

## TOXIC EFFECTS OF CHLORPYRIFOS AND Cr<sup>6+</sup> ON RED CALIFORNIAN EARTHWORM (*EISENIA FETIDA*)

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**Abstract.** *Eisenia fetida* are commonly used for water and soil ecotoxicology tests. Here, we use *Eisenia fetida* as a model organism to investigate the effect of cartap and Cr<sup>6+</sup> on the physiological and biochemical indicators, such as mortality, protein content, superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA). The results showed that with an increasing content of cartap, protein contents in *Eisenia fetida* increased in the beginning, then decreased, and finally stabilized after prolonged exposure. In the case of increasing Cr<sup>6+</sup>, protein contents in *Eisenia fetida* decreased in the beginning, then increased, and finally decreased after prolonged exposure. The SOD activity in *Eisenia fetida* was simulated under low concentration and short durations of contaminations, while the SOD activity decreased after prolonged exposure. Meanwhile, under low contaminations, CAT contents were stimulated. The MDA contents in *Eisenia fetida* were significantly negatively correlated with SOD activity. This indicated that when the organisms are subjected to pollutant stress, the activity of SOD will be rapidly induced for removing excess oxygen free radicals and producing H<sub>2</sub>O<sub>2</sub>, accompanied by enhanced activity of CAT converting H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O. With the prolonged exposure time, the SOD activity decreased, and the organisms were harmed by oxidative damage with enhanced MDA contents.

**Keywords:** *chlorpyrifos, ecotoxicity, superoxide dismutase, catalase, malondialdehyde*

### Introduction

With the rapid development of China's industrialization, heavy metal content in farmland soils and water bodies severely exceeds the standard, seriously affecting the quality of agricultural products and endangering human health (Yu and Liu, 2011). This issue gained the attention of all countries. Heavy metal pollution does great harm to aquatic and soil ecosystems, affecting the physiological activities of aquatic plants such as respiration and photosynthesis, and inhibiting their growth (He et al., 2008). Affect the metabolism and growth of aquatic animals, such as cadmium affecting fish growth, interfering with fish endocrine (Wang et al., 2015). Heavy metals cause loss of activity in proteins and enzymes in the human body, accumulating in certain organs of the human body, causing chronic poisoning (Zhao et al., 2007). Among them, Cr<sup>6+</sup> and Pb are more toxic, and they are more easily absorbed in the human body and are carcinogenic to the human body (Teng et al., 2014). When pesticides are applied, they are easy to remain on the surface of soil and agricultural products. Under the action of enzymes, metabolic processes such as hydrolysis, dechlorination and oxidation occur, and are transmitted to the human body through the food chain. In particular, some of the larger molecular weights are more soluble and easily absorbed by crops (Chen, 2014; Xia, 2008; Huang, 2002). When pesticides are applied, they are easy to remain on the surface of soil and agricultural products. Under the action of enzymes, metabolic processes such as hydrolysis, dechlorination and oxidation occur, and are transmitted to the human body

through the food chain. In particular, some of the larger molecular weights are more soluble and easily absorbed by crops (Xiao and Zhao, 2005). In addition, crops also absorb some residual pesticides from the soil and enter the crops through enrichment, which affects crop safety (Liu et al, 2002).

Traditional chemical analysis methods can accurately quantify the content of major components in pollutants, but can not directly reflect the environmental and biological toxicity of various toxic substances (Su et al, 2011). The biotoxicity method is a rapid monitoring method that reflects the overall toxicity of various toxic pollutants to the environment (Liao and Chen, 2007). Is a oligochaete terrestrial animal, one of the most biomass soil organisms, and a source of food for many birds and other organisms. It is located in the terrestrial ecosystem at the lowest level of the bio-chain and is easier in the food chain. The transfer of pollutants plays an important role in maintaining the stability of farmland ecosystems and the entire soil ecosystem, but it is likely to have more enrichment of heavy metals and pesticides in the body. In addition, alfalfa can improve the soil structure through feeding, excavation, etc., help the degradation of organic decay substances in the soil, maintain soil fertility, and affect soil physical and chemical properties. Because the soil moisture content, pH value and other changes can affect the cockroaches, cockroaches are highly sensitive to pollutants, and are highly susceptible to pollutants leading to changes in survival, genetics, etc. Therefore, cockroaches as ecotoxicology indicator organism for facilitating related research (Wang et al, 2012). Thus, our study aimed at (i) studying the toxic effects of Cr<sup>6+</sup> on *Eisenia fetida*, and (ii) determining whether *Eisenia fetida* is suitable as an indicator of soil ecotoxicity.

## Materials and methods

### *Acute toxicity test*

Chlorpyrifos, with a purity of 98.8%, from Shanghai Institute of Pesticide; potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, Purchased from Sinopharm Chemical Reagent Co., Ltd. Before the experiment, the chlordane and potassium dichromate were prepared and formulated into a 1000 mg·L<sup>-1</sup> stock solution, which was sealed and stored at 4 °C for use. *Eisenia fetida*, Purchased from a professional carp farm in Jiangsu (*Fig. 1a*). Before the test, it was pre-cultured for a period of time in the laboratory. During the test, adult mature ticks with a body weight of about 0.3 g were selected.

### *Experiment method*

#### *Clean up the gut of the earthworm*

Pick up the sexually mature adult cockroaches in the pre-cultivated red scorpion, wash them with ultrapure water and put them into a beaker. The bottom of the beaker is covered with filter paper and moistened with appropriate amount of water. Seal with plastic wrap and use the anatomical needle. Make holes in the cling film. Place the beaker in a constant temperature incubator at 20 ± 1 °C, humidity 80 to 85%, and clear the intestines for 1 day and night in the dark.

#### *Contamination*

The median expected toxicity was determined according to the literature. Pre-experiment was performed before the formal test to find the maximum lethal

concentration LD<sub>100</sub> and the minimum lethal concentration of the pollutants, and the formal test concentration was set according to the preliminary experiment. The double-layer double-circle qualitative filter paper was placed in a glass culture dish, and 4 mL of the test concentration reagent was accurately absorbed on the filter paper, and 10 replicates with one test organism were set for each treatment, and a blank control group was set at the same time. Rinse the sputum after the intestines and blot the excess water on the surface and place one in each glass dish for the test. Seal with plastic wrap, place the glass culture dish in a constant temperature incubator at  $20 \pm 1$  °C, adjust the humidity to 80% to 85%, culture in the dark for 72 h, and at 24 h, 48 h and 72 h recorded the survival of sputum.



**Figure 1.** (a) The test organism *Eisenia fetida*; (b) sexually mature adult worms; and (c) toxicity test

### **Data processing**

The dose-effect model (DRC) of the toxicity of mixed pollutants was nonlinearly fitted by origin 8.5 software, and the LC<sub>50</sub> values of the earthworms 24 h, 48 h and 72 h were calculated by nonlinear regression equation and passed SPSS software. Calculate a 95% confidence interval. The significance of different treatment was tested using Duncan's multiple-range test at  $p < 0.05$  after one-way analysis of variance (ANOVA) in SPSS 20.2.

