Antagonistic Effect On The Lethality of Earthworm (*Eisenia Fetida* L.) Being Exposed To Binary Mixtures of Herbicides

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Abstract

Frequent use of herbicides might impose a risk for non-target species. The objective was to test the combined toxic effect of binary herbicide mixtures: metribuzin:halosulfuron and metribuzin:flumioxazin on non-target earthworms in two test systems, a filter paper, and a soil toxicity test system. The joint action experiments were independently run twice to substantiate the findings. The most potent individual herbicide was metribuzin with a 50% lethal concentration (LC_{50}) of 17.17 μg ai. cm^{-2} at 48 h in filter paper test. The toxicity of the individual herbicides on filter paper test was ranked as metribuzin > halosulfuron > flumioxazin. In a soil test, metribuzin and halosulfuron had high toxicity with the LC_{50} 8.48 and 10.08 mg ai. kg^{-1} on day 14. Thus, the individual herbicide ranking did not change between the filter paper and artificial soil tests. The herbicides' mixed effect showed in both test systems consistent antagonistic effect relative to a Concentration Addition reference model. It means that the mixtures retracted the herbicides' action in the earthworms.

1 Introduction

Weed infestation reduces yield and product quality and increases production costs (Zarea and Karimi 2012). One of the most efficient tools to control weeds is the use of selective herbicides. According to Travlos et al. (2017), herbicides represent the highest load of active ingredients per ha than any other pesticides. The use of herbicides either alone or in mixtures might effectively control weeds but may affect non-target organisms within and outside the treated field. Often less than 0.1% of an herbicide reaches the target plants, and the remaining is absorbed by be the crop or left on the soil or contaminate the environment (Gill and Garg 2014).

Soil-dwelling animals like earthworms might be affected even though the herbicide's site of action is not targeted animals (Zhang et al. 2017). Earthworms are important macroinvertebrates that make up more than 80% of the terrestrial invertebrates' biomass. They have critical functions in soil structure, soil characteristics (pH, organic matter, nitrogen, and granulometry), nutrient immobilization, nitrogen mineralization of organic matter, soil permeability, and microbial community activity (Kumar and Kumawat 2018). They are used as test species to measure the biological effect of heavy metals and pesticide residues in soil due to their high sensitivity to soil pollutions (Wang et al. 2012; Chen et al. 2018).

The *Eisenia fetida* is currently used as test species in ecotoxicology (Hirano and Tamae 2011), probably because it is easily raised and bred in the laboratory and, therefore, a common species in laboratory experiments. Finally, the use of it is recommended in ecotoxicological studies by European Union (EU), Organisation for Economic Co-operation and Development (OECD), and U.S. Environmental Protection Agency (EPA) (Nahmani et al. 2007 a,b; Correia and Moreira 2010).

Herbicide mixtures are commonly used to control diverse weed floras in crops because mixtures widen the control spectrum. The pesticide mixtures have received a great deal of attention in recent decades, particularly their effect outside the arable land (Mehler et al. 2008; Ohlsson et al. 2010). The joint action of herbicide mixtures compounds is generally being analysed by either Concentration Addition (CA) and Independent Action (IA) reference models. The two models are essentially covering various views on the intrinsic mechanism of joint action of compounds in an organism.

This paper solely focuses on CA, which assumes that two compounds do not interfere with each other in an organism (Cedergreen et al. 2008). Biochemically, they do not interfere with each other at the target enzyme binding site or impair or enhance each other uptake and translocation in the plant. CA requires we know the relative potency (strength) at any response level between the two herbicides in a mixture, applied singly. If the effect of binary mixtures at any response level, say LC_{50}, in fixed ratios diverts from the CA LC_{50} Isobole, the effect is classified as either antagonistic or synergistic, respectively (Streibig and Jensen 2000). If a mixture follows the CA isobole, viz follow straight line CA isoboles the herbicides do not affect each other's action (Wang et al. 2016) in *Eisenia fetida.* If the mixtures do not follow CA the mixtures can be either acting synergistic or antagonistic.

Metribuzin, halosulfuron, and flumioxazin are used in potatoes (Alebrahim et al. 2012; Grichar et al. 2003; Hutchinson 2007). Metribuzin [4-amino-6-*tert*-butyl-3-methylsulfanyl-1,2,4-triazin-5-one] (Sencor WP 70%) belongs to systemic triazine herbicides and is a photosystem II inhibitor. It is used in potatoes, tomatoes, soybean, and carrot (Alebrahim et al. 2012). The halosulfuron methyl (methyl 3-chloro-5-[(4,6-dimethoxypyrimidin-2-yl) carbamoylsulfamoyl]-1-methylpyrazole-4-carboxylate) belongs to the sulfonylureas and is an inhibitor of the enzyme acetolactate synthase (ALS) (Chand et al. 2014). Halosulfuron methyl is a selective and systemic herbicide (Vencill 2002). The flumioxazin (2-(7-fluoro-3-oxo-4-prop-2-ynyl-1,4-benzoxazin-6-yl)-4,5,6,7-tetrahydroisoindole-1,3-dione) is a N- phenylphalimide (Mossler and Langeland 2006) and it is an inhibitor of enzyme protoporphyrinogen oxidase (PPO or Protox) (Vasilakoglou et al. 2013).

It is important to note that the herbicides do not affect the same metabolism in animals as their primary modes of action are solely targeting plant metabolism, not existing in animals.

