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ORIGINAL PAPER

Toxicological effects of microcystin-LR on earthworm (*Eisenia fetida*) in soil

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Abstract Microcystin-LR (MC-LR) is a cyclic heptapeptide toxin produced by cyanobacteria in eutrophic water. It can be transferred into soil–crop systems via irrigation and cyanobacterial paste fertilization. No studies have examined the potential toxicity of MC-LR to soil animals. Therefore, in the present study, the toxicological effects of MC-LR on earthworm (*Eisenia fetida*), including survival, growth, reproduction, oxidative stress, and cell viability, were investigated. The LC_{50} of MC-LR was 0.149 μg cm^{-2} at 72 h based on a filter paper test and 0.460 mg kg^{-1} at 14 days based on an acute soil test. MC-LR seriously affected the reproduction of earthworms. Based on hatchability, the EC_{50} of MC-LR was 0.268 mg kg^{-1}, similar to environmentally relevant concentrations of microcystins. The changes in activities of superoxide dismutase, guaiacol peroxidase, catalase, and glutathione peroxidase, together with the levels of glutathione and malondialdehyde, indicated that oxidative damage and lipid peroxidation played significant roles in MC-LR toxicity. In addition, the toxicity of MC-LR in earthworms increased despite degradation of MC-LR in soil over time, possibly due to the formation of toxic metabolites of MC-LR or the bioaccumulation of MC-LR in earthworms. A reduction in the neutral red retention time along with an increase in coelomocyte apoptosis with increasing MC-LR concentrations indicated a severe damage to viability. These results suggest that environmentally relevant MC-LR concentrations in agricultural soil may cause reproductive, biochemical, and cellular toxicity to *Eisenia fetida*. This information can be used in ecological risk assessments on MC-LR in soil.

Keywords MC-LR · Earthworm · Soil · Toxicological effect · Reproduction toxicity · Coelomocyte apoptosis

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Introduction

Microcystins (MCs) are a group of cyclic heptapeptide hepatotoxins generated by aquatic cyanobacteria in eutrophic freshwater, which have been increasingly reported worldwide (Merel et al. 2013). Exposure to MC-contaminated water or food can cause liver failure in fish and other animals and primary liver cancer in humans (Carmichael 2001; Falconer 2005). More than 100 MC variants have been identified, differing primarily in two *L* amino acids in their molecular structure (Puddick et al. 2014). MCs are frequently detected in water, and their concentrations often exceed the World Health Organization admonitory level of 1 μg L^{-1}, even reaching several milligrams per liter (Cook et al. 2004; Wood et al. 2006). Furthermore, MCs can be transferred into the soil–crop system via irrigation with contaminated water and fertilization using harvested toxic cyanobacterial paste (Chen et al. 2006). MCs have a cyclic peptide structure that enhances their chemical stability, with a half-life of about 56 days in agricultural soil (Corbel et al. 2014). The concentrations of MCs in cropland soil were observed up to 273.2 μg kg^{-1} (Chen et al. 2012; Li et al. 2014; Li 2015), which may lead to adverse effects in organisms and the

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structure and function of soil ecosystems. Therefore, it is essential to evaluate the potential ecological risk of MCs in soil.

MC-LR (-D-Ala-L-L-D-MeAsp-L-R-Adda-D-Glu-Mdha), one of the main MCs, is of particular concern due to its common occurrence and high toxicity. The adverse effects of MC-LR on various aquatic species have been extensively reported in risk assessment of MC-LR to aquatic environments (Papadimitriou et al. 2012; Zhang et al. 2013a; Zhao et al. 2015). In addition, the toxic effects of MC-LR on rats and human have also been investigated (Falconer 2005; Gupta et al. 2003; Wang et al. 2013; Zhou et al. 2012). However, to our knowledge, no research concerning the toxic effects of MC-LR in soil on organisms has been reported.

Earthworm is the most prevalent animal species in soil, representing high proportion of the soil faunal biomass. Earthworms are critical in maintaining the ecological functions of soil (Andriuzzi et al. 2016; Saint-Denis et al. 1999) and promoting soil fertility and nutrient cycling (Hoang et al. 2016). They are often used as an important model organism for soil ecological risk assessments of toxic substances (Capowiez et al. 2005; Xu et al. 2010).

MC-LR exposure in aquatic organisms can enhance the production of reactive oxygen species (ROS), including free radicals, hydrogen peroxide, and singlet oxygen (Zhang et al. 2013a). The overproduction of ROS can cause lipid peroxidation, cell death, and oxidative stress and has a vital role in the toxicity of MC-LR, as reported in several studies (Amado and Monserrat 2010; Li et al. 2010a; Zhang et al. 2013a). Meanwhile, earthworms elicit sensitive, informative, and reproducible biochemical responses that are considered as early warning indices of individual reactions to environmental stress (Liu et al. 2011). To prevent oxidative injury to tissues and cells, endogenous enzymatic [e.g., superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), and glutathione peroxidase (GSH-Px)] and non-enzymatic [e.g., glutathione (GSH) and malondialdehyde (MDA)] antioxidant defense systems in organisms work to scavenge excess ROS and alleviate their deleterious effects (Song et al. 2009; Xu et al. 2013). SOD, CAT, POD, and GSH-Px activities are often considered as good bioindicators of contaminant-induced oxidative stress that reflect adverse effects in organisms (Gomes et al. 2015; Liu et al. 2011). MDA is a product of lipid peroxidation and is often regarded as a convenient biomarker for evaluating oxidative stress (Lin et al. 2010). GSH, an important water-soluble antioxidant, has a crucial role in cellular detoxification metabolism (Li et al. 2010b; Zhang et al. 2013b). Therefore, changes in the levels of MDA, GSH, and enzymatic activities may indirectly reflect the toxic effects of pollutants on earthworms. At the subcellular level, the stability of lysosomal membranes in coelomocytes can be used as a biomarker of the toxicological effects of contamination (Svendsen et al. 2004). Flow cytometric techniques offer substantial advantages for revealing detailed alterations in earthworm coelomocytes (Duan et al. 2015).