### **Experiment on earthworm physiological and biochemical indexes**

#### **Pollutant addition method**

##### **1. Blank control of experimental methods**

In the blank test, 4 mL of deionized water was accurately absorbed and uniformly poured onto the glass culture dish with double layer and double coil qualitative filter paper.

##### **2. Method of adding pyrrolidin**

Accurately weigh the standard product of pyrrolide 1.02 g, dissolve with deionized water at a constant volume of 100 mL volumetric flask, configured into 10,000 mg·L<sup>-1</sup> pyrrolide standard product mother liquid, is now used. Deionized water was used to dilute the mother solution of pyrrolidin with the concentration gradient of 0.5, 1.0, 1.5, 2.0 and 2.5 mg·L<sup>-1</sup>, respectively. Double layer and double coil qualitative filter paper was placed in a 9 cm diameter glass culture dish, and the diluted solution 4 mL was uniformly poured on the filter paper.

### 3. Add methods of Cr<sup>6+</sup>

Accurately weigh the dried potassium dichromate standard 0.565 g, dissolve it in water, use deionized water to fix the volume of 100 mL volumetric flask, and configure it into 1000 mg·L<sup>-1</sup> potassium dichromate standard storage liquid. The mother solution of potassium dichromate was extracted and diluted with deionized water into 5 experimental solutions with concentration gradient of 1.0, 2.0, 3.0, 4.0 and 5.0 mg·L<sup>-1</sup>. Double layer and double coil qualitative filter paper was placed in a 9 cm diameter glass culture dish, and the diluted solution 4 mL was uniformly poured on the filter paper.

#### *Earthworm in vivo biochemical index test experiment*

##### 1. Test for determination of protein content

Earthworm samples from each treatment group at 24 h, 48 h and 72 h of exposure were collected, and the weight of the tissue to be measured was accurately weighed. 0.86% normal saline was added according to the proportion of weight (g): volume (mL) = 1:9. Mechanical homogenation was performed in ice water bath. The supernatant was diluted into 2% tissue homogenate with normal saline at 1:4, and was to be tested.

Blank tube, standard tube and measuring tube were set up in the experiment, in which double steaming water was added into the blank tube, protein standard substance 0.563 g/L was added into the standard tube, and homogenized earthworm tissues were placed in the measuring tube. In the test, the sample was first added with coomath bright blue color developing liquid, mixed and set for 10 min, then the absorbance value of each tube was measured at 595 nm by a colorimetric cup with a light diameter of 1 cm with ultraviolet spectrophotometer and double steaming water for zero adjustment. According to the measured absorbance, the protein content of the tissue homogenate with 2% concentration of earthworm sample was determined by the calculation formula shown below:

$$\text{Protein Concentration in the Sample (gprotj/L)} = \frac{\text{Measured value (OD)} - \text{Blank value (OD)}}{\text{standard value (OD)} - \text{Blank value (OD)}} \times \text{Standard concentration} \times \text{Dilution multiple before sample determination} \quad (\text{Eq.1})$$

##### 2. Determination of total superoxide dismutase activity

Respectively collected 24 h, 48 h and 72 h exposure when the earthworm samples of each treatment group accurately according to weight of the organization, by weight (g): the ratio of volume (mL) = 1:9 add 0.86% saline, ice water bath conditions, mechanical preparation of 10% homogenate and serum, use refrigerated centrifuge (Thermo Multifuge X1R) homogenate good earthworm tissue fluid under the condition of 2500 r/min centrifuge for 10 min. Remove the supernatant and test.

Test tube and set up the determination of the tube and the care, determination of the tube with samples, the care of the same volume of double steaming water, room temperature static 10 min, the use of ultraviolet spectrophotometer (Multiskan™ GO, Thermo Scientific), double steaming water zero, light diameter 1 cm colorimetric cup at the wavelength of 550 nm, determine the absorbance value of each tube. Because SOD has a specific inhibitory effect on superoxide anion radicals, resulting in the formation

of nitrite reduction, colorimetric determination of the tube absorbance value is lower than the care of the absorbance value, substituted into the formula to calculate SOD activity. The calculation formula is as follows:

$$\begin{aligned} \text{Total vitality (U/mgprot)} = & \frac{\text{Control value (OD)} - \text{Measured value (OD)}}{\text{Control value (OD)}} \div 50\% \\ & \times \frac{\text{Total volume of reaction liquid}}{\text{Sampling quantity}} \\ & \div \text{Protein Concentration in the Sample to be Measured (mgprot/ml)} \end{aligned} \quad (\text{Eq.2})$$

### 3. Determination of catalase (CAT) activity

Respectively collected 24 h, 48 h and 72 h exposure when the earthworm samples of each treatment group accurately according to weight of the organization, by weight (g): the ratio of volume (mL) = 1:9 add 0.86% saline, ice water bath conditions, mechanical preparation of 10% homogenate and serum, use refrigerated centrifuge homogenate good earthworm tissue fluid under the condition of 2500 r/min centrifuge for 10 min. Take supernatant and normal saline (0.86%) and prepare 5% tissue homogenate according to 1:1 volume ratio.

The care and measurement tubes were operated according to the kit purchased by Nanjing Jiancheng Institute of Biological Engineering.

Ultraviolet spectrophotometer, double evaporation water zero, light diameter 0.5 cm colorimetric cup at the wavelength of 240 nm colorimetric, determine the absorbance value of each tube. The calculation formula is as follows:

$$\begin{aligned} \text{CAT vitality (U/mgprot)} = & \frac{(\text{Control value (OD)} - \text{Measured value}) \times 271}{\text{Protein Concentration in the Sample to be Measured}} \\ & \times \frac{1}{60 \times \text{Sampling quantity}} \end{aligned} \quad (\text{Eq.3})$$

### 4. Determination of malondialdehyde (MDA)

Earthworm samples from each treatment group at 24 h, 48 h and 72 h of exposure were collected, and the weight of the tissue to be measured was accurately weighed. 0.86% normal saline was added according to the proportion of weight (g): volume (mL) = 1:9. Mechanical homogenation was performed in ice water bath. Take the supernatant and dilute it with normal saline at 1:4 to 2% tissue homogenate for testing.

