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Toxicity effects in zebrafish embryos (*Danio rerio*) induced by prothioconazole☆

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ABSTRACT

Triazole fungicides are widely used in agriculture production and have adverse impacts on aquatic organisms. As one of the triazole fungicides, prothioconazole has been reported to cause many toxicological effects, but its risks to aquatic organisms are unknown. In this study, we systematically explored the toxicity effects of prothioconazole exposure on zebrafish embryos (*Danio rerio*) involving in developmental toxicity, oxidative damage and metabolism disorders. The results showed that prothioconazole exposure to zebrafish embryos produced a series of toxic symptoms, including hatching inhibition, shortening of body length, pericardial cyst and yolk cyst. In addition, prothioconazole exposure caused significant lipid peroxidation and oxidative damage. Particularly, we also found that metabolites and genes involved in lipid metabolism also showed significant changes. This study may provide theoretical basis for systematically assessing the potential risks of zebrafish embryos with prothioconazole exposure.

1. Introduction

Triazole fungicides, are widely used in agricultural production as the second most important class of fungicides (Kahle et al., 2008). With the widespread use of triazole fungicides, it could enter the water cycle ecosystem through leaching and surface runoff (Bromilow et al., 1999). Specifically, propiconazole, fluconazole, and tebuconazole had been detected at Swiss Midland lakes with concentrations up to 0.009 μg/L (Kahle et al., 2008). Propiconazole was also detected in agricultural surface water (0.291–1.150 μg/L) (Battaglin et al., 2011). Moreover, the residue of tebuconazole was as high as 175–200 μg/L in some surface water of European streams (Wang et al., 2011). Additionally, previous studies have shown that toxicological effects on aquatic organisms could be caused by triazole fungicides. Tebuconazole had obvious effects on zebrafish such as lethality, teratogenicity and delayed development (Ge et al., 2018). Similarly, diniconazole exposure may interfere with amino acid, energy and lipid metabolism of zebrafish (Wang et al., 2017b). Difenoconazole had also been reported to affect triglyceride levels, cause estrogen endocrine disruption, and produce adverse reactions, which could be transferred to offspring (Teng et al., 2018a). Moreover, exposure to propiconazole could affect enzymes involved in oxidative damage on juvenile rainbow trout (*Oncorhynchus mykiss*) (Li et al., 2013). Obviously, the impact of triazole fungicides on aquatic organisms cannot be ignored.

Prothioconazole, as a highly effective broad-spectrum triazole fungicide, could be used to prevent and cure various diseases through inhibiting the C-14α-demethylase enzyme involved in the biosynthesis of fungal sterols (Casida and Durkin, 2017). It is developed by Bayer Crop Science and listed in 2004, and now covers more than 60 countries around the world (Jautelat et al., 2004; Markets, 2017). However, studies on prothioconazole have focused on activity evaluation and residual dynamics at present (Lin et al., 2017; Paul et al., 2019; Sun et al., 2018), and there are few studies on toxicological effects. Previous studies have shown that prothioconazole reduced the GH3 cell proliferation and inhibited the basal AhR activity (Ghisari et al., 2015). The in-vitro assay shows the proliferation of bovine peripheral blood lymphocytes was

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almost completely inhibited for 48 h following the 30 μg/ml prothioconazole exposure (Schwarzbacherova et al., 2015). There has even been evidence that prothioconazole induced malformation in rabbit fetuses (Rossi, 2011). However, prothioconazole is highly water soluble (300 mg L^{-1}) (Deb et al., 2010), but its mechanism of developmental toxicology of prothioconazole in aquatic organisms was not clear at present. Therefore, it is necessary to explore the toxic effects of prothioconazole on aquatic organisms.

As we all know, zebrafish (*Danio rerio*), is widely used in toxicology research, as an important aquatic model organism, and readily spawning under appropriate photoperiod conditions (Lele and Krone, 1996). Zebrafish embryos are simple to operate, optically transparent, and have high sensitivity to toxicant exposure. Therefore, zebrafish embryos has been used to evaluate the toxicity and mechanisms of action in the development of early life stages (Zou et al., 2017). Metabolomics examines the relevance of metabolites to pathological changes and reveals complex physiological pathways through qualitative and quantitative analysis of endogenous metabolites in biological individuals (Jia et al., 2019). As an analytical tool, it has the characteristics of whole, dynamic and comprehensive analysis (Wang et al., 2017a).

In this study, we systematically explored the toxic effects on zebrafish embryos after exposure to prothioconazole for four days, including developmental toxicity, oxidative damage, and metabolism disorders. Specifically, we observed behavioral activities during the development period, and measured changes in oxidative damage-related indicators and genes expression related to the lipid metabolism. In addition, we analyzed the metabolic characteristics of zebrafish embryos using ^{1}H NMR-based metabolomics. The study aimed to comprehensively analyze the potential risks of prothioconazole on zebrafish embryos and provide a better comprehension of its potential mechanisms.

