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Bioaccumulation and Elimination of the Herbicide Clomazone in the Earthworms *Eisenia fetida*

Jia Cao[1,2,3] · Ping Li[4] · Qing X. Li[5] · Pengfei Zheng[1,2] · Xiaoping Diao[1,2,4]

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Abstract Acute toxicity, bioaccumulation, and elimination of herbicide clomazone in the earthworm *Eisenia fetida* were investigated in the different exposure systems. The LC_{50} values of clomazone on earthworms were 5.6 μg cm^{-2} in the contact filter paper test (48 h), 174.9 mg kg^{-1} (7 days) and 123.4 mg kg^{-1} (14 days) in artificial soil test, respectively. Clomazone could rapidly bioaccumulate in earthworms and reached the highest concentration after 3 days exposure, with the maximum concentrations of 9.0, 35.3 and 142.3 mg kg^{-1} at 10.0, 40.0 and 160.0 mg kg^{-1} of clomazone, respectively. Clomazone uptake showed a good correlation with exposure concentration. After the 14th day, clomazone declined to minimum value. About 74 %–80 % of accumulated clomazone was eliminated within 1 day after exposed to clomazone-free soil. However, a trace amount of clomazone persisted for a relatively long time in earthworms.

Keywords Clomazone · Earthworm · Pesticide residue · Acute toxicity

✉ Xiaoping Diao
diaoxip@hainu.edu.cn

[1] College of Agriculture, Hainan University, Haikou 570228, Hainan, China

[2] Haikou Key Laboratory of Environment Toxicology, Hainan University, Haikou 570228, Hainan, China

[3] College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China

[4] College of Plant Protection and Environment, Hainan University, Haikou 570228, Hainan, China

[5] Department of Molecular Bioscience and Bioengineering, University of Hawaii, 1955 East-West Road, Honolulu, HI 96822, USA

Clomazone (2-[(2-chlorophenyl) methyl]-4, 4-dimethyl-3-isoxazolidinone), as a soil-application herbicide, is currently used for weed control in cultivation of soybeans, cotton, tobacco, and various vegetable crops. Clomazone is highly water-soluble (1100 mg L^{-1}), minimally volatile ($P_v = 1.44 \times 10^{-4}$ mm Hg), resistant to hydrolysis under a wide range of pH values, and weakly adsorptive to soil ($K_D = 0.47–5.30$) with sorption dependent upon organic carbon content ($K_{OC} = 300$ mL g^{-1}) (Hu et al. 2011; Cao et al. 2013a). This herbicide is highly watersoluble, which increases its potential for contamination (Menezes et al. 2014). In addition, it is more persistence in soil and has the potential risk to crop production (Mills et al. 1989). The fate of clomazone in soils was studied under controlled laboratory conditions (Mervosh et al. 1995; Li et al. 2004; Quayle et al. 2006). Soil moisture, temperature, microorganisms and pH have been shown to affect the degradation of clomazone in soil (Mervosh et al. 1995).

Earthworms are important soil organisms that play a significant role in soil ecology and terrestrial food chains. They have been used to monitor pesticides and other chemical contaminants in soils (Paoletti 1999; Olvera-Velona et al. 2008). Some reports focused on the accumulation of pesticides such as metalaxyl, avermectin and p,p-DDE in earthworms (Sun et al. 2005; Kelsey et al. 2008; Xu et al. 2011). They may be used to monitor herbicide clomazone too. In the recent years, accumulation of clomazone in different organisms was studied, which included fish, rice and watergrass (Clasen et al. 2012; Lazartigues et al. 2013; TenBrook and Tjeerdema 2006).

In this study, we report on the acute toxicity of clomazone to earthworms in different exposure systems with artificial soil and filter paper. We also investigated the bioaccumulation of clomazone in earthworms and its elimination in clomazone-free soil.

Materials and Methods

The earthworm (*Eisenia fetida*) was purchased from an earthworm production farm in Beijing, China. Mature health earthworms with body weight of 0.3–0.6 g were used for all experiments. The worms were active when being used in the experiment. Clomazone (purity, 97.3 %) was purchased from Shandong Cynda Chemical (Group) Co., Ltd. (Shandong, China). Double-distilled water was used for high performance liquid chromatography (HPLC) analysis. HPLC grade methanol was purchased from Dikma Limited (China). All other chemicals and solvents were analytical grade.

