Ecotoxicology and Environmental Safety 264 (2023) 115471

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Generation and application of a novel transgenic zebrafish line Tg (GAcyp1a:eGFP/Luc) as an in vivo assay to sensitive and specific monitoring of DLCs in the environment

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ARTICLE INFO

Edited by Dr Hyo-Bang Moon

*Keywords:*
Mosquitofish
Transgenic zebrafish
*cyp1a* promoter
DLCs
Environmental level

ABSTRACT

CYP1A is the most commonly used biomarker and transgenic fish which carrying a *cyp1a* promoter to drive a reporter gene can be used as reliable way to monitor dioxin/dioxin-like compounds (DLCs) in the environment. Here, we cloned the *cyp1a* promoter of *Gambusia affinis* and this promoter showed stronger transcriptional activity than that of zebrafish. Then, a Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish line was first constructed with the *G. affinis cyp1a* promoter driving eGFP expression using meganuclease I-SceI mediated transgenesis technology. The Tg(GAcyp1a:eGFP/Luc) larvae at 72 h post-fertilization (hpf) were tested by exposing to TCDD for 72 h, and induced GFP was mainly expressed in the liver with low background. The Tg(GAcyp1a:eGFP/Luc) zebrafish showed high sensitivity (limit of detection of 0.322 ng/L TCDD and 0.7 TEQ-ng/L PCDD/Fs) and specificity (insensitive to responses to PAHs and PCBs). In addition, the transgenic line showed a low detection concentration of the DLCs contaminated environmental samples (as low as 1.8 TEQ-ng/L), and the eGFP fluorescence intensity and the chemical-TEQ values were closely correlated. In conclusion, a sensitively and specifically transgenic zebrafish line was established to convenient and effective to detect DLCs in the environment.

## 1. Introduction

With the massive industrial development and increasing human activities, the severity of the global environmental pollution problem has increased (Chen et al., 2019a). Among them, the persistent organic pollutants (POPs) pose a serious threat to human health and ecological owing to the features of long-range transport, persistence, toxicity and bioaccumulation (Sun et al., 2020; Nadal et al., 2015). Among the POPs, dioxins and dioxin-like compounds (DLCs) (including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and co-planar polychlorinated biphenyls (PCBs)) and polycyclic aromatic hydrocarbons (PAHs) are high toxicity and extremely hazardous to humans and animals (Trinh et al., 2018; Sany et al., 2015). DLCs and PAHs can cause a wide range of toxicological and biological effects through activation of the aryl hydrocarbon receptor (AHR), including developmental toxicity, endocrine disruption, immunotoxicity, carcinogenicity, and impaired reproduction (Chen et al., 2019b; Freese et al., 2017; Chivittz et al., 2016; Xu et al., 2015a). In particular, 2, 3, 7, 8-tetrachlorodibenzo-para-dioxin (TCDD) can induce all types of cancers and is known as the most toxic anthropogenic substance (Steenland et al., 2004). Once DLCs and PAHs bind to AHR, the AHR activated complex is transferred to the nucleus to bind aryl hydrocarbon receptor nuclear translocator (ARNT). And then, the AHR-ARNT complex is recruited to the dioxin-responsive element (DRE) to up-regulate the downstream xenobiotic-metabolizing enzyme genes, such as cytochrome P450 1 gene family (Yoshida et al., 2020; Neavin et al., 2018). Therefore, the

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https://doi.org/10.1016/j.ecoenv.2023.115471
Received 12 July 2023; Received in revised form 6 September 2023; Accepted 10 September 2023
Available online 18 September 2023

expression level of the *cyp1a* gene is commonly used as the most effective biomarker to evaluate the DLCs and PAHs contamination in the aquatic ecosystem (Yoshida et al., 2020). Transgenic fish which carrying a *cyp1a* promoter to drive a reporter gene can be used as reliable way to monitor DLCs and other AHR ligands in the aquatic environment (Jones et al., 1991; Operana and Tukey, 2007). During recent years, several transgenic zebrafish and medaka lines which using the *cyp1a* promoter to drive a fluorescent protein gene have been generated for the detection of DLCs and PAHs (Xie et al., 2018; Kim et al., 2013; Ng and Gong, 2013; Xu et al., 2015b). However, these transgenic zebrafish lines are not sufficient to detect low levels of dioxin pollutants in the environment (Shen et al., 2018). So, a truncated *cyp1a* promoter and a 12DRE *cyp1a* promoter have been used to generate a highly sensitive transgenic fish line to detect the DLCs and PAHs pollutants (Luo et al., 2018a; Zhou et al., 2020a).

The western mosquitofish (*G. affinis*) was introduced in many countries for mosquito control, however, it became one of the worst invasive species attributing to its notable adaptability to harsh conditions (Pyke, 2008). In our previous study, to test the possibility of using *G. affinis* to assess PAHs contamination in the aquatic environment, the full-length nucleotide sequence of *cyp1a* mRNA was identified and assessment the time- and dose-dependent *cyp1a* induction in *G. affinis* after exposing to the PAHs (Xie et al., 2020). In this study, we cloned the *cyp1a* promoter of *G. affinis* and found that it has higher transcriptional activity (~2.4 times) compared with zebrafish (AB strain). So, a Tg (GAcyp1a:eGFP/Luc) transgenic zebrafish line that using a mosquitofish *cyp1a* promoter drive an enhanced green fluorescent protein (eGFP) reporter gene expression was generated by using the meganuclease I-SceI mediated genetic modification technology.

Here, we successfully generated the Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish line. Then, TCDD, nine ≥ 4-ring PAHs, two PCBs, three heavy metals, DEHP, and a mixture of 17 PCDD/Fs were selected to verify the specificity and sensitivity of the Tg(GAcyp1a:eGFP/Luc) zebrafish. Moreover, we also validated the efficiency and sensitivity of newly established zebrafish line by 9 fly ash and soil DLCs contaminated environmental samples which collected from a municipal solid waste incinerator (MSWI).

## 2. Materials and methods

### 2.1. Plasmid construction and functional verification in vitro

The pI-SceI- GA*cyp1a* promoter: eGFP/Luc plasmid contained 2780 bp mosquitofish (*Gambusia affinis*) *cyp1a* promoter, eGFP/Luc, and I-SceI elements. Mosquitofish *cyp1a* promoter (Fig. 1A) was cloned by nest-PCR base on the flanking sequence of *cyp1a* gene (MK286594) obtained from the genomic sequence (GCA_003097735.1) and the primers used in this study are listed in Table S1. PCR was performed following the manufacturer's protocol by using the KOD-Plus-Neo (TOYOBO, Japan). Then, the final plasmid pI-SceI- GA*cyp1a* promoter: eGFP/Luc (Fig. 1B) was constructed by replacing the zebrafish (AB strain) *cyp1a* promoter in the plasmid pI-SceI- ZB*cyp1a* promoter: eGFP/Luc which was constructed in our previous study with the mosquitofish *cyp1a* promoter (Xie et al., 2018).