Those herbicides can either be applied in mixtures to control important weed species or be sequentially applied. In either case, the joint action of mixtures is of interest for agronomists and in ecotoxicology to unravel the joint action on non-target species.

The objective of this paper was to evaluate the acute LC_{50} (Lethal Concentration that kills 50% of the test animals) of the herbicides either alone or in binary mixtures of metribuzin:halosulfuron and metribuzin:flumioxazin. The reference model was the Concentration Addition (CA), also denoted the Additive Dose Model (ADM) (Streibig and Jensen 2000). Deviation of the mixture isoboles from the straight-line CA isoboles was used to classify the toxicology of those mixtures on earthworm populations in two test systems. The mixture experiments were repeated twice to substantiate if the mixture deviation from the CA was consistent.

2 Materials And Methods

2.1 Test organism

The Iran earthworm company delivered the *Eisenia fetida*. They were carefully transferred to the laboratory in moist soil. Under laboratory conditions, the earthworms were kept for 5 days in boxes containing the original medium (a mixture of sand, clay loam soil, and peat (1:1:1 v/v)) and cattle manure free of veterinarian products and maintained at room temperature (20±1°C). The moisture content of the medium was adjusted to 50%. Soil water content was measured every week, and moisture was adjusted to 50% of the maximum water-holding capacity by adding distilled water. Healthy and clitellated adult earthworms (weight of 350–600 mg) with completely developed clitella were used in toxicity tests (OECD 2004).

2.2 Herbicides

Formulated metribuzin (Sencor, WP 70%) was obtained from Bayer, Persian AG, Tehran, Iran, halosulfuron (Sempra, WG 75%) from Nufarm Company and flumioxazin (Pledge, WP 50%) were from Sumitomo chemical company. The stock solutions and dilution series of metribuzin, halosulfuron were prepared in distilled water (1100 and 1630 mg/l) and for flumioxazin (1.7 mg/l) in acetone (OECD 2004).

The dilution series were made immediately before the application. A water control was for metribuzin and halosulfuron herbicides. A positive control (using the same amount of distilled water as added in each test substance assay) and a negative control (using the same amount of acetone for solvent control) were tested (OECD 2004).

2.3 Toxicity test methods

2.3.1 Filter paper test

An eight cm glass Petri dish was lined with a filter paper (Whatman filter paper no. 1) cut into 8×3 cm in diameter without overlapping. Metribuzin and halosulfuron were dissolved in distilled water and flumioxazin in acetone solvent and prepared herbicide concentrations. Two ml solutions were pipetted into the Petri dish. Control Petri dishes were also run in parallel with the herbicides. Distilled water was used as the control for metribuzin and halosulfuron and distilled water and acetone for flumioxazin. After the acetone was evaporated in an airing chamber, well-developed clitellate adult worms were randomly selected, washed, and dried. Then, they were exposed (one earthworm per Petri dish) to 2 ml of different concentrations of herbicides for 24 and 48 h. The earthworms were kept on wet filter paper for 24 h at 20±1° C in the dark to have the gut contents purged before the dose-response test, and they were washed and dried before being weighed and introduced in the test (Xue et al. 2009). The filter paper test had three replications. Every Petri dish contained ten adult earthworms. Petri-dishes were kept in the dark at 20±1°C at 80-85% relative humidity and covered with a plastic lid with holes to allow for aeration and prevent the earthworms from escaping the Petri dishes. The experiments were performed for 48 h treatment period, and the number of dead earthworms of each treatment was recorded of each Petri dish. The concentrations of metribuzin, halosulfuron, and flumioxazin applied singly were shown in **Table 1**. A Preliminary range-finding test to find an optimal dose range that caused 0-100% mortality was done for the single herbicides before the mixture experiments.

2.3.2 Soil toxicity test

An artificial soil consisted of 10% ground sphagnum peat (<0.5 mm), 20% kaolin clay (>45% kaolinite), 70% quartz sand (<0.2 mm), and a small amount of calcium carbonate to adjust soil pH at 6.0 ± 0.5 (OECD 2004). The water content was adjusted to 35%. Stock solution of metribuzin and halosulfuron in water were prepared. This stock solution was used to spike soil with the highest test concentration and further diluted for spiking soil with the lower concentrations tested. The desired amount of herbicides was dissolved in 10 mL distilled water or acetone and mixed into a small quantity of fine quartz sand for each concentration. The sand was mixed for at least 1 h to evaporate the acetone and then mixed thoroughly with the pre-moistened artificial soil in a household mixer. All soils were thoroughly mixed to achieve a homogenous distribution of the herbicides in the soil. The addition of distilled water adjusted the final moisture contents of artificial soil. About 0.650 kg of artificial soil (equivalent to 0.5 kg dry artificial soil) was added into one L glass jar for herbicides. Three glass jars, each containing ten adult earthworms, were used for each concentration. Positive and negative controls were prepared with 10 mL distilled water or acetone and no herbicide to ensure if the positive and negative control were not significantly different from each other. The earthworms were removed from the culture, rinsed with water, and placed on damp filter paper in the dark at 20±1°C, and the content of their guts was emptied 24 h before herbicide exposure (Xue et al. 2009). The earthworms were fed 5 g cattle manure. Test jars were weighed at the start, so water loss could be monitored weekly and replenished with deionized water if needed. All earthworms were weighted before test. The jars were covered with perforated plastic film to allow the exchange of air and kept at room temperature 20± 1° C with 80–85% relative humidity under 400–800 lux of constant light. The survival was recorded on 1, 7, and 14 days after the treatment. The concentrations of metribuzin, halosulfuron, and flumioxazin applied singly were shown in **Table 1.**

The earthworms were preconditioned for 24 h under the same conditions described above in the untreated soil before the dose-response test. The ten adult earthworms were purged for 24h and were washed and dried before being weighed and introduced in the test. Two preliminary range-finding test determined the concentration range of the herbicides to find an optimal dose-range to caused 0-100% mortality.

