WITHDRAWN: Transcriptome analysis reveals hepatotoxicity in zebrafish induced by cyhalofop-butyl

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Research Article

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Abstract

Cyhalofop-butyl is a highly effective aryloxyphenoxypropionate herbicide and widely used for weed control in paddy fields. With the increasing residue of herbicides, it poses a threat to the survival of aquatic organisms. Here, we evaluated the effect of cyhalofop-butyl on zebrafish to explore its potential hepatotoxic mechanism. The results showed that cyhalofop-butyl (0.1, 0.2 and 0.4 mg/L) induced hepatocyte degeneration, vacuolation and necrosis of larvae after embryonic exposure for 4 days and caused liver atrophy after exposure for 5 days. Meanwhile, the activities of enzymes related to liver function named alanine transaminase (ALT), aspartate transaminase (AST) were significantly increased by 0.2 mg/L cyhalofop-butyl and higher. And the contents of triglyceride (TG) involved in lipid metabolism was significantly decreased by 0.4 mg/L cyhalofop-buty. The effects of cyhalofop-butyl on zebrafish larvae were further demonstrated by GO (Gene Ontology) and KEGG pathway analysis. Cyhalofop-butyl (0.1, 0.2, 0.4 mg/L) altered the expression of 116, 154, 397 genes in liver these genes are mainly enriched in metabolism (such as lipid metabolism, amino acid metabolism), immune system (Toll-like receptor signaling pathway) and endocrine system (PPAR signaling pathway). Furthermore, the expression of key genes related to liver development combined with RNA-Seq results, indicated that cyhalofop-butyl might damage the liver development of zebrafish larvae and cause metabolic disorders. To sum up, our research results reveal the physiological and molecular responses of zebrafish liver to cyhalofop-butyl and provide new insights for further studying the mechanism of cyhalofop-butyl toxicity to aquatic organisms.

Highlights

- Cyhalofop-butyl induced liver damage of zebrafish larvae and caused liver atrophy
- Cyhalofop-butyl affected liver function by increasing the activities of ALT and AST.
- Cyhalofop-butyl disturbed the lipid metabolism of liver in zebrafish via inhibition TG levels.
- Cyhalofop-butyl exposure induced metabolism and immune system disturbance at transcriptional levels.

1. Introduction

Aryloxyphenoxypropionate herbicides (AOPPs) are one of the most widely used herbicides in the world with 4-oxyphenoxypropionic acid as the skeleton structure (Zhou et al., 2018). Cyhalofop-butyl [butyl (R) -2-[4-(4-cyano-2-fluorophenoxy) phenoxy] propionate] as the most widely used AOPPs, which is a postemergence herbicide for barnyard grass control in rice fields (Ruiz-Santaella et al., 2006; Wu et al., 2014). Due to its widespread use, cyhalofop-butyl can easily remain in paddy fields and resulting in widespread aquatic environmental pollution. For example, 2.017 mg/L was detected in rice fields in southern China after 10 % fenoxaprop-pethyl•cyhalofop-butyl EC herbicide was sprayed at 135 g a.i./ha (Guo et al., 2008). In Japan, the concentration of cyhalofop-butyl in water samples from bulk drainage was 0.01-0.08 μg/L (Cao et al., 2016; Phong et al., 2010). Up to date, many studies have shown

the toxicity of cyhalofop-butyl to aquatic organisms. For instance, the effect on the development and apoptosis of the yellow river carp embryo (Xia et al., 2018), the acute toxicity to the rana limnocharis tadpole is high (Wu et al., 2011), cyhalofop-butyl can induce developmental toxicity, oxidative stress and immunotoxicity in zebrafish (Cao et al., 2016; Cheng et al., 2021; Zhu et al., 2015), the 96 h LC_{50} of cyhalofop-methyl to misgurnus anguillicaudatus was 6.6237 mg/L, which changed the liver tissue structure and led to apoptosis of liver cells of misgurnus anguillicaudatus (Shang et al., 2019). In addition, the International Union of Pure and Applied Chemistry (IUPAC) data show that cyhalofop-ethyl is a potential gall bladder, liver and kidney toxicant for human health concerns (Lewis et al., 2016). However, the underlying mechanism involved in the toxicity of cyhalofop-butyl is still unknown and warrants further research, especially in the aspect of liver toxicity.

Since the liver is the main site of material metabolism in vertebrates and has a role in biotransformation, the metabolism is mainly carried out by cytochrome P450 (CYP) enzymes, which are mainly located in the liver. And zebrafish liver contains enzymes that can metabolize a variety of endogenous and exogenous compounds similarly to the human liver (Bambino et al., 2019; Chu and Sadler, 2009; Vliegenthart et al., 2014; Zaret and Grompe, 2008). The classic study of zebrafish liver development comes from Stainier and colleagues (Field et al., 2003; Ober et al., 2003) using the transgenic Tg (Xla.Eef1a1: GFP) (gut: GFP) fish for observation and analysis. The liver of zebrafish begins to specialize about 24 hours post-fertilization (hpf), expressing liver precursor cells marker molecules such as proxl and hhex. By 36 hpf, bile ducts are formed. After 48hpf, the zebrafish liver develops into an outgrowth process, during hepatocyte differentiation and growth, a bile duct network is also gradually formed, and blood vessels also begin to appear, and their function is fully functional by 72 hpf. At 96hpf, the liver morphology is basically formed, and liver function can be exercised (Cox and Goessling, 2015; Field et al., 2003; Katoch and Patial, 2021; Ober et al., 2003). In the early stage of life, zebrafish larvae have the advantages of rapid liver development, high fecundity, transparent embryo and feasibility of high-throughput screening, and the morphological changes of the liver can be observed by naked eyes without dissection (Howell et al., 2012; Vliegenthart et al., 2014). Therefore, it is crucial to study liver toxicity using zebrafish as a model.

Therefore, in this study we used zebrafish embryos to investigated the hepatotoxicity of cyhalofop-butyl and involved mechanisms by phenotypic, histopathological, and biochemical analysis. In addition, transcriptome was applied to further explore the molecular mechanism. This study may illustrate the aquatic risk of cyhalofop-butyl and provide a new sight to the pathogenesis of liver disease.

2. Materials And Methods

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

2.1. Materials and reagents

Cyhalofop-butyl (97.5%, CAS) was purchased from Jiangsu Zhongqi Technology Co., Ltd (Jiangsu, China). A stock solution of cyhalofop-butyl was dissolved with dimethyl sulfoxide (DMSO), and diluted with DMSO (below 0.01% (v/v) to the appropriate concentration according to the experimental design. The reconstituted water as the standard water for embryo experiments was prepared. All other reagents used in this experiment were analytical grade.

