Combined Developmental Toxicity Of Cyhalofop-butyl And Quizalofop-p-ethyl On The Zebrafish (Danio rerio) Embryos

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Abstract

Aryloxyphenoxypropionate herbicides have the characteristics of high efficiency, low toxicity, and safety to subsequent crops, and occupy an important position in the world herbicide market. Cyhalofop-butyl and quizalofop-p-ethyl are two representative herbicides, which are widely used in weed control. However, there is limited information on their combined toxicity to aquatic organisms. In this study, the developmental toxicity of cyhalofop-butyl and quizalofop-p-ethyl exposure in combination on zebrafish embryos was valuated to better understand the interaction between the that. The 96 h-LC_{50} (50% lethal concentration) of cyhalofop-butyl and quizalofop-p-ethyl on zebrafish embryos were 0.637 $mg{\cdot}L^{-1}$ and 0.248 $mg{\cdot}L^{-1}$, respectively. The combined effect of cyhalofop-butyl and quizalofop-p-ethyl was an antagonistic effect, and the 96 h-LC_{50} of zebrafish embryos was 1.043 $mg{\cdot}L^{-1}$. Morphologically distinct pericardial edema and yolk cysts were observed after combined exposure, with significant effects on body length and heart rate in zebrafish embryos. At the same time, the mRNA levels of gene related to apoptosis and cardiac development also changed significantly. Therefore, we speculate that changes in genes related to apoptosis and cardiac development should be responsible for the abnormal development during embryonic development following co-exposure of cyhalofop-butyl and quizalofop-p-ethyl.

Keywords: Cyhalofop-butyl; Quizalofop-p-ethyl; Combined toxicity; Developmental toxicity; Apoptosis ; Cardiac development

Highlights

- Combined exposure caused deformities in zebrafish.
- Combined exposure caused apoptosis in zebrafish.
- Combined exposure altered the expression of apoptosis and cardiac-related genes in zebrafish.

1. Introduction

Like most other water pollutants, pesticides can enter hydrological systems through diffusion pathways (non-point sources) from specific release points (point sources) and deposition and surface runoff (Chang et al., 2020; Hladik et al., 2014). While aquatic organisms are often exposed to a variety of pollutants, a variety of pesticides combine to produce different toxic effects. However, most of the current studies have only assessed the effects of a single compound on aquatic organisms (Zhu et al., 2015). Therefore, when assessing the harm of pesticides to aquatic organisms, the effect of combined exposure to pesticides should be fully considered. Aryloxyphenoxypropionate compounds are a class of highly active herbicides, and their mechanism of action is to inhibit the activity of acetyl-CoA carboxylase (ACCase) in gramineous plants, and occupy an important position in the world herbicide market (Cummins and Edwards, 2004; Kukorelli et al., 2013). Studies have shown that the combined toxicity of fenoxaprop-ethy and cyhalofop-butyl was evaluated, and it was found that these two herbicides could reduce the content of chlorophyll a and b in duckweed (Fan et al., 2006). The fenoxaprop-ethy toxicity is highly toxic and has certain genotoxicity to grass carp (Chen et al., 2006). Therefore, it is of great significance to strengthen the research on the toxicology of aryloxyphenoxypropionate herbicides.

Cyhalofop-butyl is a new type of highly effective aryloxyphenoxypropionic acid herbicide widely used for weed control in paddy fields (Zuo et al., 2016). Unfortunately, cyhalofop-butyl in rice fields will inevitably cause this herbicide to enter the aquatic environment and disturb the balance of farmland ecosystem (Xia et al., 2018; Yuan et al., 2019). Previous studies have shown that 7-day exposure of 0.662 mg/L cyhalofop-butyl to *misgurnus anguillicaudatus* will lead to vacuolation and swelling of liver cells, and with the extension of exposure time, cell lysis, nuclear deformation and nuclear pyknosis will also appear (Shang et al., 2019).

Quizalofop-p-ethyl is a selective transport agent for stems and leaves of aryloxyphenoxypropionate (Li et al., 2012). Some studies have pointed out that

quizalofop-p-ethyl will pollute water bodies, possibly affecting the aquatic ecological environment and aquatic organisms (Belfroid et al., 1998). At present, the biotoxicity of quizalofop-p-ethyl, such as genotoxicity (Mustafa and Arikan, 2008), reproductive toxicity (Zhu et al., 2017) and hepatotoxicity (Elefsiniotis et al., 2007) has been reported. For example, some studies have found that quizalofop-p-p-ethyl can affect the biological indicators such as molting and reproduction of the parent daphnia magna (Yang-Guang et al., 2013). The research team also found that the acute toxicity of quizalofop-p-p-ethyl to zebrafish adults was high, and both of them affected the activity of ATPase (Yang-Guang, 2012). Zhu et al. (2017) revealed for the first time that quizalofop-p-ethyl as an endocrine disrupting chemical (EDC) destroyed the endocrine system of zebrafish. In addition, quizalofop-p-ethyl aggravated cardiotoxicity due to inflammatory reaction, which affected the development of zebrafish embryos (Zhu et al., 2022).

