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Quantitative ecotoxicity analysis for pesticide mixtures using benchmark dose methodology

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ABSTRACT

Pesticide mixtures can often be found on crops and in the natural environment due to the usage of multiple pesticides in crop production. However, the toxicity of pesticides is mostly evaluated individually but not jointly. Many studies have pointed out that pesticide mixture may have elevated toxicity compared with its individual counterpart, therefore, it is important to quantify the change in toxicity. Such quantification can provide invaluable information for environmental and ecological risk assessment, and further support risk management to develop appropriate means to mitigate the risk.

The objective of this study is to quantify the ecotoxicity of pesticide mixtures composed of different combinations of four pesticides (i.e., Acetemiprid, Carbendazim, Chlorpyrifos, Cyhalothrin) to (1) understand if the co-presence of multiple pesticides will affect the toxicity and (2) to quantitatively approximate the change in toxicity. We first conducted acute toxicity testing and avoidance response testing using earthworms to obtain dose-response data for two different endpoints; then the benchmark dose (BMD) methodology was applied to estimate the toxicity values for the active ingredients of these four pesticides and their mixtures. The BMD analysis results suggest that the ecotoxicity of the active ingredients of the pesticides is very likely to increase when two or more pesticides are used simultaneously, highlighting the importance to consider toxicity of mixtures in the regulatory decision making process. This study demonstrates that the benchmark dose methodology can be a useful tool to quantify the toxicity of chemical mixtures and support cumulative risk assessment accordingly.

1. Introduction

It is very common to find that multiple pesticides are used concurrently in crop production which may cause combined toxicities to organisms and the natural environment (Altenburger et al., 2000; Backhaus et al., 2004). However, chemicals including pesticides are often tested and evaluated individually, so the toxicity values used for the purposes of risk assessment and regulation establishment are usually only available for individual chemicals. Therefore, the co-existence of pesticides mixtures has attracted much attention in recent decades (Altenburger et al., 2000; Backhaus et al., 2000; Tyler et al., 2008).

There are two key components in this study: (1) toxicity testing using earthworms and the active ingredient of four pesticides (i.e., Acetemiprid, Carbendazim, Chlorpyrifos, Cyhalothrin), and (2) the benchmark dose methodology to estimate the toxicity values for the pesticides and their mixtures. Acetemiprid is a commonly used neonicotinoid insecticide to control sucking-type insects on vegetables, fruits and other crops (US EPA, 2002). Acetamiprid may cause generalized, nonspecific toxicity in mammals through chronic exposure but has limited toxicities in soil organisms due to its moderate to high mobility and relatively short half-life in soil (US EPA, 2002). Carbendazim is a broad-spectrum systemic agricultural and horticultural fungicide being used for the control of a wide range of fungal diseases in a variety of crops. Damage to male reproductive tissues and the liver, and some birth defects can be caused by exposure to Carbendazim in laboratory animals, meanwhile, carbendazim could significantly induce DNA damage to the earthworm coelomocytes (HSDB, 2018a). Chlorpyrifos is an

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insecticide being used in both agricultural plants and non-agricultural areas to control pests, including corns, soybeans, fruit and nut trees, golf courses, turf, etc. Chlorpyrifos can cause cholinesterase inhibition in humans leading to an overstimulated nervous system and affects cardiac cholinesterase (ChE) activity and muscarinic receptor binding in rats (HSDB, 2018b). On the other hand, earthworms can be relatively well recovered from the moderate acute exposure to chlorpyrifos due to a rapid elimination rate (HSDB, 2018b). Cyhalothrin is an insecticide in the pyrethroid chemical class of pesticides and used to control a wide range of pests on a variety of plants, such as wheat, tomato, and peach (EFSA, 2014). Cyhalothrin has been shown limited toxicity in mammalian species because it has been found to be extensively metabolized and then eliminated as conjugates (IPCS, 1990). On the other hand, Cyhalothrin is highly toxic to fish and aquatic invertebrates but a low risk was concluded for earthworms, soil macro- and microorganisms (EFSA, 2014). The reason to choose these four pesticides is that they have been most frequently found on bayberries with a relatively high level of residual concentration in a recent survey to investigate pesticide residuals on bayberries in Zhejiang Province, China. In particular, carbendazim was detected in 46.5% of the 157 analyzed samples with a mean residual concentration level of 0.094 mg/kg, followed by Chlorpyrifos, Cyhalothrin and acetamiprid for 17.83% (0.06 mg/kg), 14.65% (0.418 mg/kg) and 7.01% (0.044 mg/kg), respectively. Actually, except carbendazim, none of the other three pesticides have been registered for use on bayberries.

