Mepanipyrim and cyprodinil induce divergent temporal patterns of AhR-mediated responses in zebrafish (*Danio rerio*) embryos and larvae

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Research Article

Keywords: pesticides, *Danio rerio*, AhR agonists, cyp1a, EROD

Posted Date: October 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-776853/v1>

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Abstract

Mepanipyrim and cyprodinil are widely used to control and/or prevent fungal diseases in fruit culture. They are widely detected in aquatic environment and numerous food commodities including fruit and fruit products. Different from TCDD, mepanipyrim and cyprodinil are more easily degraded and metabolized in the environment. However, the *in vivo* analysis of their metabolic dynamics is unclear and need to be further confirmed. In this study, zebrafish embryos were constantly exposed to 100 µg/L mepanipyrim or cyprodinil for 7 days. The temporal pattern of CYP1A and AhR2 expression and EROD enzyme activity at different time frames during embryonic and larval development of zebrafish were investigated. Our results showed that mepanipyrim and cyprodinil tend to accumulate in zebrafish during early embryonic developmental stages. Meanwhile, mepanipyrim and cyprodinil exposure could increase the expression level of *cyp1a* and *ahr2* genes and EROD activity by a dynamic pattern in different developmental stages of zebrafish. Besides, their metabolites, which may accumulate in the zebrafish larvae, have strong AhR agonistic activity and showed strong AhR binding ability. Importantly, the risk of exposure to pesticides in embryo stage is huge, and should be paid more attention.

1. Introduction

Pesticides are proverbially used in agriculture to control insect pests for enhancing the yields and quality of crops (Sapbamrer & Thammachai, 2020; Egbe et al., 2021). Globally, more than 1100 pesticides are marketed and developed for targeting specific pests in aquaculture, horticulture, agriculture and household (Meftaul et al., 2020; El-Nahhal, 2020). These pesticides include fungicides, herbicides, insecticides, rodenticides, etc. (Meftaul et al., 2020). Anilinopyrimidines are a chemical class of fungicides which can control and/or prevent fungal diseases in fruit culture (Castro et al., 2020). Mepanipyrim and cyprodinil belong to the anilinopyrimidine chemical family, and are widely used on the world-wide (Sieiro-Sampedro et al., 2019a; Chen et al., 2018; Tang et al., 2020). Their are broad-spectrum fungicides to target various phytopathogenic fungi by affecting the biosynthesis of hydrolases and methionine (Sieiro-Sampedro et al., 2019b; Shen et al., 2020). Due to their large usage, mepanipyrim and/or cyprodinil have been widely detected in aquatic environment, fruits and fruit products, and numerous food commodities (Esteve-Turrillas et al., 2015; Shen et al., 2020; Tang et al., 2020). For example, the residues of cyprodinil have been detected between 0.736–2.898 µg/L in the aquatic environment in Chile (Climent et al., 2019; Tang et al., 2020). Besides, cyprodinil residues have been detected between 41–907 µg/L in commercial strawberry samples (Esteve-Turrillas et al., 2015). The residual levels of mepanipyrim in grapes and clarified wines are 2.0 and 0.2–0.7 mg/L, respectively (Briz-Cid et al., 2019; Thais et al., 2019; Shen et al., 2020).

The aryl hydrocarbon receptor (AhR), a highly conserved ligand activated transcription factor, is one of the helix-loop-helix-containing transcription factors (Bugel et al., 2013; Eichbaum et al., 2014). It is vital for numerous hazardous chemicals to initiate many of their developmental toxicities and represents a crucial biomarker for risk assessment of organic contaminants (Bugiak et al., 2010; Meyer-Alert et al., 2019). It is well known that the AhR signaling pathway mediates cellular responses to extrinsic

environmental contaminants, including benzo(a)pyrene (BaP), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polybrominated diphenyl ethers (PBDEs), etc. (Norman Haldén et al., 2011; Du et al., 2015; Wang et al., 2015). The inactive form of AhR is located in the cytoplasm and forms a complex with heat-shock-protein 90 β (HSP90 β) dimer, one molecular of AhR-interacting protein (AIP) and protein 23 (p23) (Meyer-Alert et al., 2018; Meyer-Alert et al., 2019). Upon ligand binding, the AhR dissociates from complex in the cytosol, and the nuclear localization signal of AhR is uncovered (Kudo et al., 2018; Meyer-Alert et al., 2018; Roy et al., 2020). Then the AhR immediately translocates into the nucleus and ultimately alters its co-factors with AhR-nuclear-translocator (ARNT) (Meyer-Alert et al., 2019). The AhR/ARNT heterodimer can bind to dioxin-responses elements (DREs) and regulate the transcription of genes related to xenobiotic metabolism (Roy et al., 2019; Jin et al., 2020).