2.3.3 Mixture toxicity

On the basis of the measured LC_{50} values of the individual herbicides (A and B), the relative potency (r) was determined for the herbicides applied alone. LC_{50}s concentration (μg ai. cm^{-2} or mg ai. kg^{-1}) reduced the live earthworm number by 50%.

$$r = LC_{50A}/LC_{50B} \qquad (1)$$

Eq. 1 expresses the biological exchange rate between the herbicides, metribuzin, halosulfuron, and flumioxazin within the filter paper and the soil toxicity test. The LC_{50}'s were derived from the dose-response regression model (Eq. 2) based on equation 1; the fixed mixture ratios were determined to ensure evenly distributed LC_{50} values for mixtures along the CA isobole (Gessner 1995). The mixture ratios were (100:0), (10:90), (25:75), (50:50) and (0:100)% for the metribuzin:halosulfuron mixtures and for metribuzin:flumioxazin they were (100:0), (4:96), (12:88), (29:71) and (0:100)% on filter paper test. The dose-response mixture experiments were independently repeated twice. Like the filter paper test, the mixtures ratios for the soil test were based upon the relative potencies of the individual herbicides. The mixtures were for metribuzin:halosulfuron (100:0), (72:28), (46:54), (22:88), (0:100)% and for metribuzin:flumioxazin they were (100:0), (25:75), (10:90), (4:96), (0:100)%. A positive water control was tested for metribuzin:halosulfuron and two water and acetone control for metribuzin:flumioxazin for filter paper and artificial soil tests. In neither case were there significantly difference between the positive control water only and negative control water plus acetone.

2.4 Statistical analysis

The dose-response data were analyzed using the R program (Version 3.6.1). The lethality of earthworms in response to herbicides is classical in toxicology. The binomial response, dead or alive, was assessed at various times during the experiments. The log-logistic regression was used to assess the acute toxicity of metribuzin, halosulfuron and flumioxazin, and the metribuzin:halosulfuron and metribuzin:flumioxazin mixture ratios (Eq. 2). The add-on R package drc (Version 3.6.1) was used to fit the log-logistic curves, and the LC_{50} parameters for each curve of fixed ratio mixtures illustrated the deviation from the straight line isobole of the CA reference model (Ritz et al. 2015).

The non-linear regression analysis of the log-logistic model (Ritz et al. 2015) is seen below.

$$y = \frac{1}{1+\left(\frac{x}{LC_{50}}\right)^{b}} \qquad (2)$$

Where *y* is the binomial response, dead divided by total number of earthworms in a petri dish or artificial soil, *x* denoted herbicide concentration (µg ai. cm^{-2} or mg ai. kg^{-1}) of any mixture ratios defined as the sum of the actual doses. It means we fitted a total of five dose response curves per experiment for the binary mixtures. The experimental design was a so-called ray design (Gessner 1995). LC_{50} is the concentration (µg ai. cm^{-2} or mg ai. kg^{-1}) that kill 50% of the total number of earthworms, and *b* is the slope of the curve around LC_{50}. The dose-response fitted reasonably well to the data (See test for lack of fit **Table 2**).

The mixtures of metribuzin:halosulfuron and metribuzin:flumioxazin reference model CA at the equivalent LC_{50} doses can be expressed by (Streibig et al. 1998):

$$Z_1 = rZ_2 = z_m = z_1 + rz_2 \qquad (3)$$

Z_1 and Z_2 are the LC_{50} of herbicide 1 and 2 applied singly, and z_1 and z_2 are the LC_{50} of herbicide 1 and 2 in a mixture adjusted by the relative potency, r (Eq. 1) herbicide 1 and 2 is applied alone in Eq. 1.

3 Results

Table 2 gives the parameters LC_{50} of the herbicides applied singly taken at various times after herbicide applications as well as the test of lack of fit of the dose-response curves. Only the metribuzin reading at 24 h had a significant lack of fit. In an LC_{50} isobolograms (Figs. 1, 2, 3, 4), the X and Y axes are the dose axes of each herbicide in a mixture. Metribuzin is the dose of the X-axis, and halosulfuron or flumioxazin the dose on the Y-axis. The solid lines for each LC_{50} connecting the herbicides applied singly are the theoretical CA isobole.

If the herbicides in a mixture do not interact, the mixtures follow the straight line isobole and thus comply with the CA reference model (Eq. 3). When herbicides are less effective than expected, they show antagonistic action: it means one must use higher doses of each herbicide in a mixture to produce the same effect as that of the herbicides applied alone. When herbicides are more effective than expected, they show synergistic action: it means one must use lower doses of each herbicide in a mixture to produce the same effect as that of the herbicides applied alone.

