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The Monitoring and Assessment of Cd²⁺ Stress Using Zebrafish (*Danio rerio*)

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Abstract

Pollution on the Earth is ubiquitous across ecosystems from the land to the ocean. Various sources contribute to pollution including industrial (e.g. chemicals), agricultural (e.g. pesticides) and domestic (e.g. transportation) pollutants' ecosystems and substrate environment (e.g. contamination in water). The extensive use of chemicals in agriculture, forests and wetlands may impair biological communities. Due to the lack of target specificity, these chemicals can cause severe and persistent toxic effects on nontarget aquatic species, including bacteria, invertebrates and vertebrates. Different degrees of biological response have been presented according to intensities of different chemicals. The cadmium (Cd²⁺) contamination in aquatic environment has attracted more and more attention due to its toxic characteristics, for example, accumulation in environment, nondegradability and the potential threat to the ecosystem. Knowledge and understanding of these conditions have led to the development of new monitoring and assessment technologies based on biological and chemical methods. This chapter covers new monitoring technologies and environment assessment of Cd²⁺ stress using zebrafish (*Danio rerio*), which include the behaviour responses, metabolism and electrocardiogram (ECG).

Keywords: zebrafish, behaviour responses, metabolism, electrocardiogram (ECG), cadmium stress

1. Introduction

With the development of industrialization, the efforts of decreasing pollutants from the natural environment cannot satisfy the fundamental requirement of health and safety due to the large and increasing amounts of waste materials [1]. Environmental pollution has become an

increasingly serious problem in recent years [2, 3], and water pollution has caused more and more concern. The extensive use of chemicals in agriculture, forests and wetlands may impair biological communities [4, 5].

Due to the lack of target specificity, most contaminants can cause severe and persistent toxic effects on non-target aquatic species, including invertebrates and vertebrates [6, 7]. Knowledge and understanding of these conditions have led to the development of new monitoring, analysis and assessment technologies based on biological, physical and chemical methods [8–10]. Campanella et al. [11] achieved the monitoring of the evolution of photosynthetic oxygen (O_2) and the detection of alterations due to toxic effects caused by environmental pollutants using a sensor by coupling a suited algal bioreceptor to an amperometric gas diffusion electrode. Glasgow et al. [12] illustrated that real-time remote monitoring and sensing technology which is a more important tool for evaluation of water quality. Zhang et al. [13] developed an online behaviour monitoring system to assess the toxic effects of carbamate pesticides on medaka (*Oryzias latipes*), a small fish native to East Asia. Alexakis [14] assessed the water samples based on chemical indexes such as sodium adsorption ratio, sodium percentage and residual sodium carbonate. These methods greatly contribute to environmental stress assessment and water resource management.

Heavy metal pollution caused worldwide attention due to their bioaccumulation. Cadmium (Cd) is one of the most easily accumulated toxic substances in humans and organisms [15]. Cd pollution commonly occurs in food and environment (e.g. water, air), and also Cd has a long half-life period in human and animal bodies. Hansen has investigated that rats accumulated Cd^{2+} at a greater rate firstly and the kidney toxicity due to Cd^{2+} needed 6–8 weeks [16]. The accumulation of cadmium could be observed in different organs in fish [17] (**Figure 1**).
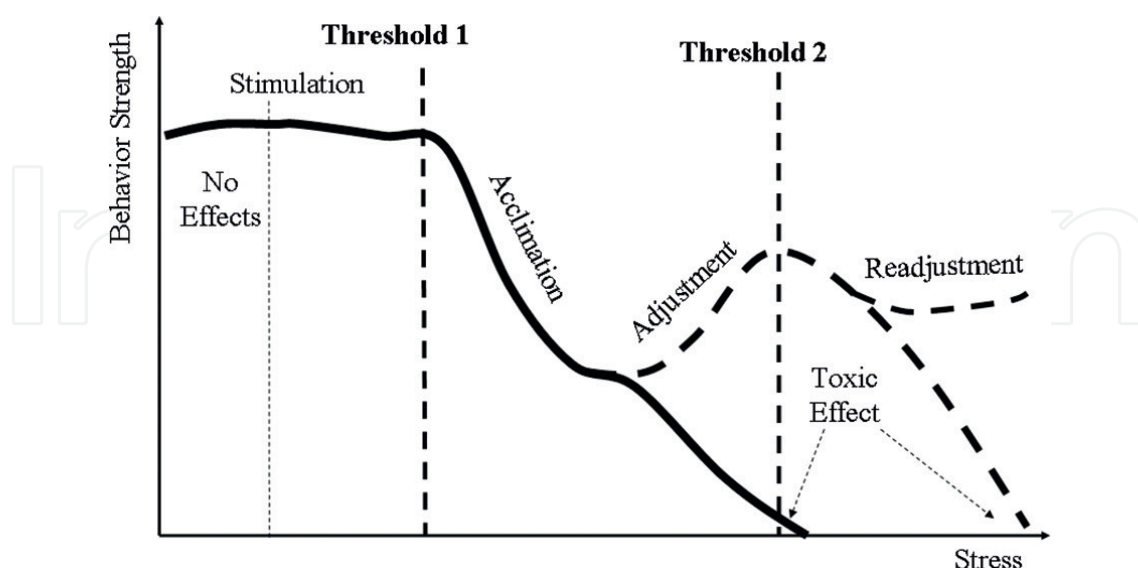

Figure 1. Step-wise behaviour responses according to previous reports: The model showed a series of different behaviours including "No effect," "Acclimation," "Adjustment (Readjustment)" and "Toxic effect". Threshold, the point at which a physiological effect begins to be specifically manifested on the individuals after exposure to stressors, decided the behaviour responses.

In the ecological assessment of environmental stress, many methods in different levels (molecular, cell, physiological and individual) are used [18]. Behaviour responses to toxic effects have been specifically observed [19, 20]. It has been reported that *Daphnia magna*, medaka (*Oryzias latipes*) and rare minnow (*Gobiocypris rarus*) are aquatic homeostatic organisms [21, 22] showing evident step-wise behaviour response, including "No effect", "Acclimation", "Adjustment (Readjustment)" and "Toxic effect" [23–25]. Wang et al. [26] reported a sequence of intoxication and recovery processes through data transformation (i.e. integration) at the time progressed after exposure to toxic chemicals and suggested that the behaviour response is a good indicator for aquatic organisms to assess the water quality.