The Dual-Glo® Luciferase Assay System was used to comparative analysis of the transcriptional activity of zebrafish and mosquitofish *cyp1a* promoters. The two plasmids pI-SceI- GA*cyp1a* promoter: eGFP/Luc and pI-SceI- ZB*cyp1a* promoter: eGFP/Luc were transiently transfected into human liver hepatocellular carcinoma (HepG2) cell respectively by using the X-tremeGENE HP DNA transfection reagent (Roche,

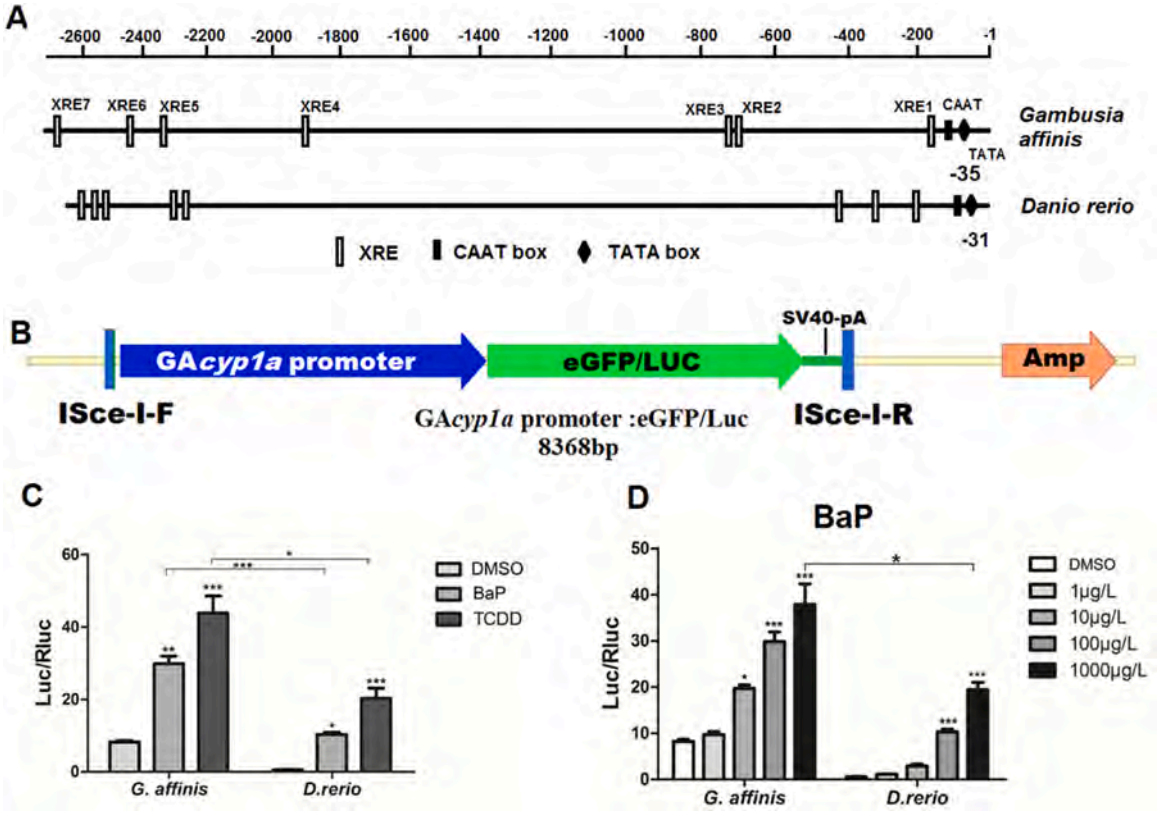



**Fig. 1.** (A) Comparison of *cyp1a* promoter/enhancer regions between the zebrafish and mosquitofish. The locations of specific XREs are shown in relation to the location of the putative transcriptional start site. (B) Schematic diagram showing the structure of the pI-SceI- GA*cyp1a* promoter: eGFP/Luc plasmid. (C) The Dual Luciferase reporter assay for zebrafish and mosquitofish *cyp1a* promoter. The two plasmids were transiently transfected into HepG2 cells and exposed to the same concentration of BaP (100 μg/L) and TCDD (1 nM) for 24 h. (D) The dual luciferase reporter assay for elucidating the comprehensive effect of BaP exposure (1, 10, 100, 1000 μg/L) on the mosquitofish and zebrafish *cyp1a* promoter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Mannheim, Germany), and then exposed to the same concentration of BaP (100 μg/L) and TCDD (1 nM) for 24 h. The HepG2 cell line was routinely cultured in high-glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C with 5% $CO_2$ in a saturated humidity incubator (Choi et al., 2015). Furthermore, to test whether there was a concentration-dependent effect of mosquitofish promoter like the zebrafish promoter, the dual luciferase analysis was also performed under exposure to 1, 10, 100, and 1000 μg/L BaP for 24 h.

### 2.2. Generation of Tg (GAcyp1a: eGFP/Luc) zebrafish

The plasmid pI-SceI-GA*cyp1a* promoter: eGFP/Luc (50 ng/μl) and ISce-I restriction endonucleases (0.5 unit/μl) were mixed, and then 1–1.5 nL was injected into each embryo by using a FemtoJet 4X pressure injector (Eppendorf, Germany). The injected embryos were raised to 2 months as founders P0. Then, the positive fish named F0 were screened out by tail-clipping test using the primer GA*cyp1a*-test-F and GA*cyp1a*-test-R (Table S1). The F0 founders were further cultured to sexual maturity and then crossed with wild type zebrafish to obtain F1 embryos. The intensity of the green fluorescent signal of the F1 embryos which enhanced after exposure to1nM TCDD were chose as the positive F1 founders and continued to be cultured to 2 months old. The positive fish in the F1 founders were screened by tail-clipping test using the primer GA*cyp1a*-test-F and GA*cyp1a*-test-R. F2 generation was obtained by self-crossing of positive F1 generation. The F2 generation was cultured to sexual maturity and the embryos of F2 generation were used for the following experiment. The zebrafish husbandry and ethical statement were showed in the supporting information Text S1.

### 2.3. Chemicals

Dimethyl sulfoxide (DMSO) was purchased from Sangon Biotech (Shanghai, China). TCDD was dissolved in DMSO for $10^{-4}$ mol/L that purchased from Wellington Laboratories, Inc. (Ontario, Canada). 2,2′,3,5′-tetrachlorobiphenyl (PCB44) and 3,3′,4,4′-tetrachlorobiphenyl (PCB77) were purchased from the Beijing CQC Biotechnology Co. (Beijing, China), and the n dissolved in DMSO to prepare 1 mg/mL stock solutions. Nine PAHs, including Pyrene (Pyr), chrysene (Chr), benzo[*g*,*h*,*i*]perylene (BghiP) benzo[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), dibenzo[*a*,*h*] anthrancene (DahA), and indeno[1,2,3-cd]pyrene (IcdP) were purchased from Shanghai Aladdin Biochemical Technology (Shanghai, China), and then dissolved in DMSO to prepare 1 mg/mL stock solutions. All the stock solution were stored at 4 ℃. The Di-(2-ethylhexyl)-phthalate (DEHP) solution (1000 μg/mL in methanol), cadmium chloride, copper nitrate and lead nitrate were also purchased from the Shanghai Aladdin Biochemical Technology (Shanghai, China). The mixture of PCDD/Fs was the $^{13}C_{12}$-labeled PCDD/F internal standards (EPA 1613CS5) which purchased from the Cambridge Isotope Laboratories, Inc (CIL, USA). The detailed concentration of each PCDD and PCDF in the PCDD/Fs were showed in Table S2.

### 2.4. Tg(GAcyp1a: eGFP/Luc) zebrafish exposure to chemicals and environmental samples

For chemicals exposure, usually, forty Tg(GAcyp1a:eGFP/Luc) zebrafish larvae at 72hpf were placed into a 90 mm diameter petri dish containing 20 mL of exposure freshwater solutions. Three replicates were set up for each chemical and each exposure concentration. The detailed exposure concentrations for each chemical were shown in the figure notes. For control group, the same volume of DMSO was added into 20 mL of exposure freshwater solutions. All the dishes were incubated at 28 °C. The fluorescence expression of embryos was observed and imaged under a fluorescence microscope (SMZ18, Nikon, Japan), and the excitation wavelength was 488 nm. The DLCs contaminated samples of the fly ash and soil were collected from a MSWI in Guangzhou suburb. The detail methods of the sample collection, sample preparation and HRGC/HRMS (Agilent 6 890 N/Waters AutoSpec Premier, Shanghai, China) analysis of PCDD/Fs were provided in the supporting information Text S2. The quality control/quality assurance (QA/QC) are provided in the supporting information Text S3. For environmental samples exposure, each 90 mm plates contain 20 mL water and an appropriate amount of DLCs contaminated samples extract. Each sample had three replicates.

