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Toxicity of atrazine and metribuzin herbicides on earthworms (*Aporrectodea caliginosa*) by filter paper contact and soil mixing techniques

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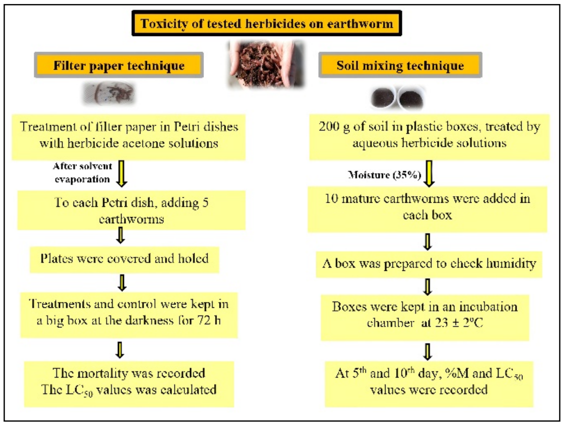
ABSTRACT

Herbicides used on a regular basis could endanger non-target species like earthworms. The aim of this work was to test the toxic effect of atrazine and metribuzin on *Aporrectodea caliginosa* by filter paper contact and soil mixing techniques. Atrazine had the highest intrinsic toxicity to earthworms, with LC_{50} of 0.026 µg mL^{-1} after 72 hr of treatment. While the LC_{50} of metribuzin was 0.063 µg mL^{-1} after 72 hr by filter paper contact test. LC_{50} was reduced from 11.121 to 3.118 and 164.824 to 19.113 µg g $soil^{-1}$ in clay soil, from 32.221 to 17.33 and 324.141 to 41.028 µg g $soil^{-1}$ in soil (1:1) and from 41.234 to 30.804 and 462.255 to 70.902 µg g $soil^{-1}$ in sandy clay loam soil of atrazine and metribuzin after 5 and 10 day. Generally, atrazine is more toxic than metribuzin in both tests.



Graphical Abstract

1. Introduction

Over the last few decades, modern farming techniques and fast urbanisation have resulted in massive soil and water pollution, with severe harmful effects on persons and ecosystems. Soil pollution has been accelerated by the extensive and

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ineffective use of pesticides that exceed the soil's potential to self-purify. Herbicides had the highest ratio (40%) across diverse pesticide categories, followed by insecticides (18%) and fungicides (10%).[1]

Atrazine (CAS No. 1912-24-9) is a systemic herbicide that is taken mostly through the roots, but also through the foliage, with acropetal xylem translocation and accumulation in the apical meristems and leaves. To manage broadleaf and grassy weeds, atrazine is a commonly used herbicide that can be used before and after planting. Atrazine belongs to the triazine family of chemicals, which also contains simazine and propazine. Because of its low cost and simplicity of application, atrazine is one of the most frequently used pesticides in the world.[2] It is largely utilized in agriculture with maize, sorghum, and sugarcane being the most common crops. It is used on residential lawns and golf courses to a lesser extent, notably in the Southeast United States. Atrazine is considered a moderately persistent chemical compound in environment with a half-life ranging from days to months.[3] It has mobility in soils is greater than that of several other herbicides.[4] In most soils, biotic changes are considered a primary route for atrazine decomposition.[5] In amphibians, atrazine has caused significant hormonal disruptions and tumors in rats.[6,7] Accumulation of atrazine in agricultural soil has the potential to produce major environmental concerns as well as human health risks. Vermicomposting is an environmentally benign method of hastening atrazine biodegradation.[8]

Metribuzin (CAS No. 21087-64-9) is a systemic herbicide that is absorbed mostly through the roots, but also through the leaves, with acropetally translocation in the xylem. At 0.07-1.45 kg ha^{-1}, metribuzin is used to manage a variety of grasses and broad-leaved weeds in soyabeans, potatoes, tomatoes, sugar cane, alfalfa, asparagus, maize, and cereals. Wettable powder, emulsifiable concentrate, water dispersible granules (dry flowable), and flowable concentrate are examples of formulations. Aerial, chemigation, and ground application methods are all used to apply metribuzin. Metribuzin has been demonstrated to have a low acute toxicity in tests with laboratory animals. It is slightly harmful when taken orally or inhaled, and is classified as Toxicity Category III (the second lowest of four categories) because of this. It is classified as Toxicity Category IV because it is basically non-toxic when ingested through the skin (the lowest of four categories).[9] In the soil environment, metribuzin has a moderate persistence.[10] Microbial degradation is the primary process through which metribuzin is lost from soil.[11]