As an endpoint in the physiological level, organisms' metabolism plays an important role in the environmental stress assessment. Metabolism is the fundamental process of organisms, and it is related to energy assimilation, transformation and allocation that strongly influence the rate of individual growth and reproduction [27]. Standard metabolic rate (SMR), which is the minimum metabolic level of fish in the state of rest and starvation, means the metabolism level of organisms, which could be analysed directly by oxygen consumption (OC) monitoring system [28]. Respiration is an important physiological characteristic of the metabolic activities of fish, which can reflect the adaptation of fish to the external environment. Metabolism in the characteristic of SMR is ordinarily equal to OC [29] on the condition of no food supplied during the monitoring of fish respiration. Usually, there are many factors that can affect OC of fish including water temperature, body mass, body size, dioxide oxygen, sanity, atmospheric pressure, injury and disease [28].

Heart ECG provides a chart that represents the electrical activity of the heart, and it also provides a time voltage of the heartbeat [30]. The notation of ECG waveform suggested by Einthoven [31] is still in use today and has been used to detect and monitor disease in different animals [32–34]. ECG waves (P, Q, R, S and T) and the time intervals (PR, QRS, ST and QT) have been used to differentiate healthy and diseased fish [35]. Therefore, fish ECG can be a good tool to assess water quality.

Zebrafish (*Danio rerio*), whose ether-a-go-go-related gene (zERG) which has high similarities in the protein sequence with the human gene (hERG) [35] has been frequently used as a representative in the toxicologic assessment of chemicals [36], providing sensitive, economical, practical and biological monitors for aquatic pollutants [37, 38]. First, it is economic to use zebrafish to assess water quality. Second, zebrafish can be a tool as good as higher vertebrates on toxic testing. Third, the use of zebrafish with other fish can realize the potential toxicity analysis [39]. Therefore, new monitoring technologies based on the behaviour responses, metabolism and electrocardiogram (ECG) of zebrafish can be applied to realize the monitoring and assessment of Cd²⁺ stress.

2. Methods

In the recording of the behaviour responses, metabolism and ECG, room temperature is controlled at 26 ± 2°C with a photoperiod of 16 h light and 8 h dark. Nonchlorinated water

(hardness based on $CaCO_3$: 250 ± 25 mg/L, pH 7.8 and temperature 26 ± 2°C) is used. No food is provided to test organisms during the assessment.

2.1. The recording of behaviour responses

An online monitoring system, built in the Research Center for Eco-Environmental Science, Chinese Academy of Sciences [40], is used to analyse the continuous swimming behaviour (**Figure 2**). Behaviour data are collected by behaviour sensors, which are made up of two pairs of electrodes that sent a high-frequency signal of alternating current by one pair and then received another. The behaviour strength is sampled automatically every second, and the average behaviour strength data are taken twice an hour in the first 2 h. Behaviour strength that changes from 0 (losing the ability of movement) to 1 (full behaviour expression) is applied to represent the differences of behavioural responses [13].

Test zebrafish were placed in behaviour sensors (10 cm long, 7 cm in diameter). These sensors were closed off on both sides with 250 μm nylon nets, and three replicates per concentration were used. Flow rate of each test channel was controlled about 2 L/h. Temperature and light conditions were the same as stock rearing. The flow rate in each test chamber was controlled to about 1 L/h in the online mixing system, and then the flow rate in the online monitoring system of behaviour strength was about 2 L/h.
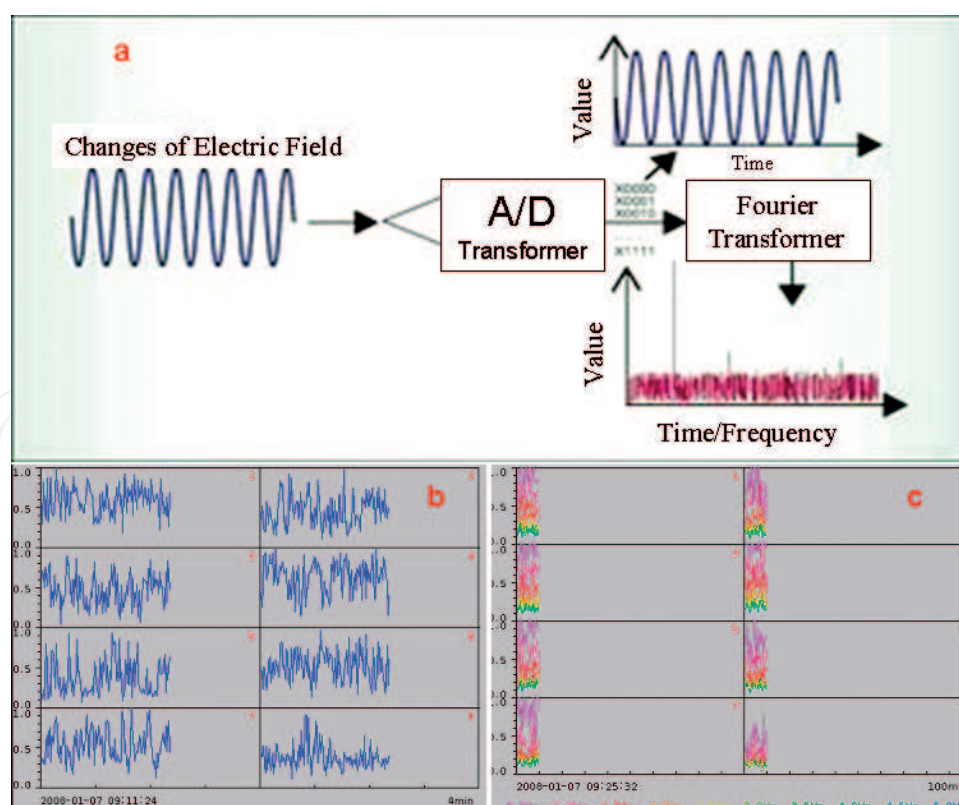

Figure 2. Signal acquisition and transmission of the behaviour responses: (a) the signal acquisition, (b) the normal signal analysis, and (c) the signal analysis after Fast Fourier Transform.

2.2. The recording of metabolism

An online monitoring system based on OC is used to record the metabolism of zebrafish (**Figure 3**). The metabolism monitoring system is made up of water tank, flow-through sensor, peristaltic pump, three-way valve, digital control unit, data acquisition unit, dissolved oxygen (DO) sensor, thermometer, pressure meter and water tubes.

