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Toxic effects of crude-oil-contaminated soil in aquatic environment on *Carassius auratus* and their hepatic antioxidant defense system

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Abstract

Under the indoor simulant conditions, toxic effects of crude-oil-contaminated soil which was put into aquatic environment on the young fishes *Carassius auratus* and their hepatic antioxidant system after a 20-d exposure were investigated. Results showed that the relationship between the mortality of *C. auratus* and the exposed doses could be divided into 3 phases: fishes exposed to the low dose groups (0.5–5.0 g/L) were dead due to the ingestion of crude-oil-contaminated soils in aquatic environment; at the medium dose groups (5.0–25.0 g/L) fishes were dead due to the penetration of toxic substances; at the high dose groups (25.0–50.0 g/L) fishes were dead due to environmental stress. The highest mortality and death speed were found in the 1.0 g/L dose group, and the death speed was sharply increased in the 50.0 g/L dose group in the late phase of exposure. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) and the content of malaondialdehyde (MDA) in the hepatic tissues of *C. auratus* were induced significantly. The activity of SOD was increased and then decreased. It was significantly inhibited in the 50.0 g/L dose group. The activity of CAT was highly induced, and restored to a level which is little more than the control when the exposed doses exceeded 10.0 g/L. The activity of GST was the most sensitive, it was significantly induced in all dose groups, and the highest elevation was up to 6 times in the 0.5 g/L dose group comparing with the control. The MDA content was significantly elevated in the 50.0 g/L dose group, and the changes of the MDA content were opposite with the changes of GST activity.

Key words: crude-oil-contaminated soil; ecotoxicology; aquatic environment; *Carassius auratus*; antioxidant defense system
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Introduction

Petroleum is a common pollutant in both aquatic and soil ecosystems. There are many reports and literature focusing on the toxic effects of oils on aquatic organisms including fishes as well as their microscopic mechanisms (Perez *et al*., 2008; Weng *et al*., 2000; Zhou *et al*., 2004). These relevant studies showed that the toxic effects on fishes were caused by extracting and accumulating oil by respiration, metabolism, and penetration, finally impacting the food chain. The tiny emulsified oil in water would also affect the respiration of fishes because they could adhibit to fish gilles. Furthermore, oil pollution can decrease the amount and livability of spawns and other aquatic organisms, and increase the abnormality and mortality of larval fishes (Gonzalez-Doncel *et al*., 2008).

Antioxidative responses of fishes exposed to xenobiotics have been extensively explored (Clarke *et al*., 2008; van der Oost *et al*., 2003; Zhu *et al*., 2008). Numerous free radicals and reactive oxygen species (ROS) could be induced, and then accumulated when oxidative stress overcomes the capacity of antioxidant defense systems. These ROS can react with proteins and DNA, inactivate enzymes, break DNA strands, and form DNA adducts.

With increasing demand for oil mining, transportation and application, more soils are being contaminated by crude oil. Simultaneously, more crude-oil-contaminated soils are entering aquatic environment via surface runoff, thus affecting the aquatic organisms and their habitat more frequently. Generally speaking, fishes are good indicator for aquatic ecotoxicology, particularly assessing toxic effects of crude-oil-contaminated soils in aquatic environment. However, there are few studies on the toxic effects, especially antioxidant systems of fishes, which were exposed to crude-oil-contaminated soils in aquatic environment.

The aim of the present study was to investigate the toxic effects of elevated concentrations of crude-oil-contaminated soils in aquatic environment on the activity of antioxidant enzymes and lipid peroxidation of the larval *Carassius auratus*. In particular, these antioxidant enzymes studied include superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST). Lipid peroxidation was determined using malonaldehyde

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(MDA), a byproduct of lipid peroxidation. It was expected that the results from these studies would provide an experimental basis – whether the hepatic antioxidant defense system can serve as an early biomarker of fishes exposed to crude-oil-contaminated soils in aquatic environment.

