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Ecotoxicological effects of binary mixtures of siduron and Cd on mRNA expression in the earthworm *Eisenia fetida*

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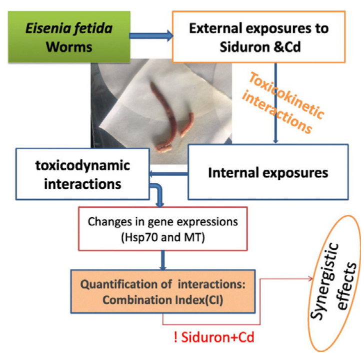
HIGHLIGHTS

- Evaluation of joint effects of siduron/Cd mixtures on expressions of MT and Hsp70 in earthworms
- Application of CI-isobologram model revealed synergistic interactions between siduron and Cd
- Consideration of only single-substance toxicity data may underestimate the potential risk posed by chemical mixtures

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to investigate the eco-toxicological responses of earthworm (*Eisenia fetida*) exposed to combined siduron (herbicide) and cadmium (Cd). *Eisenia fetida* gene expressions including metallothionein (MT) and heat shock protein70 (Hsp70) were analyzed using real-time Polymerase Chain Reaction after individual and combined siduron (0.90, 1.80, 3.60 and 7.20 μg cm^{-2}) and Cd (0.225, 0.45, 0.90 and 1.80 μg cm^{-2}) sublethal exposures. Where, the nature of the toxicological interactions between siduron and Cd in inducing or suppressing MT and Hsp70 expression was determined by applying the Combination index (CI)-isobologram model. The results revealed significant variations in MT and weak changes in Hsp70 expression when the earthworms were exposed to individual Cd. The individual siduron exposure exhibited a significant down-regulation ($p < 0.01$) in MT during all treatments and in Hsp70 expression only at 7.20 μg cm^{-2} concentration; while the mixtures of siduron and Cd exposures resulted a significant down regulation ($p < 0.05$) in both MT and Hsp70 expressions. Moreover, the combined siduron and Cd exposure revealed nearly additive effect (CI = 1) at the lower effect levels and significant synergistic effect (CI < 1) at the higher effect levels for both MT and Hsp70 expression. The synergistic effects of combined siduron and Cd suggest that there might be a potential risk connected to the co-occurrence of these chemicals in the environment.

1. Introduction

Around the world environmental pollutants are continued to be released in various ecosystems and the organisms are suggested to be

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exposed to a large number of diverse and complex contaminants mixtures. The combination of less toxic compounds may strengthen their toxicity level resulting in combined synergistic effects (Cedergreen, 2014). Majority of toxicological researchers have reported much about the toxicity of individual contaminants, although in the environment, pollutants usually accumulate in complex mixtures of chemicals. Thus risk characterization based on single chemical exposure may results in over or under-estimation of potential risk that a certain chemicals mixture may pose to the environmental health. It has been confirmed that the presence of a particular substance in a combination and its known toxicity apart does not give a guarantee of knowing its true effects when it is in combination with other substances. The researchers have shifted toward considering all the components of a chemical mixture; however, there is still a big gap in our knowledge to be filled concerning the toxic effects caused by exposure to mixtures of compounds (Heys et al., 2016). The prediction of combined effects of mixtures is usually done from reference models based on non-interaction among the individual compounds i.e. concentration addition and response addition (Backhaus and Faust, 2012; Meyer et al., 2015). When a joint toxicity of combined contaminants becomes similar to the sum of separate components effect is named as additive, otherwise the joint toxicity of different contaminates maybe stronger or weaker than expected toxicity from separate exposures (Dondero et al., 2011). When combined toxicity is higher or lower than the sum of the separate contaminants, the combined effect is called "synergistic" or "antagonistic," respectively (Cedergreen, 2014; Heys et al., 2016). Several studies have shown that joint toxicity of most chemical combinations is either additive or synergistic, while few combinations exhibit antagonistic toxicity (Heys et al., 2016; Binderup, 2008; Kepner, 2004; Yang et al., 2007).