Before the assessments, test zebrafish volumes are measured by 5 ml graduated glass cylinder (Va), and the body mass of test zebrafish is weighted as follows: A beaker (50 ml) with 10 ml water is weighted by a precision electronic balance (FA2204N, Shanghai Jinghai Instrument), which is regarded as a baseline weight. Then, test individuals are put into the beaker to get the wet weight (m). Test zebrafish are placed into the flow-through sensors (200 ml, Vr) with nylon nets (250 μm) at both sides to prevent test zebrafish running into water tubes. The water flow rate of the sensor is controlled by a peristaltic pump at approximately 2 L/h. The whole cycle of the three-way valve is designed as 150 s flushing phase (to ensure DO in test chamber is enough for test individuals) after 300 s circulation phase (to make measurement, regarded as t). During the circulation mode, the three-way valve is energized to close the loop. In this mode, the water flows from the flow-through sensor to the DO sensor. When the organisms breathe, the DO sensor can realize the measurement. In this phase, water tubes are installed in the correct direction, and all connections are sealed completely to ensure no external oxygen enters the system. During the flushing mode (dotted lines), the three-way valve will pump water (black dot) from water tank (brown box) to test chamber, and the water body will flow through chamber into water tank again (red dot). The flushing and circulation cycle are repeated until the end of the experiment. Oxygen concentrations of both inflow and outflow water are detected by DO sensor, and then these data are recorded as DO_i and DO_o. Absolute room pressure is provided to ensure the stability of DO in the system. Prior to the start of the experiment, all sensors are warmed up for 5 min, and then the OC data begin to be collected. Water temperature is recorded to remain constant through the experiments.
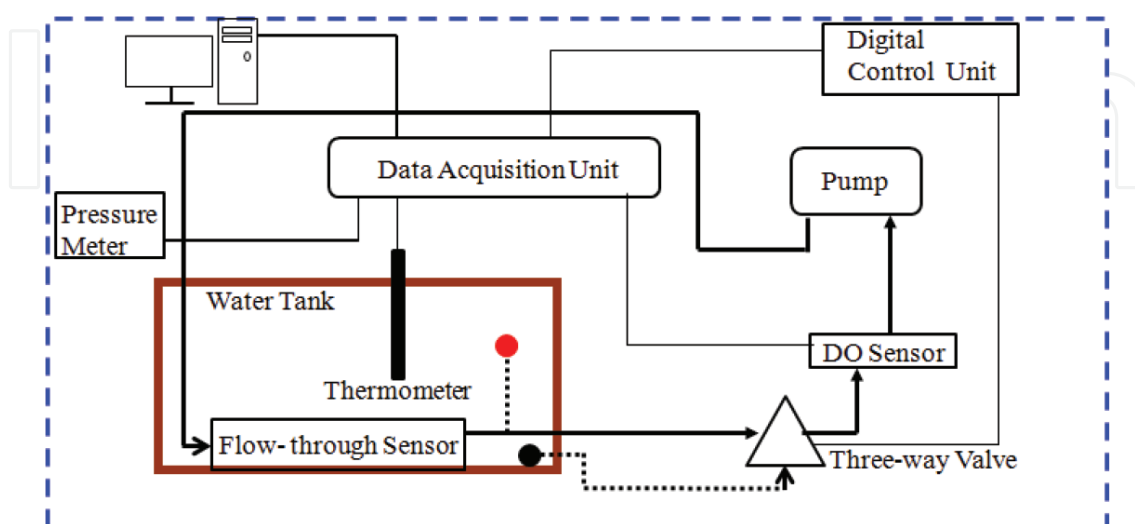

Figure 3. Online monitoring systems of metabolism based on oxygen consumption (OC).

DO (mg/L) data in the metabolism monitoring system are converted to OC using Eq. (1):

$$VO_2 = \frac{(DO_i - DO_o) \times (Vr - Va)}{m \times t}$$

(1)

in which VO_2: OC of test zebrafish (mg/kg/h); DO_i: DO concentration of water body flowing into SMR sensors (mg/L); DO_o: DO concentration of water body flowing out SMR sensors (mg/L); V_r: the volume of flow-through sensor (L) and it is 0.2 L for zebrafish; V_a: test zebrafish volume (L); m: test zebrafish mass (kg); and t: time of circulation cycle (h).

2.3. The recording of ECG

The ECGs are detected by RM-6240C Multichannel Physiological Signal Acquisition and Processing System (ChengDu Instrument Factory, China). During ECG acquisition, zebrafish
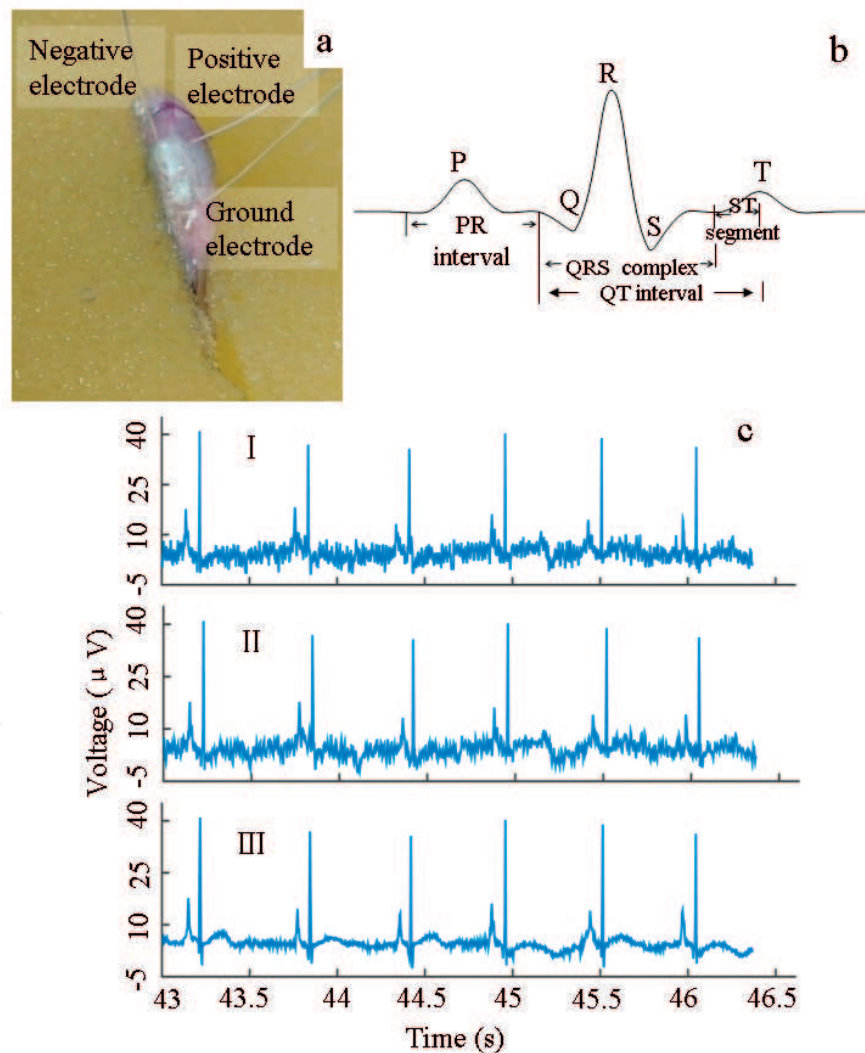

