



Original Research

Common antimicrobials disrupt early zebrafish development through immune-cardiac signaling

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ABSTRACT

The global production and use of antimicrobial chemicals surged during and after the COVID-19 pandemic, yet their developmental toxicity in aquatic organisms at environmentally relevant concentrations remains poorly understood. Here, we investigate and compare the developmental effects of two restricted antimicrobial chemicals—triclosan (TCS) and triclocarban (TCC)—and three alternative antimicrobials—benzalkonium chloride (BAC), benzethonium chloride (BEC), and chloroxylenol (CX)—on zebrafish embryos (*Danio rerio*) at concentrations of 0.4, 4, and 40 $\mu\text{g L}^{-1}$. We find that BAC induces the most severe reduction in hatching rates, followed by TCS, TCC, BEC, and CX. BAC also exhibits the strongest inhibition of heart rate, with toxicity levels comparable to those of TCS and TCC. All tested chemicals, except CX, cause significant teratogenic effects. Transcriptomic analysis reveals substantial disruptions in immune-related coagulation cascades and mitogen-activated protein kinase signaling pathways. Further validation via protein-protein interaction network analysis and real-time quantitative polymerase chain reaction confirms that altered expression of key hub genes in these pathways impacts bone and heart development, as well as immune system function, potentially driving developmental toxicity. This study provides the first systematic comparison of developmental toxicity among currently used antimicrobials at environmentally relevant concentrations, revealing that the alternative antimicrobial BAC poses greater developmental risks than the banned TCS and TCC. These findings raise concerns about the safety of BAC as a widespread substitute and highlight the necessity for more rigorous environmental risk assessments of alternative antimicrobials before their large-scale application.

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1. Introduction

Antimicrobial chemicals have broad-spectrum activity against bacteria, fungi, and viruses; therefore, they are commonly used in home and personal care products and pharmaceuticals, as well as employed in the agricultural, food processing, and clinical health care settings, to control the spread of environmental pathogens [1–3]. For example, triclosan (TCS), triclocarban (TCC), benzalkonium chloride (BAC), benzethonium chloride (BEC), and

chloroxylenol (CX) are antimicrobial chemicals commonly used in existing disinfection products [3]. Regulatory actions, such as the Food and Drug Administration's ban on TCS and TCC in antibacterial soaps, have prompted a shift to alternatives, such as BAC, BEC, and CX [3,4]. The coronavirus disease 2019 (COVID-19) pandemic markedly increased their use in disinfection practices across diverse settings, including hospitals, homes, and workplaces, to curb the transmission of severe acute respiratory syndrome coronavirus 2 [5–7]. The widespread production and use of these antimicrobial chemicals have resulted in their common detection in various environmental matrices, such as indoor residential dust, surface water, sewage sludge, sediments, and human tissue [8–15]. The reported concentrations of TCS and TCC in surface water have reached up to 40 and 6.75 $\mu\text{g L}^{-1}$, respectively [16,17]. Moreover, while data on BEC concentrations in environmental matrices are

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limited, recorded levels of BAC (up to $99.6 \mu\text{g L}^{-1}$ [15]) and CX (up to $9.57 \mu\text{g L}^{-1}$ [14]) in surface water have exceeded those of TCC. Concerns about the potential environmental risks and ecological impact of antimicrobial chemicals in aquatic ecosystems have been raised, as they are frequently detected in surface waters.

Developmental stages are key phases in the life cycles of aquatic organisms, and during their early developmental stages, these organisms are particularly sensitive to environmental contaminants [18]. The impairment of early development may affect the growth and organ development of individual organisms and the health of future generations [18]. Previous studies have found that several antimicrobial chemicals induce developmental toxicity in aquatic organisms at high concentrations. For instance, TCS at 0.5 mg L^{-1} induces developmental malformation, such as spinal deformities and pericardial edema 96 h post fertilization (hpf) [19]. Furthermore, $225 \mu\text{g L}^{-1}$ TCC results in high embryonic mortality, delayed hatching, yolk resorption, and increased malformations [20]. It was also found that $0.05\text{--}5 \text{ mg L}^{-1}$ of BAC, BEC, and CX cause zebrafish embryo hatching delay or inhibition and morphological abnormalities and that the acute toxicity of BAC exceeds that of banned TCS and TCC [21]. These studies provide valuable toxicological information on antimicrobial chemicals' impact on aquatic organism development. The developmental toxicity of antimicrobial chemicals directly reflects their ecological risks and is associated with various biological responses, from genes to tissues [22,23]. However, whether several common antimicrobial chemicals cause developmental toxicity and differences in toxicity to zebrafish embryos at environmentally relevant concentrations remains uncertain.

The mechanisms underlying the developmental toxicity of several of these widely used antimicrobial chemicals to aquatic organisms are currently poorly understood. Prior investigations employing conventional methods that focus on a restricted set of genes and proteins as biological endpoints have failed to fully encompass the comprehensive range of biological responses elicited by the toxicity of antimicrobial chemicals. The interaction between pollutants and endogenous molecules is the cause of toxicity [24]. Pollutants disrupt the normal functions of endogenous signaling molecules, leading to abnormalities in intracellular signaling pathways and thus affecting various physiological processes. The abnormal phenotype of signaling molecules affected by pollutants can be used to evaluate the mechanism of pollutant-induced teratogenicity [25,26]. Nontargeted transcriptome analysis, which comprehensively evaluates gene transcription in cells, is an important emerging tool that can address the above issues. It has been employed to clarify how pollutants adversely affect fish development at the molecular level and determine their modes of action in fish at early life stages [27,28]. Transcriptomic analysis has been used to identify the key genes affected by pollutants and to determine the functional pathways and interaction networks of these genes, thereby revealing the molecular mechanisms of pollutants' toxic effects [29,30]. The zebrafish (*Danio rerio*) embryo is a widely used model for studying the developmental toxicity of chemicals because of its sensitivity to toxic substances and the availability of a wide range of developmental parameters that serve as endpoints for assessing embryonic toxicity; examples of these parameters are delayed hatching, heart rate, pericardial edema, yolk sac edema, and morphological abnormalities [24].

In this study, we identified and compared the developmental effects of five widely used antimicrobial chemicals on zebrafish embryos exposed to these chemicals through short-term exposure experiments. Transcriptomic analysis elucidates the underlying molecular mechanisms through which the chemicals adversely affected zebrafish embryo development. This study advances our understanding of the toxicity exerted by antimicrobial chemicals on

aquatic organisms and provides novel insights into the underlying mechanisms involved. By investigating developmental toxicity, we contribute to assessing the environmental safety of widely used antimicrobials and their alternatives, ultimately providing a novel perspective on the potential ecological implications of these chemicals.