3.1 Filter paper test

Table 2 shows the LC_{50} at 24 and 48 hours. The LC_{50} declined between 24 and 48 hours for metribuzin and flumioxazin. The toxicity of herbicides was ranked as metribuzin > halosulfuron > flumioxazin, and the ranking did not change between the time of measurement (Table 2**).** The LC_{50} of halosulfuron was the same 52.95 µg ai. cm^{-2} at 24 and 48 hours. The test for lack of fit (Ritz et al 2015) was significant for metribuzin at 24 h but alarmingly small in any other instances. The lack of fit test is rather weak and was supported by compared residual plots (to illustrating the residuals plots (data not shown) (Ritz et al. 2015). The LC_{50} estimates were not significantly different for water control and acetone control for flumioxazin in both times (Student t-test; p = 0.374 on 24 and p = 0.158 on 48 day).

The binary mixture experiments demonstrated for both experiments a clear antagonistic effect at both sampling times and for both binary mixtures (Figs. 1 and 2). The LC_{50} estimates were not significantly different for water control and acetone control for metribuzin:flumioxazin in both experiments (Student t-test; p = 0.116 on 24 and 48 day in first experiment and p = 0.374 on 24 and p = 0.205 on 48h in second experiment). It is also important to emphasize that the mixtures ratios were almost evenly distributed along the CA isoboles.

3.2 Soil toxicity test

Earthworms exposed to metribuzin, halosulfuron, and flumioxazin also showed changes in LC_{50} in sampling time. The results demonstrated an increase in exposure time decreased the LC_{50}, particularly for flumioxazin (Table 2). The test for lack of fit was in no instances significant, meaning that the dose-response curves fitted reasonably to the data. The LC_{50} estimates were not significantly different for water control and acetone control for flumioxazin (Student ttest; $p = 0.374$ on 1, 7 and 14 day). The toxicity of herbicides was ranked as metribuzin > halosulfuron > flumioxazin (Table 2) and the ranking did not change among the time of measurement.

The binary mixture experiments demonstrated for both experiments a clear antagonistic effect at all three sampling times and for both binary mixtures (Figs. 3 and 4). The LC_{50} estimates were not significantly different for water control and acetone control for metribuzin:flumioxazin at different mixture ratios in both experiments (Student t-test; $p = 0.374$, 0.116 and 0.116 on 1, 7 and 14 day in first experiment and $p = 0.374$, 0.374, 0.158 on 1, 7 and 14 day in second experiment). It is also important to emphasize that the mixtures ratios were almost evenly distributed along the CA isoboles.

4 Discussion

Mortality of *Eisenia fetida* is typically used in studying chemical toxicity compounds on earthworms (Iordache and Borza 2011; Pelosi et al. 2014). The contact filter paper test is a fast, simple, and inexpensive test to screen for toxicity (Wang et al. 2012). It also applies to the soil test, but it is obvious that the potency of the herbicides and herbicide mixtures were notorious different between the two test media. However, the LC_{50} gave the same order of toxicity metribuzin > halosulfuron > flumioxazin (Table 2). The herbicides are designed to kill green plants, and therefore, the sites and modes of action are well known in plants but not in animals. When it comes to the site and mode of action in animals, the cause-and-effect relationships between mortality and specific action sites become more uncertain and need to be further investigated.

The increase of LC_{50} and exposure time increased in both test systems. For metribuzin and halosulfuron 100% mortality at high doses was observed 3–4 hours after exposure on filter paper. The LC_{50} of the PS II inhibitor, metribuzin, had the highest toxicity in both test systems. It means the compound may have some unknown effect on the earthworm. On the other hand, flumioxazin, with its particular site of action on heme and chlorophyll metabolism, is known to affect the soil biome (Pertile et al. 2020).

Chemicals with LC_{50} values of 10–100 µg cm^{-2} on filter paper were classified as “very toxic” (Landrum et al. 2006). According to this standard, metribuzin and halosulfuron toxicity to earthworms can be classified as high. However, the halosulfuron, an ALS inhibitor, is used at small field rates, so perhaps the effect of this herbicide in the field is not so severe as is metribuzin.

Herbicides affected the earthworms adversely through skin contact. Earthworm mortality by the presence of metribuzin was caused by increased mucous secretion to a high concentration (laboratory observations). The earthworms exposed to metribuzin exhibited surface wounds and extrusion of coelomic fluid. It caused bloody lesions on the posterior part of the body and ultimately death. Fragmentation of the body was also observed (laboratory observations).

The singly and binary mixtures applied to the soil test, but it is obvious that the potency of the herbicides and herbicide mixtures were notorious different between the two test media. The ranking of the herbicides was the same in both test systems with rather low metribuzin and halosulfuron LC_{50} compared to flumioxazin, particularly in the soil test. However, we have to bear in mind the field rate of metribuzin, halosulfuron and flumioxazin are 700–1000, 250 and 100 g ha^{-1}. Mortality is related to an earthworm strategy for decreasing food consumption to avoid toxins. This strategy is used for heavy metals (Burrows and Edwards 2002) and pesticides (Wang et al. 2012). Soil ingestion and dermal absorption are the most crucial uptake paths of soil contaminants by earthworms. Earthworms can absorb and accumulate pollutants in their body tissues through the skin and digestive system (Shan et al. 2014).

It should also be noted that increasing mortality might correlate with the high persistence of metribuzin and halosufuron in soil or the slow degradation in the earthworms and, subsequently, less metabolite elimination. Researchers have documented that flumioxazin has a soil half-life between 11.9 and 17.5 days (Vencill 2002). So short half-life influences the toxicity of a compound, particularly in the artificial soil experiment done here.