The parental zebrafish (AB wild-type strain) were purchased from Beijing Hongda Gaofeng Aquarium Department. The zebrafish cultured in the flow-through facility (Esen Corp) at a temperature of 27±1 ℃, and a photoperiod of 14:10 h (light: dark), and feed with live brine shrimp three times a day. Zebrafish embryos were obtained by mixing male and female adults in a 1:1 sex ratio in spawning boxes, examined for viability under a stereomicroscope (Olympus BH-2 dissecting microscope), and normally developing embryos were used for subsequent experiments.

2.2. Embryonic exposure

Embryo exposure experiments were performed according to OECD guidelines (OECD, 1998), and each group was evaluated with 3 replicates (n=3). Every 300 embryos (healthy embryos approximately 2 h post-fertilization) were placed in a glass culture dish containing 600 mL cyhalofop-butyl solution (0.1,0.2,0.4 mg/L) and solvent control (0.01% dimethyl sulfate oxide in water, v/v). After 96 hours of exposure, 20 larvae per replicate were placed in 50 mL small beakers and continued exposure to cyhalofop-butyl for another 24 hours. During the experimental exposure, the exposure solution was changed daily and the dead embryos were removed in time. All samples for biochemical analysis were snap-frozen in liquid nitrogen (stored at -80°C).

2.3 RNA-seq assay and transcriptomic analysis

At 4 dpe (days post exposure), fifty larvae were collected from each replicate used for RNA-seq. Total RNA extraction, RNA quality assessment and cDNA library construction were shown in supplementary materials, Kyoto Encyclopedia of Genomes and Genomes (KEGG) (http://www.genome.jp /KEGG/) was analyzed for complex biological functions, and Gene Ontology (GO) enrichment analysis were carried out to describe the gene functions of differentially expressed genes (DEGs). 21 representative DEGs were selected for RT-qPCR to validate the RNA-Seq results, and the primer sequences were shown in Table S1.

2.4 Morphological and histopathological analysis of liver

At 4 dpe, 10 zebrafish were randomly selected from each concentration (3 replicates) and anesthetized with 0.03% MS-222. The size of the liver was measured using a stereo microscope (Zeiss Axio Vert.A1., Germany). The analysis method was based on previous studies (Martins et al., 2021), and hematoxylin and eosin (HE) were used for staining. Briefly, the preparation of paraffin sections is based on the preparation method reported by predecessors (Yang et al., 2016), all samples were sectioned at a 4um refrigerated microtome (ERMA INC., Japan), a cross-section of the liver of each fish was randomly selected and examined with a light microscope (Olympus BH-2, Japan).

2.5 Biochemical analysis

Fifty larvae were collected in each petri dish for the determination of alanine transaminase (ALT), aspartate transaminase (AST) triglyceride (TG) and total cholesterol (TC) activities. The larvae used in the enzyme activity assay were ground on ice with physiological saline, centrifuged at 2500 rpm for 4 minutes (4°C), and the supernatant was collected for corresponding biochemical parameter analysis. Total protein levels were determined by the Bradford method (Bradford, 1976; Hildebrandt et al., 2008), ALT, AST, TG, TC were determined by enzyme activity kit (Nanjing Jiancheng Bioengineering Institute, China), and the experimental operation followed the manufacturer's instructions.

2.6 RT-qPCR analysis

Total RNA was extracted with Trizol Reagent (Invitrogen), and details of qPCR are provided in supplementary materials. Primers for key genes in zebrafish liver development (Table S2) and genes to be validated are shown in supplementary materials (Table S1). The housekeeping gene beta-actin (β-actin) was used as the internal reference gene, and the relative quantification was carried out by the $2^{-\Delta\Delta Ct}$ method.

2.7 Determination of cyhalofop-butyl in water samples

The actual concentration of cyhalofop-butyl in aqueous solution was determined at 0 h and 24 h after exposure, respectively (3 replicates per treatment). The analysis was carried out by Shimadzu LC-2010AHT high performance liquid chromatography system equipped with Ultimate XB-C18 column (4.6 mm x 150 mm, 5um). The analysis conditions were provided in supplementary materials.

2.8 Data analysis

SPSS 17.0 software (SPSS, USA) was used for statistical analysis. Shapiro-Wilk test and Levene's test were used to verify the normality and homogeneity of variance of data. The differences between the control and cyhalofop-butyl exposed groups were tested by independent sample T-test or a one-way analysis of variance (ANOVA) with Dunnett's post hoc test. All evaluations were expressed as mean ±standard deviation (SD). $P<0.05$ indicates that the difference between groups was statistically significant.

3. Results

3.1. Quantification of cyhalofop-butyl

Since all exposure solutions in this study were updated daily and the deviation between nominal and detected concentrations were within ± 20% (Table S3), nominal concentrations could represent actual concentrations.

3.2 Quantitative analysis of liver morphology

After 5 dpe, cyhalofop-butyl induced liver atrophy of zebrafish larvae (Fig. 1-A). Quantitative image analysis of zebrafish larval liver size (Fig. 1-B) revealed a concentration-dependent decrease of liver area in the treatment groups with increasing concentrations, and a significant decline in 0.4 mg/L groups.

3.3 Liver histopathology

H&E staining showed that the liver of zebrafish larvae after exposure to cyhalofop-butyl for 4 d was damaged (Fig. 2). Normal zebrafish liver cells were closely arranged, with clear nuclei, uniform cytoplasm and clear edges. In treatment group, the liver area shrank. A small amount of hepatocyte nucleus was concentrated and the nuclear to cytoplasm ratio increased (0.1 mg/L). The morphology of hepatocytes was irregular (0.2 mg/L). And vacuolation necrosis and unclear structure of hepatocyte were observed in 0.4 mg/L.

3.4 Liver function analysis

The activities of ALT and AST were increased with increasing cyhalofop-butyl treatment concentrations (Fig. 3A-B). The ALT and AST levels were significantly increased by 195.6% and 181.1% in 0.2 mg/L, and at the highest concentration (0.4 mg/L) ALT and AST levels were significantly increased by 269.4% and 410%, respectively. Compared with the control group, TG levels declined with increasing cyhalofop-butyl exposure concentrations and were significantly decreased by 22% in the 0.4 mg/L cyhalofop-butyl - treated group (Fig. 3C). In addition, TC levels showed a downward trend after exposure (Fig. 3D).