Because zebrafish has the advantages of small size, low feeding cost, strong reproduction ability, transparent embryo, short test cycle and high similarity between genes and human genes, it is widely used in water quality testing experiments for environmental pollutant toxicity assessment (Howe et al., 2013). The zebrafish model used to study human diseases is widely accepted in various fields, including developmental genetics, toxicology, cancer and regeneration (Shaukat et al., 2011; Wolfram and Sadler, 2018; Yan et al., 2016). In this study, the acute lethal and developmental toxicity effects of cyhalofop-butyl, quizalofop-p-p-ethyl and binary mixtures on zebrafish embryos were detected. The purpose of this study is to reflect the potential threat of joint toxicity of pesticides to aquatic organisms, so as to provide necessary basic data for comprehensive assessment of pesticide compound pollution risk.

2. Materials and methods

This study was approved by the research ethics from Chongqing University. The study was carried out under the Guidance of the Care and Use of Laboratory Animals

in China.

2.1 Materials and reagents

98% cyhalofop-butyl (CAS: 122008-85-9) and 95% quizalofop-p-ethyl (CAS: 100646–51–3) was obtained from Anhui Futian Agrochemical CO., Ltd and Shandong China Jingbo Agrochemicals CO., LTD. Preparation of drug exposure stock solution with acetone AR, and all reagents used in the study are analytical grade.

2.2 Maintenance of zebrafish

The parental zebrafish were AB wild type. In the zebrafish culture system, the temperature was 27±1□, and the light-dark period was 14 h:10 h. During the breeding period, the mortality rate was kept below 5%, and the shrimp were fed twice a day. Embryo acute toxicity and developmental toxicity tests were performed according to the previous reported method (Mu et al., 2016).

2.3 Toxicity test of zebrafish

2.3.1 Acute toxicity test of cyhalofop-butyl and quizalofop-p-ethyl

In the acute toxicity experiment, embryos within 2 hours after fertilization (hpf) were collected according to the OECD standards (OECD, 1992) and exposed to cyhalofop-butyl and quizalofop-p-ethyl 96 hpf. The combined exposure was based on the LC_{50} values of single cyhalofop-butyl and quizalofop-p-ethyl determined, and a ratio of toxicity units of 1:1 was used in the experimental design. All test solutions were made in 150ml small beakers, each containing 100 ml of exposure solution for 20 larvae. There were three replicates for each concentration group. The recombinant water without cyhalofop-butyl and quizalofop-p-ethyl was used as a blank control (0 mg/L), and a solution with the same acetone content as the highest concentration was arranged as the solvent control, see Table 1 for the specific concentration setting. The deformity and death of larvae were observed every 24 h, and the dead individuals were removed in time. The LC_{50} was calculated by replacing 3/4 volume of liquid every 24 h. Combined toxicity assessment was performed according to Marking's Additional Index (AI) method for combined effects of aquatic toxicology (Marking, 1984). The formula is as follows:

184 $S = (Am/Ai) + (Bm/Bi) + (Cm/Ci)$

185 $S<1.0$: $AI=(1/S)-1$; $S\geq1.0$: $AI= -S+1.0$

186 S: total biological activity. A, B and C: the compounds in the mixture, i :
187 individual LC50 value for A, B, or C. m: LC_{50} of A, B, or C in mixture. The AI value
188 can be obtained by substituting the calculated S value into the formula. When AI < 0,
189 the joint toxic effect of the mixture is antagonistic, when AI=0, it is additive, and
190 when AI > 0, it is synergistic, and the larger AI value, the stronger the synergistic
191 effect.

192 2.4 Embryonic development toxicity of co-exposure

193 According to the LC_{50} of cyhalofop-butyl and quizalofop-p-ethyl, two
194 concentrations and one solvent control were set, and each concentration was set in
195 triplicate. The concentrations of cyhalofop-butyl were set to 0.166 and 0.333 mg/L,
196 and the concentrations of quizalofop-p-ethyl were set to 0.065 and 0.129 mg/L. The
197 combined exposure concentration of cyhalofop-butyl + quizalofop-p-ethyl was set to
198 0.166 mg/L + 0.065 mg/L, 0.333 mg/L+0.129 mg/L. For each concentration, 200
199 normally developing embryos were randomly selected and placed into a 1000 mL
200 beaker containing 600mL of exposure solution. The experimental group and control
201 group were exposed to 27±1 □ incubator for 96 h, the light-dark cycle was 14 h: 10 h,
202 and the reagent was changed every 24 h. The spontaneous movement of 24 hpf,
203 hatching rate of 96 hpf, heart rate, body length of larvae (Aigo ge-5, China) and
204 deformity rate (Olympus BH-2 dissecting microscope) were recorded. At the end of
205 exposure, 50 zebrafish larvae at each concentration were randomly selected for the
206 determination of apoptosis enzyme activity and 30 zebrafish larvae for the
207 determination of genes related to apoptosis and heart development.

208 2.5 Measurement of apoptosis enzyme activity

209 The caspase-3, caspase-9 activity and the total protein of samples was measured
210 by caspase assay kit and BCA protein kit according to the manufacturer's method
211 (Beyotime Institute of Biotechnology, Haimen, China).

