Chapter 2

Sorption of Terbuthylazine in Organic Matter Amended Soils: Effects on Eisenia Fetida and Lumbricus Terrestris

Manuel Tejada, Marina del Toro, Paloma Osta, Juan Parrado and Isidoro Gómez

Additional information is available at the end of the chapter

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1. Introduction

Pesticides are important tools in agriculture that help to minimize economic losses caused by weeds, insects, and pathogens. Although their use has helped to increase crop yields and value, they may also contribute to environmental degradation [1].

Pesticides are recognized as a source of potential adverse environmental impacts and their persistent in surface and ground waters has grown considerably [2]. Some soil applied herbicides reach surface and ground waters by the losses associated with runoff and leaching processes [3]. These losses are attenuated by the natural process of sorption, since degradation, transport, and biological activity of pesticides are greatly influenced by sorption on soil constituents [2].

In order to compensate for the losses caused by transport and degradation processes, some pesticides applicators are exceeding limits on labels which greatly exceed those required for control of the target organisms, and the excessive quantities added increase the environmental impact of these compounds [4, 5]. Because of this, public concern over the residues of pesticides in environment, food and related commodities has increased over the last decades. In Europe, pesticides are considered hazardous substances in accordance with current legislation regarding water [6, 7].

Traditional agricultural requires the use of herbicides, and prevention of ground water pollution is much cheaper than restoring polluted aquifers. Thus, it is of maximum interest that development of agricultural strategies continues to be directed to the decrease in pesticide movement [8].

Triazine herbicides have been largely used in agriculture worldwide for selective and non-selective control of broadleaf and small seeded grass weeds in diverse crops, such as cotton (*Gossypium hirsutum*, L.), maize (*Zea mays,* L.), soybean (*Glycine max*, L.), groundnut (*Arachis hypogea*, L.) and vineyards (*Vitis vinifera*, L.). However, due to their physicochemical properties (in particular, their relatively long persistence) there have been numerous reports of their presence in surface or ground waters [3, 9]. Several bioremediation strategies have been proposed to reduce the presence of pesticides in soil from which they can reach groundwater, such as remediation by enhancing the microbial population able to degrade specifically the target compounds. This strategy has been approached by addition of organic exogenous matter of different origin [9, 10, 11].

However, the influence of organic matter on soil properties and sorption process depends upon the type, amount and dominant components of the added organic materials [12, 13]. This aspect is of great interest, since it supposes an important advance in the behavior of the herbicides in the soil after the addition of different sources of organic matter.

Earthworms are one of the important components in decomposer communities and contribute significantly to the organic decomposition, nutrient cycling and soil formation [14]. Continuous application of pesticides may present risks to lead to soil pollution affect soil fauna [15]. For this reason, the use of earthworms for toxicity testing is highly recommended by the European Communities [16] and are considered as preferred bioindicators for assessing the environmental health status of chemical pollution [17, 18]. Earthworm species such as *Eisenia fetida* is considered as a suitable biomonitors to determine the ecological hazard of heavy metals and chemicals contaminated soil because of its low cost, easy culturing and the standardization of the acute and sub-chronic ecotoxicological tests [14, 19].

Of the potential biomarkers, earthworm glutathione-S-transferase and cellulalse enzymes are shown to respond to toxin exposure [14]. Glutathione-S-transferase is an important detoxification enzyme and its activity has been used as a potential bioindicator and biomarker of earthworms for heavy metals, pesticides and PAHs exposure [14, 15, 20]. Also, cellulase activity of earthworms indicates their role in the decomposition of plant litter and other cellulosic materials. It has been used as a biomarker of a pesticide contamination on earthworms [14, 20].

However, the biological relevance of *Eisenia fetida* is still open to debate [21] since they are often less susceptible to pollutants than other species [22] and rarely found in conventionally tilled agricultural soils. In this respect, Ma and Bodt [23] found and different levels of chlorpyrifos insecticide sensitivity to earthworms (*Eisenia* sp.< *Aporrectodea* sp.< *Lumbricus* sp.).

Few studies have been performed comparing different sources of organic matter types on the sorption and mobility of herbicides. For this reason, the objective of this study was to investigate the sorption and mobility of terbuthylazine herbicide in a soil amended with three organic amendment and their effects on acute toxicity and morphological alterations in two earthworm species (*Eisenia fetida* and *Lumbricus terrestris*).

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2. Material and methods

2.1. Soil, organic amendment and herbicide characteristics

The soil used in this experiment is a Plagic Antrosol [24]. The main soil characteristics are shown in Table 1.

