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AVOIDANCE BEHAVIOR OF EISENIA FOETIDA TO ACETONE, DELTAMETHRIN AND GLYPHOSATE

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SUMMARY

Earthworms are perhaps the most important soil organisms in terms of their influence on organic matter breakdown, soil structural development, and nutrient cycling. The importance of earthworms in soil ecotoxicology has greatly evolved in the last decade. Although significant progress has been made on acute and chronic test methods, little information is available on avoidance response, behavioral tests. In this paper we will present the results of a six chamber behavioral test battery spiked with acetone, deltamethrin and glyphosate. As test subjects we used one of the common red worms *Eisenia foetida*.

Keywords: earthworms; soil ecotoxicology; *Eisenia foetida*; avoidance response; six chamber battery.

INTRODUCTION

Soil is a dynamic and complex system functioning as habitat for microorganism, flora, animals and humans [1]. In soil ecotoxicology acute and chronic standardized tests have been develop using soil dwelling invertebrates, like earthworms (ISO, 1998a; ISO, 1998b, etc.). Edaphic invertebrates play a crucial role in maintaining the structure and fertility of soils, recycling nutrients, increasing aeration and drainage, and can constitute an important component of the diet of birds, reptiles or small mammals [2].

Earthworms are common test organisms in terrestrial ecotoxicology. Both acute earthworm toxicity test and reproduction test, with mortality and reproduction (cocoon production and juvenile hatching) endpoints have been standardized and well described. Other endpoints, such as behavioral changes are occasionally reported [3].

The avoidance response behavior test was carried out on using the earthworm *Eisenia foetida*, exposed to three substances: acetone; deltamethrin (insecticide); and glyphosate (herbicide).

Behavioral changes such as substrate avoidance can be also ecological relevant. The migration of earthworms and the subsequent loss of their beneficial functions in soil, can lead to a degradation of the soil qualities, and the entire ecosystem [4].

The results of an avoidance response behavior test can increase the sensitivity of an evaluation, by quickly assessing an endpoint that is not measured by any other test [5]. Nevertheless, avoidance response tests are not aimed to replace other ecotoxicological tests, being just a complementary to existing tests, aiming to improve soil contamination evaluation [6].

MATERIALS AND METHODS

TEST ORGANISMS

Earthworms are sensitive to the presence of chemicals in the soil due to the chemo receptors distributed on their body surface [7]. This characteristic associated with their locomotory abilities, renders them the chance to avoid contaminated areas, therefore making them the perfect choice in avoidance response behavioral tests.

The selected test organism for this ecotoxicological battery is the earthworm *Eisenia foetida*. Three ecological types of earthworms can be identified: litter dwelling epigeic species; mineral soil dwelling endogeic species; and deep vertical borrowing, litter-feeding anecic species [8].

Eisenia foetida is an ultra epigeic species (living almost entirely in organic matter) currently used as the standard earthworm in terrestrial ecotoxicology tests in the European Union [9]. *E. foetida* combines sensitivity, economic importance, and ecological relevance, therefore it can be seen as the selected earthworm species for routine toxicity testing [10]

All the individuals used in these tests where adults, or near the stage of adulthood, all the earthworms presenting a developed clitella.

TEST SUBSTRATE

In the present study, we used one natural soil and one artificial soil. The natural soil was collected from fresh ant/moll heaps, in an area that has not been used for agricultural or any industrial purposes; therefore the soil has not been tainted with pesticides or any other chemicals.

In general it has been widely accepted that the soils of temperate Europe are suitable for conducting ecotoxicological assays with invertebrates [11], namely avoidance tests [12]. There is a controversy about the nature of the substrate to be used in toxicological batteries, natural or artificial soil. Many authors believe that by using a natural soil, will help induce a closer similarity to the natural environment, and its factors. Some believe that for a better transparency of the test and there reproduction, artificial standard soil should be used. Opinions are divided.

The artificial soil was a common garden soil bought from a gardening appliances store. The artificial soil was not mixed with any chemicals or nutrients so it wouldn't interfere with the test.

TEST CHEMICALS

Three chemical substances where selected to be tested: acetone; deltamethrin and glyphosate.

Acetone is a manufactured chemical that is also found naturally in the environment. It is a colorless liquid with a distinct smell and taste. It evaporates easily, is flammable, and dissolves in water. It is also called dimethyl hetone, 2-propanone, and beta-ketopropane. It occurs naturally plants, trees, volcanic gases, forest fires, and as a product of the breakdown of body fat. It is present in vehicle exhaust, tobacco smoke, and landfill sites [13]. Industrial processes discharge more acetone into the environment than natural processes. In 2009, about 5.1 million tones of acetone were produced worldwide [14].

