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Transcriptome reveals the immune and antioxidant effects of residual chlorine stress on *Cyclina sinensis*

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Residual chlorine is a common by-product of warm drainage in coastal nuclear power plants. when accumulating to some limit, it may threaten marine ecosystem especially for benthic clam. However, there are few studies on the molecular mechanisms related to immunity and antioxidant of residual chlorine stress on clams. In this study, the clam (*Cyclina sinensis*) was exposed for 96 h at different concentrations (0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/L) of residual chlorine to observe its mortality, measure the activity of antioxidant and immune-related enzymes, and analyses the gene expression level in the hepatopancreas by using the transcriptome sequencing. The results showed that the mortality rate increased with the increase of stress time and concentration, and the mortality rate in the 400, 450 and 500 mg/L groups reached 100% at 96 h. The tolerance to residual chlorine of *C. sinensis* decreased with the increase of chlorine dioxide concentration, and the LC_{50} of 96 h was 217.6 mg/L by linear regression method. After residual chlorine stress, the activity of antioxidant-related enzymes (T-AOC and SOD) in the hepatopancreas showed a trend of first increase and then decrease with the extension of stress time. The immune-related enzyme activities of AKP and LZM showed a downward trend between 0 and 96 h, while the ACP enzyme activity showed a trend of first rising and then decreasing. Transcriptome analysis showed that residual chlorine stress significantly changed the expression levels of immune-related molecules associated with signal transduction, prophenoloxidase cascade, cell apoptosis and pattern recognition protein/receptor. Moreover, glutathione S-transferase (*GST*), heat shock protein (*HSP*) and other antioxidant-related genes were significantly affected under residual chlorine stress. This study provided valuable information for understanding the effects of residual chlorine stress on survival, physiological metabolism and molecular mechanisms of immune and antioxidant functions of *C. sinensis*.

KEYWORDS

Cyclina sinensis, residual chlorine, immunity, antioxidants, transcriptome

1 Introduction

Chlorine residual is a common by-product of the warm water discharge from coastal nuclear power stations. In order to prevent seawater organisms from damaging the cooling system, seawater needs to be electrolysed so that the cooling water contains a certain concentration of chlorine, thereby killing all kinds of organisms in the seawater. The seawater enters the cooling system and carries away the waste heat from the nuclear reaction to become warm water, but the residual chlorine in the water also enters the ocean with the warm water. Chlorination of seawater is the main cause of killing organisms in cooling water, while the thermal shock from waste heat in nuclear power plants has less impact (Zeng, 2008). At the same time, in shellfish seedling production, the washing and scrubbing of ponds after transfer, the disinfection of bait cultivation and the daily cleaning of equipment and tools such as nets and bags often involve the use of disinfectants such as bleach. Although the whole process requires strict control, omissions and misuse are inevitable, allowing residual chlorine to flow into the seedling pond to varying degrees, with unpredictable effects on the production of *Cyclina sinensis* seedling (Xu et al., 2007; Wu et al., 2018).

In response to environmental changes, organisms need to use their own antioxidant and immune systems to counteract external stimuli (Eppley et al., 1976; Dempsey, 1986; Pan et al., 2006; Alexandre et al., 2022). The antioxidant response of aquatic organisms to acute or chronic stimuli has been studied mostly in fish and shrimps (Hegazi et al., 2010; Liang et al., 2016; Liu C et al., 2022). In the available studies, fish death under residual chlorine stress is mainly caused by asphyxiation (Jiang et al., 2009), which is also consistent with the mechanism of toxicity of chlorine damage to the respiratory systems of humans and animals. Residual chlorine causes lesions in the gill tissue of aquatic organisms, such as hyperplasia, hypertrophy and accumulation of mucus (Li et al., 2017; Nikolaivits et al., 2020). Swelling of the gill filaments separates the gill filaments from the capillaries and prevents oxygen from entering the capillaries properly in gill tissue, which causes reduced oxygen supply and lower blood oxygen levels in the organism. This phenomenon increases the respiratory rate in order to provide the oxygen levels required for activity. The free chlorine in residual chlorine has strong oxidizing property and can enter the bloodstream through the gills, where it can undergo redox reactions with haemoglobin in the blood. Thereby it disrupts the oxygen transport role of haemoglobin and reduces the oxygen content in the fish blood, inhibits the ability of the fish blood to transport oxygen (Zeitoun, 1977). The immune and antioxidant enzyme activities of bivalves are important physiological parameters of shellfish. Hepatopancreas are important detoxigenic organs and the first barrier against the interference of the whole organism (Pawert et al., 1996; Opstvedt et al., 2000; Zhao L et al., 2016). However, only a few studies have combined molecular and biochemical procedures to describe the relationship between the transcription of specific immune and antioxidant genes and their activity (Qiu et al., 2018; Chen et al., 2021; Qiao et al., 2022), and the response mechanism of shellfish to residual chlorine stress is also rarely studied.

Cyclina sinensis is a common buried shellfish, also known as the venus clam and black clam, and is one of the main cultured shellfish in China's aquaculture industry (Dong et al., 2021). The clam is very widely distributed in the coastal areas, from Korea to the north and south coasts of China, mainly in coastal mudflat areas, with river inlets and is a common shellfish species in China's coastal areas (Dong et al., 2021). Due to the advantages of strong resistance to adversity, tasty meat and high culture yield, the clam has become an ideal cultured shellfish with huge production and sales in China (Zhang, 2014; Shi et al., 2019). As a typical burial-type economic shellfish, *C. sinensis*, is good bioindicator for coastal pollution investigations and has been widely cultivated and proliferated in China (Wei et al., 2020; Ge et al., 2021; Liao et al., 2022). Therefore, *C. sinensis* could be used as a model species to understand the potential toxicity effects of marine bivalves exposed to residual chlorine.

In the present study, we observed the mortality of *C. sinensis* under residual chlorine stress, determined the antioxidant and immune-related enzymes and analysed transcriptome. This study will provide insight into the mechanism of residual chlorine toxicity in *C. sinensis* and provide some theoretical references for the understanding of the toxic effects of residual chlorine on bivalves.

2 Materials and methods

2.1 Animals

Juvenile clam (shell length: 1.20 ± 0.10 cm, weight: 0.70 g ± 0.2 g) was collected from a reproduction farm along coastal of Lianyungang China. Juvenile clam was acclimated for one week in an indoor circulating water system. One week before the experiment, we selected the healthy clams, cleaned their surface stains and disinfected, and then temporarily reared them in natural seawater (salinity: 22 ppt, DO: 8.00-9.00 mg/L, pH: 7.40-7.75) and kept aerating and no bait during the temporary rearing period to enable the clam to expel internal dirt. Meanwhile, we removed the deformed and diseased individuals during the temporary rearing period. All clams were treated in strict accordance with the guidelines for the care and use of experimental animals established by the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China and approved by the Committee on Experimental Animal Management of the Jiangsu Ocean University.

2.2 Chlorine dioxide concentration challenge

The *C. sinensis* were randomly divided into ten groups (10 individuals in each group) and were subjected to different chlorine dioxide (chlorine dioxide effervescent tablets, South Ranch) concentration levels of 0 mg/L (C_0), 50 mg/L (C_1), 100 mg/L (C_2), 150 mg/L (C_3), 200 mg/L (C_4), 250 mg/L (C_5), 300 mg/L (C_6), 350 mg/L (C_7), 400 mg/L (C_8), 450 mg/L (C_9) and 500 mg/L (C_{10}) with C_0 set as control. Three parallel experiments were conducted for each chlorine dioxide concentration level, and used a residual chlorine meter (Pocket Colorimeter II, DR 800, HATCH) to measure the residual chlorine concentration in each group. The treatment continued for 96 h.

2.3 Mortality and median lethal concentration (LC_{50}) analysis and tissue sampling

The mortality of *C. sinensis* was measured after 24, 48, 72, and 96 h of treatment. The death of individuals in each group was recorded. The mortality rate was calculated as $D_f \times O_i^{-1} \times 100\%$, where D_f is the number of the clams killed at the end of each stress in each group and O_i is the total number of the clams in each group at the start of the stress. The LC_{50} of chlorine dioxide for *C. sinensis* at 96 h were analyzed by linear regression analysis on the basis of mortality data. The calculation formula and method were referred from Li et al. (2012). The safe concentration (*SC*) was calculated as following: SC = 0.1 × 96 h LC_{50}.

Choosing 10%, 25% and 50% of the LC_{50} of chlorine dioxide at 96 h for *C. sinensis* obtained from above, i.e., 21.76 mg/L, 54.4 mg/L and 108.8 mg/L as stress concentrations, while as the concentrations could not be set precisely due to the large size of the culture water. So we set three stress concentrations of 20 mg/L (a), 50 mg/L (b) and 100 mg/L (c), and three replicates in each group (10 individuals in each group) in this experiment. The hepatopancreas samples from each individual in groups a, b and c were collected at 0, 3, 6, 12, 24, 48, 72, and 96 h of treatment for enzyme activity determination and only hepatopancreas at 96 h were collected for transcriptomic analysis, with three biological replicates for each experiment.

2.4 Determination of antioxidant and immunity enzymes activity

During sampling, 5 clams from each replicate were pooled together to eliminate any effect of tank, and each clam was collected hepatopancreas at each point of sampling time, immediately frozen in liquid nitrogen and stored at -80°C. The frozen samples were ground in liquid nitrogen, diluted with physiological saline and stored in a refrigerator at 4°C before the assay for subsequent biochemical determination. The activities of enzymes including Lysozyme (LZM), Alkaline phosphatase (AKP), Acid phosphatase (ACP), Superoxide dismutase (SOD) and Total antioxidant capacity (T-AOC) were determined through biochemical analysis kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.5 RNA extraction and transcriptomic sequencing

Selecting the hepatopancreas of *C. sinensis* treated with different concentrations (a, b, c) for 96 h in Section **2.3** described as samples, marked as H-96a, H-96b and H-96c, respectively, and the 0 h stress group was used as the control, marked as H-0. Total RNA was extracted from the hepatopancreas using Trizol method, and genomic DNA was removed with DNase I (Takara). Subsequently, the integrity and purity were estimated by a NanoDrop 2000 (Thermo Fisher Scientific Inc., USA), respectively. Only high-quality RNA samples (OD260/OD280 ranged 1.8-2.2, RIN ≥8.0) were used to construct the sequencing library. Then, these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500 or Illumina HiSeq X Ten), and 125 bp/150 bp paired-end reads were generated. After removing adaptor and low-quality sequences, the clean reads were assembled into expressed sequence tag clusters (contigs) and *de novo* assembled into transcripts by using Trinity with the paired-end method (Grabherr et al., 2011). The longest transcript was chosen as an unigene based on the similarity and length of a sequence for subsequent analysis.