The test set blank tube, standard tube, measuring tube and pair of care, according to the operation of the kit, the use of ultraviolet spectrophotometer, double steaming water zero, light diameter 0.5 cm colorimetric cup at the wavelength of 405 nm colorimetric, determine the absorbance value of each tube. The calculation company is as follows:

$$\begin{aligned} \text{MDA content (nmol/mgprot)} = & \frac{\text{Measured value (OD)} - \text{Blank value (OD)}}{\text{standard value (OD)} - \text{Blank value (OD)}} \\ & \times \text{Standard concentration} \\ & \div \text{Protein Concentration in the Sample to be Measured} \end{aligned} \quad (\text{Eq.4})$$

## Results

### *Toxicity test of chlorpyrifos and Cr<sup>6+</sup> on earthworms*

The toxicity test results of chlorpyrifos and Cr<sup>6+</sup> on earthworms are shown in *Table 1*. The results show that both drugs have a certain degree of toxicity on earthworms, and the toxicity increases with the extension of exposure time. The experimental results showed that the survival rate of earthworms was 100%. When the poisoning time was 24 h, the single toxicity value of chlorpyrifos and Cr<sup>6+</sup> on earthworms was significantly different, and the order of toxicity was > Cr<sup>6+</sup> of chlorpyrifos. Among them, the most toxic was pyridin, with a 24-h LC<sub>50</sub> value of 14.30 (13.57-15.02) mg·L<sup>-1</sup>, followed by Cr<sup>6+</sup>, and a 24-h LC<sub>50</sub> value of 104.42 (99.20-109.64) mg·L<sup>-1</sup>. Two drugs are longer duration of canister and toxic, canister for 48 h, chlorpyrifos, Cr<sup>6+</sup> + 48 h-LC<sub>50</sub> were 12.36 (11.74 12.98), 71.53 (67.95 75.11) mg·L<sup>-1</sup>, toxicity increased 1.16 and 1.46 times respectively, chlorpyrifos toxicity is still the strongest, is 5.79 times that of Cr<sup>6+</sup> infected time for 72 h., chlorpyrifos, Cr<sup>6+</sup> toxicity continue to strengthen, the strongest toxicity is still chlorpyrifos, 72-h LC<sub>50</sub> value was 10.43 (9.91-10.95) mg·L<sup>-1</sup>, and the toxicity of cyanilon and Cr<sup>6+</sup> was 1.19 times and 1.78 times stronger than that of cyanilon 48 h after exposure, respectively.

**Table 1.** Single toxicity of cartap, Cr<sup>6+</sup> on earthworm

	24 h LC <sub>50</sub> mg·L <sup>-1</sup>	95% confidence interval mg·L <sup>-1</sup>	48 h LC <sub>50</sub> mg·L <sup>-1</sup>	95% confidence interval mg·L <sup>-1</sup>	72 h LC <sub>50</sub> mg·L <sup>-1</sup>	95% confidence interval mg·L <sup>-1</sup>
Methyl ethyl chloride	14.30	13.57-15.02	12.36	11.74-12.98	10.43	9.91-10.95
Cr <sup>6+</sup>	104.42	99.20-109.64	71.53	67.95-75.11	40.21	38.20-42.22

### *Effects of pyridin, Cr<sup>6+</sup> and the biochemical indexes on earthworms*

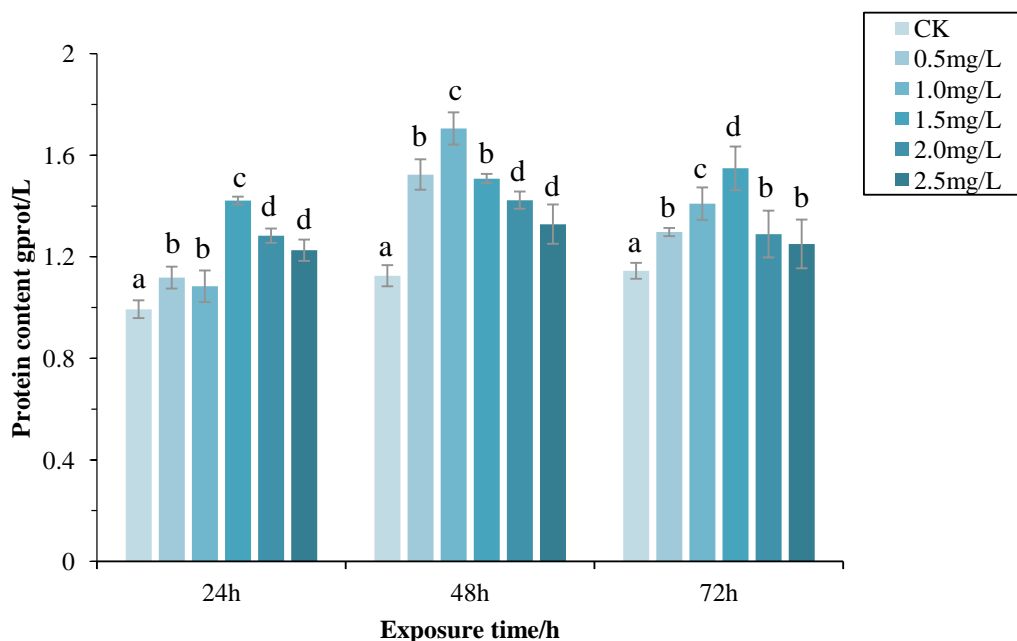
#### *Effects of pyridin and Cr<sup>6+</sup> on the protein content of earthworms*

Protein is composed of 20 kinds of amino acid, is an important part of the cell is the foundation of many life processes, is responsible for performing the life activities of the main functional molecules, easily happened due to the impact of certain factors in degeneration or other changes, thus could be obtained by using the studies of the changes of protein material toxicity mechanism of relevant information. Studies have shown that soil contaminated with pesticides and heavy metals can cause changes in the protein content of earthworms.

The experiment set up five treatment groups of 0.5, 1.0, 1.5, 2.0 and 2.5 mg·L<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h respectively, and the protein content was determined. The results were shown in *Figure 2*.

As can be seen from *Figure 2*, the protein content of earthworms changed to different degrees after the exposure of pyridin at different concentrations. The protein content of the infected earthworms was higher than that of the blank control group, which may be caused by the stimulation of pollutants and the stress proteins produced by the earthworm body, such as c-reactive protein (CRP) and fibrin connective protein (Fn), etc., which may lead to the increase of the protein content of earthworms (Ling, 2004). When the exposure time of 24 h, the earthworm body protein content increased with the

increase of concentration of test presents a trend of reducing the rise, were relatively blank control group increased by 12.9%, 12.9%, 43.57%, 29.59% and 23.8%, the concentration of 1.5 mg·L<sup>-1</sup> when the maximum, the protein content of 144% times as blank group, then gradually reduce the protein content, may be due to chlorpyrifos has strong tag and stomach poison, start acting on earthworm skin table, send the earthworm appear after stress reaction or worms infected body metabolic disorders cause the protein content is higher, With the increase of the concentration, the toxin ACTS on the stomach of earthworms and destroys the protein synthesis, resulting in the decrease of protein content (Xue and Pei, 2000).