	Soil	PM	MSW	CM
pH (H_2O)	8.6 ± 0.2	7.1 ± 0.3	6.2 ± 0.3	8.3 ± 0.2
CO_3^{2-} (g kg^{-1})	203 ± 12			
Fine sand (g kg^{-1})	142 ± 35			
Coarse sand (g kg^{-1})	387 ± 26			
Silt (g kg^{-1})	242 ± 19			
Clay (g kg^{-1})	229 ± 10			
Clay types	Smectite: 66% Kaolinite: 20% Illite: 14 %			
Organic matter (g kg^{-1})	1.1 ± 0.2	614 ± 26	469 ± 15	764 ± 29
Humic acid-C (mg kg^{-1})	18.5 ± 2.4	672 ± 1.4	1030 ± 17	461 ± 13
Fulvic acid-C (mg kg^{-1})	9.8 ± 1.1	715 ± 10	711 ± 10	631 ± 24
Total N (g kg^{-1})	0.4 ± 0.1	38.8 ± 2.9	17.3 ± 1.3	29.2 ± 2.1
Fe (mg kg^{-1})	35.8 ± 3.7	180 ± 22	815 ± 38	407 ± 28
Cu (mg kg^{-1})	9.7 ± 1.3	1.6 ± 0.3	82.6 ± 9.8	24.2 ± 1.8
Mn (mg kg^{-1})	11.3 ± 2.1	4.2 ± 0.9	75.6 ± 8.1	14.1 ± 1.2
Zn (mg kg^{-1})	8.1 ± 1.5	3.3 ± 0.8	134 ± 13	10.3 ± 1.6
Cd (mg kg^{-1})	6.5 ± 1.2	0.35 ± 0.07	1.1 ± 0.3	0.28 ± 0.09
Pb (mg kg^{-1})	0.36 ± 0.11	0.94 ± 0.12	82.4 ± 3.6	5.3 ± 0.8
Ni (mg kg^{-1})	2.9 ± 0.7	1.3 ± 0.2	13.6 ± 1.5	2.4 ± 0.6
Cr (mg kg^{-1})	5.3 ± 0.6	0.12± 0.02	19.4 ± 1.7	0.29 ± 0.04

Table 1. Characteristics of the experimental soil and organic amendment (mean ± standard error). Data are the means of four samples.

Soil pH was determined in distilled water with a glass electrode (soil:H_2O ratio 1:2.5). Soil texture was determined by the Robinson's pipette method [25] and quantification and dominant clay types were determined by X-ray diffraction. Total carbonates were measured by estimating the quantity of the CO_2 produced by HCl addition to the soil [26]. Soil organic matter was determined by the method of Yeomans and Bremner [27]. Humic and fulvic acids-

fractions were extracted with 0.1 M sodium pyrophosphate and 0.1 M sodium hydroxide at pH 13 [28]. The supernatant was acidified to pH 2 with HCl and allowed to stand for 24 h at room temperature. To separate humic acids-fraction from fulvic acids-fraction, the solution was centrifuged and the precipitate containing humic acids-fraction was dissolved with sodium hydroxide [27]. After the removal of humic acids-fraction, the acidic filtrate containing the dissolved fulvic acid-fraction was passed through a column of XAD-8 resin. The adsorbed fulvic was then recovered by elution with 0.1 M NaOH, desalted using Amberlyst 15-cation-exchange resin, and finally freeze-dried. The carbon content of humic and fulvic acids-fractions were determined by the method described. Total N was determined by the Kjeldhal method [26]. After nitric and perchloric acid digestion, total Ca, Mg, Fe, Cu, Mn, Zn, Cd, Pb, Ni and Cr concentrations were determined by atomic absorption spectrometer and K was determined by atomic emission spectrometer, according to MAPA methods [26].

The organic amendment applied were the organic fraction of a municipal solid waste (MSW), poultry manure (PM) and cow manure (CM). The general properties of the organic amendment are shown in Table 1. Organic matter was determined by dry combustion, according to the official methods of the Spanish Ministry of Agriculture [26]. Humic and fulvic acids-fraction were extracted, separated and determined by the methods previously described. Total N was determined by the Kjeldhal method [26]. After nitric and perchloric acid digestion, total Ca, Mg, Fe, Cu, Mn, Zn, Cd, Pb, Ni and Cr concentrations were determined by atomic absorption spectrometer and K was determined by atomic emission spectrometer, according to MAPA methods [26].

Table 2 shows the acidic functional group contents of humic acids isolated from both organic amendment. The carboxyl group content was estimated by direct potentiometric titration at pH 8, the phenolic hydroxyl group content was estimated as two times the change in charge between pH 8 and pH 10, and the total acidity was calculated by addition [29].