In the present study, the responses of earthworms (*E. fetida*) to exposure to various concentrations of MC-LR were investigated using different endpoints to evaluate the toxicity of MC-LR in soil. The acute, reproductive, and antioxidant defense responses, as well as coelomocyte apoptosis and the stability of lysosomal membranes, were determined. The purposes of this study were to provide essential data for comprehensive evaluation of the toxic effects of MC-LR on earthworms in soil and to provide a scientific basis for delineating the potential ecological risks of MC-LR in terrestrial ecosystems.

Materials and methods

Soil preparation

The paddy soil (0–20 cm) used in the present study was collected from an experimental farm at the South China Agricultural University, Guangdong Province, China. Air-dried soil was mixed well and sieved through a 2-mm mesh screen. The physicochemical properties of the soil that were determined as described previously (Lu 2000) are as follows: pH, 6.2; water holding capacity, 36%; organic matter content, 27.1%; cation exchange capacity, 20.26 cmol kg^{-1}; sand (>50 μm), 26%; silt (1–50 μm), 58%; and clay (<1 μm), 16%.

Chemicals and reagents

MC-LR (purity >95%) was purchased from Taiwan Algal Science Inc. (Taoyuan, Taiwan). HPLC-grade acetonitrile and methanol were obtained from Fisher Co. (Fair Lawn, NJ, USA). The solid-phase extraction system with 24-port vacuum manifolds was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sep-Pak C_{18} cartridges were purchased from Waters Corporation (Milford, MA, USA). Trifluoroacetic acid (≥99%) was obtained from Aladdin Reagent Company (Shanghai, China). All other chemicals were of analytical grade and purchased from Chemical Reagent Factory (Guangzhou, China).

Earthworms

Earthworms (*E. fetida*) were obtained from an earthworm-culturing organic farm located in Qingyuan, southern China, and maintained on a cow dung diet in an incubation chamber at 20 ± 1 °C. Under laboratory conditions, healthy adult earthworms with singular for clitellum and weights of about 300–400 mg were selected for acclimatization in paddy soil at 20 ± 1 °C for 7 days. Then, the earthworms were removed from the culture, rinsed with distilled water to remove soil and cow dung, and placed on damp filter paper for 24 h in the dark at 20 ± 1 °C to minimize their gut contents prior to exposure.

Acute toxicity tests

Filter paper contact test

The filter paper contact test was performed based on the Organisation for Economic Co-operation and Development (OECD) guideline 207: a paper contact toxicity test (OECD 1984). Filter paper was cut to a suitable size, placed in a flat-bottomed glass vial (Ø 4.3 × 6.0 cm), and treated with MC-LR dissolved in 1 mL of methanol. The control group was treated with the same volume of methanol. The exposure concentrations of MC-LR on filter paper were 62.5, 125, 250, 500, 1000, and 2000 ng cm^{-2}. Methanol was evaporated until dry under a slow nitrogen stream, followed by the addition of deionized water (1 mL) to moisten the filter paper. Each vial contained one worm and each treatment included ten replicates. All vials were maintained at 20 ± 1 °C in the dark under 80–85% relative humidity. The earthworms were regarded as dead if they had no response to mechanical stimulus on the front end. Earthworm mortality was assessed, and the concentration eliciting 50% mortality (LC_{50}) was calculated after incubation for 48 and 72 h.

Soil contact test

The paddy soil test was conducted according to OECD guideline 207: an artificial soil test (OECD 1984). A range of concentrations (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mg kg^{-1} dry weight) were included in this acute toxicity test based on preliminary analyses. MC-LR was dissolved in methanol and fully mixed into 500 g (dry weight) of paddy soil at each prescribed concentration. The control group was mixed with the same volume of methanol. After a period of evaporation to remove the methanol, the soil moisture contents were adjusted to a 60% water holding capacity with distilled water. Ten mature earthworms were placed in each beaker containing 500 g of dried soil spiked with MC-LR, and the treatments were performed in triplicate. All containers were maintained at 20 ± 1 °C under 80–85% relative humidity. Earthworm mortality was assessed, and the LC_{50} was determined after incubation for 7 and 14 days.

Reproduction

The reproduction test was performed in accordance with OECD guideline 222 (OECD 2004). Based on the results of the acute toxicity test, a range of concentrations (0.05, 0.1, 0.2, 0.3, and 0.4 mg kg^{-1} dry weight) were included to investigate the effects of MC-LR in soil on earthworm reproduction. Ten adult earthworms were placed in each beaker, and each treatment was performed in four replicates. Approximately 5 g of sterile cow manure were added to the surface of the soil in each container weekly to provide food to the earthworms. The containers were kept in an incubator at 20 ± 1 °C under 80–85% relative humidity and a 600-lx light/dark regime of 16:8 h. Adult earthworms were removed from each container and counted on day 28 of the experiment. Furthermore, the numbers of cocoons and juveniles were counted and returned to the test vessels for incubation for an additional 4 weeks without feeding. At the end of the second 4-week period, the numbers of cocoons and juveniles in the test soil were counted. Hatchability, cocoons per earthworm, hatchlings per cocoon, and cocoon weight were calculated.

Biochemical assays

According to the acute toxicity and reproduction tests, a range of concentrations (0.025, 0.05, 0.1, 0.2, and 0.3 mg kg^{-1} dry weight) were used to study the effects of MC-LR in soil on the antioxidant defense system. Earthworms were treated as described in reproduction test. Three replicates were included per treatment. After 3, 7, 14, 21, and 28 days of exposure, the earthworms were collected and left for 24 h to remove their gut contents. After measuring the biomass, five live earthworms from each container were selected for homogenization in an amount of Tris-HCl buffer (100 mmol L^{-1}, pH 7.5) equal to four times their bodyweight and centrifuged at 9000*g* at 4 °C for 30 min. After centrifugation, the supernatant was collected and stored at −80 °C until analysis. The levels of MDA and GSH and activities of CAT, SOD, POD, and GSH-Px were determined using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (China) as described elsewhere (Xu et al. 2013; Zhang et al. 2015).