### 2.5. Epifluorescence and confocal microscopic analysis of eGFP intensity

The fluorescent signal of the Tg(GAcyp1a:eGFP/Luc) larvae were observed and captured by a fluorescence stereo microscope (SMZ18, Nikon, Japan) equipped with a digital camera (MSX10, Mshot, China). Prior to observation, the larvae of the Tg(GAcyp1a:eGFP/Luc) were anesthetized with MS222 (Aladdin, Shanghai, China).

For confocal microscopic analysis, 30 dpf zebrafish were fixed overnight at 4 ℃ with 4% paraformaldehyde. The fixed zebrafish were placed in 15% and 30% sucrose solution for 4 ℃ refrigerator dehydration, respectively. And then, the samples were embedded in cryo-section medium (Tissue-Tek, Sakura Finetek USA) and cryo-sectioned at 10 μm thickness (CRYOSTAR NX50, Thermo, USA). Next, the embedding medium in the sections were removed by washing with 1 × PBS. Then, the sections were added DAPI staining solution dropwise and incubate at room temperature in the dark for 10 min. The sections were washed with 1 × PBS for three times and mounted the slides with anti-fluorescence quenching medium. A confocal microscope (Eclipse Ti, Nikon, Japan) was used to observe and photograph the frozen sections.

### 2.6. RNA isolation and quantitative PCR

Twenty zebrafish larvae for each group were used to extract the total RNA by using 600 μl RNAex Pro Reagent following the manufacturer's protocol (Accurate Biology, Hunan, China). All the samples were homogenized by using a freeze grinder (Jingxing, Shanghai, China). After sample extraction, RNA was transcribed to cDNA using the Evo M-MLV Reverse Transcription Kit with gDNA Eraser (Accurate Biology, Hunan, China) according to instructions. A Bio-Rad CFX Connect™ Real-Time System (Bio-Rad, USA) was used to perform the qPCR with the SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Hunan, China). PCR reactions consisted of an initial denaturing cycle at 95 °C for 30 s, followed by 40 amplification cycles of 5 s at 95 °C, 30 s at 60 °C. For validation purposes, melting curves were recorded. Then, the PCR products were sequenced (Sangon Biotech, Shanghai, China) and their size were verified by a 1% agarose gel. The *β-actin* was used as the housekeeping gene. The primers of *cyp1a* and *β-actin* were showed in Table S1.

### 2.7. Western blots

The protein extraction of the larvae used the RIPA buffer containing inhibitor cocktail (Servicebio, Wuhan, China). Each sample was separated through 12% SDS-PAGE, and then transferred to PVDF membranes. Next, the membranes were blocked with 5% skimmed milk powder which dissolving in Tris-based saline-Tween 20 (TBST). The membranes were next incubated in the primary anti-CYP1A antibody (1:1000; Abcam) or anti-β-actin antibody (1:1000; Proteintech) at 25 °C for 2 h. After washed with TBST, the membranes were incubated with the HRP-labeled goat anti-mouse antibody (1: 3000; Servicebio) or goat anti-rabbit antibody (1: 3000; Servicebio). The super enhanced chemiluminescence (ECL) detection reagent (Yeasen Biotechnology, Shanghai, China) was used for chemiluminescence detection in a chemiluminescence imaging (Bio-Rad, USA) according to the preset program Colorimetric. Proteins levels were then analyzed by Image J software (Bethesda, MD, USA).

### 2.8. Statistical analyses

The Image-Pro Plus 6.0 software was used to quantify the intensity of the eGFP. The results were analyzed by SPSS 20 software (SPSS Inc., Chicago, IL, USA), and the exposure experimental data were expressed as mean ± standard error (SE). Statistical significance between different groups was determined by one-way ANOVA, using the Dunnett's and LSD tests. The WHO-TEFs$_{2005}$ was used to calculate the Toxic equivalents (TEQs) of the ΣPCDD/Fs (Van den Berg et al., 2006). The linear regression and Pearson correlation coefficient analysis was used to assessment the correlation between fluorescence intensity and chemical-TEQ (Zhou et al., 2020b).

## 3. Results

### 3.1. Plasmid construction and functional verification in vitro

The detailed nucleotide sequence of the mosquitofish *cyp1a* promoter is shown in Fig. S1, with a total of 2780 bp. The promoter contains 7 predicted xenobiotic response elements (XRE), one less than the zebrafish *cyp1a* promoter (Fig. 1 A). The final structure of the pI-SceI-GA*cyp1a* promoter-eGFP/Luc plasmid containing 2780 bp mosquitofish *cyp1a* promoter, eGFP/Luc, and I-SceI elements is presented in Fig. 1B. The results of the comparative analysis of the transcriptional activity of the two *cyp1a* promoters showed that the mosquitofish *cyp1a* promoter had a stronger inducing effect (~2.4 times) than the zebrafish *cyp1a* promoter under the same concentration BaP exposure (Fig. 1C). In addition, the results of the dual luciferase reporter assay of the mosquitofish *cyp1a* promoter under a different concentrations of Bap exposure showed that the Luc/Rluc ratios increased with increasing exposure concentration (Fig. 1D) and showed a clear concentration-dependent effect.

### 3.2. Generation and Screening of Tg(GAcyp1a: eGFP/Luc) Zebrafish for TCDD tests

Five Tg(GAcyp1a:eGFP/Luc) transgenic F0 founders were initially identified, and only 2 of their F1 transgenic larvae induced GFP expression in the liver under 1 nM TCDD exposure for 48 h (Fig. 2A). In addition, the line L2 was induced stronger fluorescence than line L1 under the same exposure concentration (Fig. 2A). Therefore, the line L2 was used for the subsequent experiments. In order to prove that the fluorescence was induced expression in the liver, the juveniles (30dfp) of line L2 were exposed to 1 nM TCDD for 72 h, and then histologic assessment including the laser confocal microscopy and H.E staining were performed to analysis of the fluorescence expression locations in the L2. The results showed that the GFP were mainly induced and expressed in the liver (Fig. 2B a, c and d) and gills (Fig. 2B b, e and f) region.

### 3.3. Effect of TCDD on Tg(GAcyp1a:eGFP/Luc) zebrafish

The above results show that the eGFP was mainly induced and expressed in the liver of Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish larvae after TCDD treatment. Then, 72 hpf Tg(GAcyp1a:eGFP/Luc) larvae were exposed to 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 nM TCDD for 24, 48, and 72 h to analyze whether the expression of eGFP was dependent on TCDD exposure concentrations and time (Fig. 3). The results showed that the intensity of the eGFP gradually increased with increasing of TCDD exposure concentrations and time (Fig. 3A and B). In addition, the eGFP was significantly induced and clearly observed under 0.001 nM TCDD exposure for 72 h. Finally, the expression of *cyp1a* gene was also detected and the level of *cyp1a* expression increased with the increase of exposure concentration.

