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AVOIDANCE BEHAVIOUR TESTING OF *EISENIA ANDREI* IN BIODEGRADABLE PLASTIC ENVIRONMENT

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Abstract

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Avoidance behaviour test with the earthworms (ISO 17512-1:2008) is a rapid screening test for the evaluation of soil and the influence of pollutants and chemicals on the behaviour of earthworms. The purpose of the testing is to determine the avoidance behaviour of earthworm (in this case *Eisenia andrei* was used) which can be used as an organism for the composting and occur naturally in soil environment. The methodology was modified according to the needs of the avoidance behaviour testing of earthworms in biodegradable plastic environment. It is a biodegradable thermoplastic material Mater-Bi, which is produced from corn starch. Californian earthworm (*Eisenia andrei*) was chosen as a test organism. The two-chamber test was used in testing. 10 earthworms were used, which were exposed to a number of concentrations of the test substance, which was mixed into the compost environment. It was recorded both a positive result, avoidance reaction, as well as a negative result, non-avoidance reaction and also there was a case, where individuals prefer both substrates equally. Organisms showed no escape reaction and were fairly evenly distributed in both halves of the test vessel, it can be assessed that organisms prefer both substrates equally. In testing, the mortality was zero, none of the individuals died, at the conclusion of the test there were not found any dead individuals. Avoidance higher than 80% didn't occur; it cannot be said that the substrate is toxic or degraded.

avoidance behaviour, *Eisenia andrei*, biodegradable plastic

Avoidance behaviour test belongs to relatively new test in soil ecotoxicology, which was first introduced over 10 years ago. Avoidance can potentially indicate sub-lethal stress in a short period of time, testing is easily done in a soil matrix, and an avoidance test has the potential for specialized applications for soil testing. Avoidance behaviour proved in most cases is a more sensitive indicator of chemical contamination than acute tests. (Yeardly *et al*., 1996).

The principle of this test consists of the fact that the accurate number of adults in the same time is exposed to a control and tested natural soil or soil which has been contaminated with the test substance. However, the both soil are contained in one test vessel. Test duration may vary depending on the organism (from several hours to several days), but mostly this test lasts 48 hours. With these tests a first assessment of the toxicity of a (contaminated or spiked) soil is possible in just 48 hours. (Amorim *et al*., 2005).

Tests on the earthworms are the oldest contact tests, thus they have a sophisticated methodology and are also enshrined in the relevant legislation. Toxicity test to earthworms is listed in Commission Regulation (EC) No. 440/2008 of 30 May 2008 laying down the test methods pursuant to Regulation of the European Parliament and Council Regulation (EC) No. 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals, Part C. This regulation is based on the standard OECD 207. Eco-toxicity tests on earthworms can be carried out according to ISO standards (ISO 11268-1-3 and ISO 17512-1), OECD

(OECD 207,222) and US EPA (US EPA 850.6200) (Škarková, 2012).

Act No. 350/2011 Coll., on chemical substances and mixtures and its implementing Decree No. 222/2004 Coll., as amended, which for chemicals and chemical mixtures, laying down the basic methods for testing the physical-chemical properties, explosive properties and hazardous properties to the environment. As required eco-toxicological tests the acute test with *Eisenia foetida* is presented (including methodology), further tests of effects on soil microbial activity during the transformation of nitrogen and carbon, test of effects on aerobic and anaerobic transformation in soil, which is essentially a transfer methodology of OECD tests. Act No. 185/2001 Coll., as amended, on waste and its implementing Decree No. 376/2001 Coll., as amended, on classification of hazardous properties of waste reported as a dangerous property the eco-toxicity and as hazardous waste is assessed the waste, which the aqueous extract shows, in the acute toxicity tests according to the standards set out in paragraph 7 of Annex 3, for at least one of the test organisms at a given time of exposure tested waste. Contact waste testing is neglected, although it has for solid waste incomparably more relevant power, especially in the case of sludge and sediment, applied to agricultural soils (Hofman, 2009).

Aiming to evaluate a possible loss of soil habitat function after amendment with organic wastes, a digested sewage sludge or derived composts, where biologically tested in the laboratory using soil animals (e.g. *Eisenia andrei*). Avoidance tests did not reveal any impact of composts to soil biota (Moreira *et al*., 2008).

