INDIVIDUAL AND COMBINED EFFECTS OF A FUNGICIDE (VALETTE) AND AN HERBICIDE (OSCAR) ON THE GROWTH AND MORTALITY OF EARTHWORM *LUMBRICUS TERRESTERIS* (LINNAEUS, 1758)

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ABSTRACT

Earthworms are valuable soil invertebrate mainly used to monitor environmental quality. Thus, the present study was undertaken to provide insights on the individual and combined toxicological effects of two commonly used pesticides in Souk Ahras city (Northeast Algeria), namely Valette (fungicide) and Oscar (herbicide) on a soil invertebrate – earthworm (Lumbricus terresteris) as a bio-indicator of soil pollution. The invertebrates were subjected to increasing concentrations of each chemical (0, 2, 4, 6, 8, 12.5, 25 and 50 mg/500 mg soil) for four treatment periods (24, 48, 72 and 96 hours). Physicochemical analysis revealed that the soil pH is alkaline, and water and KCl pH are respectively 8.05 and 7.72, in addition to a sandy-loamy soil texture with a saturation rate of 49.43%, and high level of organic matter (11%). Additionally, both chemicals caused a significant weight decrease (p<0.05) from the concentration of 12.5 mg/mg soil, and noteworthy, no mortality was recorded during the entire experimental period, either for the individual or combined exposure. Furthermore, the histopathological observations showed no signs of alteration or damage to epidermal cells and muscle fibers during the treatment periods with either chemical.

Keywords: *Lumbricus terresteris,* Valette, Oscar, Individual effect, Combined effect, Growth, Mortality, Histology

INTRODUCTION

Soil health was reported as the ability of soil to function as a vital living system within the ecosystem boundaries to support plant and animal productivity, maintain or enhance the quality of soil and water environment and contribute to the health of plants and animals. As a result, healthy soils are essential for healthy ecosystems, economies and people (Lehmann *et al.,* 2020; Fierer *et al.,* 2021). In recent decades, soil pollution becomes one of the main causes of soil breakdown, and can be a great concern to the human food chain and ecosystem sustainability (Rodriguez-Eugenio *et al.* 2018).

Human, industrial and agricultural activities using a variety of pollutants (organic and inorganic chemicals), including pesticide residues are considered the principal sources of soil pollution (Cachada *et al.,* 2018). Several studies conducted in many countries around the world have reported that the presence of these pesticides in soil mixtures is a serious environmental threat to soil quality, and soil animal health, in particular, soil earthworms (Bhandari *et al.,* 2020). Recently, scientists are interested to find out valuable ways of using a variety of biological approaches to monitor the pesticide pollution impacts on soil earthworms (soil pollution bio-indicator) (Bart *et al.,* 2019 a,b). Also, recent studies investigating the potentially toxic effects of pesticides on non-target organisms suggested the importance of the routine systematic monitoring of the pesticide contaminated environment and their risks for the non-target organisms (Pelosi *et al.,* 2014). In this regard, the present study aimed to assess the individual and combined toxicological impact of a fungicide (Valette) and an herbicide (Oscar) in a concentration and time-dependent manner on earthworm (*Lumbricus terresteris* Linnaeus, 1758, Oligochaeta: Lumbricidae).

MATERIALS AND METHODS

Sampling Sites: The soil earthworms were obtained from the Forest of University Mohamed El-Cherif Messaadia located in Souk Ahras City, Northeast of Algeria (Figure 1).

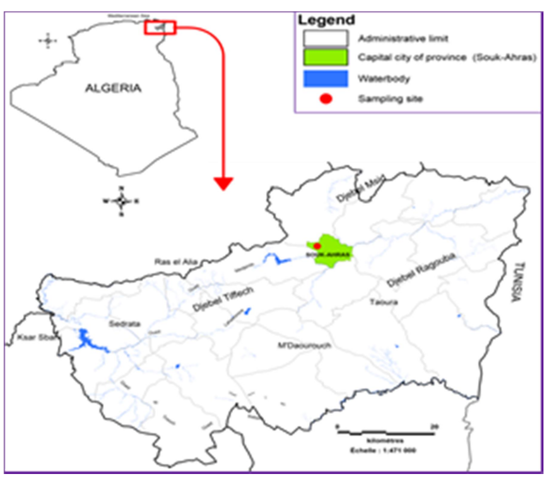

Figure 1: Map of the sampled site - Forest of University Mohamed El-Cherif Messaadia located in Souk Ahras City, Northeast of Algeria

It is surrounded by El-Taref City from the East, Guelma City from the west, Tébessa City from the south, and Oum El-Bouaghi City from the southwest.