212 2.6 Gene expression analysis

RNA was extracted from each replicated zebrafish embryo using Trizol (Tiangen Biotech, China) and reverse transcribed by a quant cDNA (First strand complementary DNA) synthesis RTase Kit (Tiangen Biotech, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed SuperReal PreMix Plus kit (Tiangen Biotech, China) by ABI 7500 Q-PCR system (Applied Biosystems, USA). Reaction system conditions and primer sequences are detailed in the Supplementary Material. The housekeeping gene beta-actin (β-actin) was used as the reference gene and was quantified by $2^{-\Delta\Delta\ Ct}$ method. All measurements were performed in triplicate.

2.7 Statistical analysis

SPSS 22.0 (SPSS, Chicago, IL, USA) was used to analyze the experimental data. One-way analysis of variance was used for significance analysis, and Dunnett test was used to evaluate the significant differences between different groups. $P< 0.05$ was considered statistically significant, and values were expressed as mean ± standard deviation (SD).

3. Results

3.1 Acute toxicity of cyhalofop-butyl and quizalofop-p-ethyl

The results showed that the toxicity of cyhalofop-butyl and quizalofop-p-ethyl on zebrafish were middle toxicity, and the LC_{50} value of 96 h were 0.637 mg/L and 0.248 mg/L, respectively. When the toxicity ratio of cyhalofop-butyl and quizalofop-p-ethyl was 1:1, the AI value of 96 h of combined exposure was -1.086, which had an antagonistic toxicity effect (Table 2).

3.2 Embryonic development toxicity

Zebrafish embryos were exposed to cyhalofop-butyl , quizalofop-p-ethyl and their co-exposure for 24 h, the voluntary movement was inhibited (Figure 1-A). In the co-exposure treatment group, the inhibitory effect increased with the increase of exposure concentration. At 48 hpf, the single treatment group of cyhalofop-butyl and quizalofop-p-ethyl had no significant effect on the fetal heartbeat of zebrafish, while

the co-exposure group significantly inhibited the fetal heartbeat of zebrafish. At 72 hpf and 96 hpf, the fetal heartbeat of zebrafish was significantly inhibited in all treatment groups except the low-concentration cyhalofop-butyl treatment group (0.166 mg/L) (Figure 1-B). After 96 hpF exposure, the hatchability of zebrafish treated with the highest concentrations of cyhalofop-butyl (0.333 mg/L) and quizalofop-p-ethyl (0.129 mg/L) in the co-exposure group was significantly inhibited (Figure 1-C). In addition, compared with the control group, the body length of zebrafish larvae in all treatment groups decreased with the increase of exposure concentration, and the body length of zebrafish larvae in the co-exposure group decreased significantly (Figure 1-D). Single and co-exposure of cyhalofop-butyl and quizalofop-p-ethyl induced pericardial edema and yolk sac edema deformity in zebrafish embryonic development (Figure 2 A-B). The results showed that the malformation rate in all treatment groups was significantly increased by dose effect. At 48 hpf and 96 hpf, the embryo malformation rate of zebrafish in the co-exposed high concentration treatment group significantly increased to 36.11% and 36.71%, respectively (Figure 2-C).

3.3 Apoptosis analysis

Compared with the control group, the activity of caspase-3 and caspase-9 in cyhalofop-butyl, quizalofop-p-ethyl and their co-exposure treatment groups increased with the increase of exposure concentration. The enzyme activities of caspase-3 and caspase-9 increased by 224.09% and 235.63% respectively in the treatment group with the highest concentration of co-exposure (Figure 3). There was no significant change in caspase activity of embryos in other treatment groups.

At 96 hpf, the related genes apoptosis protein activating factor 1 (*apaf1*), Bcl-2 associated X protein (*bax*), B-cell lymphoma-2 (*bcl-2*), the relative expressions of cysteinyl aspartate specific protease 3 (*caspase-3*), cysteinyl aspartate specific protease 9 (*caspase-9*), murine double minute 2 (*mdm2*) and *p53* increased significantly in the co-exposure treatment group (Figure 4). Compared with the control group, *apaf1* (2.644 and 2.683 times), *mdm2* (2.968 and 6.510 times) and *p53* (3.349 and 2.36 times) were increased significantly with co-exposure (0.166+0.065

mg/L, 0.333+0.129 mg/L). *Bcl-2* was significantly expressed in quizalofop-p-ethyl (0.065 mg/L, 0.129 mg/L) and Co-exposure (0.166+0.065 mg/L, 0.333+0.129 mg/L) treatment groups, which were up-regulated by 1.892, 2.490, 2.119 and 3.279 times respectively.

3.4 The expression of genes related to heart development

At 96hpf, the expressions of *Nkx2.5*, *Tbx5* and *VEGF* were significantly up-regulated with the increase of concentration in all treatment groups, especially in the high co-exposure concentration group (0.333+0.129 mg/L). The expression of *Gata4* was down-regulated in cyhalofop-butyl and quizalofop-p-ethyl treatment groups, but it was significantly increased with the increase of concentration in co-exposure treatment group. The expression of *vmhc* gene was significantly induced in all treatment groups, and the up-regulation level increased with the increase of concentration (Figure 5).

4. Discussion

In this study, the acute toxicity (96 hpf-LC_{50}) of cyhalofop-butyl and quizalofop-p-ethyl to zebrafish were 0.637 and 0.248 mg/L respectively, which is consistent with previous result (Zhu et al., 2015; Zhu et al., 2022). In addition, the 96 h-LC_{50} of cyhalofop-butyl was 0.664 mg/L, and the 96 h-LC_{50} of quizalofop-p-ethyl was 0.259 mg/L under combined exposure. The LC_{50} values were 1.04 times of those under single exposure. The results calculated by the additive index method showed that the combined exposure (toxicity ratio 1:1) had an antagonistic effect on zebrafish embryos. However, different proportions of mixed pollutants will also produce different types of toxic effects.