### 3.4. Effect of different pollutants on Tg(GAcyp1a:eGFP/Luc) zebrafish

To analyze the pollutant detection range of Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish, the eGFP expression were detected under 9 PAHs (BaA, BbF, BkF, BaP, BghiP,Chr, DahA, IcdP, and Pyr), 2 PCBs (PCB44 and PCB77), DEHP, and 3 heavy metals ($Pb^{2+}$, $Cu^{2+}$ or $Cd^{2+}$) exposure. The results showed that among those compounds, only BkF and PCB77 can significantly induced the expression of eGFP (Fig. 4A and Fig. S2). In addition, the Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish have strong background fluorescence in the abdomen after exposure to BaP and IcdP (Fig. 4A and B). Therefore, we also tested BaP and IcdP with WT zebrafish and found that WT zebrafish also have strong background fluorescence (Fig. S2). According to our previous research results,

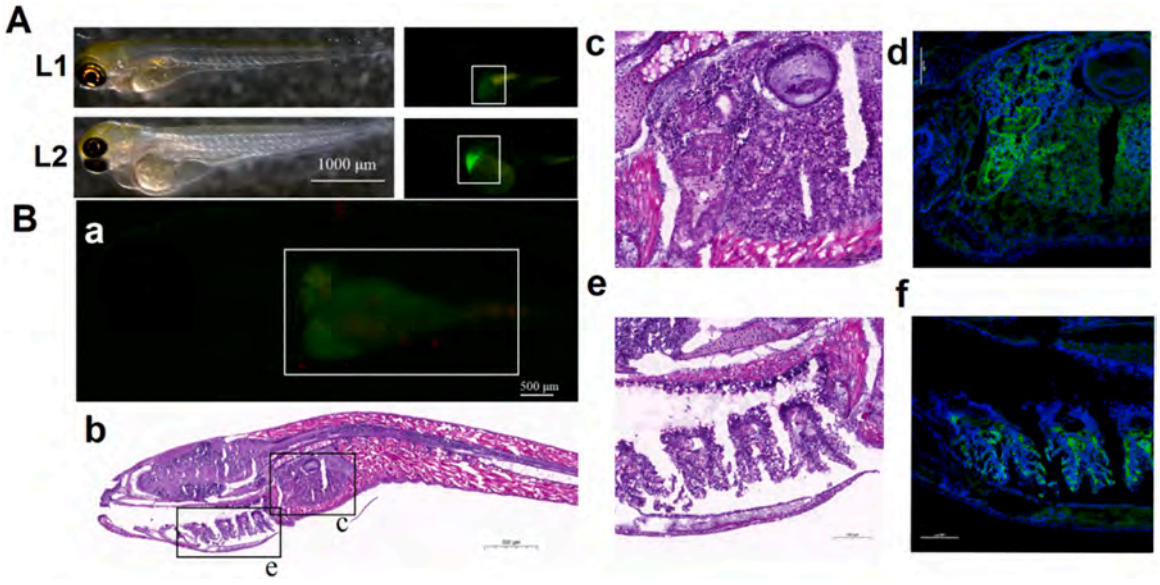



**Fig. 2.** The eGFP expression in Tg(GAcyp1a:eGFP/Luc) Zebrafish (A)The larvae of L1 and L2 were exposed to 1 nM TCDD and observed by fluorescent microscope. (B) the juveniles (30dfp) of line L2 were exposed to 1 nM TCDD for 72 h (a), and then histologic assessment including the H.E staining (b, c and e) and laser confocal microscopy (d and f) were performed to analysis of the fluorescence expression locations in the L2. The induced GFP fluorescence signals were mostly located in the liver (c and d) and gills (e and f) region.

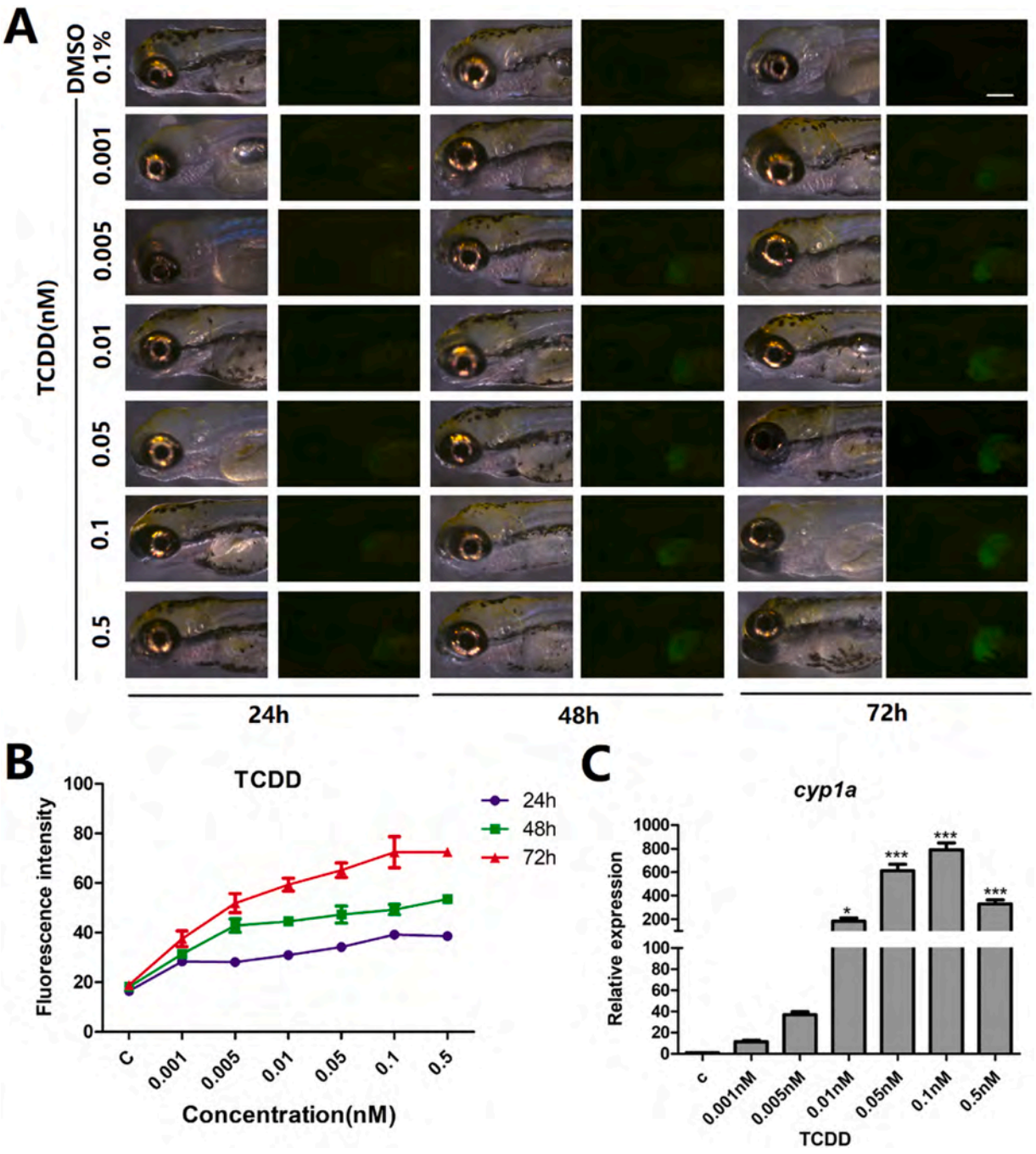



**Fig. 3.** the effect of TCDD on Tg(GAcyp1a:eGFP/Luc) zebrafish. (A) to test whether eGFP expression was TCDD time and concentration dependent, 72 hpf Tg (GAcyp1a:eGFP/Luc) larvae were exposed to 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 nM TCDD for 24, 48, and 72 h. Then, the eGFP were detected and observed. Scale bar = 500 μm. (B) the intensity of eGFP in the liver from the time and concentration response analysis of TCDD (n = 6). (C) RT-PCR analysis of *cyp1a* expression. The larvae in Fig. 2A which exposed for 72 h were collected to analyze the *cyp1a* expression.

pollutants that significantly induced *cyp1a* gene expression were able to induce fluorescence expression (Xie et al., 2018). Therefore, we first comparatively analyzed the expression levels of *cyp1a* in zebrafish under 9 PAHs, 2 PCBs and 2 low concentrations of TCDD exposure by using RT-PCR (Fig. 4C). The results showed that in addition to BkF and PCB77, other 5 PAHs including BaP, BaA, DahA, BbF and IcdP also significantly induced high expression of *cyp1a*. Then, the expression levels of *cyp1a* under these 5 PAHs were also examined by western blot and the results showed that the expression levels of *cyp1a* genes were higher than that under 0.001 nM TCDD exposure (Fig. 4D and E).