Figure 4. The acquisition and the analysis of zebrafish ECG.

are fixed in a damp sponge with the ventral side exposed for electrode placement (**Figure 4a**). Two micro-electrodes are positioned at 90° to the animal's epidermis near the heart and inside the medial pectoral fin; the positive electrode is on the right side and the negative electrode is at a relatively lower position on the left side. The grounding electrode is closer to the top of the pelvic fins as presented in **Figure 4a**. The relative distance between grounding electrode and other two electrodes is 8–10 mm. All electrodes are inserted into the skin to approximately 1 mm in depth. The exact position of the positive and negative electrodes was adjusted slightly to obtain the maximum voltage signals. ECG signals are characterized by the following parameters: waves P, Q, R, S, T and intervals PR, QRS, ST, QT (**Figure 4b**). ECG signals of each zebrafish are obtained over a 3 min period. Baseline ECG signals are recorded 24 h before assessment. The positive and negative electrodes are attached to the atrioventricular junction of the zebrafish, and the ECG signals are recorded at a sampling frequency of 4 kHz with the time constant 0.02 s [**Figure 4c(I)**]. The recorded signals were digitally processed by MATLAB (MATLAB R2009a, The MathWorks Inc., Natick, MA, USA). First, a zero-phase digital filter is used [**Figure 4c(II)**], and then the wavelet decomposition is acquired by performing a 10-level 1D wavelet analysis using coif5 wavelet. With the wavelet decomposition structure and the coif5 wavelet, the coefficients of the filtered signals are further reconstructed. The de-noised signals [**Figure 4c(III)**] are finally obtained from the above-reconstructed wavelet decomposition structure using the principle of Stein's unbiased risk; soft threshold, level-dependent estimation of level noise and coif5 wavelet at level 10 are used to analyse the ECG changes.

3. Results and discussion

3.1. The behaviour responses

According to the acute toxicity experiment following the guidelines for fish acute toxicity from OECD 203 [41], 48 h median lethal concentration (LC_{50}–48 h) of cadmium chloride ($CdCl_2$) to zebrafish after probit analysis in MATLAB with 95% confidence interval is 42.6 mg/L. The LC_{50}–48 h value (42.6 mg/L) is regarded as 1.0 TU, and then the exposure concentrations are 4.26, 42.6 and 85.2 mg/L in 0.1, 1.0 and 2.0 TU $CdCl_2$ treatments, respectively.

The behaviour responses based on behaviour strength (BS) of zebrafish are shown in **Figure 5**. The average BS in control kept about 0.8, and the higher the chemical concentrations, the lower is the behaviour strength. These results suggest that the toxic effects of $CdCl_2$ on the BS of zebrafish are closely related to the concentrations, which was defined as dose-effect relationship [42]. It is noteworthy that lower BS values could be observed in the dark period at 13–21 and 37 and 45 h in all treatments. Considering the ups and downs in BS curves matched to photo and scoot phase, this may suggest a possibility of circadian rhythm in test organisms after being exposed to the chemical [43].

The behaviour activity (BA) as integrated BS was obtained by linear regression (**Figure 6**). If BA is in the positive range, the BS would be higher than the accumulated average of BS and could be considered as in active state [44]. The BA values were mostly negative at initial stage. The crossing times of BA between positive and negative values were commonly observed which show the circadian rhythms. The control group showed the high value between 21 and 37 h,
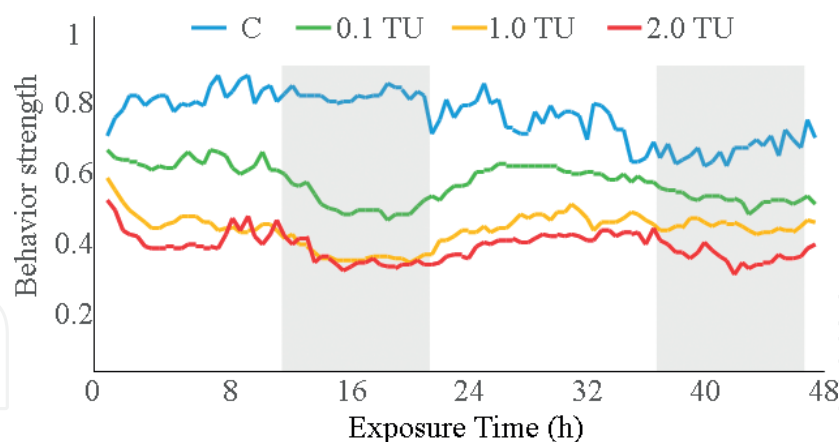
Figure 5. Behaviour responses of zebrafish in the treatments of $CdCl_2$. Grey shadow means the dark periods as the experiment started from 9 o'clock in the morning.
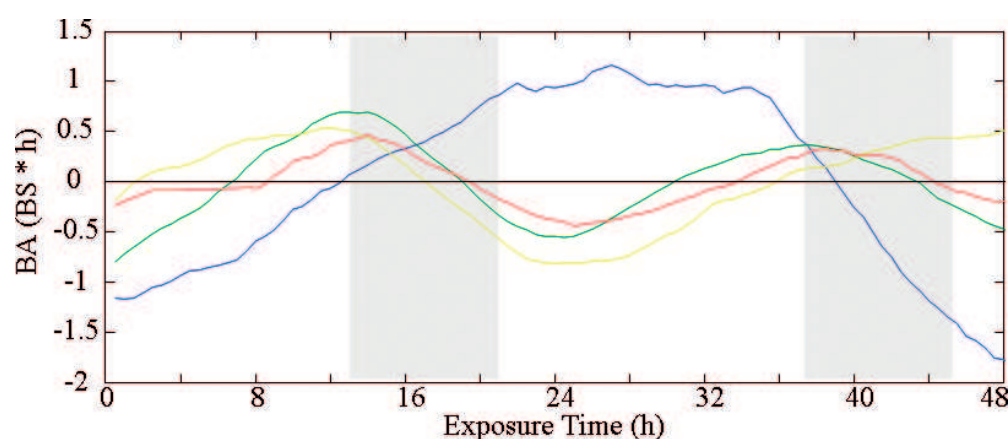