Studies have shown that the combination of imidacloprid, acetochlor and tebuconazole has synergistic toxicity to zebrafish with the toxicity ratios of 1:2:2, 1:4:4, 2:4:1 and 4:1:4, while the combination of 1:1:1, 1:1:2, 2:1:2, 2:2:1 and 4:2:1 has antagonistic toxicity to zebrafish. Therefore, the toxic effects of cyhalofop-butyl and quizalofop-p-ethyl on zebrafish embryos under different toxic ratios need to be

301 further studied. The mechanism of the effect of cyhalofop-butyl and
302 quizalofop-p-ethyl on zebrafish embryos in the ratio of 1:1 is still unclear, so we
303 explored the preliminary toxic mechanism of combined exposure on zebrafish
304 embryos in the ratio of 1:1 antagonism. Cyhalofop-butyl can induce developmental
305 toxicity, oxidative stress and apoptosis of zebrafish embryos (Zhu et al., 2015), while
306 quizalofop-p-ethyl has developmental toxicity, cardiotoxicity and reproductive
307 toxicity to zebrafish (Zhu et al., 2022; Zhu et al., 2017).

308 In this study, the results of joint developmental toxicity test showed that
309 co-exposure caused a series of adverse effects during zebrafish embryo development,
310 including abnormal voluntary movement, decreased heart rate, hatching inhibition,
311 growth inhibition and various teratogenic effects, including yolk cyst and pericardial
312 edema. Heart rate change is an important index to evaluate cardiac toxicity. Many
313 studies have found that exposure to chemicals can affect the heart of zebrafish
314 embryos. For example, study pointed that exposure to boscalid can cause the heart
315 malformation of zebrafish embryos, and significantly inhibit the embryo heartbeat
316 (Qian et al., 2018). It has been reported that difenoconazole can cause a lot of
317 symptoms during the development of zebrafish embryos, such as slow heart rate,
318 morphological abnormality and hatching inhibition (Mu et al., 2013). Dioxin and
319 dioxin-like compounds can cause morphological abnormalities of zebrafish embryos,
320 including pericardial edema and craniofacial abnormalities (Tokunaga et al., 2016).It
321 was found that the heart function of zebrafish embryos was impaired, the heart rate
322 decreased obviously, and serious changes such as pericardial edema, yolk sac edema
323 and yolk sac deformation occurred during the time when zebrafish embryos were
324 exposed to cyhalofop-butyl and quizalofop-p-ethyl, and the co-exposure aggravated
325 these changes. In addition, abnormalities mainly occur in cardiac areas, such as
326 pericardial edema. This finding indicated that abnormal yolk sac edema and
327 pericardial edema may be caused by heart damage, and the combined exposure of
328 cyhalofop-butyl and quizalofop-p-ethyl makes the heart damage more serious, which
329 makes the effects of abnormal voluntary movement, decreased heart rate, hatching
330 inhibition, growth inhibition and various teratogenic effects after combined exposure

more severe.

Apoptosis is an active cell death process with obvious morphological characteristics and energy dependence (Abrahams et al., 2014). Many environmental pollutants, such as pesticides (Qian et al., 2018), heavy metals (Kp et al.), polycyclic aromatic hydrocarbons (Deng et al., 2009b), etc., disturb the expression of protein and nucleic acid in cells in the process of contacting organisms, thus inducing apoptosis. Therefore, taking apoptosis as a detection index can reflect the early toxic effect of pollutants on organisms, which is of great value for the evaluation of environmental pollutants. Apoptosis may lead to early developmental malformation. For example, zebrafish larvae exposed to hexabromocyclododecane (HBCD) were triggered by apoptosis genes, which led to the increase of deformity and the decrease of survival rate (Deng et al., 2009a). After being exposed to boscalid, zebrafish embryos suffered from developmental toxicity, such as slow heartbeat, cell edema and spinal deformation, through apoptosis and lipid metabolism (Qian et al., 2018). In this study, the single or combined exposure of cyhalofop-butyl and quizalofop-p-ethyl caused a series of gene expressions related to apoptosis in zebrafish embryos, such as *bax*, *bcl-2*, *p53*, *mdm2*, *caspase9*, etc., and the activities of caspase-3 and caspase-9, the important executors of apoptosis, were also significantly induced. This conclusion proves that apoptosis can induce a series of adverse effects (heart rate drop and deformity, etc.) during embryo development. The expression of apoptosis-related genes is related to the process of apoptosis signal pathway, such as cytochrome C, *bcl2*, *bax*, *p53*, *apaf1* (Zhao et al., 2009). Cytochrome C was released from mitochondria to cytoplasm during apoptosis stimulation (Hildeman et al., 2003). The activation of *p53* expression indicates that the cells are in an apoptotic state (Jin et al., 2012). In addition, Bcl-2 family proteins are composed of anti-apoptosis (e.g., bcl-2) and pro-apoptosis members (e.g., bax), which play an important role in inhibiting or promoting apoptosis mainly through mitochondrial pathway (Hildeman et al., 2003; Li et al., 2009). The activation of *caspase-9* is triggered by the release of cytochrome C in mitochondria and its interaction with *apaf1*(Pallardy et al., 1999; Yoshida et al.), and the activated *caspase-9* will further activate regulatory factors such as *caspase3*

downstream (Lindenboim et al., 2000).

