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Combined effects of four pesticides and heavy metal chromium (VI) on the earthworm using avoidance behavior as an endpoint

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ABSTRACT

In natural ecosystems, organisms are commonly exposed to chemical mixtures rather than individual compounds. However, environmental risk is traditionally assessed based on data of individual compounds. In the present study, we aimed to investigate the individual and combined effects of four pesticides [fenobucarb (FEN), chlorpyrifos (CPF), clothianidin (CLO), acetochlor (ACE)] and one heavy metal chromium [Cr(VI)] on the earthworm (*Eisenia fetida*) using avoidance behavior as an endpoint. Our results indicated that CLO had the highest toxicity to *E. fetida*, followed by Cr(VI), while FEN showed the least toxicity. Two mixtures of CPF + CLO and Cr(VI) + CPF + CLO + ACE exhibited synergistic effects on the earthworms. The other two quaternary mixtures of CLO + FEN + ACE + Cr(VI) and Cr(VI) + FEN + CPF + ACE at low concentrations also displayed synergistic effects on the earthworms. In contrast, the mixture of Cr(VI) + FEN had the strongest antagonistic effects on *E. fetida*. Besides, the quinquenary mixture of Cr(VI) + FEN + CPF + CLO + ACE also exerted antagonistic effects. These findings highlighted the importance to evaluate chemical mixtures. Moreover, our data strongly pointed out that the avoidance tests could be used to assess the effects of combined effects.

1. Introduction

Pesticides such as insecticides, herbicides and fungicides are chemicals that kill, repel, or regulate the growth of harmful organisms. In agricultural fields, insecticides and herbicides are often co-applied to control weeds and insect pests (Huseth et al., 2015). However, such practice also presents the risks of soil pollution and adverse effects on soil fauna (Wang et al., 2016). Additionally, the release of heavy metals into the environment from anthropogenic activities also poses potential risks to non-target organisms (Jaishankar et al., 2014). Pesticides and heavy metals may disturb soil ecosystems, leading to impaired structure of soil invertebrate communities (Uwizeyimana et al., 2017). It is a challenge to assess the combined effect of pesticides and heavy metals as mixtures for environmental toxicology and human health studies (Heys et al., 2016). Recently, an increasing number of studies have investigated mixtures of contaminants to soil organisms (Cang et al., 2017), while most of these studies have only focused on traditional toxic endpoint, such as mortality and reproduction (Chen et al., 2015).

As common soil invertebrates in most soil environments (Uwizeyimana et al., 2017), earthworms can improve structure and fertility of soil ecosystems by increasing aeration and drainage through their burrowing, feeding and casting activities (Liu et al., 2017). It has been indicated that earthworms may represent up to 60–80% of the total animal biomass in soil (Wang et al., 2016). Unlike other soil organisms, earthworms are very susceptible to soil pollutants since they are not protected by thick cuticle on the exterior of their bodies (Liu et al., 2018). Most toxicants absorbed or ingested by earthworms would undergo bioaccumulation, and be passed on to animals at subsequent levels of the food chain (Chevillot et al., 2017). Bioaccumulation of chemicals in earthworm may not lead to significant effects on the

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animal itself, but such bioaccumulation may result in serious damages to the higher animals (Šmídová et al., 2015). Therefore, earthworms are suitable bioindicators of soil contamination (OECD, 1984; Beaumelle et al., 2017; Shi et al., 2017).

Avoidance behaviors are ecologically relevant because soil functions are reduced when organisms retreat from contaminated soils (Brami et al., 2017). Earthworm avoidance tests use the presence of chemoreceptors on the anterior segments and sensory tubercles on the body surface, which can detect a wide range of contaminants (Scheffczyk et al., 2014). There are clear advantages of using avoidance behavior tests to the earthworm over other sub-lethal (reproduction and growth) and acute tests (Xie et al., 2013; Lowe et al., 2016). For instance, a shorter time is required for avoidance tests (2 days) compared with reproduction (56 days) or acute tests (14 days) (OECD, 1984, 2004; ISO, 2008). Such a test is a valuable tool for rapid screening of large areas of potentially contaminated soil or large numbers of soil samples (Gao et al., 2016). Extensive avoidance tests using earthworms have been conducted in recent years, but most of them have focused on effects of single toxicants (García-Santos and Keller-Forrer, 2011; Scheffczyk et al., 2014; Brami et al., 2017). However, combined effects of chemical mixtures on earthworms remain largely unclear (Loureiro et al., 2009; Amorim et al., 2012; Uwizeyimana et al., 2017).

Three insecticides fenobucarb, chlorpyrifos and clothianidin, and one herbicide acetochlor with different mode of action (MOA) have been widely used in agriculture worldwide (Wood and Goulson, 2017; Yang et al., 2017). Moreover, heavy metal chromium is a widespread occupational and environmental toxicant with high ecotoxicity, and it is recognized as one of the most hazardous pollutants to ecosystems (Basha and Latha, 2016). The sources of chromium contamination include leather tanning, metallurgy, mining, fossil fuel combustion, wood preservatives, cooling installation effluents, and electroplating industries (Gutierrez et al., 2010). Chromium exists in the environment primarily in two valence states, trivalent chromium and hexavalent chromium. The trivalent chromium is less toxic to humans and the environment, while hexavalent chromium, a strong oxidizer, is considered toxic (Basha and Latha, 2016). Pesticides and heavy metals usually co-exist in the same soil sample. Therefore, there are growing concerns regarding their interactive toxicities to terrestrial organisms (Uwizeyimana et al., 2017). In the present study, we aimed to evaluate the combined effects of four pesticides and heavy metal chromium on the earthworms using avoidance behavior as an endpoint. Our findings provided a better understanding of the interactions of chemical mixtures against soil organisms.