While the herbicides were well mixed in the soil test, in the field, however, most of the herbicide residues were found in the upper 5 cm of the profile (Hyzak and Zimdahl 1974). The results also indicated the low toxicity of flumioxazin in filter paper and artificial soil tests and could be attributed to the rapid elimination in the animals. Travlos et al. (2017) investigated the survival of *E. fetida* in soil toxicity bioassays of benfluralin, metribuzin and propyzamide. They reported that the highest mortality was found after the treatment with double the recommended field rate of metribuzin (1500 g ai. ha^{-1}) at three weeks after treatment. However, this is only a single field rate that cannot be referred to the test systems used here.

The classic model for predicting mixture toxicity, such as Concentration Addition (CA), is based on simple assumptions on the mode of toxic action (Streibig and Jesnsen 2000;Teuschler, 2007). However, synergism or antagonism can occur irrespective of the primary site and mode of action (Chou, 2006). Detracted action, also denoted antagonistic action was rather consistent in the experiments. It means that to obtain the same effect level (e.g., LC_{50}) one needs a higher concentration of the mixture than that of the individual compounds applied singly. The results was indeed confirmed as we did two independent experiments (Figs. 1–4). There is no evidence of metribuzin:halosulfuron and metribuzin:flumioxazin interaction in the literature.

Our results of the filter paper test are in line with the artificial soil test of individual and combined herbicides toxicity and confirmed the work by Stepić et al. (2013). it is well known that the laboratory test results cannot be extrapolated to the field (Lowe and Butt 2007; Svendsen and Weeks 1997). However, in some rare cases, the field and laboratory outcomes are comparable (Heimbach 1984; Culy and Berry

1995; Holmstrup 2000). The complementarity between field investigations and laboratory tests is a perpetual discussion in the literature. When introducing various mixtures, one needs both methods, as noted by Svendsen et al. (2005).

The results in our experiments demonstrated that the ranking of the toxicity of the herbicides was the same whether we used filter paper test or soil test, and the lethality increased in course of time of exposure. The herbicide mixtures all consistently acted antagonistically whether the mortality was assessed 24 or 48 h or up to fourteen days after the initiation of the experiments on filter paper and in soil test, respectively.

Declarations

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Availibility of data and material Data are available by contacting ESK (samadielham@uma.ac.ir).

Code availability The used R code is available by contacting ESK (samadielham@uma.ac.ir)

Authors' contributions Mohammad Taghi Alebrahim conceived the ideas as a supervisor. Elham Samadi Kalkhoran assembled the data, analyzed the data with help from Jens Carl Streibig. Akbar Ghavidel helped to interpret the data. Hamid Reza Mohammaddust Chaman Abad discussed about relationships between applying herbicide and environment. Elham Samadi Kalkhoran wrote the manuscript. Jens Carl Streibig, Mohammad Taghi Alebrahim and Akbar Ghavidel edited the manuscript. All authors improved and approved the manuscript.

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Tables

Table 1 Concentration used in filter paper and artificial soil tests

Herbicide	Concentration for filter paper test (μg ai. cm^{-2})
Metribuzin	0, 0.156, 0.312, 0.625, 1.25, 2.5, 3.5, 5, 7, 10, 14, 28, 56, 112, 224
Halosulfuron	0, 0.0156, 0.0312, 0.0625, 0.125, 0.250, 0.500, 1, 2, 4, 8, 16, 32, 64, 128, 256
Flumioxazin	0, 10, 20, 40, 80, 160, 320, 640
Concentration for artificial soil test (mg ai. kg^{-1})	
Metribuzin	0, 0.359, 0.718, 1.436, 2.872, 5.744, 11.488, 22.976
Halosulfuron	0, 0.2051, 0.410, 0.820, 1.640, 3.281, 6.563, 13.126
Flumioxazin	0, 4.1026, 8.205, 16.41, 32.82, 65.64, 131, 262

Table 2 Estimated sigmoidal parameters for metribuzin, halosulfuron and flumioxazin in filter paper and artificial soil tests. Standard errors in parantheses

Filter paper						
Herbicide	LC_{50} (μg ai. cm-^{2})			Lack of fit		
	24h		48h	24h	48h	
Metribuzin	34.00 (2.23)		17.17 (1.17)	0.003	0.97	
Halosulfuron	52.95 (9.58)		52.95 (9.58)	1	1	
Flumioxazin	241.64 (31.62)		127.42 (16.61)	0.08	0.06	
Artificial soil						
Herbicide	LC_{50} (mg ai. kg)			Lack of fit		
	1 day	7 day	14 day	1 day	7 day	14 day
Metribuzin	25.75 (12.44)	11.72 (4.36)	8.48 (3.36)	0.95	0.95	0.08
Halosulfuron	27.63 (13.38)	14.26 (4.26)	10.08 (2.76)	0.97	0.98	0.94
Flumioxazin	315.09 (115.05)	157.67 (40.49)	74.84 (13.79)	0.95	0.94	0.42