Dioxin-like compounds (DLCs) may induce cytochrome P450-dependent monooxygenases (CYPs) activity by binding to the AhR (Aguirre-Martínez et al., 2017). CYPs induction can reduce the danger of xenobiotic toxicity by accelerating the metabolism and excretion of xenobiotics to lower xenobiotic contents in the plasma or organisms (Loerracher & Braunbeck, 2020). Cytochrome P450 1A (CYP1A) plays a crucial role in the detoxification and metabolism of organic pollutants (Zhao et al., 2017). For the determination of DLCs activity, ethoxyresorufin-*O*-deethylase (EROD) activity is usually regarded as a biomarker of AhR-mediated induction of CYP1A, and is a highly sensitive indicator of dioxins and structurally related compounds (Whyte et al., 2000; Eichbaum et al., 2014; Kais et al., 2017; Kais et al., 2018). As a strong AhR ligand and the most toxic one of planar halogenated aromatic hydrocarbons (PHAHs), TCDD can continuously maintain the induced levels of CYP1A and EROD at a plateau for sustaining periods and is difficult to degrade and metabolize *in vivo* (Zodrow et al., 2004; Ortiz-Delgado et al., 2008; Chen & Chan, 2018). This can lead to the accumulation of reactive oxygen species (ROS) in cells or organisms, and eventually result in oxidative damage of cell biomembrane, cell apoptosis, and death of the organisms (Lee & Yang, 2010; Kalaiselvan et al., 2016; Liu et al., 2019). Previous studies have shown that mepanipyrim and cyprodinil as AhR agonists could induce the expression of AhR-regulated genes via AhR signaling pathway (Tang et al., 2020; Zhu et al., 2021). Different from TCDD, mepanipyrim and cyprodinil are more easily degraded and metabolized in the environment. However, the metabolic dynamics of them *in vivo* is unclear and need to be further confirmed.

Zebrafish (*Danio rerio*) has become a prevalent vertebrate model in several concernful lines of biological research, including environmental toxicology, drug discovery, disease models, developmental biology and neurobiology etc. (Carten et al., 2011; Zhang et al., 2016; Brugman, 2016; Kithcart & MacRae, 2017; Anselmo et al., 2018; Shams et al., 2018). The embryos and larvae of zebrafish, as a valuable models in aquatic ecotoxicology, have gained increasing popularity to assess the effects of various pollutants (Velki et al., 2017; Shen & Zuo, 2020). Besides, zebrafish screenings involving metabolite analysis at the different life stages of zebrafish are also common (Saad et al., 2017; Anselmo et al., 2018). This study was designed to analyze whether the activation of the detoxification metabolism through CYP1A could continuously be induced by mepanipyrim and cyprodinil exposure in zebrafish larvae and embryos. For this aim, we have detected the residues of mepanipyrim and cyprodinil in the embryos and larvae of zebrafish. At the same time, the temporal patterns of CYP1A and AhR2 expression and EROD enzyme

activity at different time frames during embryonic and larval development of zebrafish were investigated (24, 48, 72, 96, 120, 144, and 168 hours post fertilization (hpf)). In addition, we also predicted the AhR agonistic activity of several possible metabolites of mepanipyrin and cyprodinil by molecular docking method.

2. Materials And Methods

2.1. Chemicals and reagents

Mepanipyrin (CAS 110235-47-7; exceed 99% purity) and cyprodinil (CAS 121552-61-2; 99.9% purity) were acquired from Dr. Ehrenstorfer (Augsburg, Germany). TCDD (CAS 1746-01-6; 50 µg/ml) was acquired from Wellington Laboratories, Inc. (Ontario, Canada). Dimethyl sulfoxide (DMSO; 99.7% purity) was acquired from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents with analytical grade were acquired from mercantile origins.

