33–0.54)	1.82 (1.37–2.26)	2.50
72	2.24 (1.41–3.19)	9.64 (5.03–13.61)	0.26 (0.19–0.28)	1.09 (0.85–1.21)	3.41
96	1.44 (1.01–1.91)	2.97 (1.56–4.69)	0.14 (0.091–0.19)	0.28 (0.19–0.42)	4.24

[a] The LC_{50} (95% fiducial limit) for phoxim or deltamethrin individually

[b] The LC_{50} (95% fiducial limit) for phoxim or deltamethrin in the mixture
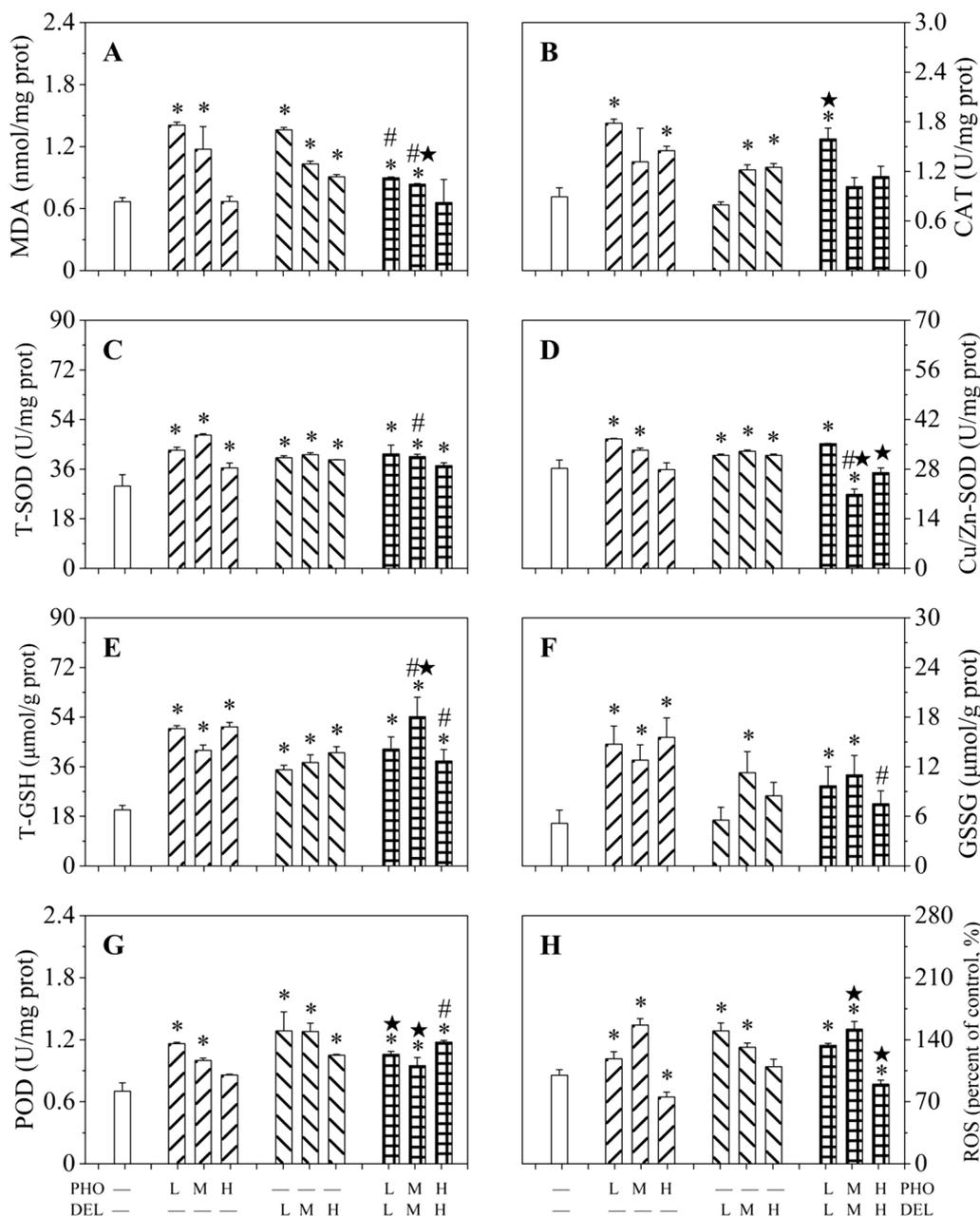
[c] AI additive index

Fig. 1 The oxidative responses in zebrafish treated with PHO, DEL, and PHO + DEL combinations. **A** MDA; **B** CAT; **C** T-SOD; **D** Cu/Zn-SOD; **E** T-GSH; **F** GSSG; **G** POD; **H** ROS. The results are presented as the means ± SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose

Similar to the T-SOD activity, the level of T-GSH was apparently increased under all the treatments relative to the baseline value. Additionally, a conspicuous rise was also observed under the moderate dose of MIT treatment relative to the corresponding PHO or DEL treatment. However, its level was obviously decreased under the

high dose of MIT treatment relative to the corresponding PHO treatment (Fig. 1E). The GSSG activity was obviously enhanced under all PHO and MIT treatments (apart from the high dose of MIT treatment) relative to the baseline value. In addition, a clear increase was also found under the moderate dose of DEL treatment relative

to the baseline value. Nevertheless, its level was distinctly reduced under the high dose of MIT treatment relative to the corresponding PHO treatment (Fig. 1F). The POD activity was considerably rised under all the treatments (except for the high dose of PHO treatment) relative to the baseline value. Moreover, a conspicuous elevation was also seen under the high dose of MIT treatment relative to the corresponding PHO treatment. Conversely, its activity was conspicuously reduced under the low and moderate doses of MIT treatment relative to the corresponding DEL treatment (Fig. 1G). The ROS level was apparently elevated under all the treatments (except for their treatments at the high dose) relative to the baseline value. An apparent up-regulation was also seen under the moderate dose of MIT treatment relative to the

corresponding DEL treatment. Conversely, its level was apparently suppressed under the high dose of MIT treatment relative to the baseline value and corresponding DEL treatment (Fig. 1H).

The activities of apoptotic and detoxification enzymes

The activity of Caspase 3 was pronouncedly increased under the moderate dose of PHO treatment and the low dose of DEL treatment relative to the baseline value. In contrast, a pronounced reduction was monitored under the high doses of PHO and DEL treatments and the low dose of PHO treatment relative to the baseline value. Its activity was pronouncedly decreased under the low dose of MIT treatment relative to the corresponding DEL treatment (Fig. 2A). The activity of Caspase 9 slightly
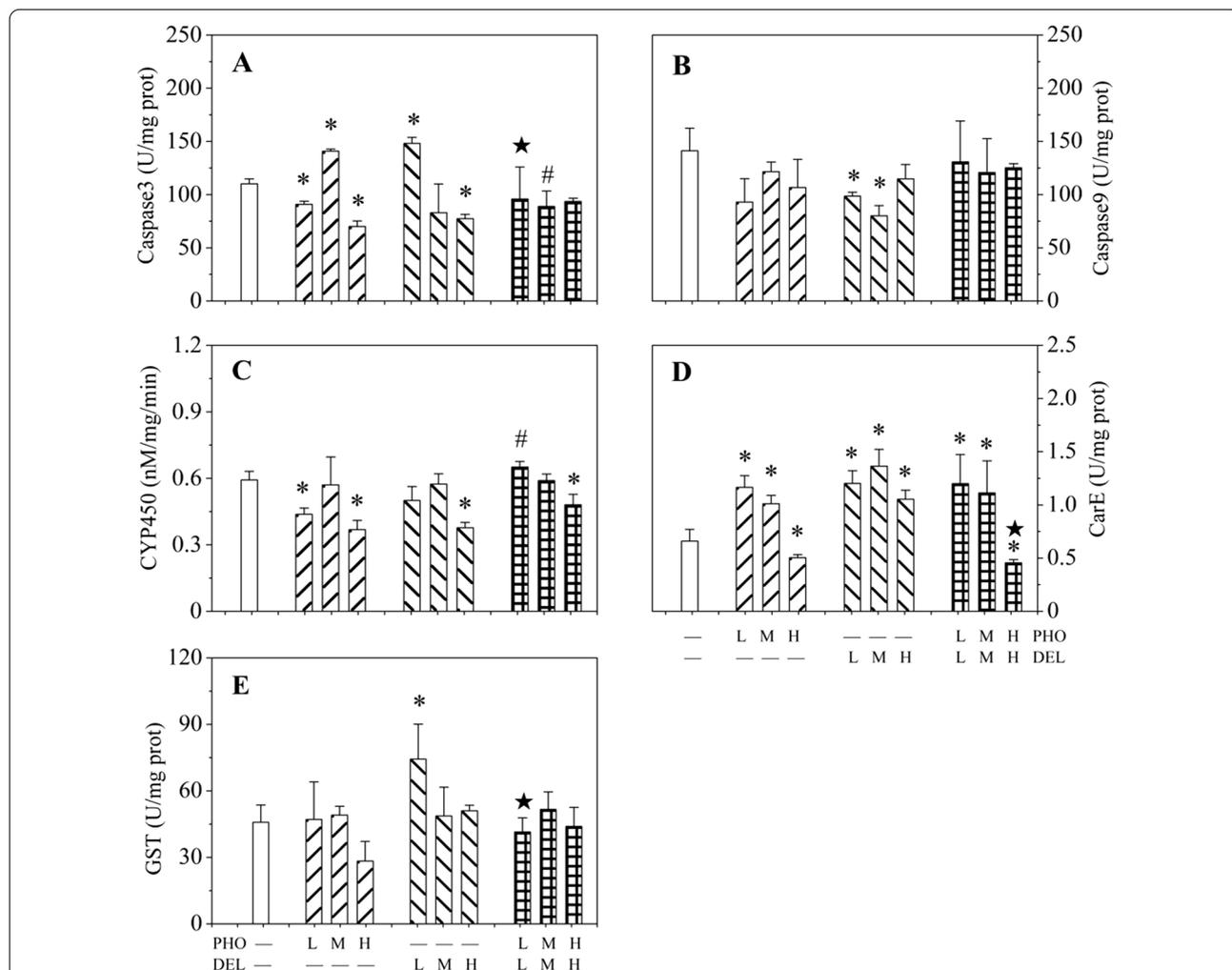