Soil Physicochemical Analysis: Soil samples collected randomly from the study site were physic-chemically analyzed based on the major soil characteristic parameters. The pH of the soil was measured according to the method of Lag *et al.* (2008), where 10 g of fine soil was mixed with 50 ml of distilled water, shacked for 10 minutes, left to stand for 30 minutes, and the pH value read by inserting the electrode of the pH metre into the solution. The pH-KCl was determined by mixing 50 ml of KCl with 20 g of fine soil, and stirred for 5 minutes followed by 30 minutes of standing at room temperature, and the mixture was read using a pH metre.

The electrical conductivity (EC) was determined by the method of Okalebo *et al.* (2002), in which a solution composed of 10 g of fine soil and 50 ml of distilled water was agitated, left to stand for 30 minutes, and read by putting the rinsed electrode of the EM38 EC metre into the solution. The EC value displayed on the digital screen was recorded. The field capacity and the soil saturation capacity were measured according to the experimental protocol of Balze (2000). This method consists of adding water drop by drop to 50 g of fine soil to have a saturated paste. The mixture was kept standing for one hour, and then placed in an oven at 105 °C for 24 hours to enable the determination of the following indices: Weight of dry soil; SR (Saturation rate), and FC (Field capacity) = saturation rate ÷ 2.

In addition, the soil texture was determined according to the method of Lag *et al.* (2008) based on measuring the percentage of humidity and comparing it to a scale determining the corresponding textures: 12 % (sandy soil), 12 – 24 % (sandy loam), 24 – 45 % (sandy loam), 45 – 75 % (clay-loamy), and 75 % (clayey soil). Furthermore, the soil organic matter content was estimated by the loss-on-ignition method as described by Bonnefont *et al.* (1980), where the samples were placed for 16 hours in an oven at 400°C, and consequently the loss of weight after calcination was used to calculate the proportions of organic matter.

Biological Materials

Sampling and acclimatization of earthworms: Earthworm (*L. terresteris)*, represent a major component of soil macro-fauna and are considered very important animal species in the recycling of soil matter. Also, earthworms are recognized as an effective biomonitoring tool for chemicals including pesticides and heavy metals contaminated soils. Thus, they are commonly used in the laboratory and/or in *in-situ* studies (Robidoux *et al.,* 2002).

The sampling of adult earthworms was carried out according to the physical method as previously reported (Bouche, 1972). This method consists of digging the ground to a depth of 20 to 30 cm enabling the collection of earthworms, which were placed into plastic bins (17 cm wide and 45 cm deep) containing soil and decayed plant residues, and then placed in a cool place for 10 – 14 days of adaptation and air conditioning.

Chemicals: In this study, two test chemicals were used; a systematic fungicide named Fosetyl-aluminum (Valetta, chemical formula; $C_6H_{18}A_lO_9P_3$), an organometallic compound of phosphonates group, having a protective and curative action, was introduced in 1978, and commonly used against late blight and other fungal diseases of specialized crops (NCBI, 2023a). The second used chemical was a sulfonylurea herbicide named tribenuron-methyl (Oscar) ($C_{15}H_{17}N_5O_6S$) and mainly used to control weeds in spring wheat, durum wheat, and barley by ground application (NCBI, 2023b).

Experimental Design

Treatments: The soil earthworms received eight increasing concentrations (0, 2, 4, 6, 8, 12.5, 25 and 50 mg/500 mg soil) of each test chemical prepared in distilled water, where each concentration was used in three replicates (5 individuals/replicate).

The treatment was carried out by direct spraying of 300 ml Oscar or Valette solutions on 500 mg of soil before the earthworms being placed in soil samples for 24, 48, 72 and 96 hour since the control earthworms were sprayed with distilled water (Boukhili and Kharrachi, 2013). The growth rate was determined by daily weighing of the earthworms from 0 hours to 96 hours, and the percentage of mortality was determined by counting dead individuals (immobilized individuals) every 24 hour.

