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Growth, reproduction and biochemical toxicity of chlorantraniliprole in soil on earthworms (*Eisenia fetida*)

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ABSTRACT

Diamide insecticides have become the fourth most commonly used insecticide class in the world. Chlorantraniliprole (CAP) is a first-generation diamide insecticide with broad application potential. In this experiment, the eco-toxicity of CAP in soil at 0.1, 1.0, 5.0 and 10.0 mg/kg on earthworms (*Eisenia fetida*) was evaluated during a 42 d exposure. More specifically, the environmental fate and transport of CAP between soil and earthworms was monitored during the exposure period. The present results indicated that the CAP contents of 0.1, 1.0, 5.0 and 10.0 mg/kg treatments decreased to no more than 20% in the soil after 42 d of exposure. The accumulation of CAP in earthworms was 0.03, 0.58, 4.28 and 7.21 mg/kg earthworm (FW) at 0.1, 1.0, 5.0 and 10.0 mg/kg after 42 d of exposure. At 0.1 mg/kg and 1.0 mg/kg, CAP had no effect on earthworms during the exposure period. The weight of earthworms was significantly reduced at 5.0 and 10.0 mg/kg at 28 and 42 days after CAP application. After the 14th day, CAP induced excess production of reactive oxygen species (ROS) at 5.0 and 10.0 mg/kg, resulting in oxidative damage to biomacromolecules. We believe that CAP has a high risk potential for earthworms when used at 5.0 and 10.0 mg/kg.

1. Introduction

Diamide insecticides are among the most recently developed class of systemic insecticides, which have become the fourth most commonly used insecticides in the world, accounting for 8% of total global insecticide sales (Sparks and Nauen, 2015). Diamide insecticides act on the ryanodine receptor (RyR) of insects and cause the excessive release of Ca^{2+}, resulting in insect death by muscle paralysis (Qi et al., 2014; Sparks and Nauen, 2015). Chlorantraniliprole (CAP), 3-bromo-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloropyridin-2-yl)-1H-pyrazole-5-carboxamide, is a first-generation diamide insecticides with activity against *Lepidopteran* and *Coleopteran* pests (Dong et al., 2011). Moreover, CAP provides an alternative mode of action to control pests that are resistant to pyrethroid and neonicotinoid insecticides (Malhat, 2012). Additionally, CAP has high security for mammals because the RyR structure differs between insects and mammals (Malhat, 2012; Wang et al., 2012). Due to its safety and efficacy, CAP has been widely applied in different crop systems around the world using a variety of methods (Schwarz et al., 2011; Zhang et al., 2012; Cui et al., 2014).

As CAP becomes more commonly used, trends suggest it will eventually be discharged into the soil during agricultural production. Studies have shown that the half-life of CAP in soil ranges from 30 to 1130 days due to some factors, such as soil properties, application dose and climate conditions (EPA, 2008; FAO, 2008; Lavtižar et al., 2016). Therefore, CAP may be a potential soil pollutant. Moreover, the improper use of pesticides occurs frequently around the world, enhancing the risk of CAP to the soil environment. However, previous studies on CAP have mainly focused on its synthesis and application (Cui et al., 2014; Chen et al., 2015; Sparks and Nauen, 2015). There is little data on the environmental behaviour and risk assessment of CAP in the soil environment. Although some studies have showed that CAP has little influence on parasitic wasps (Brugger et al., 2010), predatory insects (Gontijo et al., 2015), isopods (Lavtižar et al., 2016), enchytraeids and oribatid mites (EPA, 2008; Lavtižar et al., 2016), one study showed that CAP may pose a special risk to non-target soil arthropods, such as springtails (Lavtižar et al., 2016). Therefore, it is necessary to investigate the environmental fate of CAP in the soil environment and the influence of CAP on additional soil organisms.

Earthworm is an important soil organism that maintains soil nutrients by converting unstable organic waste, such as industrial waste and animal waste to nutrient rich vermicast (Datta et al., 2016). Earthworm can affect the soil structure and soil nutrient cycling through burrowing activity (Rodriguez-Campos et al., 2014). Additionally, earthworm is an important ecological toxicology indicator organism and has been widely used to evaluate the toxicity of

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pollutants (Chen et al., 2011b; Wang et al., 2015; Ye et al., 2016).

Biomarkers, such as growth inhibition ratio, reproductive rate, reactive oxygen species (ROS) level, antioxidant enzyme activity, lipid peroxidation level and DNA damage degree have increasingly been used in ecological toxicology to investigate the adverse effects of contaminants on organisms (Geric et al., 2014; Xia et al., 2015; Guo et al., 2016; Lemos et al., 2016).

In this experiment, we evaluated the ecotoxicity of CAP to earthworms using several biomarkers at a series of environmental background concentrations. Meanwhile, we monitored the effective concentrations of CAP in soil and earthworms over the entire exposure period. The present study could provide some data on risk assessment of CAP in soil environment.

2. Materials and methods

2.1. Materials

CAP with 99% purity (CAS NO. 500008-45-7) was purchased from Dr. Ehrenstorfer (Ausberg, Germany).

Earthworms (*Eisenia fetida*) were purchased from an earthworm breeding base (Qingdao, China). Earthworms were put in a controlled climate chamber at 20 ± 1°C with a 12/12-h photo-period for 2 weeks and fed with cow manure. Healthy earthworms with clitellum (350 ± 20 mg, fresh weight) were selected for the experiment.

The soil used in the present study was prepared using the OECD standard method, which contained 10% of sphagnum peat moss, 20% of kaolin clay and 70% of sand (OECD, 1984). The pH of the soil was adjusted to 6 ± 0.5 using calcium carbonate ($CaCO_3$), and the water content was adjusted to 35% of the total soil content using distilled water.