The function of assembled unigenes was annotated by alignment of the unigenes with the NCBI nonredundant (NR), SwissProt, and Clusters of Orthologous Groups for Eukaryotic Complete Genomes (KOG) databases using Blastx with a threshold E-value of 10^{-5}. GO and Pfam annotations were performed by Blast2GO and HMMER software, respectively. Database annotation was performed with KEGG. The gene expression level was calculated by using the FPKM (fragments per kb per million reads) method. To identify differentially expressed genes (DEGs) across samples, $|\log2(\text{fold change})| > 1$ and P-value < 0.05 were set to be the thresholds for significantly different expression levels. The gene expression profiles were compared among the four groups, control (H-0, 0 mg/L), a (H-96a, 20mg/L), b, (H-96b, 50 mg/L) and c, (H-96c, 100 mg/L) treatments, and then all DEGs in each comparison were submitted to GO functional and KEGG pathway enrichment analysis using the GO database and KEGG database, respectively.

2.6 qPCR verification

According to the transcriptome sequencing results, 10 immune and antioxidant related genes were randomly selected to detect Illumina by qRT - PCR. The reliability of sequencing was verified. All the 10 target gene primers were designed using Primer 5.0 and were synthesized by Shanghai Sangon Biological Company. Each pair of primers was validated by gel electrophoresis prior to qPCR. Primers used in the experiment are shown in Table 1.

The cDNA of hepatopancreas of *C. sinensis* treated with different residual chlorine concentrations for 96 h was used as the template for qPCR. TransGen Biotech's PerfectStart® Green qPCR SuperMix kit was used for quantitative analysis of gene expression levels. The qRT-PCR assays were performed with three replicates, and the β-actin gene was used as an internal control to normalize the expression level of the target genes The reaction system with a volume of 10 μL was as follows: 2×PerfectStart® Green qPCR SuperMix (5.0μL), Passive Reference Dye (50×) (0.2μL), primer F (0.2μL), primer R (0.2μL), cDNA template (1μL) and Nuclease-free Water (3.4 μL). The qPCR analyses were performed in triplicate on a StepOnePlus Real-Time PCR (Applied Biosystems, USA) as follows: was used, and the reaction procedure was: predenatured at 94 °C for 30 s; followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. Using the $2^{-\Delta\Delta Ct}$ method to calculate relative quantification of qRT-PCR data.

2.7 Statistical analysis

SPSS 26.0 software was used for data analysis in this study. Use One-way ANOVA and Duncan's analysis to compare the difference of the data. Differences were considered significant at $P < 0.05$.

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TABLE 1 Primer sequence for qRT-PCR verification.

Gene	Primer sequence (5′ - 3′)	Primer length (bp)	Tm (°C)
β-actin	CCTGGTATTGCCGACCGTAT	20	59.9
	TTGGAAGGTGGACAGTGAAGC	21	59.9
CLE	GAAGTCAAACTGCGACCTCA	20	55.8
	CAATCTTTACCCCACTCCATA	20	54.6
CYP450	ATGGCAAATGCGACAGAGAA	20	58.9
	AAGTGGTGGAACCCAAGGAC	20	58.5
CTL6	ACACAGGACAGCGTGGTTCT	20	57.5
	TTCATTCTCTCTGCTACTGGTAAG	24	55.8
Itgbl1	AAGTTTGATGTTTCCCCCAG	20	56.5
	AGTTTATTTCGTAGCCGTGC	20	55.2
TIRB	CCAGCACCAAGCAGTTTATT	20	55.6
	TTTCAAGCTCATCTCGACCA	20	56.4
Bcl - 2	TTACGGGATAGAATACACGACC	22	60.7
	CGCACAAACACCAAGCACAG	20	58.4
T151B	CAGGAATAAACGGCAGGACT	20	57.0
	CTTGAAAGTAAAGCCAGACCA	21	55.1
LPR4	GAACTGGTATTCTGCCTAACG	21	55.6
	CCCATCTATTCTCACCTCTCG	20	56.4
samA	TGCTGAACAACACAGACAAGA	21	54.5
	GTAGATACCAAACGACGACACA	22	55.4
RDE2	GACATACAGAACACCCCGCT	20	57.2
	CCATTTTGCCTCCCAAGATT	20	59.1

3 Results

3.1 Effect of chlorine dioxide stress on the mortality of *C. sinensis*

The mortality rate of *C. sinensis* after chlorine dioxide treatment and residual chlorine concentration for different chlorine dioxide concentrations are shown in Table 2. As can be seen, the mortality rate in the control group (C_0) remained zero during the whole experiment period, indicating the clam individuals used were all in good health.

At the same time, there was a concentration-time effect on the resistance of *C. sinensis* to residual chlorine. The results showed that the mortality increased with time, and as the concentration of chlorine dioxide increased, the tolerance of *C. sinensis* to residual chlorine decreased. Among the different concentration groups, only the groups of C_8, C_9 and C_{10} showed mortality at 24 h. At 48 h, no death occurred in the C_0 group and C_1 group, but all the other groups occurred dead individuals, but the mortality was less than 50%. At 72 h the mortality rates of the C_8, C_9 and C_{10} groups exceeded 50%, with the mortality rate in the C_{10} group reached 83.33%, and the mortality rate in the C_8, C_9 and C_{10} groups reached 100% at 96 h.

As can be seen from the concentration-mortality curve of chlorine dioxide on clams (Figure 1), using the linear regression method, the 96 h LC_{50} was 217.6 mg/L, corresponding to a residual chlorine concentration of 0.22 mg/L. The safe concentration (*SC*) was 21.76 mg/L, corresponding to a residual chlorine concentration of 0.01 mg/L.

3.2 Antioxidant and immune enzymes activity in hepatopancreas of *C. sinensis* under different concentration of residual chlorine

The activities of T-AOC and SOD in hepatopancreas of clams subjected to residual chlorine stress showed a wavy trend from 0 to 96 h (Figure 2). The maximum value of T-AOC in 20 mg/L stress group at 72 h was significantly different from that at 0 h ($P < 0.05$), the enzyme activity at 96 h was the lowest, and lower than that at 0 h, but the difference was not significant ($P > 0.05$). The T-AOC activity in 50 mg/L stress group also reached the maximum value at 72 h, and the difference was significant compared with 0 h ($P < 0.05$), the activity at the 6 h was the lowest, and there was no significant difference from the initial value ($P > 0.05$). The T-AOC in the 100 mg/L group

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TABLE 2 Experimental results of acute lethal effect of chlorine dioxide on *C. sinensis*.

Treatment	Chlorine dioxide concentration (mg/L)	Corresponding residual chlorine concentration (mg/L)	Mean mortality at different times of stress (%)			
			24 h	48 h	72 h	96 h
C_0	**0**	0	0.00	0.00	0.00	0.00
C_1	**50**	0.03 ± 0.06	0.00	0.00	0.00	10.00
C_2	**100**	0.08 ± 0.16	0.00	3.33	0.00	16.67
C_3	**150**	0.13 ± 0.22	0.00	3.33	6.67	26.67
C_4	**200**	0.20 ± 0.81	0.00	6.67	3.33	33.33
C_5	**250**	0.71 ± 0.43	0.00	10.00	10.00	56.67
C_6	**300**	1.52 ± 0.35	0.00	10.00	16.67	66.67
C_7	**350**	2.33 ± 0.19	0.00	13.33	36.67	73.33
C_8	**400**	2.94 ± 0.31	3.33	26.67	63.33	100
C_9	**450**	3.57 ± 0.21	6.67	33.33	76.67	100
C_{10}	**500**	6.81 ± 0.37	6.67	23.33	83.33	100

reached its maximum value at 48 h and was significantly different from that at 0 h ($P < 0.05$), while it was lowest at 6 h and 96 h with no significant difference from the initial value ($P > 0.05$). Compared with the other two groups, the maximum T-AOC took a shorter time in the 100 mg/L group than that in the other two groups.

The SOD enzyme activity of all three concentration groups showed an increasing trend from 0 to 12 h, after which the SOD enzyme activity started to decrease. The SOD enzyme activity of the 20 mg/L group showed an overall increasing trend, and started to decrease at 24 h, and the enzyme activity was still higher than the initial value, but was not significantly different from the initial value ($P > 0.05$), and started to increase again at 48 h. At 96 h, the SOD enzyme activity decreased significantly ($P < 0.05$) and reached the lowest value. The SOD enzyme activity in the 50 mg/L group reached its maximum value at 12 h and was significantly different from the initial value ($P < 0.05$), then the enzyme activity decreased slightly, but was still higher than the initial value and significantly different from the initial value ($P < 0.05$) and the lowest value was reached at 6 h, which was not significantly different from the initial value ($P > 0.05$).

The effect of residual chlorine stress on ACP activity in the hepatopancreas of *C. sinensis* showed a general trend of increasing and then decreasing, as seen in Figure 3A. In the 20 mg/L group, the ACP activity showed fluctuating changes, reaching two peaks at 6 h and 48 h respectively, with a maximum value at 48 h, which was significantly different from the initial value ($P < 0.05$), and the lowest ACP activity at 96 h, which was lower than the initial value, but not significantly different from the initial value ($P > 0.05$). The ACP activity in the 50 mg/L group showed a trend of increasing and then decreasing, and reached a maximum value at 48 h, which was significantly different from the initial value ($P < 0.05$), and a minimum value at 96 h, which was lower than the initial value, but not significantly different from the initial value ($P > 0.05$). The ACP activity in the 100 mg/L group showed a tendency to increase and then decrease, and reached a maximum value at 6 h, which was significantly different from the initial value ($P < 0.05$), and a minimum value at 72 h, which was lower than the initial value but not significantly different from the initial value ($P > 0.05$).

As seen in Figure 3B, the effect of residual chlorine stress on AKP activity in the hepatopancreas of *C. sinensis* showed fluctuating changes. The AKP activity in the 20 mg/L group reached its maximum value at 12 h and was significantly different from the initial value ($P < 0.05$), and reached its lowest value at 48 h and was lower than the initial value and the difference was significant compared with the initial value ($P < 0.05$). The AKP enzyme activity in the 100 mg/L group also showed fluctuating changes, but the enzyme activity was lower than that of the control group in all period of time and was significantly different from that of the control group ($P < 0.05$). The lowest value was found at 72 h.

The effect of residual chlorine stress on LZM enzyme activity in the hepatopancreas of *C. sinensis* showed fluctuating changes as can be seen in Figure 3C. The LZM activity in the 20 mg/L group showed fluctuating changes, reaching a maximum value at 12 h with no significant difference from the initial value ($P > 0.05$) and a minimum value at 48 h with significant difference ($P < 0.05$). The LZM activity in the 100 mg/L group was lower than the initial value after 0 h and was significantly different from the initial value ($P < 0.05$), with the lowest value occurring at 72 h.