	Total acidity (mol kg^{-1})	**COOH**	**Phenolic OH**
PM	3.99 ± 0.13	2.99 ± 0.09	0.99 ± 0.05
MSV	4.29 ± 0.04	3.19 ± 0.03	1.10 ± 0.03
CM	2.81 ± 0.02	2.00 ± 0.03	0.80 ± 0.01

Table 2. Acidic functional group contents (mean ± standard errors) of humic acids isolated from PM, MSW and CM

The herbicide used in this experiment was the terbuthylazine. Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) is a selective herbicide for the control of broadleaf and grass weeds in forestry, lucerne (*Medicago sativa,* L.), maize (*Zea mays,* L.), sweetcorn (*Zea mays,* L. var. *rugosa*), peas (*Pisum sativum*, L.), orchard and non-cropland, with a water solubility of 8.5 mg l^{-1} at 20 ºC. It is absorbed by roots and inhibits Hill reaction and CO_2 sorption in the chlorophyllic function [30].

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2.2. Incubation procedure

Two kg of soil were pre-incubated at 25 ºC for 7 days at 30–40% of their water-holding capacity, according to Moreno et al. [31], prior to the treatments. After this pre-incubation period, soil samples were treated with three concentrations of terbuthylazine (1, 10 and 50 μg terbuthylazine g^{-1} soil) and treated with MSW at a rate of 10% or PM at a rate of 7.6% or CM at a rate of 5.8%, respectively, in order to applying the same amount of organic matter to the soil. A non-mended treated as well as a amended non-treated soil were used as controls.

The incubation treatments are detailed as follows:

1. C1, control soil, soil non-polluted and non-organic amended
2. C2, soil treated with 1 μg terbuthylazine g^{-1} soil and non-organic amended
3. C3, soil treated with 10 μg terbuthylazine g^{-1} soil and non-organic amended
4. C4, soil treated with 50 μg terbuthylazine g^{-1} soil and non-organic amended
5. MSW1, soil non- treated and amended with MSW
6. MSW2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with MSW
7. MSW3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with MSW
8. MSW4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with MSW
9. PM1, soil non- treated and amended with PM
10. PM2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with PM
11. PM3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with PM
12. PM4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with PM
13. CM1, soil non- treated and amended with CM
14. CM2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with CM
15. CM3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with CM
16. CM4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with CM

Triplicate treatments were kept in semi-closed microcosms at 25 ºC for 3, 15, 45 and 90 days, respectively.

Twenty two earthworms of the species *Eisenia fetida* (approximately 210 mg fresh weight) and *Lumbricus terrestris* (approximately 190 mg fresh weight) were included in each microcosm. Each microcosm was covered with fine nylon mesh to prevent the soil loss and to keep earthworms from escaping. *Lumbricus terrestris* were collected in the field, in an area that has not been treated with pesticides for 20 years, whereas *Eisenia fetida* were bred in laboratory cultures on organic amendment materials, vermicomposts principally.

2.3. Adsorption studies

For adsorption studies the treatments used were:

1. S, non-organic amended control soil (10 g of soil)
2. S+CM, soil amended with CM at rate of 10% (10 g of soil + 1 g of CM)
3. S+PM, soil amended with PM at a rate of 12.4% (10 g of soil + 1.24 g of PM)
4. S+MSW, soil amended with MSW at a rate of 16.3% (10 g of soil + 1.63 g of MSW)

Terbuthylazine sorption was determined according to Cabrera et al. [32] criteria. Triplicate samples (5 g) of the non-amended and organic amended soil (S, S+CM, S+PM, S+MSW) were treated with 10 ml of terbuthylazine (50%:50%, v/v) solution (initial concentrations, Ci, ranging from 5 to 50 μM in 0.01 $CaCl_2$). Previously, it was determined that equilibrium was reached in less than 24 h, and that no measurable degradation occurred during this period. Equilibrium concentrations (Ce) in the supernatants were determined by HPLC. Sorption isotherms were fitted to Freundlich equation ($Cs=Kf \times Ce^{1/nf}$) and sorption coefficients Kf and 1/nf were calculated.

2.4. Herbicide analysis

Herbicide was extracted twice with methanol (Merck, Darmstadt, Germany) at 1:2 soil/solution ratio for 15 min. Extracts were mixed and rotary-vacuum evaporated almost to dryness at 40 ºC. The residue was dissolved in 2 ml of methanol and analyzed by HPLC [30]. Terbuthylazine was analyzed using a Beckman, System Gold, Autosampler 508 HPLC chromatograph coupled to a Waters 2996 diode-array detector. The analytical conditions were: Nova-Pack C18 column (159 mm length X 3.9 mm internal diameter), eluent mixture, 50:50 acetonitrile/water at a flow rate of 1 ml min^{-1}, 25 μl injection volume, and UV detection at 220 nm [33]. External calibration curves with four standard solutions between 0.2 and 26 μM were used in the calculations.

2.5. Earthworm analysis

Earthworm cocoon production was determined after 30 days of exposure. Cocoons were collected by hand sorting and weighed, and then incubated for four additional weeks as described by Maboeta et al. [34]. Cocoons were cultured in Petri dishes at 25±1 ºC covered with three moist filter papers. According to Xiao et al. [14], the filter papers in these dishes were changed every three days to prevent bacterial growth. At the end of the experiment (30 days), the weight of per cocoon and number of juveniles per cocoon were determined.