Laboratory tests are available in the form of standards (ISO, OECD, ASTM, EU, national legislation), or tests, which procedure has not yet been completely standardized. Recently, during the introduction of such new method, it is focused mainly on miniaturization, shortening of the tests, reducing the volume of test vessels, cheaper tests, as well as much closer to the real environment, participate as many trophic levels as possible in the test (test with multiple types of organisms), involvement of the entire battery of tests (test with microorganisms, higher plants, annelids, micro-arthropods, etc.) and the introduction of alternative eco-toxicity tests (microbiotests, tests escape behaviour).

These tests are relatively new and in the last few years it goes through their design relatively rapid development (Priessnitz, 2008).

MATERIAL AND METHODS

Avoidance behaviour test with the earthworms (ISO 17512-1:2008) is a rapid screening test for the evaluation of soil and the influence of pollutants and chemicals on the behaviour of earthworms. This test may be designed as a two-chamber or multi-chamber. The two-chamber test was used in testing. It is a quick way of testing, which reflects the bioavailability of contaminants in soils. *Eisenia foetida* and *Eisenia andrei* can be used as a test organism (Škarková, 2012).

The purpose of the testing is to determine the avoidance behaviour of earthworm (in this case *Eisenia andrei* was used) which can be used as an organism for the composting and occur naturally in soil environment. The methodology was modified according to the needs of the avoidance behaviour testing of earthworms in biodegradable plastic environment. Compost with pieces of plastic was chosen as a contaminated environment, which in this case was a contaminant in an amount of 0.1 g–4 g per 500 g of compost. Considering the simulation the real environment and test conditions as much as possible the test was modified to the environment of the compost, which was contaminated only with biodegradable plastics. The chosen biodegradable plastic sample degraded in a several weeks during the controlled composting. This test follows the series of tests with biodegradable plastics where the biodegradability was proved and represented a laboratory experiment simulating the collection of biological waste that would have been collected in biodegradable plastic bags. The test was adapted to the laboratoryand normative conditions.

Plastic as contaminant is from biodegradable plastic bags ENVIRA, which are intended for collecting kitchen and garden waste (e.g. peel, shell, leaves of vegetables and others). After filling the bag it is also possible to compost that waste including the bag or the bag inserts into the collecting container for organic waste. It is a biodegradable thermoplastic material Mater-Bi, which is produced from corn starch. Some various additives are added to the corn starch to achieve the desired technological and mechanical properties. There are individual forms of Mater-Bi; the form which contains the starch, which is in a complex with the variable amount of biodegradable polymer complexing reagents, is a representative sample of this kind of plastic.

The basic characteristics of a good test should be standardization and repeatability. It also depends on economics, therefore, the practical feasibility, cost and speed. The test should be sensitive enough. Its informative value and applicability to environmental protection is also important. The concept of ecological relevance is also presented, which means that the test should respect the ecology of the organism. Monitoring of the response should be ecologically relevant and indicate the status and function of the organism (survival, growth, reproduction, feeding and mobility) for the ecosystem. Exposure ways, bioavailability and concentrations should simulate the real conditions (Škarková, 2012).

Californian earthworm (*Eisenia andrei*) was chosen as a test organism. 10 earthworms were used, which were exposed to a number of concentrations of the test substance, which was mixed into the compost environment.

1: *Start of the earthworm test*

The vessel was divided into two halves by partition and it was filled with the appropriate substrate to a height of 5 cm–6 cm. Within one half came the test substrate to the second half the control substrate was placed. Partition was removed and into this area 10 earthworms are placed (Fig. 1).

The vessel was equipped with a lid (against escape of individuals) and it was placed in a temperature-controlled area. During the test organisms were not fed. During the testing a determined contaminant the concentration range was used to get better results. The test was performed in several repetitions for each concentration. After 48 hours, the test was terminated. Before the vessel was took out of a tempered area the partition had to be inserted back to avoid subsequent migration of the earthworms, which would be caused by changing of the temperature and light. The number of individuals was determined in both halves of the vessel. A lot of toxic substances can cause the mortality. This type of effect is beyond the range of the avoidance test, because the dead earthworms are counted as missing. So they are not counted towards the total number of earthworms in the vessel.