2. Materials and methods

2.1. Chemicals

Prothioconazole (98% purity), obtained from the China Ministry of Agriculture Institute for Control of Agrochemicals, was dissolved in acetone before use and stored at 4 °C. Standard solutions were prepared in the lab for use as the formula of ISO-7346-3.

2.2. Zebrafish maintenance and embryo collection

The AB-wild type adult zebrafish (5 months old) were purchased from the aquarium department (Beijing Hongda Gaofeng, China). All adult fish were maintained in feeding equipment (Esen Corp.) with a 14 h/10 h (light/dark) photoperiod and acclimation as described in previous work (Teng et al., 2017). During breeding, the male and female fish were individually placed in a spawning box (with a glass partition in the spawning box) at a 1:1 ratio. Then the male and female fish were mixed and laid eggs in the early morning. The normally-developed embryos were collected for experimental research.

2.3. Developmental toxicity test of embryos

According to the Organization for Economic Co-operation and Development (OECD), 0.0375, 0.0750, 0.1500 mg/L of prothioconazole were made based on the acute toxicity experiment data (Table S1), and set acetone as the solvent control according to the maximum concentration of the prothioconazole. The collected embryos were randomly selected and transferred to a 24-well plate after 2 h post-fertilization (hpf), one embryo and 2 ml of prothioconazole solution were placed in each well. Twenty wells were used for each plate (n = 3, replicates). Six embryos were randomly selected from each treatment group to observe the developmental toxicity during the exposure period daily by a light microscope (Olympus BX43), including spontaneous movements at 24 hpf, hatching rate and heartbeats at 48 and 72 hpf in 20s. Additionally, the body lengths of embryos were recorded by an Aigo GE-5 digital microscope (Beijing, China) at 96 hpf. All test solutions were replaced every 24 h. The dead embryos were cleaned up and recorded in time. After 4 days exposure, zebrafish embryos were collected from all treatment groups respectively, and stored at −80 °C for the next experiment.

2.4. Analysis of biochemical parameters

Zebrafish larvae of different treatment groups (30 embryos, n = 3 replicates) removed from −80 °C were homogenized in 100 μL ice-cold saline and centrifuged subsequently at 3500 rpm for 10 min. Then, supernatants were collected to detect the contents of total protein, the levels of glutathione (GSH) and malondialdehyde (MDA), the activities of superoxide dismutase (SOD) and catalase (CAT) according to the manufacturer's instructions using corresponding assay Kit (Jiancheng, Nanjing, China). The levels of triglycerides (TG) and total cholesterol (T-CHO) were measured by assay kits (Jiancheng, Nanjing, China).

2.5. Analysis of zebrafish larvae metabolomics

One hundred zebrafish larvae of different treatment groups (n = 5 replicates) removed from −80 °C were homogenized, and then extracted with 600 μL precooled methanol/H_2O (2:1, v/v). The supernatants were collected after the samples were centrifuged at 12,000 rpm for 10 min at 4 °C, the process was repeated twice, and then combined supernatants were merged and lyophilized. The dried samples were reconstituted with 550 μL of phosphate buffer (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, pH = 7.4) containing D_2O and TSP-d4 (1 mM, final concentration), and centrifuged at 15,000×*g* for 10 min at 4 °C subsequently. Finally the supernatant was transferred into a 5 mm NMR tube for further analysis (Meng et al., 2019). Other specific instrument parameters were described in the supporting information S1.

2.6. Analysis of the mRNA expression

Total RNA was extracted from larvae of each group (30 embryos, n = 3 replicates) by TRIzol regent (Tiangen Biotech, China). The concentrations of total RNA were measured using a spectrophotometer (DS-11, DeNovix, USA), and 1.5 μg of RNA was used to synthesize 20 μL of cDNA with Fast Quant RT Kit (Tiangen Biotech, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the SuperReal PreMix Plus (SYBR Green) (Qiagen, China) and the Bio-Rad CFX 96 PCR system (Bio-Rad, USA) respectively. β-actin was used as the reference gene and all sequences of primers ordered from Sangon Biotech (Shanghai, China.) and were shown in Table S2.

2.7. Data analysis

2.7.1. Multivariate statistical analysis

Multivariate statistical analysis was performed using SIMCA-P (Version 13.0, Umetrics, Sweden). Firstly, the data to be processed is imported into the SIM software, and principal component analysis (PCA) is performed to observe the overall distribution of the sample. A partial least squares discriminant analysis (PLS-DA) is performed to determine the altered metabolites based on the Variable Importance in Projections (VIPs). The significant contents

of identified metabolites were assessed by SPSS 19.0 (IBM, USA) using a parametric test [ANOVA] (Kruskal-Wallis). Finally, metabolites with VIP >1 and $P < 0.05$ were selected for further analysis.

2.7.2. Statistical analysis

Statistical differences were undertaken by ANOVA using SPSS 19.0 (IBM, USA). Graphical illustrations were determined using GraphPad Prism version 6.0 (GraphPad Software, Inc., USA). The error bars of graphics were presented as mean with ±SEM. Asterisk (*) indicates statistically significant difference between the control group and other 3 prothioconazole-treated groups ($p < 0.05$).