Figure 6. BA values for $CdCl_2$ across different treatments. Control (blue), 0.1TU (green), 1.0 TU (yellow) and 2.0 TU (red). Shadows mean dark periods.

which matched photophase. However, BS values were not high at either previous (0–13 h) or next (45–48 h) photoperiod. Remarkably, the circadian rhythms appeared in different treatments, being substantially different with the control group. Except the last phase with 1.0 TU, rhythmic activity was clearly observed at dark phases, 13–21 and 37–45 h, which indicated that the pollutant is a stimulating agent to resume the circadian system in test organisms. BS was higher at scoot phase. It was noteworthy that the rhythm was disrupted with 1.0 TU at the end of the experiment, whereas the lower (0.1 TU) and higher (2 TU) levels showed clear rhythms. The circadian rhythms of the test group were quite different from the control group, indicating that the environmental stress stimulated the biological clock of zebrafish and made it more clear in pollutants than in control [45], which is consistent with the result of **Figure 5**.

3.2. The changes of metabolism

As presented in **Figure 7**, the exposure of $CdCl_2$ significantly affected the overall oxygen consumption (OC) of zebrafish ($p < 0.05$). OC at 0.1 TU (463.11 ± 44.21 mg/kg/h) was significantly ($p < 0.05$) lower than the control (617.39 ± 30.48 mg/kg/h) and higher than 1.0 TU (314.40 ± 40.04 mg/kg/h) and 2.0 TU (229.07 ± 28.66 mg/kg/h). These results could be
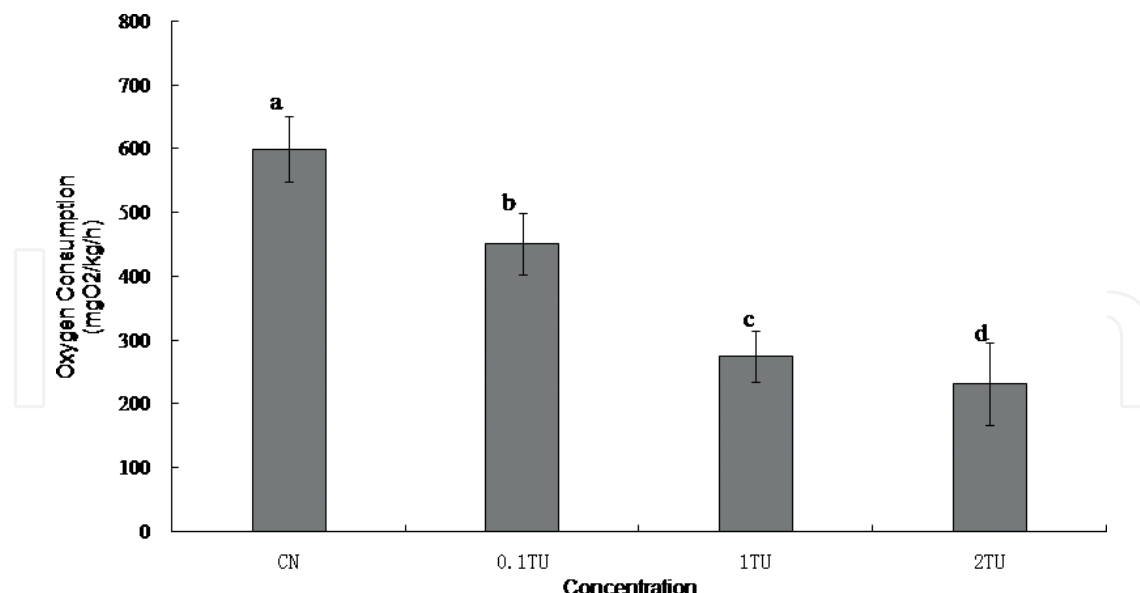
Figure 7. Changes in average oxygen consumption (mgO2/kg/h) after exposure to CdCl$_2$ across different toxic concentrations, 0TU, 0.1TU, 1TU and 2TU. The bars in the histogram represent the mean ± SD of oxygen consumption in different TUs. Different alphabets on the bar represent statistical significance based on multiple comparison ($p < 0.05$).

supported by a previous report on the effects of CdCl$_2$ on the respiration of fresh water crab [46], which is a fundamental physiological function in organisms that can affect other metabolic processes, for example, feeding, food absorption and excretion [47]. In the toxic environment of CdCl$_2$, the internal environment of the cell changed, and all the related energy processes were changed correspondingly [48].

Figure 8 shows the continuous changes of zebrafish OC during 48 h exposure of CdCl$_2$, which suggested that the total tendency of the continuous results had the same order as presented in **Figure 7**. In control, a high respiration rate was maintained at about 617.39 mg/kg/h during the observation period. In different treatments, the observed levels of OC decreased with the increase in concentrations, for example, OC was approximately 500 mg/kg/h in 0.1 TU, 300 mg/kg/h in 1.0 TU and 200 mg/kg/h in 2.0 TU.
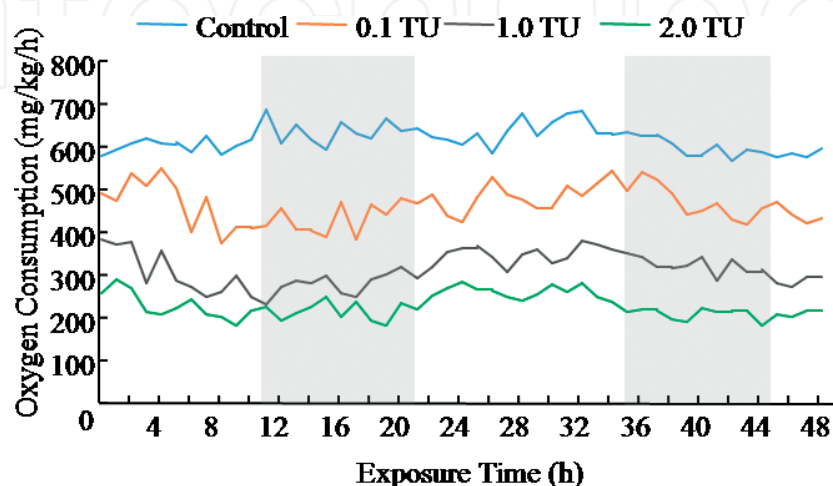

Figure 8. Continuous changes of zebrafish OC during 48 h exposure in differentCdCl$_2$treatments.