Earthworms are the representative species of soil animals with the biggest terrestrial faunal biomass, and they are an important component of soil ecosystems.[12] They also contribute to nutrient cycling, organic matter breakdown, soil porosity, and microbial activity.[13] Because earthworms are quickly influenced by soil pollutants such as metals, organic pollutants, and pesticides, they have been included in a number of studies.[14] Their propensity to absorb hazardous compounds through their skin or through ingestion of significant volumes of soil, in particular, qualifies them as bio-indicators for detecting soil contaminants and studying the toxic effects of a variety of chemicals under diverse settings.[15] To estimate the potential food chain impacts of soil pollution, it is necessary to understand the negative effects of pesticides on earthworms. A wide range of alternatives can be used for remediation of pesticide contaminated soil, such us chemical oxidation/reduction, washing with extract ants and bioremediation. Some of these techniques are effective but limited by their applicability at the macro level in the agriculture field. Application of amendments is frequently regarded as a cost-effective strategy for pesticide-polluted soil remediation. Various modifications capable of converting and immobilizing pesticides, such as rice husk, fruit peel, straw wastes, or biochar, are included in these remediation technologies.[1] The toxicity of herbicides atrazine and metribuzin on earthworms, as well as toxicity studies for these pesticides, are reviewed in this work.

2. Materials and method

2.1 Materials

2.1.1 Tested herbicides

Atrazine (6-chloro-4-*N*-ethyl-2-*N*-propan-2-yl-1,3,5-triazine-2,4-diamine). It was obtained from the Central Laboratory of Pesticides in Giza, Egypt, and was of technical grade (98 %). Solubility (20 ºC): Water 33 mg/L. Atrazine has high to slight mobility in soil. **Metribuzin (**4-amino-6-terbutyl-3-methylsulfanyl-1,2,4-triazin-5-one, Triazinone). Technical 97.0% a.i. Solubility (20 ºC): Water 1200 mg/L. Production Company: Egyptian Chemical Industries Kima, Egypt. **Fig. 1** depicts the chemical structures of various herbicides.

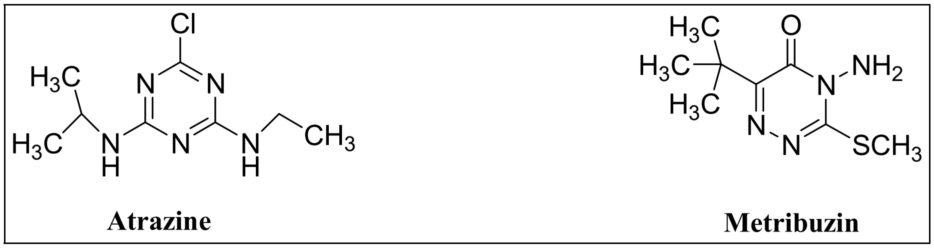


Fig. 1. Chemical structures of tested pesticides**.**

2.1.2 Tested soil

Alluvial and calcareous soils are the two most typical types of Egyptian soil. The soil samples were collected from the surface layer (0-20 cm) from different locations that have no history with the pesticides. The alluvial soil was collected from the Agricultural Research Station, Abis farm of the Faculty of Agriculture, University of Alexandria and the calcareous soil collected from the Elnahda region, Elamria, Alexandria Governorate. The physical and chemical properties were determined at the Department of Soil and Water Sciences, Faculty of Agriculture, University of Alexandria and the data are presented in **Table 1**.

Table 1. Physical and chemical properties of the tested soils.