3. Results

3.1. Effects of prothioconazole on developmental toxicity in zebrafish embryos

Although exposure to prothioconazole had no significant effects on the spontaneous movements and heartbeats of zebrafish embryos compared with the control group (Fig. 1A, B, D). The hatching rates were significantly decreased at 72 hpf in 0.0750 and 0.1500 mg/L prothioconazole treatments. The mortalities of 48, 72 and 96 hpf were significantly increased with elevating exposure concentrations (Fig. 1C, E). The body lengths at 96 hpf were apparently inhibited by prothioconazole at concentrations of 0.0750 and 0.1500 mg/L. In addition, the deformity symptoms were produced at 48hpf in the groups treated with prothioconazole, including pericardial oedema (Pe) and yolk sac oedema (Yse) (Fig. 2B). As the concentration increases, the symptom of Pe and Yse became serious increasingly (Fig. 2A), the rate of Pe had a significant increase at 0.0750 and 0.1500 mg/L treatment groups, and the rate of Yse increased apparently in the 0.1500 mg/L treatment group (Fig. 2B).

3.2. Effects of prothioconazole on oxidative stress in zebrafish larvae

The alterations of oxidative stress in zebrafish larvae were showed in Fig. 3. Compared with the control group, the activities of SOD and CAT showed no statistically significant differences (Fig. 3B and C). There was a significant decrease of the levels of GSH in the 0.1500 mg/L treatment group (Fig. 3A). Additionally, the levels of MDA of zebrafish larvae were significantly increased with exposure the 0.0375, 0.0750 and 0.1500 mg/L of prothioconazole (Fig. 3D).

3.3. Effects of prothioconazole on metabolism profiles in zebrafish larvae

The representation of 600 MHz ^{1}H NMR spectra of zebrafish larvae was shown in Fig. S1. A trend of good separation was shown in the 0.1500 mg/L treatment group by the PCA score plot as a whole (Fig. 4), which indicated that the metabolism profiles of zebrafish larvae were disturbed with prothioconazole exposure. But the significant separation was not shown in other two lower concentrations treatment groups. A total of 23 metabolites were identified, which were shown in Fig. S1 (Jia et al., 2019; Qiao et al., 2019; Teng et al., 2018b; Yan et al., 2018). Then, the heatmap with cluster analysis was generated by 19 significantly altered metabolites based on the VIP values obtained from the PLS-DA model and