After 3, 15, 45 and 90 incubation days for each treatment, three worms were selected and placed on wet filter paper in Petri dishes for 24 h to clear gut contents, and their weights were recorded after blotting them dry on paper towels. Earthworms were digested in the 1:1 nitric-perchloric extract after digestion at 450 ºC for 6 h. The terbuthylazine was measured by the method previously mentioned. Cellulase activity was measured as described by Mishra and Dash [35], and glutathione-S-transferase activity was measured according to the method described by Habig et al. [36] and Saint-Denis et al. [37].

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2.6. Statistical analysis

Two-ways analysis of variance (ANOVA) was performed for all parameters, considering two variables involved (incubation time and the terbuthylazine concentration applied to the soil) using the Statgraphics v. 5.0 software package [38]. The means were separated by the Tukey's test, considering a significance level of $P<0.05$ throughout the study. For the ANOVA, triplicate data were used for each treatment and every incubation day.

3. Results

3.1. Sorption studies

Sorption isotherms of terbuthylazine on soil, soil+CM, soil+PM and soil+MSW are shown in Figure 1. The results indicated that sorption of terbuthylazine on organic amended soils significantly increased compared to non-organic amended soil. For each organic amended soil, the herbicide sorption with MSW was higher than with PM and CM.

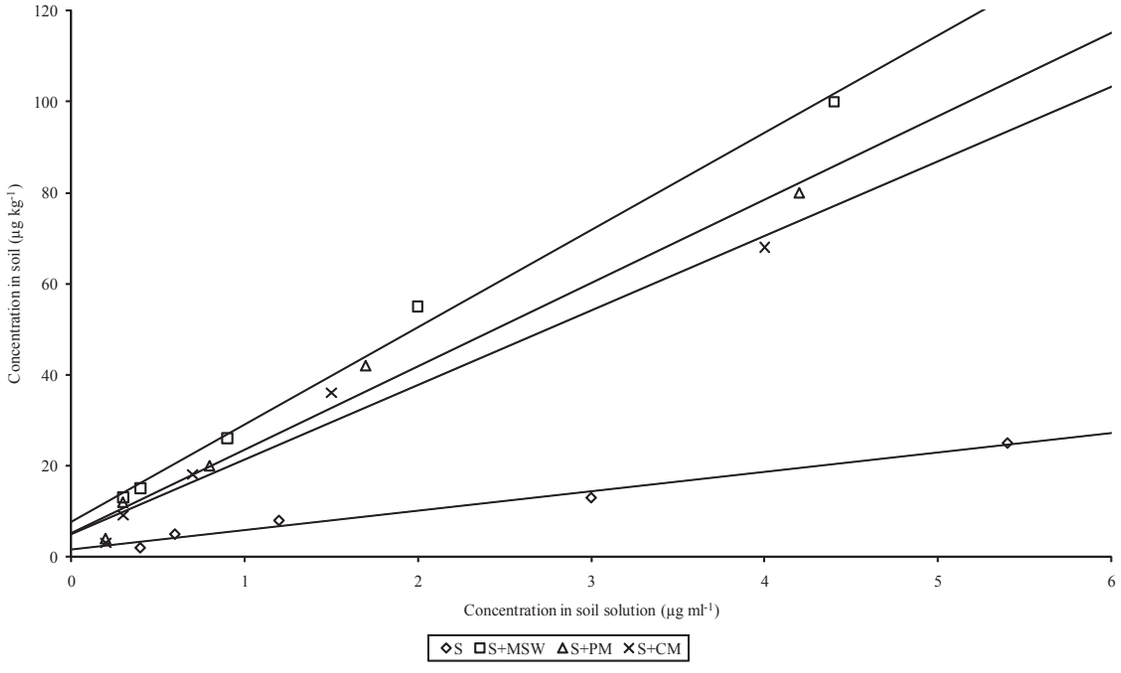

Figure 1. Terbuthylazine sorption isotherms in non-amended and organic amended soils. Symbols are experimental data points, whereas lines are the Freundlich-fit sorption isotherms.

Sorption isotherms were fit to the Freundlich equation and sorption coefficients Kf and 1/nf were calculated (Table 3). The results indicated that Kf values significantly increased in organic amended soils than for non-organic amended soils. However, terbuthylazine sorption increased by a factor of 4.5 upon amendment with MSW, whereas for PM and CM, the factor increased 4 and 3.4, respectively. Again, the results indicate significant differences between S

+MSW and S+CM treatments. Also, the 1/nf coefficients significantly decreased in organic amended soils than for non-organic amended soil. For organic amended soils, the 1/nf coefficient was higher in the soil amended with MSW, followed by PM and CM, respectively.