In the last, in order to test the LOD of BkF and PCB77, the exposure concentration of BkF and PCB77 were gradient reduced. The results showed that with decreasing exposure concentrations and time, the eGFP fluorescence intensity gradually decreased (Fig. 5). The eGFP fluorescence intensity was already very weak, when the exposure concentration was 25 μg/L.

### 3.5. Effect of Tg(GAcyp1a:eGFP/Luc) zebrafish in response to DLCs

To further analyze the effect of Tg(GAcyp1a:eGFP/Luc) on the detection of DLCs, the expression level of eGFP in the Tg(GAcyp1a:eGFP/Luc) larvae were detected under a mixture of DLCs standards which including 7 PCDDs and 10 PCDFs exposure. The detail concentration of 7 PCDDs and 10 PCDFs were showed in Table S1. Then, we

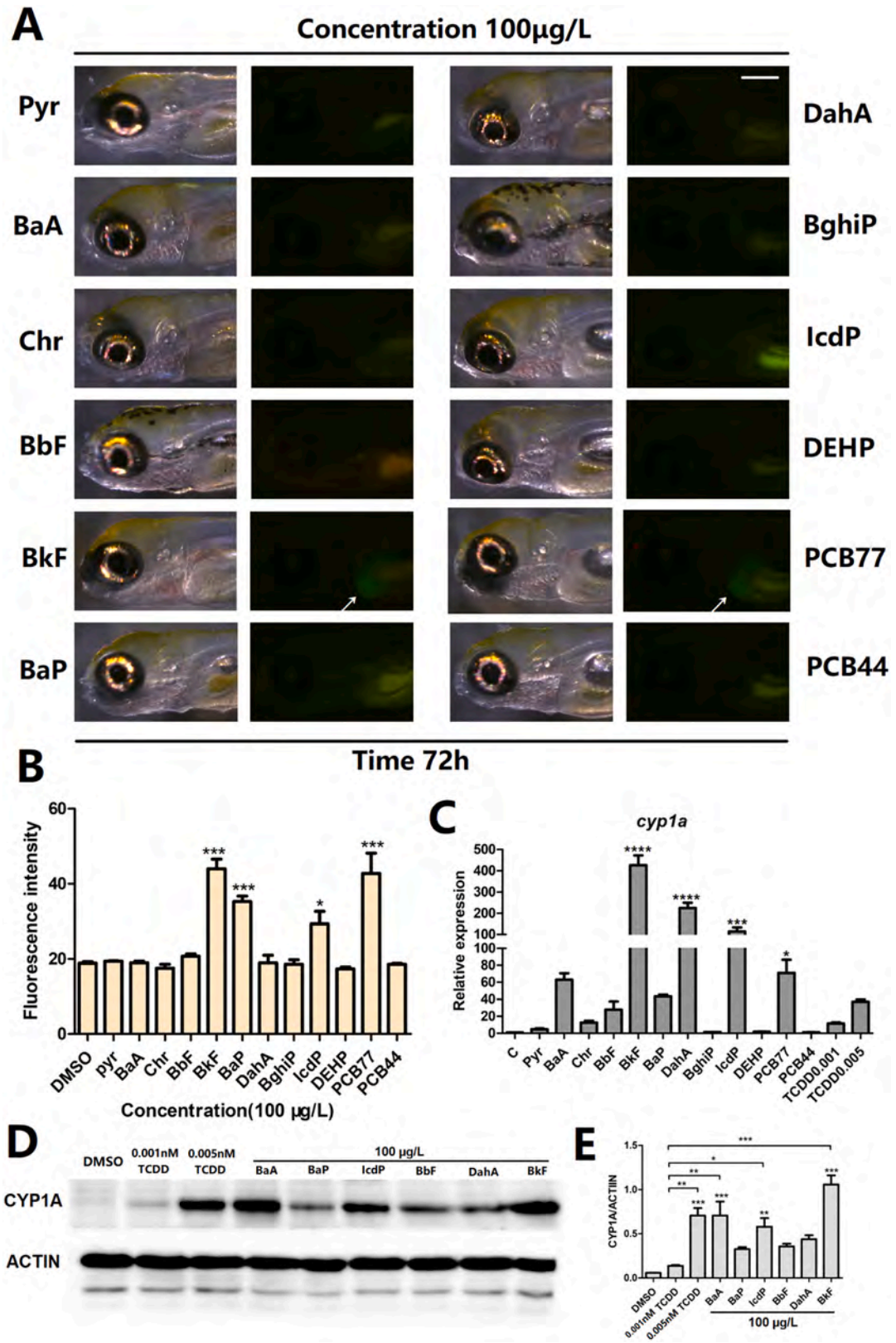



**Fig. 4.** Analysis of the effectiveness of the Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish under 9 PAHs (Pyr, BaA, Chr, BbF, BkF, BaP, DahA, BghiP, and IcdP), 2 PCBs (PCB44 and PCB77), and DEHP exposure for 72 h. The exposure concentrations for all compounds were 100 μg/L. (A) the eGFP expression were observed by fluorescent microscope. Scale bar = 500 μm. (B) the analysis of the eGFP intensity in the liver under different compounds exposure. (C) RT-PCR analysis of *cyp1a* expression. The larvae in A were collected to analyze the *cyp1a* expression. (D and E) the difference in expression of *cyp1a* under the exposure of 6 PAHs (100 μg/L) and two low concentrations of TCDD was analyzed by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

