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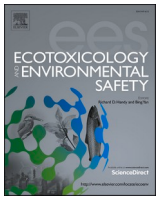

Effects of chlorantraniliprole-based pesticide on transcriptional response and gut microbiota of the crucian carp, *Carassius carassius*

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ABSTRACT

Chlorantraniliprole (CAP) is a presentative diamide pesticide utilized in agricultural area and as well as rice-fish co-culture system for pest control. However, the understanding of toxic effects of CAP on fish species is still incomplete. In the present study, we performed an integrated study of the acute toxicity and bioaccumulation of CAP on the crucian carp, *Carassius carassius*, a fish species widely distributed in freshwater area in China and commonly farmed in the rice-fish co-culture systems. Besides, biochemical changes, transcriptional responses and gut microbiota of fish were investigated upon sub-chronic CAP exposure. The results showed that CAP is low toxic to crucian carp with a 96 h LC_{50} of 74.824 mg/L, but has considerable accumulation in the fish muscles when exposed to 3 mg/L of CAP for 14 d and still detectable after 18 d recovery in fresh water. For sub-chronic test, fish were exposed to CAP at 0, 0.3, 3 and 30 mg/L respectively for 14 d. CAP induced oxidative stress and detoxification inhibition in the liver of fish by decreasing antioxidative and detoxicated enzymes activities and downregulating relevant genes expression. In addition, disrupted gut flora composition was found in all experimental groups by the 16 S rRNA sequencing data, indicating the gut microbiota dysbiosis in crucian carp and potential adverse host effect. All the results suggest that CAP at sublethal concentrations has prominent toxic effect on crucian carp and more attentions should be paid especially using directly in an integrated aquaculture system.

1. Introduction

Pesticides were massively used in agricultural industry for pest control and due to spray drift, surface runoff or unintended leaching, there're rising concerns about the ecotoxic effects of pesticides on aquatic environment (Stinson et al., 2022). Among thousands of pesticides in present, diamides are newly developed for control of many lepidopteron pests (Yang et al., 2018). They act as activator of the ryanodine receptors resulting in an uncontrolled release of calcium stores and leads to feeding cessation, contractile paralysis and finally death of insects (Cordova et al., 2006). Chlorantraniliprole (CAP), one of the main anthranilic diamide firstly developed by DuPont Crop Protection company, is registered in many countries worldwide and comprised of the 30% sale of total pesticides (Song et al., 2019). It has been introduced to China since 2007 and was extensively used in rice, coffee, and fruit farms (Wang and Wu, 2012).

To better achieve the goal of sustainable and organic agriculture, the plant-animal co-cultivation mode is developed rapidly in recent decades, particularly the rice-fish co-culture system (Lu and Li, 2006). Rice is a globally important staple food crop and the rice-fish farming started since 1700 years before in China (Kangmin, 1988). Taking advantages of mutualism in this system, rice-fish co-culture makes much more profit than monoculture systems and therefore, the area of paddy used for rice-aquaculture increased markedly that there's 40-fold increase in 2010 s compared to 1980 s (BFMA, 2017). However, to further increase the yield of rice in the co-culture system, application of pesticides is inevitable to inhibit a variety of harmful insects and weeds. Due to the high selectivity to harmful insects and low toxicity to human, CAP was considered 'safe' and its ecotoxicity in aquatic environment has been ignored before (Wu et al., 2017). However, growing numbers of reports

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demonstrate high acute toxicity of CAP to aquatic invertebrates (Cui et al., 2017), which could potentially affect the whole aquatic ecosystem. Notably, the direct use of pesticides in rice-fish co-culture systems likely has effect on non-target aquatic animals like fish species. Therefore, the pesticidal contaminants emergence and how it influences the quality and safety of aquatic products in the coculture systems need to be well studied.

Although CAP is considered slightly-to-virtually non-toxic to fish species (Seben et al., 2021), it is classified as highly dangerous to the environment based on its hazardous impact (Saglam et al., 2013). To our knowledge, there're very limited number of reports about the CAP toxicity on freshwater fish. Song reported the 96 h LC_{50} of CAP on zebrafish is 36.94 mg/L, indicating the low toxicity of the chemical according to the national standard of toxin (Song, 2020). Studies from Rathnamma and Bantu showed that the 96 h LC_{50} of CAP on grass carp, *Ctenopharyngodon Idella* and *Channa Punctatus* are 11.008 mg/L and 14.424 mg/L, respectively (Bantu and Vakita, 2013; Rathnamma and Nagaraju, 2014). The LC_{50} values above are several orders of magnitude higher than the values in aquatic invertebrates (Lavtižar et al., 2015), also far beyond the environmentally realistic concentrations (Song et al., 2019). It implies the trivial impact of CAP on fish species in aquatic ecosystem. However, several reports demonstrated bioaccumulation, oxidative stress, biochemical alteration and induction of gene expression in fish including common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), sliver catfish (*Rhamdia quelen*) and *Pimephales promelas*, when exposed to sublethal or environmentally realistic doses of CAP (Clasen et al., 2018; Rathnamma and Nagaraju, 2014; Seben et al., 2021; Stinson et al., 2022). Indeed, how CAP influence the physiological status of non-target vertebrates, especially freshwater fish is poorly understood so far. It could not only decrease the fishery yield of the co-culture system but also poses a challenge to human health through food consumption. Therefore, adverse effect of CAP on fish species is worthy to be fully uncovered.

Herein, we investigated the acute toxicity and bioaccumulation of CAP on the commercially important freshwater fish, crucian carp (*Carassius carassius*), which are wide distributed in rivers and lakes in China and also commonly farmed in rice-fish co-culture systems (Li et al., 2018). A comprehensive study with biochemical parameters detection, transcriptome and microbiome analysis was conducted in the present study. We are aiming to assess the toxic effect of CAP on crucian carp during sub-chronic exposure and unravel the underlying mechanisms.

2. Materials and methods

2.1. Chemicals and animals

A formular chlorantraniliprole (CAP) suspension concentrate (20%) was purchased from local distributor (DuPont Agricultural Chemicals Ltd., Shanghai) and prepared as 100 mg/L stock solution with distilled water. The CAP reference standard was purchased from Sigma Aldrich (USA). Other chemicals used in this study were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Juvenile crucian carp, C. *carassius* weighing 20–25 g were purchased from a commercial farm in Xichang City, China. For acclimation, fish were maintained at 28 ℃ in an aquarium system with aerated water under a 12:12 h light/dark photoperiod. Animals were feed with commercial fish ration (Tongwei, China) once a day for both acclimation and sub-chronic exposure test. Healthy fish without any wound were picked for the test. For acute toxicity test, 90 fish in total were used and divided into 6 different concentration groups. For sub-chronic test, 48 fish were used and divided into 4 groups. All experimental protocols were reviewed and authorized by the Animal Bioethics Committee, Xichang University, China.

