Determination of median lethal concentration of two common heavy metal pollutants in rivers, potassium chromate and cadmium nitrate, to red zebrafish and their mortality causes

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Abstract. In order to investigate the toxicological properties of river pollutants, potassium chromate (K₂CrO₄) and cadmium nitrate tetrahydrate (Cd(NO₃)₂·4H₂O) were selected as they are commonly discharged from industry to nearby rivers. This paper studied the Median Lethal Concentration (LC₅₀) of the pollutants to the adult red zebrafish by acute toxicity experiments within 96 hours. 120mg/L, 130mg/L, 140mg/L, 150mg/L, 160mg/L, 170mg/L potassium chromate solutions and 16mg/L, 20mg/L, 24mg/L, 28mg/L, 32mg/L, 36mg/L cadmium nitrate tetrahydrate solutions were prepared with triplicates to determine the LC₅₀. The results of the preliminary experiments and the calibration curves suggested that the LC₅₀ of potassium chromate and cadmium nitrate tetrahydrate were 165 mg/L and 28 mg/L respectively. The toxicity of thresholds and LC₁₀₀ were also evaluated. Through the observations of the tested subjects in morphological, ethological and anatomical manners, behavioural alterations, oxidative stress, and immunotoxicity were probably their major causes of death.

1. Introduction

Heavy metal pollution is one of the vital environmental issues in China, a large portion of the issue is attributed to cadmium and chromium contaminations. Currently, hexavalent chromium is used in dye manufacture, paints, metal coating, and divalent cadmium is largely used pigment and battery production. China is encountering great challenges caused by cadmium pollution. The Chinese government reported that nearly half of the rice samples taken in a market survey in Guangzhou contained cadmium above permissible levels (Sameer, 2013). Environmental contamination with hexavalent chromium and the associated health effects of Chromium exposure on humans are growing problems both in the U.S and developing countries (Banu et al., 2016). With the great threat cadmium and chromium contaminations pose to human health, it is essential to understand the toxicological characteristics of them and the corresponding symptoms that may encounter once overdosed. Current studies suggested that hexavalent chromium and divalent cadmium can cause developmental defects, physiological and physical disorders (Erika et al., 2012 & Francisco et al., 1999). Therefore, this study predominately aimed at measuring the Median Lethal Concentration (LC₅₀) of potassium chromate and cadmium nitrate tetrahydrate to red zebrafish by using gradient experiments and plotting logarithmic dose-probit curves, comparing their toxicities and determining the causes of deaths through morphological, ethological and anatomical observations. This study was critical since only a few studies carried out comparisons of the toxicities of these two substances. As
interdisciplinary research, this study could be a reference for the future studies on the assessments of their influences on human health.

2. Methodology

2.1. Apparatus and materials

Adult red zebrafish in similar size were used as tested subjects in the study. As it was sensitive to various environmental contaminants (Tanguay, 2018), zebrafish was a prominent vertebrate model for diseases which had already contributed to several examples of successful phenotype-based drug discoveries and toxicity tests (MacRae & Peterson, 2015).

2.2. Experimental procedures

According to the results gained from preliminary gradient experiments with four concentrations, the LC$_{50}$ of cadmium nitrate tetrahydrate and potassium chromate were estimated to be 24mg/L and 150mg/L respectively within 96 hours. In order to specifically determine the LC$_{50}$ within 96 hours, the formal experiments set up gradient experiments with six consecutive concentrations in triplicates. Concentration gradients were set from 120mg/L to 170mg/L with 10mg/L increment for potassium chromate, and from 16mg/L to 36mg/L with 4mg/L increment for cadmium nitrate tetrahydrate. All weights were measured by electric balance, and the prepared solutions were temporarily contained in 1000mL volumetric flasks. 1000mL beakers were used as containers in the experiments, each beaker contained 1000mL solution with 10 adult red zebrafish in similar size. The initiation time, the used substances, the corresponding concentrations and the serial numbers were labelled on the beakers. During the experiment, the number of dead zebrafish in each beaker was recorded every 24 hours. The dead zebrafish were collected and dissected immediately to observe the changes in their body and to prevent the interferences that might be caused by the rotten remains. Additionally, the changes of the living environments (e.g. the turbidity of the water), the morphological and ethological changes of the tested subjects were observed and recorded at the same time.