2. Materials and methods

2.1. Chemicals and reagents

As benzalkonium chloride C12 (BAC-C₁₂; C₂₁H₃₈NCl) is widely used and is the BAC most commonly detected in the environment, it was the target analyte in this study [10,31,32]. This BAC (99% purity), together with BEC ($\geq 98\%$ purity) and CX (99% purity), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Triclosan (99% purity) and TCC (98% purity) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All five antimicrobial chemicals were solubilized in methanol (chromatographic grade) to obtain a master solution placed at 4°C to ensure antimicrobial solubility and stability, and the resulting solutions were then diluted with dimethyl sulfoxide (DMSO; chromatographic grade) to obtain the desired test concentrations. For more information on the reagents and materials used in this study, refer to the Supporting Information.

2.2. Zebrafish maintenance and embryo collection

Adult wild-type AB zebrafish (approximately six months old) were obtained from the State Key Laboratory of the Chinese Research Academy of Environmental Sciences. The adult zebrafish were maintained in a flow-through system at a water temperature of $27 \pm 1^\circ\text{C}$ and relative humidity of $50 \pm 10\%$ under a photoperiod of 14 h:10 h (light:dark). The dissolved oxygen concentration in water was maintained above 7.72 mg L^{-1} , and the conductivity was $480 \mu\text{S cm}^{-1}$. Zebrafish were fed newly hatched brine shrimp (*Artemia nauplii*) twice daily for 14 consecutive days of adaptive feeding. Fertilized eggs were obtained from the natural spawning of adult zebrafish (*Danio rerio*). The night before the experiment, healthy adult zebrafish were randomly selected and placed into spawning tanks; the bottom of each was equipped with a stainless steel spawning tray covered by a fine net with a mesh size that could be penetrated by eggs. The male and female fish were separated by a partition, and the water temperature was kept at $27 \pm 1^\circ\text{C}$. The next morning, the clapboard was removed, and the resulting influx of light stimulated spawning. Fertilized eggs were collected within 2 h of spawning in sterile Petri dishes, and healthy embryos with a normally developed blastula were selected under a microscope for exposure experiments [33]. All experiments followed the guidelines for the Care and Use of Laboratory Animals of the Chinese Research Academy of Environmental Sciences.

2.3. Zebrafish embryo exposure design

At 2–4 hpf, 25 blastula-stage embryos per replicate were randomly transferred using clean pipettes to Petri dishes containing 50 mL of an appropriate concentration of an antimicrobial chemical. Three concentrations (0.4 , 4 , and $40 \mu\text{g L}^{-1}$) of each antimicrobial chemical were evaluated to ensure that these amounts in all exposure groups fell within the range of actual environmental water concentrations [10,34]. A solution containing 0.001% DMSO was used as a blank control, and five replicates of each concentration were used. The embryos were incubated at $28 \pm 1^\circ\text{C}$, with a 14 h light period followed by a 10 h dark period. The embryos were maintained in a semi-static solution for seven days, with the solution being refreshed every 24 h. The hatchability,

mortality, and progression of larvae were assessed at 24 h intervals during the complete exposure period. Daily monitoring included recording the number of deceased embryos and assessing the stage of embryonic development. An embryo without a heartbeat under microscopic observation was considered dead. At 48 hpf, five live embryos from each replicate were selected for heart rate measurement. The number of heartbeats in 20 s was recorded using ZEISS Vert. Axiovert 5 microscope (Jena, Germany). For pigmentation assessment, we followed established protocols in the literature [23,24,35]. We randomly selected five live embryos from each replicate to be visually inspected under a microscope at a consistent angle to assess the degree of pigmentation. Given that zebrafish are three-dimensional, and planar photographs capture only a partial view of the embryo, we based our qualitative scoring on the intensity of pigmentation observed outside the embryo. Five randomly selected live embryos from each replicate were also examined under the same microscope to assess morphological abnormalities, including yolk sac edema, spinal malformations, and pericardial edema, and to calculate the number and incidence of zebrafish presenting with each morphological change. After a seven-day exposure period, the larvae were collected, thoroughly rinsed, and sterilized with phosphate-buffered saline. Next, the larvae were promptly frozen in liquid nitrogen and stored at -80°C for subsequent experimental analysis.

2.4. RNA isolation, cDNA (Complementary DNA) library construction, and sequencing

The zebrafish larvae were collected and pooled for RNA (Ribonucleic Acid) -seq analysis. Total RNA was extracted from five replicates of each treatment using an RNeasy Mini Kit (Qiagen, Basel, Switzerland). The concentration and purity of the extracted RNA were examined using a Nanodrop 2000 (Thermo Scientific, USA), RNA integrity was assessed by performing agarose gel electrophoresis, and RNA integrity numbers were determined using an Agilent 2100 (Agilent, Santa Clara, CA, USA). A single-cell RNA library was constructed in which the total amount of RNA per cell was at least $1\text{ }\mu\text{g}$, the concentration of RNA per cell was at least $35\text{ ng }\mu\text{L}^{-1}$, the optical dispersion at 260 and 280 nm ($\text{OD}_{260/280}$) was at least 1.8, and the $\text{OD}_{260/230}$ was at least 2.0. Sequencing experiments were performed using an Illumina TruSeqM RNA Sample Prep Kit method for library construction, based on an Illumina NovaSeq 6000/HiSeq X Ten (Illumina, USA) sequencing platform. Five replicates per treatment group of all mRNAs transcribed were sequenced, and all mRNAs transcribed were sequenced.

2.5. Transcriptomics sequence (RNA-seq) analysis

First, to ensure the accuracy of subsequent bioinformational analysis, the original sequencing data were filtered using fastp (version 0.19.5) to remove unreliable readings, and the resulting data were subjected to quality assessment to obtain high-quality reads. This quality assessment involved eliminating discarded adapters, sequences with lengths of less than 30 base pairs after mass pruning, ambiguous sequences with an abundance of more than 5%, and sequences containing over 20% of low-quality bases (quality value < 10). Table S1 (Supplementary Material) lists the total numbers of raw and clean reads obtained following the quality assessment of each sample. The processed clean reads were sequence aligned with a zebrafish reference genome (version GRCz11; source: http://asia.ensembl.org/Danio_rerio/Info/Index) using HISAT2 (version 2.1.0) to obtain the mapped reads for subsequent transcript assembly and expression calculations. The mapped reads were spliced and assembled using StringTie (version 2.1.2) and then compared with the original genome annotation

information for complementation and refinement. RNA-Seq by Expectation Maximization (version 1.3.3) was used to quantitatively analyze the overall expression levels of genes/transcripts. DESeq2 (version 1.24.0) was employed to analyze differentially expressed genes (DEGs) using thresholds of a fold change equal to or greater than 2 and a p -value less than 0.05 [36]. Table S2 (Supplementary Material) lists the total numbers of DEGs (comprising upregulated and downregulated genes) for each treatment. GOATOOLS (version 0.6.5) was used for conducting gene ontology (GO) enrichment analysis on the identified DEGs, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-based Annotation System (version 2.1.1) was employed for performing functional enrichment analysis of KEGG pathways; this was used to distinguish the differentially expressed pathways between the control and treatment groups.