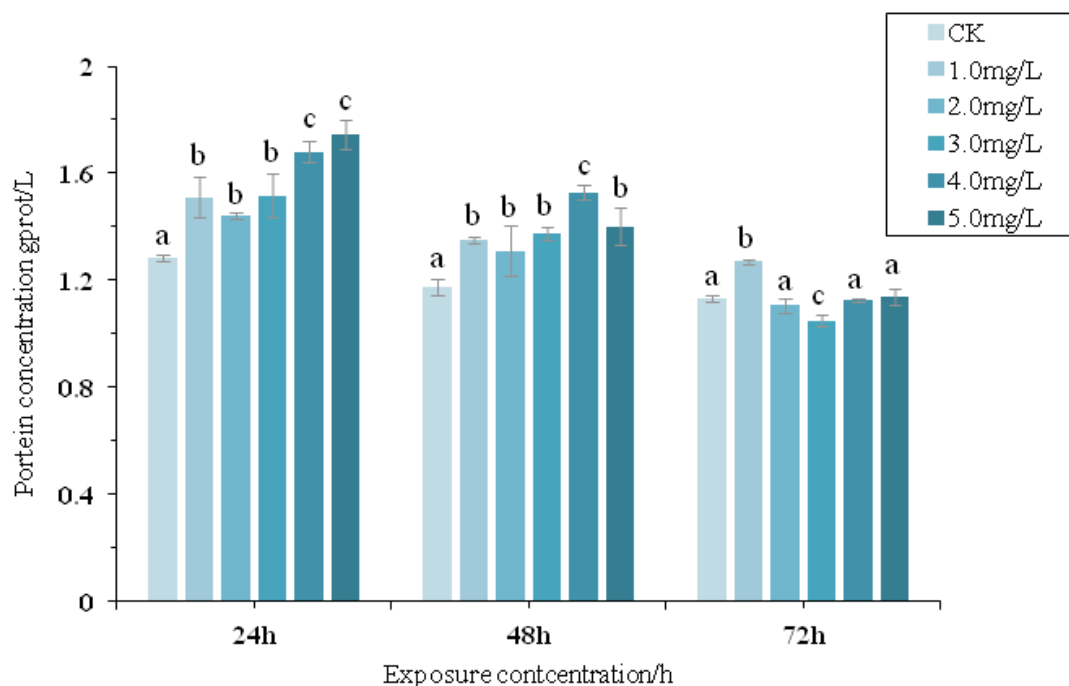


**Figure 2.** Effect of cartap with different concentrations on protein contents of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

Earthworms were treated with Cr<sup>6+</sup> solution of 1, 2, 3, 4 and 5 mg·L<sup>-1</sup>, and earthworms were collected at 24, 48 and 72 h for protein content determination. The results are shown in *Figure 2*.

As can be seen from *Figure 3*, the protein content of blank treated earthworms decreased slightly with the exposure time and was lower than that of Cr<sup>6+</sup> infected earthworms, which may be related to the stress protein produced by the body stress response of earthworms after being poisoned. When the exposure time was 24 h, with the increase of Cr<sup>6+</sup> concentration, the protein content of earthworms presented an increasing trend, indicating that earthworms produced more stress proteins in the short term, which led to the increase of protein content in the body. When the exposure time was 48 h, the content of earthworm in vivo was first decreased, then increased and then decreased. When the Cr<sup>6+</sup> concentration was 4 mg·L<sup>-1</sup>, the maximum value was 1.52 gprot/L, which was 1.30 times higher than that of the blank group. At 72 h of exposure, the change trend of protein content first decreased and then increased to a stable level, and it was lower than the blank value when the Cr<sup>6+</sup> concentration was

3 mg·L<sup>-1</sup>, which may be due to the fact that with the extension of exposure time, earthworms were stressed and the absorption of digestive tract cells was destroyed (Sun et al, 2011). This leads to decreased protein content.



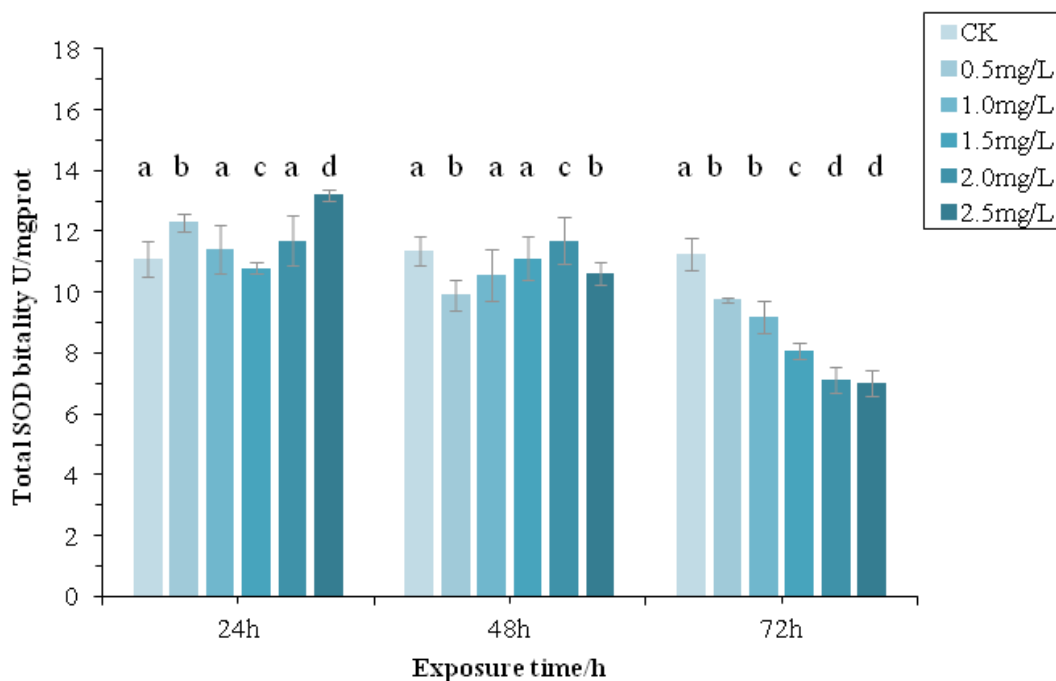
**Figure 3.** Effect of Cr<sup>6+</sup> with different concentrations on protein contents of earthworm. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

#### Effect of pyroridin and Cr<sup>6+</sup> on SOD content in earthworms

The experiment set up five treatment groups of 0.5, 1.0, 1.5, 2.0 and 2.5 mg·L<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h, respectively, to determine the content of SOD in vivo.