2.2. Acute toxicity of CAP on crucian carp

After a trial test (data not shown), CAP concentrations (0, 50, 60, 70, 80, and 90 mg/L) were selected. 15 fish were used for each group and were divided into 3 separate aquarium tanks (rectangular, 120 cm × 45 cm × 60 cm) as replicates. A static acute toxicity test was conducted for the determination of LC_{50} of CAP on crucian carp. Test water was half changed every 24 h to maintain the desired CAP concentrations. A regression Probit analysis in SPSS V21.0 software was performed to calculate the 96-h LC_{50} values and 95% confidence limits.

2.3. Sub-chronic exposure of CAP

Based on the acute toxic test results in the present study, three nominal concentrations of 0.3, 3 and 30 mg/L of CAP, representing low (L), medium (M) and high (H) dosages of pesticides respectively, were used for the sub-chronic exposure test. In addition, fish without receiving any CAP were used as control (C). In total, 48 fish were divided into the four groups mentioned above (C, L, M and H) and 12 fish were plotted for each group. In each group, every 4 fish were placed into a replicate tank (rectangular, 120 cm × 45 cm × 60 cm) and 3 replicate tanks were used. After 14 d exposure, fish from each group were collected and anaesthetized by ice bath. Liver and intestine from each fish was taken and tissues from every 3 fish were pooled into one sample to meet the experimental requirement. The liver was prepared as three aliquots, one for transcriptional assay, one for antioxidant activity assay and one for microsomes preparation. Intestinal contents were collected in a clean bench for 16 S rRNA sequencing analysis. All livers and intestinal contents were stored at −80 ℃ until further use.

2.4. CAP residual in water and bioaccumulation assay

During acute test, water samples were collected right after exposure (regard as 0 h) and before next renewal of solution (regard as 24 h), to assess the actual CAP concentrations in test water. Approximately 2 mL of water sample was centrifuged at 5000 rpm for 5 min and mixed 1:1 with acetonitrile (with 0.1% formic acid in water, 40:60). Then the mixture was filtered through a polytetrafluoroethylene membrane (0.22 μm). For the determination of CAP levels in water, a high-performance liquid chromatography coupled with mass spectrophotometry (HPLC-MS) system (Agilent 1290-G6495A) was adopted with the multiple reaction monitoring (MRM) programme. A ZORBAX Eclipse Plus C18 column (2.1 ×50 mm, 1.8 μm, Agilent, USA) was used to separate the target analytes at 40 ℃. The liquid phase consisted of solution A (0.1% formic acid water solution) and solution B (acetonitrile) with gradient elution at a ratio of 90: 10 initially, 50: 50 at 2 min, 10: 90 at 4.5 min with a flow rate of 0.3 mL/min. The mass spectrophotometer equipped with an AJS electrospray ionization (ESI) source.

To evaluate the bioaccumulation of CAP in crucian carp, a new batch of fish from the same stock used in toxicity tests were exposed to 3 mg/L of CAP with same procedure above but sampled every 2 days until 14 days. Afterwards, the rest of fish were transferred to tanks with only aerated water for recovery for another 18 days. During the recovery period, fish were sampled every 3 days. Fish muscles were collected and every three fish muscle samples were pooled into one replica to minimize the individual difference. The residual level of CAP in fish muscles was determined following a QuEChERS method described by Clasen et al. (2018), with little modification. 2 g of muscle samples were homogenized in 10 mL acetonitrile and centrifuged at 4200 rpm for 5 min. Then, 6 mL of supernatant was added into a falcon tube with 200 mg C18, vortex for 5 min and centrifuged again at 4200 rpm for 5 min 2 mL of supernatant was filtered through a polytetrafluoroethylene membrane (0.22 μm) and analysed by the HPLC-MS with similar instrument conditions above.

2.5. Biochemical measurement

Livers samples from sub-chronic test described in Section 2.3 were homogenised in prechilled PBS and centrifuged at 13000 rpm for 20 min. The supernatants were collected and used for SOD, CAT, GST and T-AOC activities detection. SOD, CAT, GST and T-AOC activities in liver were determined spectrophotometrically at 550 nm, 405 nm, 412 and 405 nm respectively with a microplate reader (iMark, Bio-Rad, USA), by using several commercial detection kits from Nanjing Jiancheng Bioengineering Institute, China. Detailed detection protocols were described elsewhere in a previous study (Hong et al., 2020). Tissue total protein content was quantified by Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Liver microsomes were prepared according to the method described by (Zhu et al., 2014) with little modification. Liver samples were homogenised in ice-cold 0.1 M potassium phosphate and centrifuged at 10, 000 g, 4 ℃ for 30 min to remove mitochondria and nucleus. Then, supernatants were collected and centrifuged again at 100, 000 g for 60 min at 4 ℃. The supernatant was discarded and sediments were used for detection of total cytochrome P450 and b5 levels. The P450 activity was determined by an ELISA detection kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China) and b5 content was determined by a spectrometric method at 425 and 490 nm respectively, with a b5 detection kit (Nanjing Jiancheng Bioengineering Institute, China). Aminopyrine N-demethylase (APND) and erythromycin N-demethylase (ERND) was detected according to the colorimetric method described in our previous study (Hong et al., 2020). Activities of APND and ERND were calculated by measuring the formation of methanol at 420 nm. Aminopyrine was used as the reaction substrate for APND and erythromycin for ERND.

2.6. RNA-seq analysis

Another aliquot of liver samples from sub-chronic test were used for RNA-seq analysis. The total RNA from liver of crucian carp was obtained by Trizol (Invitrogen) extraction method and quality checked by both Nano drop and agarose gel electrophoresis, as described in previous paper (Hong et al., 2020). High-throughput mRNA sequencing was performed on a Novaseq PE-150 platform (Illumina, USA). Clean reads were mapped to *C. auratus* genome (ASM336829v1) and |log2 fold change|>1 and $P < 0.05$ were selected as screening criteria for differential expression genes (DEGs) analysis. All data mining and figure presentation processes were implemented by R software with the help of Shanghai Personal Biotechnology Co., Ltd (China).

2.7 mRNA level detection by qRT-PCR.

The liver RNA retro-transcription to cDNA was conducted by the PrimeScript™ RT reagent Kit (Takara, Japan). Based on the transcriptome analysis data, four target genes (*Cu/Zu-SOD*, *GST*, *CYP1A1* and *CYP2B19*) and an internal control gene *β-actin* were selected. The relative mRNA expression level was detected by using SYBR green dye method. Detailed information of primers for each gene was listed in Table S2. A total amount of 20 μL Mixture containing SYBR Green, dNTP, primers, ddH_2O and cDNA templates was added into a 96-well transparent qPCR plate and programme was performed as described before (Hong et al., 2020).