2.6. Real-time quantitative polymerase chain reaction (RT-qPCR) validation

Key DEGs from the enriched pathways were subjected to RT-qPCR to validate the reliability of the transcripts acquired from transcriptomic sequencing. The mRNA primer sequences were obtained from published literature and synthesized by Sangon Biotech (Shanghai, China). Table S3 (Supplementary Material) provides the sequences of the primers. Many studies have demonstrated the stability of β -actin expression in zebrafish by normalizing the expression data of different genes [37–42]; β -actin was thus used as a reference gene [43]. The methods employed to extract and isolate total RNA from each sample were the same as those used in the transcriptomic analysis. Real-time quantitative polymerase chain reaction was carried out in a Fluorescent Quantitative PCR Detection System (LineGene 9600, China) using the SuperReal Premix Plus (SYBR Green) Kit (Tiangen Biotech, Beijing, China). For each gene, qRT-PCR reactions were conducted with three technical replicates for every biological replicate ($n = 3$). Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, and the results were compared with transcriptomic sequencing data using linear regression analysis in Origin 2023 (OriginLab, USA) to afford Pearson correlations.

2.7. Data statistics and analysis

Data were graphed and analyzed using GraphPad Prism 9.0 (San Diego, CA, USA) and Origin 2023. Before statistical analysis, the normality of the data was assessed using the Shapiro–Wilk test. The normality test results indicated that the dataset conformed to a normal distribution. Therefore, one-way ANOVA (analysis of variance) was performed to analyze the hatching rate, heart rate, and rates of four typical deformities. Dunnett's test determined the differences between the control and treatment groups. The data on the differences between the various groups are expressed as means $\pm$ standard deviations. A p -value less than 0.05 indicated a significant (*) difference, while a p -value less than 0.01 indicated an extremely significant (**) difference.

3. Results and discussion

3.1. Effects of the five typical antimicrobial chemicals on the hatching rate of zebrafish

Hatching is a key step in the transition from an embryo to a larval fish; therefore, the hatching rate is commonly used to evaluate whether a chemical harms the development and growth of an organism [44]. The hatching rates of the control group at 48, 72, and 96 hpf were 50.0%, 91.7%, and 98.3%, respectively. Compared with

the hatching rate of the control group, the hatching rates of the TCS, TCC, and BAC treatment groups were significantly reduced, while the hatching rates of the BEC and CX exposure groups were not significantly reduced (Fig. 1). Triclosan and BAC had the greatest inhibitory effects on hatching. Specifically, there were significantly decreased hatching ratios in all TCS and BAC exposure groups as early as 72 hpf. Among the five tested antimicrobial chemicals, only BAC inhibited hatching at 48 hpf, with an environmentally relevant lowest effective concentration of $0.4 \mu\text{g L}^{-1}$. These findings suggest that BAC significantly affects zebrafish embryo developmental processes, particularly hatching. Although the hatching rate does not directly reflect acute toxicity, the results indicate that BAC may be the most potent of the five chemicals regarding developmental toxicity. This is consistent with previous studies reporting significant concentration-dependent hatching inhibition by BAC at higher concentrations ($0.1\text{--}0.5 \text{ mg L}^{-1}$), in which it also exhibited greater toxicity compared to TCS and TCC [21]. This study is the first to report that short-term exposure to BAC inhibits zebrafish embryo hatching at environmentally relevant concentrations and that BAC exhibits higher toxicity than other widely used disinfectants. These findings highlight the need for more stringent regulation and monitoring of BAC concentrations in aquatic environments to protect organisms. Our results suggest that exposure to certain antimicrobial chemicals may adversely affect zebrafish embryonic development, potentially negatively affecting survival and population size.

3.2. Effect of typical antimicrobial chemicals on the heart rate of zebrafish

Alterations in zebrafish embryo heart rates during hatching were further evaluated. Significant dose–response decreases in heart rate were observed at 48 hpf in embryos exposed to the chemicals, with the lowest effective concentrations being $4 \mu\text{g L}^{-1}$

for TCS, TCC, and BAC and $40 \mu\text{g L}^{-1}$ for BEC and CX (Fig. 2). Compared with the two other replaced chemicals, namely, CX and BEC, BAC had a greater effect on the heart rate of the embryos. At an exposure concentration of $4 \mu\text{g L}^{-1}$, the BAC-treated group showed an 89.4% reduction in heart rate compared with the control group. This reduction was similar to those observed in the groups treated with the banned chemicals TCS and TCC, which were 84.3% and 89.8%, respectively. This result indicates that BAC, TCS, and TCC are similarly toxic to embryos regarding heart rate. Consistent with the hatching rate results, BEC and CX had a lower heart rate impact than TCS, TCC, and BAC. At a concentration of $40 \mu\text{g L}^{-1}$, BEC and CX reduced heart rates by 87.8% and 88.6%, respectively. By contrast, TCS, TCC, and BAC induced similar adverse effects at a much lower concentration of $4 \mu\text{g L}^{-1}$. The heart is the first organ to form and function during zebrafish development, and the heart rate is an important indicator of heart function [45]. Thus, the significant decreases in heart rate caused by exposure to these chemicals indicate that they can negatively affect the development and function of the heart in the early developmental stages of zebrafish, leading to developmental defects and phenotypic abnormalities in zebrafish embryos [46].