During 48 h exposure, OC in most treatments had some circadian rhythms according to the average values in different periods (**Table 1**) because OCs were significantly (p < 0.05) higher during photo phase (D1 and D2) than scoot period (N1 and N2).

When zebrafish were exposed to 0.1 TU and 1.0 TU CdCl$_2$, OCs were significantly ($p < 0.05$) different between sooct phase and photo phase. This indicated that the response of zebrafish to pollutants was stronger and had great effects on diurnal variation during 48 h exposure. There was a greater fluctuation of OC in CdCl$_2$ exposures than in the control. In most treatments, higher values were observed during photo phases (0.1, 1 and 2 TU). At the beginning, OC decreased until the 11th h and then recovered during 21–36 h. In the end, OC decreased at all concentrations during the exposure of CdCl$_2$ in 36–48 h.

3.3. The changes of ECG

Figure 9 shows the average values of zebrafish ECG characteristics in different treatments. The values of all exposure time points during the 48-h exposure are assigned to the corresponding concentration groups. After exposure to CdCl$_2$, the amplitudes of all waves (P, Q, R, S and T) and interval durations (PR, QRS, ST and QT) showed some difference with significance ($p < 0.05$ or $p < 0.01$). In the control group, the amplitudes of all waves were the largest, and they were almost the smallest in different intervals. Overall, the amplitudes of waves tended to show negative relationships, and intervals showed positive relationship with CdCl$_2$ concentrations. The changes of waves that Q, T, QRS and QT intervals showed clearly observed dose-effect relationship. There were some exceptions: S and ST did not show an obvious dose-effect relationship, and R showed a reverse effect to other waves. These results suggested that it was not sufficient to analyse the toxic effects of environmental stress on zebrafish ECG alone, depending on the average values of these characteristics.

To assess water quality using zebrafish ECG characteristics, the continuous changes based on the de-noised ECG signals were applied (**Figure 10**). The difference of zebrafish ECG characteristics after two-way ANOVA is shown in **Table 2**. Some characteristics (Q, R, S and T) showed some significant difference ($p < 0.05$ or $p < 0.01$) at different exposure times in the control group (**Figure 10b–e**), but the differences of P, PR, QRS, ST and QT showed no significant changes, which suggests that P, PR, QRS, ST and QT can serve as normal control in the analysis of CdCl$_2$ toxic effects on zebrafish ECG (**Figure 10a**, f, g, h, i).

Time periods	Control	0.1 TU	1.0 TU	2.0 TU
D1	610.47 ± 25.69*aBb	460.38 ± 52.88	310.53 ± 52.92	236.69 ± 32.28*b
N1	632.18 ± 23.31*Ab	436.54 ± 36.44*B	285.47 ± 21.01*Bb	214.81 ± 22.22*B
D2	633.20 ± 33.03*Ab	488.10 ± 37.48*a	342.35 ± 27.01*ab	246.67 ± 23.02*ab
N2	590.88 ± 17.78*AaB	458.77 ± 30.42	309.99 ± 22.47*aB	207.22 ± 12.83*AB

D1, the first 0 day results, from 0 to 11 h and from 21 to 24 h; D2, the second-day results, from 24 to 36 h and from 46 to 48 h; N1, the first-night results, from 11 to 21 h; N2, the second-night results, from 36 to 46 h exposure. Data are shown as M ± S.D.* $p < 0.05$, A, B, a and b mean the significant difference with D1, D2, N1 and N2, respectively.

Table 1. OC of zebrafish in different treatments during both photo phase (D) and scoot period (N).
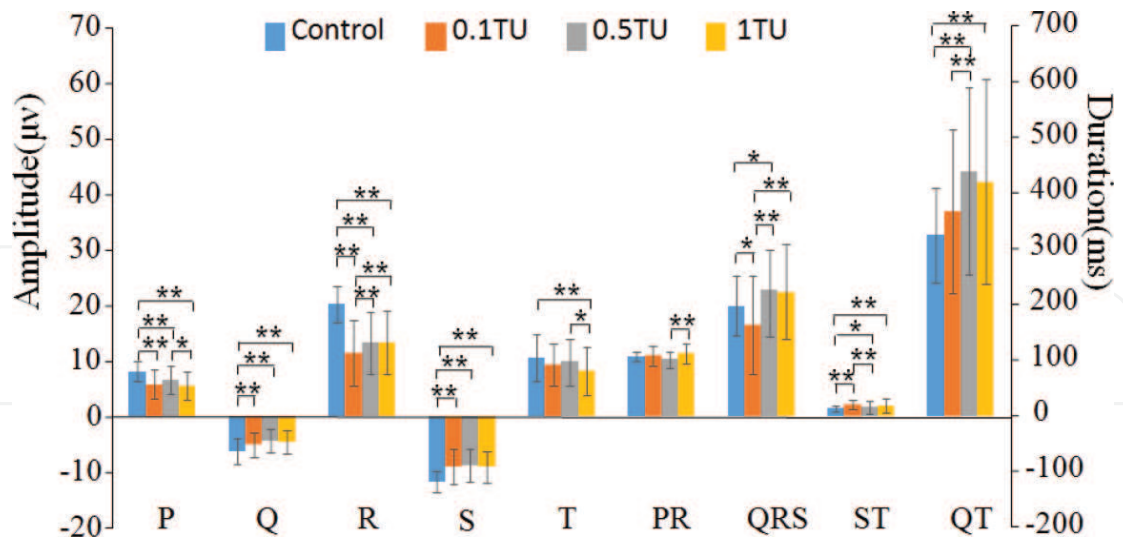
Figure 9. Comparison of zebrafish ECG characteristics after treated with CdCl$_2$. The unit of wave amplitude (P, Q, R, S and T) is μv, the unit of duration (PR interval, ST segment, QRS complex and QT interval) is ms. Vertical bars indicate mean values and the solid lines are standard deviation. The data of every ECG parameter are representative of at least four independent experiments. — represents two experimental groups indicating the ends of a line segment. *$p < 0.05$, **$p < 0.01$.

The observed continuous changes of ECG suggested that signals of P, Q, T and PR were disordered, in which the data of amplitudes and durations showed no clear regularity depending on either exposure time or concentrations. According to the analysis of the tendency (**Table 2** and **Figure 10**), R, QRS, ST and QT in different treatments showed dose dependency; however, due to some fluctuation changes at 8, 16 and 32 h, ST and QT did not show clear time dependence.