2. Materials and methods

2.1. Test organisms

Adult earthworms (*Eisenia fetida*) with well-developed clitellum (0.35–0.50 g) were obtained from a commercial breeder in Hangzhou, China. They were fed with pig manure for 2 weeks at 20 ± 1 °C under a 16 h/8 h light/dark cycle with a relative humidity of 80 ± 10% in order to meet the conditions of the test guidelines of the Organization for Economic Co-operation and Development (OECD, 1984, 2004). The worms were acclimatized in OECD artificial soil for 1 day before testing (OECD, 1984, 2004). Experiments were conducted in OECD artificial soil consisting of 70% quartz sand, 20% kaolin clay and 10% sphagnum peat. Calcium carbonate was added to adjust the pH to 6.0 ± 0.5, and distilled water was added to achieve a humidity of 50 ± 10%. The experimental soils were prepared by adding appropriate chemicals on a dry weight basis.

2.2. Test chemicals

Five chemicals were tested in this study. Chlorpyrifos (CPF, CAS: 2921-88-2, purity of 96%) was obtained from Yangnong Agrochemical Group (Yangzhou, Jiangsu, China), and its exposure concentration to earthworms ranged from 14.25 to 456 mg a.i. kg^{-1}. Fenobcarb (FEN, CAS: 3766–81-2, purity of 98.1%) was purchased from Changlong Chemical Industrial Group (Changzhou, Jiangsu, China), and its exposure concentration to earthworms ranged from 18.38 to 588 mg a.i. kg^{-1}. Clothianidin (CLO, CAS: 210880-92-5, purity of 96.5%) was donated by Bide Biochemistry Science Company (Linxiang, Hunan, China), and its exposure concentration to earthworms ranged from 0.54 to 17.34 mg a.i. kg^{-1}. Acetochlor (ACE, CAS: 34256–82-1, purity of 93%) was provided by Qiaochang Chemical Co., Ltd. (Binzhou, Shandong, China), and its exposure concentration to earthworms ranged from 13.94 to 446 mg a.i. kg^{-1}. $K_2Cr_2O_7$ [Cr(VI), CAS: 7440–47-3, purity of 99.8%] was supplied from Guanghua Chemical Group (Guangzhou, Guangdong, China), and its exposure concentration to earthworms ranged from 3.72 to 118.8 mg a.i. kg^{-1}. The desired amount of each pesticide was dissolved in 10 mL acetone and mixed with a small quantity of fine quartz sand. The acetone was evaporated by mixing for 1 h, and then the fine quartz sand was combined with the artificial soil in a household mixer. Cr(VI) was dissolved with distilled water and appropriately added to the soils. The nominal concentrations of Cr(VI) were used in this study. After these chemicals were separately added to the same soil, which was then diluted by clean soil to get 5–7 concentrations. Table 2 presents the individual concentrations of the five chemicals in all the mixtures.

2.3. Avoidance response test

Avoidance response test was carried out according to ISO test guideline with minor modifications (ISO, 2008). Briefly, two-chamber rectangular plastic containers (16 cm × 10 cm, 7 cm height) with removable dividers were filled with 500 g control soil (untreated with the test compounds) on one side, while the other side was filled with the same amount of contaminated soil. The divider was removed, and 15 earthworms were placed on the soil surface at the midline of the container. To reduce the water evaporation and earthworm escape, the test containers were covered with a transparent plastic lid, on which pinholes were made to facilitate air exchange. The trays were incubated for 2 days after treatment. A double-control test was carried out (ISO, 2008) to guarantee the homogeneous distribution of earthworms throughout the two compartments of each container, and to avoid the influences by the surroundings or some other factors. Uncontaminated soil was placed in both compartments with five replicates. While the procedures for treated soil were the same as uncontaminated soil, and the expected mean distribution of worms in a given compartment was 40–60%.

To obtain AC_{50} (concentration inducing an avoidance rate of 50%), the clean soil spiked with five to seven concentrations of 2-fold serial dilutions were used for each chemical. The test was valid if the number of dead or missing worms was $< 10\%$ per treatment.

2.4. Experimental design of chemical mixtures

In order to assess the interaction of equitoxic mixtures, earthworms were treated with serial dilutions of each chemical with a fixed equitoxic constant mixture ratio (the same effect came from each individual chemical) based on the measured individual AC_{50} values. Five to seven dilutions (with a serial dilution factor of 2) of each chemical with their binary, ternary, quaternary and quinquenary mixtures were tested. Besides, a control was tested in three independent experiments with replicate samples. The binary combinations included Cr(VI) + CLO, CPF + CLO, CLO + ACE, Cr(VI) + ACE, Cr(VI) + FEN, FEN + CLO, FEN + ACE, Cr(VI) + CPF, CPF + ACE and FEN + CPF. A total of 10 ternary mixtures were designed as follows: Cr(VI) + CPF + CLO, FEN + CPF + CLO, FEN + CLO + ACE, CPF + CLO + ACE, Cr(VI) + CPF + ACE, Cr(VI) + FEN + CLO, Cr(VI) + FEN + ACE, Cr(VI) + CLO + ACE, Cr(VI) + FEN + CPF