It can be seen from *Figure 4* that the SOD activity of earthworms infected with chlorpyrifos at different concentrations showed different trends in different poisoning time. SOD activity first decreased and then increased at 24 h after poisoning, then increased at 48 h and then decreased at 72 h after poisoning. Exposure time of 24 h, the low concentration of chlorpyrifos examination in the SOD activity of SOD vitality is higher than the blank group, were higher than that of blank control group 10.93%, 3.02%, 2.06%, 5.51% and 19.04%, obviously when the concentration of 1.5 mg·L<sup>-1</sup> basic comparative with the SOD vitality of blank control group, with the increase of concentration, the higher SOD vigor, chlorpyrifos is mainly applied to earthworms in the stomach, in the early infected by epidermal cells after absorption, the body's antioxidant defense response, SOD activity was induced. Infected time 48 h, chlorpyrifos role in earthworms in the stomach, earthworms affects the body metabolism, inhibit SOD activity, lower overall than 24 h, and SOD activity in vivo in contrast to the 24 h trends, the concentration of 2.0 mg·L<sup>-1</sup>, reach the maximum, then the SOD activity decreased, but speculation is that as the extension of exposure time, SOD is consumed, and enzyme activity reduced. Exposure time of 72 h, the earthworm

body total SOD vigor decreasing trends, and much lower than the blank control group, who is probably the most prolonged and the increase of concentration, SOD activity significantly reduced, because the earthworm when suffer less stress, increased the amount of oxygen free radical, in order to remove excess oxygen free radicals and reduce the membrane lipid peroxidation, induction of SOD content increases, the stress levels increase, the body is not enough to resist oxidation, severely damage, enzyme activity is also reduced (Xue and Pei, 2000).

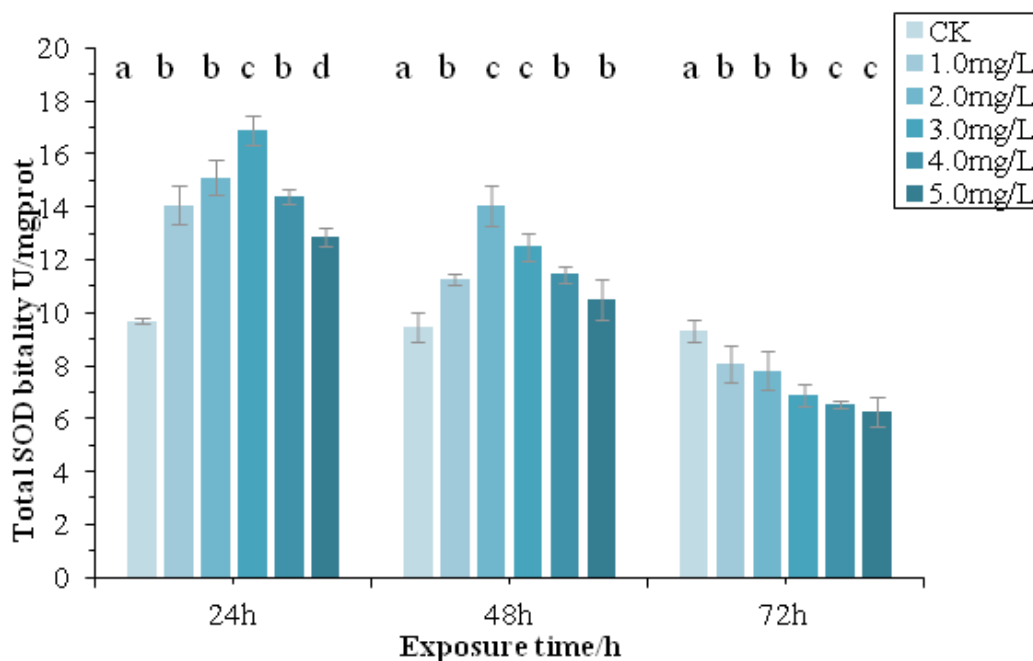


**Figure 4.** Effect of cartap with different concentrations on SOD activity of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

In the experiment, Cr<sup>6+</sup> was set as the concentration of 1.0, 2.0, 3.0, 4.0 and 5.0 mg·L<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h, respectively, to determine the content of superoxide dismutase (SOD) in vivo.

By Figure 5, exposed to the Cr<sup>6+</sup>, earthworms + pollution, the SOD activity in the body as the canister to the extension of time is reduced, the highest SOD activity in 24 h, may be due to the superoxide dismutase (SOD) is the first line of organism to resist oxidation, so under pollution stress the body to make quick oxidation reaction, induction of enhanced SOD activity, remove excess oxygen free radicals, avoid lipid oxidation reaction. Infected when the time for 24 h, according to the processing of SOD activity were blank control group 145%, 156%, 174%, 148% and 133%, with the increase of concentration of Cr<sup>6+</sup> processing in earthworm body SOD activity showed a trend of lower after the first rise, the maximum SOD activity was in Cr<sup>6+</sup> concentration of 3.0 mg·L<sup>-1</sup>, compared with the blank control group, SOD activity increased by 74.12%. SOD activity decreased at 72 h after exposure compared with that at 24 h and 48 h after exposure, which may be because earthworms were severely affected by Cr<sup>6+</sup>

stress, the body was seriously damaged, and SOD activity decreased. The above results showed that Cr<sup>6+</sup> pollution could significantly induce SOD activity in earthworms at the early stage of infection. When the exposure time was more than 48 h, the SOD activity in earthworms decreased significantly. Under the stress of heavy metal pollution, earthworms produce a large number of oxygen free radicals in the body. In order to reduce the damage caused by oxygen free radicals to themselves, antioxidant enzyme activity increases. However, with the extension of the exposure time, the body is seriously damaged, and various internal balances are broken, and enzymes are gradually consumed to maintain the steady state, thereby reducing the SOD activity.



**Figure 5.** Effect of Cr<sup>6+</sup> with different concentrations on SOD activity of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

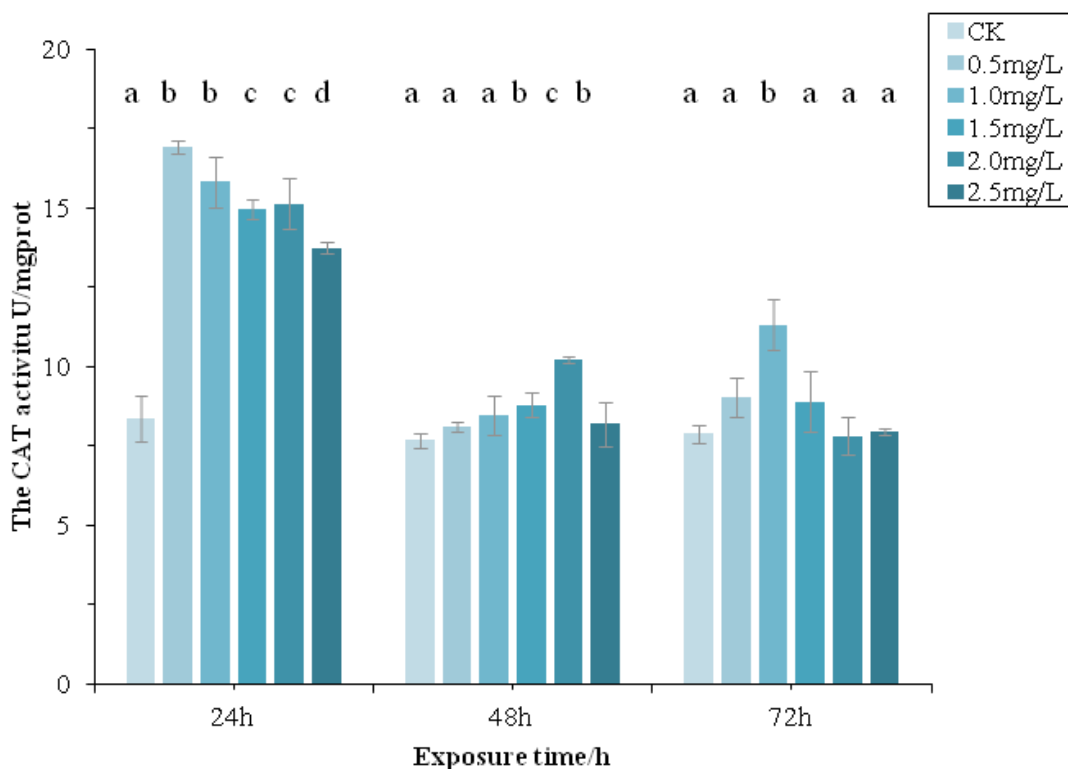
#### Effect of pyroridin, Cr<sup>6+</sup> and Pb on the content of CAT in earthworms