3.3 Assembly, function enrichment analysis and identification of differentially expressed genes

In this study, the H-0 group was used as the control, and the number of differential genes in H-96a, H-96b and H-96c groups were counted (Figures 4, 5). The ILLUMINA data were submitted to the National Center for Biotechnology Information (NCBI, PRJNA904083). The results showed that compared with the control group, 13, 17 and 16 unigenes were up-regulated and 23, 34 and 13 unigenes were down-regulated in group H-96a, H-96b and H-96c, respectively. Compared with group H-96a at 96 h of residual chlorine stress, 19 and 23 unigenes were up-regulated and 17 and 16 unigenes were down-regulated in group H-96b and group H-96c, respectively. Finally, 23 and 27 unigenes were up-regulated and down-regulated in

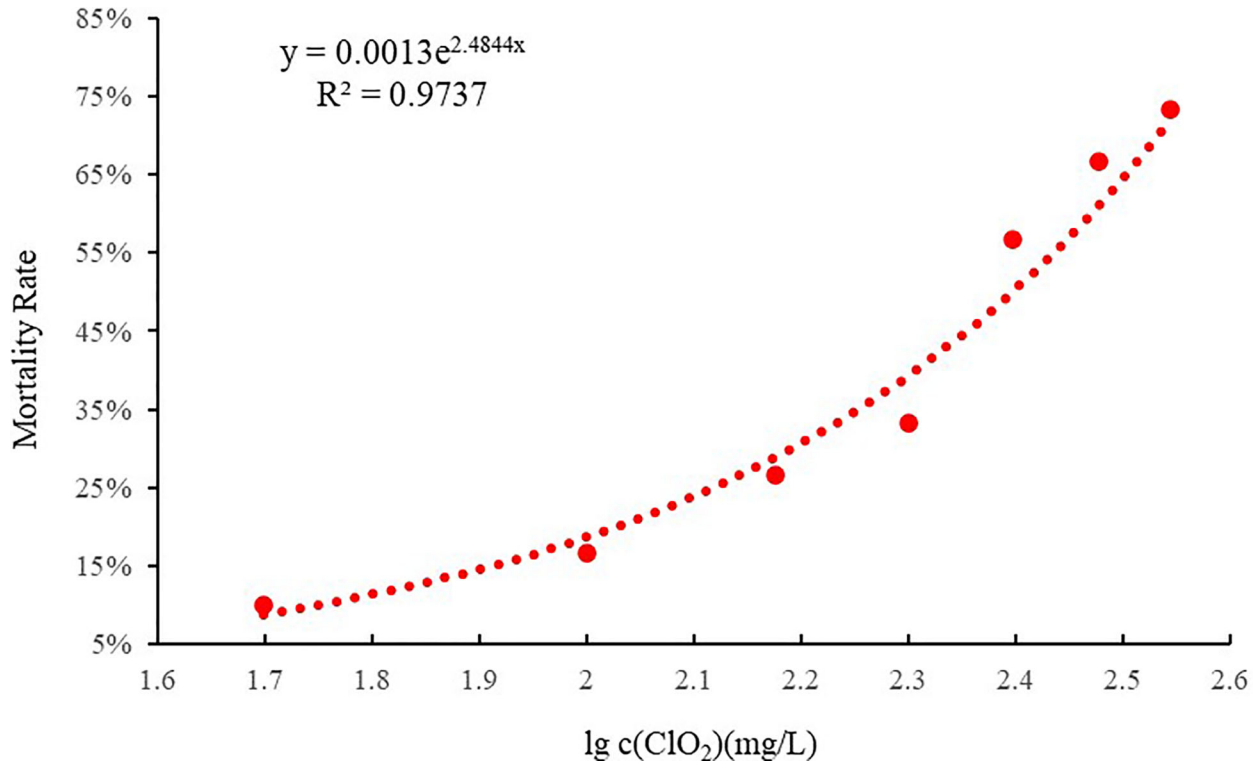

FIGURE 1
The Concentration - Mortality curve of chlorine dioxide to *C. sinensis* at 96 h (R^2 = 0.9737).

group H-96c, respectively, compared with group H-96b at 96 h. Most of the genes in groups H-96a, H-96b, and H-96c were down-regulated compared with the control group, and most of the genes in groups H-96a, H-96b, and H-96c were up-regulated compared with each other.

To analyse the functions of these DGEs, GO assignments were made. These DGEs were assigned to major GO categories (level 3), i.e., biological process, cellular component, and molecular function (Figure 6). KO annotation of transcriptome results classified differentially expressed genes (referred to as differential genes) into 5 categories based on metabolic pathways categories, which belong to Metabolism, Organismal systems, Cellular Processes, Genetic information processing, and Environmental information processing. The annotation of the number of differential genes is shown in Figure 7: In KEGG annotation, the most single gene pathways among groups were: cofactors in metabolic pathways and signal transduction in vitamin metabolism and environmental information processing; Transport and catabolism in cellular processes and Signal transduction in environmental information processing.

3.4 Key genes involved in immunity and antioxidation response to residual chlorine stress in *C. sinensis*

In the present study, DEGs associated with immune defence were divided into 5 groups (Table 3). There were 31 genes associated with signal transduction, the most of the 5 groups, followed by other immune molecules (10), pattern recognition proteins/receptors (8), apoptosis (8), antimicrobial peptides (8) and genes associated with the pre-phenol oxidase cascade (3).

Compared with the control group H-0, the expression levels of genes in H-96a related to signal transduction, such as caspase 9 (*CASP9*), HRAS-like suppressor 3, Thrombospondin-4, Dual oxidase 2-like isoform X2, C-terminal-binding protein-like isoform X1 were significantly up-regulated, while the expression levels of phospholipid phosphatase 3-like, pantetheinase-like, Follistatin, ADP-ribosylation factor-like and other genes were significantly down-regulated. In addition, residual chlorine at group H-96a down-regulated the expression levels of genes in the C-Type Lectin family of pattern recognition proteins/receptors, such as C-type lectin domain family member isoform crab 3, C-type lectin domain family 6 member A, and genes in the prophenoloxidase cascade system, such as serine protease, Kazal-type serine protease genes. The expression levels of Baculoviral IAP repeat-containing protein 7-B were significantly upregulated in genes related to apoptosis, while the expression levels of cathepsin L, inhibitor of apoptosis protein-like were significantly downregulated. In addition, the expression levels of pantetheinase-like, deleted in malignant brain tumors 1 protein-like, gelsolin-like protein 2 and legumain-like were also altered in the concentration treatment group compared to the control group.

Compared to the control group, the expression levels of genes related to signal transduction such as guanylate cyclase soluble subunit beta-2-like, MMP-19 and mitogen-activated protein kinase/p38 were significantly up-regulated in the H-96b group, while the expression levels of phospholipid phosphatase 3-like, ADP-ribosylation factor-like, Protocadherin Fat 4, and fatty acid synthase were significantly down-regulated. At the same time, the expression

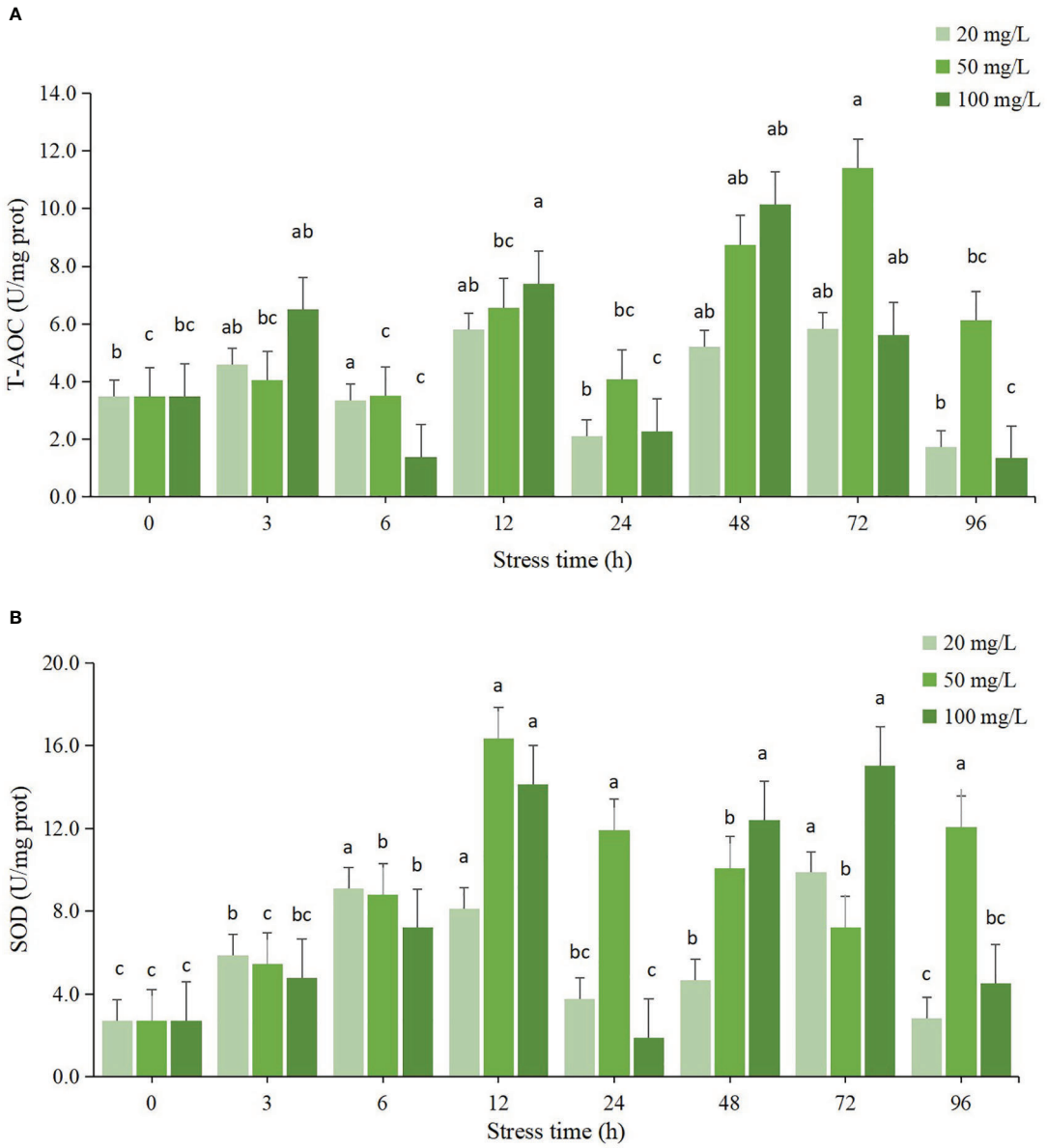

FIGURE 2
Changes of antioxidant enzyme activities in hepatopancreas of *C sinensis* at different time points after different residual chlorine stress. **(A)** T-AOC activitiy, **(B)** SOD activitiy. Different letters indicate statistically significant differences between different time points for the same treatment using one-way ANOVA and Tukey's comparison of the means test ($P < 0.05$).

levels of leptin precursor 4, C-type lectin domain family 6 member A and C-type lectin were significantly down-regulated in genes related to pattern recognition proteins/receptors (*PRPs*), while the expression levels of mitogen-activated protein kinase/p38 were significantly up-regulated. The expression levels of mitogen-activated protein kinase/p38 were significantly up-regulated. The expression of cathepsin L, Bcl-2 like 2 protein and baculoviral IAP repeat-containing protein 5-like, which are genes related to apoptosis, were all down-regulated. In addition, the expression levels of genes such as guanylate cyclase soluble subunit beta-2-like and Full=G2/mitotic-specific cyclin-B were also altered in the b concentration treatment group.