2.7. Microbiota analysis

Total DNA of intestinal contents was extracted and quality checked by agarose gel electrophoresis. General primers 338-forward (5`-ACTCCTACGGGAGGCAGCA-3`) and 806-reverse (5`-GGACTACHVGGGTWTCTAAT-3`) were used for amplification of bacterial 16 s rRNA. All protocols used are identical from our previous study. Cluster the denoised sequences into OTUs using the Vsearch plugin in QIIME2 and assign taxonomy to the OTUs using a reference database. Evaluate alpha diversity levels for each sample based on the distribution of OTUs and then calculate the distance matrices of each sample for beta diversity analysis. Bacterial community composition in each sample was shown from phylum to genus levels as relative abundance to the mean total abundance.

2.8. Statistical analysis

SPSS V21.0 software was used for all statistical analysis. The mean values of the three replicates with their standard deviations were presented in all tables and figures. One-way analysis of variance (ANOVA) with Tukey's post hoc test was applied to determine any significant differences between the treatment and control groups. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Acute toxicity of CAP and bioaccumulation in fish muscle

As shown in Table S2 in the sup file, the 24 h and 96 h LC_{50} of CAP on crucian carp were 84.206 and 74.824 mg/L, respectively. The CAP residual concentrations at 24 h were 0.028, 2.948 and 26.996 mg/L in low concentration (L), median concentration (M) and high concentration (H) groups. A rapid degradation of CAP at 0.3 mg/L was observed after 24 h, but not at 3 and 30 mg/L. There're 90.64%, 18.90% and 15.95% decrease respectively, comparing to the CAP concentrations at the beginning (Fig. 1 A). Therefore, the CAP concentrations used in this study were presented as nominal concentrations. The CAP level in the muscle of crucian carp in 3 mg/L group was monitored during 14 days exposure and as shown in Fig. 1 B, it increased in a time-dependent manner and peaked at 14 d with around 830 μg/Kg. After transferring to drug-free water, the CAP residual in fish muscles decreased to about 152 μg/Kg at 32 d.

3.2. Toxic effects of CAP on antioxidation and detoxication of crucian carp

After 14 d exposure, SOD activities in liver of crucian carp significantly decreased in all three concentration groups but for CAT and T-AOC, there's no significant difference between low concentration group (L) and control (C). As for GST activity, it decreased significantly only in H group (Fig. 2 A). In addition, we also checked the level of detoxification enzymes of cytochrome family. The total P450 and b5 levels in liver decreased significantly in M and H groups. Similarly, the ERND and APND activities were restrained upon CAP exposure at medium and high concentrations (Fig. 2 B).

3.3. Transcriptome analysis and qRT-PCR

High-throughput transcriptome analysis was conducted to identify the transcriptional profile of liver under sub-chronic CAP exposure. After data filtration, a mean number of 38,564,895 clean reads was obtained for each sample. As shown in the Venn diagram (Fig. 3 A) and also the volcano plots (Fig. 3 C, D, E), there're 1658 (701 up and 957 down), 2290 (893 up and 1397 down) and 3017 (1356 up and 1661 down) DEGs in L, M and H groups compared to control respectively. There're only 173 shared DEGs among each comparison. To explore the common effects of CAP exposure on fish, the 173 shared DEGs were clustered and more down-regulation genes than up-regulation genes was found (Fig. 3 B). Then, the shared DEGs were subjected to functional enrichment analysis. KEGG analysis data showed that up-regulated DEGs primarily enriched in several important cell signalling pathways, for example, the MAPK pathway, ErbB pathway and Toll-like signalling pathway (Fig. 3F). In addition, apoptosis pathway was also up-regulated. On the other hand, most down-regulated genes were enriched in metabolism pathways especially the cytochrome P450 metabolism. These results indicate that CAP exposure could markedly

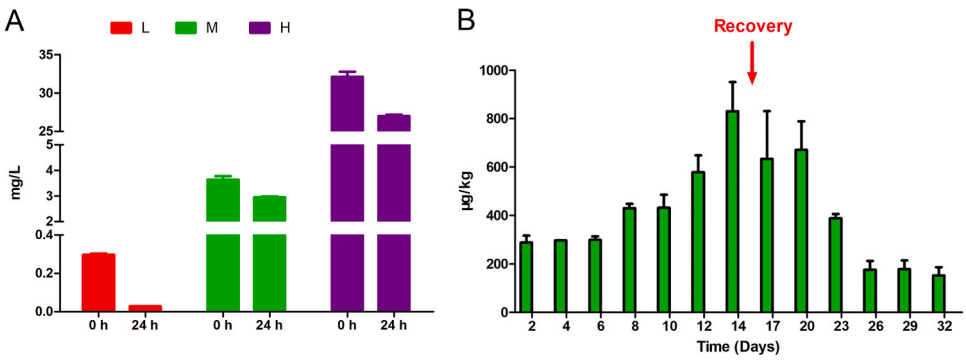

Fig. 1. CAP concentrations in water and bioaccumulations in muscles of crucian carp. A: CAP residual concentrations in water samples after 0 h and 24 h exposure. B: CAP residual concentrations in muscles of crucian carp when exposed to 3 mg/L of CAP. Red arrow indicates the recovery of fish by transferring fish to clean tanks without any CAP. Data represent the mean ± SD, n = 3.

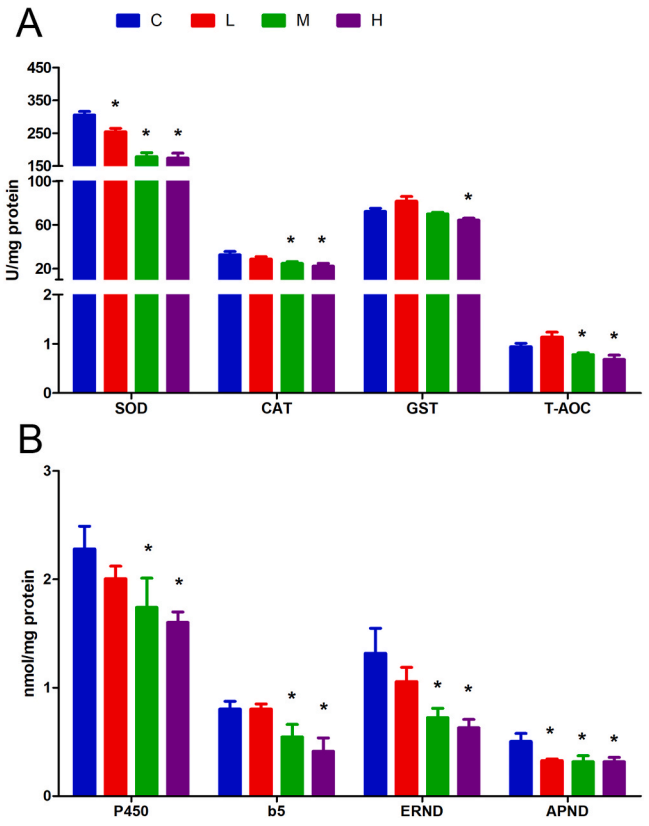

Fig. 2. Effects of CAP on biochemical parameters of crucian carp, C. *carassius*. Data represent the mean ± SD, n = 3. Asterisk (*) represents the significant difference between treatment and control ($P < 0.05$).

alter the transcriptional profile in liver of crucian carp even at 0.3 mg/L.