Soil type	Particle size distribution (%)			Texture class	Water holding capacity	EC	pH	OM%	Total carbonate (%)	Cations conc. (meq/L)	Anions conc. (meq/L)
	Clay	Silt	Sand								
Alluvial	42	18	40	Clay	46	1.4	8.3	3.3	7.9	18.7	13.3
Calcareous	20	13	67	Sandy clay loam	38	5.1	8.2	1.5	44.7	60.3	50.3

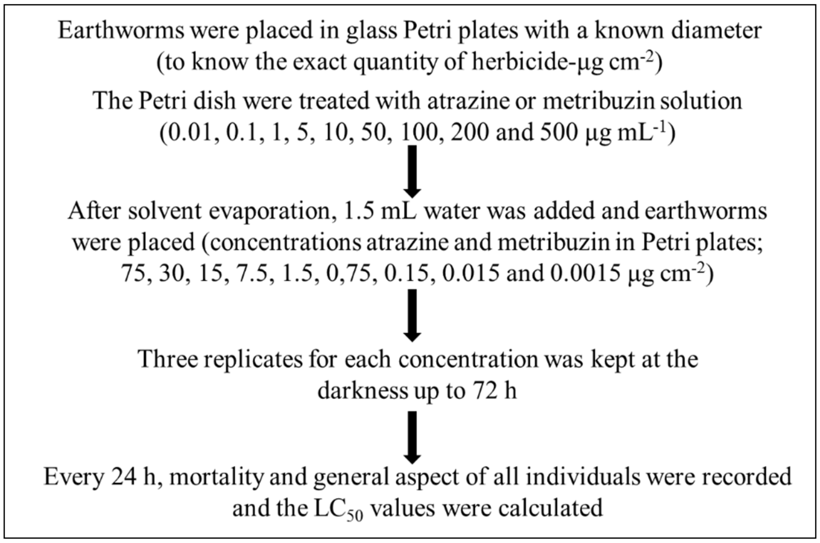
2.1.3 Earthworm

Earthworm used in this study belonged to a species commonly found in Egypt (*Aporrectodea caliginosa*). Individual worms were collected from fields around Alexandria Governorate and acclimatization was done in the laboratory for 15 days on clay soil to sandy clay loam soil in a ratio of 1:1 and the addition of 100 gm peat moss per kg of soil. Earthworms used in this study were adults. Because earthworms are hermaphrodites, there are no sexual distinctions to consider. The adults were removed from the soil 24 h before use and stored in Petri dishes on damp filter paper (in the dark at 24 ± 2˚C) to void gut contents.[16,17]

2.2 Experiments

2.2.1 Contact toxicity of tested herbicides on earthworm by filter paper contact test

The standard OECD Guidelines for contact toxicity protocol[18] was followed, glass Petri dishes and filter paper, 10 cm diameter were used.[19] The filter papers were treated with tested atrazine (Technical 98 %) and metribuzin (Technical 97 %) solutions in acetone (0.01, 0.1, 1, 5, 10, 50, 100, 200 and 500μg/ml) to obtain final concentrations of herbicides in filter paper; 75, 30, 15, 7.5, 1.5, 0,75, 0.15, 0.015 and 0.0015 μgcm^{-2}. Following the evaporation of the solvent, 1.5 mL water was added, and four earthworms were utilized as replicate. Plates were coated and pierced with a plastic film to allow for interior aeration. Three replicates of each tested concentration, as well as a control, were maintained in a large box for 72 hours in the dark at room temperature. Every 24 hours, mortality and general aspect of all individuals was recorded and the LC_{50} values were calculated by LdP line software in **Fig. 2**.[20,17]



Earthworms were placed in glass Petri plates with a known diameter (to know the exact quantity of herbicide-μg cm^{-2})

The Petri dish were treated with atrazine or metribuzin solution (0.01, 0.1, 1, 5, 10, 50, 100, 200 and 500 μg mL^{-1})

After solvent evaporation, 1.5 mL water was added and earthworms were placed (concentrations atrazine and metribuzin in Petri plates; 75, 30, 15, 7.5, 1.5, 0,75, 0.15, 0.015 and 0.0015 μg cm^{-2})

Three replicates for each concentration was kept at the darkness up to 72 h

Every 24 h, mortality and general aspect of all individuals were recorded and the LC_{50} values were calculated

Fig. 2. Schematic diagram of LC_{50} assessment of atrazine and metribuzinon earthworm by residual film technique.