3.5 Transcriptomic analysis

Transcriptomic analysis indicated that the whole gene expression profile of zebrafish larvae exposed to 0.1, 0.2, 0.4 mg/L cyhalofop-butyl was significantly changed compared with the control group (differential gene volcano plot Fig. S1A-C). A total of 116 DEGs were found in the 0.1 mg/L treatment group, including 67 up-regulated DEGs and 49 down-regulated DEGs; a total of 154 DEGs were found in the 0.2 mg/L treatment group, including 64 up-regulated DEGs and 90 down-regulated DEGs, a total of 397 DEGs were found in the 0.4 mg/L treatment group, including 154 up-regulated DEGs and 243 down-regulated DEGs (Fig. S2). The increase in the number of differentially expressed genes may roughly indicate that more biological processes were deregulated with increasing cyhalofop-butyl exposure concentrations. In addition, hierarchical clustering analysis was performed on the screened differentially expressed genes, and a heatmap dendrogram further described the expression of DEGs in zebrafish larvae (Fig. 4A-C), which indicated strong clades between groups.

The effects of cyhalofop-butyl on zebrafish larvae were further demonstrated by GO (Gene Ontology) and KEGG pathway analysis. Compared with the control group, the results showed that the GO Term included 226, 362, 584 Biological Processes (BP), 51, 45, 94 Cellular Compositions (CC) and 97, 168, 282 Molecular Functions (MF) (0.1, 0.2, and 0.4 mg/L). Among the top 30 biological processes with the most significantly enriched GO terms (p-value < 0.05) in cyhalofop-butyl-treated groups, immune-inflammatory response, small molecule biosynthesis process, fatty acid biosynthesis and metabolism process, bile

secretion, cytochrome P450 pathways, redox process and lipid metabolism process were the most significant (Fig. S3A-C). The most significantly enriched KEGG pathways were mainly involved in metabolism (such as carbohydrate metabolism, lipid metabolism, amino acid metabolism and nucleic acid metabolism), immune system (Toll-like receptor signaling pathway) and endocrine system (PPAR signaling pathway) (Fig. 5A-C). RT-PCR was used to verify the expression of 21 genes identified by RNA-Seq analysis mainly involved in predicted pathways such as liver detoxification metabolism (cytochrome P450 enzymes) and inflammatory response (Fig. 6A-C). Although expression levels of 3 genes (*vegfbb*, *alpi. 2*, *hbae5*) were different from the RNA-Seq data, expression levels of 18 genes were similar to the RNA-Seq results.

3.6 Expression of genes related to liver development

The expression profiles of genes (*hhex*, *fox*, *tnf-α*, *mmp9*, *Igf2bp1*, *pparb*, *dkk2* and *dkk1*) related to liver development of zebrafish larvae was shown in Figure 7. After exposure to cyhalofop-butyl (0.1, 0.2, 0.4 mg/L) for 4 days, *hhex* and *fox* mRNA expression were decreased significantly in larvae (0.2 and 0.4 mg/L). In the 0.1 mg/L group, cyhalofop-ethyl caused significant downregulation *tnf-α* and *mmp9* mRNA expression at 4 dpe, and increased *tnf-α* and *mmp9* mRNA at 0.2 and 0.4 mg/L Cyhalofop-ethyl. Although *Igf2bp1* mRNA decreased with increasing exposure concentration, no significant difference in *Igf2bp1* mRNA expression was observed at 4 dpe ($p > 0.05$). The mRNA expression of *pparb*, *dkk2*, and *dkk1* remained stable at 4 dpe in the 0.1 and 0.2 mg/L treatment groups, but a significant increase was observed in the 0.4 mg/L treatment group.

4. Discussion

In recent years, the developmental toxicity and immunotoxicity of cyhalofop-butyl have been reported successively in aquatic animals, such as zebrafish (Cheng et al., 2021; Zhu et al., 2015), Yellow River carp (Xia et al., 2018), misgurnus anguillicaudatus (Shang et al., 2019), Chinese toad and tadpole (F. Huang et al., 2007). However, the hepatotoxicity of cyhalofop-butyl on aquatic organisms and involved mechanism are still unclear. As the most important organ of metabolism and detoxification in fish xenobiotics, damage or dysfunction of the liver can lead to destructive changes in a series of physiological functions (such as metabolic disorders and inflammation), and is more susceptible to poison-induced damage (Jia et al., 2022). Similar to the mammalian liver, the zebrafish liver is an important metabolic organ in the body and plays an irreplaceable role in metabolism, detoxification and homeostasis (de Souza Anselmo et al., 2018; Menke et al., 2011; Tao and Peng, 2009). Many studies have shown that exposure to environmental pollutants can lead to liver damage of zebrafish and metabolic abnormalities. For example, thiophanate-methyl induces hepatotoxicity through zebrafish apoptosis and oxidative stress, and then leads to a metabolic imbalance of zebrafish liver (Jia et al., 2020). Oxyfluorfen damaged the lipid and sugar metabolism in the liver of zebrafish larvae, and the liver injury caused by metabolic dysfunction eventually affected the early development of zebrafish (Li et al., 2021). The combined hepatotoxicity in imidacloprid and microplastics caused the changes of glucose

and lipid metabolism and the expression of inflammation-related genes in zebrafish (Luo et al., 2021). Therefore, the hepatotoxicity of pollutants to zebrafish should be paid attention to.

Previous data have clearly shown that exposure to cyhalofop-butyl can lead to various morphological malformations during development (such as pericardial edema and yolk sac edema), and induce embryo teratogenicity in a dose-dependent manner (Zhu et al., 2015). The same deformity has also been reported in zebrafish embryos, high concentration difenoconazole can cause teratogenic effects such as pericardial edema and yolk sac edema, which may be caused by liver atrophy and liver degeneration of zebrafish larvae caused by difenoconazole (Jiang et al., 2020). The yolk sac is mainly composed of lipids, which are absorbed by the liver and metabolized in the liver. If the liver is damaged, the absorption of yolk sac will be disturbed. Therefore, the absorption of yolk sac can also reflect the degree of liver injury (Sant and Timme-Laragy, 2018; Wang et al., 2019; Zhang et al., 2021). When the liver is seriously affected or chemicals induce hypertrophy or atrophy, the liver size may change (Zhang et al., 2014). Normal zebrafish hepatocytes were uniformly distributed, with nuclei in the center of cells in a regular circle, and cells were tightly connected. In our analysis, cyhalofop-butyl exposure caused liver damage and atrophy in larvae, liver degeneration, vacuolation and necrosis of zebrafish liver hepatocytes, and liver damage and atrophy were more severe with increasing concentrations. The results showed that exposure to cyhalofop-butyl may pose a potential threat to early liver development and growth in zebrafish.