Fig. 2 The apoptotic and detoxification enzyme activities in zebrafish treated with PHO, DEL, and PHO + DEL combinations. **A** Caspase 3; **B** Caspase 9; **C** CYP450; **D** CarE; **E** GST. The results are presented as the means ± SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose

changed under all the treatments, apart from the low and moderate doses of DEL treatment, in which its activity was markedly weakened relative to the baseline value. No significant change was detected under all MIT treatments relative to the corresponding PHO or DEL treatments (Fig. 2B).

The activity of CYP450 was significantly reduced under all the treatments at the high dose relative to the baseline value. A noticeable reduction was also seen under the low dose of PHO treatment relative to the baseline value. Contrastingly, its activity was conspicuously elevated under the low dose of MIT treatment relative to the corresponding PHO treatment (Fig. 2C). The activity of CarE was markedly elevated under all the treatments (apart from the high doses of PHO and MIT treatments) relative to the baseline value. Conversely, significant inhibition was observed under the high dose of PHO and MIT treatments relative to the baseline value. Its activity was also dramatically inhibited under the high dose of MIT exposure relative to the corresponding DEL exposure (Fig. 2D). The activity of GST was conspicuously increased under the low dose of DEL exposure relative to the baseline value. Contrarily, its activity was remarkably decreased under the low dose of MIT exposure relative to the corresponding DEL exposure (Fig. 2E).

Contents of THs and VTG

The content of T3 was conspicuously rised under the moderate dose of PHO treatment relative to the baseline value. A distinct enhancement was also found under all MIT treatments (apart from the moderate dose of MIT treatment) relative to the baseline value, as well as corresponding PHO and DEL treatments. Nevertheless, its activity was obviously reduced under the high dose of DEL treatment relative to the baseline value (Fig. 3A).

The content of VTG was remarkably reduced under all PHO treatments relative to the baseline value. A marked reduction was also seen under the low dose of MIT treatment relative to the baseline value and corresponding DEL exposure. In contrast, its content was conspicuously rised under the moderate and high doses of MIT exposure relative to the corresponding PHO exposure (Fig. 3B).

Gene expression analysis

Expressions of antioxidation- and cell apoptosis-related genes

The expressions of antioxidation-related genes (*Mn-sod* and *Cu/Zn-sod*) were analyzed in our study. The expression level of *Mn-sod* was positively correlated with the dose of PHO, and there was a significant induction under all PHO treatments relative to the baseline value. Moreover, a dramatic elevation was found under the moderate dose of DEL and MIT treatments relative to the baseline value. Under the MIT treatment, a distinct elevation was also detected in all doses (apart from the moderate dose of MIT) relative to the baseline value, as well as corresponding PHO and DEL treatments (Fig. 4A). The expression level of *Cu/Zn-sod* was distinctly enhanced under the moderate and high doses of PHO treatment and the moderate dose of DEL treatment relative to the baseline value. Additionally, a marked up-regulation was also detected under all doses of MIT treatment relative to the baseline value. Meanwhile, it was also up-regulated under the low and high doses of MIT treatment relative to the corresponding PHO and DEL treatments (Fig. 4B).

The expressions of apoptotic genes (*p53*, *cas3*, and *cas9*) were analyzed in the present study. Relative to the baseline value, the expression of *p53* was dramatically rised under the moderate dose of PHO treatment and low dose of DEL treatment. Nevertheless, a noticeable reduction
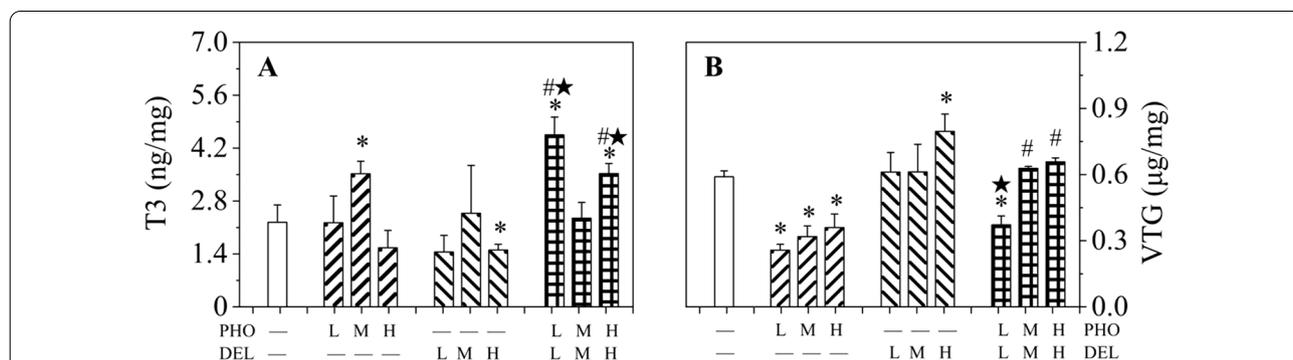

Fig. 3 **A** T3 and **B** VTG levels in zebrafish treated with PHO, DEL, and PHO+DEL combinations. The results are presented as the means±SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose

Lv *et al. Environmental Sciences Europe*　(2022) 34:90

Page 8 of 16

Fig. 4 Impacts on expressions of genes involved in oxidative stress and cellular apoptosis in zebrafish treated with PHO, DEL, and PHO + DEL combinations. **A** *Mn-sod*; **B** *Cu/Zn-sod*; **C** *p53*; **D** *cas3*; **E** *cas9*. The results are presented as the means ± SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose
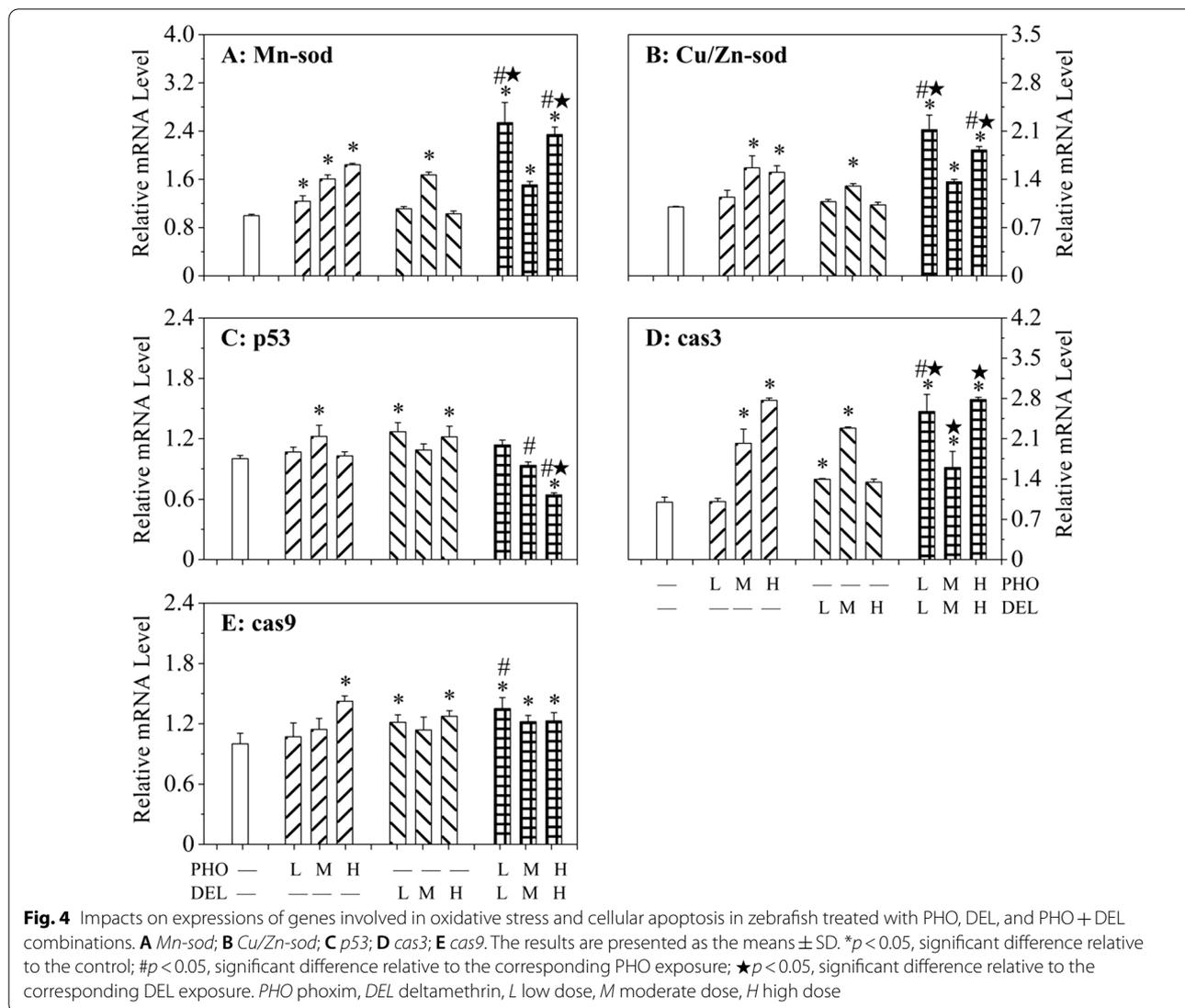
was seen under the high dose of MIT treatment relative to the baseline value and corresponding individual treatments (Fig. 4C). The expression of *cas3* was apparently rised under all treatments (except for the low dose of PHO and high dose of DEL) relative to the baseline value. Meanwhile, a marked elevation was also seen under the low dose of MIT treatment relative to the corresponding PHO and DEL treatments. A noticeable reduction was seen under the moderate dose of MIT treatment relative to the corresponding DEL treatment (Fig. 4D). The expression level of *cas9* was markedly induced under all DEL and MIT treatments (apart from the moderate dose of DEL treatment) relative to the baseline value. A noticeable rise was also observed under the high dose of PHO treatment relative to the baseline value. Additionally, the expression of the *cas9* gene was also markedly induced

under the low dose of MIT treatment relative to the corresponding PHO treatment (Fig. 4E).

Expressions of the genes related to HPT and HPG axes in the endocrine system

The mRNA expressions of genes in the HPT axis (*TRα*, *dio1*, and *tsh*) were analyzed (Fig. 5). The expression of *TRα* was remarkably reduced under the moderate dose of DEL treatment relative to the baseline value. In contrast, a significant increase was seen under the low and high doses of DEL treatment relative to the baseline value. Its expression was also conspicuously rised under the moderate dose of MIT treatment relative to the corresponding DEL administration (Fig. 5A). The expression of *dio1* was up-regulated under all administrations (apart from the low and high doses of DEL treatment) relative to the baseline value. Besides, a positive correlation was
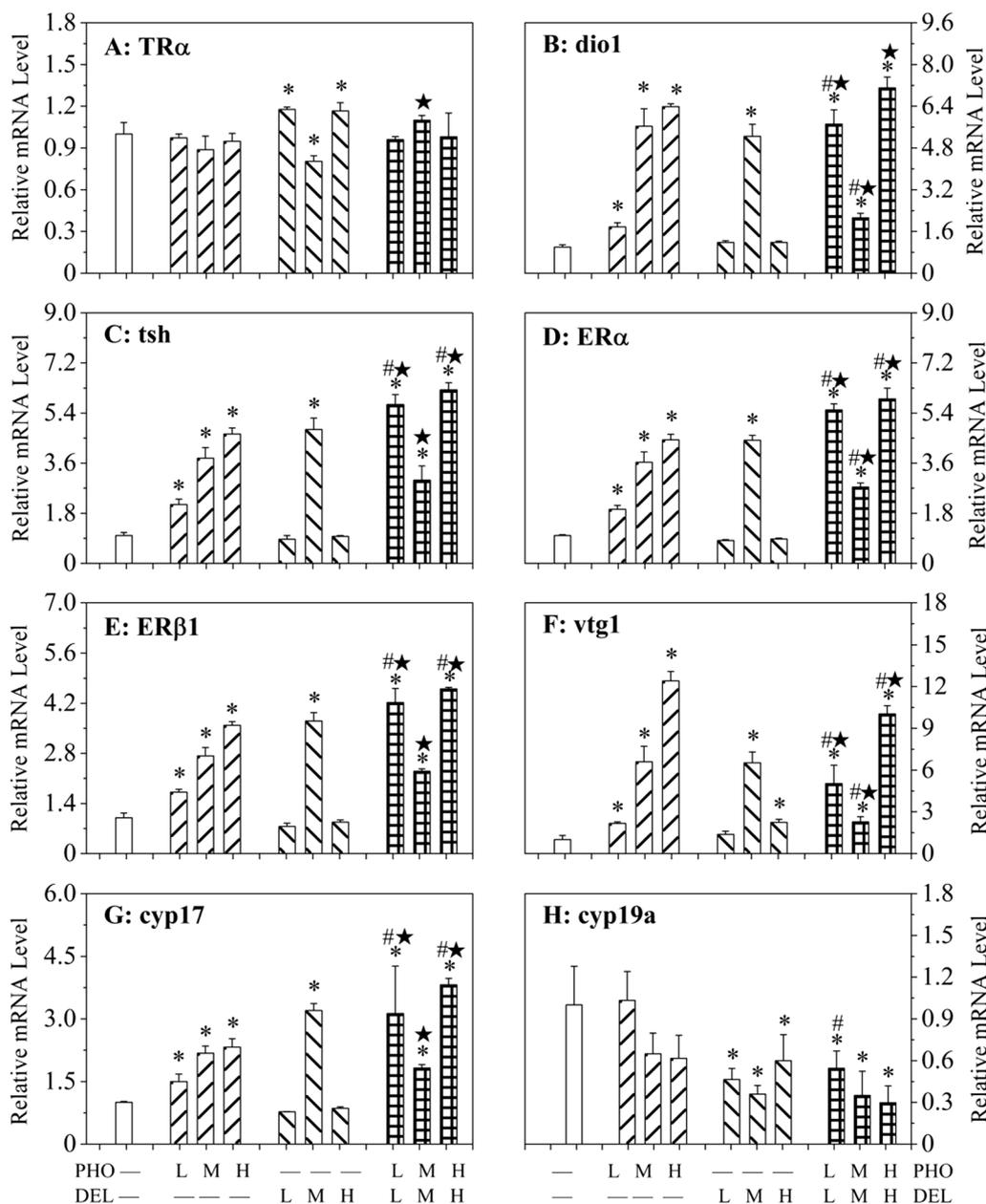
Lv *et al. Environmental Sciences Europe* (2022) 34:90

Page 9 of 16

Fig. 5 Impacts on expressions of genes involved in the HPT and HPG axes of zebrafish treated with PHO, DEL, and PHO + DEL combinations. **A** *TRa*; **B** *dio1*; **C** *tsh*; **D** *ERa*; **E** *ERβ1*; **F** *vtg1*; **G** *cyp17*; **H** *cyp19a*. The results are presented as the means ± SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose

found between the expression of *dio1* and the dose of the PHO. A large increase was seen under the low and high doses of MIT treatment relative to the corresponding DEL administration. Contrastingly, a conspicuous reduction was observed under the moderate dose of MIT treatment relative to the corresponding PHO and DEL administrations (Fig. 5B). The expression of *tsh* was

increased dramatically under the PHO administration in a dose-dependent manner relative to the baseline value. A significant increase was seen under the moderate dose of DEL administration relative to the baseline value, and the same change was observed under the MIT administration. The expression was markedly rised under the low and high doses of MIT administration relative to the

Lv *et al. Environmental Sciences Europe*   (2022) 34:90

Page 10 of 16

corresponding PHO and DEL administrations, respectively. Nevertheless, a pronounced inhibition was seen under the moderate dose of MIT administration relative to the corresponding DEL administration (Fig. 5C).