2.2.2 Toxicity of tested formulated atrazine and metribuzin on earthworm by soil mixing test

In the soil mixing bioassay, the earthworms were adapted in the laboratory using soil for 15 days. Using the tested clay soil, sandy clay loams soil and clay soil: sandy clay loams soil (1:1), the boxes were treated with aqueous solutions of herbicides, atrazine (80% WP) and metribuzin (70% WP) to obtain 1000, 500, 200, 100, 10 and 1 μg g soil^{-1}. Mature individuals weighing between 0.7 and 0.8 g were selected. Four prewashed and ventilated mature earthworms were then inserted into each box (three duplicates for each concentration), covered with Parafilm and pierced for aeration, and housed in an incubation chamber at 23 2oC with a 12:12 photoperiod. The control was prepared in a similar way except that only water was added to the soil. During the assessment, lost moisture was replenished on a lost weight basis, and the lost weight was replaced with distilled water. The percentage of mortality was monitored after 5 and 10 days (**Fig. 3**). Also, the LC_{50}value of herbicides was calculated by LdP line software.[21,17]

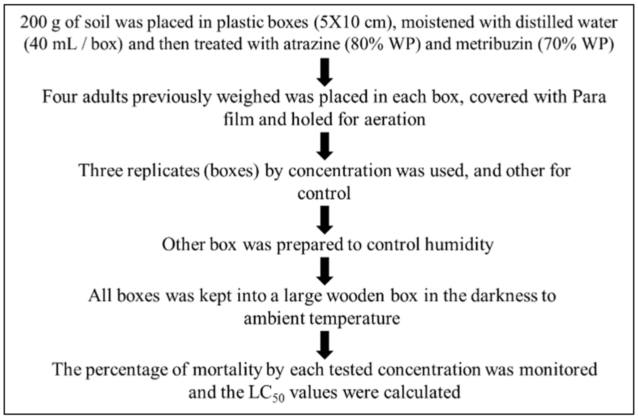


Fig. 3. Schematic diagram of LC_{50} assessment of tested atrazine and metribuzin on earthworm by soil mixing technique.

2.3 Statistical analysis

Experimental data presented as LC_{50} statistical analysis was performed by the Ldp line software.[22] A probit analysis developed before[23] was employed to assess the acute toxicity of atrazine and metribuzin to earthworm.

3. Results

3.1 Toxicity of tested herbicides on earthworm by filter paper contact test

The results of acute toxicities of two tested herbicides (atrazine and metribuzin) by filter paper contact test demonstrated that widely varied in their contact toxicities to *Aporrectodea caliginosa* shown in Table 2. Obviously, the LC_{50} declined between 24, 48 and 72 hours in two herbicides.Atrazine showed the highest intrinsic toxicity to the worms with an LC_{50} value of 0.026 (0.079-0.007) µg a.i mL^{-1} at 72-hours compared to LC_{50} at 48 h was 0.23 (0.543-0.066) µg a.i mL^{-1} and LC_{50} at 24 hours was 21.78 (26.568-17.846) µg a.i mL^{-1}. While metribuzin showed the highest intrinsic toxicity to the worms with an LC_{50} value of 0.063 (0.162-0.023) µg a.i mL^{-1} at 72-hours compared to LC_{50} at 48 hours was 1.693 (4.479-0.507) µg a.i mL^{-1} and LC_{50} at 24 hours was 65.62 (97.957-43.833) µg a.i mL^{-1}.The results demonstrated that an increase in exposure time was a factor that increased the mortality in the filter paper test media. The toxicity of herbicides was ranked as atrazine>metribuzin (**Table 2**) and the ranking did not change between the time of measurement.

Table 2. Toxicity indices and their parameters for atrazine and metribuzin on earthworm (*Aporrectodea caliginosa*) by filter paper contact test.