Figures

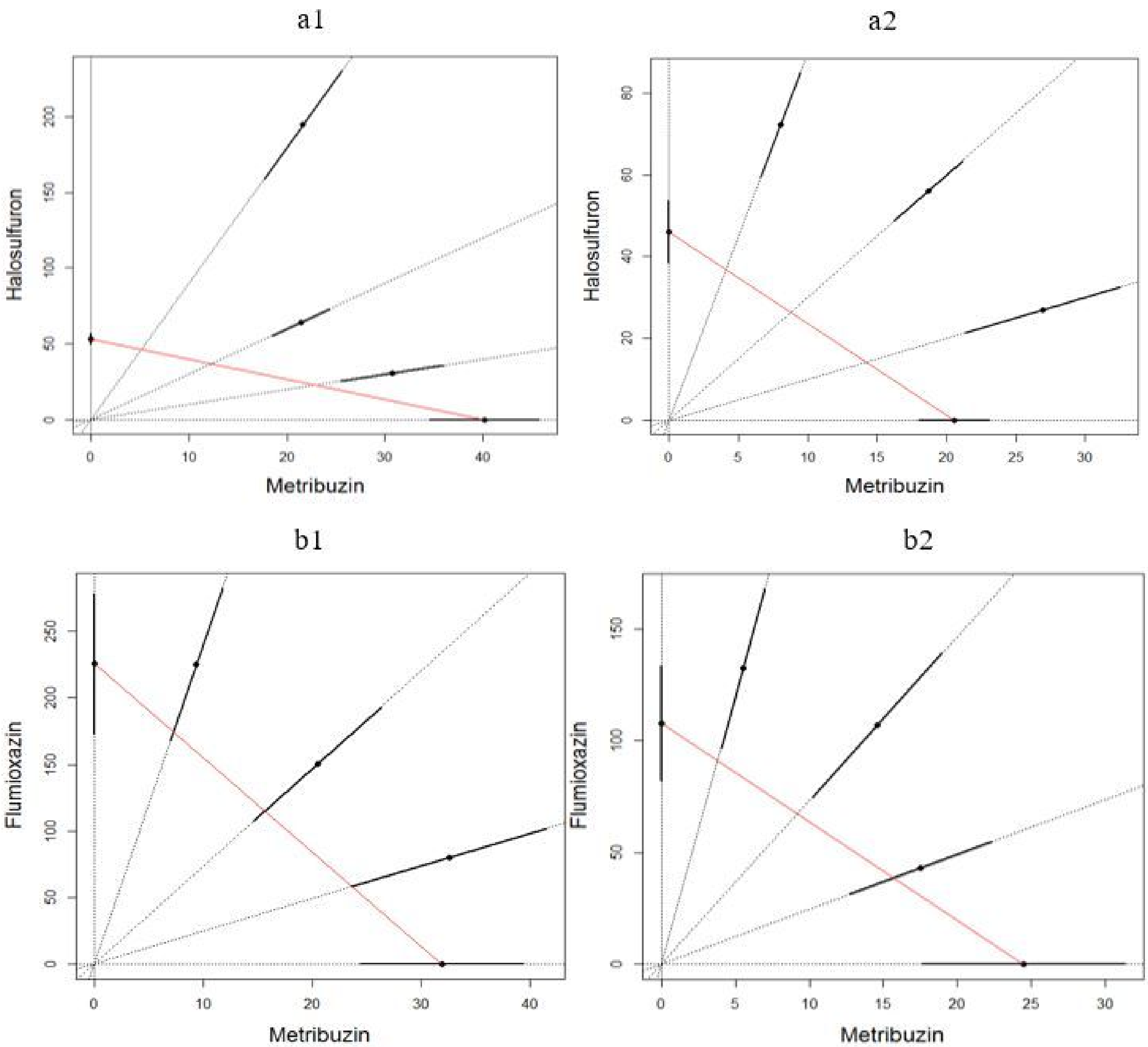

Figure 1

LC50 isobologram showing the toxicological interactions of metribuzin:halosulfuron (a) and metribuzin:flumioxain (b) on Eisenia fetida in first experiment of filter paper test at 24 (1) and 48h (2). The straight line of isobologram indicates additivity. The lines around the mixture points are 95% confidence intervals.