We chose this substance because of its availability and is global production and usage. The acetone (30%) was diluted with tap water. 2 ml of acetone were mixed with 18 ml of water, resulting 20 ml of acetone solution. In each of the test chambers 1, 2, 3, 4, 5 (6 witness) was introduced the equal amount of substance to the number of the chamber.

The second substance tested was the insecticide named Decis, which actives substance is deltamethrin (50 g/l), one of the most common and used insecticides today.

Deltamethrin is in the chemical class of pyrethroids. Pyrethroids are synthetic chemicals modeled after the pyrethrin components of pyrethrum. Unlike other pyrethroids deltamethrin consists of one pure compound. Other names for deltamethrin include (S)-α-cyano-3-phenoxybenzyl, and the former, rejected name decamethrin [15].

The insecticide Decis, based on delthamehrin, was also diluted. In each test chamber a solution of 5 ml water plus X drops of active substance was added. X represents the number of the test chamber and implicit the number of delthamethrin drops.

The third substance used is a common herbicide named Roundup, which has glyphosate (360g/l) as is active substance. Glyphosate, the isopropylamine salt of N-(phosphonomethyl) glycine, is a non-selective post emergence herbicide for controlling weeds in agriculture (cropped and non cropped), forestry, rights-of-way and aquatic systems. At low doses, it is used as a plant growth regulator [16].

The herbicide glyphosate was diluted in a 5:1 ratio, water to active substance. The solution was formed with 25ml water and 5 ml herbicide. The distribution of solution in the five test chambers was made the same as in the case of acetone.

TEST DESIGN

A round plastic container (30 cm in diameter, 10 cm in height) with six different chambers connected to a central chamber (6 cm in diameter) was used as a test container. (see Figure 1). All of the six chambers were connected to the central chamber with an arch (1cm wide,1 cm height).

At the start of each experiment 50 earthworms were put in the soil free central chamber. Because of their negative tactical reaction, the earthworms moved quickly into the soil filled chambers. To prevent worms from escaping, the test container was covered with a plastic lid. The lid had small halls in it to provide some oxygen.

All six chambers were field with about 400 ml of soil. The arrangement of the substances in the test chambers was alternate, having a different concentration of the studied substance in each of the chambers, except for one chamber which was used as a reference, where no chemical was added.

At the end of the exposure period of 7 days, each test compartment was isolated from the main chamber to prevent further movement of worms amongst the compartments. The location of worms in the test chambers was determined by removing the soil from each compartment and recording the number of worms present, and their condition (alive or immobilized/dead).

We must mention that in the first test with acetone, the substrate was natural soil, and in the other two tests involving delthamethrin and glyphosate, artificial soil was used. It is also worth mentioning that in each of the test chambers, around 5 ml of tap water was added to raze the humidity of the soil.

The battery was kept in a dark closed place at room temperature, except for the series in which the herbicide Roundup was tested.

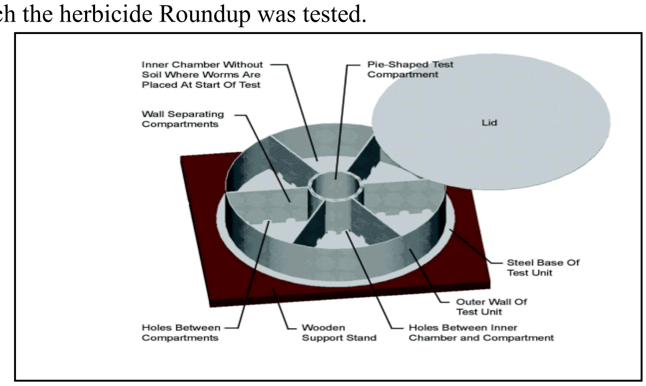

Figure 1. Representation of the six chamber avoidance response behavioral test battery.

DATA ASSESSMENT

Results in percent (%) substrate preference were shown to give an impression of the effect level compared to the uncontamined control substrate. For each replicate the net response (NR) (expressed as percentage) was calculated as NR =((C -T)/50)*100, where C = sum of earthworms observed in control soil, T = sum of earthworms observed in treated Soil, 50 = total number of earthworms. A positive (+) net response indicates avoidance and a negative net response (-) indicates an attraction to the chemical tested in that given concentration. According to Annex E of the ISO guideline17512 (2006), describing the testing of chemicals, attraction reactions (= the worms prefer the soil treated with the test chemical) have to be considered as 0% of avoidance [17].