The expression levels of signal transduction-related protein crumbs homolog 1-like and MASP-related molecule Type 1 genes were significantly down-regulated in group H-96c compared to the H-0, while in the phenol oxidase progenitor system, the expression levels of serine/threonine-protein kinase TBK1 like isoform X1 were significantly up-regulated. Meanwhile, the expression levels of inhibitor of apoptosis 1, a gene related to apoptosis, were significantly up-regulated, while the expression levels of C-type lectin, electin precursor 4 and toll-like receptor 13, a gene related to pattern recognition protein/receptor, were significantly down-regulated. In addition, the c-treatment group also caused deleted in malignant brain tumors 1 protein-like, proteasome activator complex subunit 3-like, Full=G2/mitotic-specific cyclin-B and NRAMP gene expression levels were altered.

Meanwhile, several DEGs are involved in various processes of animal antioxidant mechanisms in the hepatopancreas (Table 4). Compared with the H-0 treated group, the expression levels of omega

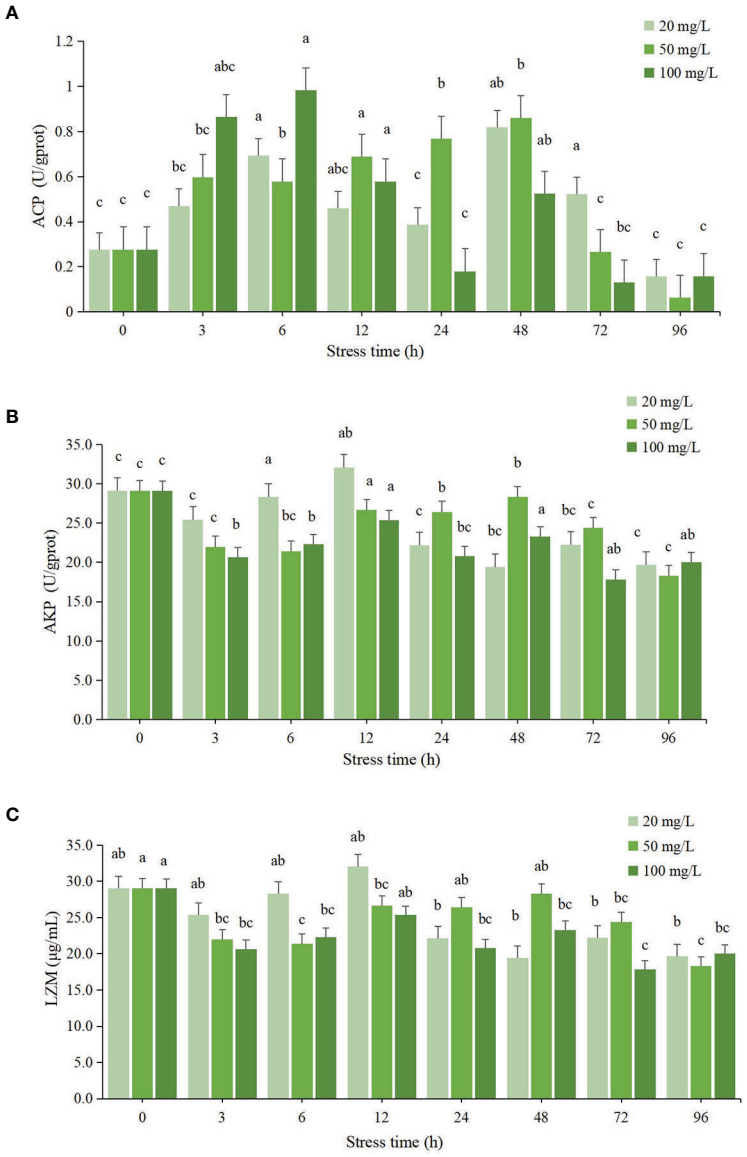

FIGURE 3
Changes of immune enzyme activities in hepatopancreas of *C sinensis* at different time points after different residual chlorine stress. **(A)** ACP activitiy, **(B)** AKP activitiy and **(C)** LZM activitiy. Different letters indicate statistically significant differences between different time points for the same treatment using one-way ANOVA and Tukey's comparison of the means test ($P < 0.05$).

glutathione S-transferase, glutathione S-transferase and mu class glutathione S-transferase genes associated with antioxidant were significantly up-regulated in the H-96a group, while the expression levels of Thioredoxin-like fold genes were significantly down-regulated in the H-96a group. The expression levels of alanine—glyoxylate aminotransferase 2, pi-class glutathione S-transferase, and thioredoxin genes were significantly up-regulated in the H-96b group, while the expression levels of genes such as ribosyldihydronicotinamide dehydrogenase, thioredoxin-like fold, and asparagine synthetase were significantly downregulated. The expression levels of omega glutathione S-transferase, pi-class glutathione S-transferase, and pi-class glutathione S-transferase were significantly up-regulated in the H-96c group, while the expression levels of Thioredoxin-like fold and peroxiredoxin V genes were significantly down-regulated. As a response to environmental stress, chaperone genes in the organism showed significant variation at the transcriptional or translational level. In the present study, the expression levels of heat shock protein were significantly up-regulated in group H-96a compared to the H-0; in group H-96c, the expression levels of heat shock protein 70 and heat shock protein were significantly up-regulated, while the expression levels of heat shock protein 60 gene were significantly down-regulated. In addition, residual chlorine stress also altered the expression levels of metal-binding proteins, including heavy metal-binding protein HIP-like, metallothionein and ferritin subunit.

3.5 Validation of gene expression by qPCR

The transcriptome results were verified by qPCR (Figure 8), and selected 10 immune and antioxidant related genes (5 up-regulated and 5 down-regulated) randomly. The verification results were

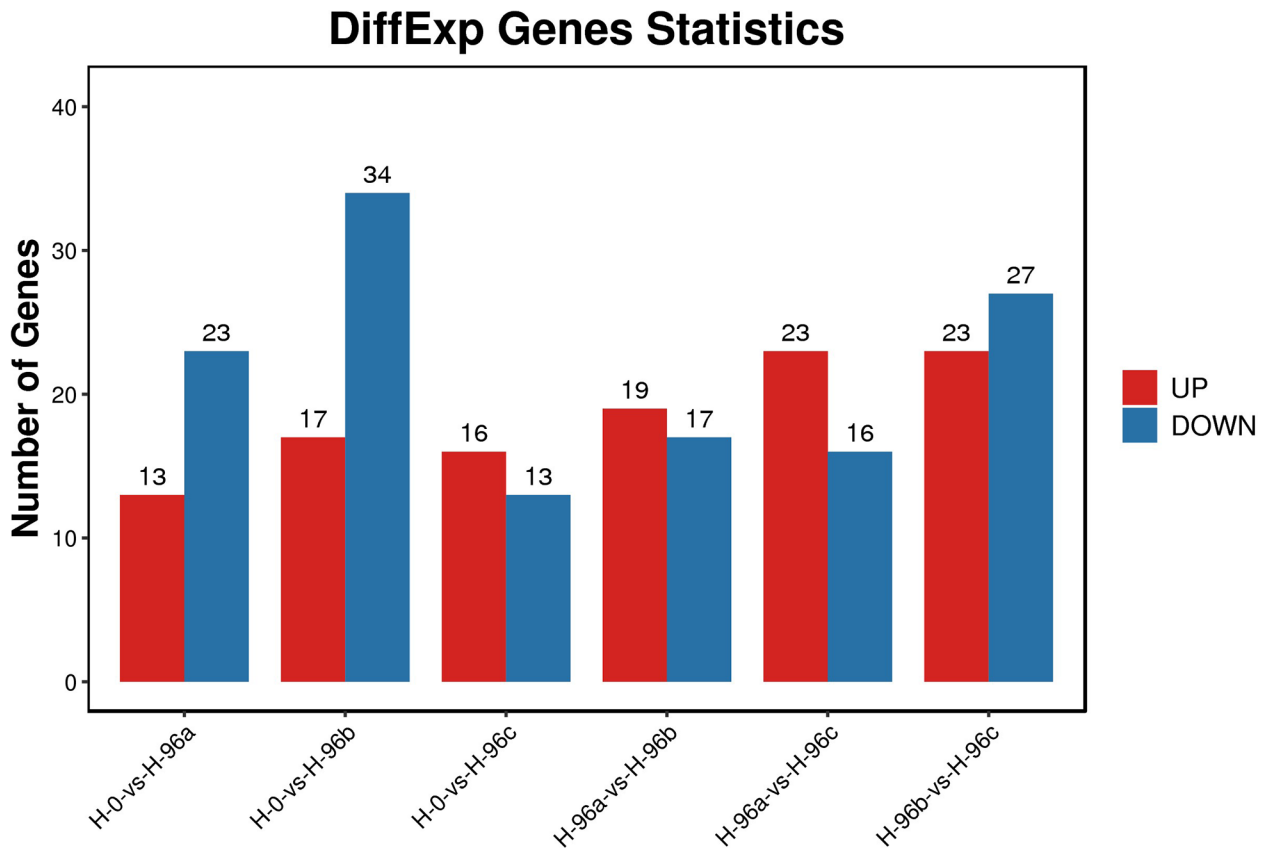

FIGURE 4
Statistical chart of DEGs. H-0: Control group; H-96a: concentration of 20 mg/L group; H-96b: concentration of 50 mg/L group; H-96c: concentration of 100 mg/L group. Up- and down-regulated genes are shown in red and blue, respectively.

consistent with the sequencing results, indicating that the transcriptome sequencing results were authentic and reliable.

4 Discussion

4.1 Effects of acute residual chlorine on the mortality of *C. sinensis*

The mortality of aquatic organisms can be affected by changes in the water environment and chemical stimuli, such as temperature (Casas et al., 2020; Syafaat et al., 2021), salinity (Nie et al., 2018; Zhang Y et al., 2020), pH (Swezey et al., 2020; Liew et al., 2022), and feed (Willer and Aldridge, 2019; Mohan et al., 2019). Chlorine and compounds in water can undergo complex chemical reactions to synthesise a range of compounds under different conditions, such as the chloramine family of compounds. Electrolytic chlorine for the disinfection of cooling water in nuclear power plants is usually free chlorine, which is unstable in its state and very easily dissipated in seawater (Venkatnarayanan et al., 2021). The main sources of chlorine for production activities are several common disinfectants such as bleach, chlorine dioxide and trichloroisocyanuric acid. Also, during the shellfish culture and nursery phase, the cleaning and disinfection of various types of equipment and ponds inevitably involves the use of disinfectants such as bleach and chlorine dioxide, in which residual chlorine is inevitably produced.