CAT as a biological enzyme systems is an important antioxidant defensive function, existence of red blood cells in most organisms, and some cells of oxygen in the body, usually with the GSH-Px synergy, the product of the free radicals SOD disproportionation H<sub>2</sub>O<sub>2</sub> into harmless H<sub>2</sub>O and O<sub>2</sub>, eliminate free radical and lipid peroxide formation, pollutants stress can lead to the active content changes, so the test in earthworm body of CAT as a biomarker research.

The experiment set up five treatment groups of 0.5, 1.0, 1.5, 2.0 and 2.5 mg·L<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h, respectively, to determine the catalase (CAT) content in vivo.

From Figure 6, you can see that when infected 24 h, the earthworm treated by chlorpyrifos induced rapidly rising, the CAT activity was obviously in the kill moth when chlorpyrifos is 0.5 mg·L<sup>-1</sup> peak, with the increase of chlorpyrifos concentration, the CAT inside its body content is on the decline, probably because with the increased

concentration of cells in the body chlorpyrifos, nature is the CAT activity. When infected time for 48 h, earthworm body CAT activity increased with the increase of concentration of chlorpyrifos was reduced after the first rise trend, when chlorpyrifos is 2.0 mg · L<sup>-1</sup> peak, compared with 24 h, slash, CAT content decrease respectively 52.18%, 46.51%, 41.24%, 46.51% and 41.24%, may be due to the early infected cells absorb less chlorpyrifos, vicarious induced the CAT activity increased, with the extension of time, the activity of CAT back to normal levels. CAT activity increased first and then decreased with the increase of pyrrolidan concentration at 72 h, and reached the maximum when pyrrolidan concentration was 1 mg·L<sup>-1</sup>. Compared with 48 h, CAT activity was more stable, which may be due to the adaptive reaction of earthworms to pyrrolidan, and the enzyme activity reached a stable state.

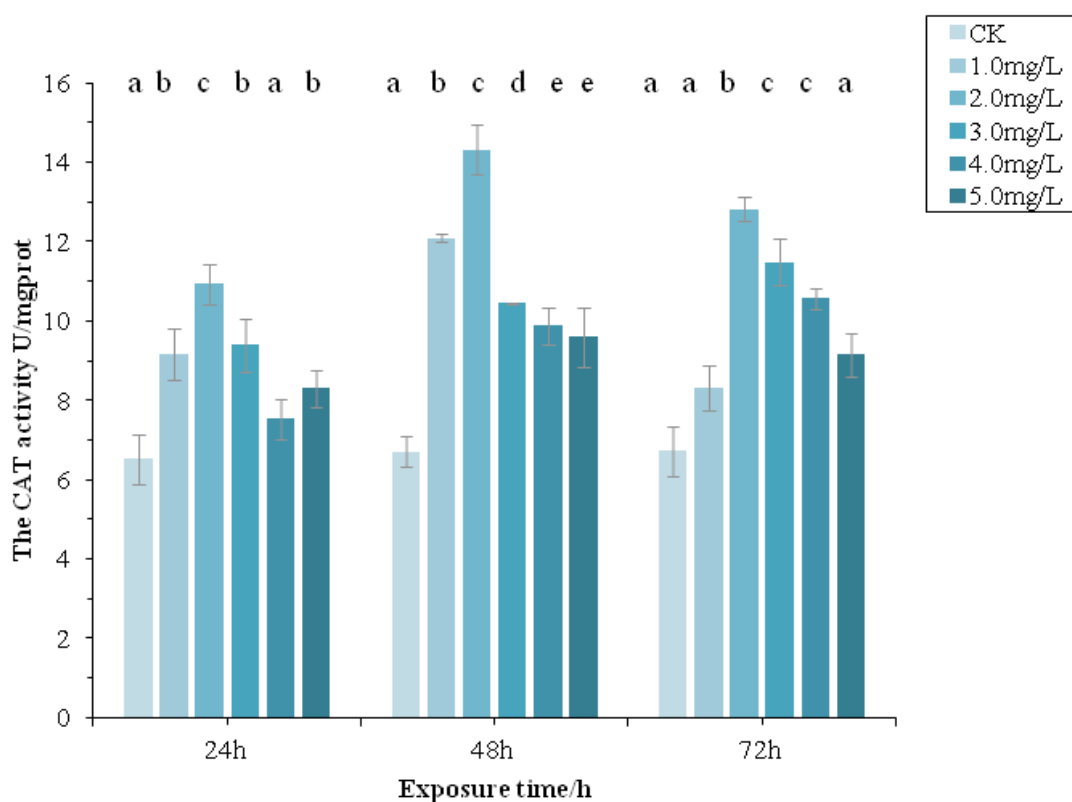


**Figure 6.** Effect of cartap with different concentrations on CAT activity of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan’s multiple-range test at  $p < 0.05$

In the experiment, Cr<sup>6+</sup> was set as the concentration of 1.0, 2.0, 3.0, 4.0 and 5.0 mg·l<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h, respectively, and the CAT content in vivo was determined, as shown in Figure 7.