Time (h)	**24**	**48**	**72**
Herbicide		**Atrazine**	
LC_5 (µg/mL)	11.031	0.005	0.0008
Upper/Lpower	16.585-7.292	0.000-0.022	0.009-0.000003
LC_{50} (µg/mL)	21.780	0.230	0.026
Upper/Lpower	26.568-17.846	0.543-0.066	0.079-0.007
LC_{95} (µg/mL)	43.007	10.260	0.910
Upper/Lpower	61.555-30.190	114.106-2.749	8.688-0.167
Slope	5.567±1.878	0.975±0.042	1.060±0.092
χ^2	3.050	1.625	2.467
P	0.220	0.797	0.292
Herbicide		**Metribuzin**	
LC_5 (µg/mL)	15.122	0.030	0.003
Upper/Lpower	38.429-5.799	0.162-0.0004	0.019-0.0003
LC_{50} (µg/mL)	65.620	1.693	0.063
Upper/Lpower	97.957-43.833	4.479-0.507	0.162-0.023
LC_{95} (µg/mL)	284.684	131.345	1.420
Upper/Lpower	635.893-130.120	4193.509-24.745	14.361-0.248
Slope	2.581±0.476	0.871±0.048	1.213±0.118
χ^2	6.780	6.440	3.165
P	0.030	0.523	0.419

3.2 Toxicity of tested atrazine and metribuzinon earthworm by soil mixing test

The toxicity of atrazine (80% WP) and metribuzin (70% WP) on earthworm by soil mixing technique expressed as LC$_{50}$ was increased when the exposure time was increased. Regarding to atrazine, the LC$_{50}$ was reduced from 11.121 (28.757-3.960) to 3.118 (9.757-0.736) µg g soil^{-1} in clay soil, from 32.221 (77.098-12.834) to 17.33 (43.727-6.471) µg g soil^{-1} in clay soil : sandy clay loam soil (1:1) and from 41.234 (105.425-15.306) to 30.804 (70.352-13.072) µg g soil^{-1} in sandy clay loam soil at 5 and 10 days after treatment, respectively (Table 3). Also, the LC$_{50}$ of metribuzin in clay soil was decreased from 164.824 (404.127-66.793) to 19.113 (66.143-4.879) µg g soil^{-1}, from 324.141 (1038.847-104.837) to 41.028 (126.294-12.756) µg g soil^{-1} in clay soil : sandy clay loam soil (1:1) and from 462.255 (1637.472-138.207) to 70.902 (184.040-26.922) µg g soil^{-1} sandy clay loam soil at 5 and 10 days, respectively (Table 4). The lower the LC$_{50}$ value the more toxic the chemical. The toxicity of tested herbicides was greater (lower LC$_{50}$) in clay soil at both time intervals than that in clay soil : sandy clay loam soil (1:1) and sandy clay loam soil. Ranking of toxicity in soil types for two herbicides was clay soil > clay soil : sandy clay loam soil (1:1) > sandy clay loam soil. In general, atrazine is more toxic than metribuzine in all three types of soil (**Tables 3** and **4**).

Table 3. Toxicity indeces and their parameters for herbicide atrazine earthworm (*Aporrectodea caliginosa*) by soil mixing test.

Soil Types	Clay Soil	Clay soil : Sandy Clay Loam Soil (1:1)	Sandy Clay Loam Soil
Time (day)		**5th day**	
LC$_5$	0.315	1.968	1.629
Upper/Lpower	1.944-0.030	12.427-0.248	13.838-0.148
LC$_{50}$	11.121	32.221	41.234
Upper/Lpower	28.757-3.960	77.098-12.834	105.425-15.306
LC$_{95}$	393.290	527.539	1043.984
Upper/Lpower	2321.135-95.973	2107.056-150.981	5183.188-245.627
Slope	1.063±0.054	1.355±0.114	1.173±0.091
Chi Square	1.616	0.481	1.227
Probability (P)	0.808	0.787	0.542
Time (day)		**10th day**	
LC$_5$	0.068	0.484	1.423
Upper/Lpower	0.779-0.002	2.868-0.054	6.557-0.242
LC$_{50}$	3.118	17.330	30.804
Upper/Lpower	9.757-0.736	43.727-6.471	70.352-13.072
LC$_{95}$	144.701	621.459	666.940
Upper/Lpower	1979.929-26.801	3456.357-151.080	2820.379-189.737
Slope	0.987±0.075	1.059±0.048	1.232±0.062
Chi Square	0.279	2.473	2.066
Probability (P)	0.871	0.702	0.737