The LC_{50} is an important index to measure the toxin tolerance of an organism. This study calculated the 96-h LC_{50} of juvenile *C. sinensis* to chlorine dioxide exposure, based on linear regression method ($y = 0.0013e^{2.4844x}$, $R^2 = 0.9737$, where y = % mortality and x = lg c (ClO_2)) was 217.6 mg/L. The results of the present studies were similar to those reported in other aquatic organisms. The LC_{50} of residual chlorine (Cl^-) in STEL water for brood-stock and 2-mo-old shrimp were 2.3 and 3.2 ppm, respectively (Park et al., 2004). The Manila clam *Ruditapes philippinarum* exhibited strong tolerance to hypoxia as the 20-day LC_{50} for dissolved oxygen (DO) was estimated to be 0.57 mg L^{-1} (Li et al., 2019). The 96-h LC_{50} at 22 and 26°C values was 28.591 and 11.761 mg/L, respectively, for $NiCl_2$ exposure in the abalone (Min et al., 2015).

Studies on the effects of residual chlorine on mussels showed that the time of death of mussels at the same concentration depended on the size of the mussel, with smaller mussels taking less time to reach 100% mortality than larger mussels. In addition, the death time of mussels with the same size depends on the residual chlorine concentration, with higher concentrations of residual chlorine taking less time to reach 100% mortality than lower concentrations (Zhang et al., 2000; Masilamoni et al., 2002). In the present study, the mortality of bay scallop (*Argopectens irradias*) parents (Chen, 2009) and yellow clam (*Corbicula fluminea*) (Zou and Cheng, 2003) showed a gradual increase in mortality with increasing chlorine dioxide concentration in the water column and the extension of soaking time. In this study, chlorine dioxide concentration and duration of stress directly affected the survival and growth of first instar clams,

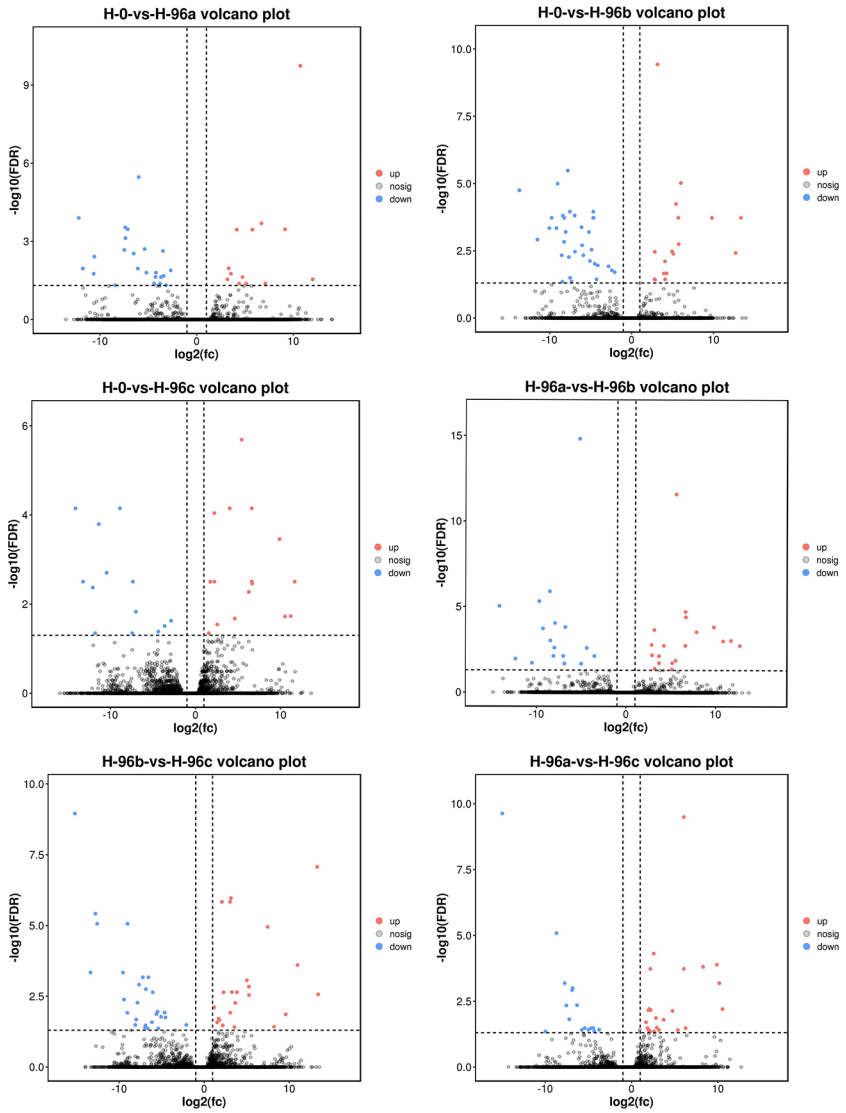

FIGURE 5
Difference comparison volcano map. H-0: Control group; H-96a: concentration of 20 mg/L group; H-96b: concentration of 50 mg/L group; H-96c: concentration of 100 mg/L group. Up- and down-regulated genes are shown in red and blue, respectively.

and the mortality rate of clams increased with increasing chlorine dioxide concentration and duration of stress.

4.2 Effects of residual chlorine on physiological metabolism of *C. sinensis*

The living environment of the *C. sinensis* is near the estuary of the freshwater river, and the outlet of the warm water drainage of the coastal nuclear power plant is one of the main habitats of *C. sinensis*. When faced with changes in the external environment, the immune system of the clam undergoes stress behaviour, resulting in the production of large amounts of reactive oxygen species (ROS), and the sharp increase in ROS places a great burden on the organism, generating oxidative stress and causing a decrease in immune function (Liang et al., 2016). The antioxidant system consists of antioxidant enzymes (Lian et al., 2019; Dong et al., 2022; Holen et al., 2022) (e.g., SOD, CAT, T-AOC), which have a strong detoxifying effect on ROS. The activity of antioxidant enzymes also reflects the physiological state of aquatic organisms in response to environmental changes to a certain extent. The immune system of shellfish consists of immune-related enzymes (e.g., ACP, AKP, LZM) and changes in the activity of immune-related enzymes also show changes in immune function.

T-AOC reflects the total antioxidant capacity of organisms. In this study, it can be seen that the T-AOC in the three concentration groups tended to increase significantly at the beginning of the experiment, with the 20 mg/L and 50 mg/L groups reaching their maximum values at 72 h and the 100 mg/L group reaching its maximum value at 48 h. The T-AOC activity of the three concentration groups remained at a higher level at 96 h and was significantly different compared to the initial value ($P < 0.05$). It is speculated that high concentration of chlorine stimulates the stress response of *C. sinensis* to produce a large number of ROS. In order to prevent its own damage, the body improves the enzymatic reaction, secretes a large amount of SOD and antioxidant-related enzymes, and improves the total antioxidant capacity. The changes in SOD enzyme

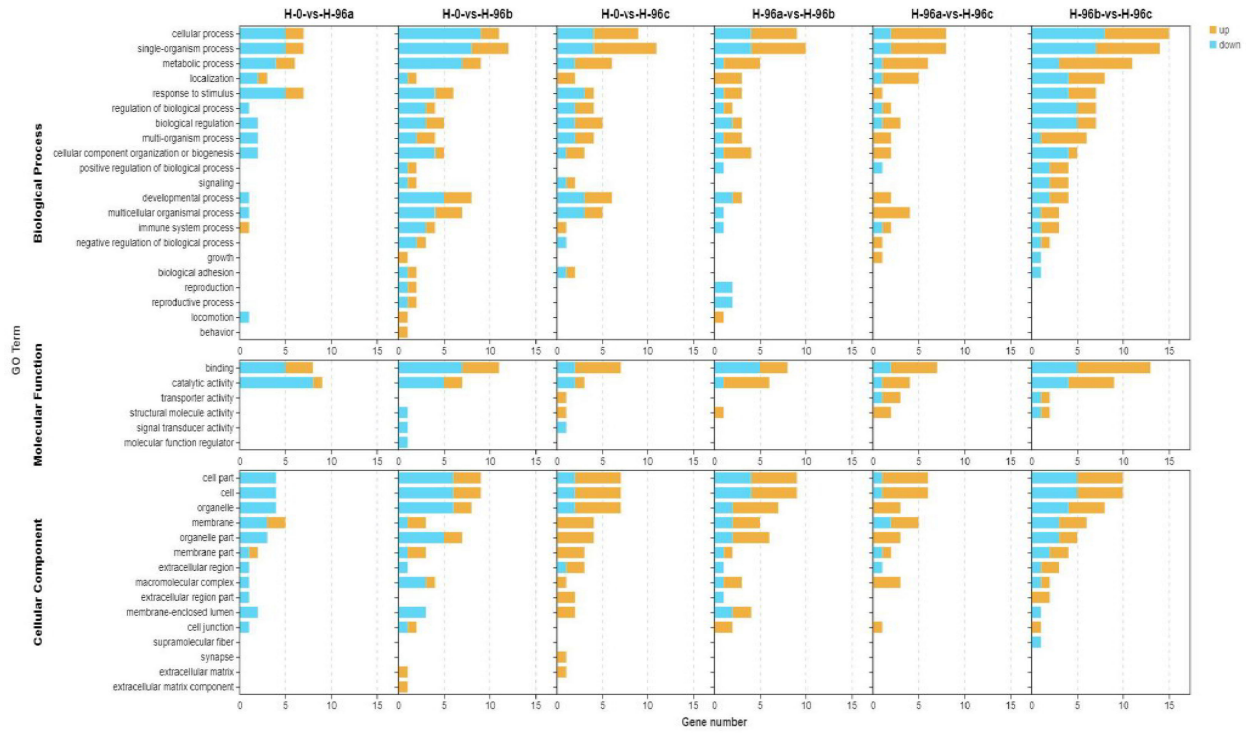

FIGURE 6
GO enrichment analyses of differentially expressed genes (DEGs) in hemolymph transcriptome. H-0: Control group; H-96a: concentration of 20 mg/L group; H-96b: concentration of 50 mg/L group; H-96c: concentration of 100 mg/L group.

activity were the same as those in *T*-AOC, showing a trend of increasing, then decreasing and then increasing. The SOD activity of all three concentration groups reached its maximum at 12 h, and the activity remained at a high level afterwards, which was significantly different from that of the control group ($P < 0.05$). This result was also consistent with the results of T-AOC.