By Figure 7 you can see that when infected time for 24 h, the earthworm body CAT activity of Cr<sup>6+</sup> treatment was obviously higher than that of blank control group, obviously with the increase of concentration of Cr<sup>6+</sup> CAT activity is “S” shape change, Cr<sup>6+</sup> concentration of 2 mg·L<sup>-1</sup> peak, is at the beginning of the canister, the earthworm intimidation, induction of SOD and CAT activity, CAT is mainly used to produce the H<sub>2</sub>O<sub>2</sub>, consumption of SOD with elevated Cr<sup>6+</sup> concentration, the cell absorption Cr<sup>6+</sup>

increase, SOD activity continues to strengthen, to produce a large amount of H<sub>2</sub>O<sub>2</sub>, The activity of CAT was inhibited. With the increase of concentration, SOD activity decreased and CAT activity recovered. When the time of exposure was 48 h, the time of exposure was prolonged and the stress of the body was enhanced. The CAT activity of earthworms in each group was increased to reduce the oxidative damage of the body, and the change trend was first increased and then decreased. The maximum CAT activity also appeared when the Cr<sup>6+</sup> concentration was 2mg·L<sup>-1</sup>. When the time of exposure was 72 h, the change trend was similar to that of 48 h, and the overall decrease was relatively small compared with that of 48 h. With the extension of exposure time, the CAT activity of earthworm in the blank control group was relatively stable without obvious fluctuation. The exposure time of the group treated with Cr<sup>6+</sup> at different concentrations was basically inverted u-shaped at 24 h, 48 h and 72 h.



**Figure 7.** Effect of Cr<sup>6+</sup> with different concentrations on CAT activity of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

#### Effects of chlorpyrifos and Cr<sup>6+</sup> on MDA content in earthworms

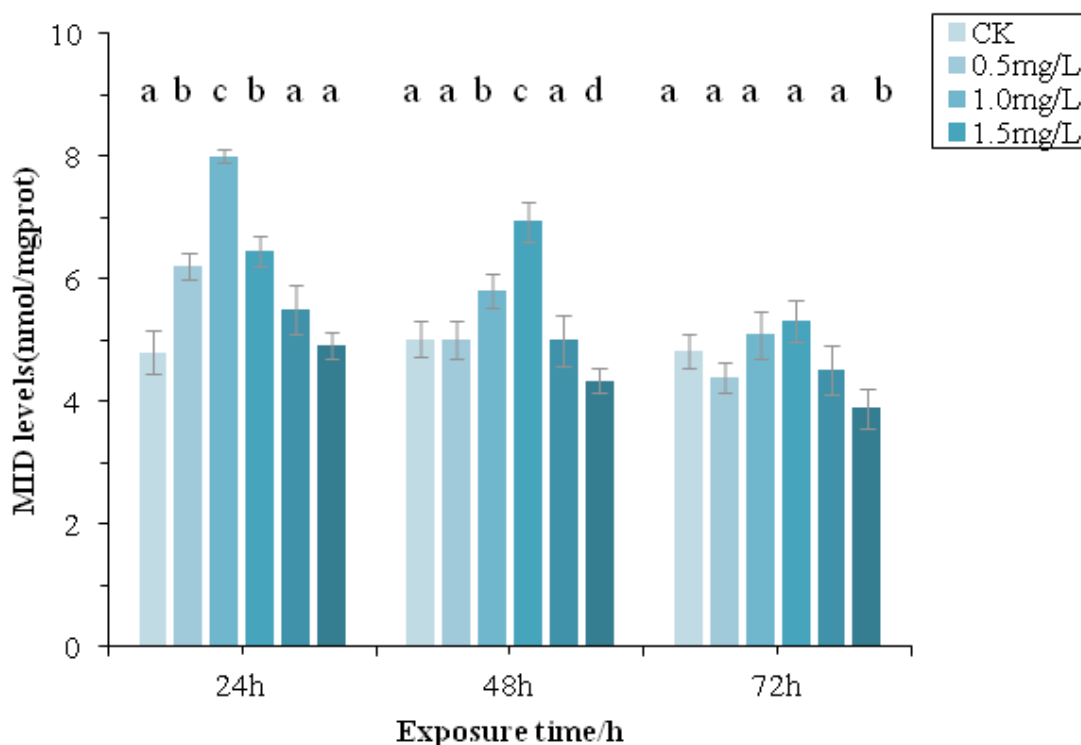
Earthworm under environmental stresses can produce ROS metabolism, the body through a series of antioxidant way to eliminate, when the degree of oxidation than compensatory ability to eliminate the oxidation and anti-oxidation system will lose the original balance appear oxidative stress response, at this point, the antioxidant enzyme system (SOD, CAT, POD, GSH-Px) such as enzyme activity, enhance antioxidant capacity. Malondialdehyde (MDA) is an excess ROS oxidative lipid product that

directly reflects the degree of peroxidative injury. Therefore, malondialdehyde (MDA) in earthworm was used as an important indicator of peroxidation level in organism.

### 1. Effect of pyrrolidan on MDA content in earthworms

In the experiment, pyrrolidin was set as 0.5, 1.0, 1.5, 2.0 and 2.5 mg·L<sup>-1</sup> concentrations. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h respectively, and malondialdehyde (MDA) content in vivo was determined. The results were shown in *Figure 8*.

By *Figure 8* can be concluded that chlorpyrifos infected by different concentration of earthworms in infected time after 24 h, 48 h, 72 h, the overall trend of decrease the MDA content in the body, speculated that this phenomenon is because at the beginning of the virus, worm surface stress by chlorpyrifos, antioxidant defense system is not fully, the body by oxidative damage, leading to lipid oxidation product MDA content rise rapidly, as the antioxidant defense system open, all kinds of antioxidant enzyme activity, eliminates the excessive oxygen free radicals, reduce MDA content.

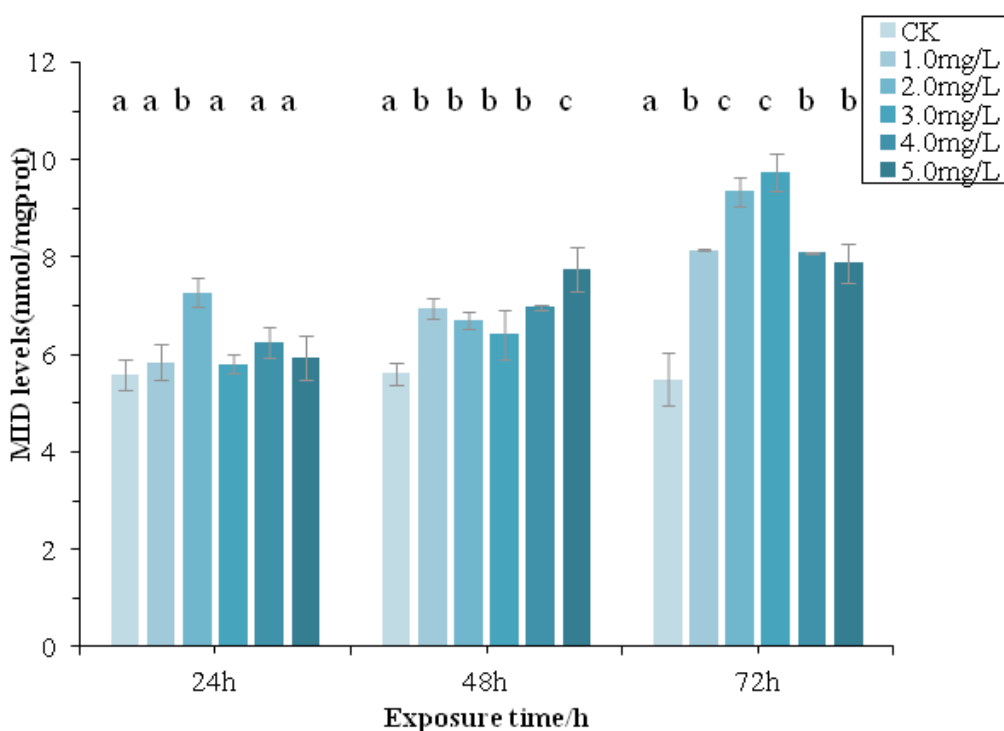


**Figure 8.** Effect of cartap with different concentrations on MDA content of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