The occurrence of a combined pollution from Pesticides and metal ions is imminent because these two types of contaminants are commonly present in most of the soil environmental systems (J.H. Wang et al., 2012; Y. Wang et al., 2012). Mixtures of pesticides and the additional input of metal ions (from industries) in agricultural fields are common. The present study focuses on siduron also known as Tupersan or l-(2-methylcyclohexyl)-3-phenylurea, which is a popular preemergent herbicide used for the control of certain annual grass weeds in the newly seeded or established plantings of some cool season grasses (Belasco and Reiser, 1969). It is usually applied in golf courses, ornamental turf grass, lawns, parks, sport facilities and roadsides (Lewis and Gilbert, 1966). In soil environment siduron slowly degraded into 2-methylcyclohexylamine and two other compounds which haven't been identified yet. Previous studies have reported that siduron is may toxic to earthworms and aquatic organisms (Uwizeyimana et al., 2017; Jones et al., 2003). This herbicide is commonly used in Tianjin northern China where the surface water has been shown to contain higher concentrations of it (average of 0.20 μg/L) (Kong et al., 2015). The presence of siduron in surface water of Tianjin was credited to the application in control of weeds on yards in nearby residential areas. In Vietnam, siduron is among the 12 herbicides that were detected in surface water systems of Hanoi and other cities (in the range of 0.02–1.2 μg/L) (Chau et al., 2015). Siduron is also widely applied for controlling weeds in the USA. Collins and Sprague (2005) have detected siduron in the Cache la Poudre River in Colorado, they ascribed this detection to the application of siduron for controlling weeds along the Highway 14 and on the yards of surrounding residential areas (Collins and Sprague, 2005). Reports show that in the year 1992, siduron was used on sod crops at a rate of 4000 lbs/acre/year in states of Connecticut, New York and New Hampshire and the active ingredient of this herbicide for each state was estimated to 156, 475 and 113 lbs, respectively (Gianessi and Anderson, 1996).

Whereas, cadmium (Cd) is a widespread metal pollutant that is found in most environmental systems including industrial parks, residential areas (yards), mining and waste water irrigation areas (Pruski and Dixon, 2002; Järup, 2003). The combination of siduron and Cd is likely to occur following the uncountable application of siduron in environments that may be contaminated by Cd pollution especially in urban soils. The great challenge still remains on understanding the toxicity of siduron singly and in combinations with other environmental contaminants. Studies on combined pollution from herbicides and Cd are also still scarce; therefore any study that will focus on the evaluating combined effects caused by combinations of herbicides and metal ions would be a great contribution the environmental protection.

Several studies have measured the acute and chronic toxicity of chemicals both at species and populations levels by considering the classical ecotoxicological endpoints such as survival, growth, reproduction and behavioral changes (Boverhof and Gollapudi, 2011; Connon et al., 2012). In recent years there has been an increase in using biomarkers for the assessments of chemical effects on the environment (Peakall, 1994). Biological responses include molecular, biochemical and physiological alterations in organisms following the exposure to pollutants. Contrary to traditional tests, biomarker responses are directly related to the bioavailability of toxic chemicals and maybe a suitable early warning of pollutant stress before the appearance of sublethal effects such as inhibition of growth or reproduction (Van Gestel and Van Brummelen, 1996). The use of biochemical indicators (biomarkers) such as enzyme activities, variations of expression levels of proteins (e.g. Heat shock proteins, Metallothioneins) and DNA damage, has been reported in previous studies to be the best tool for early assessments of single and joint toxic effects of chemical mixtures including those formed by metals and pesticides (J.H. Wang et al., 2012; Santos et al., 2010; Zhou et al., 2013; Eichler et al., 2006). Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins known to be involved in metal detoxification and homeostasis, thus forming an obvious target for investigation. It has been proved that the expression of MT could be modified by both natural and anthropic factors (Pytharopoulou et al., 2008; Santovito et al., 2012), and this has led to several studies focusing on MT expression to assess ecotoxicity (Bernard et al., 2010; Brulle et al., 2008). Heat shock protein 70 (Hsp70), is also another type protein found in major classes of molecular chaperones that play a decisive, protective role in cellular stress responses (Scheil et al., 2010),and the stress-induced of Hsp70 protein make it a great useful molecular biomarker to monitor the impact of pollutants on earthworms (Homa et al., 2005). Earthworm is one of the most common soil organisms in most environments, and plays an important role in the functioning of soil ecosystems (Spurgeon et al., 2003). This broad presence of earthworms in most environmental systems make them suitable bioindicators of soil contamination, and can be used to provide safety step for pesticides application and metal regulation. There are many studies regarding the use of earthworm biomarkers to assess toxic effects of individual pesticides or metals (Song et al., 2009; Dittbrenner et al., 2012; Zang et al., 2000; Nakashima et al., 2008; Muangphra and Gooneratne, 2011; Maity et al., 2008), however, data on biomarker responses in earthworms exposed to mixtures of pesticides and metal ions (such as Cd^{2+}) are scarcely found.

Therefore, the present study aimed to evaluate the ecotoxicological effects caused by individual/mixture of siduron and Cd on MTs and Hsp70 expression in *Eisenia fetida*.

2. Materials and methods

2.1. Chemicals and reagents

Siduron [*N*-(2-methylcyclohexyl)-*N*′-Phenylurea] commercially known as Tupersan with active ingredient of 48% was obtained from the Institute of Ping An Garden and plant protection technology in Zhengzhou, China. Cadmium Chloride ($CdCl_2$) was obtained from Sinopharm Chemical Reagent Co., Ltd.