The expressions of HPG axis genes (*ERα*, *ERβ1*, *vtg1*, *cyp17*, and *cyp19a*) were analyzed in our study. In the PHO administration, the expression level of *ERα* was remarkably rised relative to the baseline value, which was positively correlated with the administration dose. An apparent increase was also observed under the moderate dose of DEL treatment and all doses of MIT treatment relative to the baseline value. The expression was markedly increased under the low and high doses of MIT treatment, while it was conspicuously decreased under the moderate dose of MIT administration relative to the corresponding PHO and DEL administrations (Fig. 5D). The expression level of *ERβ1* was noticeably induced under all doses of PHO treatment, the moderate dose of DEL treatment, and all doses of MIT treatment relative to the baseline value. Moreover, a conspicuous increase was observed under the low and high doses of MIT treatment relative to the corresponding PHO and DEL administrations, while a distinct down-regulation was seen under the moderate dose of MIT treatment relative to the corresponding DEL administration (Fig. 5E).

The expression of *vtg1* was significantly increased under all administrations (apart from the low dose of DEL treatment) relative to the baseline value. Additionally, its expression under the high dose of PHO treatment was over 12 times higher than the baseline value. A significant induction under the low and high doses of MIT treatment and a significant reduction under the moderate dose of MIT treatment were detected relative to the corresponding PHO and DEL administrations (Fig. 5F). The expression level of *cyp17* was noticeably induced under all PHO administrations relative to the baseline value. Additionally, a distinct increase was monitored under the moderate dose of DEL and MIT treatments relative to the baseline value. The expression of *cyp17* was also markedly increased under the low and high doses of MIT treatment relative to the baseline value, as well as corresponding PHO and DEL administrations (Fig. 5G). The expression of *cyp19a* was conspicuously suppressed under all doses of DEL and MIT treatments relative to the baseline value, and its expression was negatively correlated with the dose of MIT treatment. Meanwhile, a conspicuous decrease was observed under the low dose of MIT treatment relative to the corresponding PHO administration (Fig. 5H).
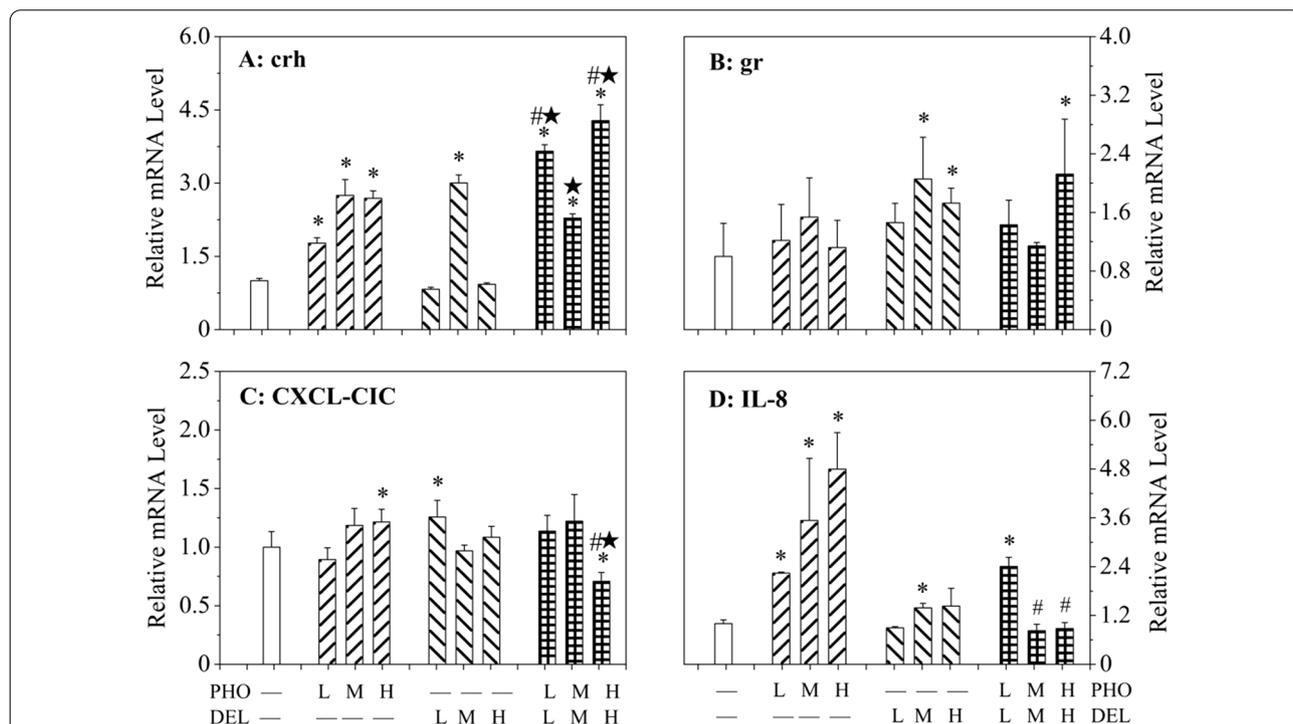

Fig. 6 Impacts on expressions of genes related to HPA axis and immune system of zebrafish treated with PHO, DEL, and PHO + DEL combinations. **A** *crh*; **B** *gr*; **C** *CXCL-CIC*; **D** *IL-8*. The results are presented as the means ± SD. *$p < 0.05$, significant difference relative to the control; #$p < 0.05$, significant difference relative to the corresponding PHO exposure; ★$p < 0.05$, significant difference relative to the corresponding DEL exposure. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose
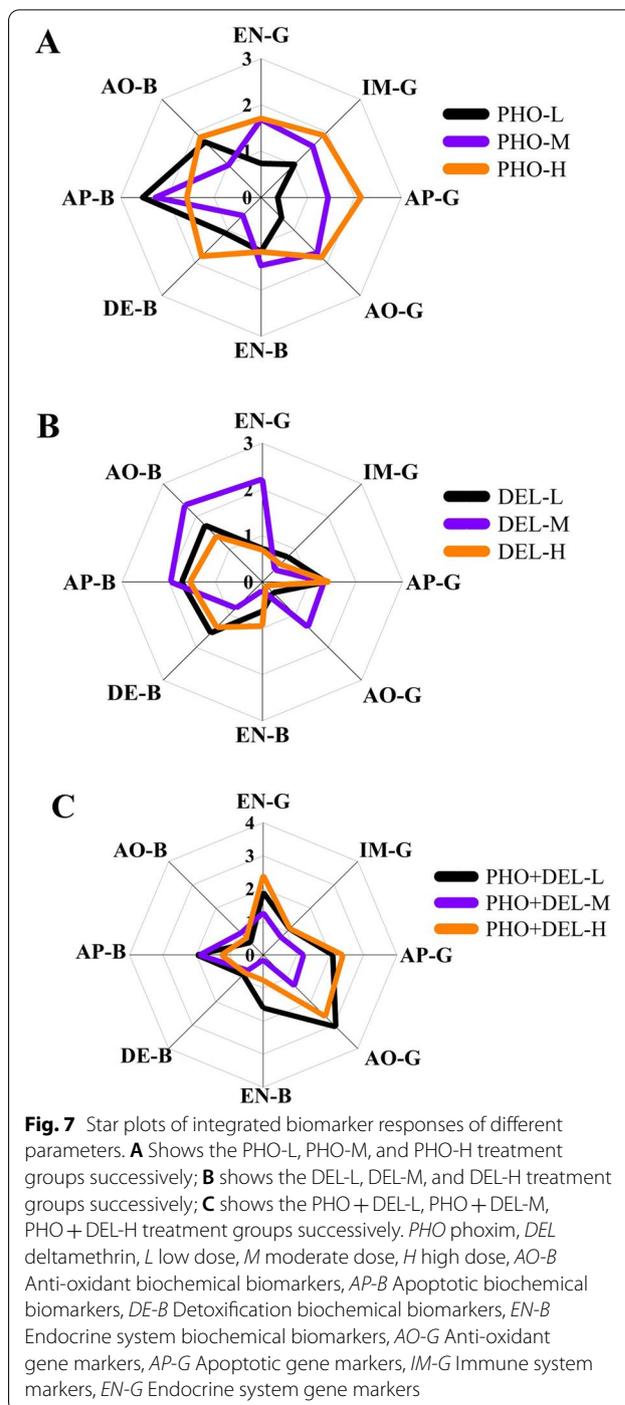
Expressions of the genes related to HPA axis and immunity system

The expressions of the HPA axis genes (*crh* and *gr*) were analyzed (Fig. 6). The expression of *crh* was conspicuously enhanced under all administrations (apart from the low and high doses of DEL treatment) relative to the baseline value. A conspicuous increase was also detected under the low and high doses of MIT treatment relative to the corresponding PHO and DEL administrations, while a conspicuous reduction was seen under the moderate dose of MIT treatment relative to the corresponding DEL administration (Fig. 6A). The expression of *gr* gene was conspicuously rised under the high dose of DEL and MIT treatments relative to the baseline value. A dramatic increase was also observed under the moderate dose of DEL treatment relative to the baseline value (Fig. 6B).