1 Materials and methods

1.1 Chemicals

L-glutathione (reduced) (GSH) and sodium dodecylsulfate (SDS) were purchased from the Amresco Chemical (USA); bovine serum albumin (BSA), and Coomassie Brilliant Blue G250 were obtained from Sigma (USA); pyrogallol was bought from the Acros Organics (Belgium); 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Chemical Industry (Japan); 1-chloro-2,4-dinitrobenzene (CDNB), 2-thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were obtained from the International Laboratory (USA). Other chemicals and reagents were purchased from the Tianjin Chemical Reagent Company (China). All chemicals and reagents were of analytical grade.

1.2 Experiment animals and exposure conditions

Larval fishes *C. auratus* were selected in this study according to its availability, commercial importance (fisheries and aquaculture), and distribution throughout China. The fishes with the weight of 9.15 ± 0.92 g and the body-length of 8.0 ± 0.5 cm were purchased from a market in Tanggu, Tianjin, China. They were initially acclimated in tanks with dechlorinated water for 7 d in laboratory. The total mortality of the fishes was less than 5.0%.

Soil samples were collected from a typical field in the Victory Oil Field, Dongying, Shandong Province, China. The soil was polluted by crude oil and had a water content of 30.5% and pH 6.5. Crude oil from the Victory Oil Field was a heavy crude oil characterized by a high density of 0.91 kg/L. It was revealed by analyses that this oil contained: 68% saturated hydrocarbons, 13.5% aromatic hydrocarbons, 10.3% polar substances, and 8.2% asphaltenes. The content of oil in the crude-oil-contaminated soil used in this work was up to 12.48%.

The fresh samples were passed through a sieve of 1.7 mm and then put into glass aquaria, each contained 35 L water. The final tested concentration was set at 0, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 25.0, and 50.0 g/L, respectively. Tested fishes were randomly added to each dose groups (n = 12) with a fish/water ratio of about 3 fish/L. All glass aquaria had meshed net on top to keep fishes in tanks. The fishes were fed by commercial assorted feed once a day. Everyday the soil and water were mixed with a glass stick to simulate the disturbance in nature. This step can effectively avoid harming the tested fishes in aquatic environment, when they would swim away to escape from touching with the glass stick. This method is also of great importance because it can rapidly facilitate the exchange of the contaminant from soil to water. In nature environment, this disturbance is frequent.

Dead fishes were immediately taken out to prevent from water contamination. All water and soil were changed every five days. During the experiment, the water temperature was maintained at 15 ± 2°C and water pH was 6.0–8.0. The dissolved oxygen was kept at more than 5.0 mg/L by continuous aeration. Fishes were randomly sampled from each treatment group after exposure for 20 d.

1.3 Biochemical analysis

Fishes were dissected and their livers were separated after rinsing in ice-cold physiological brine (0.9% NaCl). Samples were wrapped by aluminum foil and frozen immediately after collection at –80°C before use.

Liver homogenates were prepared at a 1/10 (*W/V*) ratio in chilled Tris-HCl buffer (0.01 mol/L Tris, 0.25 mol/L sucrose, 0.1 mmol/L EDTA, pH 7.5) in glass homogenizer in ice bath and centrifuged at 10000 r/min for 15 min at 4°C to obtain the supernatant, which was used as the source of enzymes and MDA.

The activity of SOD was determined by measuring the inhibition of autooxidation of pyrogallol using the modified method by Li *et al*. (2007). One unit of enzyme activity was defined as the amount of enzyme exhibiting 50% inhibition of oxidation rate of pyrogallol in 1 mL solution at 25°C. The activity of CAT was assayed according to the method suggested by Xu *et al*. (1997). One unit of enzyme activity was defined as the amount of an enzyme to be reduced by half of the concentration of H_2O_2 in 100 s at 25°C. The activity of GST was evaluated with CDNB as substrate, following the formation of the conjugate with GSH at 340 nm according to Habig *et al*. (1974). The GST activity was expressed as μmol/(min·mg protein). The level of MDA (nmol/mg protein) was determined as described by Luo *et al*. (2008). The concentration of protein in the supernatant was determined by the Bradford procedure (Bradford, 1976) using bovine serum albumin as a standard. The absorbance at UV and visible wavelength was monitored using a TU-1901 2 UV-Vis spectrophotometer (PGenaral, Beijing, China).

