Monitoring low-level mercury contamination by zebrafish school behavioral responses

Y Huang 1,4, J S Zhang 2, F J Mi 3, G H Zhang 1 and J Sun 1

1 Xi'an Technological University, No.2 Xuefu Road, Xi'an, China
2 Shenzhen Water (Group) Co., Ltd., No.1019 Shennan Middle Road, Shenzhen, China
3 Xi'an Municipal Facilities Administration, Xi'an, 710038, China
4 E-mail: huangyiwater@xatu.edu.cn

Abstract: Low-level pollution accidents are keeping increasing and difficult to monitor in real time. In our current study, zebrafish are used as a common freshwater model to monitor low-level concentrations of 0.05 mg·L⁻¹ mercuric chloride (HgCl₂). Avoidance (swimming increased and closely gathered) was the first response, but those abnormal behaviour just lasted 15~20 min and then recovered to the original level with much more fluctuation. In order to quantitative evaluate the first stress responses of fish exposed to lower concentration of toxicant, entropy is proposed for detecting the first responses. The use of entropy contributed to the reliability and precision for detecting toxicant at lower concentration pollution.

Key words: Swimming Behavior, Toxic Responses, Biomonitoring, Avoidance, Entropy

1. Introduction
Among different environmental toxins, it is worth mentioning mercury, mainly because of their high toxicity, carcinogenic and non-biodegradable [1]. Both elemental and ionic mercury can be converted by sulfate-reducing bacteria to methyl mercury, which can easily bio-accumulate through the food chain [2,3]. Through damage to the central nervous system, DNA, mitosis, and the endocrine system, mercury may cause serious human health problems even at very low concentrations, such as prenatal brain damage, serious cognitive and motion disorders [4]. Therefore, it is highly desirable to a sensitive and selective method for Hg²⁺ ions detection in aquatic environments.

Compared with traditional physico-chemical method, real time biomonitoring by fish behavior is the most optimal way to ensure an appropriate and timely response [5]. Fish have a keen sense of chemoreception, which not only enables a fish to feel changes in various environment but also to respond to those changes [6,7]. Changes in behavior are extremely very sensitive to the stress imposed on fish by pollution and widely applied to monitoring heavy metals [2], cyanide [8], insecticides [9] and other chemical pollutants [10-12].

The most widely studied behaviors for water quality monitoring include the following three behavioral endpoints: 1) Swimming or locomotion. The main endpoints include velocity, acceleration, rate of change in orientation, turning angles, time spent swimming, as well as vertical distribution [9,13]. 2) Respiration behavior. Ventilation rate, cough rate (gill purge), respiratory frequency and respiratory depth are measured for respiration changes assessment [14,15]. 3) Social interactions. Motion trajectories are converted into fractal dimension, distance between fish, dispersion, the
Numerical approaches have been performed to automatically characterize behaviour in fish. Some thresholds for behavior parameters are predefined and generated an alarm if surpassed the threshold [17]. Artificial neural networks have been introduced for pattern recognition in the trajectory analysis and behavioral responses of fish [18,19]. The coefficient of variation has been used to determine the trout behavioral differences between untreated river-water and the effluent exposure periods [20]. Fukuda et al [21] proposed and applied the entropy of vertical position to detect behavioural response in Japanese Medaka. However, most of them were used to sublethal or lethal concentration and for longer time intervals (e.g., 5-30min) to evaluate their applicability. Further studies on fish exposed to lower concentrations of toxicants should be conducted, in addition to select a simple method matching the fish behavioral changes would benefit the reliability and precision of real-time monitoring.

In the current study, zebrafish was used as a common freshwater model to evaluate the effects of a low concentrations of 0.05 mg·L⁻¹ mercuric chloride (HgCl₂) on school behavior. To identify a first glance of fish, entropy was used to characterize the behavioral stress responses to HgCl₂.

2. Experimental

2.1. Test species and Test chemical
Adult zebrafish were captured from the Institute of Hydrobiology and followed the OECD Guideline TG 203 in semi static test condition [22]. Dechlorinated water maintained at 28.5±1°C and with the concentration of DO at the range of 6.8±0.5 mg/L. Mercuric chloride (HgCl₂), purity 99.5%(m/v), was purchased from company (Sigma, USA). Stock solution of 100 mg/L HgCl₂ was prepared.