The test conditions represented a temperature of 20 °C ± 2 °C, substrate pH 7.78 and the illumination of normal daylight in the laboratory conditions.

RESULTS AND DISCUSSION

For each vessel separately the number of surviving individuals is counted. For each concentration the average number of individuals in testing soil is compared to the number of individuals in the control soil. If less than 20% of the total number of live individuals is placed in the testing soil at the end of the test, this soil is classified as toxic to organisms. For the validation of the test, it has to meet certain criteria, such as mortality of individuals in test has not to exceed 10% in a control soil at the end of the test (ISO 2008).

In early tests of avoidance behaviour a simple comparison of occurrence measure in figures or percentages was performed. Currently a more sophisticated method of expression is used, namely the method of net response. The net response (NR), which is expressed as a percentage, is calculated according to the following formula (Amorim *et al.*, 2005):

$$NR = \frac{(C - T)}{N} \times 100,$$

C.... observed individuals in control soil
T.... observed individuals in testing soil
N... total number of individuals.

During the test a constant temperature of 20 °C (± 2 °C) was maintained and in each test vessel 10 organisms was placed and for each concentration

there were two repetitions. First the substance of determined concentration was applied in test vessel and subsequently the earthworms were added. The test was realized according to a determined procedure, and under the exact conditions. Other authors (Loureiro *et al*., 2005) used similar tests, where a rapid avoidance behaviour test was used as a screening tool with the earthworm *Eisenia andrei*, where organisms were exposed during 48 hours to several chemicals and substances.

In the period of 48 hours the organisms can choose between the control soil and the other soil – a contaminated or spiked or another soil with different physics-chemical properties (Amorim *et al*., 2005), which was utilized in the testing experiment.

Start of the test: 17. 9. 2012
End of the test: 19. 9. 2012
Time duration of test: 48 hours
pH at the start (Sample No. 1, 2, 3, 4): 7.78
pH at the end: Sample No. 1: 8.32
Sample No. 2: 8.00
Sample No. 3: 7.96
Sample No. 4: 8.08
Temperature: 21 °C

The number of individuals in the both testing environment is calculated for the first testing (Tab. I) and is also calculated for the second testing (Tab. II).

Biostatistics, science dealing with the evaluation of biological data is a very important and quite significant part of each experiment. The statistical processing of data can examine the significance of the observed processes such as the differences between the versions, the concentration, the differences versus control, etc.

IV: *Avoidance and Non-Avoidance Reaction Calculation in first testing*

Sample No.	Plastic Sample Weight [g]	NR [%]
1	0.1	20
2	1	–40
3	3	–40
4	4	–20

V: *Avoidance and Non-Avoidance Reaction Calculation in first testing*

Sample No.	Plastic Sample Weight [g]	NR [%]
1	0.1	–20
2	1	0
3	3	–60
4	4	0

A positive result (+) indicates a avoidance and negative (–) means non-avoidance reaction (or the attractiveness of the test soil to the organism). Both reactions were noticed (Fig. 2, 3).