To validate the reliability of RNA-seq results and further quantify the oxidative stress and detoxification status of fish as well, four representative genes, including two antioxidant genes and two P450 genes were selected for qRT-PCR assay. The results showed that except *Cu/Zn SOD* expression in M group and *GST* expression in L group, all four genes were significantly down-regulated after CAP exposure in a dose-dependent manner (Fig. 4).

3.4. Effects of CAP on gut microbiome

In total 1049,863 high quality sequences from all intestinal content samples were collected after data filtration. After clustering and alignment, OTUs was used for alpha diversity analysis. Results of alpha diversity were provided in Fig. 5 A. There is significant decrease of Observed species and Shannon indexes between H group and control (one-way ANOVA, $P < 0.05$). Besides, PCoA results showed that samples from low concentration group (L) was close but M and H groups were distinct from control (Fig. 5 B). The Bra-Curtis distance calculated in pairwise comparison showed significantly difference between control and treatment (one-way PERMANOVA, $P < 0.05$). In addition, only 136 shared OTUs were identified, indicating the difference of microbial community composition under CAP exposure.

To further analyse the effects of CAP on microbial structures, normalized sequences were aligned to Greengenes database and clustered into different taxonomic levels. At phylum level, the dominant bacteria in intestine of crucian carp were *Fusobacteria* (25.92%), followed by *Proteobacteria* (24.80%), *Firmicutes* (21.95%) and *Tenericutes* (16.77%). After CAP exposure, the relative abundance of *Proteobacteria* and *Tenericutes* increased to 44.55% and 26.15% respectively in H group, whereas the abundance *Fusobacteria* and *Firmicutes* significantly decreased to 5.15% and 4.48% respectively (Fig. 5 D). Similar decrease was found in M group but not in L group. Besides, there's more than 3-fold increase of *Actinobacteria* abundance in M and H groups. At genus level, the relative abundance of *Cetobacterum* decreased significantly in all treatments ($P < 0.05$), whereas increase of Rhodobacter abundance was recorded except in M group. In addition, the relative abundance of *Bacterioides* was found decreased in all CAP treatment groups (Fig. 5 E). The enriched metabolic pathways in different groups were presented in Fig S1 according to the microbial function prediction. Biosynthesis is the main category in all samples. The top enriched metabolic pathways include amino acid biosynthesis, vitamin biosynthesis and nucleoside and nucleotide biosynthesis.

4. Discussion

In previous research, the ecological risk of CAP has been assessed by exploring the degradation dynamics in both soil and water (Meng et al., 2015; Wu et al., 2021). The half-life of CAP in different natural water ranges from 3.55 to 4.46 d. Residual concentrations of CAP was detected to a max of 10.2 μg/L in the agricultural surface water in California, U.S. A (Stinson et al., 2022). Besides, the level of CAP in paddy water of a rice-crab co-culture system was 5.7 μg/L after 7 d spraying (Song et al., 2019). The long existence of CAP in water body rises the contamination risk to non-target organisms, particularly fish in the co-culture system. In this study, the 96 h LC50 of CAP on crucian carp is 74.824 mg/L, which is higher than other fish species investigated before (Bantu and Vakita, 2013; Seben et al., 2021). It indicates a high tolerance of crucian carp to CAP. The HPLC-MS results showed obvious bioaccumulation of CAP in the muscle of crucian carp and it was still detectable with around 0.15 mg/Kg after recovery to 32 d. Similarly, study from Meng et al. (2022) showed bioaccumulation of CAP in zebrafish with