3.3. Toxic effects of antimicrobial chemicals on the early development of zebrafish

Damage or disruption to the heart may affect the overall health and development of zebrafish [46]. Since embryo heart rates decreased at 48 hpf because of antimicrobial chemical exposure, further evaluations were conducted to assess whether there were any developmental or other physiological defects in the treatment groups compared with the control group. Developmental toxicity tests showed that treatment with TCS, TCC, BAC, or BEC, but not CX, had significant teratogenic effects in zebrafish. For example, spinal deformation was observed in the groups treated with TCS, TCC,

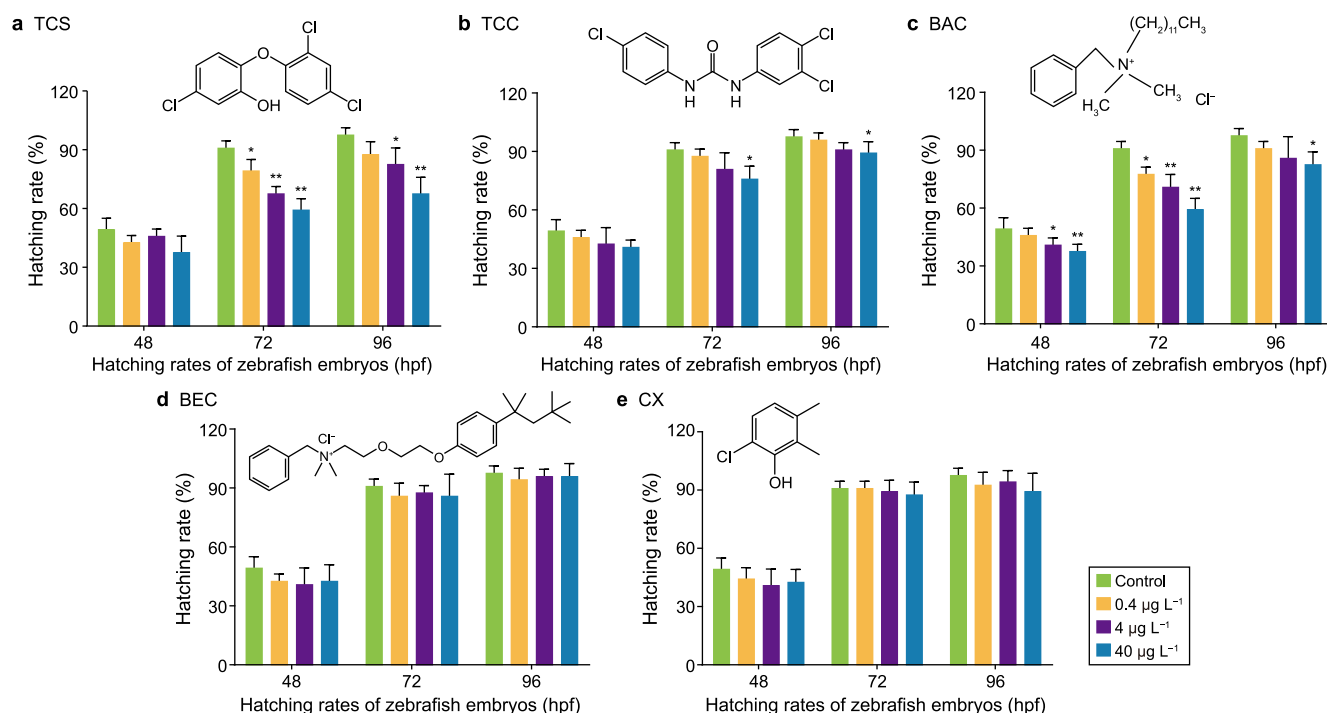


Fig. 1. Chemical structures of triclosan (TCS, **a**), triclocarban (TCC, **b**), benzalkonium chloride (BAC, **c**), benzethonium chloride (BEC, **d**), and chloroxylenol (CX, **e**), along with their effects on the hatching rates of zebrafish embryos at 48, 72, and 96 h post-fertilization (hpf). Data shown as mean $\pm$ standard deviation. Asterisks indicate significant differences from control (* $p < 0.05$; ** $p < 0.01$; Dunnett's test).

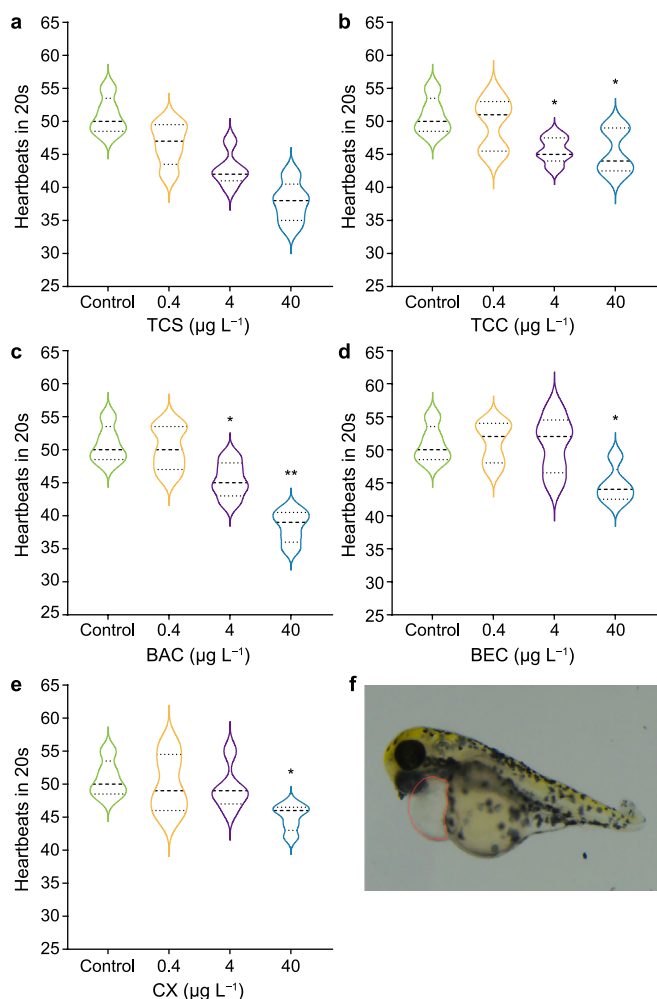


Fig. 2. a–e, Heart rate (number of heartbeats in 20s) of zebrafish embryos at 48 h post-fertilization after exposure to triclosan (TCS, **a**), triclocarban (TCC, **b**), benzalkonium chloride (BAC, **c**), benzethonium chloride (BEC, **d**), and chloroxylenol (CX, **e**). Box plots show median (center line) and interquartile range (25th–75th percentiles). Asterisks denote significant differences between treatments and the control (determined by a Dunnett post hoc comparison; results are shown as means $\pm$ standard deviations (* $p < 0.05$, ** $p < 0.01$)). **f**, Location of zebrafish heart (red circle).