Histological study: At the end of the treatment, three earthworms, one from each replicate, were taken for the histopathological examination following the routine histological procedure of Drury and Wallington (1980). Worm samples were washed with distilled water, transfer into boxes containing 01% jelly agar-agar, and left to stand for 96 hours to get rid of the soil out of the digestive tract. After that, the earthworms were cut into two parts, placed in specimen bottles and fixed with Bouin's fluid for 12 hours before subjecting it to histological procedures of serial alcohol dehydration, embedding in paraffin wax, sectioning and staining with Haematoxylin-Eosin for microscopic observation. The histological sections were observed by a light microscope (x40) and photographed by a digital camera (Fine pix 40i, Fuji, Japan) (Berrouk *et al.,* 2021; Houda *et al.,* 2022).

Statistical Analysis: Statistical analysis was performed using MINITAB software (version 16, PA State College, USA), and results are displayed as mean ± standard deviation (SD). The significance between multiple groups was tested using the single-criteria analysis of variance (ANOVA), and the comparison between the means was carried out using post-hoc LSD and the level of significance was considered as $p<0.05$.

RESULTS

Effects of Fungicide on Body Weight Gain: As shown in Figure 2, the body weight gain of earthworms treated with fungicide compared with controls revealed a positive fluctuation after application of the first four concentrations, and the was significantly decreased ($p<0.05$) at 12.5 mg/l.

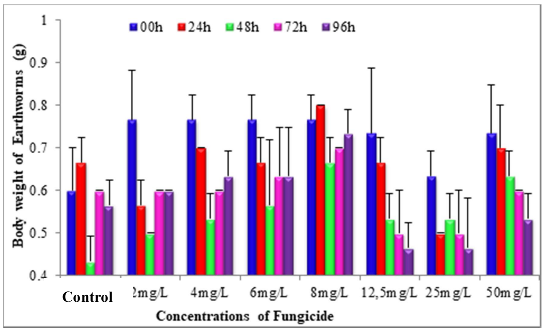

Figure 2: Effect of fungicide on earthworm body weight gain (g). *Key:* *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

Effects of Herbicide on Body Weight Gain: The test herbicide (Oscar) applied at different concentrations to the earthworms caused significant changes in the body weight of the treated individuals compared to the controls (Figure 3). Furthermore, individuals treated with the lowest concentrations (2 to 6 mg/l) of the herbicide had positive body weight gain followed by a significant drop ($p<0.05$) for individuals treated with 12.5 mg/l.

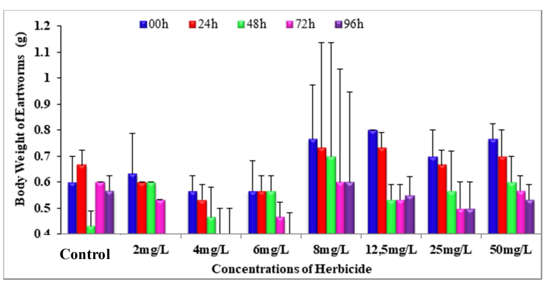

Figure 3: Effect of herbicide on earthworm body weight gain (g). *Key:* *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

Effects of Fungicide and Herbicide on Weight Gain: The mixture of the two pesticides was tested on earthworms to estimate the possible interactions between them. As shown in Figure 4, the combined treatment caused body weight loss in the treated individuals compared to the controls.

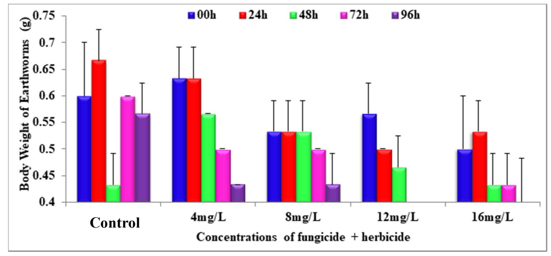

Figure 4: Effect of the combined fungicide and herbicide treatment on the body weight of earthworms. *Key:* *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

Interaction between Fungicide and Herbicide: To estimate the nature of the interaction between the two pesticide chemicals, the correlation between the individual and combined forms was studied as a function of time. As indicated in Figure 5 (A, B, C and D), the correlation between the combined treatments of the herbicide and the fungicide was more potent compared to chemical individual treatment.