	Kf	1/nf	R^2
S	6.93a ± 0.95	0.92a ± 0.05	0.963
S+MSW	31.22c ± 2.46	0.80c ± 0.06	0.943
S+PM	28.03bc ± 2.03	0.83bc ± 0.05	0.958
S+CM	23.48b ± 1.99	0.86b ± 0.04	0.977

Table 3. Freundlich sorption coefficients Kf and 1/nf and standard error for terbuthylazine in non-amended and organic amended soils. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

3.2. Effect of terbuthylazine on weight on earthworms

In treated and non-organic amended soils, the *Eisenia fetida* weight decreased during the experimental period and when increased the terbuthylazine concentration in soil (Table 4). At the end of the experiment, the worm weight decreased 23.6%, 31.4% and 39.1% in soil treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively. For *Lumbricus terrestris*, the weight decreased 25.6%, 33.6% and 41.8% in soil treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively.

The application of organic matter to non-treated soil increased the worm weight. However, this increased depended of the organic matter type. At the end of the experiment, *Eisenia fetida* weight increased 20.3%, 15.1% and 11.3% in MSW, PM and CM-amended soils, compared to control soil, whereas *Lumbricus. terrestris* weight increased 18.6%, 13.9% and 10.1% in MSW, PM and CM-amended soils, compared to control soil. However, the ANOVA analysis indicated no significant differences between these treatments.

In treated and organic amended soils, the both worms weight increased respect to the treated and non-organic amended soils. This increase was higher for MSW, followed by PM and CM-amended soils.

The non-treated and organic amended soils have the highest mean cocoon numbers (Table 5). For both worms, the cocoon numbers were highest in MSW followed by PM and CM-amended soils. Terbuthylazine treatments in organic amended soils decreased the cocoon numbers. However, this decrease was lowest in MSW followed by PM and CM-amended soils.

The average weight per cocoon was also higher in non-polluted and organic amended soils, compared to control soil. Again, the average weight per cocoon in terbuthylazine treated and organic amended soils were highest in MSW followed by PM and CM-amended soils.

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	Eisenia fetida				*Lumbricus terrestris*			
	Incubation days				**Incubation days**			
	3	**15**	**45**	**90**	**3**	**15**	**45**	**90**
C1	208ab ± 12	211b ± 14	211b ± 9	212b ± 10	192ab ± 5	190ab ± 8	195ab ± 7	199ab ± 10
C2	206ab ± 15	190a ± 20	173a ± 15	162a ± 13	188ab ± 10	172a ± 10	160a ± 8	148a ± 14
C3	201ab ± 17	180a ± 11	164a ± 16	145a ± 18	180a ± 11	163a ± 15	150a ± 10	132a ± 13
C4	197ab ± 19	164a ± 23	140a ± 12	129a ± 16	170a ± 13	156a ± 11	134a ± 8	116a ± 12
MSW1	216b ± 13	233b ± 16	244b ± 19	255b ± 14	203ab ± 11	215b ± 15	222b ± 12	236b ± 17
MSW2	215b ± 12	217b ± 18	213b ± 18	220b ± 17	198ab ± 13	195ab ± 12	197ab ± 10	201ab ± 13
MSW3	214b ± 10	215b ± 13	209ab ± 10	205ab ± 11	194ab ± 10	190ab ± 11	186ab ± 13	182a ± 14
MSW4	213b ± 13	211b ± 10	204ab ± 13	197ab ± 13	190ab ± 14	188ab ± 10	182a ± 11	179a ± 12
PM1	215b ± 14	229b ± 17	233b ± 12	244b ± 11	198ab ± 12	206b ± 15	219b ± 12	227b ± 14
PM2	214b ± 12	212b ± 13	209ab ± 18	207ab ± 18	195ab ± 10	194ab ± 12	194ab ± 8	192a ± 11
PM3	213b ± 11	210ab ± 15	199ab ± 13	189a ± 15	193ab ± 11	188ab ± 15	180a ± 16	172a ± 15
PM4	212b ± 15	205ab ± 12	190a ± 11	180a ± 11	190ab ± 12	181a ± 10	175ab ± 12	168a ± 13
CM1	213b ± 17	220b ± 15	225b ± 14	236b ± 18	196ab ± 11	202ab ± 13	210b ± 10	219b ± 12
CM2	221b ± 15	201ab ± 10	195ab ± 15	193ab ± 14	193ab ± 10	193ab ± 9	180a ± 12	186ab ± 11
CM3	210ab ± 11	195ab ± 12	180a ± 10	174a ± 11	190ab ± 8	185ab ± 10	170a ± 13	162a ± 13
CM4	209ab ± 13	190a ± 17	173a ± 12	165a ± 13	188ab ± 6	179a ± 11	164a ± 11	157a ± 14

Table 4. Changes in weight (mean ± standard error) (mg) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