The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) mainly exist in liver cells, which are highly concentrated transaminase in liver and a classic index of hepatotoxicity. And ALT and AST activity are widely used to indicate the degree of liver damage. In the case of inflammation, necrosis or poisoning, liver cells may be damaged, resulting in a significant increase in transaminase level (Ozer et al., 2008; Vliegenthart et al., 2014). In this study, the levels of ATL and AST in zebrafish significantly increased after exposure to cyhalofop-butyl, and with the increase of cyhalofop-butyl exposure concentration, the activities of ALT and AST increased. In addition, in HE staining, the morphology of zebrafish hepatocytes changed compared with the control group. As we all know, the liver plays a key role in lipid metabolism. As the main components of lipids, triglyceride and cholesterol are closely related to lipid metabolism, and the abnormality of lipid metabolism is often related to the occurrence of many diseases (Nguyen et al., 2008; Ponziani et al., 2015). Interestingly, we also observed that the TC and TG levels of zebrafish larvae exposed to cyhalofop-butyl decreased significantly, which indicated that liver lipid metabolism was also affected. According to these data, we concluded that cyhalofop-butyl destroyed the integrity of liver cells, caused the disorder of lipid metabolism in zebrafish, and damaged the liver of zebrafish.

In our study, the expression of key genes involved in liver development (*hhex*, *fox*, *tnf-a*, *dkk2* and *dkk1*) also changed significantly after cyhalofop-butyl treatment, which further supported the liver toxicity of

cyhalofop-butyl to zebrafish. After exposure to cyhalofop-butyl, the significant decrease of *hhex* mRNA expression in larvae may lead to the occurrence of small liver, thus affecting the development of liver. Similarly, it is reported that *hhex* is a key transcription factor in the developing liver during the regulation of hepatoblastoma. Ochratoxin A exposure leads to liver atrophy and inhibits *hhex* gene expression, thus inducing liver development interruption (Wu et al., 2018). At about 28 hpf, zebrafish liver begins to bud, while *foxa3* is expressed in liver primordium at a budding stage, and *igf2bpl* is the key factor for liver growth, knockout of *igf2bpl* gene can lead to smaller liver phenotype (Tao and Peng, 2009; Wu et al., 2020). The expression of *foxa3* and *igf2bpl* mRNA in larvae decreased after cyhalofop-butyl treatment, which may affect the growth of liver, thus leading to liver atrophy. In the liver, tumour necrosis factor-α (TNF) can induce apoptosis and necrosis of hepatocytes, liver inflammation and many other biological reactions(Tiegs and Horst, 2022). After exposure to cyhalofop-butyl, the expression of *tnf-α* increased significantly, which accelerated the apoptosis of hepatocytes and induced liver injury. Transcriptional matrix metalloproteinases (MMPs) are involved in the regulation of extracellular matrix accumulation (Tang et al., 2019; Zhou et al., 2013), and liver injury is often associated with complex extracellular matrix-related pathways (Duarte et al., 2015; Gong et al., 2021). It has been found that the up-regulation of *mmp2* and *mmp9* accelerates the degradation of the extracellular matrix of liver and aggravates alcohol-induced liver injury (Koneru et al., 2017). In this experiment, the increased expression of *mmp9* in larvae may be due to the degradation of the extracellular matrix of liver, which aggravated the liver injury induced by cyhalofop-butyl. Wnt-beta-catenin signaling can regulate hepatobiliary development and liver homeostasis (Perugorria et al., 2019), in our study, the mRNA of *dkk1* and *dkk2* in larvae was significantly up-regulated. Similarly, some studies showed that after carbamazepine treatment, the mRNA of *dkk1* and *dkk2* was significantly up-regulated, which led to the inhibition of the Wnt/ß-catenin signaling pathway and finally induced zebrafish hepatotoxicity (Bai et al., 2021).

The results of RNA-seq showed that there was a dose-effect on the number of DEGs, and 116,154 and 397 DEGs (Fig. S2) were identified in low, middle and high dose exposure groups (0.1, 0.2 and 0.4 mg/L cyhalofop-butyl), respectively. DEGs analysis showed that the damage of liver detoxification enzyme and inflammatory reaction pathway was the most important process leading to zebrafish hepatotoxicity. The results of GO analysis showed that these up-regulated DEGs were significantly enriched in GO terms related to lipid metabolism, amino acid metabolism, P450 pathway and immune-inflammatory reaction in the low dose group (0.1mg/L) after exposure to cyhalofop-butyl (Fig. S4). The middle dose group and high dose group (0.2 and 0.4 mg/L) mainly involved the P450 pathway, glucose and lipid transport and immune-inflammatory reaction (Fig. S5-6). In addition, all cyhalofop-butyl-treated groups significantly reduced the expression of genes encoding serine protease (endopeptidase, hydrolase, peptidase, etc.) and changed the extracellular matrix. Serine protease plays an important role in cell differentiation, tissue reconstruction, angiogenesis, embryo development and so on (Zou et al., 2006). Extracellular matrix proteins not only provide structural support to cells and tissues, but also promote cell migration (Jessen, 2015), thereby regulating various important biological processes during embryonic development and repair, which are important for development in the early stages of life (Bax et al., 2009; Gjorevski and Nelson, 2009; Nauroy et al., 2018). Therefore, it is obvious that cyhalofop-butyl poses a great risk to the

early development of zebrafish. The enrichment analysis of KEGG pathway showed that it significantly enriched after exposure to cyhalofop-butyl mainly involved metabolism (such as sugar metabolism, lipid metabolism and amino acid metabolism) and body system (such as immune system and endocrine system). The changes of signaling pathways (such as FGFR signaling pathway (0.1mg/L), toll-like receptor signaling pathway, apoptosis signaling pathway (0.2mg/L), PPAR signaling pathway (0.4mg/L), etc.) are closely involved in the growth and development of zebrafish. Fibroblast growth factor (FGF) is a secreted polypeptide ligand, and FGF signals regulate the metabolism of lipids and bile acids in the liver, the disruption of metabolic homeostasis mediated by FGF signals may indirectly affect the proliferation of hepatocytes (Seed and Hauschka, 1988; Tsai et al., 2013). In addition, bile acid metabolism and lipid metabolism were significantly enriched in KEGG pathway in the highest concentration group (0.4mg/L cyhalofop-butyl). It has also been reported that zebrafish liver injury is related to lipid accumulation (Jiang et al., 2020; Koneru et al., 2017). Toll-like receptors (TLRs) induce complex inflammatory reactions, which are widely expressed in hepatocytes. They are related to apoptosis-induced cell death, and are important sensors of the immune system, the signal transduction of TLRs in liver is related to acute and chronic liver diseases (Casanova et al., 2011; Fischer et al., 2005; Lawless and Greene, 2012; Yamamoto and Takeda, 2010). PPARα, a member of PPAR family, is mainly located in adipose tissues such as liver and kidney, and can regulate various metabolic processes in the liver, thus regulating the expression of downstream lipid metabolism-related genes and protein (Braissant et al., 1996; Hu et al., 2021; Wang et al., 2020). RNA-seq results also showed high expression of cytochrome P450 family genes (such as *cyp7a1*, *cyp2ada*, *cyp2n13*, *cyp2k18*, *cyp2k6* and *cyp2y3*) and fatty acid-related genes (such as *faah2b* and *fabp6*) changed in larvae, which further confirmed the effect of cyhalofop-butyl on liver function. To sum up, combining phenotypes of zebrafish liver atrophy and transcriptome analysis, we concluded that cyhalofop-butyl caused liver damage and metabolic imbalance (sugar, amino acids, lipids and other substances metabolism) in zebrafish liver, and various metabolic abnormalities disturbed the normal development of an organism, thus leading to abnormal liver function caused by cyhalofop-butyl.