BAC, and BEC (Fig. 3), with the lowest effective concentrations being 4 $\mu\text{g L}^{-1}$ for TCC and 40 $\mu\text{g L}^{-1}$ for TCS, BAC, and BEC. These results indicate that such chemicals may perturb the early skeletal development stages of zebrafish [23], leading to the development of spinal deformities. Although BAC and BEC caused spinal deformities at an effective concentration of 40 $\mu\text{g L}^{-1}$, the deformity rate for the BAC and BEC treatments was much lower than that for the TCS and TCC treatments. Moreover, unlike BAC, BEC, and CX, TCS and TCC induced yolk sac edema in zebrafish. The effective concentration of TCS was 4 $\mu\text{g L}^{-1}$, significantly lower than that of TCC (i.e., 40 $\mu\text{g L}^{-1}$). This result indicates that TCS was more toxic to zebrafish yolk sacs than TCC was. As the yolk sac serves as the nutrient reserve organ for the zebrafish embryo [35], yolk sac abnormalities induced by TCS and TCC may affect the nutrient supply during zebrafish development.

Furthermore, TCS and TCC had different teratogenic effects on zebrafish. Specifically, treatment with 40 $\mu\text{g L}^{-1}$ TCS inhibited pigment deposition in zebrafish, while treatment with the same concentration of TCC caused pericardial edema (Fig. 3a and b). This result suggests that these two chemicals may have different

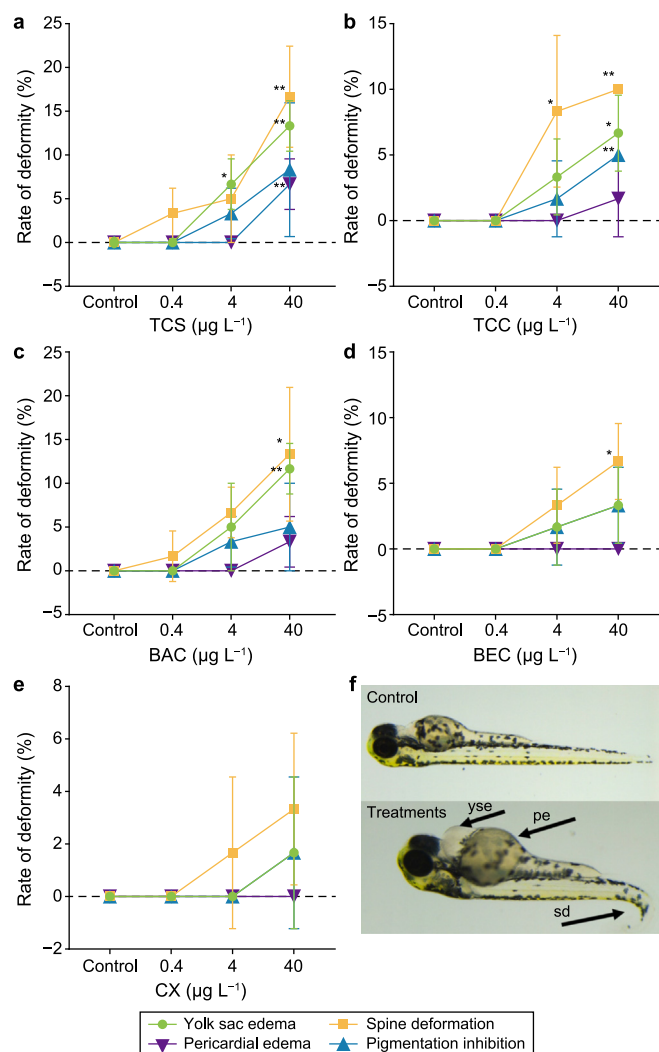


Fig. 3. a–e, Developmental abnormalities in zebrafish embryos exposed to antimicrobial compounds. Incidence rates of four typical deformities (yolk sac edema, spine deformation, and pericardial edema at 72 h post-fertilization (hpf) following exposure to triclosan (TCS, **a**), triclocarban (TCC, **b**), benzalkonium chloride (BAC, **c**), benzethonium chloride (BEC, **d**), and chloroxylenol (CX, **e**). Results are presented as means $\pm$ standard deviations. Significant differences versus the control are indicated as * $p < 0.05$, ** $p < 0.01$ (ANOVA, Dunnett's test). **f**, Representative brightfield images showing the teratogenic effects of antimicrobial chemicals at 72 hpf. yse: yolk sac edema; sd: spine deformation; pe: pericardial edema.

negative impacts on the health and survival of zebrafish during development. By contrast, treatment with CX at various concentrations had no teratogenic effects, which may be attributable to the structure of CX or the treatment concentrations. Additionally, the occurrence of multiple teratogenic changes in single fish suggests that some of these antimicrobial chemicals may exhibit broad-spectrum toxicity. That is, they adversely affect the normal development of zebrafish embryos in multiple ways. The results of the hatching rate and teratogenic tests indicate that exposure to antimicrobial chemicals adversely affects the early growth and development of zebrafish. However, the potential molecular mechanisms driving these downstream effects remain poorly understood. We employed transcriptomic analysis to clarify the molecular mechanisms of these chemicals' toxic effects during zebrafish development.

3.4. Transcriptomic analysis of zebrafish larvae

We performed RNA sequencing-based transcriptomic analysis of larvae that survived exposure to the antimicrobial chemicals to explore the potential molecular mechanisms underlying the observed developmental and physiological defects caused by exposure. After adaptor trimming and quality control, 520.62 Gb of clean reads were obtained. The clean reads of all samples totaled more than 6.01 Gb, with a Q20 percentage of over 96.46% and a Q30 percentage of over 90.67%. Sequence comparisons of the clean reads of each sample with the genome of zebrafish (*Danio rerio*) were conducted, and the matching rate ranged from 88.73% to 93.98%. This analysis detected 34,890 genes; 28,681 of these genes were known, and 6209 had not been previously characterized.

Volcano plots were drawn based on the log₂-fold changes in transcript expression in larvae and their corresponding $-\log_{10}$ significance values. These plots show the overall scattering of various transcripts and the upregulated and downregulated genes in each treatment group (Supplementary Material Fig. S1). There were dose-dependent increases in the numbers of DEGs in the groups treated with the five antimicrobial chemicals. The group treated with 40 $\mu\text{g L}^{-1}$ of TCC had the most DEGs, with 3726 being upregulated and 399 being downregulated. The groups treated with 0.4, 4, and 40 $\mu\text{g L}^{-1}$ of BEC had relatively high numbers of DEGs, 1,823, 1,988, and 1,932, respectively (Supplementary Material Table S2). A Venn diagram was drawn to illustrate the relationships between DEGs and the overlapping of DEGs in the groups treated with the five antimicrobial chemicals, revealing that 13,642 genes were differentially expressed in all groups (Supplementary Material Fig. S2a). Moreover, principal component analysis (Supplementary Material Fig. S2b) showed that the five antimicrobial chemicals had similar effects at the transcriptional level, indicating that they may have similar modes of action. Pathway annotation analysis of these common DEGs using KEGG revealed that they are typically involved in metabolic pathways associated with five categories: metabolism, environmental information processing, cellular processes, organismal systems, and human diseases. The KEGG annotations of these DEGs indicate that the five antimicrobial chemicals primarily affect the immune system and signal transduction functions in the zebrafish brain (Supplementary Material Fig. S3). The DEGs for each processing group were also functionally annotated using the GO classification standard. The GO enrichment analysis (Supplementary Material Fig. S4) revealed that the DEGs across all treatment groups were predominantly associated with cellular processes, biological regulation, and metabolism. These categories encompass extracellular structure organization, extracellular matrix organization, negative regulation of hydrolase activity, and regulation of endopeptidase activity.