RESULTS

ACETONE

The test battery in which the soil was spiked with different concentrations of acetone revealed that the earthworms were attracted to the chambers with relative high concentrations of acetone. This can be seen from the table below:

Table I. Data results from the test battery with acetone.

Chamber Number	Introduced Substance (ml)	Live subjects	Immobilized subjects	Total number of subjects	Percentage (%)	Net Response (%)
1	1	4	1	5	10	10
2	2	5	1	6	12	8
3	3	6	3	9	18	2
4	4	5	5	10	20	0
5	5	4	6	10	20	0
6	0(witness)	10	0	10	20	-
Total		34(68%)	16(32%)	50	100	

Interesting, both the percentage and the NR values indicate an attraction response in chambers 3, 4, 5 which represents the chambers with the highest concentration of acetone. It was also observed the natural and normal behavior of 20% of the worms which were found inside the reference chamber.

Although this is not a mortality test it can be obviously observed that many of the dead worms were found in test chambers 3, 4, 5, in spite of there attraction to the higher concentrations of acetone in those chambers.

DELTHAMETHRIN

Just like in the acetone scenario the avoidance response test, in which the soil was spiked with delthamethrin, revealed that most of the worms were attracted to the chambers with medium and high concentrations. This can be easily observed from the table below.

Table II. Data results from the test battery with delthamethrin

Chamber Number	Introduced Substance (ml)	Live subjects	Immobilized subjects	Total number of subjects	Percentage (%)	Net Response (%)
1	5	4	0	4	8	16
2	5	5	0	5	10	14
3	5	9	2	11	22	2
4	5	8	2	10	20	4
5	5	6	3	9	18	6
6	0(witness)	11	1	12	24	-
Total		42(84%)	8(12%)	50	100	

The mortality ratio was 84% alive to 12% dead. Which although the test does not have acute mortality endpoints, it shows at least partially that the insecticide is not dangerous to *Eisenia foetida*, and by extrapolation to other earthworm species.

Again, the most avoided chamber was number one, with a net response of 16%, in spite of the fact that it had the lowest concentration of delthamethrin.

GLYPHOSATE

The test battery in which the substrate was spiked with the herbicide Roundup, in which the active substance is glyphosate, was the most revealing.

Table III. Data results from the test battery with glyphosate

Chamber Number	Introduced Substance (ml)	Live subjects	Immobilized subjects	Total number of subjects	Percentage (%)	Net Response (%)
1	1	0	3	3	6	26
2	2	0	1	1	2	30
3	3	0	0	0	0	32
4	4	0	24	24	48	-16(0)
5	5	0	6	6	3	20
6	0(witness)	0	16	16	32	-
Total		0*	50	50	100	

* The death of all the subjects was caused by heat.

At the end of the 7 days exposure time, the head count of earthworms in each individual chamber revealed that almost half (48%) of the total number of introduced subjects were found in test chamber number 4, which represents the upper medium concentration.

The net response parameter, although calculated in the case of test chamber four as -16, has to be defined as 0% avoidance, conform to Annex E of the ISO guideline17512 (2006),

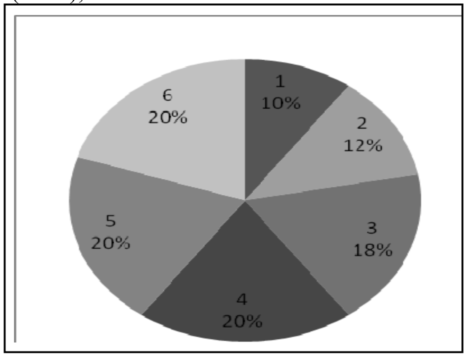

Figure 2. Representation of earthworms (%) per test chamber in the acetone test battery

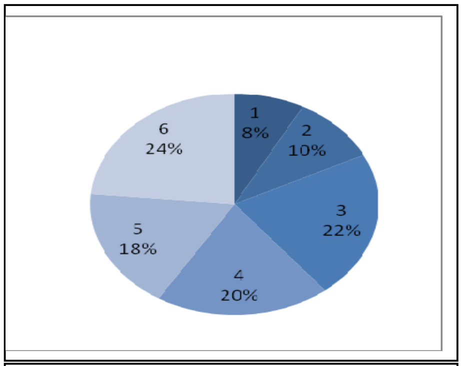

Figure 3. Representation of earthworms (%) per test chamber in the delthamethrin test battery