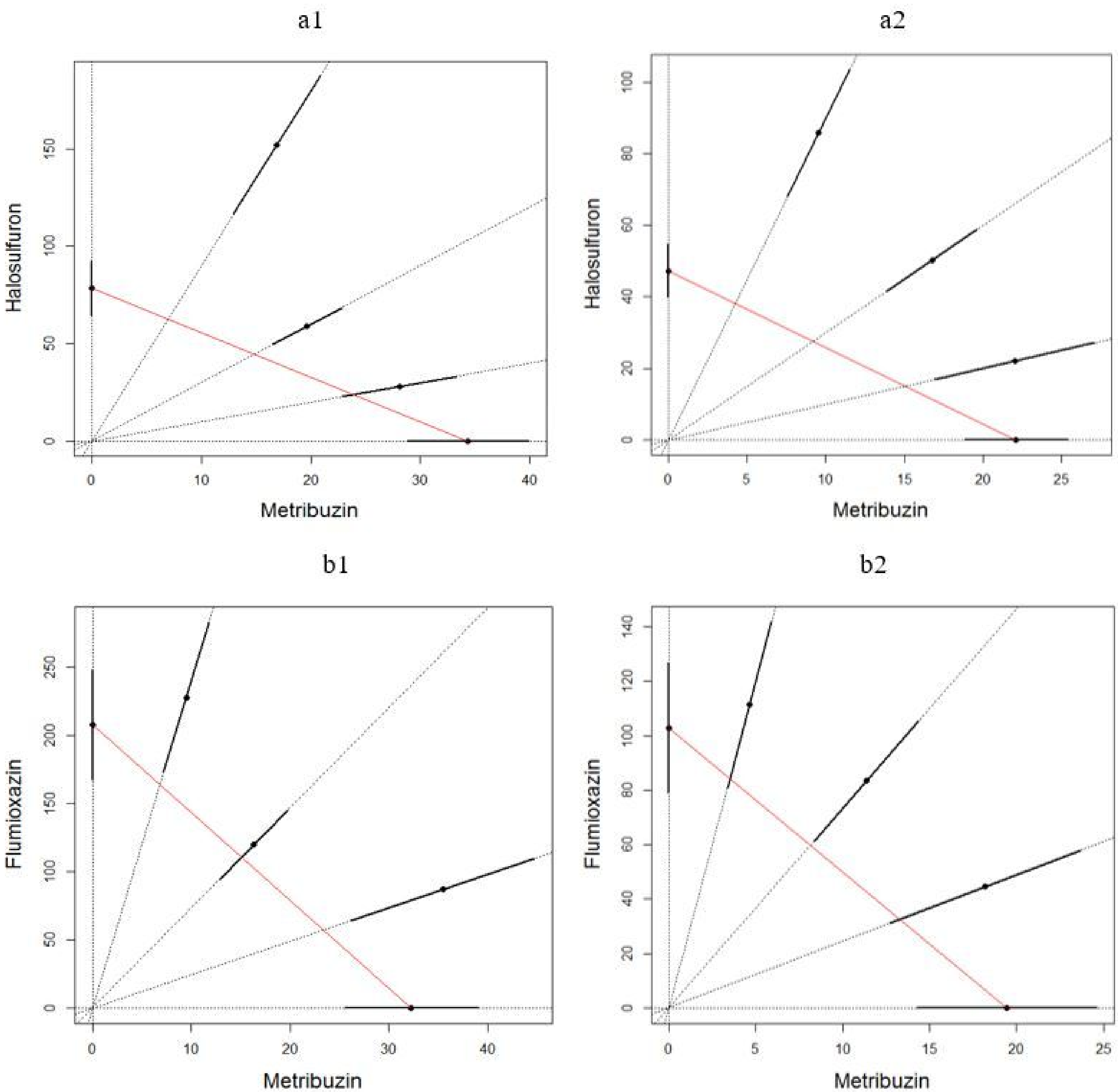

Figure 2

LC50 isobologram showing the toxicological interactions of metribuzin:halosulfuron (a) and metribuzin:flumioxain (b) on Eisenia fetida in the second experiment on filter paper test at 24(1) and 48h (2). The straight line of isobologram indicates additivity. The lines around the mixture points are 95% confidence intervals.