I: *Number of individuals in first testing*

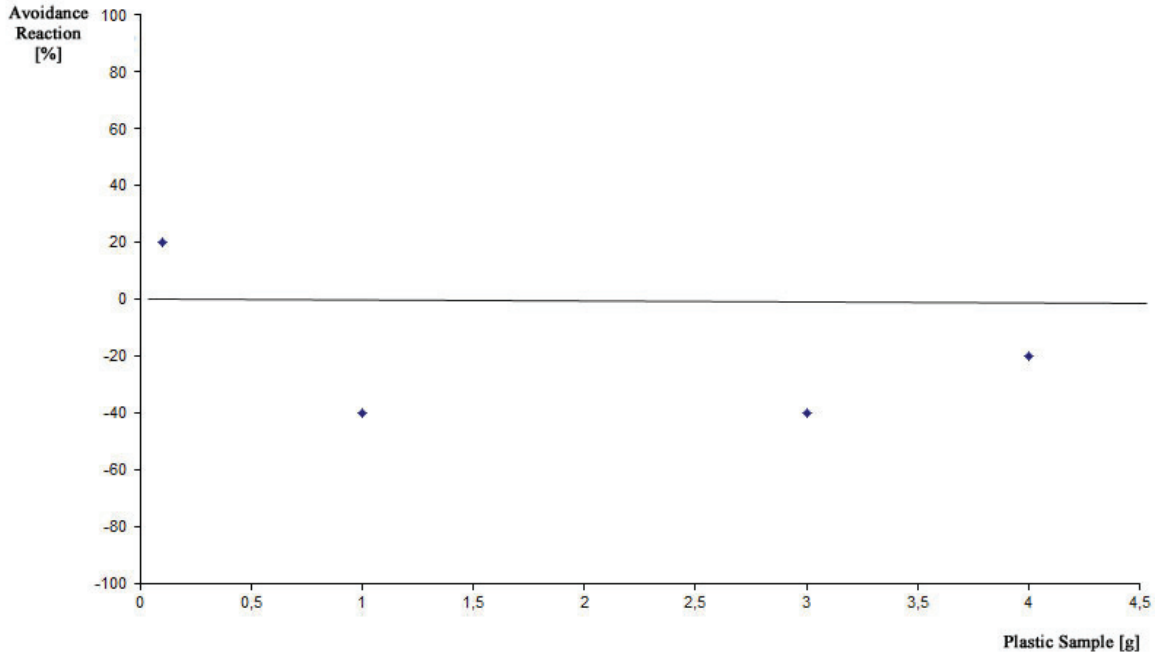
Sample No.	Plastic Sample Weight [g]	Number of earthworms in contaminated environment, number of individuals	Number of earthworms in no contaminated environment, number of individuals
1	0.1	4	6
2	1	7	3
3	3	7	3
4	4	6	4

II: *Number of individuals in second testing*

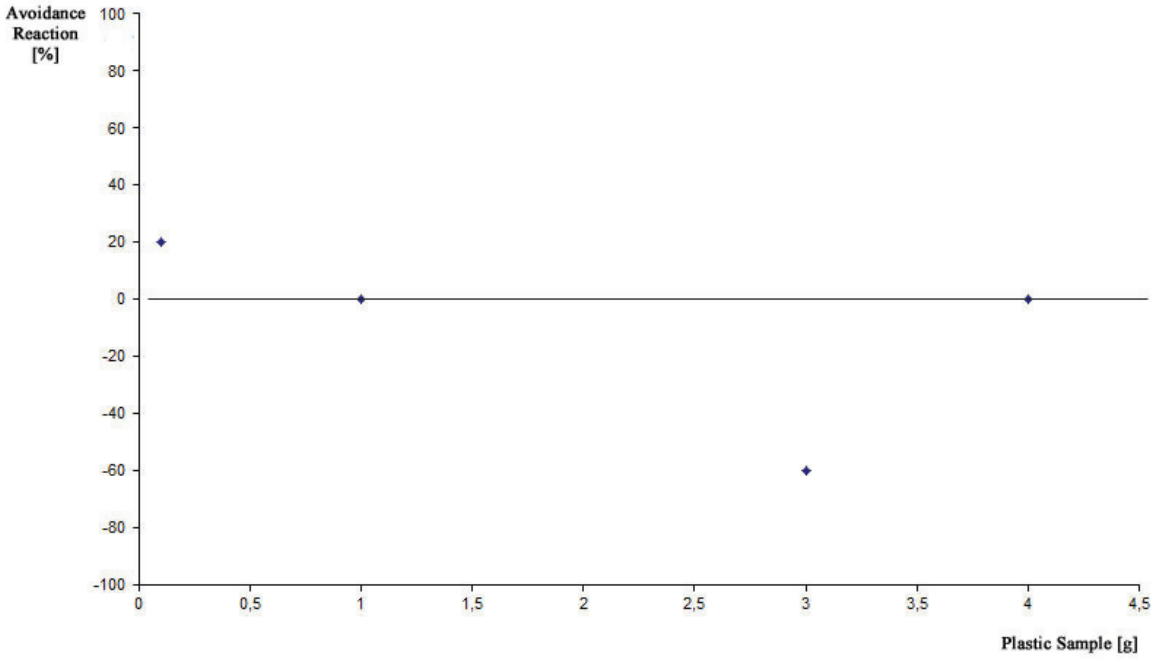
Sample No.	Plastic Sample Weight [g]	Number of earthworms in contaminated environment [Number of individuals]	Number of earthworms in no contaminated environment [Number of individuals]
1	0.1	6	4
2	1	5	5
3	3	8	2
4	4	5	5