2.2. Zebrafish maintenance and embryonic exposure

The wild-type TU zebrafish maintenance was based on our previous studies (Shen et al., 2020; Shen et al., 2021a). The mepanipyrin and cyprodinil were dispersed in DMSO to achieve stock solutions of 1 mg/mL, respectively. TCDD (1 ng/L), mepanipyrin (100 µg/L), and cyprodinil (100 µg/L) exposure solutions were prepared by entering the proper volume of the stock solutions to the zebrafish culture medium. The control group was taken over a coordinative volume of DMSO solvent (0.01%). Eighty embryos (0.5-1.0 hours post-fertilization (hpf)) were cultured in a glass dish containing 40 mL exposure solution (n = 5), which was renewed solutions once daily. The embryos were continuously exposed for 24, 48, 72, 96, 120, 144, and 168 hours to TCDD, mepanipyrin, or cyprodinil.

2.3. Residue detection of mepanipyrin and cyprodinil

The actual doses of mepanipyrin and cyprodinil in the exposure solutions were measured according to our previous study (Shen et al., 2020). In the residue detection of embryos and larvae, the embryos or larvae were homogenized in 0.2 mL acetonitrile, and then transferred to a 5 mL centrifuge tube with 1 g anhydrous sodium sulfate, 2 ml acetonitrile, and 1 ml acetonitrile-saturated n-hexane. After centrifugation, the acetonitrile layer was extracted and dried with a nitrogen blowing instrument (EYELA MGS-2200, Japan). A solution of 0.1% formic acid-methanol: water (2: 8 v/v) was used for volume determination. The separation was achieved on the chromatographic column (Kinetex 2.6u C18 100A 100*3.0 mm (Phenomenex, USA)), with a gradient elution using mobile phase consisted of ultrapure water (A) and 0.1% formic acid with acetonitrile (B) (**S-Table 1 & S-Table 2**). The flow rate was 0.25 mL/min. The injection volume was 5 µL. A series of standard concentrations of mepanipyrin and cyprodinil (0.5, 2, 10, 50, 100, and 200 part per billion) were prepared to make the standard curve for

quantification. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was performed using Agilent 1290 Infinity LC- 6490 QQQ (USA).

2.4. Real-time quantitative fluorescence PCR (RT-qPCR)

Zebrafish embryos and larvae (6 samples/group, 25 embryos or larvae/sample) were mixed for RNA isolation. Total RNA extraction, cDNA synthesis, and RT-qPCR were carried out according to our previous studies (Zhou et al., 2019; Shen et al., 2020; Shen et al., 2021a). The primer sequences used for mRNA expression analysis of genes are listed in **S-Table 3**.

2.5. Measurement of the EROD activity

Zebrafish embryos and larvae (6 samples/group, 25 embryos or larvae/sample) were collected for EROD activity detection. A total volume of 75 μ L EROD buffer (3 μ L for per embryo or larva) was added to 1.5 mL reaction tube. Next, the samples were dispersed using a dispersing device (TIANGEN Biotech CO., LTD, Beijing) until completely dissociated. The homogenized samples were centrifuged at 3000 rotation per minute (rpm) and 4 °C for 15 min. Total protein concentrations of the homogenized samples were analyzed using BCA Protein Assay Kit (EpiZyme, Shanghai, China). A resorufin (Sigma-Aldrich, Germany) standard was used for drawing the sample-specific standard curves. The supernatant of the homogenized samples were transferred to a white opaque 96-well plate to detect the EROD activity. In detail, 0.6 μ M 7-ethoxyresorufin (Sigma-Aldrich, Germany) was added to each well, and was incubated in darkness at room temperature for 10 min. Thereafter, 3.35 mM NADPH (Sigma-Aldrich, Germany) was added to each well, and was incubated in darkness at room temperature for 15 min. Lastly, 1.35 mM ice-cold acetonitrile-fluorescamine (Sigma-Aldrich, Germany) solution was added to stop the reaction. After 15 min, the fluorescence was measured by a POLARstar Omega automatic analysis system (BMG Labtech, Ortenberg, Germany) at 544 nm excitation and 590 nm emission. The EROD activity was calculated as pmol resorufin/min/mg protein.

2.6. Molecular docking

To examine the potential AhR activity of pesticides or their metabolites, AutoDock suite of programs was used to implement the molecular docking process. The ligand-binding domain sequence of AhR protein was obtained from UniProt (Entry ID: P35869). The detailed molecular docking process is based on our previous study (Tang et al., 2020; Zhu et al., 2021).