2.2. Experimental design

Studies have shown that the initial residual concentrations of CAP in soil were 0.28–4.56 mg/kg (Dong et al., 2011; Malhat et al., 2012; Zhang et al., 2012). Therefore, the experimental concentrations were 0, 0.1, 1.0, 5.0 and 10.0 mg/kg soil dry weight (DW). The CAP (0.1 g) was dissolved in methanol and a proper amount of CAP solution was added to 750 g of artificial soil (OECD, 1984). The soil was fully stirred to ensure methanol volatility and then transferred to a beaker. For the control group, the same amount of methanol was added to the soil to avoid damage to earthworms induced by solvent. The beakers were put in the fume hood for 24 h to remove all the methanol. Subsequently, twenty earthworms were placed in the beaker and cultured in a controlled climate chamber at 20 ± 1°C with a 12/12-h photo-period. The beaker was covered using a plastic wrap with small holes to limit water loss. Once a week, the water content for each beaker was compensated by weighing. For each beaker, 5 g of cow manure was placed on the soil surface, and the same amount of cow manure was provided once a week. For each concentration, 10 beakers were prepared, and half were used to determine the effective concentrations, weight change rate, cocoon production and number of juveniles. The other half was used to determine ROS level, antioxidant enzyme activities and oxidative damage degree. During the entire exposure period, no dead earthworms were found in any treatment. On days 3, 7, 14, 28 and 42, four earthworms from each beaker were randomly selected for analysis of various biomarkers. Prior to analysis, earthworms were washed using distilled water and placed on filter paper for 12 h to depurate the gut contents.

2.3. Effective concentrations of CAP in soil and earthworms

On days 3, 7, 14, 28 and 42, the CAP concentrations were determined using a high-performance liquid chromatographic-tandem mass spectrometric (HPLC- MS/MS). For each sample, 5 g of soil and four earthworms were randomly selected from each beaker. The fresh body weight of the earthworm was measured and recorded. The earthworm was homogenized in 5 mL of deionized water. The CAP contents in soil and the earthworm tissue grinding fluid were extracted using 10 mL of acetonitrile. Subsequently, a salt package (1 g NaCl and 4 g $MgSO_4$) was added to the sample and the sample was vigorously shaken for 2 min. After centrifuging at 5000 rpm for 2 min, 1.5 mL of supernatant was purified using 50 mg of C_{18}. Finally, the purified supernatant was filtered using a 0.22-μm syringe filter.

A Hypersil GOLD C_{18} column (Thermo, 2.1 × 100 mm, 3.0 μm) was used to separate CAP. The mobile phase was 0.1% formic acid water (A) and acetonitrile (B) with the flow rate of 0.25 mL/min. The gradient elution program was: 0–0.5 min, 10% B; 0.5–1.0 min, 10–60% B; 1.0–6.0 min, 60% B; 6.0–8.0 min, 60–10% B; 8.0–10.0 min, 10% B.

A triple-quadrupole mass spectrometer (Thermo TSQ Quantum Ultra, Thermo Fisher Scientific Inc., San José, CA, USA) was used to determine CAP content. The detection was performed in multiple reaction monitoring mode with positive electrospray ionization (ESI^+). The capillary temperature was 350°C and the capillary voltage was 3.0 kV. The quantitative ion pair was 483.9/452.8 (*m/z*) and the qualitative ion pair was 483.9/285.8 (*m/z*). The collision energies were 16 eV and 14 eV, respectively. In the present study, the recoveries of CAP in soil and earthworms were 90.2–104.6% and 85.6–97.3%, respectively. The concentrations of CAP in soil and earthworms were expressed as mg/kg soil dry weight (DW) and mg/kg fresh weight (FW).

Biota-soil-accumulation factors (BSAFs) were used to reflect the accumulation of CAP in earthworms and calculated by dividing the CAP concentration in the earthworms by the concentration in the soil.

2.4. Determination of weight, amount of cocoon production and number of juveniles

Before the experiment, the initial average weight of four randomly selected earthworms from each beaker was recorded as W_0. On days 3, 7, 14, 28 and 42, the average weight of four randomly selected earthworms from each beaker was recorded as W_t. The weight change rate was calculated using the formula as follows:

$$\text{Weight change rate}(\%) = \frac{W_t - W_0}{W_0} \times 100$$

After exposure, the soil was sifted using a sieve with a mesh aperture of 1 mm, and the earthworm cocoons and earthworm juveniles were collected.

2.5. Determination of ROS level

The ROS level was measured using a method described by Liu et al. (2016a). One earthworm per beaker (five per treatment) was randomly selected and homogenized. After centrifuging at 3000 g for 5 min, the sample was re-centrifuged at 20,000 g for 20 min. Subsequently, DCFH-DA (2 μM) was added into the sample, and the sample was incubated at 37°C for 30 min. Finally, the fluorescence was measured using a fluorescence spectrophotometer (F-4600, Hitachi, Japan). Meanwhile, the protein contents were determined using coomassie brilliant blue method and quantified using the BSA as the standard substance (Bradford, 1976). The ROS levels were expressed as fluorescence intensity/mg protein.

2.6. Determination of antioxidant enzyme activities

One earthworm per beaker (five per treatment) was randomly selected and homogenized. After centrifuging at 2500 g for 5 min, the sample was re-centrifuged at 3000 g for 15 min and used for various analyses.

The superoxide dismutase (SOD) activities were determined by measuring the amount required to induce reduction of nitroblue tetrazolium chloride (NBT). One unit of SOD activity was defined as the quantity of SOD causing half of the NBT photoreduction (Song et al.,