After the experiments, the remained tested subjects were inactivated in prevention of environmental contaminations and species invasions. Basic statistical values (e.g. mean values, standard deviations etc.) were calculated and analysed using the recorded data; the relationships between the dose and mortality, logarithmic dose and probit units were determined using scatter diagrams to determine the toxicological values (e.g. LC$_{50}$, threshold etc.); the causes of deaths were analysed using observation records.

3. Results

3.1. Mathematical results

In this experiment, 390 red zebrafish were used for the final experiment in total, 127 subjects died during the experiment. Sufficient required information was gained and recorded in Table 1 and Table 2. Additionally, by recording the results of each trial, the figures of dose versus mortality and the figures of Log-dose versus probit unit were plotted in scatter using the mean value of each dose, showing in Figure 1, Figure 2, Figure 3 and Figure 4.

Figure 1 and Figure 2 illustrated the tendency of the death quantity and probability as the dose increased. The trendline reached x-axis at approximately 110mg/L, reached y=10 at 195mg/L in Figure 1. A linear correlation was shown on the plot of logarithmic dose versus probit units.

Figure 3 and Figure 4 illustrated the tendency of the death quantity and probability as the dose increased. The trendline reached x-axis at approximately 13.5mg/L, reached y=10 at 38mg/L in Figure 3. A linear correlation was shown on the plot of logarithmic dose versus probit units.
Table 1. The specific results gained using potassium chromate. Exposed to the toxin for 96 hours.

|        | 120mg/L | 130mg/L | 140mg/L | 150mg/L | 160mg/L | 170mg/L |
|--------|---------|---------|---------|---------|---------|---------|
| First Trial | 9       | 10      | 8       | 9       | 4       | 4       |
| Second Trial | 10      | 8       | 9       | 6       | 7       | 4       |
| Third Trial | 10      | 9       | 8       | 7       | 6       | 6       |
| S.D.        | 0.5774  | 1.0000  | 0.5774  | 1.5276  | 1.5275  | 1.1547  |
| Log-Dose    | 2.0792  | 2.1139  | 2.1461  | 2.1761  | 2.2041  | 2.2304  |
| Mean        | 9.6667  | 9.0000  | 8.3333  | 7.3333  | 5.6667  | 4.6667  |
| Mortality   | 0.3333  | 1.0000  | 1.6667  | 2.6667  | 4.3333  | 5.3333  |
| Probability | 0.0333  | 0.1000  | 0.1667  | 0.2667  | 0.4333  | 0.5333  |
| Probit      | 3.1661  | 3.7184  | 4.0326  | 4.3771  | 4.8321  | 5.0836  |

Table 2. The specific results gained using cadmium nitrate tetrahydrate. Exposed to toxin for 96 hours.

|        | 16mg/L | 20mg/L | 24mg/L | 28mg/L | 32mg/L | 36mg/L |
|--------|--------|--------|--------|--------|--------|--------|
| First Trial | 10     | 7      | 5      | 4      | 2      | 2      |
| Second Trial | 9      | 9      | 7      | 5      | 2      | 1      |
| Third Trial | 9      | 8      | 8      | 6      | 4      | 1      |
| S.D.        | 0.5774 | 1.0000 | 1.5275 | 1.0000 | 1.5275 | 0.5774 |
| Log-Dose    | 1.2041 | 1.3010 | 1.3802 | 1.4472 | 1.5052 | 1.5563 |
| Mean        | 9.3333 | 8.0000 | 6.6667 | 5.0000 | 2.6667 | 1.3333 |
| Mortality   | 0.6667 | 2.0000 | 3.3333 | 5.0000 | 7.3333 | 8.6667 |
| Probability | 0.0667 | 0.2000 | 0.3333 | 0.5000 | 0.7333 | 0.8667 |
| Probit      | 3.4989 | 4.1584 | 4.5693 | 5.0000 | 5.6229 | 6.1108 |

Figure 1. The scatter of dose versus mortality. Exposed to potassium chromate for 96 hours.
Figure 2. The scatter of logarithmic dose versus probit unit. Exposed to the potassium chromate for 96 hours.