III: *Total mortality of individuals in testing*

Sample No.	Plastic Sample Weight [g]	Mortality, number of individuals	Mortality [%]
1	0.1	0	0
2	1	0	0
3	3	0	0
4	4	0	0



2: *Avoidance Reaction – Test No. 1*



3: *Avoidance Reaction – Test No. 2*

If the result is zero, then it means that the organisms showed no escape reaction and were evenly distributed in both halves of the test, respectively they prefer both substrates equally. If avoidance is higher than 80%, it is considered a toxic environment or reduced quality environment. (Amorim *et al*., 2005; Priessnitz, 2008). In testing the avoidance higher than 80% didn't occur. In testing the mortality was zero, none of the individuals died, which is confirmed by other authors in the avoidance test in polluted soil there was no mortality after 48 hours (de Sousa, A.P.A., de Andréa, M.M., 2011).

Because of the ecosystem consequences of behavioural effects and the fact that avoidance response tests can reveal the toxic potential of pollutants in low concentrations, such tests should be included into eco-toxicological test protocols. (Schaefer, 2003).

CONCLUSION

The diversity of individual test results shows that the selected individuals of earthworms tolerate the environment with chosen biodegradable plastics. It was recorded both a positive result, avoidance reaction, as well as a negative result, non-avoidance reaction and also there was a case, where individuals prefer both substrates equally. Organisms showed no escape reaction and were fairly evenly distributed in both halves of the test vessel, it can be assessed that organisms prefer both substrates equally. In testing, the mortality was zero, none of the individuals died, at the conclusion of the test there were not found any dead individuals. Avoidance higher than 80% didn't occur; it cannot be said that the substrate is toxic or degraded. In the ecosystem can be a significant influence of foreign substances and also their influence on the behaviour of organisms. Considering the potential for these tests to detect the toxic potential of a relatively low concentration, the avoidance test can be a good tool for this type of testing.

SUMMARY

Avoidance behaviour test with the earthworms is a rapid screening test for the evaluation of soil and the influence of pollutants and chemicals on the behaviour of earthworms. The purpose of the testing is to determine the avoidance behaviour of earthworm (in this case *Eisenia andrei* was used) which can be used as an organism for the composting and occur naturally in soil environment. The methodology was modified according to the needs of the avoidance behaviour testing of earthworms in biodegradable plastic environment. Compost with pieces of plastic was chosen as a contaminated environment. Plastic as contaminant is from biodegradable plastic bags ENVIRA, which are intended for collecting kitchen and garden waste. It is a biodegradable thermoplastic material Mater-Bi, which is produced from corn starch. Earthworm *Eisenia andrei* was chosen as a test organism. 10 earthworms were used, which were exposed to a number of concentrations of the test substance, which was mixed into the compost environment. The vessel was divided into two halves by partition and it was filled with the appropriate substrate to a height of 5–6 cm. Within one half came the test substrate to the second half the control substrate was placed. Partition was removed and into this area 10 earthworms were placed. The vessel was equipped with a lid (against escape of individuals) and it was placed in a temperature-controlled area. During the test organisms were not fed. The test was performed in several repetitions for each concentration. After 48 hours, the test was terminated. Before the vessel was took out of a tempered area the partition had to be inserted back to avoid subsequent migration of the earthworms, which would be caused by changing of the temperature and light. The number of individuals was determined in both halves of the vessel. The test conditions represented a temperature of 20° C ± 2° C, substrate pH 7.78 and the illumination of normal daylight in the laboratory conditions. It was recorded both a positive result, avoidance reaction, as well as a negative result, non-avoidance reaction and also there was a case, where individuals prefer both substrates equally. Organisms showed no escape reaction and were fairly evenly distributedin both halves of the test vessel, it can be assessed that organisms prefer both substrates equally. In testing, the mortality was zero, none of the individuals died. Avoidance higher than 80% didn't occur; it cannot be said that the substrate is toxic or degraded.

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