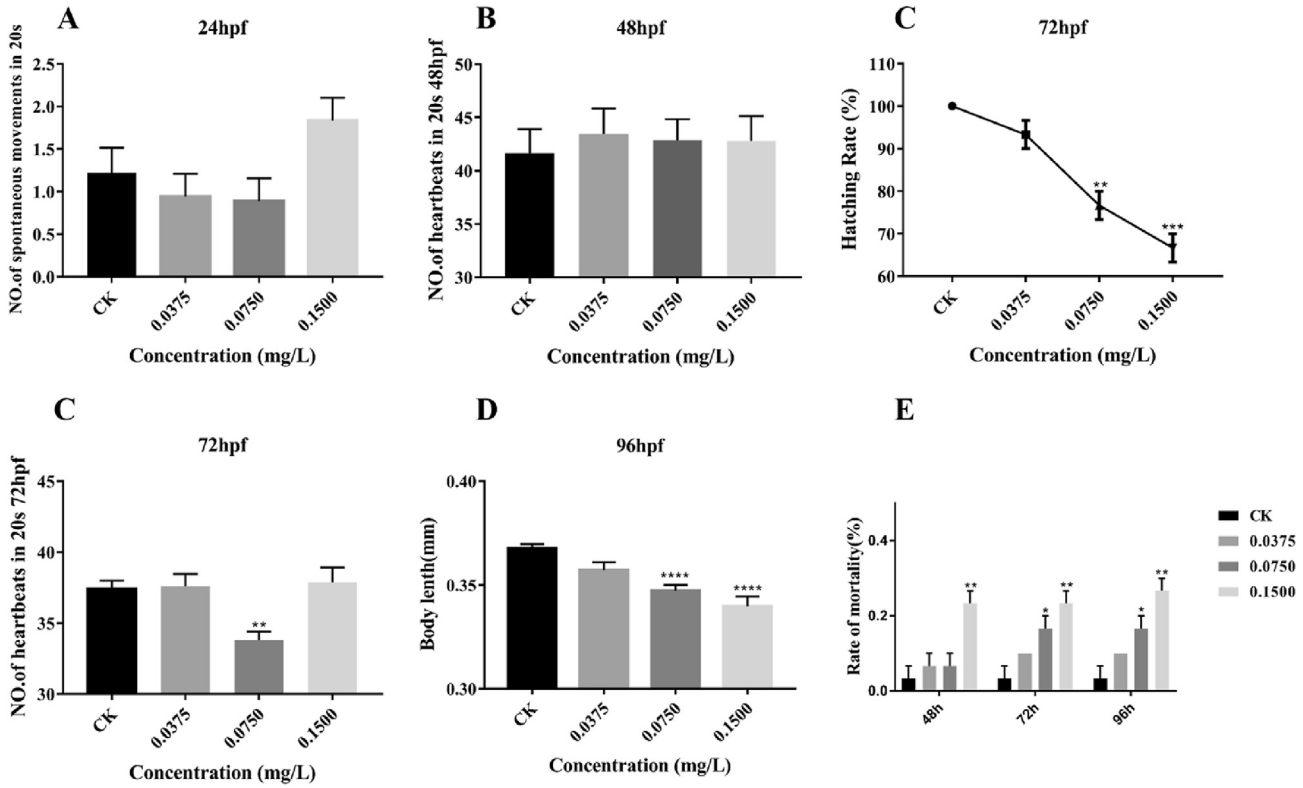

Fig. 1. Developmental effects of prothioconazole on embryos. A: The number of spontaneous movements at 24 hpf. B: Heart rates of zebrafish embryos in the control and prothioconazole -treated groups at 48hpf. C: The hatching rate at each observation time point. D: Heart rates of zebrafish embryos in the control and prothioconazole treated groups at 72hpf. E: Body lengths of hatched larvae at 96 hpf in the control and prothioconazole-treated groups. F: The hatching rate at each observation time point. Asterisks denote significant difference between the treatments and control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

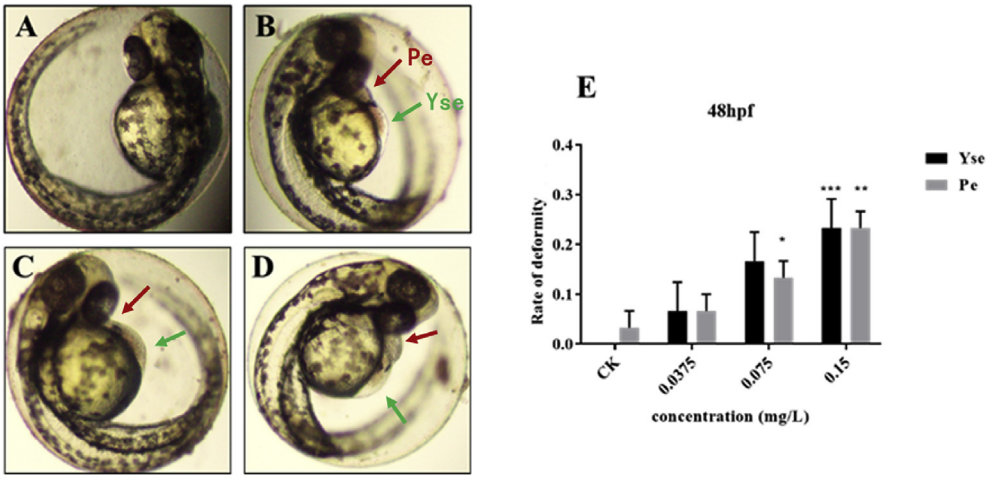

Fig. 2. Malformations of embryos after exposure for 48 hpf. A: Embryo in control group with normal yolk sac and normal pericardial; B–D: embryo exposed to 0.0375, 0.0750 and 0.1500 mg/L prothioconazole. E: Rate of Yse and Pe caused by prothioconazole at 48 hpf. Asterisks denote significant difference between the treatments and control (*p < 0.05; **p < 0.01; ***p < 0.001).