2.7. Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, Inc., USA) was employed to carry out the statistical analysis of the data. The data (mean $\pm$ standard errors, n = 3 or 6) were analysed using the Duncan's post hoc test

after one-way analysis of variance (ANOVA). *** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$; n.s. indicates no significant difference.

3. Results

3.1. The residues of mepanipyrim and cyprodinil in zebrafish embryos and larvae

The actual measured concentrations of mepanipyrim and cyprodinil in the exposure solutions were 65.60 ± 11.71 and 62.75 ± 4.94 $\mu\text{g/L}$, respectively (Table 1). In order to better understand the metabolism of mepanipyrim and cyprodinil in the developmental process of zebrafish embryos and larvae, we detected the residues of mepanipyrim and cyprodinil in different developmental stages of zebrafish. The measured concentrations of mepanipyrim in 24 and 48 hpf embryos of zebrafish were 5.16 ± 0.16 and 5.27 ± 0.88 $\mu\text{g/g}$, respectively (Fig. 1a, Table 2). However, with the development of embryos, mepanipyrim was gradually degraded and metabolized in larvae. The measured concentrations of mepanipyrim in 72, 96, 120, 144, and 168 hpf larvae of zebrafish were 1.83 ± 0.20 , 0.98 ± 0.30 , 0.15 ± 0.01 , 0.06 ± 0.02 , and 0.08 ± 0.01 $\mu\text{g/g}$, respectively (Fig. 1a, Table 2). Compared with mepanipyrim, cyprodinil was easier to accumulate in zebrafish larvae. The measured concentrations of cyprodinil in 24, 48, and 72 hpf embryos or larvae of zebrafish were 18.71 ± 1.94 , 19.56 ± 2.32 , and 18.41 ± 0.83 $\mu\text{g/g}$, respectively (Fig. 1b, Table 2). However, cyprodinil was also gradually degraded and metabolized in larvae after 72 hours. The measured concentrations of cyprodinil in 96, 120, 144, and 168 hpf larvae of zebrafish were 2.40 ± 0.13 , 0.15 ± 0.02 , 0.36 ± 0.06 , and 0.53 ± 0.07 $\mu\text{g/g}$, respectively (Fig. 1b, Table 2).

Table 1
Mean measured concentrations of mepanipyrim & cyprodinil ($\mu\text{g/L} \pm \text{SD}$) in the water samples (n = 3).

Nominal concentration ($\mu\text{g/L}$)	Measured concentration (mepanipyrim, $\mu\text{g/L}$)	Measured concentration (cyprodinil, $\mu\text{g/L}$)
0	0.11 ± 0.01	0.16 ± 0.04
100	65.60 ± 11.71	62.75 ± 4.94

Table 2

Mean measured concentrations of mepanipyrin & cyprodinil ($\mu\text{g/g} \pm \text{SD}$) in zebrafish larvae or embryos ($n = 3$).

Nominal concentration ($\mu\text{g/L}$)	Larvae exposed days (D), measured concentration ($\mu\text{g/g}$)						
	1 D	2 D	3 D	4 D	5 D	6 D	7 D
Mepanipyrin-100	5.16 ± 0.16	5.27 ± 0.88	1.83 ± 0.20	0.98 ± 0.30	0.15 ± 0.01	0.06 ± 0.02	0.08 ± 0.01
Cyprodinil-100	18.71 ± 1.94	19.56 ± 2.32	18.41 ± 0.83	2.40 ± 0.13	0.15 ± 0.02	0.36 ± 0.06	0.53 ± 0.07
D: days.							

3.2. The mRNA expression of *cyp1a* and *ahr2* genes in mepanipyrin or cyprodinil exposed embryos and larvae

We next examined the temporal pattern of *cyp1a* and *ahr2* mRNA expression during zebrafish early developmental process. The mRNA expression patterns of *cyp1a* gene after exposure to mepanipyrin or cyprodinil show differences compared with TCDD exposure. Expression of *cyp1a* gene in embryos and larvae was dynamically induced by mepanipyrin and cyprodinil (Fig. 2a). However, the expression of *cyp1a* in embryos and larvae was continuously induced by TCDD. The number of *cyp1a* transcripts was constantly increased by TCDD exposure and reached about 2000-fold change in 7 dpf larvae compared with 24 hpf solvent control embryos (Fig. 2a). At the same time, the mRNA expression of *ahr2* gene was also showed a dynamic induction by mepanipyrin and cyprodinil exposure (Fig. 2b). Nevertheless, the mRNA expression of *ahr2* gene was showed a continuous induction by TCDD exposure (Fig. 2b).