In this study, when cyhalofop-butyl (0.166 and 0.333 mg/L) and quizalofop-p-ethyl (0.065 and 0.129 mg/L) were exposed alone, we observed that *p53*, *bax*, *bcl-2* and *apaf1* were up-regulated with the increase of exposure concentration. Compared with the control group, the gene transcription of *caspase-3* and *caspase-9* was also up-regulated, and *caspase-9* was significantly up-regulated in quizalofop-p-ethyl treatment group. In the co-exposure (0.166+0.065 mg/L, 0.333+0.129 mg/L) treatment group, we observed that *p53*, *bax*, *bcl-2*, *apaf1*, *caspase-3* and *caspase-9* were up-regulated with the increase of exposure concentration, and up-regulated significantly in the highest concentration (0.333+0.129 mg/L). We also detected the apoptosis induced by zebrafish embryos in vivo by caspase activity assay. It was found that the gene expression and activity of caspase-3 and caspase-9 increased when exposed to cyhalofop-butyl and quizalofop-p-p-ethyl alone or in combination, and significantly increased in the co-exposed highest concentration (0.333+0.129 mg/L) treatment group. It is suggested that cyhalofop-butyl and quizalofop-p-p-ethyl may induce mitochondrial cytochrome C release by up-regulating pro-apoptotic factor *bax*, *caspase-3* and *caspase-9* are important executors of inducing apoptosis. On the other hand, it can induce the initiation of *p53* pathway and activate the apoptosis of zebrafish embryos. Subsequently, these enzymes promote cell apoptosis, resulting in pericardial cyst and yolk cyst, which in turn affects the heart function, and eventually leads to embryo death.

As the first organ in zebrafish embryo development (Bakkers, 2011), the formation of heart is a very complicated process (Gonzalez-Rosa et al., 2017). Many key genes, such as Tbx5 (Ingham, 2000), Nkx2.5 and gata4 (Välimäki et al., 2017), play an important role in the development and maturation of zebrafish heart. In this study, the exposure of two drugs will cause obvious cardiotoxicity to zebrafish embryos. By observing the heart rate of zebrafish embryos, it was found that the heart beats of the embryos in the control group was regular, while the heart beat rhythm of the embryos in the treatment group was irregular, and the heart rate decreased

obviously. And we found that cyhalofop-butyl and quizalofop-p-p-ethyl and binary mixed exposure could induce the expression of genes related to heart development of zebrafish embryos, such as *Nkx2.5*, *Tbx5* and *VEGF*. Compared with the control group, the expressions of *Tbx5* and *Gata4* genes were up-regulated under combined exposure, and the expressions of *Nkx2.5*, *VEGF* and *vmhc* were significantly up-regulated in the low concentration group (0.166+0.065 mg/L). Compared with the control group, the expression of these genes in high concentration group (0.333+0.129 mg/L) was significantly up-regulated. Compared with the single dose of cyhalofop-butyl and quizalofop-p-p-ethyl on the expression of heart development-related genes in zebrafish embryos, the co-exposure showed synergistic effect on the expression of *Nkx2.5*, *Tbx5*, *VEGF* and *vmhc*, and showed antagonistic effect on the expression of *Gata4*, which was significantly up-regulated with the increase of concentration. In addition, previous studies also found that cyhalofop-butyl exposure can produce zebrafish embryos with developmental abnormalities, which mainly include pericardial edema, yolk sac edema and abnormal yolk sac morphology, among which the most significant ones are pericardial edema and yolk sac edema (Zhu et al., 2015). After quizalofop-p-ethyl exposure, the embryo appeared pericardial edema, abnormal cardiac cyclization and atrial hypertrophy, and the expression of a series of genes (such as *gata4*, *Nkx2.5*, *Tbx5*, *VEGF*.) and proteins (Tbx5) related to heart development were induced to change (Zhu et al., 2022). This is consistent with our results. Generally speaking, quizalofop-p-ethyl, cyhalofop-butyl and co-exposure may induce the expression of genes related to heart development of zebrafish embryos, and further cause the malformation of zebrafish embryos such as hatching inhibition, heart rate decrease, autonomic motor inhibition and pericardial cyst. Therefore, when assessing the harm of pesticides to aquatic organisms, the combined effect of combined pollution should be fully considered.