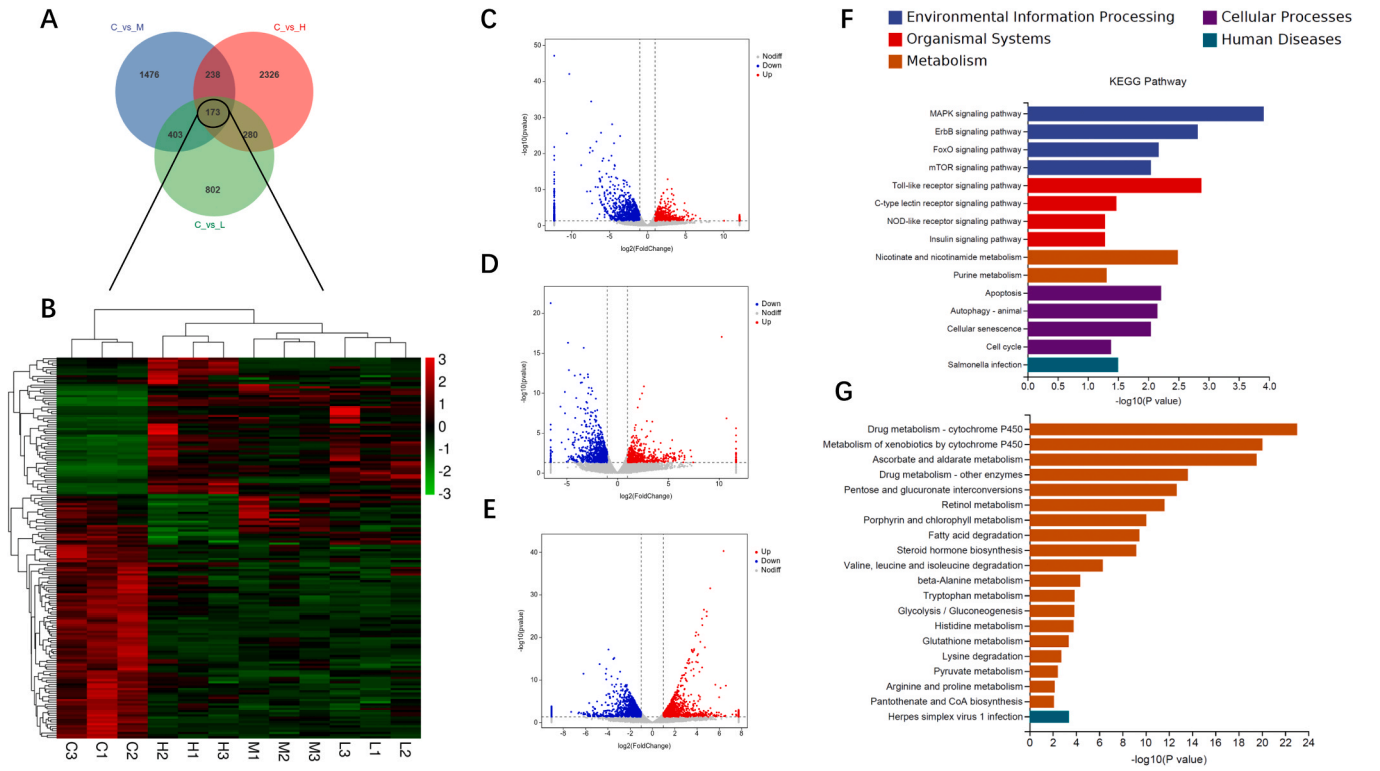

Fig. 3. RNA-seq analysis of liver of crucian carp, C. *carassius* in response to CAP exposure. A: Venn diagram shows the shared and unique DEGs of treatments in comparison to control. B: Heatmap shows the cluster analysis of shared DEGs. Colours in red represent up-regulation and in green represent down-regulation. C, D and E: Volcano plots for DEGs between L vs C, M vs C and H vs C, respectively. F: Significantly enriched KEGG pathways of up-regulated DEGs. G: Significantly enriched KEGG pathways of down-regulated DEGs.

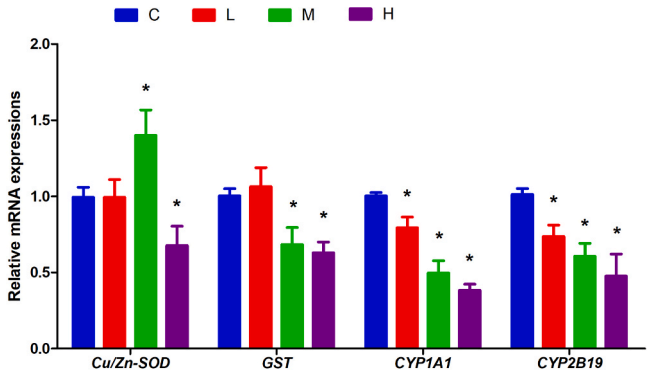

Fig. 4. Relative mRNA expression of genes detected by qRT-PCR. Data represent the mean ± SD, n = 3. Asterisk (*) represents the significant difference between treatment and control ($P < 0.05$).

0.4284–0.9043 mg/Kg when exposed to 1 mg/L CAP. As the main eatable part of fish, meat with CAP residual could greatly influence the product quality and even threat human health. However, the tested concentrations of CAP are far higher than residual concentrations detected in aquatic environment (Song et al., 2019). As a consequence, genuine toxic effect of CAP on fish in aquatic ecosystem could be not as strong as expected. Nevertheless, overuse of CAP in industry has been reported by several studies (He et al., 2019; Wei et al., 2019). Especially in the rice-fish/shrimp/crab co-culture systems, the spaying of CAP according to recommend doses could results in relatively high concentration in paddy area in a short time, leading to acute toxicity and further long-term effects (Clasen et al., 2018; Song et al., 2019; Sumon et al., 2016). Therefore, the environmentally-realistic concentrations of CAP should be precisely monitored in further investigation.

Oxidative stress is an important bioindicator of the toxic effects of environmental pollutants including pesticides on non-target organisms (Peters et al., 2021; Zhao et al., 2021). Several studies have shown that CAP could induce oxidative stress in not only insects but also freshwater fish species. For example, elevated level of oxidative products and induced antioxidative enzymes activities in different tissues were found in common carp, *C. carpio* (Clasen et al., 2018) and grass carp *C. idella* (Rathnamma and Nagaraju, 2014) respectively when exposed to CAP. In this study, significant decrease of SOD, CAT, GST and T-AOC activity was determined after 14 d CAP exposure, indicating the inhibitory effect of CAP on the antioxidation system of crucian carp. It's different with the results mentioned above but in consist with the results from Song's research that low concentration of CAP (0.1 mg/L) induced SOD activity in liver but it significantly decreased after high concentration (1 and 10 mg/L) exposure.

The P450 superfamily comprise the phase I detoxification system and build up the first enzymatic defence line to xenobiotics (Moore et al., 2003). They could respond to variety of pollutants including pesticides and therefore, were commonly used as biomarker for pesticide exposure in fish (Arellano-Aguilar et al., 2009; Goksøyr, 1995). The results in current study showed significant decrease of both P450 and b5 levels in lever of crucian carp after 14 d CAP exposure. In addition, ERND and APND, detoxification enzymes regulated by CYP families, were also decreased upon exposure. Our findings are in accordance with the results obtained from the study of King pigeons (*Columba livia*) that chronic avermectin exposure down-regulates P450 enzymes activities in time and dose-dependent manners (Zhu et al., 2014).

In addition to biochemical endpoints, the transcriptional response of crucian carp to sub-chronic CAP exposure was investigated by RNA-seq analysis. More DEGs were found along with dose increasing of CAP and there're more down-regulated DEGs than up-regulated DEGs in all treatments. It may indicate the degraded resistance of fish to CAP-induced stress. Moreover, most up-regulated DEGs were enriched in