The expressions of the genes related to immunity response (*CXCL-CIC* and *IL-8*) were analyzed in the present study. The *CXCL-CIC* gene expression level was remarkably increased under the low dose of DEL treatment and high dose of PHO treatment relative to the baseline value. On the contrary, a conspicuous down-regulation was seen under the high dose of MIT treatment relative to the baseline value as well as corresponding PHO and DEL treatments (Fig. 6C). The expression of *IL-8* was positively correlated with the dose of PHO. Moreover, its expression under the high dose of PHO treatment was approximately five times greater than the baseline value. Under the MIT administration, the expression of *IL-8* was noticeably increased under the low dose relative to the baseline value. Nevertheless, a conspicuous decrease was seen under the moderate and high doses of MIT treatment relative to the corresponding PHO administration (Fig. 6D).

Integrated biomarker response (IBR) analysis

In order to assess the toxic impacts of PHO, DEL, and their mixtures at various doses, we integrated all determined biochemical and molecular indicators using an IBR analysis. The IBR index was calculated based on the data of zebrafish embryos treated with pesticides under different doses. The toxicity of single pesticides and their combinations are depicted in the bar graphs and radar charts, as shown in Fig. 7. Among all the tested biochemical and molecular indices, the most sensitive indices for PHO, DEL, and their mixtures were apoptotic biochemical biomarkers, anti-oxidant biochemical biomarkers, and antioxidant gene markers, respectively.

Fig. 7 Star plots of integrated biomarker responses of different parameters. **A** Shows the PHO-L, PHO-M, and PHO-H treatment groups successively; **B** shows the DEL-L, DEL-M, and DEL-H treatment groups successively; **C** shows the PHO + DEL-L, PHO + DEL-M, PHO + DEL-H treatment groups successively. *PHO* phoxim, *DEL* deltamethrin, *L* low dose, *M* moderate dose, *H* high dose, *AO-B* Anti-oxidant biochemical biomarkers, *AP-B* Apoptotic biochemical biomarkers, *DE-B* Detoxification biochemical biomarkers, *EN-B* Endocrine system biochemical biomarkers, *AO-G* Anti-oxidant gene markers, *AP-G* Apoptotic gene markers, *IM-G* Immune system markers, *EN-G* Endocrine system gene markers

Discussion

Acute toxicity results provide a first approximation of the impacts of pesticides and may be helpful in the detection of dose thresholds for the sequent investigation of sublethal impacts [31]. Our results showed that both PHO and DEL possessed relatively high toxicities to zebrafish during multiple developmental stages, with the 96-h LC_{50}

Lv *et al. Environmental Sciences Europe*   (2022) 34:90

Page 12 of 16

values ranging from 0.24 to 1.44 μM and from 0.0041 to 2.97 μM, respectively. Fish are highly sensitive to DEL since this pesticide can be absorbed into gills at high rates, degraded slowly through hydrolysis, and hypersensitive to the piscine nervous system [32]. It has been reported that PHO exerts acute toxicity to most fish, such as Tilapia (*Oreochromis niloticus*), Black seabream (*Acanthopagrus schlegelii*), and Loach (*Paramisgurnus dabryanus*), which is consistent with our current findings [33–35]. Furthermore, its high toxicity to fish indicates that fish have a lower ability to metabolize PHO [36]. In the natural ecosystem, organisms are more often exposed to chemical mixtures rather than to an individual compound [3, 37]. Therefore, it is crucial to determine the mixture toxicity when evaluating the environmental quality [17, 38]. Mixtures of PHO and DEL have a synergistic effect on zebrafish embryos, implying that ecological risk assessments according to single chemicals cannot sufficiently protect aquatic environments from exposure to pesticide mixtures [39–41]. Therefore, PHO and DEL should be carefully mixed to avoid adverse impacts on the water ecosystem.

Antioxidation is a crucial topic in the research of environmental toxicology [42]. Oxidation stress is regarded as a cellular ROS imbalance that can lead to cell damage via alteration of DNA, proteins, and lipids [43]. Lipid peroxidation is a type of oxidative stress related to the instability of biofilms and can be measured by MDA quantification [7]. To resist oxidative stress from environmental contaminations, zebrafish always stimulate their antioxidation systems by increasing the expressions of enzymes, such as SOD, CAT, and POD [42]. In this study, the conspicuous increases of ROS and MDA contents in most exposures suggested that zebrafish embryos suffered from severe oxidative stress when exposed to PHO, DEL, and their mixture.

It is well known that SOD can catalyze superoxide radicals into hydrogen peroxide (H_2O_2), which is further broken down by CAT or POD into nontoxic H_2O and molecular oxygen (O_2) [44]. The levels of T-SOD and Cu/Zn-SOD under nearly all treatments were markedly enhanced relative to the baseline value, indicating that SOD was produced to transform the superoxide radicals into H_2O_2 in zebrafish embryos. It was noteworthy that the content of Cu/Zn-SOD was noticeably down-regulated under moderate and high doses of MIT treatment relative to the corresponding PHO and DEL administrations. The decrease suggested that the oxidative system was destroyed by the higher dose of combined pesticides, which might be one reason for the synergistic impact produced by the MIT administration [45]. In addition, the levels of CAT and POD were also induced under most treatments, suggesting that large amounts of

CAT and POD enzymes were required to convert excess H_2O_2 into H_2O, thereby relieving the toxicity from pesticide exposures [47]. Glutathione can occur in cells with reduced form (GSH) or oxidative form (GSSG) [48], and it is a major non-enzymatic antioxidant that contributes to removing some residual free radicals and cannot be decomposed by antioxidant enzymes [49]. In our present research, the levels of T-GSH and GSSG were clearly enhanced under most exposures relative to the baseline value. These results implied that two forms of glutathione (GSH and GSSG) played important roles in preserving the zebrafish embryos from oxidative stress.

Analyzing the expressions of antioxidation-related genes at the mRNA level will be helpful in assessing the ability of antioxidants in the organism [44]. Meanwhile, we found that the expressions of *Mn-sod* and *Cu/Zn-sod* genes were markedly rised under most administrations, which was consistent with the up-regulated activities of T-SOD and Cu/Zn-SOD. These results indicated that the up-regulation of antioxidative ability was achieved by enhancing the quality of mRNA transcription in zebrafish embryos when exposed to these pesticides [46]. The mRNA can be translated into more SOD protein in order to eliminate the negative effect of ROS, keeping the zebrafish away from oxidative stress [40]. The activity of Cu/Zn-SOD was significantly decreased in the moderate and high doses of MIT treatment relative to the baseline value, as well as corresponding PHO and DEL treatments. In contrast, significant increases in *Mn-sod* and *Cu/Zn-sod* expressions were discovered under the moderate and high doses of MIT treatment. The discrepancy between the mRNA level and enzyme activity of SOD could be explained as follows: (1) the mRNA level only represented a snapshot of antioxidant enzyme activity, and a time lag impact existed between transcription and translation; and (2) post-translation might regulate the enzymatic activity [52, 56].

Detoxification enzymes play crucial roles in excluding the exogenous contaminations to zebrafish [50]. Among these enzymes, CYP450 is one of the most important detoxification enzymes and is deemed an effective biomarker for environmental pollution detection [51]. CarE enzymes can hydrolyze ester compounds into alcohol and acid, resulting in a decline in the toxicity of ester contaminations [52]. The GST enzyme is the most critical phase II detoxification enzyme and can catalyze the conjugation of reduced glutathione (GSH) to the compounds [53]. The results of reduced activity of CYP450 in the present study implied that CYP450 would be the detoxification mechanism of zebrafish to PHO and DEL. Additionally, the activity of CarE was induced by the ester property of DEL, indicating that this enzyme was involved in the detoxification process. Nevertheless, the level of CarE

declined under the high dose of MIT treatment relative to the corresponding DEL administration. We concluded that the inhibited enzymatic activity might be attributed to the synergistic impact of the combined exposure. No apparent change in GST activity was observed, indicating that the GST enzyme was not responsible for detoxification metabolism in the present study.