We performed a systematic KEGG functional enrichment analysis to clarify the underlying mechanisms. The top 20 enriched KEGG pathways (with the most DEGs or minimum *p*-adjust values) were obtained (Fig. 4), three associated with the digestive system, three with xenobiotic biodegradation and metabolism, and three with lipid metabolism. Detailed information on the signaling pathways, categories, related genes, and numbers of DEGs can be found in Table S4 (Supplementary Material). Fig. 4a shows that the signaling pathways highly enriched in zebrafish treated with the antimicrobial chemicals were the complement and coagulation cascade pathway and the mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK signaling pathway is central to the embryonic development of vertebrates, as it aids in the regulation of hormones and in immune response and contributes to overall growth [47,48]. Similarly, the complement and coagulation cascade pathway is involved in immune response. It plays an important role

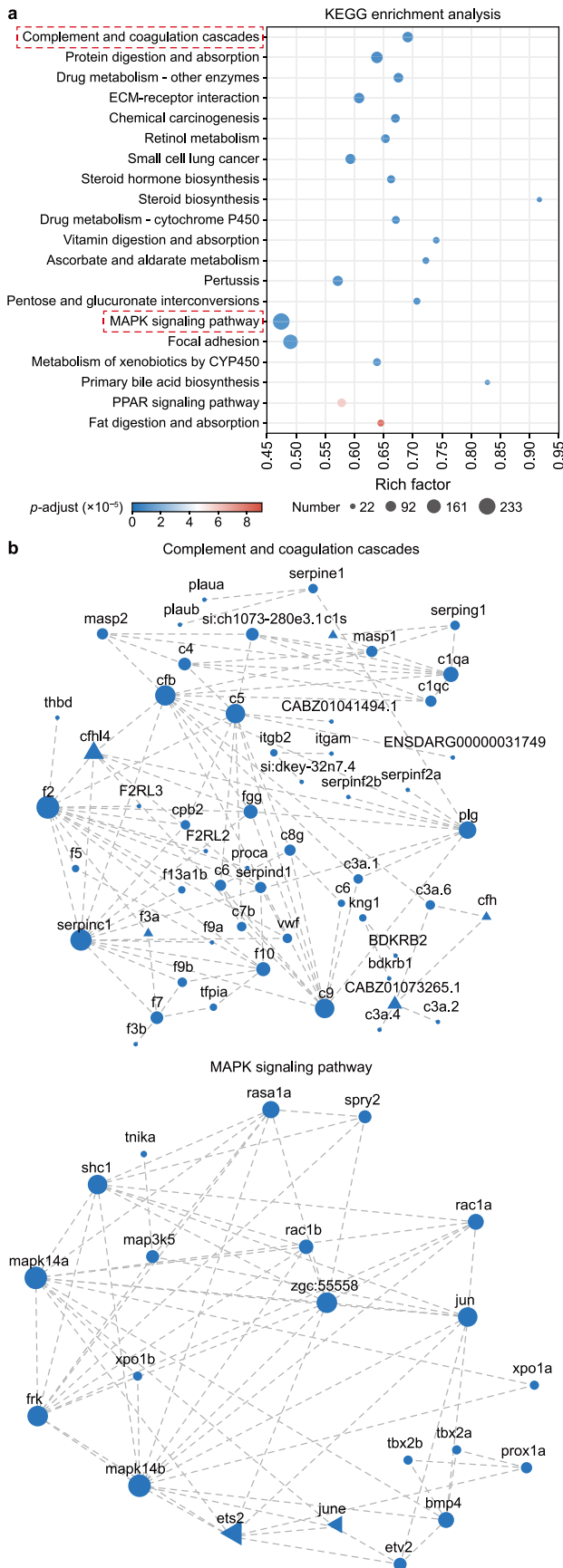
in antimicrobial defense reactions, immune regulation, and mediation of the damaging reactions of immune pathologies [49]. The observed developmental abnormalities may be linked to disruptions in these significantly enriched pathways in the treated zebrafish. However, further investigation is required to establish a more direct relationship between pathway disruptions and the toxic phenotypes, such as malformations, observed in this study.

To explore the interactions between DEGs, we used the search tool of the Retrieval of Interacting Genes/Proteins Database and Cytoscape to conduct a protein–protein interaction (PPI) network analysis of genes encoding proteins in the MAPK signaling pathway and the complement and coagulation cascade pathway, respectively. We then generated a gene expression network. Fig. 4b shows that the complement and coagulation cascade pathway network comprises 55 nodes connected by 100 edges. Five hub genes were identified, namely *f2*, *cfb*, *c5*, *c9*, and *plg*, and these may modulate the complement and coagulation cascade pathways. The MAPK signaling pathway network consists of 21 nodes connected by 61 edges. Four hub genes were identified, namely, *mapk14a*, *mapk14b*, *frk*, and *jun*, which are the core nodes of the MAPK signaling pathway. The network analysis results show that *mapk14a* and *frk* share a large number of co-regulatory genes with *mapk14b*. These hub genes play crucial roles in connecting and regulating the complex signaling pathway network, thereby affecting the network's activity and stability.

3.5. RT-qPCR validation of hub genes for the selected key pathways

The biological pathways and interaction networks associated with hub genes are important components of toxic processes. Thus, the expression levels of hub genes in the complement coagulation cascade pathway and the MAPK pathway were examined. The aim was to verify the interactions and functions of these genes in biological processes and thereby reveal the molecular mechanisms underpinning the developmental toxicity of antimicrobial chemicals in zebrafish.