Figure 3. The scatter of dose versus mortality. Exposed to cadmium nitrate tetrahydrate for 96 hours.

Figure 4. The scatter of logarithmic dose versus probit unit. Exposed to cadmium nitrate tetrahydrate for 96 hours.
3.2. Observational results
The observational results including the environmental changes of the water, the physiological and physical changes of the red zebrafish. Pictures of some process were shown in appendix section. The most significant change happened to the zebrafish was the imbalance shown in mobile state and stationary state. During the observation, zebrafish presented symptoms of turning the body in horizontal manner with the stomach facing the beaker wall rather than the bottom. The zebrafish swam in the same state with the tail swung in vertical orientations. Feces of the zebrafish in blank tended to be linear shape, yet the one exposed to heavy metal environment tended to be cotton like. Additionally, more feces were observed in the environment as the concentration of the tested substance increased. As the concentration of the tested substances increased, the water in the container tended to be more turbid which could be differentiated with unaided eyes. In dissections of the dead zebrafish, the fish scales were loosely connected with the skin, partially lost before salvage, the remained scales could be easily removed. Haemorrhages were observed before operation. The skin colour of the zebrafish was aggravated by red, large area of blood congestion observed near the fish gill, presenting dark red in colour. The liver of the tested zebrafish presented black colour. After the dissection, the rotten shapeless organs were observed. In addition, several zebrafish that naturally died in the fish tanks were also observed and dissected for comparisons. No observation of obvious skin colour change for the naturally died zebrafish. The scales of the zebrafish could still be removed, however, not as easy as the tested subjects. No blood congestion near the fish gill. No black appeared on the liver of the naturally died zebrafish. Complete shaped organs were observed after dissection.

Nevertheless, some tested zebrafish experienced different results. During the experiment, very few zebrafish gradually lost the colour on their skin, presenting yellow-white in colour. These zebrafish were more readily to die in the experiment and presented totally white colour in body after the death. At the same time, the white-coloured fish were more readily to become rotten after their deaths. Blood vessels of the dead white-coloured red zebrafish could not be seen before dissection.

4. Discussion
The discussion section discussed the analytical data in terms of mathematical results, analysed the causes of death in chemical, biological and physiological manner, then discussed the errors and limitations.

4.1. Data analysis
As the tables and figures showed in the results, the toxicities of the substances that contained heavy metals increased in exponential manner. The threshold dose of potassium chromate was 110mg/L. \( LC_{50} \) of the potassium chromate to the red zebrafish was 165mg/L, indicating that 50% of the red zebrafish exposed to the environment might die. When the dose increased to 195mg/L, the lethal concentration reached 100. The correlation coefficient of the dose – mortality plot indicated strong correlations between the mortality and the dose. Additionally, the linear correlation between the logarithmic dose versus probit units also indicated the strong correlations between one another. By using the linear correlations, the \( LC_{50} \) could also be approximate between 160mg/L and 170mg/L. The \( LC_{50} \) of cadmium nitrate tetrahydrate was 28mg/L, \( LC_{100} \) was approximately 38mg/L, and threshold was 13.5mg/L. Similar to the plot of potassium chromate shown in Figure 1 and Figure 2, both Figure 3 and Figure 4 showed strong correlations. By using the plot of the logarithmic dose versus probit units, the \( LC_{50} \) could also be gained between 24mg/L and 28mg/L yet extremely close to 28mg/L.

By comparing the dose used in the experiment, the potassium chromate was not as lethal as cadmium nitrate tetrahydrate to the subjects. There was approximately no harm to the red zebrafish before the concentration of potassium chromate reached 110mg/L but would be extremely toxic if the same amount of cadmium nitrate tetrahydrate was added to the environment. Nevertheless, the statistical results could be different under actual situations.
4.2. Causes of death

As having been shown by both in vitro and in vivo experiments in a great many reports, heavy metals can exert lethal impacts on zebrafish (Riggio, Filosa, Parisi, & Scudiero, 2003; Risso-de Faverney, Orsini, de Sousa, & Rahammi, 2004; Zhang et al., 2012). Especially, Cr\(^6+\) and Cd\(^{2+}\) both have the potential to trigger the toxicological effects. Since the rates of metabolism of heavy metals are quite low, the Cr\(^6+\) or Cd\(^{2+}\)-induced negative impacts would persist in the zebrafish for a long time (Kusch, Krone, & Chivers, 2008). Based on previous study, the death of zebrafish after the exposure of Cr\(^6+\) and Cd\(^{2+}\) can be caused by behavioural alterations, oxidative stress, and immunotoxicity (Jin et al., 2015).