1.4 Data processing

Each experiment had three replicates. Results were expressed as mean ± standard error. The data were processed by one-way ANOVA and the significance level among data was examined by Dunnet t (2-sided) post-hoc test using SPSS 13.0 software. Differences were statistically significant when $p < 0.05$.

2 Results

2.1 Effect on the mortality of *C. auratus*

At the low doses, fishes tended to eat the crude-oil-contaminated soil in aquatic environment. When the doses exceeded 5.0 g/L, fishes ate less feed than the control group, and their dorsal fins were not fully stretched. After exposed to 1.0 g/L for 6 d, however, the fishes became die. Fishes stayed at the bottom of aquarium before dying, since their abilities of swimming and balancing were

limited at that time.

As shown in Fig. 1, at the concentration of 0.5 g/L, the crude-oil-contaminated soil in aquatic environment did not have lethal effect on *C. auratus*. After a 10-d exposure, the highest mortality was found in 1.0 and 2.5 g/L dose groups. After a 15- and 25-d exposure, the mortality of fishes in the 1.0 g/L dose group was the highest among all groups. In the dose groups ranged from 5.0 to 25.0 g/L , the mortality was increased with the exposure time, while the death speed changed little. The 10 g/L dose group had the highest death speed. The death speed was sharply increased with exposure time in 50.0 g/L dose group.

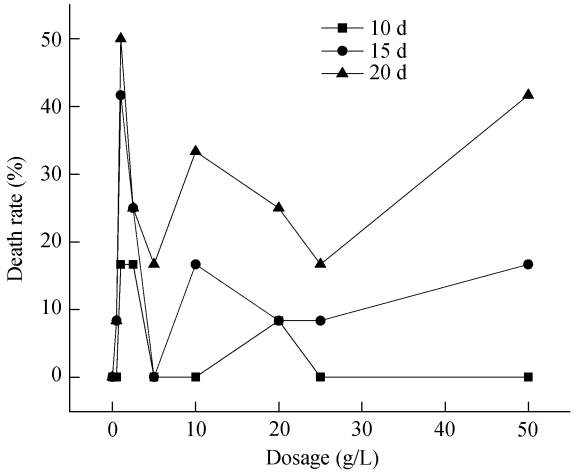

Fig. 1 Relationships between the mortality of *Carassius auratus* and the exposed dose of crude-oil-contaminated soil in aquarium.

2.2 Effect on the activity of SOD

The activity of SOD in each dose group after a 20-d exposure is shown in Table 1. The activity of SOD was induced by crude-oil-contaminated soil in aquatic environment, and the maximal induction was found in 10.0 g/L dose group, which was significantly ($p < 0.05$) higher than that in the control. Then it decreased markedly from the maximum, and the maximal inhibition was found at the 50.0 g/L dose group, which was significantly ($p < 0.05$) lower than that in the control (Fig. 2a). The highest SOD activity was 1.41 times as much as in the control, while in 50.0 g/L dose group the SOD activity was only 49% of the control group.

2.3 Effect on the activity of CAT

The activity of CAT in different dose groups after a 20-d exposure is shown in Table 1. The CAT activity was significantly ($p < 0.05$) induced even in 0.5 g/L dose group. Except for 1.0 g/L dose group, the activity of CAT in the low dose groups (0.5–5.0 g/L) was all induced significantly ($p < 0.05$) comparing with that in the control, and the highest induction was found in 2.5 g/L dose group (486% of the control). The increase in CAT activity began to fall when the dose was 5.0 g/L (Fig. 2b). In dose groups with a range 10.0–50.0 g/L, the activity of CAT fell almost to that in the control.