Apoptosis removes excess cells in the early developmental stage of zebrafish, contributing to normal tissue remodeling and morphogenesis [54]. The activities of Caspase 3 and Caspase 9 were somewhat altered under all PHO and MIT administrations, except under the high dose of PHO administration, in which the activity of Caspase 3 was apparently inhibited relative to the baseline value. An apparent inhibition of Caspase 3 activity was also observed under the moderate dose of DEL treatment relative to the baseline value. The activity of Caspase 9 was apparently increased under the low dose of DEL treatment relative to the baseline value. However, the expressions of *p53*, *cas3*, and *cas9* were markedly up-regulated under most administrations relative to the baseline value, suggesting that PHO, DEL, and their combinations would probably induce apoptosis in the cell [55]. Two reasons might cause the disparity between gene expression and enzyme activity. Firstly, there was a delayed influence between transcription and translation, causing the transcriptome not to always reflect in the proteome. Secondly, modulation of enzyme activity might occur via post-translational events, such as post-translational modification and regulation of enzyme activity [56]. Therefore, PHO, DEL, and their combinations could induce cell apoptosis, which might be responsible for the increase in the number of developmental defects in zebrafish embryos.

The immune system of fish has many chemokines that maintain a stable environment in their bodies and are important components of the immune system [48]. Environmental substances can affect the gene expressions of relevant chemokines [57]. Chemokines (*CXCL-CIC* and *IL-8*) are secreted proteins that can regulate the nature of immune responses [25]. Stress factors greatly affect the immune system during zebrafish development [58]. Therefore, the immune system is an essential indicator when assessing the toxicity of chemicals in fish [59]. The innate immune system is the only defense system against infections caused by chemicals during the early life stages of zebrafish [60]. In the current work, the expression levels of *CXCL-CIC* and *IL-8* genes were increased significantly under most of the administrations relative to the baseline value, indicating that the PHO, DEL, and MIT administrations triggered inflammatory responses, which might be explained by their intoxication-induced adaptation. The expressions of these inflammatory factors

are essential for homeostasis in the body, and excessive amounts of them can lead to cell or tissue damage and may induce serious diseases, such as cancer and metabolic diseases [48]. Besides, the expressions of *CXCL-CIC* and *IL-8* were considerably inhibited in the moderate and high doses of MIT treatment, indicating that the mixture of PHO and DEL could lead to immunity depression against various exogenous contaminant stimulations.

The endocrine systems of vertebrates are modulated by the hypothalamic-pituitary-thyroid/gonadal/adrenal (HPT/HPG/HPA) axes, which coordinate hormone synthesis, secretion, and metabolism to regulate kinetic processes [63]. Chemicals can disrupt the endocrine system, leading to impaired growth, development, and reproduction of aquatic vertebrates [61]. The current study demonstrated that the level of T3 was substantially altered after administration of PHO, DEL, and their combinations, indicating that the endocrine system of zebrafish was disturbed by exposure to these pesticides. THs regulated by the HPT axis have an important influence on the development and growth of fish [57]. We found that the expression levels of *TRα*, *dio1*, and *tsh* were obviously induced under most of the exposures relative to the baseline value. Additionally, apparent alterations of *dio1* and *tsh* were observed under most MIT exposures relative to the baseline value and the corresponding single exposures. The variation of the mRNA expression in the HPT axis indicated PHO and DEL had the possibility to induce thyroid disruption, while their mixture elicited more serious injuries relative to the single pesticides. The HPA axis is responsible for regulating the adaptive stress response in organisms [18]. The current study showed that the expression levels of *crh* and *gr* in the HPA axis were distinctly enhanced under most administrations relative to the baseline value, suggesting that PHO, DEL, and their combinations could impact the growth, metabolism, and reproduction of zebrafish.

In oviparous vertebrates, the female-specific yolk protein precursors VTGs act to transport nutrients into oocytes during the maturation of oocytes [25]. By detecting the VTG content, the effects of pesticides on hormone endocrine disruptors can be detected [16]. Our analysis showed that the VTG content was increased dramatically after DEL exposure at the high dose relative to the baseline value, indicating that DEL had an estrogen-disrupting capacity [62]. Conversely, a dramatic decrease was observed under all PHO exposures relative to the baseline value, showing that PHO had antioestrogen-disrupting capacity in *D. rerio*. However, there was no significant regularity of VTG changes under MIT treatment, implying that PHO and DEL had opposite effects on VTG levels when these two pesticides were mixed.

Lv *et al. Environmental Sciences Europe*   (2022) 34:90

Page 14 of 16

The HPG axis can regulate sex hormones, which are closely related to fish reproduction [64]. VTG is modulated by the activation of estrogen receptors (ERs), which provide the nutrients for the development of zebrafish embryos [65]. ERs have an essential function in the synthesis of *vtg* [66]. The expressions of *ERα*, *ERβ1*, and *vtg* were clearly elevated under most administrations relative to the baseline value, along with a clear elevation of VTG level under individual treatments, indicating that PHO, DEL, and their combinations had potential oestrogenic impacts on *D. rerio*. Apparent induction was also observed under most MIT exposures relative to the corresponding PHO and DEL administrations, revealing that the mixture of PHO and DEL enhanced the oestrogenic impact mainly by revising genes in *D. rerio*. We deduced that the treatment of embryonic fish with PHO, DEL, and their mixture changed the expressions of ERs, which influenced the production of VTG, leading to significant absorption of nutrients. Additionally, the expression levels of *cyp17* and *cyp19a* genes were conspicuously altered under most administrations relative to the baseline value, implying that PHO, DEL, and their combinations could affect the reproduction of *D. rerio* [67].

Our study showed that the concurrent pesticides in the aquatic environment caused greater toxicological impacts on biota than those reported from individual compounds. Therefore, evaluating an individual pesticide does not provide a realistic estimate of the impacts on aquatic ecosystems [68]. Our investigation also supported the hypothesis that the environmental interaction between various chemicals within the maximum permitted limits might harm aquatic life forms [69]. Therefore, future studies need to run an assessment of the environmental level of pollutants and test them in mixtures under laboratory conditions to clarify what is taking place in the environment. Ultimately, our findings might facilitate the selection of pesticide mixtures that are likely to exert synergistic interactions at environmentally-realistic doses.

Conclusions
Mixtures of PHO and DEL induced synergistic effects on zebrafish embryos. The activities of T-SOD, Cu/Zn-SOD, POD, and CarE were substantially altered under most treatments of PHO, DEL, and their combinations relative to the baseline value. The expressions of nine genes, including *Mn-sod*, *Cu/Zn-sod*, *cas3*, *dio1*, *tsh*, *ERα*, *vtg1*, *cyp17*, and *crh*, related to oxidative stress, apoptosis, immune response, and endocrine system were significantly altered under the mixture treatment relative to the individual pesticides. Overall, our results suggested that PHO, DEL, and their combinations caused different levels of toxic impacts on the early developmental stage of zebrafish. The enlarged adverse impacts of these chemicals in a mixture indicated that joint toxicity assessment played a vital role in developing more realistic water quality standards and monitoring guidelines.

Abbreviations
PHO: Phoxim; DEL: Deltamethrin; MIT: Phoxim + deltamethrin; LC_{50}: Median lethal concentration; AI: Additive index; L: Low dose; M: Moderate dose; H: High dose; MDA: Malonaldehyde; CAT: Catalase; T-SOD: Total superoxide dismutase; Cu/Zn-SOD: *Cu,Zn*-Superoxide dismutase; T-GSH: Total glutathione; GSSG: Oxidized glutathione; POD: Peroxidase; ROS: Reactive oxygen species; CYP450: Cytochrome P450; CarE: Carboxylesterase; GST: Glutathione-S-transferase; THs: Thyroid hormones; VTG: Vitellogenin; T3: Triiodothyronine; HPT: Hypothalamic-pituitary-thyroidal; HPG: Hypothalamic-pituitary–gonadal; HPA: Hypothalamic–pituitary–adrenal; IBR: Integrated biomarker response.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00672-6.

Additional file 1: Table S1 Detailed information about the biochemical parameters tested. **Table S2** Gene primer sequences in real time quantitative PCR reaction.