### 2. Effects of Cr<sup>6+</sup> on MDA content in earthworms

In the experiment, Cr<sup>6+</sup> was set as the concentration of 1.0, 2.0, 3.0, 4.0 and 5.0 mg·L<sup>-1</sup>. Earthworm samples were collected for pretreatment at 24 h, 48 h and 72 h respectively, and malondialdehyde (MDA) content in vivo was determined, as shown in *Figure 9*.

It can be concluded from *Figure 8* that the content of malondialdehyde (MDA) in earthworms with different concentrations of Cr<sup>6+</sup> varied in different poisoning time ranges after single exposure. Chlorpyrifos infected by different concentration of earthworms in infected time after 24 h, the MDA content in the body as a whole was reduced after the first rise trend, but with the blank control group, MDA content was not significantly change, is due to the earthworm itself is Cr<sup>6+</sup> stress and oxidative stress response, antioxidant system effectively resist Cr<sup>6+</sup> oxidative damage to the body. Infected time 72 h, with the canister to the extension of time, the earthworm body by oxidative damage degree aggravating, increased MDA content accumulation in the body, a trend of reducing the rise on the whole, were higher than the blank control group 48.23%, 69.88%, 77.12%, 47.23% and 43.21%, in Cr<sup>6+</sup> concentration of 3.0 mg·L<sup>-1</sup> MDA content is the highest, while the concentration of earthworms body oxidative damage is the most serious.



**Figure 9.** Effect of Cr<sup>6+</sup> with different concentrations on MDA content of earthworm after single exposure 24, 48 and 72 h. Different lowercase letters indicate significant differences between treatments, as revealed by one-way ANOVA with Duncan's multiple-range test at  $p < 0.05$

## Discussion

Test to select chlorpyrifos, Cr<sup>6+</sup> to the single toxicity test of earthworm, record the different influence on earthworm mortality after a single drug, and calculate the chlorpyrifos, Cr<sup>6+</sup> in 24 h, 48 h and 72 h exposure when single LC<sub>50</sub> value of earthworms, after two or three kinds of drug toxicity of earthworms size order: chlorpyrifos > Cr<sup>6+</sup>, and with the extension of exposure time and enhance the toxic. Select chlorpyrifos, Cr<sup>6+</sup> to the single toxicity test of earthworm, record the different influence on earthworm mortality after a single drug, and calculate the chlorpyrifos, Cr<sup>6+</sup> in 24 h, 48 h and 72 h exposure when single LC<sub>50</sub> value of earthworms, after two or

three kinds of drug toxicity of earthworms size order: chlorpyrifos > Cr<sup>6+</sup>, and with the extension of exposure time

Total protein content reflects the sum of all types of enzymatic and non-enzymatic cellular proteins. When the chlorpyrifos single action, the earthworm body protein changes have the regularity, as the chlorpyrifos concentration increases the protein content first increases then decreases, as the time of infection extends the protein content first increases after tends to be stable. SOD in earthworms is sometimes induced and a dynamic balance is maintained to meet the needs of organisms to eliminate O<sup>2-</sup> but the balance can be easily disrupted by the stress of pollutants (Ma et al., 2017). SOD activity in vivo was induced in a short period of time with low concentration, and decreased with the extension of the exposure time. The CAT activity of earthworm decreased with the increase of pyrrolidine concentration and decreased with the extension of time. MDA is the product of lipid peroxidation and has been used to assess oxidative stress in earthworms (Xue et al., 2009). The content of MDA in earthworms is regular. With the increase of the concentration of pyrrolidin, the content of MDA increases first and then decreases. With the extension of the poisoning time, the content of MDA decreases, possibly because the body is less damaged by oxidation. When Cr<sup>6+</sup> acted alone, the protein content of earthworm decreased first and then increased with the increase of Cr<sup>6+</sup> concentration, and the protein content of earthworm showed a trend of decrease with the extension of the duration of infection.

After single action of Cr<sup>6+</sup>, the activity of SOD in earthworms first increased and then decreased, and the activity decreased with the extension of the poisoning time. CAT activity in vivo increased first and then decreased with the increase of Cr<sup>6+</sup> concentration, and increased with the extension of exposure time. There was no obvious rule between the change of MDA content and the concentration of Cr<sup>6+</sup> in vivo. As a whole, MDA content increased with the extension of the exposure time, indicating that the body was aggravated by oxidative damage.

Generally, single toxicity test of chlorpyrifos and Cr<sup>6+</sup> on earthworms was carried out, and the effect of different drugs on the mortality of earthworms was recorded. Similar result was also reported before (Oliveira et al., 2018). The order of toxicity of two drugs on earthworms was obtained: chlorpyrifos > Cr<sup>6+</sup>, and the toxicity increased with the extension of exposure time.

Potential synergistic effects of chemicals interactions were highlighted recently because of existence of complicated synergistic and antagonistic responses, which might cause serious ecological problems (Yang et al., 2017). Here, joint effects of chlorpyrifos and Cr<sup>6+</sup> on earthworms were studied. The changes of SOD activity, CAT activity and MDA content in earthworms infected with chlorpyrifos and Cr<sup>6+</sup> at sublethal dose were significant ( $p < 0.05$ ). The results showed that when the body was under the stress of pollutants, the first antioxidant defense was activated, and the activity of SOD in the body was rapidly induced to remove excessive oxygen radicals in the body and generate H<sub>2</sub>O<sub>2</sub> (Ma et al., 2017). Subsequently, the activity of CAT was enhanced and H<sub>2</sub>O<sub>2</sub> was converted into H<sub>2</sub>O. With the extension of poisoning time, SOD activity decreased and MDA content increased, indicating that the degree of oxidative damage to the body increased. Therefore, both SOD activity and MDA content can be used as indicators for earthworms to cope with oxidative stress and the degree of oxidative damage to the body.

## Conclusion

In conclusion, distinctive toxicity effects on *Eisenia fetida* under different chlorpyrifos and Cr<sup>6+</sup> stress levels were generally indicated by levels of enzyme activity/content impact, in terms of protein content, SOD, CAT and MDA activities. Generally, enzyme activity of *Eisenia fetida* may be sensitive indicators of chemical stress. However, in order to appropriately assess the ecological risk, more studies should be focused on long-term effects of chemical mixtures at different concentrations on earthworms.

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