5. Conclusions

In conclusion, our study confirmed that cyhalofop-butyl could induce the hepatotoxicity of zebrafish larvae, resulting in smaller zebrafish larval livers, liver degeneration, and vacuolation of hepatocytes in adult livers. The levels of ALT and AST in larvae were significantly increased, while the levels of TG and TC were significantly decreased, suggesting that the hepatotoxicity caused by cyhalofop-butyl might be related to the disorder of lipid metabolism. In addition, the liver toxicity in zebrafish larvae induced by cyhalofop-butyl was further proved by the combination of gene expression related to key factors of liver development and transcriptome analysis. This study provides new insights into the harmful effects of cyhalofop-butyl on the aquatic environment, and describes the potential mechanism of cyhalofop-butyl induced zebrafish hepatotoxicity.

Declarations

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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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Figures

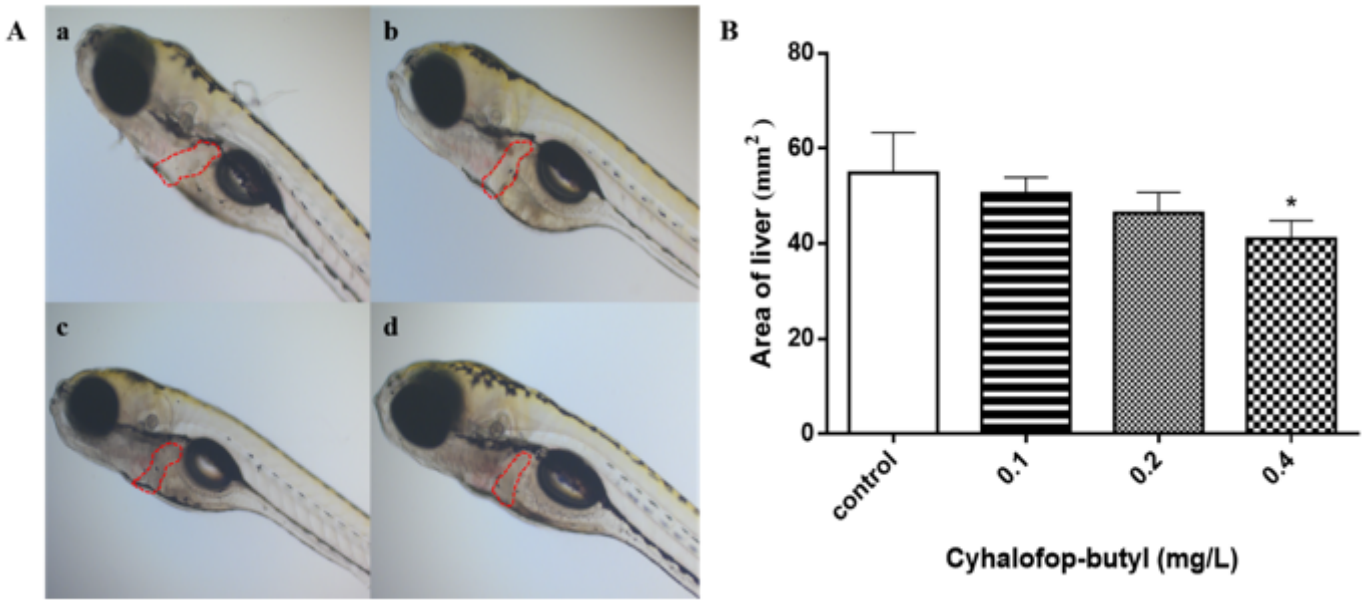

Figure 1

(A) Morphological aspects of the livers of zebrafish larvae with 120 hours. (a) zebrafish larvae in control ; (b), (c) and (d) are zebrafish larvae exposed for 120 h at 0.1, 0.2 and 0.4 mg / L of cyhalofop-butyl. (B) The quantitative area of the liver of zebrafish larvae (0.1, 0.2 and 0.4 mg/L). Arrows mark the different positions. Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, *p < 0.05; **p < 0.01; ***p < 0.001).