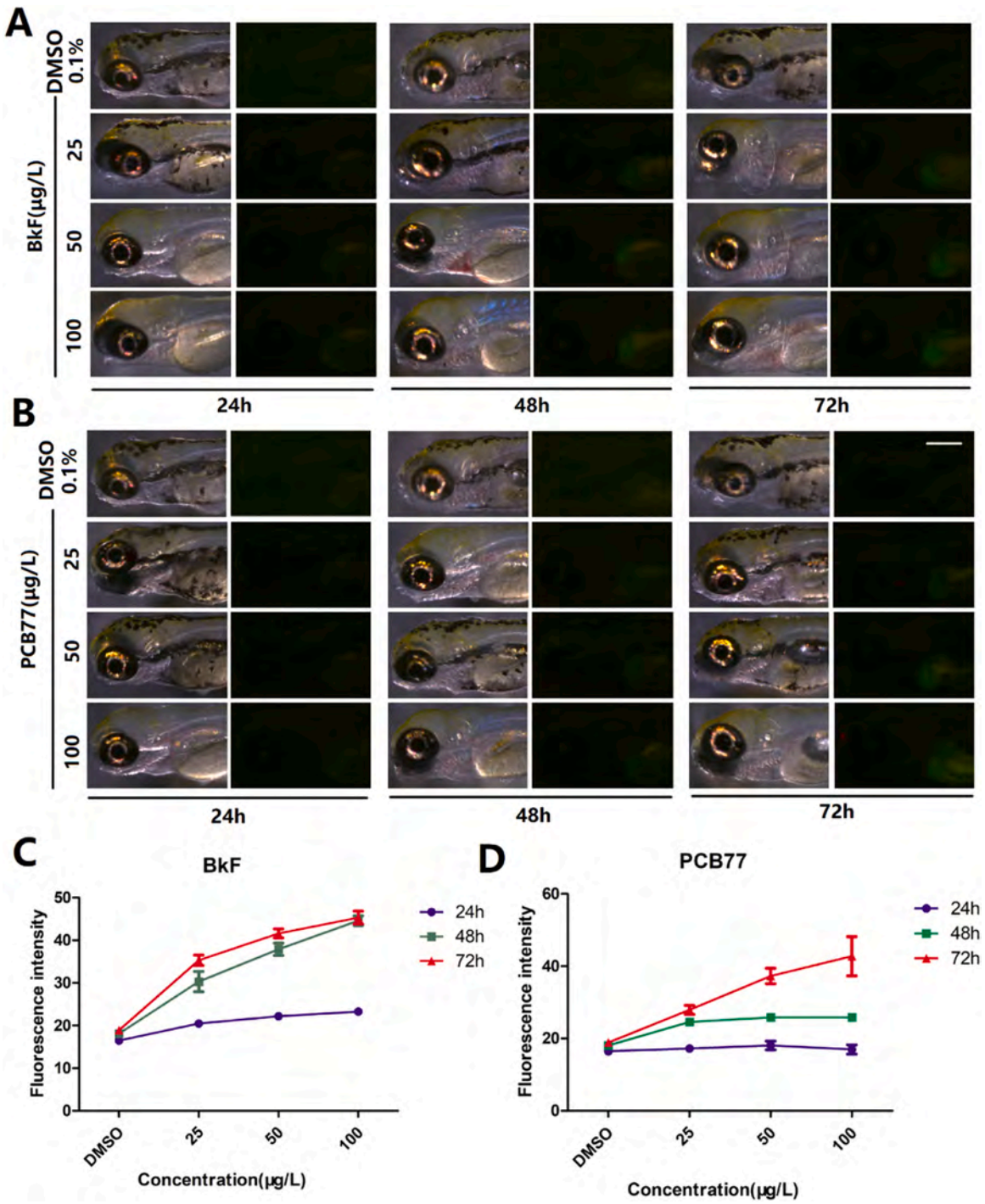



**Fig. 5.** the effect of BkF and PCB77 on Tg(GAcyp1a:eGFP/Luc) zebrafish. A and B, Time and concentration response of BkF (A) and PCB77 (B) inducible eGFP expression. Scale bar = 500 μm. C and D, the intensity of eGFP in the liver from the BkF (C) and PCB77 (D) response analysis (n = 6).

converted the actual concentration of the compound to the TEQ concentration, and then gradient exposure to Tg(GAcyp1a:eGFP/Luc) zebrafish. The results showed that the intensity of eGFP gradually increased with increasing of DLCs exposure concentrations and time (Fig. 6). When the exposure concentration was as low as 0.7 ng/L (TEQ), there was still faint fluorescence in the liver of Tg(GAcyp1a:eGFP/Luc) zebrafish (Fig. 6A).

### *3.6. Bioanalysis of DLCs contaminated samples extracts using Tg (GAcyp1a:eGFP/Luc) zebrafish*

To further analyze the effect of the Tg(GAcyp1a:eGFP/Luc) zebrafish on the detection of DLCs in environmental samples, the expression of eGFP in the Tg(GAcyp1a:eGFP/Luc) larvae were detected after exposure with DLCs contaminated samples extracts. The DLCs contaminated samples of the fly ash and soil were collected from a MSWI and named as S1-S9. The detail concentration of the 7 PCDDs and 10 PCDFs in samples extracts were showed in Fig. 7A and Tab. S3. First, we added 4 μl of extracts to 20 mL of the exposed system and the results showed that the