First, the five hub genes in the PPI network of the complement and coagulation cascade pathways were subjected to RT-qPCR validation. In general, the expression levels and regulatory trends of the genes detected using RNA-seq were consistent with the results obtained from qualification by RT-qPCR in all five chemical exposure groups (Fig. 5a). Relative to the control, the transcripts of *f2* (1.17- and 3.17-fold) and *plg* (1.42- and 2.66-fold) were significantly downregulated ($p < 0.01$) in the high-concentration treatment groups (40 $\mu\text{g L}^{-1}$). *f2* and *plg* encode two important proteins of the coagulation cascade pathway. *f2* encodes coagulation factor II, which is activated to convert prothrombin into thrombin, thereby promoting coagulation [50]. Thrombin is an essential link between inflammation and hemostasis, key processes in the body's defense against infection [51]. *plg* encodes plasminogen, a precursor protein involved in the fibrinolytic system. Plasminogen is converted into plasmin, an enzyme that plays an important role in the breakdown of blood clots [52–54]. A reduction in the expression of *f2* and *plg* induced by antimicrobial exposure may lead to a decrease in thrombin generation and impaired plasminogen function in the early development of zebrafish. This could contribute to developing inflammatory diseases and potentially lead to late-onset mortality in zebrafish [55]. The expression of the classical complement component-related genes *c5*, *c9*, and *cfb* was significantly downregulated ($p < 0.01$) in the groups treated with high concentrations (40 $\mu\text{g L}^{-1}$) of the five antimicrobial chemicals (Fig. 5a). Relative to the control, the expression of *c5* was significantly downregulated ($p < 0.05$) in the groups treated with 40 $\mu\text{g L}^{-1}$ of TCS (−0.52-fold), TCC (−1.36-fold), BAC (−1.12-fold), BEC (−1.86-fold), and CX (−1.94-fold). Similarly, relative to the



control, the expression of *c9* was significantly downregulated ($p < 0.01$) in the groups treated with $40 \mu\text{g L}^{-1}$ of TCS (-0.91 -fold), TCC (-1.49 -fold), BAC (-1.77 -fold), BEC (-1.42 -fold), and CX (-1.99 -fold). The complement system is an indispensable part of the immune response, as it plays an important role in supporting resistance to infection and regulating immune balance [56]. Animals lacking various complement components exhibit a range of phenotypes associated with suboptimal host defense, including reduced phagocytic activity, increased susceptibility to infection, impaired T and B cell responses, and other compromised immune capabilities [49]. The results of this study show that exposure to antimicrobial chemicals significantly reduces *c5* and *c9* expression, suggesting that these chemicals may impair immune function in fish. *cfb* encodes complement factor B, a crucial protein in the complement system that plays a key role in immune response by regulating inflammatory and immunomodulatory processes [57,58]. A reduced expression of *cfb* may result in the inadequate synthesis of complement factor B, thereby affecting immune regulation.

Second, the four hub genes in the PPI network of the MAPK signaling pathway were subjected to RT-qPCR analysis (Fig. 5). It was found that exposure to the five antimicrobial chemicals significantly upregulated *mapk14a* and *mapk14b* expression compared with the control group. *mapk14a* (full name: mitogen-activated protein kinase 14a; also known as p38 α) and *mapk14b* (full name: mitogen-activated protein kinase 14b; also known as p38 β) encodes p38, which is a member of the MAPK family. The activation of *mapk14a* and *mapk14b* is usually associated with apoptosis, and their corresponding proteins regulate the function of downstream target proteins through phosphorylation [59–61], thereby affecting embryonic development [62]. Thus, the increased expression levels of *mapk14a* and *mapk14b* in zebrafish treated with the five antimicrobials may be associated with the observed malformation of zebrafish larvae. *Mapk14a* regulates heart formation, differentiation, and maturation during zebrafish development, playing a crucial signaling role in cardiac development. Changes in its expression can lead to heart defects, including structural abnormalities and issues with cardiomyocyte proliferation and differentiation. Additionally, *mapk14a* is involved in the heart's adaptive response to environmental stressors, such as chemical pollutants or drug exposure [62]. Dysregulation of *Mapk14a* may be associated with heart developmental defects in zebrafish [60]. Activated *mapk14a* was previously shown to block the proliferation of adult zebrafish cardiomyocyte proliferation [63]. Therefore, the significant decrease in the heart rates of zebrafish larvae following treatment with the five antimicrobial chemicals may be attributable to the substantial upregulation of *mapk14a* expression. By contrast, compared to the control, *frk* expression was significantly downregulated ($p < 0.01$) upon exposure to $40 \mu\text{g L}^{-1}$ of TCS (-1.59 -fold), TCC (-1.05 -fold), BAC (-2.34 -fold), BEC (-1.60 -fold), and CX (-1.36 -fold) (Fig. 5). The *fyn*-related kinase (*frk*) protein encoded by *frk* regulates cell growth, proliferation, and survival. It is involved in MAPK signaling pathway activation [64]. *Frk* gene expression regulates osteoblast differentiation and bone growth during zebrafish skeletal development, playing a crucial role in bone morphogenesis and development [65]. This protein also

Fig. 4. a, A systematic Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis showing top 20 enriched pathways based on differentially expressed genes (DEGs) or the lowest p -adjust values. ECM: extracellular matrix; PPAR: peroxisome proliferator-activated receptor. b, Protein-protein interaction network of hub genes and their interacting genes in the complement and coagulation cascades pathway and mitogen-activated protein kinase (MAPK) signaling pathway. Node size indicates connectivity degree. Circular nodes represent transcription factors; triangular nodes represent non-transcription factor proteins.