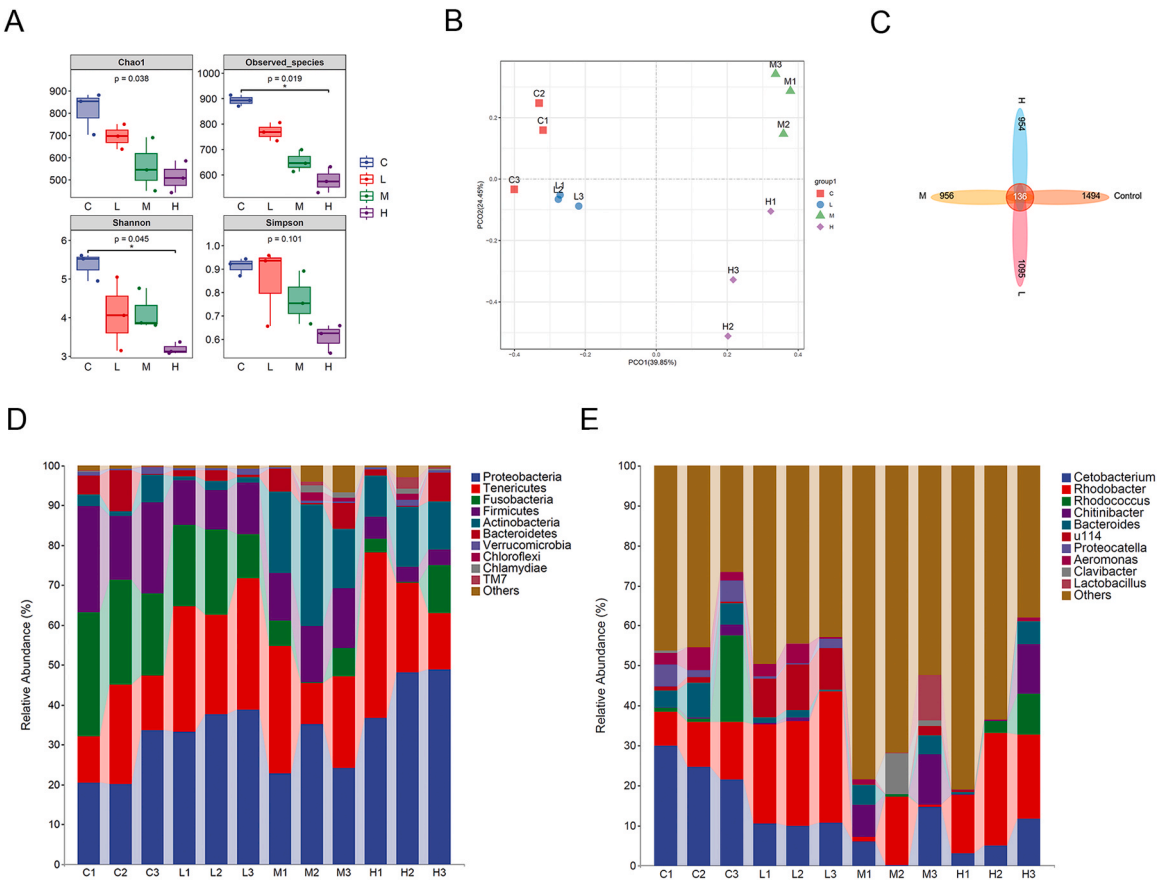

Fig. 5. Intestinal microbiota analysis by 16 S rRNA sequencing. A: Alpha diversity; B: Beta-diversity; C: Venn diagram for shared and unique OTUs in each group; D: Microbiota composition at phylum level; E: Microbiota composition at genus level, n = 3. Asterisk (*) represents the significant difference between treatment and control ($P < 0.05$).

pathways involved in 'environmental information process', for example, MAPK signalling pathway, ErbB signalling pathway and FoxO signalling pathway. MAPK signalling pathway mediates plenty of cellular responses including cell growth, development, division, death and particularly, the stress response (Qi and Elion, 2005). There're evidences that MAPK pathway participates in regulation of pesticide-induced oxidative stress and several cellular responses (Farkhondeh et al., 2020; Ki et al., 2013). The ErbB family are typical receptor tyrosine kinases that were implicated in many signalling cascades for physiological out comes including apoptosis, migration, growth, etc. The ErbB pathway is also connected tightly with MAPK signalling as up-stream regulator (Yarden and Sliwkowski, 2001). FoxO transcription factors were shown to induce apoptosis and adaptive response upon exposure of oxidative stress (Lehtinen et al., 2006). As we expected, the apoptosis pathway was also up-regulated in CAP treatments. In a word, several protein kinase related signalling pathways involving in stress response were activated upon CAP exposure in crucian carp. It revealed that sub-chronic CAP exposure leads profound alteration of transcriptional profile of fish which could result in adverse physiological outcomes like apoptosis and detoxification dysfunctions. The qRT-PCR results are in consist with the RNA-seq data that antioxidant genes, *Cu/Zn-SOD and GST*, as well as detoxification genes, *CYP1A1 and CYP2B19* were all significantly down-regulated in a dose-dependent manner. It implies the inhibition of anti-stress systems of fish upon CAP exposure, which supports our conclusion above. A research from Hasenbein et al. (2018) showed significant decrease of CYP1A and CYP3A in fathead minnow (*Pimephales promelas*) when exposed to field water containing CAP residuals. In addition, Lawrence et al. (2022) reported down-regulation of Phase II detoxification genes including GST in liver of sea lamprey (*Petromyzon marinus*) following 24 h TFM (3-Trifluoromethyl-4′-nitrophenol) exposure. Their results are similar with ours.

Gut microbiota as a bioindicator for aquatic pollutants exposure is a burgeoning field in toxicological assessment. Previous studies indicated the importance of intestinal microbiota in aquatic animals in pesticide-induced toxicity (Jin et al., 2017; Yuan et al., 2019). However, as we know there's no report about the toxic effects of diamides on gut microbiota of fish species. In the present study, sub-chronic exposure of CAP significantly affected the richness and diversity of gut microbial communities. At phylum level, the relative abundance of *Proteobacteria* and *Tenericutes* increased whereas *Fusobacteria* and *Firmicutes* decreased. *Proteobacteria* are commonly regarded as the fish dominant phylum (Holt et al., 2021). The increased abundance of *Proteobacteria* is considered as physiological signal for microbial dysbiosis and potential diagnostic criterion for disease (Shin et al., 2015). The phylum of *Tenericutes* comprised many environmental pathogens and commensal or obligate parasites of human and animals (Wang et al., 2020). Besides, *Fusobacteria* are reported involving in metabolism of fatty acid via butyrate production. *Firmicutes* is also one of the dominant phylum in fish including many probiotics (Fu et al., 2022). Therefore, the alteration of ratios between *Proteobacteria*, *Tenericutes* and *Fusobacteria*, *Firmicutes* observed in this experiment suggest an imbalance of fish gut microbiota. Similar results were found in studies of zebrafish (Li et al., 2020) and Pacific white shrimp (Fu et al., 2022) when exposed to oxytetracycline and imidacloprid respectively.

At genus level, the relative abundance of *Cetobacterium* decreased obviously while *Rhodobacter* increased in all treatments. As the core commensal microbial genus in fish species, *Cetobacterium* participates in

fat and protein metabolism (Xie et al., 2021). A report from Huang et al. (2021) demonstrated that *Cetobacterium* abundance in tadpoles was induced when exposed to atrazine, indicating an interference of fat and protein metabolism. *Rhodobacter* species are photosynthetic bacteria preferring organic compounds as hydrogen donors (Cho and Kim, 2022). Thus, they were used as antibiotics in aquaculture water for better growth of fish (Chiu and Liu, 2014). The decrease of *Cetobacterium* and increase of *Rhodobacter* indicates the reconstitution of gut bacterial community structure and impairment of beneficial flora upon CAP exposure. Moreover, the altered abundance of key flora responsible for nutrient biosynthesis and metabolism may eventually laid adverse host effect.