Determination of MC-LR in soil

Concurrent to the enzyme assays, 5 g of soil was collected from each container on days 3, 7, 14, 21, and 28. The MC-LR analysis was conducted with liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described in our previous paper (Li et al. 2013). Soil samples were thawed at room temperature and extracted using 10 mL of EDTA-$Na_4P_2O_7$ solution with 5 min of vortexing and 10 min of ultrasonic extraction, followed by centrifugation at 9000*g* for 5 min. The supernatant was collected and introduced into a C_{18} cartridge for solid-phase extraction cleanup. The extraction was repeated twice. The cartridge was rinsed with 5 mL of ultrapure water and vacuum-dried. Then, it was eluted with 5 mL of acidified aqueous methanol, and the resulting eluate was filtered through a 0.22-μm filter and concentrated to 1 mL using a nitrogen stream for LC-MS/MS detection. An external MC-LR standard was used.

Coelomocyte harvesting and analysis

According to the acute toxicity and reproduction tests, a range of concentrations (0.025, 0.05, 0.1, 0.2, and 0.3 mg kg^{-1} dry weight) were used to study the effects of MC-LR in soil on the antioxidant defense system. Earthworms were treated as described in reproduction test. Three replicates were performed for each treatment. Earthworms were allowed to depurate their guts for 24 h in Petri dishes on filter paper moistened with distilled water. Coelomocytes were harvested using a modified version of the method described by Eyambe et al. (1991). First, each earthworm was placed in a 1.5-mL Eppendorf tube and rinsed twice with 1 mL of ice-cold phosphate-buffered saline (PBS) for 3 min to remove mucus and feculence from their surface. Then, 1 mL of cold extrusion medium containing 95% normal saline, 5% ethanol, 10 mg mL^{-1} guaiacol glyceryl ether, and 2.5 mg mL^{-1} EDTA (pH 7.3) was added to stimulate the spontaneous release of coelomocytes. After 2 min, the earthworms died and were removed from the tubes. To collect the coelomocytes, the cell suspension was centrifuged at 9000*g* and 4 °C, and the precipitate was washed with the same PBS (pH 7.5) and centrifuged three times.

Lysosomal membrane stability was assessed with the neutral red retention assay. The measurement and counting for the neutral red retention time (NRRT; the time at which half of the cells were stained red) were conducted following the method described by Weeks and Svendsen (1996).

Coelomocyte samples were washed twice with cold PBS and resuspended with 200 μL of 1× binding buffer. Subsequently, 100 μL of cell suspension was transferred into a new 1.5-mL Eppendorf tube containing 10 μL of Annexin V-R-PE. After mixing by inversion, the tube was placed in an ice bath for 20–30 min in the dark. Then, 380 μL of 1× binding buffer and 10 μL of 7-aminoactinomycin dye were added to the cell suspension and mixed uniformly. Finally, the cell samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). The LC_{50} and effective concentration at which 50% of hatchability was attained (EC_{50}) combined with the associated 95% confidence intervals were estimated with probit regression analysis. Analysis of variance (ANOVA) was performed to analyze the enzyme activities of earthworms in terms of MC-LR concentration, time of exposure, and the interaction between concentration and time. The post hoc least significant difference (LSD) test (homoscedasticity has been checked) was used to identify significant differences ($p < 0.05$) between the treatment and control groups. All statistical analyses were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Acute toxicity based on the filter paper contact test

The effects of MC-LR on earthworm mortality were both concentration- and time-dependent (Table 1). Earthworm survival decreased with increasing MC-LR concentration. The LC_{50} values for MC-LR at 48 and 72 h were 0.634 and 0.149 μg cm^{-2}, respectively. According to the toxicity classification of chemicals, MC-LR was classified as a super-toxic chemical (LC_{50} < 1 μg cm^{-2}) (Roberts and Dorough 1984). Complete mortality was observed at 2 μg cm^{-2} of MC-LR after 72 h. No mortality was observed after 72 h in the control, implying that the acute toxicity test was not affected by the use of methanol as a solvent and that it met the validity criterion of the OECD test protocol.

Acute toxicity based on the soil contact test

The effects of MC-LR on earthworm mortality were positively correlated with concentration and exposure duration (Table 2). No mortality was observed after 14 days in the control, meeting the validity criterion of the OECD test protocol. The LC_{50} values for MC-LR in soil were 0.517 mg kg^{-1} at 7 days and 0.460 mg kg^{-1} at 14 days, indicating that MC-LR should be classified as a highly toxic chemical (LC_{50} = 0.1–1 mg kg^{-1}) based on the Chinese National Criterion for Pesticides in Soil (GB/T 2014). Similarly, An et al. (2015) reported the LC_{50} of MC-LR in crayfish of 0.567 mg L^{-1} after 96 h, also equivalent to classification as a highly toxic chemical (LC_{50} = 0.1–1 mg L^{-1}) based on the Chinese National Criterion for Pesticides (GB/T 2014). The LC_{50} of MC-LR in earthworms was much lower than the lowest LC_{50} values (0.9–68.1 mg kg^{-1}) of several common pesticides (Table 3), indicating that MC-LR is much more toxic than these pesticides. Furthermore, the concentration that complete mortality was observed at 7 days was 0.8 mg kg^{-1}. MC-LR was less toxic in soil (highly toxic) than on filter paper (super-toxic), which was attributed to the reduced bioavailability owing to sorption of MC-LR onto soil. Considering that the LC_{50} values determined in the present study are much higher than environmentally relevant concentrations (ca. 0.3 mg kg^{-1}) of MC-LR in