2.4 Effect on the activity of GST

The activity of GST in different dose groups after a 20-d exposure was shown in Table 1. In all exposure groups, the activity of GST was significantly ($p < 0.05$) induced comparing with that in the control (Fig. 2c). The highest increase reached 6-fold relative to the control in the group exposed to 0.5 g/L crude-oil-contaminated soil, the lowest treatment group. However, the activity of GST in 10.0 and 50.0 g/L dose groups were relatively lower than that in other dose groups. The lowest GST activity was found in the highest dose group (50.0 g/L), though still 2.95 times as much as that in the control group.

2.5 Effect on the content of MDA

The content of MDA in each dose group after a 20-d exposure was shown in Table 1. The MDA content was decreased and then increased with the increase in exposure doses (Fig. 2d). The content of MDA was insignificantly ($p > 0.05$) changed comparing with that in the control, except when the exposed dose was as high as 50.0 g/L. The content of MDA in 10.0 and 50.0 g/L dose groups was relatively higher than that in other groups.

3 Discussion

The mortality curve could be divided into 3 phases: low dose groups (0.5–5.0 g/L), medium dose groups (5.0–25.0 g/L) and high dose groups (25.0–50.0 g/L) (Fig. 1). In low dose groups, especially in the groups of 0.5 and 1.0 g/L, fishes tended to eat the contaminated soil in aquatic environment, which was not observed in other groups. The activity of CAT, SOD, and GST in 1.0 g/L group has no significant difference with other groups although

Table 1 Effect of crude-oil-contaminated soils on the hepatic enzyme activity of *Carassius auratus* after a 20-d exposure at different doses

Dose (g/L)	SOD (U/mg protein)	CAT (U/mg protein)	GST (U/mg protein)	MDA (nmol/mg protein)
0	126.46 ± 11.37	3.16 ± 0.66	169.22 ± 71.90*	5.86 ± 0.34
0.5	149.13 ± 13.40	9.05 ± 1.69*	1026.85 ± 45.05*	3.92 ± 0.40
1.0	143.19 ± 3.89	5.68 ± 0.33	845.40 ± 17.65*	3.03 ± 0.20
2.5	149.75 ± 6.97	15.37 ± 1.01*	762.00 ± 66.17*	4.73 ± 0.21
5.0	161.60 ± 18.52	9.04 ± 0.61*	896.38 ± 9.80*	6.71 ± 0.54
10.0	177.67 ± 5.41*	3.02 ± 0.71	551.99 ± 5.47*	9.19 ± 2.68
20.0	97.89 ± 3.53	4.54 ± 0.70	831.72 ± 22.93*	8.21 ± 2.31
25.0	90.99 ± 5.18	5.32 ± 0.33	733.49 ± 11.22*	5.95 ± 0.99
50.0	62.19 ± 3.24*	4.35 ± 0.14	499.18 ± 52.24*	11.78 ± 1.50*

* The mean difference is significant at the 0.05 level.