	Eisenia fetida			*Lumbricus terrestris*		
	Cocoon numbers	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**	**Cocoon numbers**	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**
C1	2.93ab ± 0.35	8.76b ± 0.47	3.09b ± 0.36	2.63b ± 0.22	8.15b ± 0.31	2.81ab ± 0.17
C2	2.05a ± 0.22	6.64ab ± 0.24	2.49ab ± 0.17	1.78a ± 0.18	6.06ab ± 0.23	2.21a ± 0.14
C3	1.74a ± 0.15	5.68a ± 0.31	2.34a ± 0.21	1.54a ± 0.24	5.20a ± 0.16	2.13a ± 0.10
C4	1.18a ± 0.17	4.88a ± 0.25	1.98a ± 0.13	1.03a ± 0.10	4.33a ± 0.13	1.77a ± 0.15
MSW1	3.42b ± 0.22	8.95b ± 0.39	3.37b ± 0.28	3.03b ± 0.34	8.31b ± 0.24	3.03b ± 0.17
MSW2	2.90ab ± 0.17	7.50b ± 0.25	2.94b ± 0.17	2.51b ± 0.19	6.76ab ± 0.17	2.60ab ± 0.13
MSW3	2.69ab ± 0.13	7.12ab ± 0.20	2.87b ± 0.13	2.33b ± 0.15	6.43ab ± 0.22	2.54ab ± 0.19
MSW4	2.37ab ± 0.13	6.97ab ± 0.22	2.55ab ± 0.11	2.06ab ± 0.17	6.24ab ± 0.25	2.22a ± 0.16

	Eisenia fetida			*Lumbricus terrestris*		
	Cocoon numbers	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**	**Cocoon numbers**	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**
PM1	3.29b ± 0.25	8.91b ± 0.45	3.29b ± 0.22	2.92b ± 0.19	8.27b ± 0.35	2.96b ± 0.19
PM2	2.68ab ± 0.20	7.39b ± 0.18	2.82b ± 0.16	2.34b ± 0.22	6.67ab ± 0.24	2.46ab ± 0.23
PM3	2.41ab ± 0.18	6.82ab ± 0.24	2.73ab ± 0.19	2.19ab ± 0.15	6.28ab ± 0.26	2.41ab ± 0.18
PM4	2.10a ± 0.15	6.38ab ± 0.21	2.39a ± 0.23	1.91ab ± 0.11	6.10ab ± 0.28	2.12a ± 0.15
CM1	3.17b ± 0.19	8.83b ± 0.53	3.21b ± 0.17	2.83b ± 0.14	8.21b ± 0.39	2.89a ± 0.29
CM2	2.47ab ± 0.10	7.09ab ± 0.21	2.68ab ± 0.19	2.16ab ± 0.12	6.35ab ± 0.22	2.38ab ± 0.21
CM3	2.19a ± 0.11	6.42ab ± 0.15	2.58ab ± 0.13	1.88ab ± 0.15	5.78a ± 0.21	2.28ab ± 0.12
CM4	1.90a ± 0.13	5.95a ± 0.13	2.23a ± 0.15	1.66a ± 0.17	5.35a ± 0.18	1.98a ± 0.16

Table 5. Cocoon production, average weight of cocoons (mg) and number of juveniles per coccon (mean ± standard error) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

The number of juveniles per cocoon decreased when terbuthylazine concentration increased. This decrease was higher for *Lumbricus terrestris* than for *Eisenia fetida*. The application of organic matter in terbuthylazine treated soils increased this parameter. Again, this increase was higher MSW followed by PM and CM-amended soils.

3.3. Biochemical assay

At the end of the experiment and for 50 μg terbuthylazine g^{-1} soil treatment, the cellulase activity of *Eisenia fetida* and *Lumbricus terrestris* worms was significantly recudec (29.4% and 31.1%) compared to the control soil (Table 6). The application of organic matter in herbicide treated soil increased the cellulase activity. At the end of the incubation day and for the higher concentration of terbuthylazine, the *Eisenia fetida* cellulase activity decreased 25%, 21.4% and 19% in soils amended with CM, PM and MSW, respectively, compared to organic amended and non-treated soils. For *Lumbricus terrestris*, cellulase activity decreased 26.8%, 23.9% and 21.5% in soils amended with CM, PM and MSW, respectively, compared to organic amended and non-treated soils.

Compared to the control soil, the glutathione-S-transferase activity of *Eisenia fetida* decreased 12.3%, 19.6% and 30.9% in soils treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively, whereas for *Lumbricus terrestris* the glutathione-S-transferase activity decreased 14%, 21% and 32.4%, respectively (Table 7). At the end of the experiment, the glutathione-S-transferase activity of both worms had higher increase in MSW, PM and CM-amended soils, respectively, compared to control soil. The application of organic matter in herbicide treated soils increased the glutathione-S-transferase activity. Again, this increase was higher in MSW followed by PM and CM-amended soils.