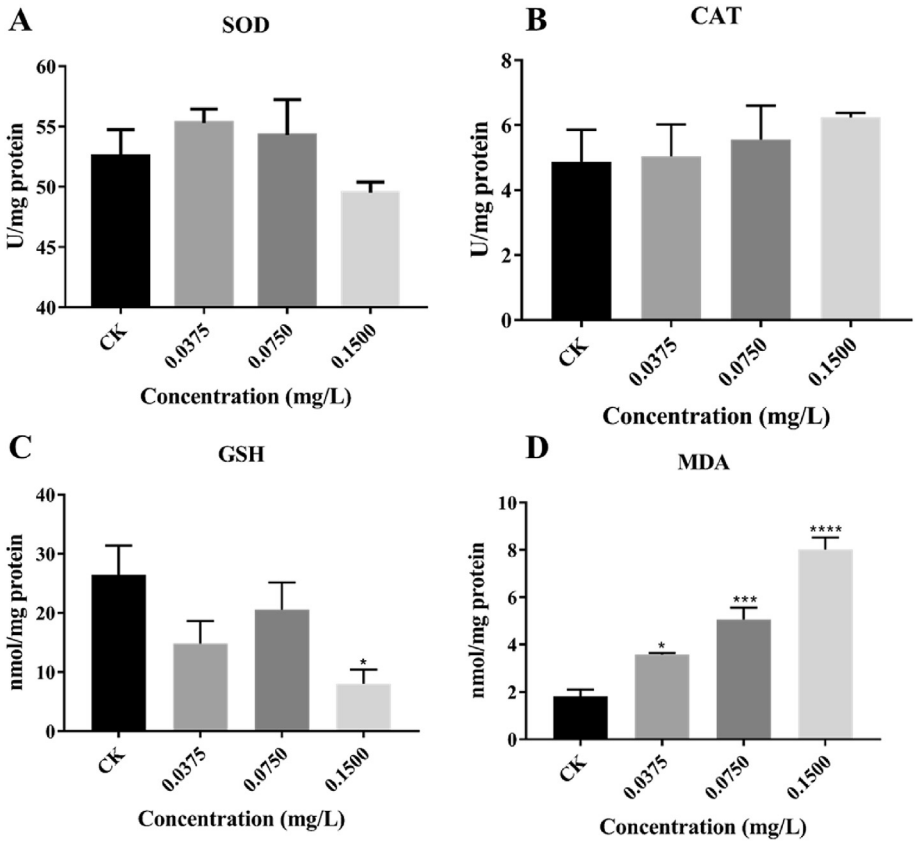

Fig. 3. Effects of prothioconazole on zebrafish larvae oxidative stress levels. A: The glutathione levels. B: The superoxide dismutase (SOD) activities. C: The catalase (CAT) activities. D: The malondialdehyde (MDA) levels. Asterisks denote significant difference between the treatments and control (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

combined with the FDR values obtained from the *t*-test of MetaboAnalyst 4.0 (Fig. 5). Specifically, compared with the control group, 10 metabolites: very low-density lipoprotein (VLDL), lipid, leucine, acetate, lysine, acetone, sarcosine, creatine, tyrosine and phenylalanine were increased significantly. Meanwhile, 9 metabolites: lactate, acetamide, O-acetyl-glycoproteins (O-AcGPs), betaine, glycine, valine, glutamate, histidine and inosine were decreased significantly. The analysis of the metabolic pathway was performed based on the 19 significantly changed metabolites by using the pathway analysis module of MetaboAnalyst 4.0. Four metabolic pathways were disrupted including the metabolism of phenylalanine, the degradation of valine, leucine and isoleucine, the biosynthesis of phenylalanine, tyrosine and tryptophan and the metabolism of D-Glutamine and D-glutamate (Fig. S3).

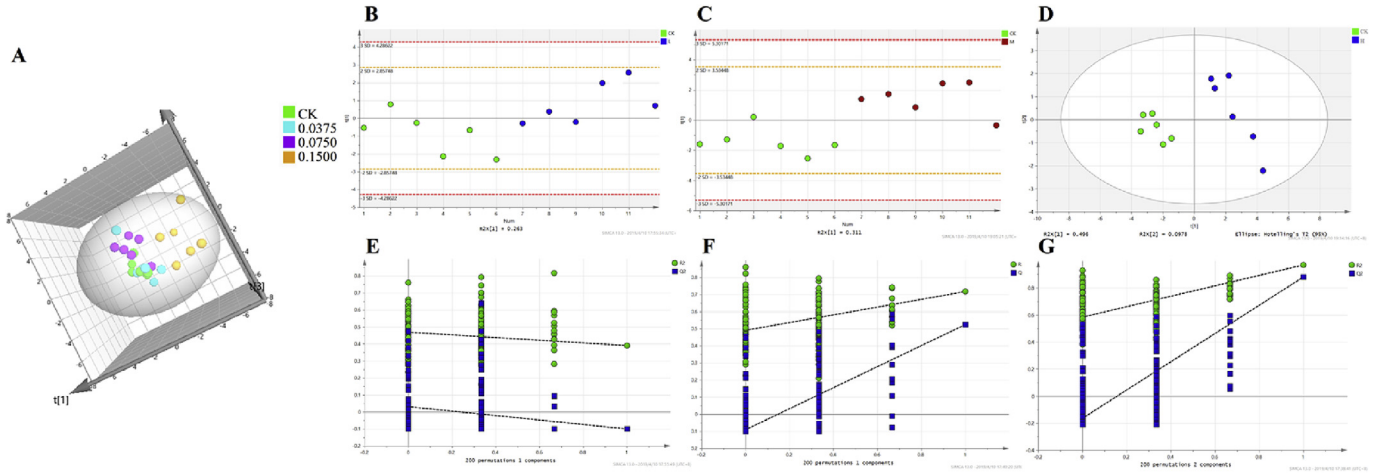

Fig. 4. The 3D PCA score plot for all groups and the PLS-DA score plots for every two groups. A: The 3D PCA score plot of zebrafish larvae metabolites for all groups. B–D: The corresponding score scatter plot of PLS-DA models between the treatments and control groups. E–G: The corresponding permutation plots of the validate models between the treatments and control groups. (n = 6 per group).