5. Conclusion

In this study, we have investigated the acute toxicity, bioaccumulation and sub-chronic toxic effects of CAP on crucian carp. The results showed that CAP is low toxic to crucian carp but accumulated in fish meat at a considerable level. Sublethal exposure of CAP induced oxidative stress and detoxification inhibition in the liver of fish. Several stress-related cellular signalling pathways were activated while drug metabolism pathways, particularly the cytochrome P450 pathway were inhibited. The gut microbiota analysis demonstrated a prominent alteration of gut microbiota composition. All these results suggest that CAP could target both liver and gut of crucian carp and form transcription inhibition of detoxification genes and dysbiosis of gut microbiota. In addition, the CYP genes and gut microbiota are sensitive to CAP and could serve as biomarkers for further toxicological research.

CRediT authorship contribution statement

Hongmei Yin: Writing – original draught preparation, Investigation. **Yi Huang:** Data curation. **Guangwen Yan:** Data curation, Investigation. **Qiang Huang:** Investigation. **Yan Wang and Hongming Liu:** Data curation. **Zhiqiu Huang:** Project administration. **Yuhang Hong:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing 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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115292.

References

Arellano-Aguilar, O., Montoya, R.M., Garcia, C.M., 2009. Endogenous functions and expression of cytochrome P450 enzymes in teleost fish: a review. Rev. Fish. Sci. 17, 541–556.

Bantu, N., Vakita, V.R., 2013. Acute toxicity of chlorantraniliprole to freshwater fish *Channa punctatus* (Bloch). Adv. Zool. Bot. 1, 78–82.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 25, 248–256.

B.F.M.A. China Fishery Statistical Yearbook. China Society of Fisheries, Beijing, 2017.

Chiu, K.-H., Liu, W.-S., 2014. Dietary administration of the extract of Rhodobacter sphaeroides WL-APD911 enhances the growth performance and innate immune responses of seawater red tilapia (*Oreochromis mossambicus*×*Oreochromis niloticus*). Aquaculture 418–419, 32–38.

Cho, K.S., Kim, J.K., 2022. A simple culture technique of *Rhodobacter azotoformans* EBN-7 for public use: application to NH4+-N removal in shrimp aquaculture water. Fish. Aquat. Sci. 25, 525–536.

Clasen, B., Loro, V.L., Murussi, C.R., Tiecher, T.L., Moraes, B., Zanella, R., 2018. Bioaccumulation and oxidative stress caused by pesticides in *Cyprinus carpio* reared in a rice-fish system. Sci. Total Environ. 626, 737–743.

Cordova, D., Benner, E.A., Sacher, M.D., Rauh, J.J., Sopa, J.S., Lahm, G.P., et al., 2006. Anthranilic diamides: a new class of insecticides with a novel mode of action, ryanodine receptor activation. Pestic. Biochem. Physiol. 84, 196–214.

Cui, F., Chai, T., Qian, L., Wang, C., 2017. Effects of three diamides (chlorantraniliprole, cyantraniliprole and flubendiamide) on life history, embryonic development and oxidative stress biomarkers of *Daphnia magna*. Chemosphere 169, 107–116.

Farkhondeh, T., Mehrpour, O., Buhrmann, C., Pourbagher-Shahri, A.M., Shakibaei, M., Samarghandian, S., 2020. Organophosphorus Compounds and MAPK Signaling Pathways. Int. J. Mol. Sci. 21.

Fu, Z., Han, F., Huang, K., Zhang, J., Qin, J.G., Chen, L., et al., 2022. Impact of imidacloprid exposure on the biochemical responses, transcriptome, gut microbiota and growth performance of the Pacific white shrimp *Litopenaeus vannamei*. J. Hazard. Mater. 424, 127513.

Goksøyr, A., 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. In: Degen, G.H., Seiler, J.P., Bentley, P. (Eds.), Toxicology in Transition. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 80–95.

Hasenbein, S., Holland, E., Stinson, S., Connon, R., 2018. Developing molecular biomarkers to assess Chlorantraniliprole and imidacloprid impacts in aquatic species (No. 16-C0084). CA Dep. Pestic. Regul.

He, F., Sun, S., Tan, H., Sun, X., Shang, D., Yao, C., et al., 2019. Compatibility of chlorantraniliprole with the generalist predator *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) based toxicity, life-cycle development and population parameters in laboratory microcosms. Chemosphere 225, 182–190.

Holt, C.C., Bass, D., Stentiford, G.D., van der Giezen, M., 2021. Understanding the role of the shrimp gut microbiome in health and disease. J. Invertebr. Pathol. 186, 107387.

Hong, Y., Huang, Y., Wu, S., Yang, X., Dong, Y., Xu, D., et al., 2020. Effects of imidacloprid on the oxidative stress, detoxification and gut microbiota of Chinese mitten crab, *Eriocheir sinensis*. Sci. Total Environ. 729, 138276.

Huang, M.-Y., Zhao, Q., Duan, R.-Y., Liu, Y., Wan, Y.-Y., 2021. The effect of atrazine on intestinal histology, microbial community and short chain fatty acids in *Pelophylax nigromaculatus* tadpoles. Environ. Pollut. 288, 117702.

Jin, Y., Wu, S., Zeng, Z., Fu, Z., 2017. Effects of environmental pollutants on gut microbiota. Environ. Pollut. 222, 1–9.

Kangmin, L., 1988. Rice-fish culture in China: a review. Aquaculture 71, 173–186.

Ki, Y.-W., Park, J.H., Lee, J.E., Shin, I.C., Koh, H.C., 2013. JNK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicol. Lett. 218, 235–245.

Lavtižar, V., Helmus, R., Kools, S.A.E., Dolenc, D., van Gestel, C.A.M., Trebše, P., et al., 2015. Daphnid life cycle responses to the insecticide Chlorantraniliprole and its transformation products. Environ. Sci. Technol. 49, 3922–3929.

Lawrence, M.J., Grayson, P., Jeffrey, J.D., Docker, M.F., Garroway, C.J., Wilson, J.M., et al., 2022. Variation in the transcriptome response and detoxification gene diversity drives pesticide tolerance in fishes. Environ. Sci. Technol. 56, 12137–12147.

Lehtinen, M.K., Yuan, Z., Boag, P.R., Yang, Y., Villén, J., Becker, E.B.E., et al., 2006. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell 125, 987–1001.