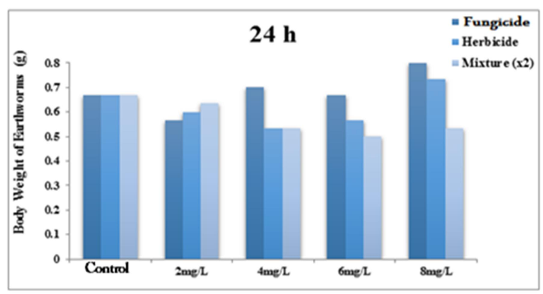

Figure 5A: Interaction between the tested fungicide and herbicide on earthworm body weight after 24 hours of exposure. *Key:* *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

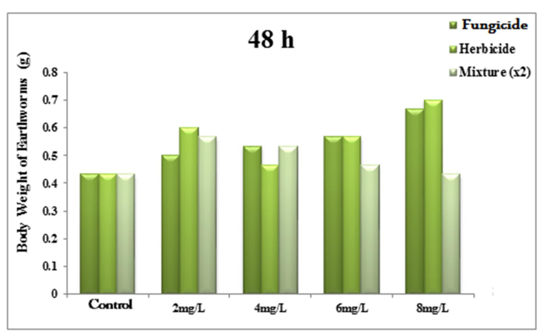

Figure 5B: Interaction between the tested fungicide and herbicide on earthworm body weight after 48 hours of exposure. *Key:* *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

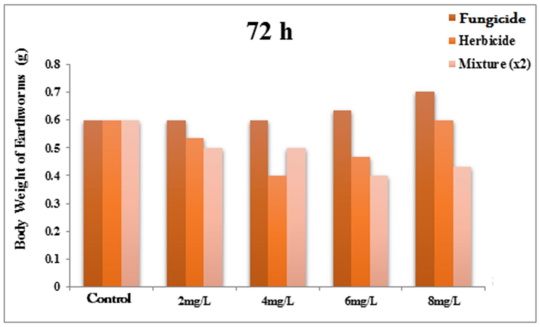

Figure 5C: Interaction between the tested fungicide and herbicide on earthworm body weight after 72 hours of exposure. ***Key:*** *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

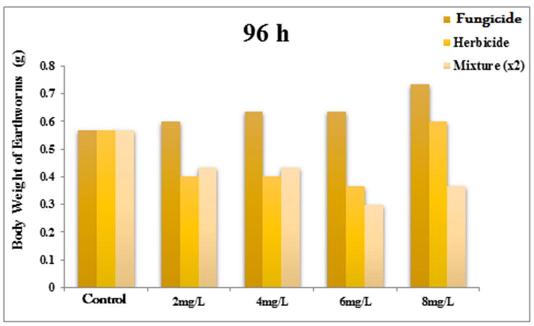

Figure 5D: Interaction between the tested fungicide and herbicide on earthworm body weight after 96 hours of exposure. ***Key:*** *Results are given in Mean ± SD of three replications for each concentration and five earthworms per replicate*

In addition, the fungicide was found to be the lower toxic chemical as compared to the other, suggesting thus the presence of a reinforcing synergy between the two test chemicals.

Herbicide, fungicide separately and combined-induced mortality in earthworms: No significant mortality was observed in earthworms received either the individual or combined treatments of the test pesticides when compared with the controls (Table 1).

Table 1: The mortality rate was recorded in earthworms treated with fungicide and/or herbicide after at different concentrations after 96 hours of exposure

Mortality	Herbicide	Fungicide	Mixture
96h mortality for all chosen concentrations	7	9	9

Histo-pathological changes: As shown in Figure 6, no histological alterations and cellular damages in the epidermal tissues and muscle fibres were noticed in pesticide-treated worms compared with the controls. Also, the longitudinal muscle fibres that make up the entire body revealed normal histological structure.