Filter paper contact test was conducted according to the standard test procedure recommended by the Organization for Economic Co-operation and Development (OECD) guideline 207 (1984). Clomazone dissolved in acetone (1 mL) was added to the filter paper using glass tubes (3 × 8 cm). Acetone was dried under a stream of high purity nitrogen followed by addition of deionized water (1 mL) to maintain moisture. The earthworms were emptied on moist filter paper for 3 h before being placed in the tube. Each tube contained a depurated earthworm. Ten earthworms were used for each concentration test. Exposure concentrations of clomazone were 0, 1, 2, 4, 8, 16, 32, 64 μg cm^{-2}, respectively.

Artificial soil test preparation: The artificial soil was prepared according to OECD guideline 207 (1984). It was comprised (by dry weight) of 10 % finely ground sphagnum peat, 20 % kaolin clay, and 70 % industrial fine sand with pH 6.5.

The concentrations of clomazone in dried artificial soil are 0, 10, 20, 40, 80, 160, 240 mg kg^{-1}. Earthworms were incubated in clomazone-free artificial soil for 24 h and then 10 of them were added to the contaminated soils with clomazone in different concentrations contained in jars according to OECD guideline 207 (1984). Three replicates for each treatment with each jar containing 10 worms were done. Earthworms in the bottle were incubated at 20 ± 1°C, moisture of 75 % for 14 days. The number of survived earthworms was counted on the 7th and 14th days. The earthworm was considered as dead when it did not respond to gentle touching and was removed immediately.

The bioaccumulation experiment was conducted in artificial soil with the different concentrations of clomazone (10, 40 and 160 mg kg^{-1} dry soil). Clomazone in the artificial soils were detected by HPLC, no clomazone detected in the reference artificial soil and the tested concentrations were closely equal to the spiked concentration at all three levels. After being exposed for 1, 3, 5, 7, 10 and 14 days, 2 g of earthworms were randomly selected from the contaminated artificial soil. On the 14th day, the earthworms were then transferred to clomazone-free artificial soil for another 14 days to observe their elimination rate. During the clearance period the earthworms were randomly sampled on days 1, 3, 5, 7, 10 and 14 to determine the concentrations of clomazone. The earthworms were washed to remove free soil and placed on moist filter paper immediately for 24 h in order to depurate their gastrointestinal contents, and then were stored at −20°C until analysis. The exposure concentration of earthworm to clomazone was determined by the HPLC method described below.

Whole frozen earthworms (2.0 ± 0.05 g) were thawed and homogenized for 3 min at full speed on a vortex mixer. The homogenized earthworm sample was extracted by 10 mL acetonitrile (ACN), followed by centrifugation at 3000*g* for 15 min (Kennedy et al. 1993). The supernatant was transferred to a round-bottom flask and dehydrated at 50°C using a vacuum rotary evaporator. The residue was dissolved in 10 mL n-hexane and was loaded to the alumina B cartridge that was previously activated with n-hexane (10 mL) at a flow rate of about 2 mL min^{-1}. The column was eluted with 6 mL of n-hexane–acetone (90:10, v/v) and the eluate was collected into a round-bottom flask and dehydrated at 50°C using the vacuum rotary evaporator. After the extract was dehydrated under a gentle nitrogen stream, 2 mL of methanol was added to dissolve the sample for HPLC analysis.

A high performance liquid chromatograph (Shimadzu LC-20A) equipped with an analytical column (250 × 4.6 mm inner diameter, 5 μm ODS) was attached to a diode array detector (DAD). The mobile phase was 70 aqueous methanol (70:30, v/v) with a flow of 1.0 mL min^{-1}. The injection volume was 20 μL. Detection was performed at 199 nm. Under such conditions, the retention time of clomazone was about 7.5 min. All measurements were carried out at room temperature. Earthworm samples were fortified with clomazone standard at concentrations of 0.01, 0.1, 0.5 and 150 mg kg^{-1}. All samples were extracted, purified, and determined as described above.

Accumulation rate was estimated from the uptake parameters during a period of exposure by the following equation:

$$R = \Delta C_{\text{worm}}/\Delta T = (C_{\text{wormn2}} - C_{\text{wormn1}}) / (t_{\text{n2}} - t_{\text{n1}})$$

where t_{n1} and t_{n2} represent two continuous sampling times, and $C_{\text{worm n1}}$ and $C_{\text{worm n2}}$ represent clomazone concentrations in earthworms at the corresponding sampling time (Zhang et al. 2009).