2.2. Experimental procedure

2.2.1. Acute Toxicity. Bumping the tail with a pincer, fish was to be considered dead if it does not move. Dead individuals were removed immediately. Zebrafish were not fed during the test period and mortality was recorded at 6, 12 and 24 h.

2.2.2. Behavioral responses. An real time biomonitroing system which was built in Shenzhen Water (Group) Co., LTD., PR China [23]. Five zebrafish were selected and placed into the continuous flow rectangular test tank (400×75×300mm), with a continuous flow (flow rate, 1 min/L). Only dechlorinated water was delivered to record the normal behavioural activity in the 1st hour and then HgCl₂ solution was introduced in the 2ed hour (Figure 1). No food was added during experiments.

Figure 1. School behavioural performance Device.
Swimming trajectories of the five zebrafish were converted into two-dimensional data, x and y coordinates, which facilitated subsequent quantification of endpoints for zebrafish school behavioral performance. Endpoints are defined in Table 1 and calculated every 10s to assess the temporal changes under the impact of toxicants.

**Table 1. Endpoints for zebrafish school behavioural responses analysis.**

| Endpoints (Units) | Definition |
|------------------|------------|
| Swimming activity | Speed (mm s⁻¹) | Average speed of movement. |
|                  | Depth (mm) | Average distance from the original place to the bottom of the test chamber. |
|                  | Turning frequency (n. 10s⁻¹) | Average frequency of the angle of two consecutive movement directions more than 90°. |
| Communication patterns | Average distance (mm) | Average distance between individual fish. |
|                  | Dispersion (%) | The proportion of the pentagon composed by fish of total dimension of the test chamber. |

2.2.3. Data analysis. Parameters were classified into four categories (Table 2). Based on this, the uniformity of the parameters across the defined categories were evaluated by the Shannon-Weaver entropy:

\[
Ent\text{ropy} = -\frac{1}{\log_2 n} \sum_{i=1}^{n} p_i \log_2 p_i \quad (i=1,2,\ldots,n)
\]

Where \( n \) is the number of categories assigned to the endpoint and \( p_i \) is the proportional frequency of endpoints in category \( i \). The values ranged from 0 to 1, where 0 indicates that data were assigned to a single category and 1 indicates that data distributed uniformly. The entropy was counted for each 3-min interval.

**Table 2. Four categories were classified for endpoints.**

| Endpoints | Categories1 | Categories2 | Categories3 | Categories4 |
|-----------|-------------|-------------|-------------|-------------|
| Speed     | 0<S₁<16     | 16<S₂<64    | 64<S₃<70    | S₄>70       |
| Depth     | 0<H₁<50     | 50<H₂<180   | 180<H₃<250  | H₄>250      |
| Turning   | 0<T₁<2      | 2<T₂<17     | 17<T₃<20    | H₄>250      |
| Distance  | 0<D₁<80     | 80<D₂<230   | 230<D₃<280  | D₄>280      |
| Dispersion| 0<Dis₁<6    | 6<Dis₂<18   | 18<Dis₃<28  | Dis₄<28     |

Assumptions of homogeneity of variance across treatments were checked by Levene’s test and one-way analysis of variance (ANOVA) analyzed by Dunett’s test. If homogeneity was not observed, differences between unexposed and exposed conditions were detected by nonparametric statistical comparisons (Wilcoxon test). Statistical tests were performed using GraphPad PRISM software (San Diego, CA).

3. Results and discussion

3.1. Acute Toxicity Test
Acute toxicity, slope and 95% confidence limits of Hg to zebrafish are listed in Table 3. The LC_{50-24h} of Hg for zebrafish is found to be 0.2mg/L. The concentration used for exposure in this study is 0.05 mg/L, about a quarter of the value of 24-h LC_{50}, but it is high enough to trigger a stimulus response.

**Table 3.** Acute static 24-h toxicity experiments of Hg for zebrafish.

| Pesticide | Slope | LC_{50-24h}(mg/L) | 95% Confidence limit |
|-----------|-------|-------------------|----------------------|
| Mercury   | 7.4129| 0.20              | 0.12                 |

![Graphs](image)

**Figure 2.** Description statistics for parameters of zebrafish school behavior exposed to 0.02mg L^{-1} HgCl\textsubscript{2} during 3-min intervals. Dechlorinated water was delivered in the 1\textsuperscript{st} h and then HgCl\textsubscript{2} solution was introduced in next hour.