Li, J., Dong, T., Keerthisinghe, T.P., Chen, H., Li, M., Chu, W., et al., 2020. Long-term oxytetracycline exposure potentially alters brain thyroid hormone and serotonin homeostasis in zebrafish. J. Hazard. Mater. 399, 123061.

Li, Z., Wang, Z.-W., Wang, Y., Gui, J.-F., 2018. Crucian carp and gibel carp culture. Aquac. China 149–157.

Lu, J., Li, X., 2006. Review of rice–fish-farming systems in China - one of the Globally Important Ingenious Agricultural Heritage Systems (GIAHS). Aquaculture 260, 106–113.

Meng, Z., Wang, P., Chen, X., Lu, C., Chen, S., Ren, L., 2015. Degradation characteristics of chlorantraniliprole and flubendiamide in different waters. J. Acta Agric. Univ. Jiangxiensis 37, 79–83.

Meng, Z., Cui, J., Liu, L., Yang, C., Bao, X., Wang, J., et al., 2022. Toxicity effects of chlorantraniliprole in zebrafish (*Danio rerio*) involving in liver function and metabolic phenotype. Pestic. Biochem. Physiol. 187, 105194.

Moore, M.J., Mitrofanov, I.V., Valentini, S.S., Volkov, V.V, Kurbskiy, A.V., Zhimbey, E. N., et al., 2003. Cytochrome P4501A expression, chemical contaminants and histopathology in roach, goby and sturgeon and chemical contaminants in sediments from the Caspian Sea, Lake Balkhash and the Ily River Delta, Kazakhstan. Mar. Pollut. Bull. 46, 107–119.

Peters, A., Nawrot, T.S., Baccarelli, A.A., 2021. Hallmarks of environmental insults. Cell 184, 1455–1468.

Qi, M., Elion, E.A., 2005. MAP kinase pathways. J. Cell Sci. 118, 3569–3572.

Rathnamma, V.V., Nagaraju, B., 2014. Oxidative stress induced by Chlorantraniliprole in various tissues of freshwater fish *Ctenopharyngodon Idella*. J. Med Sci. Public Health 2, 21–27.

Saglam, O., Athanassiou, C.G., Vassilakos, T.N., 2013. Comparison of spinetoram, imidacloprid, thiamethoxam and chlorantraniliprole against life stages of *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) on concrete. Crop Prot. 53, 85–95.

Seben, D., Salbego, J., da Silva, E.G., Gressler, L.T., Baldisserotto, B., Marchesan, E., et al., 2021. Acute Silver Catfish (*Rhamdia quelen*) exposure to Chlorantraniliprole Insecticide. Bull. Environ. Contam. Toxicol. 107, 883–888.

Shin, N.-R., Whon, T.W., Bae, J.-W., 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33, 496–503.

Song, C., Zhang, J., Hu, G., Meng, S., Fan, L., Zheng, Y., et al., 2019. Risk assessment of chlorantraniliprole pesticide use in rice-crab coculture systems in the basin of the lower reaches of the Yangtze River in China. Chemosphere 230, 440–448.

Song, Y., 2020. Bioaccumulation and toxic effects of chorantrailiprole and flubendiaide in zebrafish (*Danio rerio*). Master. Yangzhou University. Yangzhou 19–20.

Stinson, S.A., Hasenbein, S., Connon, R.E., Deng, X., Alejo, J.S., Lawler, S.P., et al., 2022. Agricultural surface water, imidacloprid, and chlorantraniliprole result in altered gene expression and receptor activation in *Pimephales promelas*. Sci. Total Environ. 806, 150920.

Sumon, K.A., Rico, A., Ter Horst, M.M.S., Van den Brink, P.J., Haque, M.M., Rashid, H., 2016. Risk assessment of pesticides used in rice-prawn concurrent systems in Bangladesh. Sci. Total Environ. 568, 498–506.

Wang, X., Wu, Y., 2012. High levels of resistance to chlorantraniliprole evolved in field populations of *Plutella xylostella*. J. Econ. Entomol. 105, 1019–1023.

Wang, Y., Huang, J.-M., Zhou, Y.-L., Almeida, A., Finn, R.D., Danchin, A., et al., 2020. Phylogenomics of expanding uncultured environmental *Tenericutes* provides insights into their pathogenicity and evolutionary relationship with Bacilli. BMC Genom. 21, 408.

Wei, Y., Yan, R., Zhou, Q., Qiao, L., Zhu, G., Chen, M., 2019. Monitoring and mechanisms of Chlorantraniliprole resistance in *Chilo suppressalis* (Lepidoptera: Crambidae) in China. J. Econ. Entomol. 112, 1348–1353.

Wu, M., Liu, J., Li, W., Liu, M., Jiang, C., Li, Z., 2017. Temporal dynamics of the compositions and activities of soil microbial communities post-application of the insecticide chlorantraniliprole in paddy soils. Ecotoxicol. Environ. Saf. 144, 409–415.

Wu, M., Li, G., Li, P., Jiang, N., Wei, S., Petropoulos, E., et al., 2021. Assessing the ecological risk of pesticides should not ignore the impact of their transformation byproducts – the case of chlorantraniliprole. J. Hazard. Mater. 418, 126270.

Xie, M., Zhou, W., Xie, Y., Li, Y., Zhang, Z., Yang, Y., et al., 2021. Effects of Cetobacterium somerae fermentation product on gut and liver health of common carp (*Cyprinus carpio*) fed diet supplemented with ultra-micro ground mixed plant proteins. Aquaculture 543, 736943.

Yang, H., You-Ning, M., Mei-Ling, Q., Shuang-Shuang, C., He, Q., Han-Tong, Z., et al., 2018. Distribution and degradation of chlorantraniliprole and chlorpyrifos in rice. J. Agric. Resour. Environ. 35, 342–348.

Yarden, Y., Sliwkowski, M.X., 2001. Untangling the ErbB signalling network. Nat. Rev. Mol. Cell Biol. 2, 127–137.

Yuan, X., Pan, Z., Jin, C., Ni, Y., Fu, Z., Jin, Y., 2019. Gut microbiota: an underestimated and unintended recipient for pesticide-induced toxicity. Chemosphere 227, 425–434.

Zhao, L., Tang, G., Xiong, C., Han, S., Yang, C., He, K., et al., 2021. Chronic chlorpyrifos exposure induces oxidative stress, apoptosis and immune dysfunction in largemouth bass (*Micropterus salmoides*). Environ. Pollut. 282, 117010.

Zhu, W.-J., Zhang, Z.-W., Wang, X.-S., Xu, S.-W., Li, M., Li, S., 2014. Effects of avermectin on microsomal cytochrome P450 enzymes in the liver and kidneys of pigeons. Environ. Toxicol. Pharmacol. 38, 562–569.