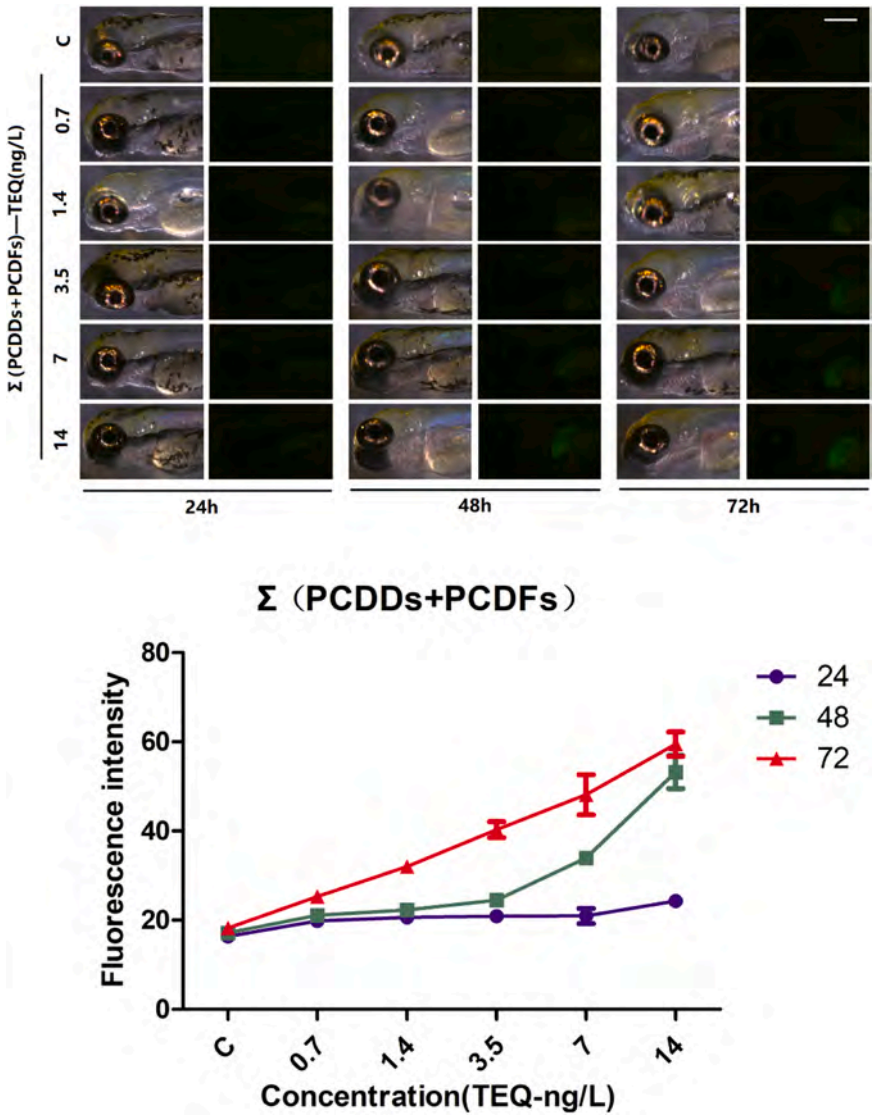


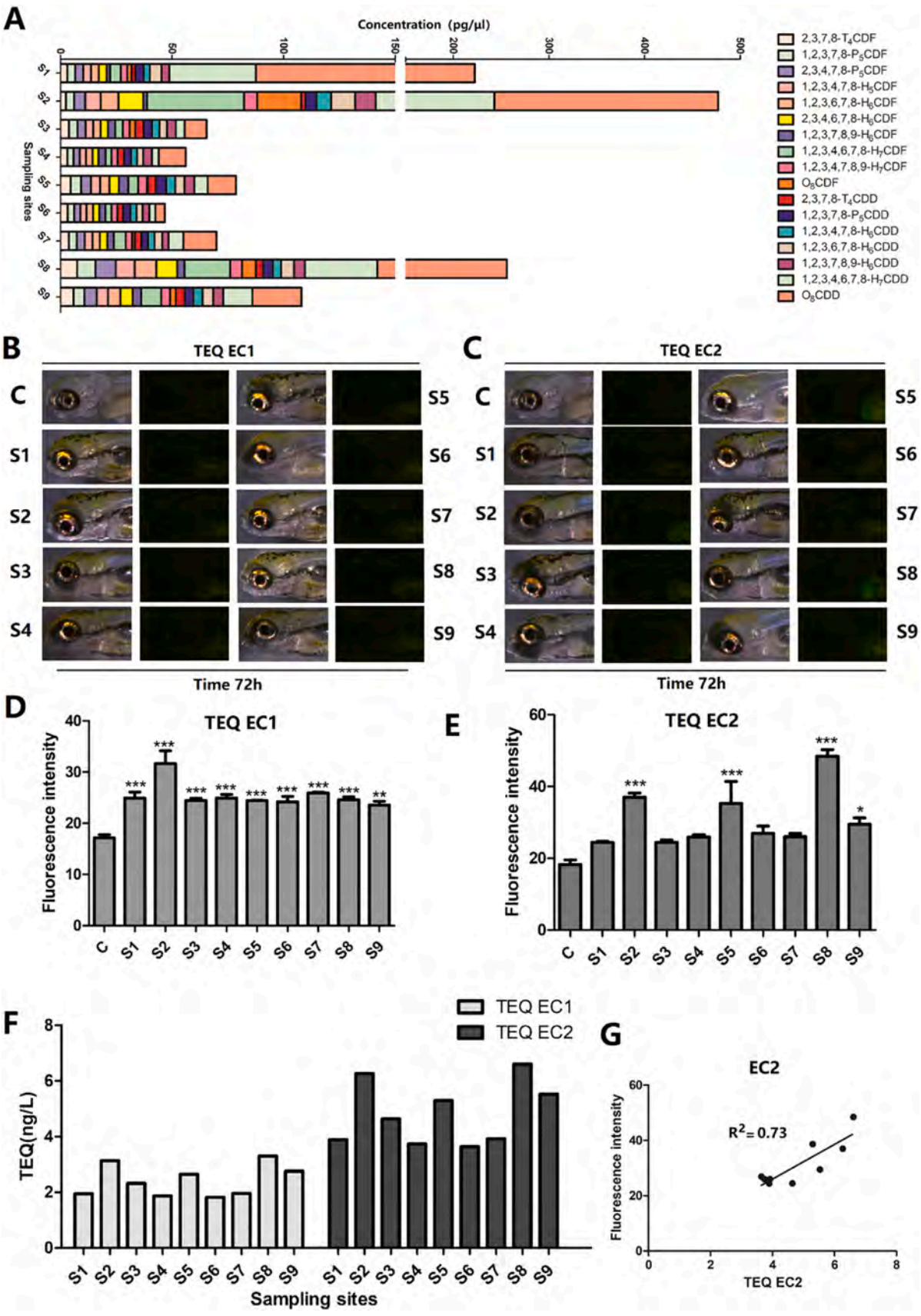

**Fig. 6.** the effect of a mixture of PCDD/Fs on Tg(GAcyp1a:eGFP/Luc) zebrafish. (A) the expression of eGFP in Tg(GAcyp1a:eGFP/Luc) zebrafish larvae were detected under different exposure time and exposure concentration of PCDD/Fs. Scale bar = 500 μm. (B) the intensity of eGFP in the liver under different exposure time and exposure concentration of PCDD/Fs were analyzed (n = 6).

eGFP in the liver of Tg(GAcyp1a:eGFP/Luc) larvae could be detected by exposure to each samples extracts for 72 h. However, the induced fluorescence intensity was very weak and difficult to observe (Fig. 7B and D). So, we increased the exposure concentration by adding 8 μl of extracts. The results showed that the expression of eGFP was significantly induced by exposure to extracts from S2, S5, S8, and S9 for 72 h (Fig. 7C and E). Then, the results of the correlation analysis showed that the fluorescence intensity and the chemical-TEQ (Fig. 7F) were found to be closely correlated (R2 = 0.73) (Fig. 7G). The detail TEQs of the analyzed ΣPCDD/Fs which calculated using the WHO-$TEFs_{2005}$ were showed in Table S2.

## 4. Discussion

In this study, a novel transgenic fluorescent zebrafish line was developed and applied to detect contamination DLCs in the environment. Here, we cloned the *cyp1a* promoter of *G. affinis* and successfully applied it to construct a transgenic zebrafish Tg(GAcyp1a:eGFP/Luc) which possessed a low limit of detection (LOD) (at least 0.001 nM TCDD, equivalent to 0.32 ng/L). Although, a number of transgenic fluorescent fish lines have been previously reported for the detection of DLCs, the Tg (GAcyp1a:eGFP/Luc) transgenic zebrafish line which constructed in this study showed several differences.

First, the results of the dual luciferase reporter assay revealed that the transcriptional activity of mosquitofish *cyp1a* promoter was significantly higher than that in zebrafish. Previous studies showed that the DLCs and PAHs can activate the AHR pathway and forming a AhR-ARNT complex to bound the XREs to up-regulate the *cyp1a* expression (Neavin et al., 2018). Here, there were seven potential XREs have been identified in mosquitofish *cyp1a* promoter, which was one less than that in zebrafish. Many studies have shown that the amount of XRE in the *cyp1a* promoter affects its transcriptional activity (Zhou et al., 2020b). Our previous studies showed that the sensitivity of the *cyp1a* promoter for AHR ligands increased with the increasing quantity of XREs (Xie et al., 2018). In addition, the results of comparative analysis of the two reconstructed zebrafish *cyp1a* promoters containing different numbers of XREs also showed that the detection sensitivity of the promoter with 12 XREs was higher than that of the promoter with 6 XREs (Shen et al., 2018). These results seemed to indicate that a high number of XREs was associated with high transcriptional activity of *cyp1a* promoter. However, the number of XREs in the mosquitofish *cyp1a* promoter is less than that of zebrafish, and its transcriptional activity is significantly higher




*(caption on next page)*

**Fig. 7.** Detecting the levels of DLCs in the fly ash and soil extracts from a MSWI in the south China (S1-S9) using Tg(GAcyp1a:eGFP/Luc) zebrafish larvae. The WHO-$TEFs_{2005}$ was used to calculate the Toxic equivalents (TEQs) of the ΣPCDD/Fs. The EC1 was added 4 μl of DLCs contaminated samples' extracts to 20 mL of the exposed system and the EC2 was added 8 μl of extracts. A, the concentrations of 17 kinds of PCDD/Fs congeners in fly ash and soil extracts; B and C, eGFP expression of Tg(GAcyp1a:eGFP/Luc) larvae after treatment with fly ash and soil extracts for 72 h and imaged under a fluorescence microscope. Scale bar = 500 μm. The TEQ EC1 and TEQ EC2 were 4 μl and 8 μl of fly ash and soil extracts added to 20 mL of exposure system, respectively. D and E, the intensity of eGFP in the liver from the fly ash and soil extracts response analysis. The data are presented as the mean ± SE. Data were analyzed by Student's t-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. F, the exposure concentrations converted to TEQ values (EC1 and EC2) base on the analyzed ΣPCDD/Fs in the fly ash and soil extracts. G, the correlation between the intensity of eGFP and chemical-TEQ was performed by using the linear regression and Pearson correlation coefficient analysis.

than that of zebrafish, which seems to be inconsistent with this result. Many studies have shown that not all of these XREs are necessarily active and the contribution of each XRE was not equal (Her et al., 2004; Zeruth and Pollenz, 2007). In addition, some researches also revealed that the presence of XREs was not the only determinant of the regulation of AHR-mediated genes (Zeruth and Pollenz, 2007). Therefore, we speculated that the higher transcriptional activity of the mosquitofish *cyp1a* promoter compared to zebrafish may be due to the presence of more active XREs or the presence of other cis-regulatory elements.

Second, the eGFP induction patterns in Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish was different from that of the previously constructed zebrafish and medaka lines. The eGFP were induced mainly in the liver and intestine in all the transgenic fluorescent fish which reported in the previous studies (Xie et al., 2018; Kim et al., 2013; Xu et al., 2015b; Zhou et al., 2020b). However, the eGFP was just induced mainly in the liver in the Tg(GAcyp1a:eGFP/Luc). Previous study showed that the binding sites (C/EBP and GATA) and the novel 15-bp element contribute to intestine-specific gene expression (Her et al., 2004). Comparative analysis of the *cyp1a* promoters in mosquitofish and zebrafish, the mosquitofish *cyp1a* promoter may lack the 15-bp key element and cannot induce fluorescence expression in zebrafish intestine.