5. Conclusions

In summary, our results showed that the single or combined exposure of

cyhalofop-butyl and quizalofop-p-p-ethyl in water had significant negative effects on the development of zebrafish embryos. During the development of zebrafish embryos, obvious developmental effects such as hatching inhibition, decreased heart rate, shortened body length and deformity were observed. The effects of apoptosis and heart development may be the responsibility of the toxicity of combined exposure to zebrafish embryo development. Aquatic organisms are often exposed to a variety of compounds, and a variety of pesticides combine to produce different toxic effects. Therefore, it is of great significance to study the combined effects of combined pollution when assessing the harm of pesticides to aquatic organisms.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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584 ## Tables and Figures

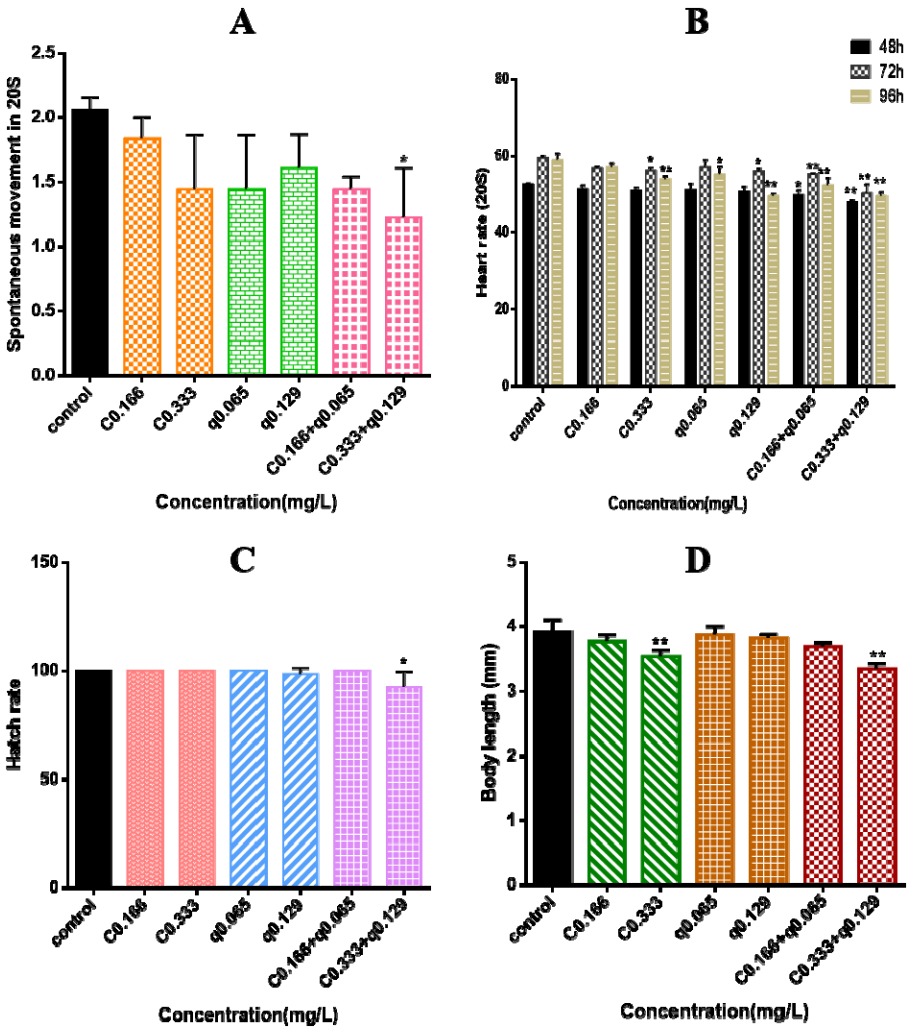

Fig. 1. Developmental effects of cyhalofop-butyl, quizalofop-p-p-ethyl and binary mixtures on embryos. Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, $^{*}p < 0.05$; $^{**}p < 0.01$).

A: Spontaneous movement of zebrafish at 24hpf. B: Heart rate at each observation time C: Hatch rate at 96 hpf. D: Body length at 96 hpf

C0.166: 0.166 mg/L cyhalofop-butyl; C0.333: 0.333 mg/L cyhalofop-butyl; q0.065: 0.065 mg/L quizalofop-p-p-ethyl; q0.129: 0.129 mg/L quizalofop-p-p-ethyl; C0.166+q0.065: co-exposure of cyhalofop-butyl + quizalofop-p-ethyl 0.166 mg/L + 0.065 mg/L; C0.333+q0.129: co-exposure of cyhalofop-butyl + quizalofop-p-ethyl 0.333 mg/L+0.129 mg/L).

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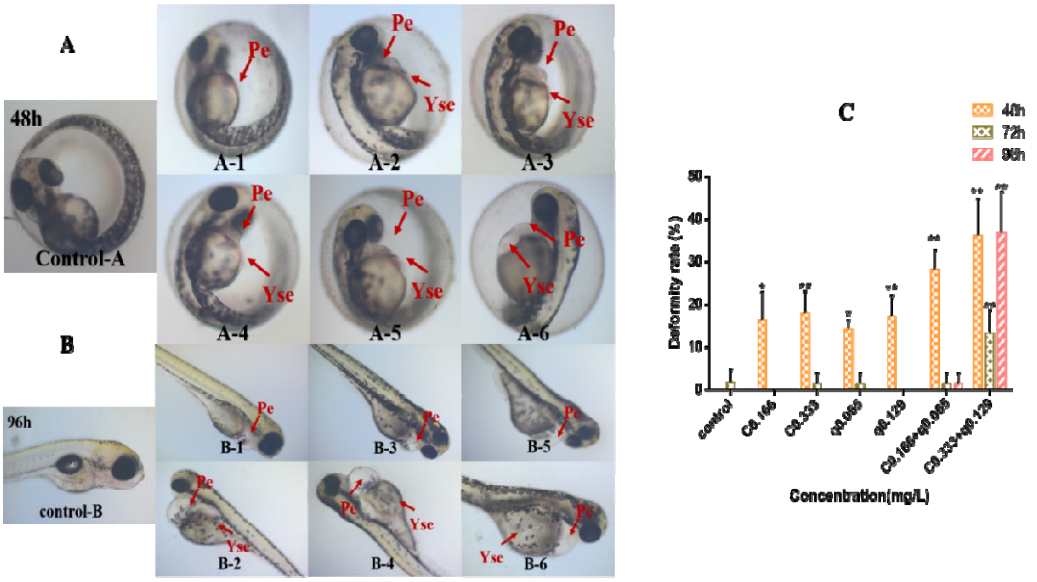