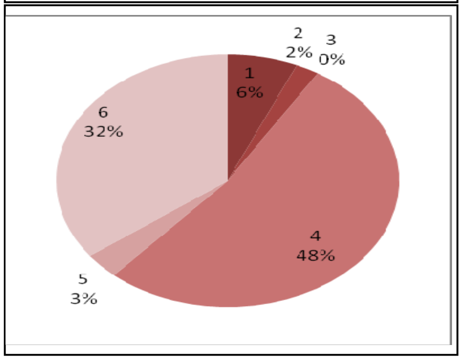

Figure 4. Representation of earthworms (%) per test chamber in the glyphosate test battery

DISCUSSION

The six chamber avoidance response tests have both benefits and drawbacks. A good example for the use of the six chamber test would be the fact that six different substances or six different concentrations of the same substance can be tested at the same time.

One of the biggest negative observation, on this type of test battery, is that after the initial orientation of the earthworms in different chambers at the beginning of the test, there movement is somewhat restricted by the soil lacking central room, that represents the only way the worms could move from a chamber to another. This is quite a paradox since is the absence of soil in the central room that starts of the experiment, by forcing the worms to leave the central chamber. Therefore it is suggested that in future experiments gateways should be made in the lateral sides of each chamber so that the earthworms movements are facilitated.

It must be mentioned that during the entire 7 days period of each of the tests, no food supply was added in the test container. Also during the acetone test, at the middle of the experiment, the substrate was spiked again with the same quantity of acetone solution as in the beginning of the test. This was done because of the acetone well known volatilization property.

The death of all the subjects in the third experiment needs to be clarified. This was not the effect of the tested chemical, but, it can be blamed on dehydration caused by excessive temperature during the last days of the experiment. None the less this does not mean that the results are invalid. We need to remember that mortality is not an endpoint in a behavior test. So we presume that by the time that the worms died, they had already choose there preferred chamber.

By comparing a behavioral with an acute test, it becomes clear that the endpoint mortality requires the highest effect concentration. This endpoint indicates maximum damage upon on organism, and consequently a high concentration of the pollutant is necessary to cause the effect [18]. Therefore acute toxicity testing provides relatively little information on actual effects on a population in the natural environment. Endpoints of chronic (reproduction) or behavioral (substrate avoidance) tests are thus more likely to detect an adverse effect in response to even a lower exposure concentration.

The obvious attraction to the medium and high concentration chambers, displayed by the worms in all three scenarios may be explain by the fact that in time chemical compounds have the tendency to degenerate in smaller and simpler compounds. These may, in some cases, be considered as nutrients by the test subjects, in this case earthworms.

One other hypothesis is that although the worms are attracted to the chambers with high concentrations, there may be some neurological side effects that prevent them from leaving. Not in the sense that they are physically immobilized but that they are unwilling to leave, more like a drug induce state.

CONCLUSION

Not only direct, but also indirect effects (like those expressed in behavioral endpoints) of chemicals on earthworms can have grave consequence on soil ecosystems.

Avoidance response tests can produce results that indicate lower thresholds effect concentration. This has been visible obvious, in all three experiments, the first two chambers presented a much bigger avoidance response from the earthworms than the other chambers with higher concentrations of chemicals.

Regarding to the preference substrate of the worms it is clear to see, especially in the first and second test, with acetone and delthamethrin, that in both cases in chambers 3,4 and 5, which represent the medium, upper medium and highest concentration of the mentioned substances, the percentage of worms/chamber was about 20%. Although in the third test with glyphosate the pattern mentioned above, has not occurred, still the fourth chamber presented an astonishing 48% attraction. This in net response represented 0% avoidance according with ISO guidelines.

Our findings confirmed that neglecting sub lethal endpoint behavioral responses to different concentrations of acetone, delthamethrin and glyphosate, might be underestimating the effects from contaminants on *Eisenia foetida*. Also the results in this study support the idea that of using the avoidance test either as a substitute or a screening test for acute tests.

The outcomes from avoidance behavior tests might bring rapid information for future decisions on the evaluation procedure of contaminant sites, terrestrial risk assessment and soil quality criteria studies.

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