Third, the Tg(GAcyp1a:eGFP/Luc) showed a sensitive and specific response to DLCs. Up to now, five zebrafish transgenic lines and two medaka transgenic lines using *cyp1a* promoter to drive fluorescence expression have been developed. The LOD of TCDD for the seven transgenic lines were shown in table S5. The Tg(*cyp1a*: gfp) zebrafish which constructed using a medaka *cyp1a* promoter showed weak GFP expression (only in the kidney) under 1 pM (0.322 ng/L) TCDD exposure and effective induce GFP under 10 pM (3.22 ng/L) TCDD exposure (Xu et al., 2015b). The Tg(T-*cyp1a*: mCherry) zebrafish using a truncated zebrafish *cyp1a* promoter which constructed in our previous study showed markedly increased mCherry expression under 5 pM (1.61 ng/L) TCDD exposure (Luo et al., 2018b). The Tg(*cyp1a*-12DRE: eGFP) zebrafish and marine medaka can effective induce GFP expression under 1 ng/L TCDD (3.106 pM) exposure (Shen et al., 2018; Zhou et al., 2020b). In the present study, we observed noticeable eGFP expression in the liver of the Tg(GAcyp1a:eGFP/Luc) zebrafish larvae under a concentrations as low as 1 pM (0.322 ng/L) TCDD for 72 h. This result indicated that the Tg(GAcyp1a:eGFP/Luc) zebrafish had high detection sensitivity.

The specificity analysis of these lines for DLCs detection have rarely been performed in the previous studies (Xie et al., 2018; Xu et al., 2015b; Zhou et al., 2020b). The results of the previous studies showed that all the reported transgenic fluorescent fish could respond to TCDD and PAHs. And, three of them were tested for heavy metals and found that these lines did not effectively induce fluorescence under heavy metals exposure (Xie et al., 2018; Xu et al., 2015b; Zhou et al., 2020b). In addition, the eGFP expression in the Tg(*cyp1a*-12DRE: eGFP) transgenic marine medaka could be effectively induced by the mixture of 16 PAHs and mixture of 12 PCBs (Zhou et al., 2020b). In present study, nine ≥ 4-ring PAHs, two PCBs, three heavy metals, and DEHP were selected to verify the specificity of the Tg(GAcyp1a:eGFP/Luc) zebrafish. The results showed that only BkF and PCB77 could effectively induce the eGFP expression in the liver of the Tg(GAcyp1a:eGFP/Luc) zebrafish. Interestingly, five PAHs including BaA, BbF, BaP, DahA and IcdP that can induce high expression of *cyp1a* do not effectively induce eGFP expression in liver. This result was different from what we expected. Numerous previous studies have stated that many PAHs exert their toxic effects through activation of the AHR pathway (Garland et al., 2020; Shankar et al., 2020, 2022). Once PAHs bind to AhR, the AhR-activated complex translocate to the nucleus, and then forming an AHR/ARNT complex. Next, the complex binds to XREs and causing the upregulation of numerous phase I metabolic enzymes, including the *cyp1a* (Van Tiem and Di Giulio, 2011). In this study, the results of the RT-PCR and western blot showed that the five PAHs (BaA, BbF, BaP, DahA and IcdP) can induce high expression of *cyp1a* in zebrafish and higher than that under 0.001 nM TCDD exposure. However, under the exposure of 0.001 nM TCDD, the Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish can effectively induce the expression of eGFP, while these five PAHs cannot, although there were seven XREs in the mosquitofish *cyp1a* promoter. Therefore, we have to suspect that there are other key cis-regulatory elements in the zebrafish *cyp1a* promoter that can significantly upregulate the expression level of *cyp1a* gene under PAHs exposure, and these elements were missing in the mosquitofish *cyp1a* promoter. However, our previous study showed that the expression of *cyp1a* can be significantly induced in the liver of the mosquitofish under PAHs exposure (Xie et al., 2020). The Dual-luciferase assay results revealed that the transcriptional activity of the mosquitofish *cyp1a* promoter can also be activated under BaP exposure in human HepG2 cell line. These results suggest that the transcription factors activated in mosquitofish and human HepG2 cells are different from those in zebrafish when exposed to PAHs. In the last, to test the LOD of BkF and PCB77, the exposure concentration of BkF and PCB77 were gradient reduced. The results showed that the eGFP fluorescence intensity was already very weak, when the exposure concentration was 25 μg/L. The above results indicated that the Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish is able to specifically and sensitively detect DLCs.

Therefore, in order to further test the detection effect of Tg(GAcyp1a:eGFP/Luc) zebrafish on DLCs, a mixture of 17 PCDD/Fs was used to gradient expose this line. The results showed that weak fluorescence can still be observed when the exposure concentration is as low as 0.7 ng TEQ/L. Then, 9 fly ash and soil DLCs contaminated samples which collected from a MSWI were used to test the detection effect of the environment samples. The results showed that weak fluorescence can still be observed when chemical-TEQ was as low as 1.82 ng/L. When the chemical-TEQ was greater than 5 ng/L, it can induce obvious fluorescence, and showed a strong correlation between the chemical-TEQ and the fluorescence intensity. E-waste recycling, municipal solid waste incinerators and chemical industries are a significant source of DLC contamination in China and some other developing countries (Dai et al., 2020; Lei et al., 2020). Dioxin-like compounds can be generated when the e-waste and municipal solid waste are incinerated at insufficient temperatures and will then be released into the atmosphere (Zhang et al., 2017). Previous study showed that the air samples taken near the "World Electronic Waste Terminal" Guiyu (Guangdong, China) in 2005 contained the highest DLCs level, with PCDD/Fs concentrations of 64.9–2765.0 $pg/m^3$ (Li et al., 2007). The atmospheric particulates are an important carrier to transport DLCs from the emission sources to other environmental matrices (Qin et al., 2019). Unsurprisingly, relatively high PCDD/Fs levels were found in soil, dust and sediment from an open e-waste burning site in Guiyu. The soil and dust TEQ levels of PCDD/Fs in Guiyu were $2.1 \times 10^4$ pg/g dry weight (dw) and $1.3 \times 10^3$ pg/ g dw, respectively (Leung et al., 2007, 2011). In addition, the sediment TEQ

levels of PCDD/Fs in Lianjiang river of Guiyu ranged from 21.2 to 35, 200.0 pg/g dw (Dai et al., 2020). The concentrations of DLCs contamination in these priority monitoring areas are much higher than the minimum monitoring concentrations for the Tg(GAcyp1a:eGFP/Luc) zebrafish which constructed in our study. Therefore, the Tg(GAcyp1a:eGFP/Luc) zebrafish can be used as a specific and sensitive model for rapid screening of environmental sample extracts for the presence of DLCs contamination.

In conclusion, a Tg(GAcyp1a:eGFP/Luc) transgenic zebrafish line using a mosquitofish *cyp1a* promoter was first constructed. This newly constructed line induced fluorescence under DLCs exposure that was only expressed in the liver and had a low fluorescence background. In addition, the Tg(GAcyp1a:eGFP/Luc) line showed high sensitivity and specificity to DLCs and could be an efficient and convenient tool for detection of DLCs in the environment.

## CRediT authorship contribution statement

**Shaolin Xie**: Writing, Construction of transgenic fish. **Bing Yang**: Writing, Exposure experiment. **Siying Li**: Exposure experiment, Data curation. **Liangjun Ge**: Data curation, Editing. **Min Li**: Chemical analysis. **Qingshi Chen**: Review, Editing. **Xian Qing**: Supervision, Chemical analysis. **Jixing Zou**: Ideas, Supervision, Fund.

## Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

## Data Availability

Data will be made available on request.

## Acknowledgment

This work received the support of Guangzhou Basic and Applied Basic Research Foundation (202201010287); National Natural Science Foundation of China (No. 21806041). Science and Technology Planning Project of Guangdong Province of China (2020B1212060058).

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115471.

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