Results and Discussion

The standard calibration curve of clomazone was constructed by plotting the analyte concentration against peak areas under the proposed chromatographic conditions. At

199 nm, a good linearity was achieved with correlation coefficients (R^2) = 0.9996. The standard curve equation was $y = 6011.43x + 241.76$, where y was peak area, and x was clomazone concentration.

The mean recoveries of clomazone at spiking levels (0.01, 0.1, 0.5 and 150 mg kg^{-1}) were from 87 % to 92 % in earthworms, and the coefficient variations (CV %) of the method ranged from 0.4 % to 1.1 %, the limit of detection (LOD) was 0.4 ng L^{-1}.

The filter paper test and artificial soil test (OECD method) results showed that the LC_{50} values of clomazone on earthworms were 5.63 μg cm^{-2} (48 h exposure) by filter paper contact test and 174.92 mg kg^{-1} (7 days exposure) and 123.44 mg kg^{-1} (14 days exposure) by artificial soil test.

No mortality was observed in the control group (deionized water and acetone control) after 48 h of exposure. Earthworms exposed to clomazone on filter paper showed concentration- and time-dependent lethal and sublethal effects. Chemical compound with LC_{50} values of 10–100 μg cm^{-2} on filter paper was classified as ''very toxic'' (Landrum et al. 2006), which suggested that clomazone (48 h-LC_{50}: 5.63 μg cm^{-2}) was highly toxic to *E. fetida*.

This study has shown that clomazone is toxic to earthworms in artificial soils. The pesticide on the acute toxicity of earthworm can be divided into three grades (Shi et al. 2009): $LC_{50} > 10$ mg kg^{-1}, low toxicity; $LC_{50} = 1$–10 - mg kg^{-1}, middle toxicity; $LC_{50} < 1$ mg kg^{-1}, high toxicity. According to this standard, clomazone toxicity to earthworms can be classified as low toxicity.

Based on the clomazone acute toxicity test in artificial soil, 10, 40 and 160 mg kg^{-1} were chosen as lower, middle and higher dose groups, respectively in the bioaccumulation study. Uptake and accumulation rates of clomazone in earthworm are shown in Figs. 1 and 2. Earthworms can take up and accumulate pollutants in their body tissues through the skin and digestive systems (Shan et al. 2014; Rodriguez-Campos et al. 2014).

The concentrations of clomzaone in soil were correlated with the concentrations in earthworms. On the first day, the concentrations of clomazone in earthworm were 4.4, 18.7 and 50.9, respectively, for the lower, middle and higher dose groups. On the third day, the concentrations reached their maximums with concentrations of 9.0, 35.3 and 142.3 mg kg^{-1} for the lower, middle and higher dose groups, respectively. The concentrations in earthworms then decreased gradually to the concentrations of 2.9, 4.6, and 18.7 mg kg^{-1} respectively on the 14th day. All clomazone concentrations in the earthworms from the three groups were lower than those in the artificial soil. The bioaccumulation results showed no significant accumulation of clomazone in earthworms, but there was a good correlation between the amounts of clomazone taken up by the earthworms and the concentrations of the chemical in soil.

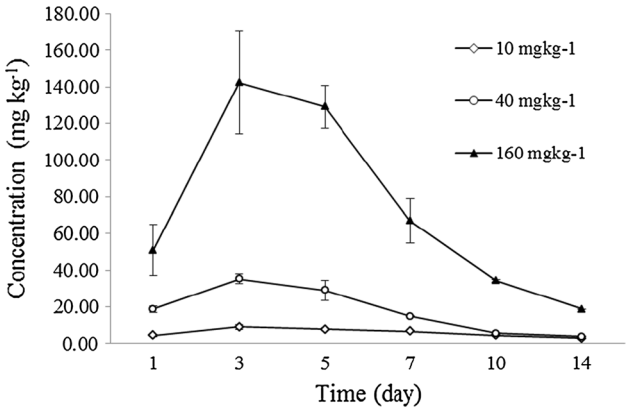

Fig. 1 The concentrations in earthworms after the exposure to clomazone fortified soil at three different concentration levels of 10, 40 and 160 mg kg^{-1}, respectively. Significant differences were observed between high clomazone treatment and the other two treatments over time ($p < 0.05$)