Figure 6A, B and C: Longitudinal sections of *Lumbricus terresteris* after 96 hours of exposure (G x 40) ***Key:*** *A) Control earthworms appearing with normal histological structure, B) Histological section from worms treated with the herbicide, C) Histological section from worms treated with the fungicide*

DISCUSSION

Physicochemical Properties of Soil: Earthworms are good bio-indicators of biodiversity and soil quality, and the environmental impact of cropping systems (Paoletti, 1999; Fründ *et al*., 2011). In this regard, the results of the soil physicochemical analysis indicated that the earthworms can live in moderately alkaline soil, and this was due to the reason that the values of pH water and pH KCl were in the interval [8.05-7.72] with a sandy-loam texture, where the saturation rate of soil was 49.43%, and the soil organic matter (OM) content was 11%. As reported by Kanianska *et al.* (2016), the physicochemical characteristics of soil can strongly affect the spatial distribution of earthworms, showing high sensitivity to their variability of the environmental conditions. Also, the influence of pedoclimatic conditions on the density and biomass of earthworms proved that earthworms are closely correlated with the state of the environment where they live. In addition, the pH of the earthworms' soil was found to be between 6.4 and 7.0, and thus lower pH values can lead to a reduction in the growth rate since the earthworms were reported to have the ability to modify their environmental conditions to maintain the pH between 6.5 and 7 (Coulibaly and Zoro, 2010; Coulibaly *et al.*, 2016; Byambas *et al.*, 2017). Sailo *et al.* (2020) have linked soil pH to precipitation variations, while soil acidity was linked with factors like phosphate, calcium, magnesium and potassium deficiencies. Unlike acidic soils (unfavorable environments for earthworms), neutral and alkaline soils are favorable environment for earthworm proliferation (Yesguer, 2015). Furthermore, Bazari (2015) reported that the distribution of *Aporrectodea caliginosa* and *Aporrectodea monticola* were related to the levels of $CaCO_3$ and Ca^{++}, as well as six species of earthworms among the 18 identified species had tendencies towards high levels of organic matter and clay, while another group of earthworms identified at the same period had preference for loam soil poor in organic matter. According to Sivasankari *et al.* (2013), *Eudrilus eugeniae* like the majority of earthworms feeds on organic matter present in the soil. The quantity, quality and location of organic matter are important factors for worms and depend above all, on its content in the agricultural plots, as demanded by the cultivated species. In this context, Lofs-Holmin (1983) reported that the quality and quantity of crop residue returned to the soil are essential for the development and growth of earthworms. Additionally, the input of the organic matter increases the number of earthworm individuals per unit area, although several authors have reported that the sandy soils were less favorable for the survival of earthworms (Laossi *et al.*, 2010; Toure *et al.*, 2017). Interestingly, the availability of organic matter in the soil stimulates the propagation of earthworms or plays an important role in their decomposition (Cesarz *et al.*, 2016; Frousz, 2018). Moreover, Six (2014) has indicated that disturbance in the environment of earthworms can have significant consequences at various scales (populations, organisms, and even cellular and molecular levels). Also Chauhan (2014) has suggested that the soil moisture content, texture, richness in organic matter, phosphorus and total nitrogen are the main factors influencing the number and distribution of earthworms in the soil. Bazari (2015) and Toure *et al.* (2017) found that the addition of nitrogen in the soil leads to an increase in the number of earthworm individuals per unit area, such as *Aporrectodea rosea* and *Aporrectodea trapezoides*, which prefer sandy and nitrogen-rich environments.

Toxicological Effects

Effect on growth rate: In this study, the body weight was markedly decreased in *L. terreteris* received fungicide and/or herbicide above the concentration of 12.5 mg/g soil. This finding was in agreement with the results of Houda *et al.* (2022) who reported decreased body weight of juveniles of *Aporrectodea giardia* treated with lead and zinc. Houda *et al*. (2021) found that the fertilizer "Activeg" does not affect the growth and mortality of *A. giardia*. Pelosi *et al.* (2014) have reported reduced body weight growth, fertility and increased mortality in pesticide treated earthworms. Halaimia *et al.* (2021) also reported the inhibitory effect of NPK fertilizer with concentrations between 400 – 1200 mg/g soil-treated on the body weight and growth of juvenile *A. caliginosa*. Similarly, Mosleh *et al.* (2002) reported a marked drop in body weight and growth of *L. terresteris* treated with sublethal concentrations of isoproturon. As earlier reported, the use of pesticides and inorganic fertilizers can both be beneficial or harmful to both crop productivity and worm populations (Edwards and Lofty, 1982; Mahajan *et al.*, 2007; Curry *et al.*, 2008).