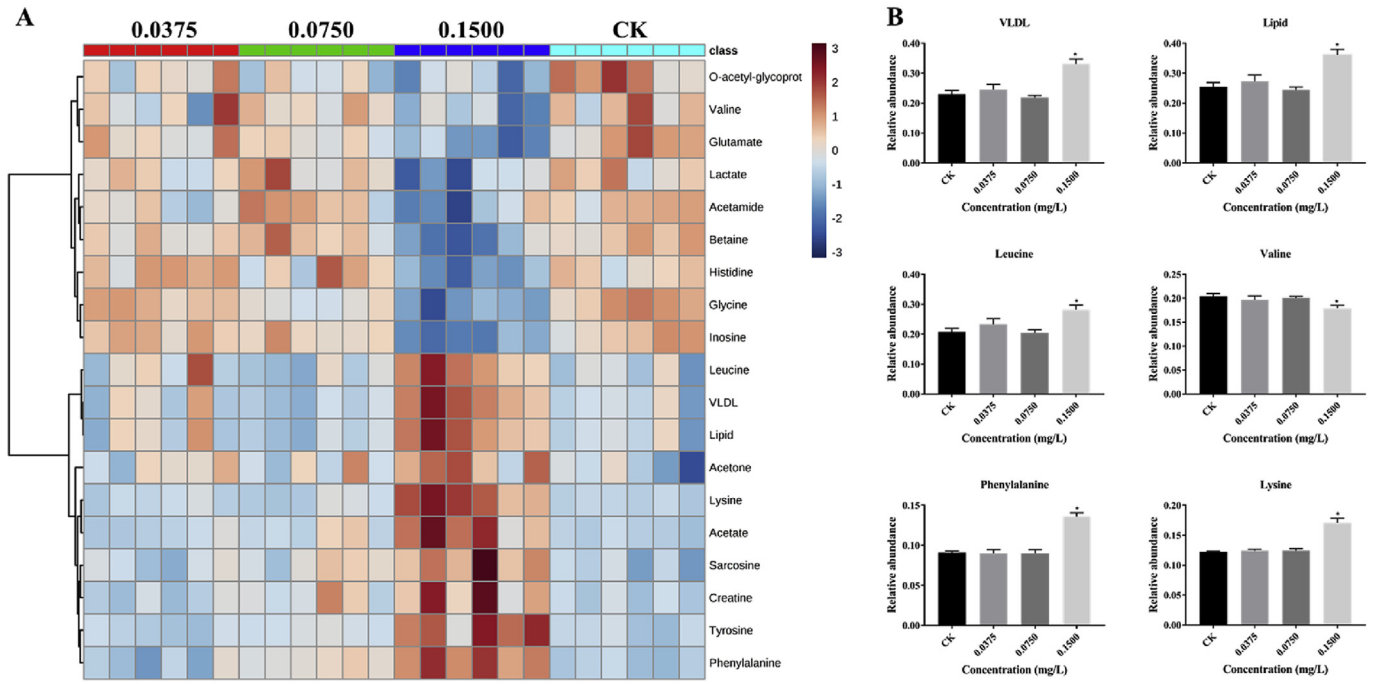

Fig. 5. Heatmap for all significantly changed metabolites in the zebrafish larvae (A) and typical significant changed metabolites (B).

3.4. Effects of prothioconazole on lipid metabolism of zebrafish larvae

The changes in the metabolic profile of zebrafish larvae prompted us to explore the effects of lipid metabolism. The TG and T-CHO levels of zebrafish larvae with prothioconazole exposure were measured and shown in Fig. 6. The levels of TG were significantly decreased in the 0.0750 and 0.1500 mg/L groups. However, the levels of T-CHO were significantly increased in the highest concentration group. The gene expression levels related to lipid metabolism of zebrafish larvae after 96 h of prothioconazole exposure were shown in Fig. 7. Specifically, these genes related to fatty acid synthesis (*Fasn*, *Hmgcra*, *Hmgcrb*, *Acox* and *Acc*), fatty acid oxidation (*Cpt1*), cholesterol metabolism (*Ppar-α*) and cholesterol synthesis (*Cyp51*). From the results of the study, the relative expression of *Cyp51* decreased in the 0.0750 and 0.1500 mg/L treatment groups significantly, and the relative expression of *Acox* also reduced in the 0.1500 mg/L treatment group.

4. Discussion

Zebrafish embryos are usually used as an animal model for monitoring water quality environments, which are very sensitive to change in the water environment and used for the monitoring of teratogens and toxic substances in water (Jin et al., 2015). In this study, some symptoms appeared after zebrafish embryos were exposed to prothioconazole, including increased mortality, hatching inhibition, decreased body length, and developmental malformations. Hatching was inhibited in the 0.075 and 0.15 mg/L treatment groups at 72 hpf significantly, its depended primarily on