3.3. The EROD activities of mepanipyrin or cyprodinil exposed embryos and larvae

EROD activity is usually regarded as a biomarker of AhR-mediated induction of CYP1A, and is a highly sensitive indicator of dioxins and structurally related compounds (Whyte et al., 2000; Eichbaum et al., 2014; Kais et al., 2017; Kais et al., 2018). To further confirm our results, we detected the EROD activity of mepanipyrin and cyprodinil exposed embryos and larvae. As shown in Fig. 3, the EROD activities of embryos and larvae were dynamically altered by mepanipyrin and cyprodinil exposure, while TCDD exposure lead strong increase in EROD activities.

3.4. The AhR agonistic activities of mepanipyrin and cyprodinil metabolites

To detect the AhR agonistic activity of potential metabolites of mepanipyrin and cyprodinil, molecular docking method was used to explore the binding energies between their metabolites and AhR. The

smaller binding energies indicating better interaction activity. As shown in Table 3, the binding energy of strong AhR agonist TCDD interacting with AhR was -8.5 kcal/mol, and the binding energy of mepanipyrim and cyprodinil interacting with AhR were -9.3 and -9.5 kcal/mol, respectively. In addition, the binding energy of two potential metabolites (No. 5 and No. 10) of cyprodinil interacting with AhR were -9.2 and -6.6 kcal/mol, respectively. And the binding energy of five potential metabolites (No. 4, No. 6, No. 7, No. 8, and No. 9) of mepanipyrim interacting with AhR were -8.3 , -8.9 , -8.9 , -6.8 , and -8.7 kcal/mol, respectively.

4. Discussion

Developing fish are one of the most sensitive organisms to TCDD and other DLCs (Teraoka et al., 2003). The CYPs play a crucial role in the metabolism and degradation of hazardous compounds (Guengerich, 2017; Loerracher et al., 2020). Among the CYPs, *cyp1a* is the best characterized and is the strongest response in TCDD bio-activation in fish (Chuang et al., 2004; Jonsson et al., 2009; Chen & Chan, 2018). As a persistent organic pollutant, TCDD is very difficult to be degraded and metabolized in organisms (Sorg et al., 2009; Sorg, 2014). Due to its persistent existence in organisms, it can constantly induce the expression of AhR targeting gene *cyp1a* (Roy et al., 2011; Hervé et al., 2010; Bock, 2018; Roy et al., 2018). CYP1A protein is first detected at 36 hpf zebrafish larvae after exposure to TCDD, and constitutive expression of CYP1A is demonstrated in the 120 hpf larval zebrafish (Andreasen et al., 2002; Otte et al., 2010). Many studies have shown that low concentration of TCDD could strongly induce the expression of *cyp1a* in many tissues of zebrafish, especially in the liver and gut (Kim et al., 2013; Shen et al., 2018; Xie et al., 2018). Similarly, we found that 1 ng/L TCDD exposure could dramatically induce the expression of *cyp1a* by a constant increasing pattern in zebrafish larvae. These results further indicate the persistence of TCDD in organisms.

CYP1A is commonly associated with the biotransformation of environmental contaminants, especially the DLCs (Cardoso et al., 2019; Licata et al., 2019). Many DLCs including pesticides belong to the substrates of CYP1A enzyme biotransformation, which are usually converted to more hydrophilic metabolites for excretion (Roy et al., 2019). Besides, CYP1A induction can also produce toxic effects by promoting the production of active metabolites (Voelker et al., 2008). We found that mepanipyrim and cyprodinil exposure could induce the expression of *cyp1a* by a dynamic pattern in different developmental stages of zebrafish. Meanwhile, the potential metabolites of mepanipyrim and cyprodinil also have AhR agonistic activity, indicating the induction of *cyp1a* expression could also be mediated by their metabolites.