Author contributions
LL: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing—review and editing. ZG: investigation, data curation. lm: investigation, data curation, writing—review and editing. XL: writing—review and editing, administration, funding acquisition. QW: investigation, resources. WS: conceptualization, methodology, software, formal analysis, investigation, resources, data curation, writing-original draft, corresponding. YW: conceptualization, methodology, writing—review and editing, project administration, funding acquisition, corresponding. All author discussed the results and commented on the articles. All authors read and approved the final manuscript.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Institute of Agro-Product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, Zhejiang, China. [2]College of Chemistry, Research Institute of Elemento-Organic

Lv *et al. Environmental Sciences Europe* (2022) 34:90

Page 15 of 16

Chemistry, Nankai University, Tianjin 300071, China. [3]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China.

References

1. Moreira RA, Daam MA, Vieira BH, Sanches AL, Reghini MV, da Silva MA, de Freitas EC, Espindola EL, Rocha O (2017) Toxicity of abamectin and difeno-conazole mixtures to a Neotropical cladoceran after simulated runoff and spray drift exposure. Aquat Toxicol 185:58–66
2. Houndji MAB, Imorou Toko I, Guedegba L, Yacouto E, Agbohessi PT, Mandiki SNM, Scippo ML, Kestemont P (2020) Joint toxicity of two phytosanitary molecules, *lambda*-cyhalothrin and acetamiprid, on African catfish (*Clarias gariepinus*) juveniles. J Environ Sci Health B 55(7):669–676
3. Shahid N, Liess M, Knillmann S (2019) Environmental stress increases synergistic effects of pesticide mixtures on *Daphnia magna*. Environ Sci Technol 53(21):12586–12593
4. Vu HT, Keough MJ, Long SM, Pettigrove VJ (2017) Toxicological effects of fungicide mixtures on the amphipod *Austrochiltonia subtenuis*. Environ Toxicol Chem 36(10):2651–2659
5. Bhagat J, Singh N, Nishimura N, Shimada Y (2021) A comprehensive review on environmental toxicity of azole compounds to fish. Chemosphere 262:128335
6. Cao Z, Huang Y, Xiao J, Cao H, Peng Y, Chen Z, Liu F, Wang H, Liao X, Lu H (2020) Exposure to diclofop-methyl induces cardiac developmental toxicity in zebrafish embryos. Environ Pollut 259:113926
7. Wei Y, Meng Y, Huang Y, Liu Z, Zhong K, Ma J, Zhang W, Li Y, Lu H (2021) Development toxicity and cardiotoxicity in zebrafish from exposure to iprodione. Chemosphere 263:127860
8. Bopp SK, Kienzler A, Richarz AN, van der Linden SC, Paini A, Parissis N, Worth AP (2019) Regulatory assessment and risk management of chemical mixtures: challenges and ways forward. Crit Rev Toxicol 49(2):174–189
9. El-Nahhal Y (2018) Toxicity of some aquatic pollutants to fish. Environ Monit Assess 190(8):449
10. Gonçalves ÍFS, Souza TM, Vieira LR, Marchi FC, Nascimento AP, Farias DF (2020) Toxicity testing of pesticides in zebrafish-a systematic review on chemicals and associated toxicological endpoints. Environ Sci Pollut Res 27(10):10185–10204
11. Martínez R, Tu W, Eng T, Allaire-Leung M, Piña B, Navarro-Martín L, Mennigen JA (2020) Acute and long-term metabolic consequences of early developmental Bisphenol A exposure in zebrafish (*Danio rerio*). Chemosphere 256:127080
12. Di Paolo C, Groh KJ, Zennegg M, Vermeirssen EL, Murk AJ, Eggen RI, Hollert H, Werner I, Schirmer K (2015) Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae. Aquat Toxicol 69:168–178
13. Abe FR, Accoroni KAG, Gravato C, de Oliveira DP (2021) Early life stage assays in zebrafish. Methods Mol Biol 2240:77–92
14. Ma X, Li H, Xiong J, Mehler WT, You J (2019) Developmental toxicity of a neonicotinoid insecticide, acetamiprid to zebrafish embryos. J Agric Food Chem 67(9):2429–2436
15. Jia M, Teng M, Tian S, Yan J, Meng Z, Yan S, Li R, Zhou Z, Zhu W (2020) Developmental toxicity and neurotoxicity of penconazole enantiomers exposure on zebrafish (*Danio rerio*). Environ Pollut 267:115450
16. Sun Y, Cao Y, Tong L, Tao F, Wang X, Wu H, Wang M (2020) Exposure to prothioconazole induces developmental toxicity and cardiovascular effects on zebrafish embryo. Chemosphere 251:126418
17. Belden JB, Brain RA (2018) Incorporating the joint toxicity of co-applied pesticides into the ecological risk assessment process. Integr Environ Assess Manag 14(1):79–91
18. Zhang Y, Xue W, Long R, Yang H, Wei W (2020) Acetochlor affects zebrafish ovarian development by producing estrogen effects and inducing oxidative stress. Environ Sci Pollut Res 27(22):27688–27696
19. Strungaru SA, Plavan G, Ciobica A, Nicoara M, Robea MA, Solcan C, Petrovici A (2019) Toxicity and chronic effects of deltamethrin exposure on zebrafish (*Danio rerio*) as a reference model for freshwater fish community. Ecotoxicol Environ Saf 171:854–862
20. Wu Y, Li W, Yuan M, Liu X (2020) The synthetic pyrethroid deltamethrin impairs zebrafish (*Danio rerio*) swim bladder development. Sci Total Environ 701:134870
21. Li H, Wang F, Li J, Deng S, Zhang S (2021) Adsorption of three pesticides on polyethylene microplastics in aqueous solutions: kinetics, isotherms, thermodynamics, and molecular dynamics simulation. Chemosphere 264(Pt 2):128556
22. ISO (1996) Water quality-determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* hamilton-buchanan (teleostei, cyprinidae)]-part 3: flow-through method. ISO, Geneva (**7346-3**)
23. OECD (1992) OECD Guidelines for the testing of chemicals, fish acute toxicity test. OECD, Paris, p 203
24. OECD (2013) OECD guidelines for the testing of chemicals, fish embryo acute toxicity (FET) test. OECD, Paris, p 236
25. Shen W, Yang G, Guo Q, Lv L, Liu L, Wang X, Lou B, Wang Q, Wang Y (2021) Combined toxicity assessment of myclobutanil and thiamethoxam to zebrafish embryos employing multi-endpoints. Environ Pollut 269:116116
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
27. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ Method. Methods 25:402–408
28. Chi H (1997) Computer program for the probit analysis. National Chung Hsing University, Taichung
29. Marking LL (1985) Toxicity of chemical mixtures. In: Rand GM, Petroceli SR (eds) Fundamentals of aquatic toxicology. Hemisphere Publishing Corporation, Washington DC, pp 164–176
30. Su LS, Yang GL, Wu SG, Pi TX, Wang Q (2016) The single and joint toxicity of tiazophos and cyhalothrin to earthworm. Asian J Ecotoxicol 11:294–301
31. Rawlings JM, Belanger SE, Connors KA, Carr GJ (2019) Fish embryo tests and acute fish toxicity tests are interchangeable in the application of the threshold approach. Environ Toxicol Chem 38(3):671–681
32. Yang C, Lim W, Song G (2020) Mediation of oxidative stress toxicity induced by pyrethroid pesticides in fish. Comp Biochem Physiol C Toxicol Pharmacol 234:108758
33. Liu ZY, Wang ZZ, Lv GT, Shao GE, Bao JM (2010) Acute toxicity of three organophosphorus pesticides to juveniles *Acanthopagrus schlegel*. J Zhejiang Ocean Univ (Nat Sci) 29(1):20–24
34. Meng SL, Qu JH, Fan LM, Qiu LP, Chen JC, Xu P (2014) Joint toxicity of pesticides methomyl and phoxim to Tilapia (*Oreochromis niloticus*). J Agro-Environ Sci 33(2):257–263
35. Guan FL, Xiong LF, Fang HS (2020) Acute toxicity of phoxim and mebendazole on *Paramisgurnus dabryanus*. Ecological Sci 39(6):25–29
36. Singh S, Kumar V, Kanwar R, Wani AB, Gill JPK, Garg VK, Singh J, Ramamurthy PC (2021) Toxicity and detoxification of monocrotophos from ecosystem using different approaches: a review. Chemosphere 275:130051
37. Hu Y, Hu J, Li W, Gao Y, Tian Y (2021) Changes of embryonic development, locomotor activity, and metabolomics in zebrafish co-exposed to chlorpyrifos and deltamethrin. J Appl Toxicol 41(9):1345–1356
38. Hernández F, Bakker J, Bijlsma L, de Boer J, Botero-Coy AM, Bruinen de Bruin Y, Fischer S, Hollender J, Kasprzyk-Hordern B, Lamoree M, López FJ, Laak TLT, van Leerdam JA, Sancho JV, Schymanski EL, de Voogt P, Hogendoorn EA (2019) The role of analytical chemistry in exposure science: focus on the aquatic environment. Chemosphere 222:564–583
39. Rodea-Palomares I, González-Pleiter M, Martín-Betancor K, Rosal R, Fernández-Piñas F (2015) Additivity and interactions in ecotoxicity of pollutant mixtures: Some patterns, conclusions, and open questions. Toxics 3(4):342–369
40. Nunes MEM, Müller TE, Murussi C, do Amaral AMB, Gomes JLC, Marins AT, Leitemperger J, Rodrigues CCR, Fiuza TL, Costa MD, Severo ES, Rosemberg DB, Loro VL (2018) Oxidative effects of the acute exposure to a pesticide mixture of cypermethrin and chlorpyrifos on carp and zebrafish—A comparative study. Comp Biochem Physiol C Toxicol Pharmacol 206–207:48–53
41. Tao MT, Bian ZQ, Zhang J, Wang T, Shen HY (2020) Quantitative evaluation and the toxicity mechanism of synergism within three organophosphorus pesticide mixtures to *Chlorella pyrenoidosa*. Environ Sci Process Impacts 22(10):2095–2103