Table 1 Acute toxic response of earthworms to MC-LR in the filter paper test

Exposure time	Toxicity regression equation	LC_{50}	95% confidence limits	R^2
48 h	Y = 2.153X + 0.426	0.634 μg cm^{-2}	0.412 ~ 1.086	0.951
72 h	Y = 1.834X + 1.514	0.149 μg cm^{-2}	0.070 ~ 0.242	0.941

Table 2 Acute toxic response of earthworms to MC-LR in the soil contact test

Exposure time	Toxicity regression equation	LC_{50}	95% confidence limits	R^2
7 days	Y = 10.037X + 2.876	0.517 mg kg^{-1}	0.459 ~ 0.572	0.953
14 days	Y = 9.685X + 3.270	0.460 mg kg^{-1}	0.403 ~ 0.511	0.976

X the logarithmic of the concentration

cropland soil (Chen et al. 2012; Li 2015), acute toxicity of MC-LR in earthworms in the environment is unlikely; however, chronic toxicity should not be neglected, because the toxicity of MC-LR in soil is affected by many factors including soil type, pH, etc. In particular, the toxicity of MC-LR may be significantly enhanced in the presence of heavy metals according to our previous research on the seed germination of plant (Zhan et al. 2013), which should be emphasized, considering ubiquitous occurrence of heavy metals in agricultural soil.

Effects of MC-LR on earthworm weight

Changes in bodyweight are regarded as the first screening biomarker to observe the effects of contaminants on organisms (Shi et al. 2007; Xu et al. 2015). The biomass of earthworms was measured to identify the effects of MC-LR on growth (Fig. 1). The bodyweight of earthworms in the control group increased significantly relative to incubation duration. Meanwhile, growth was inhibited in all treatment groups compared with that in the control, except for in the earthworms exposed to 0.05 mg kg^{-1} MC-LR at day 3. At day 7, only the growth of earthworms exposed to the highest concentration (0.3 mg kg^{-1}) of MC-LR was significantly inhibited. At days 14 and 21, the weight change rates of earthworms exposed to 0.2 and 0.3 mg kg^{-1} MC-LR in soil were significantly lower than in the control. After exposure for 28 days, the weight change rates of all treatment groups were significantly lower

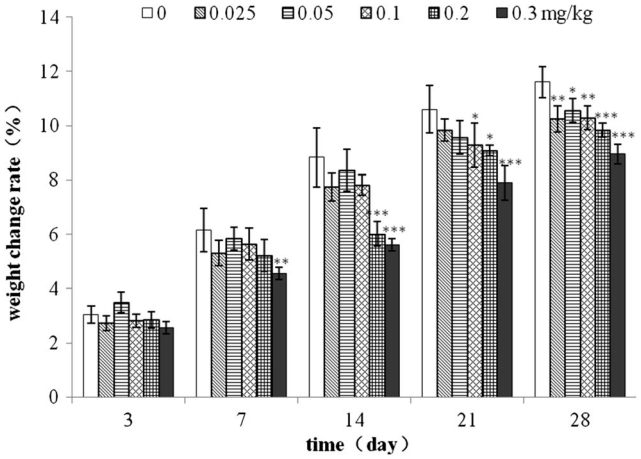

Fig. 1 Weight change rate in earthworms exposed to MC-LR. Data are expressed as mean ± sd ($n = 3$). Statistical significance versus the control group: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

than those of the control. The ANOVA results showed that the weight change rate of earthworms was affected significantly by MC-LR concentration, exposure duration, and the interaction between concentration and duration (Table 4). These results indicated that the exposure of earthworms to MC-LR at sublethal concentrations significantly affected their growth, and no hormesis was observed, even at lower MC-LR concentrations, supporting its characterization as a super-toxic chemical. Growth inhibition in earthworms exposed to pollutants may be attributable to reductions in glycogen, lipid contents, and protein contents (Shi et al. 2007; Xu et al. 2015). In addition, reduced food intake may be responsible for the decrease in growth in earthworms as a method to avoid xenobiotic uptake.

Reproductive toxicity

Increasing concentrations of MC-LR led to a significant decrease in hatchability, mean number of cocoons per earthworm, mean number of hatchlings per cocoon, and cocoon weight compared to the control, with the exception of the mean number of cocoons per earthworm at 0.05 mg kg^{-1} MC-LR (Table 5). All reproductive parameters were

Table 3 Acute toxic effects of selected pesticides in soil on earthworms

Pesticides	Time (days)	LC_{50} (mg kg^{-1})	Toxicity grade	References
Deltamethrin	14	68.1	Low	(Song et al. 2015)
Fenvalerate	14	56.3	Low	(Song et al. 2015)
Carbendazim	14	8.6	Moderate	(Huan et al. 2016)
Imidacloprid	14	3.1	Moderate	(Wang et al. 2015)
Acetamiprid	14	2.7	Moderate	(Wang et al. 2015)
Nitenpyram	14	4.0	Moderate	(Wang et al. 2015)
Clothianidin	14	0.9	High	(Wang et al. 2015)
Thiacloprid	14	2.7	Moderate	(Wang et al. 2015)

Table 4 Effects of MC-LR on the bodyweights and antioxidant levels of earthworms based on ANOVAs

Items	Concentration			Duration			Concentration*duration		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
SOD	5	20.79	0***	4	471.31	0***	20	11.20	0***
POD	5	28.56	0***	4	61.86	0***	20	8.22	0***
CAT	5	19.28	0***	4	48.56	0***	20	16.47	0***
GSH-PX	5	6.89	0***	4	56.37	0***	20	4.41	0***
GSH	5	28.87	0***	4	177.25	0***	20	6.41	0***
MDA	5	64.44	0***	4	79.63	0***	20	5.38	0***
Weight change rate	5	30.36	0***	4	579.25	0***	20	2.43	0.004**

df degrees of freedom, *F* *F*-max of Hartley

**$p < 0.01$, ***$p < 0.001$

significantly inhibited by MC-LR at concentrations above 0.2 mg kg^{-1} ($p < 0.05$), suggesting that MC-LR exerted highly toxic effects on earthworm reproduction above this concentration. The EC_{50} of MC-LR was 0.268 mg kg^{-1} (95% CI: 0.151–3.725). No difference between water control and solvent control was observed, implying that the reproduction toxicity test was not affected by the use of methanol as a solvent, and that it met the validity criterion of the OECD test protocol.