Overall, QRS, ST and QT showed prolonged effects after CdCl$_2$ exposure and wave R showed a decrease in amplitude. A prolonged ST reflects delayed repolarization of ventricular function. As it is reported, ST depression hysteresis could offer a substantially better diagnostic accuracy for coronary artery disease [49], and ST elevation could be induced by CdCl$_2$ [50]. A wide QRS reflecting left-sided intra-ventricular conduction delay and prolonged QRS reflects delayed depolarization of ventricular function too. Prolonged QT reflects delayed repolarization of action potential [51]. CdCl$_2$ is highly toxic to the cardiomyocytes, characterized by lengthening the repolarization phase of the cardiac action potential, manifested as prolongation of the QT on the surface ECG and predisposition to special arrhythmia [35]. Therefore, the QT prolonging suggested that CdCl$_2$ induced bradycardia and arrhythmia in zebrafish. The R wave voltage changes were observed with doxorubicin (DXR) treatment. On the cellular level, DXR treatment led to a decrease in V$_{max}$, with little increase or no change in resting potential and a marked prolongation in action potential duration at 50–75% repolarization levels [52].

To specify the role of ECG characteristics in the assessment of CdCl$_2$ stress, the Pearson correlation analysis between ECG characteristics and CdCl$_2$ stress was performed based on the correlation coefficient r and significance p. We first checked the correlation coefficient r to see how much they correlated ($r < 0.3$, poor correlation, $0.3 < r < 0.5$ moderate, $r > 0.5$ high correlation).
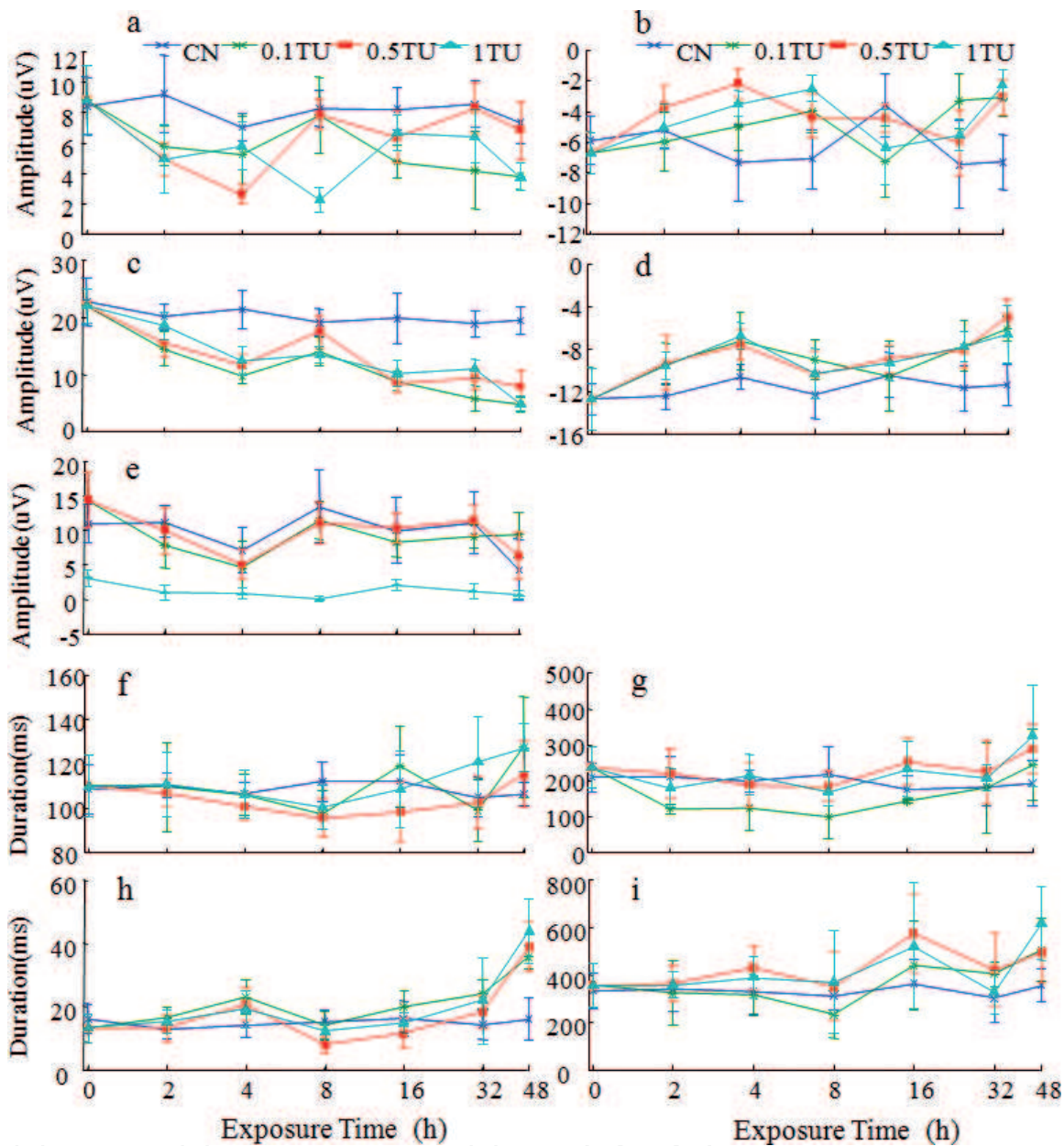
Figure 10. The continuous changes of ECG characteristics in different treatments. a-e represent the characteristics of waves P, Q, R, S and T and f-i represent the characteristics PR interval, QRS complex, of ST segment and QT interval. Log scale was used in the x-axis.

Then, we checked p value to see whether these two variables are correlated significantly ($p < 0.05$). When r is high (absolute $r > 0.5$) with significance ($p < 0.05$), it indicates that the data correlation is significant [53, 54].