As a target gene of AhR, the change of *cyp1a* expression is closely related to the successful entry of AhR into nucleus and the formation of AhR/ARNT dimer acting on dioxin-responsive elements (Denison et al., 2017; Vogel et al., 2020). AhR located in the cytoplasm remains inactive before binding ligands (Reynaud & Deschaux, 2006; AnvariFar et al., 2018). The storage of AhR in the cytoplasm always maintains a dynamic balance (Bell & Poland, 2000; Mejía-García, et al., 2015). It can play a nuclear receptor role only after ligand binding or activation by phosphorylation (Guyot et al., 2013). Many studies have reported

that DLCs can strongly induce the expression of *ahr2* (Guo et al., 2017; Zhou et al., 2020). The increased mRNA expression of *ahr2* have become an important toxic indicator of DLCs (Cheng et al., 2020). In the present study, we found that the mRNA expression of *ahr2* also showed a dynamic change by mepanipyrin or cyprodinil exposure, and was consistent with the expression of *cyp1a* in zebrafish. The continuous activation of AhR signaling pathway is a crucial condition to ensure that mepanipyrin and cyprodinil can be effectively degraded and metabolized (Hale et al., 2017).

Over-expression of CYP1A is involved in production of reactive oxygen species (ROS) (Zangar et al., 2004). Previous study found that TCDD could induce ROS production by up-regulating the expression of CYP1A, which could further cause damage to DNA (Kopf & Walker, 2010; Kalaiselvan et al., 2016; Liu et al., 2019). Besides, pesticides exposure can result in the transformation of toxic substances into ROS, which can ultimately cause cell injury (Singh et al., 2018; Shen et al., 2021b). In the present study, we found that the mRNA expression of *cyp1a* in zebrafish larvae was significantly increased by mepanipyrin or cyprodinil exposure, indicating that mepanipyrin and cyprodinil may increase the level of ROS. Under normal physiological conditions, the production of ROS can promote the growth and development of cells or organisms. However, excessive production of ROS can cause great damage to cell components, such as biomembrane and cell membrane, which will seriously affect the “dynamic balance” of cells or organisms (Suzuki et al., 2012; Bhagat et al., 2020).

Because of the short half-life, the pesticides exhibit a short term toxic effects after application and/or exposure (Korkmaz et al., 2018). During pregnancy, pesticides have the potential to pass through the placental barrier and disturb the fetal development (Dewailly et al., 2014). In addition, pesticides are likely to accumulate in the organisms in early embryonic developmental stages (Gavelle et al., 2016; Kuang et al., 2020). Our results showed that mepanipyrin and cyprodinil tended to accumulate in zebrafish during early developmental stages. With the development of zebrafish, mepanipyrin and cyprodinil began to be degraded and metabolized. These results implied that organisms were sensitive to pesticides in early life, and pesticides were more likely to accumulate in early life stages of organisms. At the same time, pesticides exposure in early life may affect the normal development of organisms. Therefore, the risk of exposure to pesticides in embryo stage is huge, and should be paid more attention. Furthermore, pregnant women, in particular, should avoid to be exposed to pesticides, such as the diets with pesticide residues during pregnancy (Dewailly et al., 2014).

The induction of EROD activity is often used to assess the AhR agonistic activity of dioxins and DLCs in organisms (Eichbaum et al., 2014; Kais et al., 2017; Ji et al., 2021). TCDD can dramatically increase the EROD activity in the liver, kidney, and gills of seabream, *Sparus aurata* (Ortiz-Delgado et al., 2008). Basal EROD enzymatic activity is detected in 6 hpf zebrafish embryos, after then, it has constantly increased by a slow pattern (Meyer-Alert et al., 2018; Meyer-Alert et al., 2019). Previously, Xu et al. (2018) found that TCDD exposure could cause prominent elevation of EROD activity in larval zebrafish. In the present study, our results showed that 1 ng/L TCDD exposure could dramatically increase the EROD activity in different development stages of zebrafish. It was consistent with the increase of *cyp1a* mRNA expression induced by TCDD. At the same time, we found that mepanipyrin and cyprodinil exposure could increase the EROD

activity with a dynamic pattern in different developmental stages of zebrafish. It also implied that the potential metabolites of mepanipyrin and cyprodinil could increase the EROD activity in zebrafish larvae.