The reproductive system is one of the most important targets of MC-LR toxicity, second only to the liver (Chen et al. 2016). Greig-Smith et al. (1992) revealed that measuring the toxic effects on reproduction might provide more sensitive results than the acute toxicity index for environmental risk assessments. In the present study, MC-LR posed highly toxic effects on earthworm reproduction. Similarly, Zhao et al. (2015) reported decreased fertilization and hatching rates in female zebrafish (*Danio rerio)* exposed to MC-LR (2, 10, or 50 μg L^{-1}) for 21 days, which was attributed to the effects of MC-LR on oogenesis and endocrine function. Considering concentrations of MCs in cropland soil up to 0.273 mg kg^{-1} (dry weight) (Chen et al. 2012; Li 2015), therefore, there is a high potential for detrimental effects of MC-LR on earthworm productivity in cropland soil, and more research is warranted. Earthworms are ecologically highly important in many soils (Van Groenigen et al. 2014). While earthworms are at the bottom of the terrestrial food chain, once earthworm populations are destroyed, both soil fertility and the entire food chain may be heavily affected, with potential severe consequences for entire ecosystems.

Effects of MC-LR on antioxidant enzymes

MC-LR hepatotoxicity is closely associated with intracellular ROS formation (Chen et al. 2005). Normally, production and elimination of ROS in organisms exist in a dynamic balance (Gomes et al. 2015). However, this balance is easily disrupted by environmental contaminants, inducing a sharp rise in ROS levels. To counteract this increase, diverse antioxidant systems are stimulated to scavenge toxic radicals. In the present study, the effects of MC-LR on antioxidant enzymes (SOD, POD, CAT, and GSH-Px) in earthworms were evaluated (Fig. 2). In general, the SOD activity of the MC-LR-treated earthworms was activated at the first 21 days, and then returned to the levels of the control at day 28 (Fig. 2a). A similar pattern was observed for POD activity (Fig. 2b). On the other hand, CAT activity was first activated at the first 14 days, and then returned to control levels (at day 21) before being inhibited (at day 28) (Fig. 2c). A similar

Table 5 Reproduction parameters measured in earthworms exposed to MC-LR

Concentrations (mg kg^{-1})	Mean cocoons per *E. fetida*	Mean hatchlings per cocoon	Mean cocoon weight (mg)	Hatchability (%)
0.00	2.08 ± 0.17	3.43 ± 0.34	13.28 ± 1.23	87.81 ± 3.38
0.05	2.18 ± 0.15	3.31 ± 0.32	13.14 ± 1.19	81.81 ± 4.11
0.10	1.83 ± 0.13	2.66 ± 0.12***	11.81 ± 1.49	67.60 ± 6.64**
0.20	1.35 ± 0.06***	2.35 ± 0.19***	11.08 ± 0.88*	59.34 ± 6.36***
0.30	1.03 ± 0.10***	2.09 ± 0.21***	9.60 ± 0.61**	51.39 ± 4.64***
0.40	0.73 ± 0.13***	1.85 ± 0.14***	7.87 ± 0.79***	35.45 ± 8.15***

Data are expressed as mean ± sd ($n = 4$). Statistical significance versus control group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