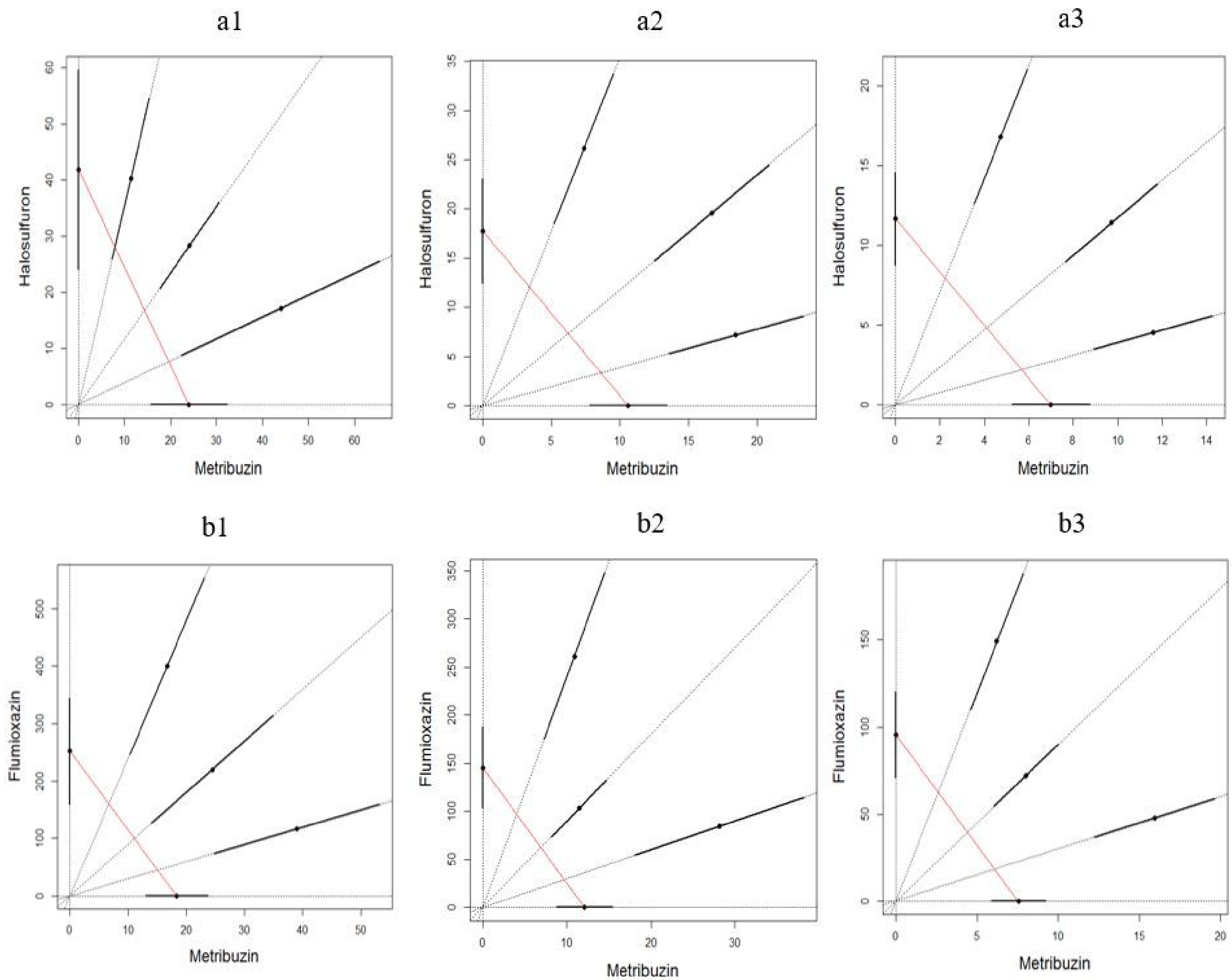

Figure 3

LC50 isobologram showing the toxicological interactions of metribuzin:halosulfuron (a) and metribuzin:flumioxazin (b) on Eisenia fetida in the first experiment of artificial soil test on 1(1), 7(2) and 14 day (3). The straight line of isobologram indicates additivity. The lines around the mixture points are 95% confidence intervals.

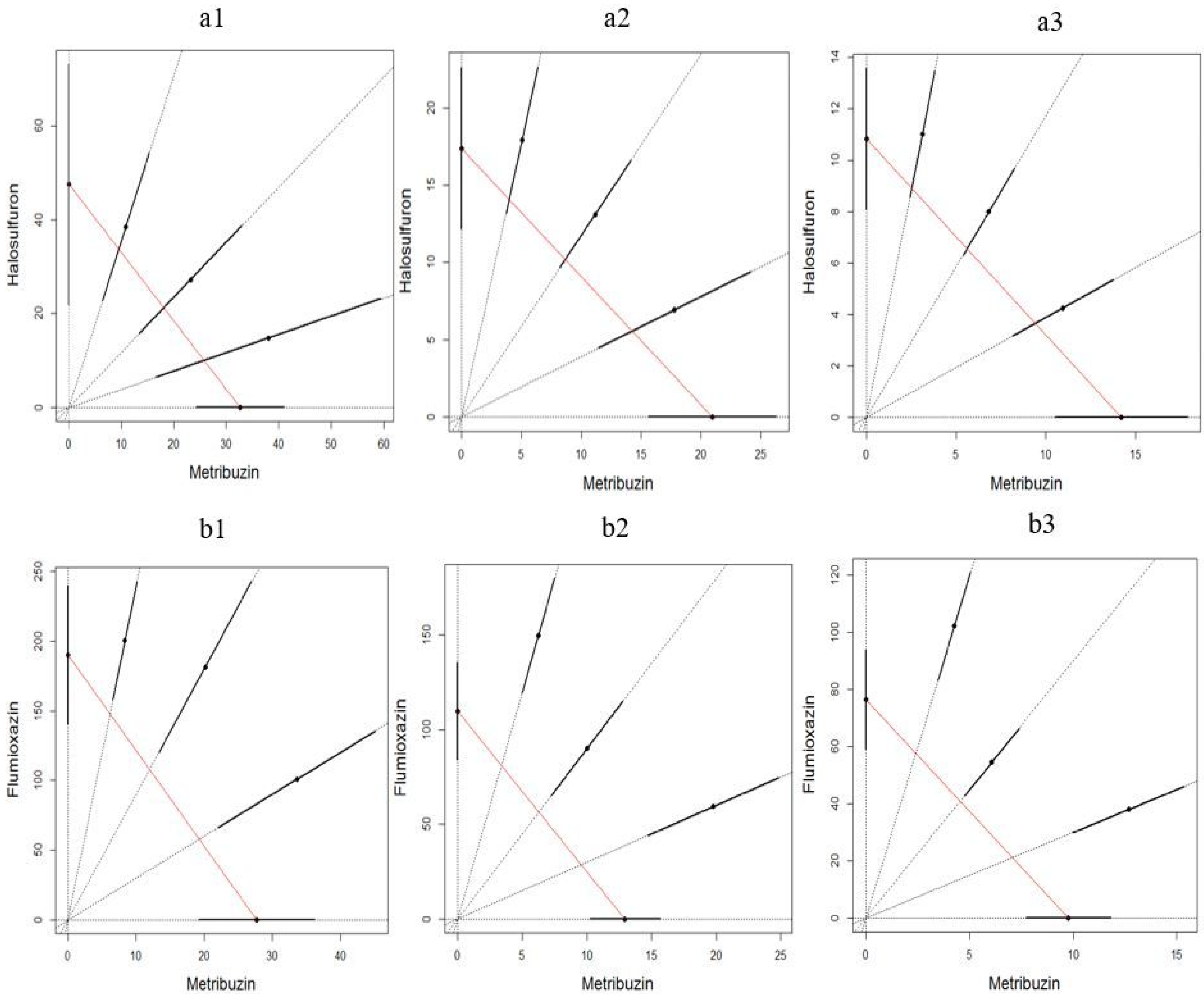

Figure 4

LC50 isobologram showing the toxicological interactions of metribuzin:halosulfuron (a) and metribuzin:flumioxazin (b) on of Eisenia fetida in the second experiment of artificial soil test at 1(1), 7(2) and 14 (3) day. The straight line of isobologram indicates additivity. The lines around the mixture points are 95% confidence intervals.