The environmental stress E is primarily decided by both chemical concentrations (C) and exposure time (t) with an exponential function as shown in Eq. (2) [55]:

$$E = e^{(C \times t)} + E_f \tag{2}$$

ECG characteristics	Treatments	Start time	Exposure time					
		0 h	2 h	4 h	8 h	16 h	32 h	48 h
P (μv)	Control	—	\	\	\	\	\	\
	0.1 TU	—	**	**	\	**	**	**
	0.5 TU	—	\	\	\	\	\	\
	1.0 TU	—	**	**	**	*	*	**
Q (μv)	Control	—	\	\	\	**	*	\
	0.1 TU	—	\	*	**	\	**	**
	0.5 TU	—	**	**	**	**	\	**
	1.0 TU	—	*	**	**	**	\	**
R (μv)	Control	—	*	\	**	**	**	**
	0.1 TU	—	**	**	**	**	**	**
	0.5 TU	—	**	**	**	**	**	**
	1.0 TU	—	*	**	**	**	**	**
S (μv)	Control	—	\	*	\	*	\	\
	0.1 TU	—	**	**	**	*	**	**
	0.5 TU	—	**	**	*	**	**	**
	1.0 TU	—	**	**	*	**	**	**
T (μv)	Control	—	\	\	\	\	\	*
	0.1 TU	—	**	**	**	**	**	**
	0.5 TU	—	**	**	*	**	*	**
	1.0 TU	—	**	**	**	*	**	**
PR (ms)	Control	—	\	\	\	\	\	\
	0.1 TU	—	\	\	*	\	\	**
	0.5 TU	—	\	\	*	*	\	\
	1.0 TU	—	\	\	\	\	\	**
QRS (ms)	Control	—	\	\	\	\	\	\
	0.1 TU	—	**	**	**	**	\	\
	0.5 TU	—	\	\	\	\	\	\
	1.0 TU	—	\	\	*	\	\	**
ST (ms)	Control	—	\	\	\	\	\	\
	0.1 TU	—	\	**	\	**	**	**
	0.5 TU	—	\	**	*	\	*	**
	1.0 TU	—	\	*	\	\	**	**

ECG characteristics	Treatments	Start time	Exposure time					
		0 h	2 h	4 h	8 h	16 h	32 h	48 h
QT (ms)	Control	—	\	\	\	\	\	\
	0.1 TU	—	\	\	*	\	\	**
	0.5 TU	—	\	\	\	**	\	**
	1.0 TU	—	\	\	\	**	\	**

The significance shows the difference between exposure times and start time (0 h) in each treatment.* $p < 0.05$, ** $p < 0.01$ and \ represents no significant differences.

Table 2. The difference of zebrafish ECG characteristics after the two-way ANOVA.

in which C is based on TU values and t presents the time with 6 min per unit (0–480) according to our previous results [13]. E_f is the environmental stress due to the effects of all other physico-chemical factors, including water temperature, turbidity, pH, dioxide oxygen and conductivity. As physico-chemical factors are controlled under experimental conditions as shown in our previous research results [56], the effects of E_f are not considered ($E_f = 0$) for simplicity of model execution in this study. Then, if $C \times t = 0$, $E = 1$, it means that the minimum value of E is 1 in a pollution-free environment [57].

The correlation between ECG parameters and CdCl$_2$ stress (E) after Pearson correlation analysis is shown in **Table 3**. The results suggest that the relationship between E and wave S, intervals PR, QRS, ST and QT, showed a high correlation with absolute $r > 0.5$ and $p < 0.05$. With the correlation coefficient $r = 0.729$ (the highest) and correlation significance $p = 0.002$ (the smallest), the relationship between E and QRS was extremely significant, which suggested that QRS could be significantly affected by CdCl$_2$ stress.

On the surface ECG, QRS reflects ventricular depolarization and propagation of the excitatory cardiac impulse throughout the ventricles [58]. Cardiac conduction system excitability was depressed preferentially in Cd^{2+} [59]. Cd^{2+} could lead to changes in ECG, which may be attributed to its effects on ventricular conduction. Yin et al. found that workers exposed to Cd^{2+} had a significantly longer QRS [60]. However, Cd^{2+} administration caused a reduction in myocardial contractile performance, slowing of heart rate and disturbances in metabolism

Pearson correlation analysis	ECG parameters								
	P	Q	R	S	T	PR	QRS	ST	QT
E Correlation (r)	−0.094	0.317	−0.487	0.517	−0.443	0.685	**0.729**	0.667	0.635
Significance (p)	0.739	0.25	0.066	0.048*	0.098	0.005**	**0.002****	0.007**	0.011*

*$p < 0.05$
**$p < 0.01$.

Table 3. The correlation between ECG parameters and environmental stress (E) after Pearson correlation analysis.

of the heart [61], which may prolong QRS. On a cellular level, depolarizing inward current passing through the voltage-gated cardiac sodium (Na^+) channel is responsible for the rapid upstroke of the ventricular action potential that initiates the conduction of the excitatory wave front throughout the ventricular wall [58]. Visentin et al. investigated the dependence of Na^+ current block by Cd^{2+} on external Na^+ concentration in ventricular myocytes. Depression of inward Na^+ current by Cd^{2+} was essentially voltage independent, in agreement with it being caused by channel block. The data show that Cd^{2+} reduces Na^+ current in Purkinje fibres and in ventricular myocytes [62]. In other words, exposure to Cd^{2+} could have an effect on Na + current in zebrafish cardiac myocytes. Cd^{2+} could block the Na^+ channel and decrease inward Na^+ currents, resulting in delayed ventricular conduction and prolonged QRS in the ECG.

4. Conclusions

As a by-product of industry, wastewater with Cd^{2+} should not be discharged into aquatic environment only after basic treatment according to the Environmental Quality Standards for Surface Water, GB3838–2002 [63], in which the limitation of Cd is 0.01 mg/L. However, some industrial wastewater may be discharged without any treatment, in which Cd^{2+}concentration might be higher than 26 mg/L [64] and the concentration of Cd^{2+} in some sediments could reach 359.8 g/kg [65].

The online behaviour responses of zebrafish showed that BS has obvious dose-effect relationship with Cd^{2+}, and the online behaviour responses could illustrate the toxicity of Cd^{2+} directly. The circadian rhythms could be observed even in higher concentrations (1.0 and 2.0 TU). Meanwhile, Cd^{2+} has an inhibitory effect on the standard metabolic rate of zebrafish, and respiratory parameter of zebrafish with ECG can be regarded as sensitive biological monitoring indexes to realize the online assessment of Cd^{2+} pollution. It is noteworthy that there is an extreme significant correlation between QRS complex and Cd^{2+} stress with the highest *r* and the smallest *p* among all ECG characteristics, and it may be a good way to monitor Cd^{2+} pollution in aquatic environment by observing and analysing QRS complex.

These results provide an objective ground for analysing complex stress response that could be applied to test the changes of organisms (exposed in different treatments) quantitatively in toxic physiology and ecology.

Acknowledgements

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