Effect on mortality: Pesticides, heavy metals and other chemical pollutants disturb the natural habitat of animals and can lead to ecological imbalance (Zeriri, 2014). According to Braham and Mansour (2017), the measure of mortality and the change in the biomass of an organism are the difference between their initial mass and their mass at the end of exposure period to a contaminant, and also are the commonly used tests to determine and to understand the eco-toxic impact of pollutants. In this context, the results of this study revealed no intense mortalities in the earthworms treated with pesticides separately and together during the four control periods, even at high concentrations. This may be explained by the short exposure period of up to 96 hours and that the toxicity effect of the two pesticides may appear after a long exposure period, where the earthworms exposed to the different concentrations may accumulate these pollutants mixing with the treated soil via either the epidermis or the digestive tract. Accordingly, Pelosi *et al.* (2014) found that the fungicide (Curprafor) had delayed effect after 12 month of exposure on *A. caliginosa*, the effect was manifested in particular on the decrease in density, and on the other hand the fungicide (Swing) had no effect on the biomass and density of the same earthworm. Wu *et al.* (2022) have reported that the fungicide tris (2-butoxyethyl) phosphate (TBOEP) can be accumulated in *Eisenia fetida* (0.09 – 76.0 µg/kg), and that the activation of the detoxification pathway of cytochrome P450 and glutathione promoted its metabolism in the worm. The toxic effects of various pesticides on worms have been well documented, including the study of Zhang *et al.* (2020) that reported the toxic effects of 24 insecticides on different earthworm species, the study of Badawy *et al.* (2013) that reported three growth regulating insecticides (Buprofezin, Lunfenuron and Triflumuron) that caused body growth reductions in *Aporrectodea calginosa*, and the study of Wang *et al.* (2016) that reported the toxic effects of the herbicide Atrazine and three insecticides, namely Chlorpyrifos, Lambda-Cyhalothrin and Imidacloprid on *Eisenia fetida* after 14 days of the exposure period. According to Zhang *et al.* (2020), Acetamiprid (insecticide), although it has been suggested as a worldwide substitute, due to its low toxicity, it has been found to affect the immune system, antioxidant defense system, and the reproductive system even with low doses. Additionally, Mosleh *et al.* (2002), Madjri and Hachad (2014) and Houda *et al.* (2021) found that the different groups of used pesticides showed harmful effects with respect to different exposed species.

Histopathology: As earlier reported, pesticides and heavy metals accumulate especially in the digestive tissues of earthworms, either orally after ingesting the contaminated soil, or through contact by the skin that does not have protective cuticle (Conder and Lanno*,* 2000; Lanno *et al.,* 2004). Morgan and Turner (2005) and Oluah *et al.* (2010) have reported histological evaluations as valuable tools for assessing the toxic effects of contaminants on several earthworm species. Indeed, the examination of the histological sections of the earthworms exposed to the two chemicals did not reveal any tissue damage. The results agreed with those of Berrouk *et al.* (2021) who reported no histological alterations in *A. giardia* exposed to Activeg fertilizer. Kılıç (2011) has reported that pollutants can cause damage and accumulate mainly in the circular muscles of earthworms, and Gao *et al.* (2013) reported that Traizole (fungicide) can cause tissue necrosis in *Eisenia foetida*. Also, Zeriri (2014) proved histological effects in *Octadrilus complanatus* after the use of Methomyl (insecticide), and Houda *et al.* (2022) have found marked damages in epidermis and muscle fibers of *A. giardia* exposed to that zinc and lead.

Conclusion: The non-selective toxic effect and the environmental behavior of different pesticides are receiving great research attention. Application of fungicides, herbicides and insecticides can increase residues in the soil, leading to environmental damage including oxidative stress and damage to sentinel organisms such as earthworms, gastropods such as snails and slugs. It should be noted that when a pesticides exhibits no short-term activity, the accumulated residues will cause long-term reproduction disruption, population decline and behavioral change in impacted organisms. The contributions of this study will help regulatory authorities understand the effects of pesticide mixtures on non-target organisms, and will also provide useful information on the interaction of different pesticides detected in the natural environment.

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