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4. Discussion

Our results indicated that terbuthylazine induced negative effects on weight, reproductive and enzymatic activities on the both earthworms. These negative effects increased with increasing herbicide concentration and/or exposure time. These results are in accordance with Brunninger et al. [39] who studied the toxicity of terbuthylazine on the growth and reproduction of *Eisenia andrei* over a period of three generations.

The weight loss may indicate a feeding inhibition situation, with the earthworms regulating the intake of the terbuthylazine by reducing consumption rate and thus affecting their subsequent growth rate. This strategy is commonly used by earthworms to avoid poisoning with herbicides and heavy metals [40].

The decrease of earthworm cellulase and gluthatione-S-transferase activities possibly is due to a physiological adaptability to compensate for pesticide stress. To overcome the stress situation, animals require high energy, and this energy demand may have led to protein catabolism [41]. Furthermore, this decrease in protein content might be a result of mechanical lipoprotein formation, which is used to repair damaged cells, tissues, and organs [40].

	Eisenia fetida				***Lumbricus terrestris***			
	Incubation days				**Incubation days**			
	3	**15**	**45**	**90**	**3**	**15**	**45**	**90**
C1	556b ± 22	624bc ± 28	610bc ± 19	590b ± 20	527b ± 19	542b ± 15	575bc ± 18	558bc ± 13
C2	550b ± 19	543b ± 18	538b ± 17	529ab ± 22	520b ± 15	508b ± 12	496b ± 17	489b ± 17
C3	544b ± 20	529ab ± 20	510ab ± 19	491ab ± 17	511b ± 17	492b ± 18	470ab ± 14	450ab ± 15
C4	530ab ± 17	498ab ± 15	456a ± 21	416a ± 13	510b ± 11	479b ± 13	425a ± 18	384a ± 12
MSW1	563b ± 26	679c ± 25	710c ± 19	748c ± 22	539b ± 10	568bc ± 18	602c ± 20	687c ± 18
MSW2	558b ± 19	652c ± 21	684c ± 17	703c ± 15	520b ± 15	547b ± 14	589bc ± 13	595bc ± 21
MSW3	549b ± 14	624bc ± 18	643bc ± 22	663c ± 19	511b ± 13	530b ± 11	559b ± 13	539b ± 19
MSW4	536b ± 17	560b ± 24	582b ± 26	606bc ± 20	505b ± 12	520b ± 15	528b ± 10	676c ± 17
PM1	570b ± 24	668b ± 26	700c ± 15	738c ± 21	528b ± 20	560b ± 17	608c ± 15	616c ± 18
PM2	552b ± 23	601bc ± 17	643bc ± 19	683c ± 18	515b ± 18	547b ± 19	590bc ± 22	568bc ± 13
PM3	540b ± 19	590b ± 15	628bc ± 22	642bc ± 16	504b ± 14	530b ± 12	546b ± 19	568bc ± 13
PM4	529ab ± 22	541b ± 20	561b ± 20	580b ± 13	493b ± 17	519b ± 17	522b ± 15	514b ± 16
CM1	575b ± 19	647c ± 24	680c ± 17	700c ± 22	524b ± 13	555b ± 15	586bc ± 15	650c ± 19
CM2	539b ± 21	565b ± 17	596b ± 23	642bc ± 17	510b ± 21	532b ± 12	558b ± 19	588bc ± 15
CM3	520ab ± 18	540b ± 18	573b ± 22	525ab ± 13	498b ± 19	517b ± 11	524b ± 18	483b ± 13
CM4	500ab ± 19	509ab ± 21	516ab ± 20	525ab ± 13	480b ± 18	496b ± 15	482b ± 19	476ab ± 11

Table 6. Cellulase activity (mean± standard error) (mg glucose mg protein hour^{-1}) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