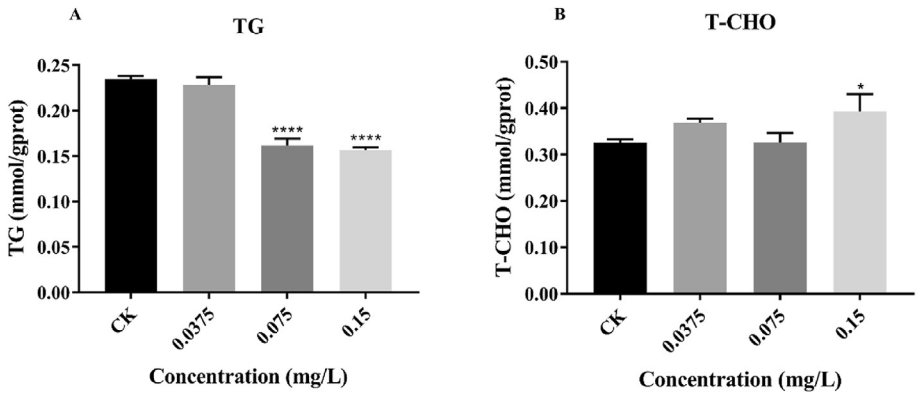

Fig. 6. Effects of prothioconazole on lipid metabolism. A and B: The content of TG (A) and TC (B) of control and prothioconazole-treated groups at 96hpf. Asterisks denote significant difference between the treatments and control (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

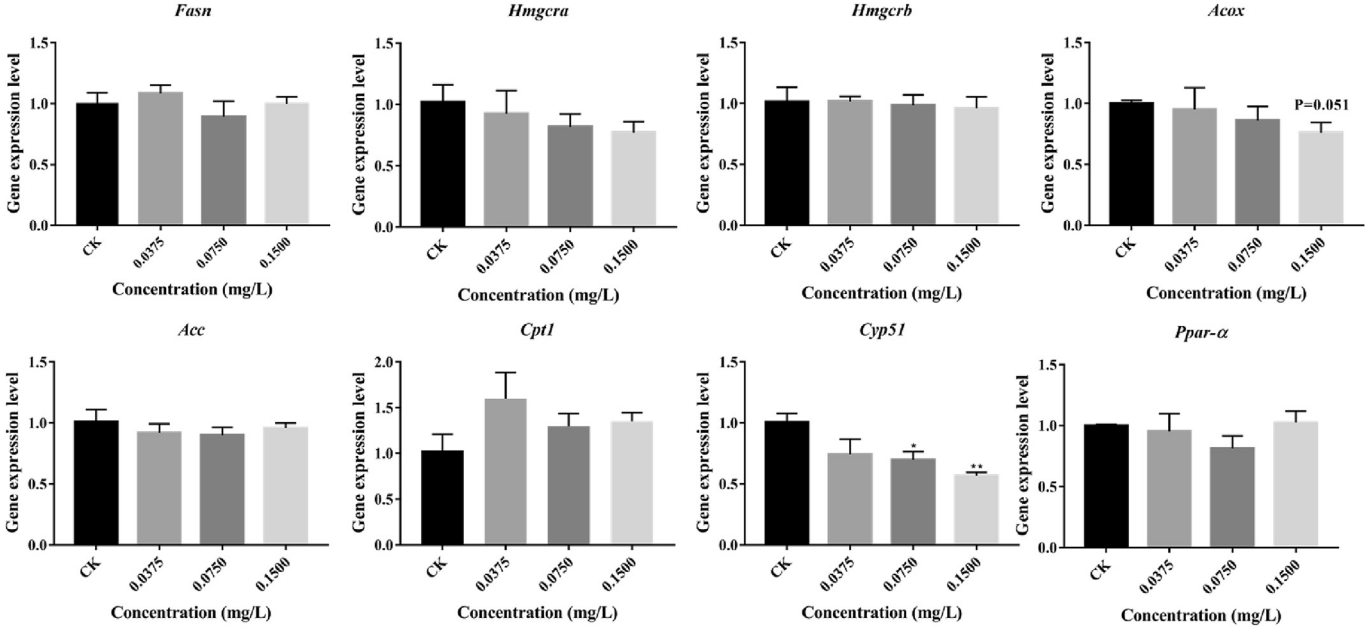

Fig. 7. Relative mRNA expression of *Fasn, Hmgcra, Hmgcrb, Acc, Ppar-α, Acox, Cyp51* and *Cpt1* in larvae exposed to prothioconazole for 96 h. The asterisks denote significant difference between the treatments and control (*p < 0.05; **p < 0.01).

enzyme activity and embryonic movement (Leung and Bulkley, 1979; Samaee et al., 2015; Teng et al., 2018a). Studies have shown that yolk sac abnormalities in zebrafish may have adverse effects on hatching glands (Zhou et al., 2009). In this study, prothioconazole may affect the development of the hatching gland by inducing yolk sac oedema, which was accompanied by a significant inhibition of hatching rate.