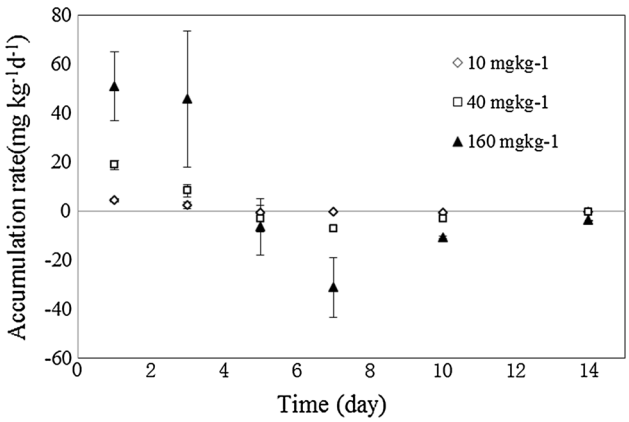

Fig. 2 Clomazone accumulation rate after exposure to clomazone fortified soil at three different concentration levels of 10, 40 and 160 mg kg^{-1}, respectively. Significant differences were observed between high clomazone treatment and the other two treatments over time ($p < 0.05$)

The earthworms were transferred to the clomazone-free artificial soil for the elimination test. Figure 3 shows elimination of clomazone from earthworms. After the end of exposure to free clomazone-treated soil, clomazone levels in earthworms decreased significantly ($p < 0.05$). Approximately 74 %, 75 % and 80 % of clomazone in the earthworms were lost within 1 day in 10, 40 and 160 mg kg^{-1} groups, respectively. After the 14-day elimination test period, the concentrations of clomazone in the earthworms exposed to uncontaminated artificial soil were 0.16, 0.35 and 0.56 mg kg^{-1}, respectively, in the low, middle and high exposure groups. In one of our previous investigations, we observed that clomazone effect on

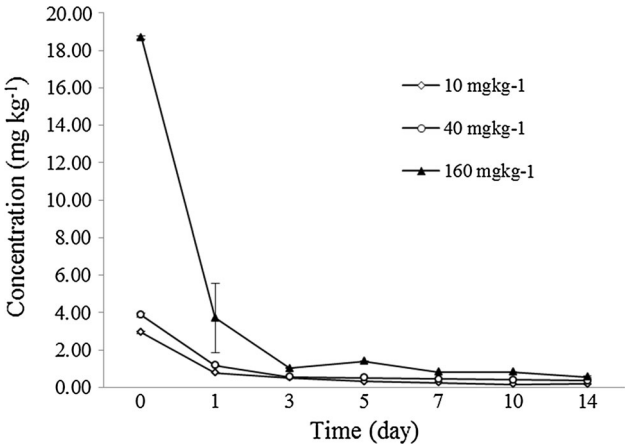

Fig. 3 Elimination in earthworms after exposure to clomazone-free soil at levels of 10, 40 and 160 mg kg^{-1}. Significant differences were observed between high clomazone treatment and the other two treatments over time ($p < 0.05$)

activity of antioxidative enzymes SOD, CAT, GSH-Px, T-AOC (Cao et al. 2013b), which may help eliminate contaminants from the body or transform them into other unknown compounds, ultimately resulting in lower accumulated concentrations in earthworms. The similar phenomenon of the elimination of avermectin B1a in uncontaminated soil was also observed (Sun et al. 2005).

Although clomazone was not significantly accumulated in the worms, clomazone was taken up rapidly, reached a highest concentration in a short period of time and then eliminated quickly. The relatively rapid elimination of clomazone from earthworm indicated clomazone are not likely to bioaccumulate in higher organisms.

In conclusion, the LC_{50} of clomazone on earthworms for 48 h of exposure was 5.6 μg cm^{-2} in the contact filter paper test. The LC_{50} values tested by the artificial soil test were 174.9 and 123.4 mg kg^{-1}, respectively, for 7 and 14 days of exposure. Clomazone was rapidly absorbed by the earthworms and the highest concentration in the earthworm was 9.02, 35.30 and 142.27 mg kg^{-1}, respectively in the low, middle and high exposure groups after 3 days of exposure, which suggests that clomazone could be absorbed rapidly by the earthworms. Clomazone uptake highly correlated with exposure concentrations. However, a trace amount of clomazone persisted for a relative long time in earthworms.

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