ACP and AKP are two common immune-related enzymes, both of which are involved in the detoxification process of the organism

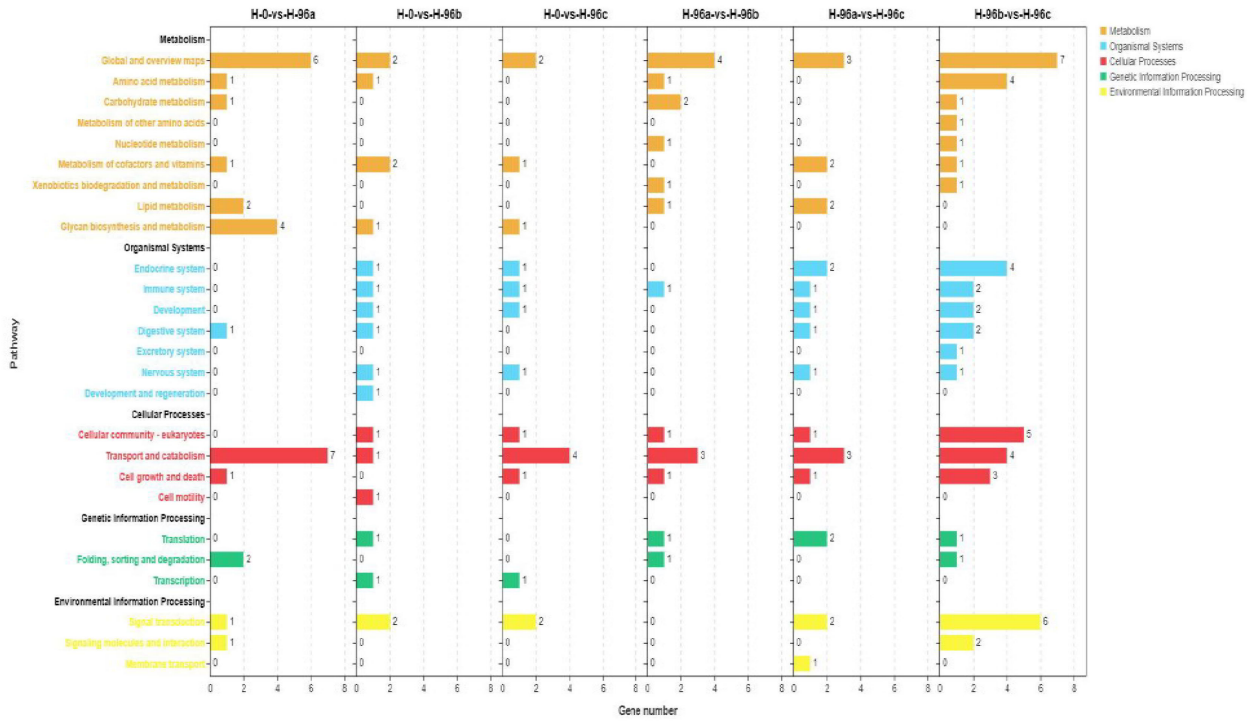

FIGURE 7
KEGG enrichment analyses of differentially expressed genes (DEGs) in hemolymph transcriptome. H-0: Control group; H-96a: concentration of 20 mg/L group; H-96b: concentration of 50 mg/L group; H-96c: concentration of 100 mg/L group.

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TABLE 3 Summary of differential expressed genes related to immune in transcriptome of *C. sinensis*.

Functional category	ID	Treatment	Fullname	log2(fc)	PValue
Prophenoloxidase cascade					
	evm.TU.Hic_asm_14.1340	H-0 vs H-96a	serine protease	-11.499	2.35E-04
	evm.TU.Hic_asm_14.1339	H-0 vs H-96a	Kazal-type serine protease	-2.517	2.48E-04
	evm.TU.Hic_asm_12.1545	H-0 vs H-96c	serine/threonine-protein kinase TBK1-like isoform X1	1.351	3.67E-02
Pattern recognition proteins/receptors					
	MSTRG.3775	H-0 vs H-96a	C-type lectin domain family member isoform crab 3	-1.133	2.66E-02
	MSTRG.16577	H-0 vs H-96a	C-type lectin 5	-1.935	6.99E-03
	MSTRG.14827	H-0 vs H-96c	C-type lectin	-5.457	4.45E-02
	evm.TU.Hic_asm_5.816	H-0 vs H-96a	lectin precursor 4	-5.535	1.30E-02
	evm.TU.Hic_asm_5.430	H-0 vs H-96a	C-type lectin	-7.141	1.02E-07
	evm.TU.Hic_asm_5.382	H-0 vs H-96b	lectin precursor 4	-6.244	5.81E-04
	evm.TU.Hic_asm_5.382	H-0 vs H-96c	lectin precursor 4	-5.115	4.31E-03
	evm.TU.Hic_asm_3.81	H-0 vs H-96a	C-type lectin domain family 6 member A	-1.665	4.55E-02
	evm.TU.Hic_asm_3.302	H-0 vs H-96b	C-type lectin domain family 6 member A	-3.322	2.56E-02
	evm.TU.Hic_asm_15.1667	H-0 vs H-96a	perlucin-like protein isoform X2	-3.786	3.78E-02
	evm.TU.Hic_asm_13.1924	H-0 vs H-96b	C-type lectin	-2.797	7.25E-03
	evm.TU.Hic_asm_12.301	H-0 vs H-96b	mitogen-activated protein kinase/p38	1.101	5.26E-02
	evm.TU.Hic_asm_11.26	H-0 vs H-96c	toll-like receptor 13	-7.409	2.07E-02
Signal transduction					
	MSTRG.7549	H-0 vs H-96a	phospholipid phosphatase 3-like	-2.040	7.06E-03
	MSTRG.7549	H-0 vs H-96b	phospholipid phosphatase 3-like	-2.598	9.09E-04
	MSTRG.7457	H-0 vs H-96c	protein crumbs homolog 1-like	-2.045	4.65E-02
	MSTRG.25534	H-0 vs H-96a	tumor necrosis factor ligand superfamily member 6-like	-2.037	3.45E-02
	MSTRG.19434	H-0 vs H-96a	pantetheinase-like	-2.024	1.34E-02
	MSTRG.1925	H-0 vs H-96a	caspase 9, partial	1.632	3.55E-02
	evm.TU.Hic_asm_8.972	H-0 vs H-96b	low-density lipoprotein receptor-related protein 1-like, partial	1.444	4.88E-02
	evm.TU.Hic_asm_7.39	H-0 vs H-96a	HRAS-like suppressor 3	3.107	8.80E-03
	evm.TU.Hic_asm_6.906	H-0 vs H-96b	guanylate cyclase soluble subunit beta-2-like	1.464	1.06E-02
	evm.TU.Hic_asm_3.1133	H-0 vs H-96b	MMP-19	1.587	2.75E-02
	evm.TU.Hic_asm_2.761	H-0 vs H-96a	Thrombospondin-4	1.643	1.75E-02
	evm.TU.Hic_asm_17.1590	H-0 vs H-96b	solute carrier family 2 facilitated glucose transporter member 1 protein	-1.298	4.04E-02
	evm.TU.Hic_asm_16.997	H-0 vs H-96a	Follistatin	-3.195	6.00E-03
	evm.TU.Hic_asm_16.161	H-0 vs H-96a	dual oxidase 2-like isoform X2	1.163	3.23E-02
	evm.TU.Hic_asm_15.13	H-0 vs H-96a	C-terminal-binding protein-like isoform X1	1.174	4.53E-02
	evm.TU.Hic_asm_12.301	H-0 vs H-96b	mitogen-activated protein kinase/p38	1.101	5.26E-02
	evm.TU.Hic_asm_12.1630	H-0 vs H-96c	MASP-related molecule Type 1	-3.027	7.05E-03
	evm.TU.Hic_asm_12.1627	H-0 vs H-96a	chymotrypsin-like protease	2.105	3.06E-02
	evm.TU.Hic_asm_12.127	H-0 vs H-96a	gelsolin-like protein 2	1.164	2.93E-02
	evm.TU.Hic_asm_11.1497	H-0 vs H-96a	serine palmitoyltransferase 1-like	1.463	1.84E-02
	evm.TU.Hic_asm_10.963	H-0 vs H-96a	probable 4-coumarate–CoA ligase 5	2.128	3.41E-02

(Continued)