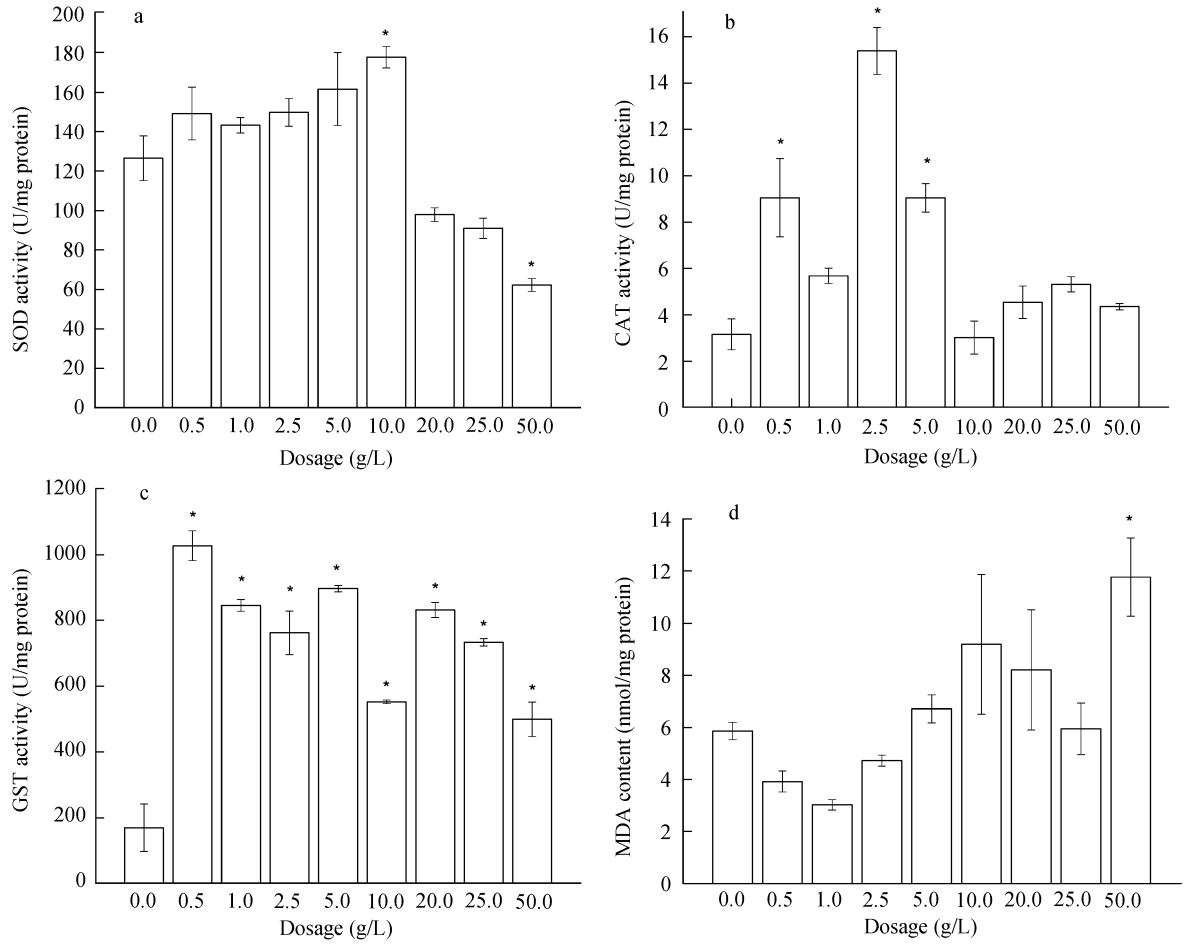

Fig. 2 Effects of crude-oil-contaminated soil on the SOD activity (a), CAT activity (b), GST activity (c), and MDA content (d) in the liver of *Carassius auratus* after a 20-d exposure at different doses in aquarium (* $p < 0.05$).

the highest mortality occurred. They were dead probably because they could not excrete the contaminated soil they ingested. The highest mortality of the fishes in the low dose group (1.0 g/L dose group) was higher than that in the medium and high dose groups (10.0 and 50.0 g/L dose groups). The accumulation of the contaminated soil in the tested fishes may be the most efficient way to cause toxic effects on them, thus resulting in the highest mortality and death speed.