First, in this study, the locomotor behaviours changed apparently while the dose reached the threshold, indicating that the movement imbalance would be related to exposure to Cr\(^6+\) and Cd\(^{2+}\). The alterations regarding swimming speed and spontaneous movement were observed, which were similar with previous findings (Begum, Venkateswara, & Srikanth, 2006; Mishra & Mohanty, 2008). The induced limited motility would be caused via two ways: one is the alteration in function and composition of the skeletal muscle fibres; another is the induction of neurotoxicity (Jin et al., 2015). The former can directly influence the swimming ability (Capriello et al., 2019). As for the latter, ChEs, a family of enzymes for neurotransmission, was inhibited in a dose-dependent way, thus disruption of nervous system occurred (Domingues et al., 2010).

Second, oxidative stress is also a critical factor in aquatic toxicity, and Cr\(^6+\) and Cd\(^{2+}\) might be involved in this process leading to the zebrafish death (Jin et al., 2015). In accordance with previous study, when exposed to a certain concentration of Cr\(^6+\) and Cd\(^{2+}\), the levels of malondialdehyde (MDA) increased and that of glutathione (GSH) decreased, indicating that Cr\(^6+\) and Cd\(^{2+}\) induced severe oxidative damage in the zebrafish (Jin et al., 2010). Moreover, exposure to Cr\(^6+\) and Cd\(^{2+}\) affected not only the activities of antioxidant enzymes such as superoxide dismutase (SOD), but also the transcriptional levels of their respective genes (Valavanidis, Vlahogianni, Dassenakis, & Scoullos, 2006). However, zebrafish have their self-protection system to prevent them from the oxidative damage by utilizing antioxidant enzymes, which can convert superoxide anions into H\(_2\)O\(_2\) and ultimately into water and oxygen (Lu, Qiao, An, & Zhang, 2018).

Last, Cr\(^6+\) and Cd\(^{2+}\) can result in immunotoxicity, which could be linked with the death of zebrafish (Jin et al., 2015). In this study, black livers and other rotten organs of dead zebrafish were observed, being as similar as those of previous studies (Forn-Cuní et al., 2017; Lu et al., 2018). Jin et al. (2015) have shown that Cr\(^6+\) and Cd\(^{2+}\) could induce or inhibit the mRNA expression of the innate immune-related cytokines such as tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) and interleukin-6 (IL-6). Due to this reason, it culminated into necrosis of the cells (Phelan et al., 2005).

Generally, these factors induced by Cr\(^6+\) and Cd\(^{2+}\) can contribute to multi-toxic effects in zebrafish. Since heavy metals normally accumulate in zebrafish gill, gut, and liver, the multi-toxic effects would trigger the inflammatory responses, such as intestinal cilia defects, liver infiltration, and fuzzy gill structures, which were also observed in this study (Tousoulis et al., 2008). Therefore, zebrafish can be reported to have a high probability of death when suffering from Cr\(^6+\)- or Cd\(^{2+}\)-induced toxicological effects.

4.3. Error & limitations

In this experiment, possible errors might be caused by laboratory equipment, human operations and control of variable conditions.

In the case of experimental instruments, the 1000mL plastic volumetric bottles might have the potential to cause errors due to the deformability of plastics. In addition, in terms of operations, the inexperienced operators could make mistakes in weighing and transferring of the substances in small quantity experiments.

As for the control of variable conditions, including the changes of temperature in the outer environment, and the water quality were considered as the key factors that might cause errors.
Additionally, the water became turbid due to the detoxification of zebrafish during the experiment, and the open container may also cause changes in dissolved oxygen in the water.