Table 4. Toxicity indeces and their parameters for herbicide metribuzin earthworm (*Aporrectodea caliginosa*) by soil mixing test.

Soil Types	Clay Soil	Clay soil : Sandy Clay Loam Soil (1:1)	Sandy Clay Loam Soil
Time (day)		**5th day**	
LC$_5$	4.079	3.118	4.366
Upper/Lpower	38.715-0.350	60.961-0.115	80.751-0.174
LC$_{50}$	164.824	324.141	462.255
Upper/Lpower	404.127-66.793	1038.847-104.837	1637.472-138.207
LC$_{95}$	6660.848	33705.740	48947.230
Upper/Lpower	68138.830-789.971	1743927-972.069	3581842-1022.254
Slope	1.024±0.085	0.816±0.079	0.813±0.085
Chi Square	1.222	0.597	0.696
Probability (P)	0.544	0.742	0.707
Time (day)		**10th day**	
LC$_5$	0.083	0.263	1.150
Upper/Lpower	1.719-0.002	3.549-0.011	8.595-0.112
LC$_{50}$	19.113	41.028	70.902
Upper/Lpower	66.143-4.879	126.294-12.756	184.040-26.922
LC$_{95}$	4438.499	6408.593	4372.326
Upper/Lpower	94223.970-416.686	109278.1-641.310	38052.05-673.879
Slope	0.696±0.030	0.750±0.032	0.920±0.042
Chi Square	3.957	4.557	4.995
Probability (P)	0.623	0.566	0.525

4. Discussion

Laboratory tests play an essential role in the risk assessment of chemicals such as pesticides toward earthworms and are considered valuable if they predict the impacts on earthworms under field conditions.[24] The contact filter paper test is a fast, simple, and inexpensive test as a screening method for assessing relative toxicity.[22] Metribuzin had high effect on earthworms biomass at 24 and 48 hours after treatment.[25] Metribuzin showed high toxicity to earthworms in the filter paper test with LC_{50} 17.17 µg a.i cm^{-2} at 48 hours.[26]

The soil mixing test was used to evaluate the toxicity of tested herbicides on earthworm, *Aporrectodea caliginosa*. The soil mixing test is more representative of the natural earthworm environment, and the chemicals are absorbed mainly by the gut in this method.[27] Thus, the soil mixing test is more practical when the pesticide toxicities to earthworms are assessed.[22] Pesticide-based soil pollution may have a detrimental impact on the break down and decomposition processes associated with microbial activities.[1] This work confirms the importance of synthetic organic compounds as effective biological agents in different fields.[28-59]

5. Conclusion

Laboratory tests play an essential role in the risk assessment of herbicides toward earthworms in filter paper test and soil mixing test. The acute toxicities of atrazine and metribuzin by filter paper contact test demonstrated that widely varied in their contact toxicities to *Aporrectodea caliginosa*. The LC_{50} values were 21.78, 0.23 and 0.026, 0.23 µg a.i mL^{-1} for atrazine, 65.62, 1.69 and 0.063 µg a.i mL^{-1} for metribuzin at 24, 48 and 72 hours, respectively. The soil mixing test is more representative of the earthworm environment and more practical when the herbicides toxicities to earthworms are assessed. The ranking of toxicity in soil types for two herbicides was clay soil > clay soil: sandy clay loam soil (1:1) > sandy clay loam soil. The results demonstrated that an increase in exposure time was a factor that increased mortality. In general, atrazine is more toxic than metribuzin, the ranking did not change between the time of measurement and test type.

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