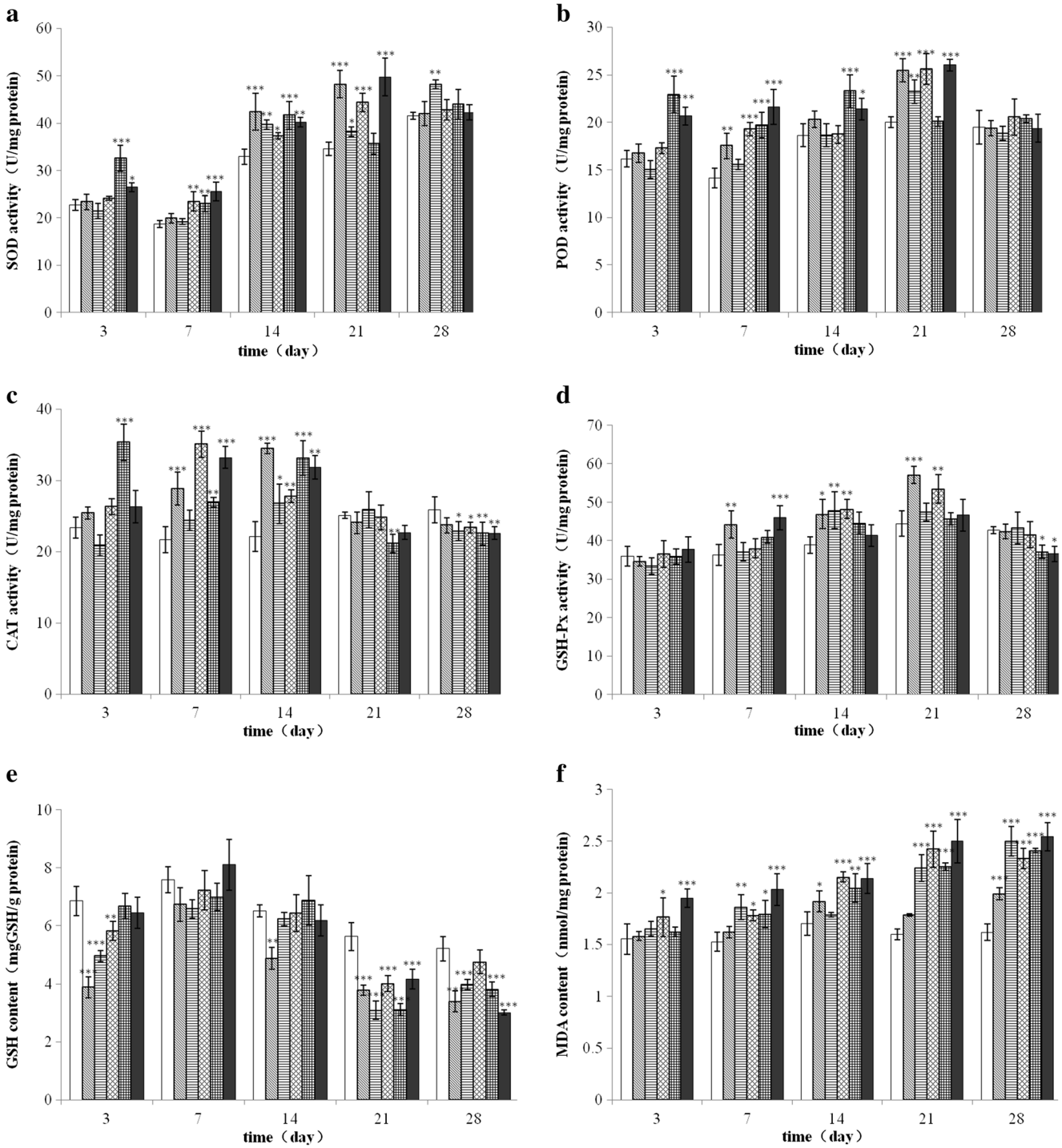

Fig. 2 Effects of MC-LR on biochemistry parameters in earthworms. **a** SOD activity, **b** POD activity, **c** CAT activity, **d** GSH-Px activity, **e** GSH content, and **f** MDA content were determined after being exposed to MC-LR at concentrations of 0.025, 0.05, 0.1, 0.2, and 0.3 mg kg^{-1}. Data are expressed as mean ± SD (n = 3). Statistical significance versus control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

pattern was observed for GSH-Px activity (Fig. 2d), i.e., GSH-Px activity was first activated at the first 21 days, and then returned to control levels (the concentration less than 0.1 mg kg^{-1} at day 28) before being inhibited (the concentration more than 0.2 mg kg^{-1} at day 28). According to the ANOVA results (Table 4), all MC-LR concentrations, exposure durations, and interactions between concentration and duration had significant effects on the activities of SOD, POD, CAT, and GSH-Px.

SOD catalyzes the transformation of superoxide anions into H_2O_2, protecting cells against oxidative damage (Lin et al. 2010). In the present study, SOD activity was stimulated in all MC-LR treatments for all exposure durations, showing that