4.4. Future directions
Since the limitations of the experiment might influence the accuracy of zebrafish toxicity test, researchers have pursued various approaches to increase the accuracy in view of the limitations, the following aspects will be discussed: (i) ADME studies; (ii) machine learning for objective morphological evaluation in toxicology tests; (iii) a systems toxicology approach for human risk assessment.

4.4.1. ADME studies. Although it has shown that zebrafish toxicological assays can attain a good level of predictivity, false negatives and false positives have been found to compromise the sensitivity and specificity of the assays used (Mesens, 2015). This is due to the importance of understanding the absorption, distribution, metabolism and excretion (ADME) of drugs between zebrafish and other mammalian models has often been overlooked, and the ADME characteristics of drugs are in turn influenced by such factors as the route of administration, the physicochemical nature of the drugs and the physiology of the fish. Thus, in order to fully realize the potential of zebrafish as a toxicological model, the knowledge gap in defining the effects of ADME on drugs and their metabolites in the zebrafish needs to be addressed.

4.4.2. Machine learning for objective morphological evaluation in toxicology tests. During the experiment, the physiological and physical changes in zebrafish were observed. Based on limited experimental equipment and knowledge, the observations were taken only by naked eyes and simple anatomy. And this kind of evaluation of morphology may be prone to subjectivity, so more accurate results need to be achieved by means of automated data acquisition methods and images obtained through defect identification and classification. Machine learning can be used to classify a variety of developmental toxins, including the degree of looseness at the skin junction, dilatation of the blood vessels near the gills and decay of the organs, especially the liver. Machine learning could also be used in detection of morphological changes.

4.4.3. A systems toxicology approach for human risk assessment. By simply making a comparison between the toxicity and morphological changes on zebrafish caused by the two heavy metals might not be enough for exploring the toxicity mechanism of human body, and a much more nuanced understanding of the parallels between the zebrafish and higher organisms in health and in disease is required.

The widely used measurement methods in systems toxicology such as transcriptomic analysis can be a useful established approach for identifying perturbed biological networks and thereby gaining mechanistic insight into the system’s response to an exposure (Sturla et al., 2014). Through performing transcriptome analysis of zebrafish exposed to chemical compounds, it was less likely to identify both commonly and selectively expressed genes induced by the toxicants, suggesting that there may be common and selective modes of action in these toxicants, which can be also helpful for further predicting the developmental toxicity.

5. Conclusion
In conclusion, based on gradient experiments of red zebrafish exposed to the potassium chromate and cadmium nitrate tetrahydrate, the toxicities and behaviours of these two chemicals in an environmentally relevant aqueous exposure setting have been characterized. The LC50 of the potassium chromate and cadmium nitrate tetrahydrate were 165mg/L and 28mg/L respectively, Thresholds, LC100 were also estimated. In addition, the environmental changes occurred in the experiments including the increased turbidity and the deformed feces, the morphological, ethological and anatomical changes of the red zebrafish had been observed and analysed to determine the causes of deaths. According to the
research, most of the dead subjects died due to behaviour alterations, oxidative stress, and immunotoxicity. The possible errors and limitations of this experiment have been discussed and categorized into instrumental errors, personal errors, and environmental errors. This study highlighted the need for a comprehensive toxicological assessment and suggested that relevant approaches need to be applied in future studies to facilitate a convincing model for human health risk assessment.

6. Appendices

Figure A.1 (a) showed the naturally dead fish from the fish tank, (b) showed the tested subject. Clear black dot shown on the tested subject while no black dot observed on naturally dead fish. The Black dot was located near the liver. (b) clearly showed the removed scales of the tested subject. The scales could be removed by merely touching the dead body.

Figure A.2 Clear differences in water turbidity could be observed by comparisons. (b) showed more turbid water. Additionally, the feces of the fish are abnormal with cotton-shapes.
Figure A.3 Most of the dead tested subjects had black colour on the liver. Some of the test subjects have faded the red colour while some enhanced.

Figure A.4 Dissections of the tested subjects. Most of the tested subjects' organs had already rotten with no shape observed. The only organ remained on the pictures was the swim bladder. The position where the organs used to locate presented dark red or black colour. Assumptions were made that the test subjects experienced organ failure before their deaths.

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