Unlike persistent organic pollutants, pesticides are easier to be degraded and metabolized (Velki et al., 2017). But their metabolites may be difficult to be degraded and could accumulate in the organism for a long time. Our study found that embryonic exposure to diuron for 7 days, diuron could quickly be degraded into its two major metabolites *N*-demethyldiuron (DCPMU) and *N*-didemethyldiuron (DCPM) in zebrafish larvae. Meanwhile, DCPMU and DCPU could gradually accumulate in the larvae with the extension of exposure time (**unpublished data**). In the present study, we found that mepanipyrin and cyprodinil could also be quickly degraded, and their residues in larvae decreased with the exposure time. Therefore, we speculated that their potential metabolites may accumulate in the zebrafish larvae. Previously, it has been reported that mepanipyrin and cyprodinil exhibit a very strong AhR agonistic activity and can dramatically induce the expression of AhR-regulated genes (Tang et al., 2020; Zhu et al., 2021). Presently, we found that mepanipyrin or cyprodinil had strong binding with AhR, even stronger than TCDD. Besides, their metabolites also had strong AhR agonistic activity and showed strong AhR binding ability. The residues of mepanipyrin or cyprodinil in 5 dpf zebrafish larvae were very low, but the *cyp1a* expression and EROD activity did not return to normal level, indicating that their metabolites could accumulate in larvae. This is very consistent with our hypothesis. Unfortunately, there are no commercial metabolites available in the market, so it is impossible to carry out substantive verification. To sum up, a direct temporal relation between CYP1A or EROD induction and mepanipyrin or cyprodinil exposure is still vaguely indicated by the present results and further research is required.

5. Conclusion

This study was designed to analyze whether the activation of the detoxification metabolism through CYP1A could be continuously induced by mepanipyrin and cyprodinil exposure in zebrafish larvae and embryos. Our results showed that mepanipyrin and cyprodinil tended to accumulate in zebrafish during early embryonic developmental stages. With the development of embryos, mepanipyrin and cyprodinil were gradually degraded and metabolized in larvae. Meanwhile, mepanipyrin and cyprodinil exposure could increase the expression of *cyp1a* and *ahr2* genes and EROD activity by a dynamic pattern in different developmental stages of zebrafish. Besides, their metabolites, which may accumulate in the zebrafish larvae, have strong AhR agonistic activity and showed strong AhR binding ability.

Declarations

Funding

This work was supported by the National Natural Science Foundation of China (No. 31971234) and the Natural Science Foundation of Fujian Province of China (No. 2018J01067).

Conflicts of interest statement

The authors declare that they have no conflict of interest.

Author contributions

Chao Shen: Conceptualization, Methodology, Investigation, Writing-Original draft preparation, Writing-Reviewing and Editing; **Chen Tang:** Methodology, Investigation; **Kongyang Zhu:** Methodology, Investigation; **Chengyong He:** Project administration, Supervision, Validation, Writing-Reviewing and Editing; **Chunyan Yang:** Supervision, Validation, Writing-Reviewing and Editing; **Zhenghong Zuo:** Funding acquisition, Project administration, Resources, Visualization, Writing-Reviewing and Editing.

Ethics approval

All experiments using zebrafish were performed according to the animal protocol approved by the guides of Animal Ethics Committee of Xiamen University.

Consent to participate and consent for publication

Not applicable.

Availability of data and materials

The obtained and analyzed data of this study are available from the corresponding author on reasonable request.

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Tables

Due to technical limitations, Table 3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Measured concentrations of mepanipyrin (a) and cyprodinil (b) (ng/g ± SD) in zebrafish larvae or embryos (n = 3). Zebrafish embryonic exposure to 100 µg/L mepanipyrin or cyprodinil for 1, 2, 3, 4, 5, 6,

and 7 days (D), the residues of mepanipyrin or cyprodinil in different developmental stages of zebrafish were detected. N.D. represents no detection.

Figure 2

The mRNA expression of *cyp1a* and *ahr2* genes in zebrafish embryos and larvae. The expression of *cyp1a* (a) and *ahr2* (b) exhibited dynamic change by 100 µg/L mepanipyrin or cyprodinil exposure, and strongly induced by 1 ng/L TCDD exposure. The data (mean ± standard errors, n = 6) were analysed using the Duncan's post hoc test after one-way analysis of variance (ANOVA). ***P < 0.001; *0.01 < P < 0.05; n.s. indicates no significant difference. D: days.

Figure 3

Detection of EROD activity in zebrafish embryos and larvae. EROD activity of embryos or larvae exhibited dynamic change by 100 µg/L mepanipyrim or cyprodinil exposure, and strongly increased by 1 ng/L TCDD exposure. The data (mean ± standard errors, n = 6) were analysed using the Duncan's post hoc test after one-way analysis of variance (ANOVA). ***P < 0.001; **0.001 < P < 0.01; *0.01 < P < 0.05; n.s. indicates no significant difference. D: days.

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