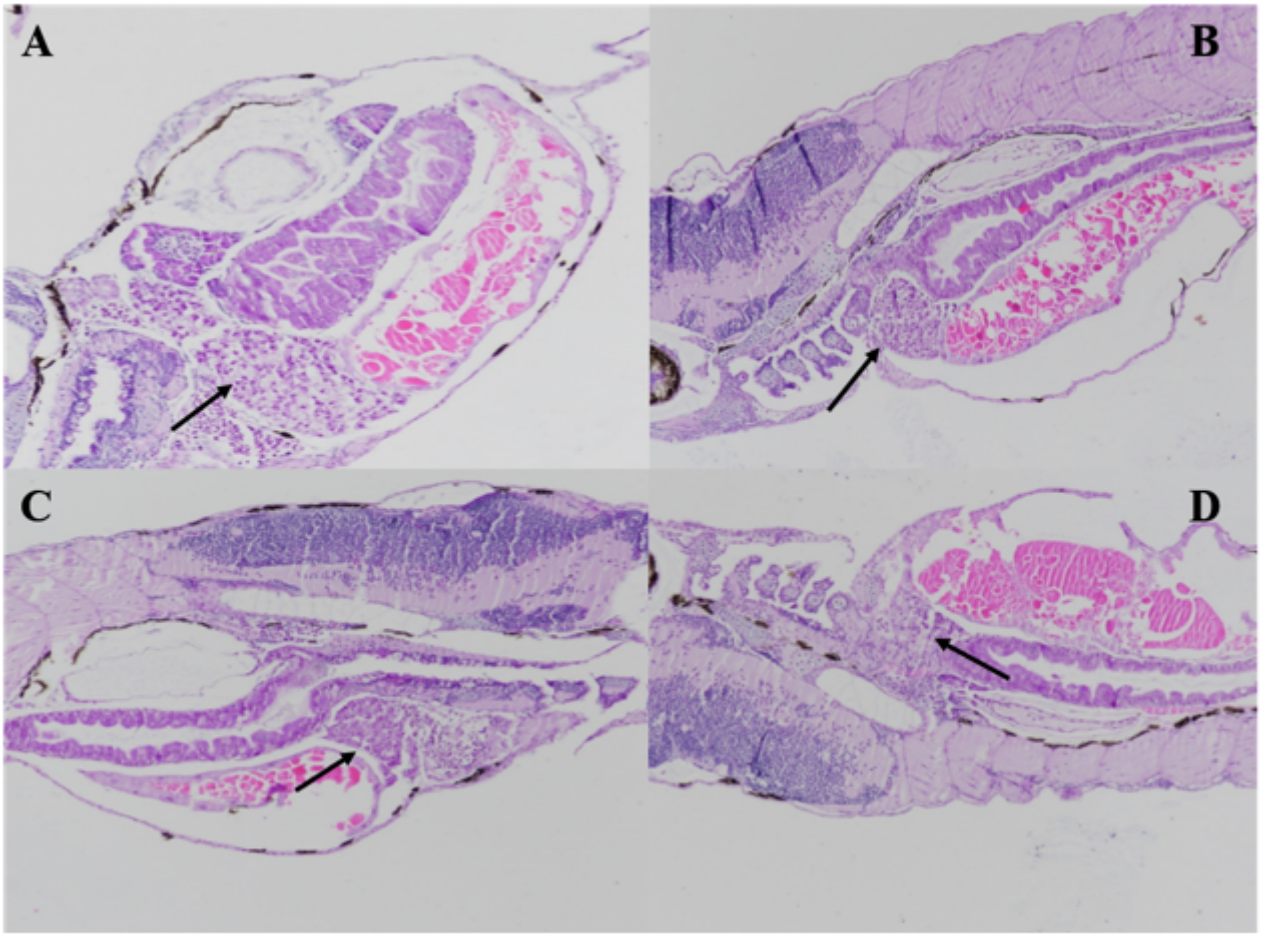

Figure 2

HE stained sections of liver in zebrafish larvae exposed to 0, 0.1, 0.2 and 0.4 mg/L cyhalofop-butyl at 4 dpe (10×). A-Control: Hepatocytes have clear nuclei, normal structure and clear edges (10×); B-0.1 mg/L: A small amount of hepatocyte nucleus is concentrated and the nuclear to cytoplasm ratio increases; C-0.2 mg/L: Irregular shape of liver cells, the overall area of the liver becomes smaller; D-0.4mg/L: Atrophy and necrosis of the liver, shrinking of the overall area, vacuolar degeneration of liver cells and unclear structure. Arrows mark the different positions. Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

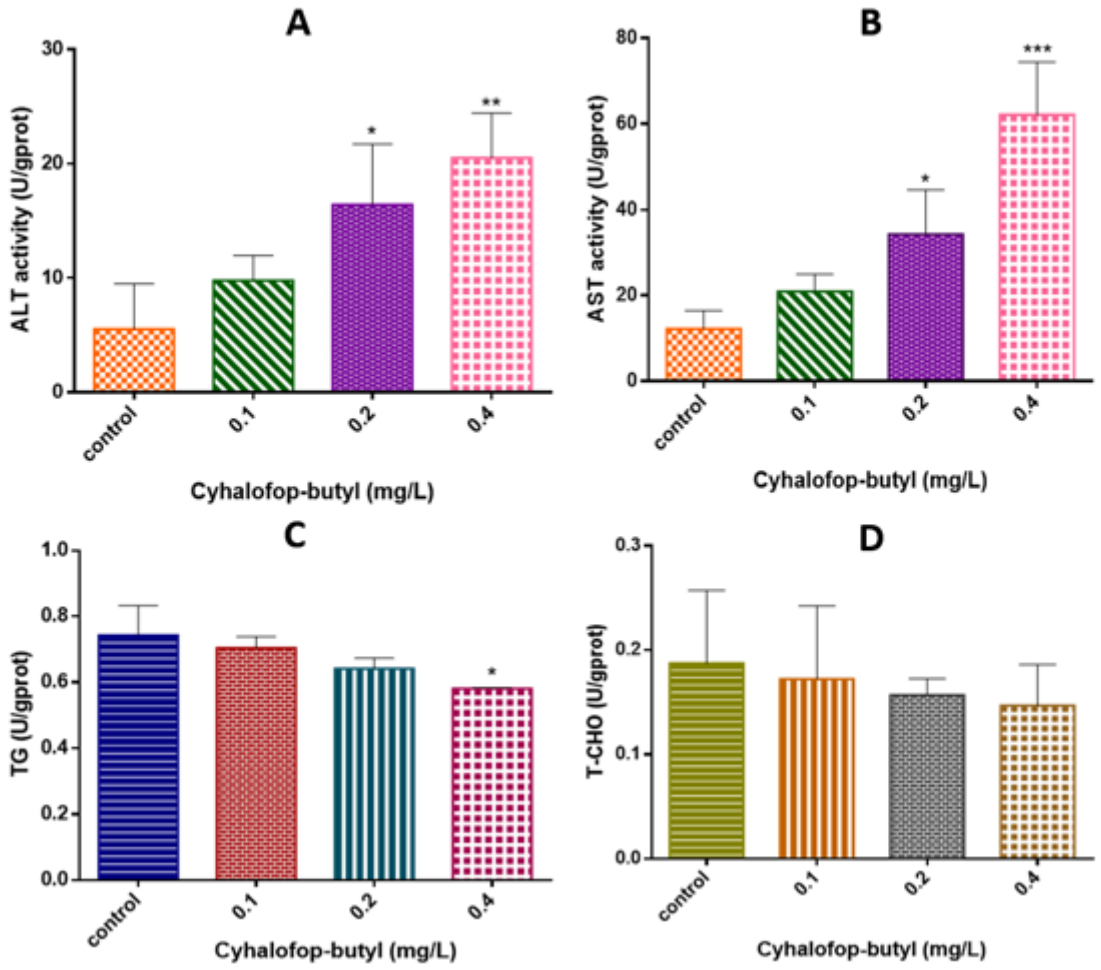

Figure 3

Enzymatic activity of alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B) triglyceride (TG) (C) and total cholesterol (TC) (D) of zebrafish larvae exposed to different concentrations of cyhalofop-butyl for 96 h. Arrows mark the different positions. Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, *p < 0.05; **p < 0.01; ***p < 0.001).