In the medium dose groups, the tested fishes did not eat crude-oil-contaminated soil, and they ate less feed than that in the control and low dose groups as well. The main pathway for the fishes exposed to the pollutants is by respiration and penetration (Zhang, 1997). Fishes in medium dose groups were mostly dead after a 10-d exposure. The dorsal fins of the fishes in the high dose groups were not fully stretched, indicating that they were impacted by the contaminated environment. The death speed was sharply increased in the 50.0 g/L dose group in the late phase of the exposure. Noticeably, toxic effects on the fishes were different with the elevated amount of crude-oil-contaminated soils. In other words, there is no dose-response relationship between the dose of crude-oil-contaminated soil in aquatic environment and the activity of GST, CAT, SOD, and MDA, perhaps because some supernatant lipids floated at the top of the homogenate of livers.

In recent years, many studies showed that oxidative stress could be induced when living organisms exposed to environmental pollutants (Vigano *et al*., 2001; Wang and Zhou, 2006). The liver is an immune organ, and is one of the target organ attacked by many contaminants. The physiological changes of livers can be an early indicator of contaminants (Cheung *et al*., 2004). Our results showed that the antioxidant system was sensitive to toxic effects of crude-oil-contaminated soil. The oxidative stress caused by exposure to xenobiotics might be one of the reasons that cause hepatic injury. After a 20-d exposure, the activity of SOD, CAT and GST was significantly ($p < 0.05$) induced, and the highest level was found in 10, 2.5, and 0.5 g/L, respectively. It indicates that the activity of SOD and CAT, especially GST in the hepatic system of *C. auratus* is sensitive, and can be considered as an indicator and early monitoring index of oil pollution in an aquatic ecosystem.

Sun *et al*. (2006) reported that the activity of SOD in *Nereis diversicolor* showed a tendency of induction at first and then inhibition after a 5-d exposure of petroleum hydrocarbons. In our study, the activity of SOD and CAT was increased and then decreased with an increase of exposure doses. The increase of the enzyme activity in response to exposure doses was possibly attributed to the de-novo synthesis of the enzymatic proteins (Dinakar *et*

al., 2008). With further increase in the dose of crude-oil-contaminated soil in an aquarium, the activity of SOD and CAT decreased (Table 1). The activity of SOD was decreased by crude-oil-contaminated soil, in particular, significantly inhibited in the 50.0 g/L dose group (Fig. 2a). Although it was reduced, the activity of CAT was still higher than that in the control. The high concentration of a pollutant may have damaged antioxidative defensive systems (Wang and Zhou, 2006).

GST catalyzes the conjugation of electrophilic intermediates and reactive oxygen species with GSH, thereby defending against oxidative damage (Wu *et al*., 2007). A significant increase of the GST activity was observed in several studies (Cheung *et al*., 2002; Zhang and Wang, 2003; Xiao *et al*., 2006). In this study, the induction of the GST activity was in an agreement with pervious studies, and the GST activity had the highest induction rate than other antioxidative enzymes (Fig. 2c).

Lipid peroxidation may be the first step of the celluar membrane damage. The existence of oxidative stress can be demonstrated by the MDA formation, however, changes in the content of MDA and antioxidative enzyme activities were not synchronous (Martinez-Alvarez *et al*., 2002). In this study, it was found that the changes in the content of MDA were opposite with the changes in the activity of GST (Figs. 2c and 2d). The high content of MDA in 10.0 and 50.0 g/L dose groups indicated severe oxidative damages, corresponding to the high mortality of fishes in the two groups (Fig. 1). Although the highest mortality was found in 1.0 g/L dose group, the MDA content was the lowest. It can be concluded that the antioxidant defense system effectively protected the cellular system from lipid peroxidation (Ye *et al*., 2004). Therefore, unlike 10.0 and 50.0 g/L dose groups, the fishes survived due to their antioxidative responses.

4 Conclusions

The above experimental results showed that the activity of SOD and CAT, especially GST in the hepatic system of *C. auratus* was sensitive to crude-soil-contaminated soil in aquatic environment, and can serve as an early monitoring index of oil pollution in an aquatic ecosystem.

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