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TABLE 3 Continued

Functional category	ID	Treatment	Fullname	log2(fc)	PValue
	evm.TU.Hic_asm_10.2188	H-0 vs H-96a	ADP-ribosylation factor-like	-3.022	1.01E-03
	evm.TU.Hic_asm_10.2172	H-0 vs H-96a	ADP-ribosylation factor-like	-3.060	6.31E-04
	evm.TU.Hic_asm_10.2172	H-0 vs H-96b	ADP-ribosylation factor-like	-3.367	2.21E-03
	evm.TU.Hic_asm_10.1800	H-0 vs H-96a	Collagen alpha-4(VI) chain	1.723	1.74E-03
	evm.TU.Hic_asm_10.1800	H-0 vs H-96b	Collagen alpha-4(VI) chain	1.202	2.55E-03
	evm.TU.Hic_asm_10.1718	H-0 vs H-96a	legumain-like	-1.174	3.66E-02
	evm.TU.Hic_asm_1.865	H-0 vs H-96b	angiotensin-converting enzyme-like isoform X4	-2.578	1.28E-02
	evm.TU.Hic_asm_0.82	H-0 vs H-96b	Protocadherin Fat 4	-3.026	1.19E-02
	evm.TU.Hic_asm_0.599	H-0 vs H-96b	acyl-CoA desaturase isoform X2	-1.919	4.28E-03
	evm.TU.Hic_asm_0.560	H-0 vs H-96b	fatty acid synthase	-3.889	2.62E-03
	evm.TU.Hic_asm_0.1830	H-0 vs H-96b	protocadherin-like wing polarity protein stan	-6.038	2.17E-06
	evm.TU.Hic_asm_0.1428	H-0 vs H-96a	lethal(2) giant larvae protein homolog 1-like	1.407	3.61E-02
	evm.TU.Hic_asm_0.1423	H-0 vs H-96a	peptidyl prolyl cis-trans isomerase A (II)	-3.253	2.00E-02
	evm.TU.Hic_asm_0.1347	H-0 vs H-96a	calcium-activated potassium channel slowpoke-like isoform X6	4.051	1.60E-03
Apoptosis					
	MSTRG.6005	H-0 vs H-96c	inhibitor of apoptosis 1	2.463	1.33E-03
	MSTRG.29423	H-0 vs H-96a	cathepsin L	-2.101	3.18E-02
	MSTRG.29423	H-0 vs H-96b	cathepsin L	-2.396	1.01E-02
	MSTRG.1925	H-0 vs H-96a	caspase 9, partial	1.632	3.55E-02
	MSTRG.1925	H-0 vs H-96b	caspase 9, partial	1.620	5.04E-03
	MSTRG.13361	H-0 vs H-96a	inhibitor of apoptosis 2, partial	-1.881	2.32E-02
	evm.TU.Hic_asm_9.458	H-0 vs H-96a	cathepsin L	-1.550	4.39E-03
	evm.TU.Hic_asm_9.458	H-0 vs H-96b	cathepsin L	-1.698	1.75E-02
	evm.TU.Hic_asm_6.810	H-0 vs H-96a	Baculoviral IAP repeat-containing protein 7-B	10.728	7.75E-15
	evm.TU.Hic_asm_16.1565	H-0 vs H-96b	Bcl-2 like 2 protein	-1.251	2.23E-02
	evm.TU.Hic_asm_11.2315	H-0 vs H-96a	inhibitor of apoptosis protein-like	-11.978	3.72E-02
	evm.TU.Hic_asm_0.1758	H-0 vs H-96b	baculoviral IAP repeat-containing protein 5-like	-1.954	3.86E-02
Other immune molecules					
	MSTRG.19434	H-0 vs H-96a	pantetheinase-like	-2.024	1.34E-02
	evm.TU.Hic_asm_6.906	H-0 vs H-96b	guanylate cyclase soluble subunit beta-2-like	1.464	1.06E-02
	evm.TU.Hic_asm_5.1466	H-0 vs H-96a	deleted in malignant brain tumors 1 protein-like	1.390	4.98E-02
	evm.TU.Hic_asm_5.1466	H-0 vs H-96c	deleted in malignant brain tumors 1 protein-like	1.568	5.55E-05
	evm.TU.Hic_asm_3.513	H-0 vs H-96c	proteasome activator complex subunit 3-like	-2.384	1.68E-02
	evm.TU.Hic_asm_17.964	H-0 vs H-96b	b(0,+)-type amino acid transporter 1-like isoform X3	-1.587	2.04E-02
	evm.TU.Hic_asm_16.193	H-0 vs H-96b	Full=G2/mitotic-specific cyclin-B	-1.844	3.02E-02
	evm.TU.Hic_asm_16.193	H-0 vs H-96c	Full=G2/mitotic-specific cyclin-B	-3.579	6.28E-04
	evm.TU.Hic_asm_15.1378	H-0 vs H-96b	uracil-DNA glycosylase-like	-2.100	2.23E-02
	evm.TU.Hic_asm_12.127	H-0 vs H-96a	gelsolin-like protein 2	1.164	2.93E-02
	evm.TU.Hic_asm_10.1718	H-0 vs H-96a	legumain-like	-1.174	3.66E-02
	evm.TU.Hic_asm_1.1054	H-0 vs H-96c	NRAMP	1.415	7.18E-04

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TABLE 4 Summary of differential and non-differentially expressed genes related to antioxidative capability in transcriptome of *C. sinensis*.

Functional category	ID	Treatment	Fullname	log2(fc)	PValue
Nrf2 signalling pathway					
	evm.TU.Hic_asm_14.463	H-0 vs H-96a	mitogen-activated protein kinase kinase kinase 7-like isoform X2	1.1162	6.65E-02
	evm.TU.Hic_asm_12.301	H-0 vs H-96a	mitogen-activated protein kinase/p38	1.1641	8.56E-02
	evm.TU.Hic_asm_11.1866	H-0 vs H-96b	kelch-like protein 30	4.1859	2.79E-02
Antioxidant					
	MSTRG.11302	H-0 vs H-96b	ribosyldihydronicotinamide dehydrogenase	-1.6087	4.83E-02
	evm.TU.Hic_asm_18.94	H-0 vs H-96a	Thioredoxin-like fold	-1.7154	1.90E-03
	evm.TU.Hic_asm_18.94	H-0 vs H-96b	Thioredoxin-like fold	-2.0399	1.80E-02
	evm.TU.Hic_asm_18.94	H-0 vs H-96c	Thioredoxin-like fold	-1.5592	4.66E-02
	evm.TU.Hic_asm_18.402	H-0 vs H-96c	glutathione S-transferase theta-1-like	0.9051	1.55E-02
	evm.TU.Hic_asm_18.377	H-0 vs H-96b	asparagine synthetase	-1.2806	4.34E-02
	evm.TU.Hic_asm_17.1421	H-0 vs H-96a	omega glutathione S-transferase	0.9259	6.86E-02
	evm.TU.Hic_asm_17.1421	H-0 vs H-96c	omega glutathione S-transferase	0.5777	4.31E-02
	evm.TU.Hic_asm_17.122_	H-0 vs H-96b	alanine–glyoxylate aminotransferase 2	1.3636	1.09E-02
	evm.TU.Hic_asm_17.1077	H-0 vs H-96a	omega glutathione S-transferase	1.9723	6.10E-02
	evm.TU.Hic_asm_14.810	H-0 vs H-96b	pi-class glutathione S-transferase	-1.3021	2.20E-02
	evm.TU.Hic_asm_14.808	H-0 vs H-96b	pi-class glutathione S-transferase	1.5357	3.00E-02
	evm.TU.Hic_asm_14.808	H-0 vs H-96c	pi-class glutathione S-transferase	1.5711	1.30E-03
	evm.TU.Hic_asm_12.1240	H-0 vs H-96a	glutathione S-transferase	2.5167	7.41E-03
	evm.TU.Hic_asm_12.1240	H-0 vs H-96c	glutathione S-transferase	2.1996	1.16E-03
	evm.TU.Hic_asm_12.1238	H-0 vs H-96a	mu class glutathione S-transferase	2.3959	2.43E-02
	evm.TU.Hic_asm_12.1235	H-0 vs H-96c	glutathione S-transferase	1.5995	1.65E-03
	evm.TU.Hic_asm_11.1942	H-0 vs H-96b	thioredoxin	3.5194	2.48E-02
	evm.TU.Hic_asm_10.1023	H-0 vs H-96c	peroxiredoxin V	-1.9552	1.14E-02
	evm.TU.Hic_asm_0.2822	H-0 vs H-96b	glutamate dehydrogenase	-1.8303	1.84E-02
Chaperones					
	evm.TU.Hic_asm_13.1719	H-0 vs H-96a	heat shock protein	1.0017	5.14E-02
	evm.TU.Hic_asm_4.634	H-0 vs H-96c	heat shock protein 70	1.8990	3.67E-03
	evm.TU.Hic_asm_16.191	H-0 vs H-96c	heat shock protein 70	3.7798	1.69E-04
	evm.TU.Hic_asm_13.392	H-0 vs H-96c	heat shock protein 70	2.4537	8.60E-03
	evm.TU.Hic_asm_13.1719	H-0 vs H-96c	heat shock protein	0.7215	1.07E-02
	evm.TU.Hic_asm_10.736	H-0 vs H-96c	heat shock protein 70	2.2761	5.50E-03
	evm.TU.Hic_asm_7.742	H-0 vs H-96c	heat shock protein 60	-1.7482	2.89E-02
Metal-binding protein					
	evm.TU.Hic_asm_5.725	H-0 vs H-96a	heavy metal-binding protein HIP-like	-4.2672	2.45E-05
	evm.TU.Hic_asm_5.725	H-0 vs H-96b	heavy metal-binding protein HIP-like	-1.8680	1.89E-01
	evm.TU.Hic_asm_5.725	H-0 vs H-96c	heavy metal-binding protein HIP-like	-2.8378	3.87E-02
	evm.TU.Hic_asm_4.572	H-0 vs H-96c	heavy metal-binding protein HIP-like	1.8727	2.87E-03
	evm.TU.Hic_asm_3.88	H-0 vs H-96a	heavy metal-binding protein HIP-like	-0.9580	2.50E-02
	evm.TU.Hic_asm_16.1511	H-0 vs H-96a	metallothionein	-2.2738	5.84E-02

(Continued)

TABLE 4 Continued

Functional category	ID	Treatment	Fullname	log2(fc)	PValue
	evm.TU.Hic_asm_16.1511	H-0 vs H-96c	metallothionein	-3.4181	7.44E-03
	evm.TU.Hic_asm_12.1610	H-0 vs H-96a	ferritin subunit	-0.9914	7.17E-02

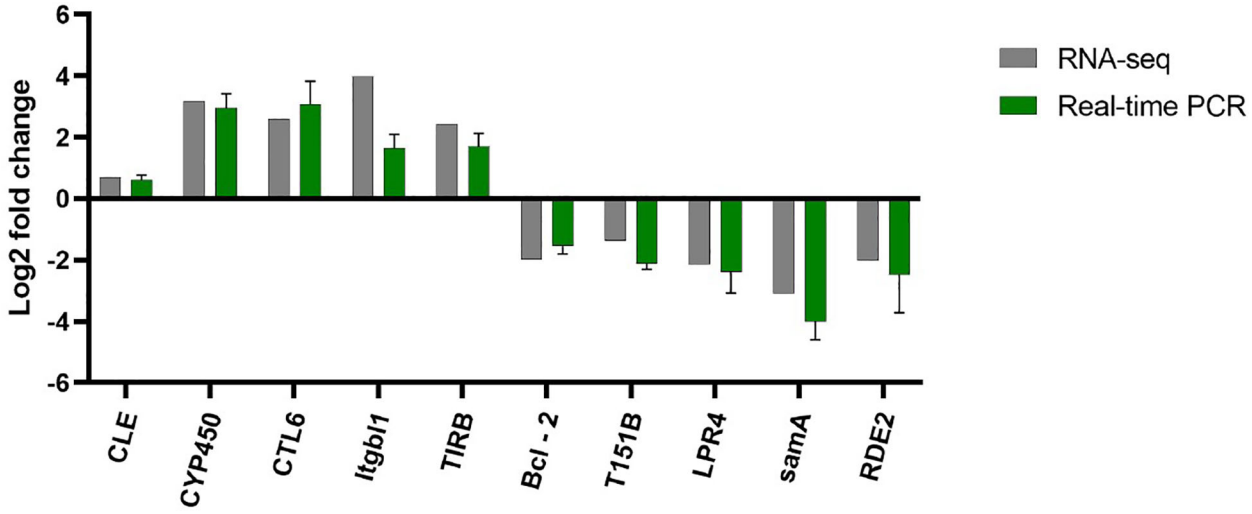

FIGURE 8
Transcriptome result validation. RNA seq and Real-time PCR are shown in grey and green, respectively.

when aquatic organisms are affected by external environmental factors (Chi et al., 2019). The study about the ACP and AKP activities in deltamethrin stressed *Macrobrachium rosenbergii* found that the ACP activity showed a wavy change and the AKP activity showed a trend of increasing and then decreasing after acute stress (Guo et al., 2021; Jiang et al., 2021). A similar conclusion was reached by Peng., in his study on the acute toxicity of the proposed cave green crab (*Cyprinus carpio*) (Peng et al., 2018). In this study, the ACP activity in the 20 mg/L group showed fluctuating changes, while the ACP *a*ctivity in the 50 mg/L and 100 mg/L groups both showed an increase followed by a decrease. There was no significant difference between the initial values. It is assumed that the prolonged survival in a high chlorine environment affected the expression of enzyme activity. The AKP activity in this study showed an overall decreasing trend, with all three concentration groups having lower AKP activity than the initial value after 96 h but no significant difference, suggesting that the AKP activity of the *C. sinensis* organism is inhibited in a high chlorine environment.