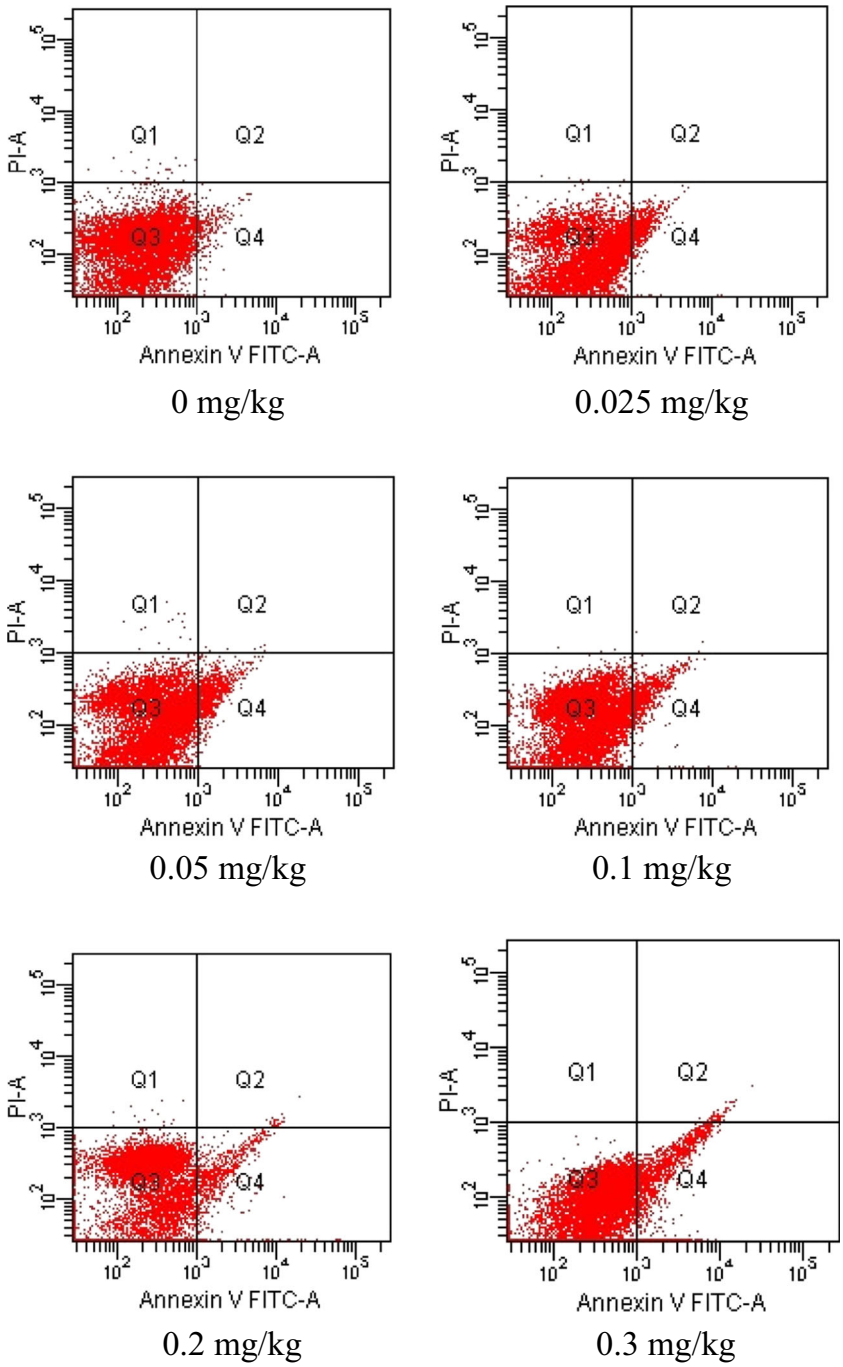

Fig. 3 Coelomocyte apoptosis in earthworms exposed to MC-LR after 14 days (Q1, necrotic cells; Q2, late/secondary apoptotic cells; Q3, live cells; and Q4, early/primary apoptotic cells)

SOD could remove excess superoxide anions effectively to reduce oxidative damage in the exposed earthworms (Zhang et al. 2015). After the dismutation of superoxide anions into H_2O_2, H_2O_2, and other free radicals are eliminated by CAT, POD, GSH-Px, and other enzymes (Liu et al. 2011). In the present study, POD activity was significantly elevated in the first 21 days and then returned to the control level at day 28, indicating that POD had the capacity to remove excess H_2O_2. Conversely, CAT and GSH-Px activities first increased, then returned to control levels, and finally decreased below control levels, showing that CAT and GSH-Px could remove excess H_2O_2 before day 14 (CAT) or day 21 (GSH-Px). However, the significant reduction in CAT activity at day 28 at exposures greater than 0.05 mg kg^{-1} could have been caused by the irreversible inactivation of the enzyme by accumulated ROS owing to their ineffective removal, decreased enzyme synthesis, or changes in enzyme subunit assembly (Du et al. 2015; Velki and Hackenberger 2013). The significant inhibition of GSH-Px activity at day 28 when exposed to MC-LR levels higher than 0.2 mg kg^{-1} could have been attributable to

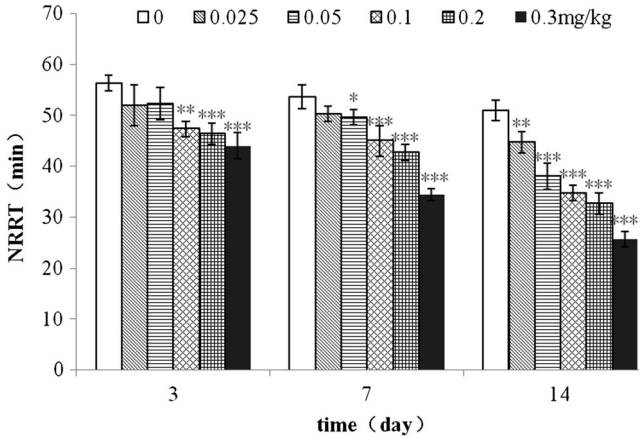

Fig. 4 The neutral red retention time (NRRT) in earthworms exposed to MC-LR. Data are expressed as mean ± SD ($n = 3$). Statistical significance versus control group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

a decline in GSH concentration because GSH-Px catalyzed the reduction of H_2O_2 at the expense of GSH. This observation was inconsistent with the effects of MC-LR on GSH-Px levels in frogs (Zhang et al. 2013b). Overall, the significant inhibition of the activities of CAT and GSH-Px in earthworms indicated substantial damage to their antioxidant defense system. Taken together, these results suggested that POD should play more important role in removing H_2O_2 caused by MC-LR exposure than those of CAT and GSH-Px, because its activity kept uninhibited.

Effects of MC-LR on non-enzymatic antioxidants

MC-LR has been reported to produce intracellular ROS by altering intracellular GSH contents and lipid peroxidation in animals (Zhang et al. 2013b). GSH is regarded as a sensitive molecular indicator of environmental pollution (Li et al. 2010b). It can act as a substrate for GSH-Px and glutathione-S-transferase (GST), and directly combine with cellular electrophilic reagents to protect against ROS and xenobiotics (Li et al. 2010b). The MC-GSH complex can be formed via GST catalysis in aquatic plants and fish, acting as the first step of MC detoxification (Pflugmacher et al. 1998). In general, the GSH contents in all treatment groups were markedly inhibited compared to the control (Fig. 2e). Similarly, the GSH contents in the dark-spotted frog (*Rana nigromaculata*) significantly decreased after exposure to 1 μg L^{-1} MC-LR for 7 and 14 days (Zhang et al. 2013a). The ANOVA results indicated that the GSH contents were significantly affected by MC-LR concentration, exposure duration, and the interaction between concentration and duration (Table 4). The reduction in GSH contents in the treatment groups indicated that GSH was significantly consumed, lowering the cellular antioxidative capacity. Once GSH was exhausted, contaminants and metabolites could not be consumed by GSH, allowing oxidative damage to occur.

Lipid peroxidation is an important parameter for evaluating oxidative stress in organisms (Radi et al. 1991). MDA is a major product of the peroxidation reaction between free radicals and unsaturated fatty acids in cellular membranes, and the MDA content can indirectly reflect the degree of intracellular injury. Figure 2f presents the alterations in MDA content after MC-LR exposure. The MDA contents in MC-LR-treated groups increased with increasing exposure duration. At day 3, the MDA contents in MC-LR-treated groups were higher than the control. At day 28, the difference in MDA contents between MC-LR-treated groups and the control was highly increased ($p < 0.001$). According to the ANOVA results, the MDA contents were markedly affected by MC-LR concentration, exposure duration, and the interaction between concentration and duration (Table 4). These results suggested that MC-LR caused major lipid peroxidation and oxidative injury in earthworms, consistent with the toxic effects of MC-LR on frog (*Rana. Nigromaculata*) (Zhang et al. 2013a). In addition, DNA adducts produced by the reaction of MDA with deoxyadenosine and deoxyguanosine in DNA could lead to mutagenesis and carcinogenesis (Bartsch and Nair 2000). Therefore, the MDA accumulated in earthworms might impair their DNA and physiological functions, subsequently reducing their growth rate.