Earthworms are an essential component of terrestrial ecosystems, acting as decomposers to improve soil structure. They can accumulate toxicants and may detrimentally affect the organisms feeding upon them consequently. As recommended by the Organization for Economic Co-operation and Development (OECD, 1984, 2004) and International Standardization Organization (ISO, 1993), earthworms such as *Eisenia fetida* are currently used for ecotoxicological assessment of substances in soil. Additionally, due to its ecological relevance in Chinese territory, *E. fetida* has been widely used by Chinese researchers to study chemical toxicity in soil (Duan et al., 2016; Zhang et al., 2015). Therefore, *E. fetida* is an appropriate test organism for ecological risk assessment in terrestrial ecosystems (Fourie et al., 2007), and used in the study for two sets of experiments to evaluate two different toxicity endpoints: mortality rate in acute toxicity study and effective rate of avoidance to contaminated soil in avoidance response test.

Unlike the traditional No Observed Adverse Effect Level (NOAEL) method which identifies a dose level where responses are statistically different from the counterpart in the control (but how much different is not defined), the benchmark dose (BMD) method explicitly defines the adversity as a certain change in response (e.g., 10% increase) and calculates the dose associated with the certain change. Because the BMD methodology uses explicit and standardized specification of change in response to define toxicity values (e.g., the BMD value), it is an invaluable tool to compare the toxicity of various chemicals given appropriate settings. In this study, we apply the BMD modeling method to quantitatively analyze the dose-response data generated from the animal experiments and estimate the toxicity values for individual pesticides and their mixtures.

The goal of this study is to quantify the ecotoxicity of pesticide mixtures to understand if the co-presence of multiple pesticides will affect the toxicity. The contributions of this study include two major parts: (1) the demonstration of BMD methodology to estimate and compare the toxicity of pesticide mixtures, and (2) the toxicity analyses of four commonly seen and used pesticides with their mixtures. The results obtained from this study can provide important evidence for environmental/ecological risk assessment regarding pesticide mixtures and potentially support pesticide regulations.

2. Materials and methods

2.1. Test organisms and environment

Eisenia fetida (*E. fetida*) was used as the test species for assessing the ecotoxicity of the four pesticides and their mixtures following the guidelines of the Organization for Economic Cooperation and Development (OECD, 1984 and 2004). As suggested by the OECD testing guideline, adult *E. fetida* weighted between 300 and 500 mg with well-developed clitellum were cultured in artificial soil for two weeks with pig manure as food in the laboratory. The ambient temperature of the laboratory was strictly controlled at 20 ± 2 °C, the light-dark cycle was controlled at a ratio of 16 h: 8 h, and the relative humidity was set at 80 ± 10%. These worms were acclimatized for 24 h in artificial soil (introduced in detail in Section 2.3) before tests.

2.2. Test chemicals and mixtures

2.2.1. Single test chemicals

The four pesticides investigated in this study include one pyrethroid insecticide (cyhalothrin), one benzimidazole fungicide (carbendazim), one neonicotinoid insecticide (acetemiprid) and one organophosphate insecticide (chlorpyrifos). The selected pesticides are widely used in agriculture around the world. Cyhalothrin [96% technical product (TC)] was purchased from Zhejiang Yifan Chemical Co., Ltd. (Wenzhou, Zhejiang, China). Carbendazim (95% TC) was obtained from Jiangsu Huifeng Agrochemical Group (Dafeng, Jiangsu, China). Acetamiprid (96.2% TC) was donated by Hebei Weiyuan Chemical Industrial Group (Shijiazhuang, Hebei, China). Chlorpyrifos (97% TC) was provided by Zhejiang Xinan Chemical Industrial Group (Jiande, Zhejiang, China). Since our study aimed to evaluate and document the toxicity of the chemical compounds but not the adjuvants added to the commercial products, active ingredients were used instead of commercial formulations.

2.2.2. Test mixtures

Based on the LC_{50} values estimated from the single chemicals in the acute toxicity test (or the AC_{50} values for the avoidance response test), 11 different compounds (including their binary, ternary and quarternary combinations listed in Table 1) were prepared according to a mixture ratio of equal median effect. For example, if the LC_{50} of pesticides A, B, C were 1 mg/L, 2 mg/L, and 3 mg/L respectively, then in the mixture of A + B + C, the ratio of concentration of A, B and C is 1:2:3. The ratio was kept unchanged when the same combination was prepared at multiple concentration levels for the toxicity testing. The four single pesticides were tested simultaneously, therefore, together with the mixtures (listed in Table 1 below) there were 15 compounds in

Table 1
Tested pesticide mixtures.

2-Component mixtures	3-Component mixtures	4-Component mixture
Acetemiprid + Carbendazim	Acetemiprid + Carbendazim + Chlorpyrifos	Acetemiprid + Carbendazim + Chlorpyrifos + Cyhalothrin
Acetemiprid + Chlorpyrifos	Acetemiprid + Carbendazim + Cyhalothrin	
Acetemiprid + Cyhalothrin	Acetemiprid + Chlorpyrifos + Cyhalothrin	
Carbendazim + Chlorpyrifos	Carbendazim + Chlorpyrifos + Cyhalothrin	
Carbendazim + Cyhalothrin		
Chlorpyrifos + Cyhalothrin		