LZM protects the organism from foreign bacteria by hydrolyzing the mucopolysaccharides in bacteria (Yin et al., 2022). In a study by Zhou Y., it was found that LZM activity increased during stress after starvation stress in *Oratosquilla oratoria* and began to decrease beyond a certain range (Zhou, 2014). In the present study, the LZM activity of *C. sinensis* showed a decrease, then an increase and then a decrease after chlorine stimulation, and all three concentration groups showed this wavelike change. At 96 h, the LZM activities of all three concentration groups were significantly lower than the initial values ($P < 0.05$). It is hypothesized that the immune function of the *C. sinensis* was reduced after high chloride stress and the LZM activity was inhibited.

4.3 Transcriptome analysis of the effects of residual chlorine stress on hepatopancreas of *C. sinensis*

Studies have shown that residual chlorine stress affects the growth and survival of organisms and their physiological changes, but the molecular mechanisms involved are not well understood. In this study, the transcriptome of the hepatopancreas of *C. sinensis* stressed with residual chlorine for 96 hours was sequenced using RNA-seq technology. These transcriptional data provide a resource for further studies on the molecular mechanisms by which residual chlorine affects immune responses in molluscs. The data from this study indicate that residual chlorine stress has an effect on both the immune and antioxidant systems of the clam.

Serine proteases (*SP*) are a group of widely distributed protein hydrolases that perform a range of important physiological functions, including participation in digestion, blood coagulation, embryonic development and immune response processes (Yin S et al., 2022; Liu H et al., 2022), and play an important role in the innate immune defence of invertebrates (Hu et al., 2018; Xu et al., 2018). When invertebrates are stimulated or infected by microorganisms (Faisal et al., 1998; Xue et al., 2006), *SP* in their bodies can rapidly activate the body's immune system to produce the corresponding immune effects and kill the invading pathogens. However, the benzoquinone and ROS produced during the reaction can cause cellular damage, and serine protease inhibitors (*SPI*) can precisely regulate this process to avoid serious damage to the body (Ding et al., 2011; Chen et al., 2020). In this study, *SP* expression level was down-regulated at safe concentrations compared to the control group, while the expression

levels of *SP* in the high residual chlorine stress group were not significantly different from the control group, which is consistent with previous findings (Zhang et al., 2016; Mao et al., 2018), indicating that that residual chlorine stress induces *SP* in the clam to protect the organism by activating cascade processes and phenol oxidase pathways.

The C-type lectin family is an important class of pattern recognition receptors that trigger processes such as agglutination of microorganisms, induction of phagocytosis and activation of complement by recognizing glycan structures on the surface of microorganisms (Vasta et al., 2004; Osorio and Sousa, 2011) and participate in the regulation of specific immunity with the emergence of specific immunity (van Vliet et al., 2008), playing an important role in shellfish innate immunity (Wang et al., 2007; Zhu et al., 2008; Hu et al., 2011), but has rarely been studied in *C. sinensis*. The results of the present study showed that the expression levels of C-type lectins were significantly down-regulated in both groups a and b after 96 h of residual chlorine stress compared to the control group, with no significant difference in expression levels in group c. This is consistent with the previous findings that the expression of the C-type lectin gene tends to decrease when the stimulation time exceeds 72 hours (Shen et al., 2018), indicating that the clam is affected by residual chlorine and adapts to the environment by regulating the secretion of C-type lectin in the body to protect itself from the stimulus. The regulatory mechanism of the body becomes slower when the stimulation time is too long, and the higher the concentration of residual chlorine, the higher the level of gene expression.

Cathepsin L (*Cts L*) is a major member of the papain C1 family of cysteine proteases. Studies have shown that *Cts L* is involved in a variety of physiological and pathological processes in the organism, such as antigen presentation, hormone protein maturation and processing, tumour invasion and metastasis, apoptosis, bone resorption, individual development and tissue differentiation. In addition, *Cts L* may also mediate the immune defence of the organism (Ma et al., 2010; Peng et al., 2012). Consistent with previous studies (Wang et al., 2013; Liu S et al., 2014) in the present study, *Cts L* expression was significantly down-regulated in both groups a and b after 96 h of residual chlorine stimulation compared to the control group, while no significant difference was observed in group c. This may imply that residual chlorine stress induced an inflammatory response in the organism, which was in a post-infection recovery period after 96 h.

It has been shown that apoptosis is a highly regulated programmed cell death and that the Caspase family plays a pivotal role in apoptosis, with Caspase 9 (*CASP9*) being the initiator of apoptosis (Yu, 2017; Lu et al., 2021; Zhu et al., 2022). In the present study, *CASP9* expression was up-regulated at a and b stress concentrations compared to the control, while no significant difference was observed in group c. This suggests that residual chlorine stress initiated an apoptotic response, but the initiation of the response became slower with increasing residual chlorine concentrations after 96 hours of stress. Previous studies on stress in aquatic animals such as carp (*Ctenopharyngodon idella*) (Luan et al., 2022), yellow catfish (*Pelteobagrus fulvidraco*) (Li et al., 2020) and grouper bream (*Megalobrama amblycephala*) (Zhang, 2015) are consistent with the results of this experiment, suggesting that *CASP9* is associated with apoptosis induced by stress.

The effects of Glutathione S-transferase (*GST*) molecule are both antidotal and antioxidant. When shellfish are stimulated by environmental pollutants, the *GST* activity in their organism is significantly increased (Sandamalika et al., 2019) and protects cells from external stressful environmental damage by catalyzing the redox reaction of hydrogen peroxide and scavenging oxygen radicals and peroxides generated by metabolic reactions in the body (Samaraweera et al., 2019). pi type is a typical representative of many *GST* isoforms and is widely involved in the metabolism of toxins in immune cells and the production of lipophilic compounds and their derivatives (Liu et al., 2020). The *GST* in marine shellfish is basically of the pi type (Liu H et al., 2014). Under the stimulation of pathogenic bacteria, the respiratory burst of cells increases significantly and induces the production of large amounts of ROS. ROS protect the organism from oxidative damage by killing bacteria (Zhang et al., 2017). The antibacterial process of ROS refers to that overly high concentration of ROS breaks the antioxidant defense mechanism in cells and reacts with genetic materials, enzymes, proteins and other substances in bacteria to cause oxidative damage, or reacts with structural components of cell membrane (wall) to cause lipid peroxidation in cells, causing damage and death of bacteria (Zhou et al., 2021). However, excess ROS can cause damage to host cell structure and function, oxidizing cell membrane phospholipids and leading to alterations in cell membrane fluidity and permeability. *GST*s, as an important antioxidant enzyme, play an important role in detoxification of exogenous organisms and protection of cells from excess effects, and ROS regulate cell function by affecting the end products formed from macromolecules damaged by oxidative stress. Therefore, the antioxidant function of *GST*s is essential for the survival of molluscs, as these enzymes (Hu et al., 2012) can rapidly scavenge excess ROS. In the present study, the expression of *GST* was significantly higher in the H-96a, H-96b and H-96c treated groups than in the control group, which is consistent with previous findings (Zheng et al., 2019; Zhang H et al., 2020). This suggests that *C. sinensis* are sensitive to residual chlorine stress and that *GST* are involved in the detoxification process of residual chlorine, thus maintaining the stability of the organism.

Heat shock proteins (*Hsp*), also known as stress proteins, are a group of highly structurally conserved peptide protein family molecules that perform important physiological functions under both normal and stressful conditions. *Hsp70*s are members of a family of 70 kDa heat shock proteins that are widely present in prokaryotic and eukaryotic cells (Gong et al., 2020). *Hsp70* is an inducibly expressed protein that is one of the first protective molecules to appear in response to cellular injury, which helps denatured proteins to recuperate and clear permanently denatured proteins, and its expression increases significantly in response to stimulation by contaminants (Guo et al., 2020; Wang et al., 2020; Cui et al., 2022; Roh and Kim, 2022). In this study, the expression level of *Hsp70* gene was significantly increased in the H-96c group compared to the control group, while there was no significant change in the other two groups, which is consistent with the results of previous studies (Shao and Zhang, 2015; Zhao H et al., 2016; Jing et al., 2019), indicating that the stress response of hepatopancreatic cells increased

with the increase of residual chlorine concentration and the expression level of *Hsp70* gradually increased.

This study showed that residual chlorine stress significantly altered the expression of antioxidant and immune-related genes in the hepatopancreas of *C. sinensis*, providing evidence at the gene transcriptional level for a potential mechanism by which excessive residual chlorine residues in the culture environment affect basal immunity in molluscs. Among the DEGs between the control and three different residual chlorine concentration treatment groups, 68 DEGs were associated with immune responses and 29 DEGs were involved in antioxidant mechanisms. Specifically, genes associated with the proPO cascade system, *PRRs* and signal transduction play an important role in residual chloride-stimulated immune defence to maintain the normal physiological state of the green clam. In addition, molecules associated with the *GST*-dependent antioxidant system are highly involved in antioxidant defence against residual chlorine stress. Thus, these results not only provide some evidence that residual chlorine affects immune homeostasis in aquatic animals, but also contribute to further understanding of the molecular mechanisms and potential pathways of immune function and antioxidant capacity in larval *C. sinensis* under residual chlorine stress.

5 Conclusion

The study showed that the mortality rate of the *C. sinensis* increased with the increase of stress time and concentration. The antioxidant-related T-AOC and SOD activities and immune-related ACP, AKP and LZM enzyme activities of hepatopancreas of *C. sinensis* after residual chlorine stress were significantly changed between 0 and 96 h ($P < 0.05$). Meanwhile, transcriptome analysis showed that residual chlorine stress significantly altered the expression levels of immune and antioxidant related genes. Thus, this study provided valuable information for understanding the effects of residual chlorine stress on survival, physiological metabolism and molecular mechanisms of immune and antioxidant functions of the *C. sinensis* and provided some theoretical references for the understanding of the toxic effects of residual chlorine on bivalves.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

All clams were treated in strict accordance with the guidelines for the care and use of experimental animals established by the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China and approved by the Committee on Experimental Animal Management of the Jiangsu Ocean University.

Author contributions

SW: validation, writing - original draft, visualization, data curation. GR: formal analysis, data curation. DL: data curation, writing - review and editing. SF: data curation, writing - review and editing. SY: data curation, writing - review and editing. JS: data curation, writing - review and editing. ML: supervision, project administration. ZD: data curation, writing - review and editing, funding acquisition, project administration, conceptualization. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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