Effects of MC-LR on cell viability

Coelomocyte apoptosis indicates the loss of cell viability and beginning of death. After incubation for 14 days, coelomocyte apoptosis (including early and late apoptosis) increased markedly in MC-LR-treated groups compared with the control, indicative of severe toxicity and

Table 6 Degradation of MC-LR in soil during the experiment

Exposure (day)	25 μg kg^{-1}	50 μg kg^{-1}	100 μg kg^{-1}	200 μg kg^{-1}	300 μg kg^{-1}
3	21.53 ± 1.62	44.20 ± 1.12	87.79 ± 2.77	164.41 ± 8.98	262.23 ± 1.70
7	18.78 ± 1.03	37.09 ± 1.38	76.93 ± 1.01	146.93 ± 9.26	241.99 ± 2.78
14	16.53 ± 0.98	33.11 ± 2.33	67.05 ± 0.52	133.91 ± 2.64	211.75 ± 2.94
21	12.94 ± 0.73	25.52 ± 1.25	53.24 ± 4.24	110.12 ± 4.79	179.82 ± 10.74
28	10.06 ± 0.37	21.18 ± 1.98	44.72 ± 2.06	94.63 ± 1.94	143.33 ± 3.39

Data are expressed as mean ± sd ($n = 3$)

substantial damage to coelomocytes (Fig. 3). The coelomocyte apoptosis rate increased from 2.2 to 12.6% with increasing MC-LR concentrations, mainly comprising coelomocytes in early apoptosis.

At the initial stage of apoptosis, phosphatidylserine in the cell membrane is transferred from the inside to the outer surface of the cell membrane. Then, the function of mitochondria begins to change due to the alteration of the membrane potential, which is followed by the release of various mitochondrial proteins (e.g., cytochrome C and Smac protein), resulting in the activation of caspase and DNA fragmentation (Ma et al. 2016). Coelomocyte apoptosis might be the result of MC-LR-induced oxidative stress causing lipid peroxidation, which could damage cell membranes, altering their permeability (Wang et al. 2007). The severe lipid peroxidation caused by MC-LR (see the section of non-enzymatic antioxidants) could explain the damage to coelomocytes driving apoptosis and could indirectly reflect the degree of intracellular damage (Wang et al. 2007). These results demonstrated that realistic concentrations of MC-LR in soil (Chen et al. 2012; Li 2015) could cause severe levels of coelomocyte apoptosis in earthworms.

Lysosomal membrane stability in coelomocytes is a potential cellular biomarker used widely to study the toxicological effects of contaminants, which can be evaluated using the neutral red retention time (NRRT). It is regarded as a sensitive biomarker in earthworms that meets the needs of soil pollution risk assessments (Svendsen et al. 2004). In the present study, the NRRT of coelomocyte lysosomal membranes was evaluated after exposure to MC-LR-polluted soil (Fig. 4). The NRRT in MC-LR-treated groups was significantly inhibited compared with the control, especially after exposure for 14 days. NRRT inhibition increased with increasing MC-LR concentration and exposure duration. This inhibition was indicative of a decrease in lysosomal membrane stability and severe damage to earthworm coelomocytes after exposure to MC-LR-polluted soil. MC-LR seems to enhance the catabolic activity of cells in earthworms, which may help explain the decrease in bodyweight after exposure to it. However, the mechanism driving the changes in lysosomal membrane stability is not well understood. Evidence from previous studies suggests that cellular ROS production has an important role in pollutant-induced cellular responses and affects various cellular organelles and their repair systems (Markad et al. 2012). The results of the present study support this cognition.

MC-LR degradation in soil

The recovery rate of MC-LR in soil was 89–94%. Only 82–88% and 40–48% of the MC-LR doses were detected at day 3 and day 28, respectively (Table 6). These results indicated that the actual concentrations of MC-LR in the tests were significantly reduced compared with the nominal concentrations at longer exposure durations. However, the acute toxicity, growth inhibition, reproduction toxicity, oxidative damage, and cytotoxicity in the earthworms exposed to MC-LR in soil increased with prolonged exposure time, possibly due to the formation of toxic metabolites of MC-LR or the bioaccumulation of MC-LR in earthworms (Corbel et al. 2016). Regardless, it is worrying that MC-LR concentrations of several treatments in tests, equivalent to levels observed in agricultural soils (Chen et al. 2012; Li 2015), showed adverse effects on *Eisenia fetida*. In particular, such concentrations of MC-LR might cause greater toxicity (or even death) to other earthworm species that are often more sensitive than *Eisenia fetida*.

Conclusions

This is the first study to examine the acute and subacute toxic effects of MC-LR on earthworms in soil. Several indicators that were used to evaluate the toxicity of MC-LR in soil demonstrated that MC-LR was classified as a highly toxic or super-toxic chemical, showing various adverse effects including acute toxicity, growth inhibition, reproduction toxicity, oxidative damage, and cytotoxicity on earthworm at environmentally relevant concentrations. Especially, earthworm reproduction was strongly impacted at a concentration as low as 0.2 mg kg^{-1}, implying the potential for a reduction in earthworm populations and adverse effects on soil ecosystems. This study proved the potential risk of MC-LR at low concentrations, which should be considered in ecological risk assessments of MC-LR in soil. It is worrying that realistic MC-LR concentrations in agricultural soil in China could pose a serious threat to earthworms, and possibly other soil animals. More researches are eagerly needed and even governmental actions are necessary in the future.

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