Oxidative stress is associated with developmental toxicity during zebrafish embryogenesis, and it is thought to contribute to the abnormal development of embryos (Carney, 2013; DeMarco et al., 2008; Wells et al., 2009). Subsequently, the activities of SOD and CAT and the levels of MDA and GSH were measured to evaluate whether prothioconazole induced oxidative stress in zebrafish embryos. The results of this experiment showed that the levels of GSH were significantly reduced with prothioconazole exposure, which indicates that antioxidant system of zebrafish embryos was impacted. Actually, oxidation and antioxidants maintain a dynamic balance in normal living organisms (Dogan et al., 2011). Specifically, O^{2-} is converted to produce H_2O_2 by SOD. Subsequently, H_2O_2 could be metabolized to produce oxygen and water with the participation of CAT, and this process is catalyzed by GSH (Haruta et al., 2009; Mu et al., 2017; Yang et al., 1996). However, when the body's antioxidant system cannot maintain a dynamic balance, oxidative stress occurs. In addition, MDA is the main product of lipid peroxidation, and its changes can reflect the extent of biological oxidative damage (Draper et al., 1990). Compared with the control group, the levels of MDA were significantly elevated in all treatment groups, indicating that prothioconazole exposure caused significant lipid peroxidation and oxidative damage in zebrafish embryos. Similarly, other triazole fungicides had been reported to induce oxidative damage in zebrafish embryos. The relative expression level of zebrafish embryos was significantly reduced, and the oxidative stress occurred after exposure to difenoconazole (Mu et al., 2016). Additionally, the SOD activity of rare minnow (*Gobiocypris rarus*) embryos was decreased at high concentration of myclobutanil, fluconazole, flusilazole, triflumizole, and epoxiconazole, which also indicated that the antioxidant system of embryos might be destroyed (Zhu et al., 2014).

In order to explore the toxicity of prothioconazole in zebrafish embryos more comprehensively, metabolomics based on ^{1}H NMR were used in our study. And we found that there were significant changes in 19 metabolites in the 0.1500 mg/L treatment group. This included a variety of amino acid deregulation, in which phenylalanine acts as a precursor to neurotransmitters in the zebrafish brain (Jeong et al., 2015; Peng et al., 2013), and elevated phenylalanine indicates that prothioconazole exposure may cause brain damage, therefore prothioconazole has potential to cause apoptosis and oxidative damage, leading to an adverse effect on the central nervous system (CNS), which consistent with the results of zebrafish exposure to Methyl-triclosan (Fu et al., 2019). Therefore, the dysregulation of phenylalanine can actually be a potential cause of oxidative damage caused by exposure of zebrafish embryos to prothioconazole. In particular, some metabolites related to lipid metabolism have also changed. The relative levels of the lipid and VLDL increased, suggesting that lipid metabolism may be interfered by prothioconazole in zebrafish embryos (Zhang et al., 2016). VLDL can transport endogenous triacylglycerol and cholesterol synthesized by the liver to tissues such as fat and heart muscle and transform into low-density lipoprotein (LDL) (Otis et al., 2015). Besides, the relative levels of leucine and valine also significantly change. Predecessor studies have shown that changes of branched-chain amino acids are usually accompanied by lipid accumulation (Du et al., 2012; Zhang et al., 2007). Which insinuated that prothioconazole may interfere with lipid metabolism in zebrafish embryos.

Furthermore, we measured indicators related to lipid metabolism. Lipid plays an important role in energy supply and maintenance of normal metabolic activity (Qian et al., 2019). TG and T-CHO are the main components of lipids, and their synthesis and metabolism can affect lipid metabolism (Dong et al., 2016). The contents of TG and T-CHO were quantified to assess disorders of lipid metabolism in zebrafish embryos. Consistently, prothioconazole exposure significantly changed the contents of TG and T-CHO. Then we measured the genes involved in lipid metabolism. Among them, *CYP51* is an important member of the P450 supergene family (Eberle et al., 2004). The 14α-demethylase encoded by this gene is one of the key enzymes in cholesterol synthesis (Shimano, 2001). The expression level of *CYP51* was significantly reduced with prothioconazole exposure. Taken together, the alterations of genes related to cholesterol resulted in significantly decreased levels of T-CHO following exposure to prothioconazole, which suggesting that lipids metabolism in zebrafish embryos has been disturbed.

Particularly, Lipids are one of the main sources of energy required for embryo formation and hatching (Tocher, 2003). Lipid metabolism disorders may lead to growth retardation and developmental deformity in zebrafish embryos and larvae (Moran, 2007). This was consistent with the apparent inhibition of the body length of zebrafish larvae after exposure to prothioconazole. Previous studies have shown that fatty acids are important for the growth and development of organisms in the early stages of life, which may cause delayed development (Teng et al., 2019). All in all, the results suggest that lipid metabolism disorders may be an important cause of developmental toxicity in the early life stages of zebrafish.

5. Conclusions

In summary, we comprehensively explored the toxic effects of prothioconazole exposure on zebrafish embryos. The results showed that the developmental toxicity, oxidative damage and disorders of lipid metabolism were induced after exposure to prothioconazole, which means prothioconazole may pose a potential risk to aquatic organisms. Therefore, we suggest that the potential environmental and health risks of prothioconazole should be considered, especially at the early life stages of aquatic organisms. Furthermore, these findings provide us with further insight into the developmental toxicity mechanisms combined with metabolism disorders following exposure to environmental pollutants.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113269.

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