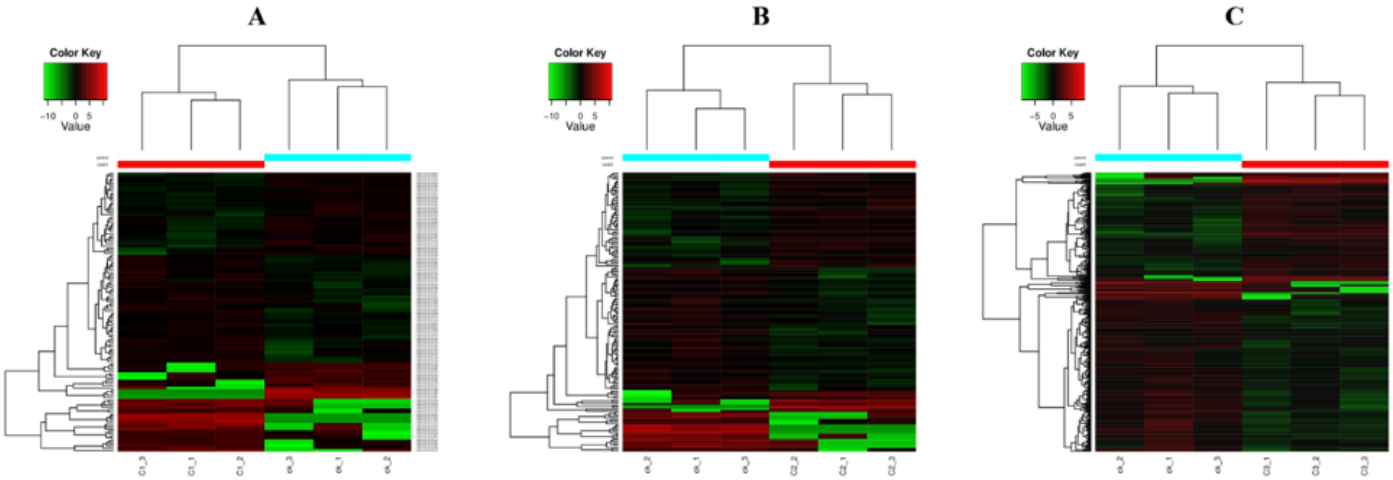

Figure 4

Heatmap obtained from the hierarchical clustering of DEGs in zebrafish embryos at 96 hpf. Green, black and red shading indicate the standardized values of gene expression (estimated by FPKM). Notes: ck-1, ck-2 and ck-3 represent three parallel samples of the control group; A:C1-1, C1-2 and C1-3 represent three parallel samples of 0.1 mg/L cyhalofop-butyl treatment group; B: C2-1, C2-2 and C2-3 represent three parallel samples of 0.2 mg/L cyhalofop-butyl treatment group; C: C3-1, C3-2 and C3-3 represent three parallel samples of 0.4 mg/L cyhalofop-butyl treatment group.

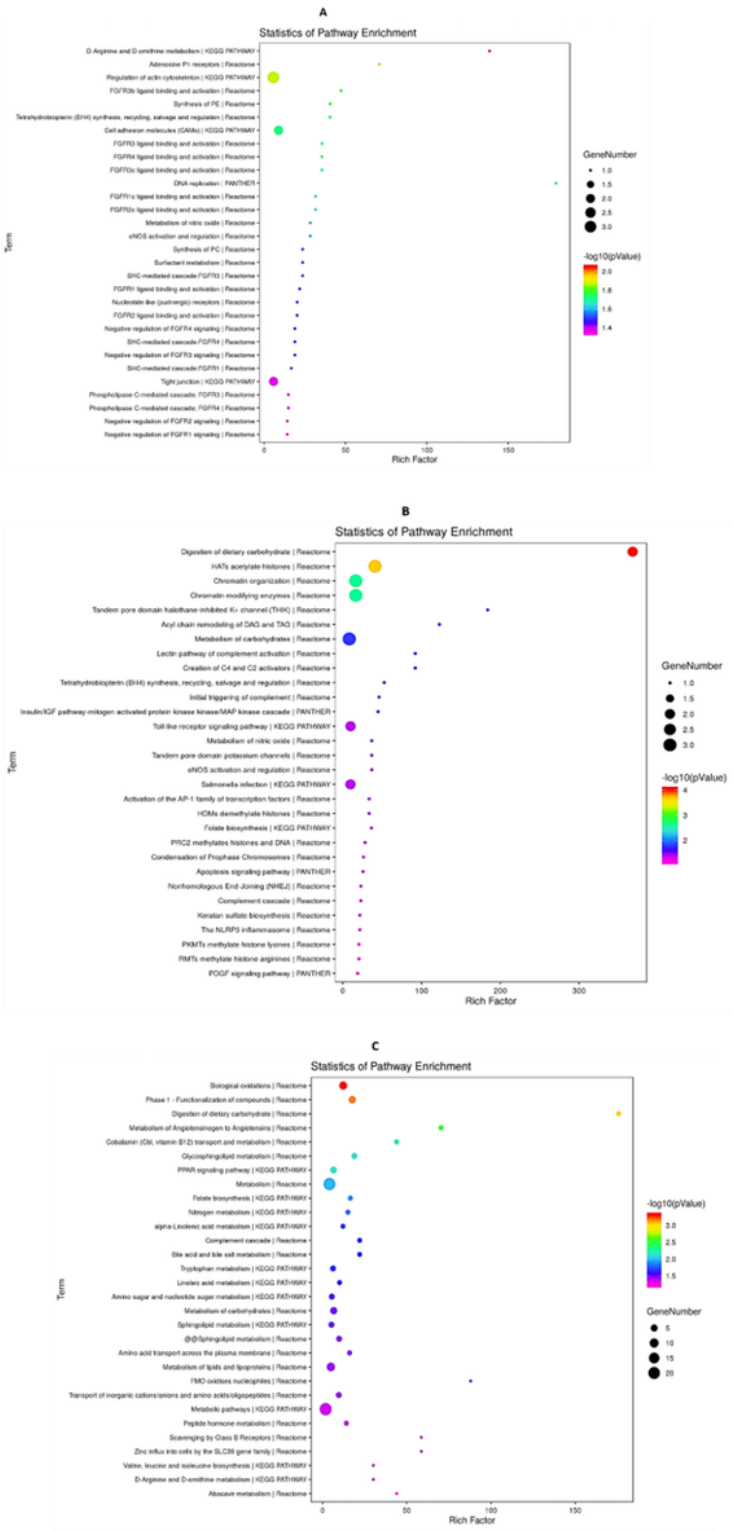

Figure 5

KEGG enrichment analysis of DEGs in the cyhalofop-butyl-treated group (A:0.1 mg/L; B:0.2 mg/L; 0.4 mg/L).