42. Chagas TQ, Freitas ÍN, Montalvão MF, Nobrega RH, Machado MRF, Charlie-Silva I, Araújo APDC, Guimarães ATB, Alvarez TGDS, Malafaia G (2021) Multiple endpoints of polylactic acid biomicroplastic toxicity in adult zebrafish (*Danio rerio*). Chemosphere 277:130279

43. Li S, Jiang Y, Sun Q, Coffin S, Chen L, Qiao K, Gui W, Zhu G (2020) Tebuconazole induced oxidative stress related hepatotoxicity in adult and larval zebrafish (*Danio rerio*). Chemosphere 241:125129

44. Cong B, Liu C, Wang L, Chai Y (2020) The impact on anti-oxidant enzyme activity and related gene expression following adult Zebrafish (*Danio rerio*) exposure to dimethyl phthalate. Animals 10(4):717

45. Yan S, Wang J, Zhu L, Chen A, Wang J (2015) Toxic effects of nitenpyram on anti-oxidant enzyme system and DNA in zebrafish (*Danio rerio*) livers. Ecotoxicol Environ Saf 122:54–60

46. Perumal S, Gopal Samy MV, Subramanian D (2021) Developmental toxicity, anti-oxidant, and marker enzyme assessment of swertiamarin in zebrafish (*Danio rerio*). J Biochem Mol Toxicol 35(9):22843

47. Song Z, Zhang Y, Zhang H, Rajendran RS, Wang R, Hsiao CD, Li J, Xia Q, Liu K (2020) Isoliquiritigenin triggers developmental toxicity and oxidative stress-mediated apoptosis in zebrafish embryos/larvae via Nrf2-HO1/JNK-ERK/mitochondrion pathway. Chemosphere 246:125727

48. Park S, Lee JY, Park H, Song G, Lim W (2020) Bifenthrin induces developmental immunotoxicity and vascular malformation during zebrafish embryogenesis. Comp Biochem Physiol C Toxicol Pharmacol 228:108671

49. Han Y, Song S, Wu H, Zhang J, Ma E (2017) Anti-oxidant enzymes and their role in phoxim and carbaryl stress in *Caenorhabditis elegans*. Pestic Biochem Physiol 138:43–50

50. Loerracher AK, Braunbeck T (2020) Inducibility of cytochrome P450-mediated 7-methoxycoumarin-O-demethylase activity in zebrafish (*Danio rerio*) embryos. Aquat Toxicol 225:105540

51. Manikandan P, Nagini S (2018) Cytochrome P450 structure, function and clinical significance: a review. Curr Drug Targets 19(1):38–54

52. Gaaied S, Oliveira M, Le Bihanic F, Cachot J, Banni M (2019) Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-dichlorophenoxyacetic acid herbicide. Chemosphere 224:289–297

53. Wu H, Gao C, Guo Y, Zhang Y, Zhang J, Ma E (2014) Acute toxicity and sublethal effects of fipronil on detoxification enzymes in juvenile zebrafish (*Danio rerio*). Pestic Biochem Physiol 115:9–14

54. Zhao W, Hu N, Ding D, Long D, Li S, Li G, Zhang H (2019) Developmental toxicity and apoptosis in zebrafish embryos induced by low-dose γ-ray irradiation. Environ Sci Pollut Res 26(4):3869–3881

55. Shan B, Pan H, Najafov A, Yuan J (2018) Necroptosis in development and diseases. Genes Dev 32(5–6):327–340

56. Hossain MM, Huang H, Yuan Y, Wan T, Jiang C, Dai Z, Xiong S, Cao M, Tu S (2021) Silicone stressed response of crayfish (*Procambarus clarkii*) in anti-oxidant enzyme activity and related gene expression. Environ Pollut 274:115836

57. Xu C, Li X, Jin M, Sun X, Niu L, Lin C, Liu W (2018) Early life exposure of zebrafish (*Danio rerio*) to synthetic pyrethroids and their metabolites: a comparison of phenotypic and behavioral indicators and gene expression involved in the HPT axis and innate immune system. Environ Sci Pollut Res 25(13):12992–13003

58. Liang X, Wang F, Li K, Nie X, Fang H (2020) Effects of norfloxacin nicotinate on the early life stage of zebrafish (*Danio rerio*): developmental toxicity, oxidative stress and immunotoxicity. Fish Shellfish Immunol 96:262–269

59. Campos-Sánchez JC, Esteban MÁ (2021) Review of inflammation in fish and value of the zebrafish model. J Fish Dis 44(2):123–139

60. Aksakal FI, Ciltas A (2019) Impact of copper oxide nanoparticles (CuO NPs) exposure on embryo development and expression of genes related to the innate immune system of zebrafish (*Danio rerio*). Comp Biochem Physiol C Toxicol Pharmacol 223:78–87

61. Zhu B, Han J, Lei L, Hua J, Zuo Y, Zhou B (2021) Effects of SiO$_2$ nanoparticles on the uptake of tetrabromobisphenol A and its impact on the thyroid endocrine system in zebrafish larvae. Ecotoxicol Environ Saf 209:111845

62. Lu J, Wu Q, Yang Q, Li G, Wang R, Liu Y, Duan C, Duan S, He X, Huang Z, Peng X, Yan W, Jiang J (2021) Molecular mechanism of reproductive toxicity induced by *beta*-cypermethrin in zebrafish. Comp Biochem Physiol C Toxicol Pharmacol 239:108894

63. Chen J, Zheng L, Tian L, Wang N, Lei L, Wang Y, Dong Q, Huang C, Yang D (2018) Chronic PFOS exposure disrupts thyroid structure and function in zebrafish. Bull Environ Contam Toxicol 101(1):75–79

64. Sun D, Chen Q, Zhu B, Zhao H, Duan S (2021) Multigenerational reproduction and developmental toxicity, and HPG axis gene expression study on environmentally-relevant concentrations of nonylphenol in zebrafish. Sci Total Environ 764:144259

65. Luo Y, Chen H, Li D, Zhan M, Hou L, Dong W, Luo Y, Xie L (2020) The effects of norethindrone on the ontogeny of gene expression along the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes in zebrafish (*Danio rerio*). Sci Total Environ 747:141554

66. Guo D, Liu W, Yao T, Ma M, Wang Q, Qiu J, Qian Y (2021) Combined endocrine disruptive toxicity of malathion and cypermethrin to gene transcription and hormones of the HPG axis of male zebrafish (*Danio rerio*). Chemosphere 267:128864

67. Kim DJ, Seok SH, Baek MW, Lee HY, Na YR, Park SH, Lee HK, Dutta NK, Kawakami K, Park JH (2009) Benomyl induction of brain aromatase and toxic effects in the zebrafish embryo. J Appl Toxicol 29(4):289–294

68. Ilyushina NA, Egorova OV, Masaltsev GV, Averianova NS, Revazova YA, Rakitskii VN, Goumenou M, Vardavas A, Stivaktakis P, Tsatsakis A (2020) Genotoxicity of mixture of imidacloprid, imazalil and tebuconazole. Toxicol Rep 7:1090–1094

69. Fu Y, Wang Q, Zhang L, Ling S, Jia H, Wu Y (2021) Dissipation, occurrence, and risk assessment of 12 pesticides in *Dendrobium officinale* Kimura et Migo. Ecotoxicol Environ Saf 222:112487

Publisher's Note