	Eisenia fetida				*Lumbricus terrestris*			
	Incubation days				**Incubation days**			
	3	**15**	**45**	**90**	**3**	**15**	**45**	**90**
C1	118ab ± 10	116ab ± 11	121ab ± 10	120ab ± 12	110ab ± 9	113ab ± 11	117ab ± 10	115ab ± 11
C2	116ab ± 9	111ab ± 13	108a ± 9	105a ± 10	110ab ± 7	106a ± 10	104a ± 11	98.9a ± 6.2
C3	113ab ± 8	108a ± 12	103a ± 11	96a ± 8	108a ± 10	104a ± 11	95.3a ± 8.6	90.9a ± 5.8
C4	110a ± 11	102a ± 9	90a ± 10	82.9a ± 6.9a	104a ± 6	99.6a ± 7.8	82.4a ± 7.2	77.7a ± 4.9
MSW1	130ab ± 12	140ab ± 14	152b ± 15	161b ± 13	123ab ± 13	131b ± 12	142b ± 10	154b ± 10
MSW2	127ab ± 10	134b ± 8	140b ± 10	148b ± 16	118ab ± 12	127b ± 11	135b ± 9	140b ± 12
MSW3	122ab ± 11	130ab ± 10	135b ± 13	141b ± 15	115ab ± 11	120ab ± 13	126b ± 11	132b ± 11
MSW4	118ab ± 12	121ab ± 11	123ab ± 11	126ab ± 11	109a ± 7	112ab ± 10	115ab ± 12	117ab ± 10
PM1	128ab ± 13	136b ± 17	147b ± 10	156b ± 11	120ab ± 10	129ab ± 11	140b ± 11	148b ± 11
PM2	124ab ± 10	130ab ± 12	135b ± 12	142b ± 13	117ab ± 10	124ab ± 12	130b ± 10	134b ± 12
PM3	120ab ± 8	125ab ± 9	129ab ± 11	134b ± 15	112ab ± 9	118ab ± 11	120ab ± 11	124ab ± 10
PM4	115ab ± 9	119ab ± 10	121ab ± 9	120ab ± 11	107a ± 9	111ab ± 9	107ab ± 9	109ab ± 9
CM1	124ab ± 12	130ab ± 15	140b ± 12	150b ± 10	117ab ± 10	125b ± 10	136b ± 12	142b ± 13
CM2	123ab ± 10	128ab ± 11	133b ± 10	135b ± 12	113ab ± 11	116ab ± 8	123ab ± 9	127b ± 11
CM3	118ab ± 11	122ab ± 10	123ab ± 12	125ab ± 13	106a ± 8	113ab ± 7	115ab ± 9	118ab ± 10
CM4	111ab ± 9	112ab ± 10	113ab ± 11	113ab ± 10	100a ± 9	104a ± 9	108a ± 8	106a ± 9

Table 7. Glutathione-S-transferase activity (mean ± standard error) (nmol mg protein min^{-1}) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

However, these negative effects were higher in *Lumbricus terrestris* than for *Eisenia fetida*. Therefore, the sensitivity of each worm is different to the terbuthylazine herbicide. According to Ma and Bodt [23] this can be due to some physiological property of the worms or to factors governing the exposure to herbicide. Studies on the effect of benomyl and carbofuran on earthworms have similarly shown that the toxicity is much greater to *Lumbricus terrestris* than for *Eisenia fetida* when tested under standardized conditions in soil substrates [42].

The addition of organic matter to the herbicide treated soil increased the earthworm weight, reproductive and enzymatic activities probably due to the sorption of terbuthylazine with the organic matter. These results are in agreement with Dolaptsoglou et al. [43] and Cabrera et al. [30, 32], who found a decrease of terbuthylazine in the soil solution after the addition of organic matter to soil due to the herbicide sorption.

The terbuthylazine sorption isotherms and Freundlich sorption coefficients obtained in this study, suggested that organic matter play a fundamental role in the sorption of the herbicide

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in agricultural soils, probably as a result of the humic substances containing several major functional groups, such as carboxyl, phenolic, alcohol and carbonyl [44, 45]. However, our results also suggested that the chemical composition of the organic matter influenced in the terbuthylazine sorption.

Several studies of metal complexation with organic matter indicated that the sorption of heavy metals increased when the humic acid-fraction content increased in the organic matter, compared to the fulvic acid-fraction content, probably due to the humic acid-fraction possess a higher number of carboxylic groups than fulvic acid-fraction [12, 13].

The terbuthylazine sorption isotherms and Freundlich sorption coefficients indicated higher herbicide sorption in MSW-amended soils, followed by PM and CM. Therefore, and similar to the heavy metals complexation, the sorption of herbicide increased with the humic acid-fraction content in the organic amendment applied to the soil. The higher sorption probably caused a larger decrease of herbicide in the soil solution, and therefore, lowest availability of terbuthylazine availability for earthworms. This fact probably is the responsible of the increase in earthworm weight, reproductive and enzymatic activities.

5. Conclusions

It can be concluded that the sensitivity of earthworm to pesticides differ depending on the taxonomic species, *Lumbricus terrestris* being more sensitive than *Eisenia fetida* to terbuthylazine herbicide. The application of organic matter have a positive effect on reducing the toxic effect of terbuthylazine on both *Eisenia fetida* and *Lumbricus terrestris*, which is attributable to their capability of absorbing the pesticide decreasing its concentration in soil solution. This positive effect will depend on the organic amendment characteristics, those with higher amount or reactive humic acid being the most effective.

Author details

Manuel Tejada[1*], Marina del Toro[1], Paloma Osta[1], Juan Parrado[2] and Isidoro Gómez[1]

*Address all correspondence to: mtmoral@us.es

1 Grupo de investigación "Edafología ambiental". Departamento de Cristalografía, Mineralogía y Química Agrícola, E.U.I.T.A. Universidad de Sevilla, Sevilla, Spain

2 Departamento de Bioquímica y Biología Molecular. Facultad de Farmacia. Universidad de Sevilla, Sevilla, Spain

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