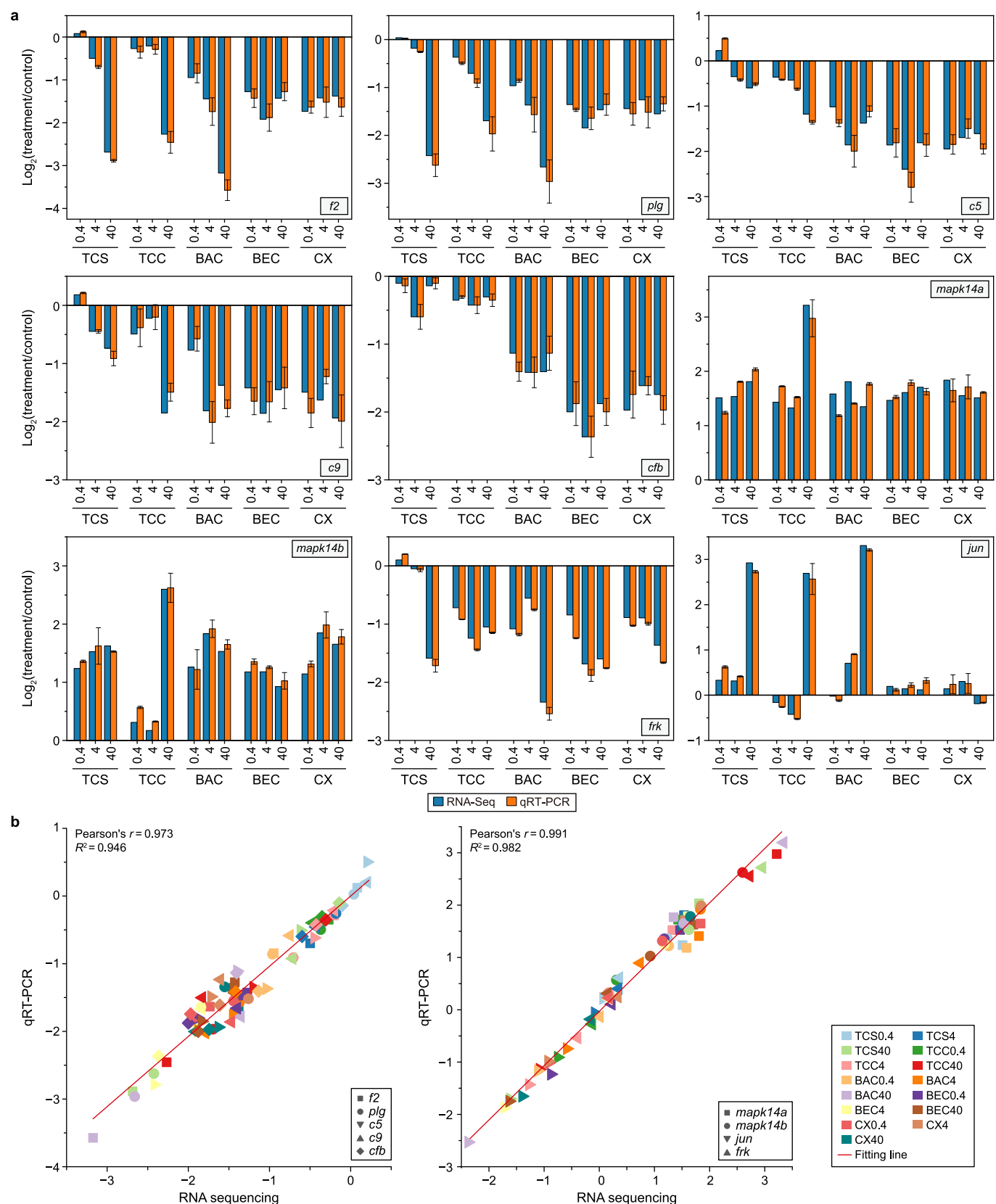


Fig. 5. a, Validation of RNA sequencing data by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of selected target genes, shown as $\log_2$ -fold changes, was consistent with RNA sequencing results across exposure treatments compared to the control. TCS: triclosan; TCC: triclocarban; BAC: benzalkonium chloride; BEC: benzethonium chloride; CX: chloroxylenol. b, Correlation analysis between RNA sequencing (x-axis) and qRT-PCR data (y-axis) for genes in the complement and coagulation cascades and mitogen-activated protein kinase (MAPK) signaling pathways. Pearson correlation analysis revealed significant positive correlations ($p < 0.01$) between the two datasets.

significantly impacts the embryonic and craniofacial development of zebrafish [65]. Therefore, the downregulation of *frk* expression by exposure to antimicrobial chemicals may lead to developmental deficits and malformation in zebrafish larvae, affecting their overall health and development. Compared with *jun* expression in the groups treated with 40 µg L⁻¹ of BEC and CX, respectively, *jun* expression was significantly increased in the groups treated with 40 µg L⁻¹ of BAC, TCS, and TCC. Jun (also commonly referred to as c-Jun) is a key component of the MAPK pathway that is activated by phosphorylation and mediates apoptosis [66,67]. Therefore, the significantly increased expression of *jun* in larvae induced by BAC, TCS, or TCC exposure might have led to an increase in cell apoptosis. However, this finding needs further experimental verification. In line with the above-described results, bisphenol A was previously shown to induce apoptosis in zebrafish by affecting the expression of *jun* [68]. Overall, our transcriptomic sequencing findings established the relationship between the developmental toxicity of zebrafish larvae treated with the five antimicrobial chemicals and the coagulation cascade and MAPK signaling pathways, with these relationships reflected in the morphological results.

Furthermore, the correlations between the sequencing results of the hub genes in the complement and coagulation cascade pathway and the MAPK pathway and the results of RT-qPCR were analyzed, and their statistical significance was determined by performing Pearson correlation tests. Fig. 5b shows the correlation data of the hub genes detected by RNA sequencing and RT-qPCR. The results show that the qualitative consistencies between the upregulated and downregulated genes in the complement and coagulation cascade pathway ($R^2 = 0.946$) and between those in the MAPK pathway ($R^2 = 0.982$) were strong, indicating that the correlations between RNA sequencing data and RT-qPCR data were highly statistically significant.

4. Conclusion

In this study, we compared the development toxicological effects of five common antimicrobial chemicals on zebrafish during their early life stages and explored the potential mechanisms underlying these effects using transcriptomic analysis. The zebrafish embryos were more adversely affected by the replacement antimicrobial chemical BAC than by the banned chemicals TCS and TCC, exhibiting delayed or inhibited hatching, embryo death, reduced heart rate, and developmental morphology deformities.

By contrast, zebrafish were less affected by the BEC and CX replacement antimicrobial chemicals than BAC, TCS, and TCC. Transcriptomic analyses revealed that the interactions between hub genes in the MAPK signaling and coagulation cascade pathways may be crucial in influencing zebrafish development. The downregulation of hub genes in the coagulation cascade pathway weakens immune function, while the upregulation of hub genes in the MAPK signaling pathway may trigger cell apoptosis, potentially contributing to increased mortality. These molecular alterations suggest a mechanistic link to developmental toxicity.

Our findings comprehensively compare the adverse effects of five commonly used antimicrobial chemicals on zebrafish development, offering a possible mechanistic explanation for their developmental toxicities. These results highlight the potential environmental risks these chemicals pose to aquatic ecosystems.

CRediT authorship contribution statement

Yueyue Liu: Writing - Review & Editing, Writing - Original Draft, Software, Methodology, Formal Analysis, Data Curation. **Chen Wang:** Writing - Review & Editing, Supervision, Funding Acquisition, Conceptualization. **Zhiyou Fu:** Methodology, Formal Analysis.

Yingchen Bai: Formal Analysis, Data Curation. **Guomao Zheng:** Methodology, Data curation. **Fengchang Wu:** Writing - Review & Editing, Supervision, Funding Acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

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