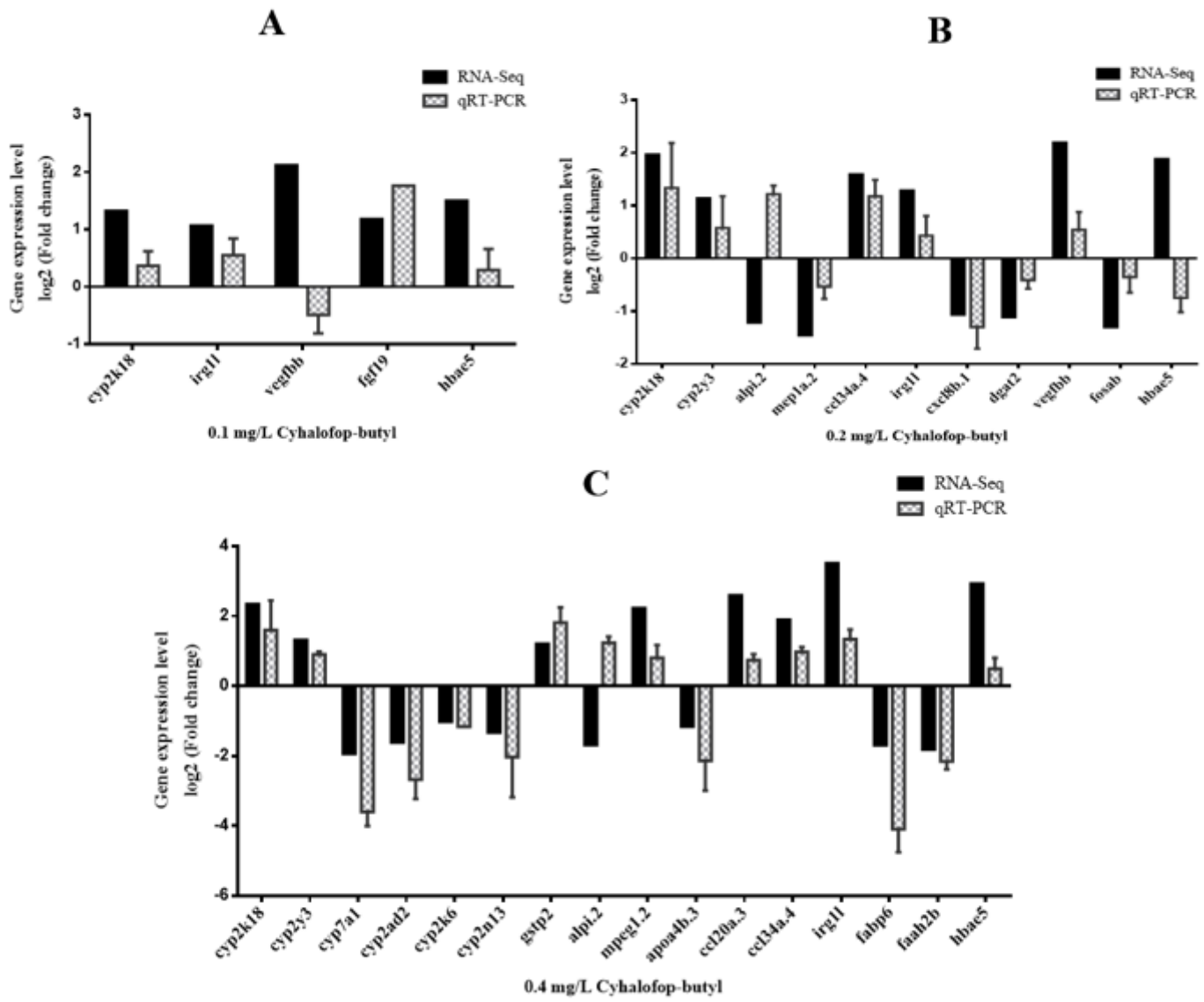

Figure 6

Validation of the RNA-Seq analysis with RT-qPCR after cyhalofop-butyl exposure. (A:0.1 mg/L; B:0.2 mg/L; 0.4 mg/L). Relative expression levels were calculated according to the $2^{-\triangle\triangle CT}$ method with the control group as a calibrator. β-actin gene was used as internal control gene.

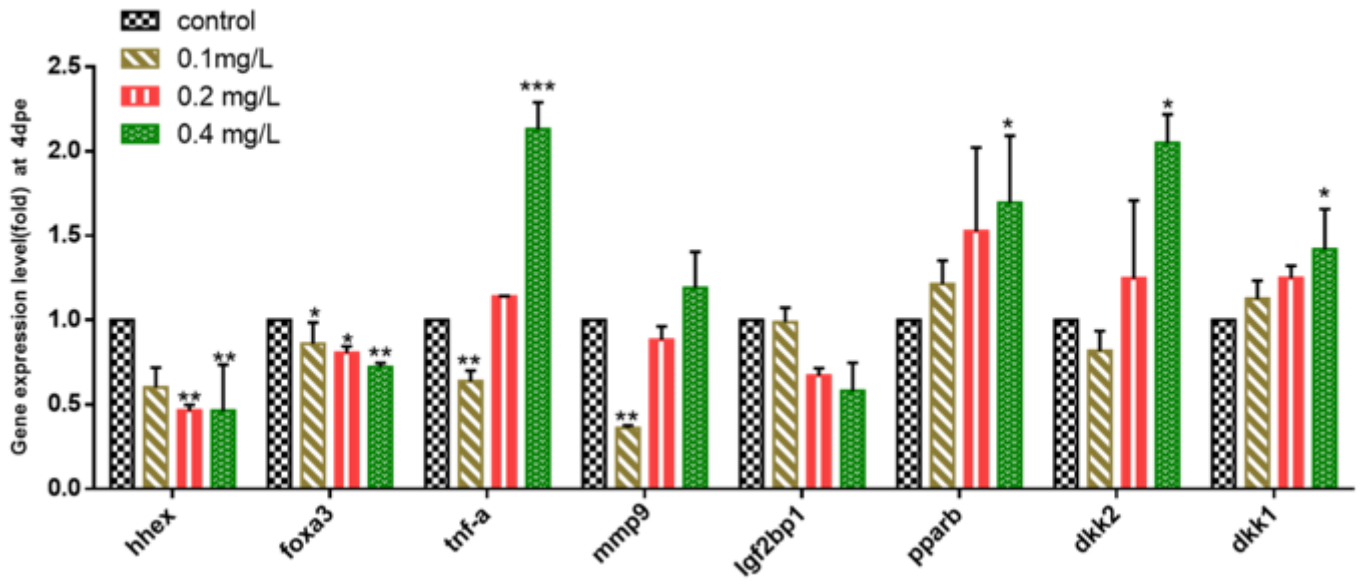

Figure 7

The expression of genes involved in liver development in larvae after exposure to 0, 0.1, 0.2 and 0.4 mg/L cyhalofop-butyl for 4 d (n = 3). Asterisks denote significant difference between the treatments and control (determined by Dunnett post-hoc comparison, *p < 0.05; **p < 0.01; ***p < 0.001).

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