3.2. Performance and reliability of fish behavior

3.2.1. Behavioral responses. Although variations exist between individuals, the value of behaviour endpoints remained stable, which consistent with Categories 2. These ranges differ from the study of others [15,24] in that school behavior varies between species and water inlet velocity. Either median
or maximum did not change significantly throughout the experimental period under unexposed condition ($p>0.5$) (Figure 2). Hence, the range was regarded as the criterion of normal variation in zebrafish to determine if there are any abnormal behaviours after exposure.

3.2.2. Effects of HgCl$_2$ on zebrafish school behaviour. As shown in Figure 2, there were significant increases in the median of swimming speed ($p<0.05$) and turning frequency ($p<0.001$) during the the period from the 60th to 78th min. The maximum swimming speed rise to 83.5 mm/s and the maximum turning frequency reached to 21.1 n./(10s), but fluctuation range becomes smaller. Meanwhile, the median of distance ($p<0.001$) and dispersion ($p<0.05$) decreased significantly from 65~78 min, and fluctuated in a narrow range. The first stress response with hyperactivity and aggregation lasted only for 15~20 minutes. Then slowly recovered to the level of unexposed condition. No obvious changed in depth. These results match with previous results reported by Zhang et al[24], which showed that in lower concentration treatments test organism was more significant, including stimulation, acclimation and adjustment.

Avoidance as well as increased swimming and aggregation distribution as a first response and then recovery movement behaviour strength to the original level with a substantial increase in fluctuation, as shown in Figure 3. Fish had an obvious behavioral regulation to avoidance and difficulty adjusting to the new environment by physiological function[9,15]. Yet, there was no injury to the fish bodies during that time period, only "purpose avoidance"[25]. Due to 0.05 mg/L concentration is too low to trigger changes in respiration, swimming depth did not immediately change when fish were exposed to Hg. Mercury may be uptake by zebrafish and then gradually accumulated in gills, which caused respiratory damage at last[26]. Increased ventilation can be interpreted as a way to detoxification, but some time was necessary for biological accumulation to cause respiratory damage. The ventilatory rate and amplitude of zebrafish increased significantly after one hour exposure to a series of HgCl$_2$ concentrations[27]. Swimming depth changes lagged behind other parameters for 15min in this experiment.

![Figure 3](image_url). The analysis of the effects of HgCl$_2$ on zebrafish in time series.

3.3. Entropy for detecting behavioural response. Swimming speed, which had a positive correlation to turning frequency, was recognized as the effective indictor for stress response to other toxicants[28].The entropy of speed and turning frequency increased significantly ($p<0.05$) compared to it under the unexposed condition, even up to 0.9. Despite variations between groups, the sum of entropy remained stable over the unexposed period. After exposure to HgCl$_2$, the sum entropy experienced a significant increase ($p<0.001$), even up to 1.72, and then decreased rapidly to the range from 0~0.7 (Figure 4).

Under unexposed conditions, the value of entropy was lower than 0.3 due to swimming parameters changed within the second categorical range, which was equivalent to the based range under unexposed condition. After exposure, the data of the parameters were out of the normal range and distributed randomly across the categories resulting in entropy increased. Since the behaviour change
occurred almost in the same time, the sum of those five parameters showed more reliable and sensitive. The sum of the entropy of five parameters increased immediately and detected the first stress response of zebrafish successfully when exposure to 0.05 mg·L\(^{-1}\) HgCl\(_2\).

Figure 4. The entropy of swimming speed, depth during each 3-min interval, and changes in the sum entropy of speed, depth, turning frequency, distance and dispersion.

4. Conclusions
It is proved that the behavioral responses of zebrafish are rapid and sensitive when exposed to HgCl\(_2\) at 0.05 mg L\(^{-1}\). Avoidance with increased swimming and aggregation distribution is the first stress response and those performances last only 15–20 min. The median of zebrafish school behavior parameters calculated by 3-min intervals is more reasonable and is generally representative of the overall results. Increased swimming for individual fish and aggregation distribution for fish school, the sum entropy of five parameters was a more reliable and sensitive indicator than using a single parameter. It is concluded that entropy contribute to detect toxicants at early stages with biological early warning system.

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