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598 **Fig. 2.** Malformations of embryos after exposure to cyhalofop-butyl,

599 quizalofop-p-p-ethyl and binary mixtures.

600 A: embryos with morphological deformation after exposure at 48 hpf. B: embryos

601 with morphological deformation after exposure at 96 hpf (-1, 0.166 mg/L

602 cyhalofop-butyl; -2, 0.333 mg/L cyhalofop-butyl; -3, 0.065 mg/L

603 quizalofop-p-p-ethyl; -4, 0.129 mg/L quizalofop-p-p-ethyl; -5, co-exposure of

604 cyhalofop-butyl + quizalofop-p-ethyl 0.166 mg/L + 0.065 mg/L; -6, co-exposure of

605 cyhalofop-butyl + quizalofop-p-ethyl 0.333 mg/L+0.129 mg/L). C: Rate of deformity

606 of embryos after exposure for 48,72 and 96 hpf. Arrows mark the different positions.

607 Asterisks denote significant difference between the treatments and control

608 (determined by Dunnett post-hoc comparison, $^{*}p < 0.05$; $^{**}p < 0.01$).

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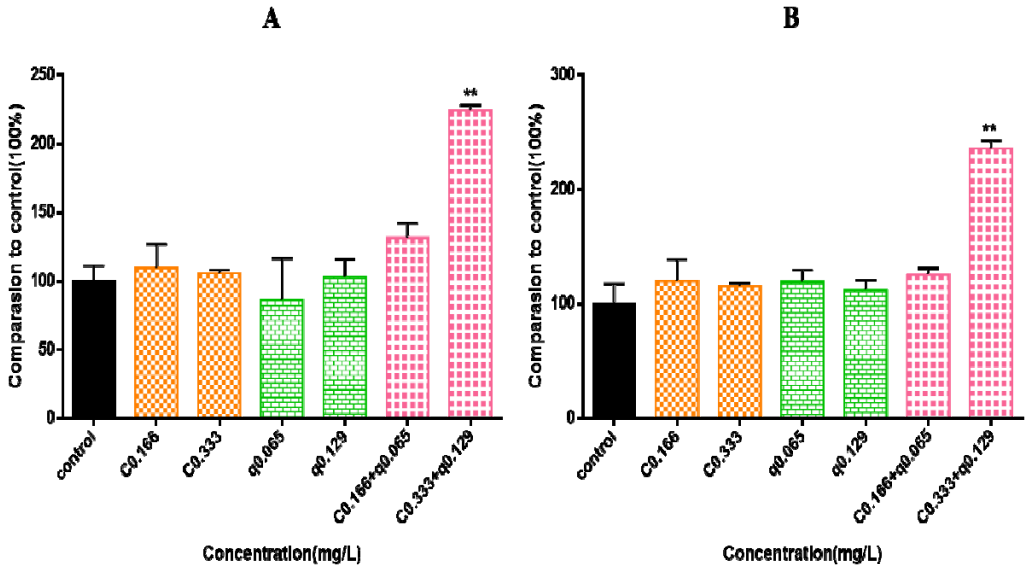

Fig. 3. Cell apoptosis induced by cyhalofop-butyl, quizalofop-p-p-ethyl and binary mixtures. A and B: The activity of caspase-3 (A) and caspase-9 (B) of control and treated groups at 96 hpf. Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, $^{*}p < 0.05$; $^{**}p < 0.01$).

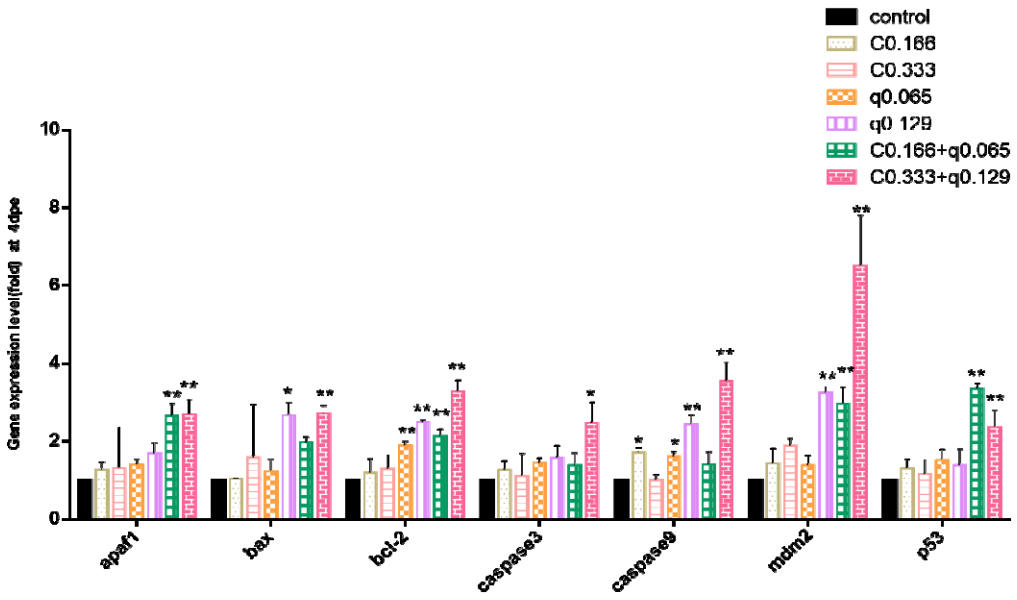

Fig. 4. Transcription of apoptosis related genes in embryos after 96 h exposure to cyhalofop-butyl, quizalofop-p-p-ethyl and binary mixtures. Results are presented as mean ± SD of three replicate samples (determined by Dunnett post-hoc comparison, $^{*}p < 0.05$; $^{**}p < 0.01$).

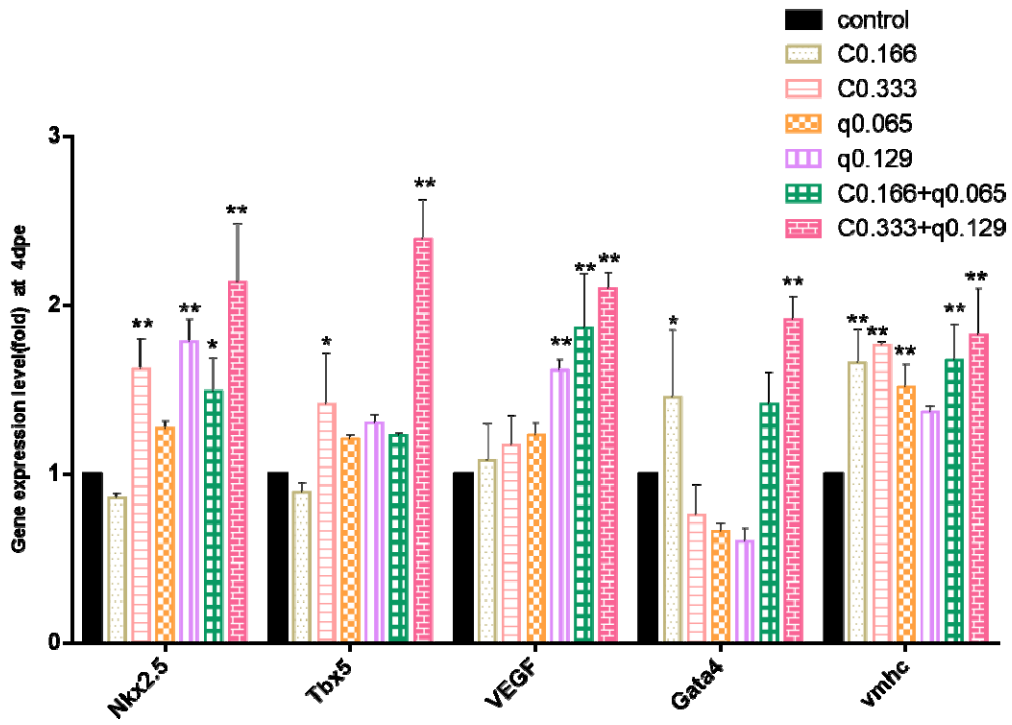

Fig. 5. The expression level of key cardiac genes (*Nkx2.5*, *Tbx5*, *VEGF*, *Gata4*, *vmhc*) in control and treated zebrafish groups for 96 hpf. Results are presented as mean ± SD of three replicate samples (determined by Dunnett post-hoc comparison, $^{*}p < 0.05$; $^{**}p < 0.01$).

645 **Table 1.** Concentration of cyhalofop-butyl and quizalofop-p-ethyl in acute toxicity

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Number	Cyhalofop-butyl/(mg/L)	Quizalofop-p-ethyl/(mg/L)	Cyhalofop-butyl+Quizalofop-p-ethyl/(mg/L)
1	1.52	0.94	1.096+0.427
2	1.01	0.52	0.911+0.355
3	0.68	0.29	0.758+0.295
4	0.45	0.16	0.637+0.248
5	0.30	0.09	0.529+0.206
6	0.20	0.05	0.440+0.171

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648 **Table 2.** Acute toxicity test of cyhalofop-butyl, quizalofop-p-ethyl and co-exposure

649 on zebrafish embryos.

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Chemical	Regression equation	96h-LC_{50} (mg /L) (95% CL^a)	R^{2b}	AI	Effect evaluation
(Cyhalofop-butyl)	y=0.634+3.243x	0.637 (0.551~0.728)	0.937	-1.086	Antagonistic effect
(Quizalofop-p-ethyl)	y=1.496+2.469x	0.248 (0.212~0.291)	0.96		
Cyhalofop-butyl+Quizalofop-p-ethyl	y=-0.252+13.662x	1.043 (1.001~1.085)	0.988	\	\

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652 CL^a: Confidence limit; R^{2b}: Coefficient of determination

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