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Soil quality — Effects of pollutants on earthworms —

Part 3: Guidance on the determination of effects in field situations

Qualité du sol — Effets des polluants vis-à-vis des vers de terre —

Partie 3: Lignes directrices relatives à la détermination des effets sur site

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 11268-3:1999), which has been technically revised.

ISO 11268 consists of the following parts, under the general title *Soil quality — Effects of pollutants on earthworms*:

— *Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei*

— *Part 2: Determination of effects on reproduction to Eisenia fetida/Eisenia andrei*

— *Part 3: Guidance on the determination of effects in field situations*

Introduction

The earthworm field test is based on a method being developed by the German Federal Biological Research Centre for Agriculture and Forestry for the testing of pesticides.[6] Later, it was internationally standardized by the International Organization for Standardization (ISO), taking into account results and recommendations of an international workshop in 1991 in Sheffield, United Kingdom, [7] "Ecotoxicology of Earthworms", as a tool for characterizing soil quality. Growing experience has shown that the practical performance of the test can be improved. In two meetings organized by the Federal Biological Research Centre for Agriculture and Forestry (Braunschweig, 2002) and by the German Federal Agency for Consumer Protection and Food Safety (Lille, 2005), an ad-hoc working group of experts from various countries and institutions proposed recommendations that should be taken into account if revision has been approved by voting in the periodical review. A report of the discussions, comments, and recommendations has been published.[8]

In cases where earthworms and other organisms are used as bioindicators to assess the soil quality of a site as a habitat for soil organisms, guidance for extraction procedures and advice for planning a survey is given in ISO 23611-1 to ISO 23611-6.

Soil quality — Effects of pollutants on earthworms —

Part 3:
Guidance on the determination of effects in field situations

1 Scope

This part of ISO 11268 specifies techniques for determining the effects of substances on earthworms in the field and provides a basis for determining the effects of chemicals applied to or incorporated into soil, including soil injections or drilled pelleted formulations.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11274, *Soil quality — Determination of the water-retention characteristic — Laboratory methods*

ISO 11277, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

ISO 23611-1, *Soil quality — Sampling of soil invertebrates — Part 1: Hand-sorting and formalin extraction of earthworms*

3 Units

Rates of application of test substances are expressed in kilograms per hectare (kg/ha) or litres per hectare (l/ha) of the substance applied. When this is a formulated material, the application rate is expressed in terms of the amount of active ingredient applied.

The concentrations of test substances incorporated in the soil are given in mg active ingredient (a.i.)/kg soil dry mass, d_m. The same units are used when comparing the results of this field test with those gained in laboratory studies.

4 Principle

Species, numbers, and biomass of earthworms collected by sampling plots treated with a test substance are compared with those collected from treated control and reference plots. Sampling is performed as specified in ISO 23611-1. The duration of the study depends on the characteristics of the test substance but is usually of one year's duration. Sampling dates are chosen to lie within the periods of activity of the earthworms.

The test is of a randomized complete block design with four replicates per treatment. Statistical analysis of numbers of each species collected at each sampling occasion is used to determine the effects of treatments by comparing abundance, biomass, and diversity between control and treated plots.

NOTE The test also generates samples of earthworms from treated plots for residue analysis where such information is appropriate.

5 Reagents and material

5.1 Formalin [formaldehyde solution, 4 % (volume fraction)].

5.2 Formalin [formaldehyde solution, 37 % (volume fraction)].

5.3 Ethanol, 70 % (volume fraction).

5.4 Carbendazim, e.g. applied as Derosal®[1)] formulation (360 g a.i./l) as reference substance.

6 Apparatus

Use standard laboratory equipment and the following.

6.1 Plastic vessel, (250 ml and 500 ml) for storing the worms.

6.2 Plastic hand gloves.

6.3 Forceps.

6.4 Piece of thick plastic (1 m^2 to 2 m^2).

6.5 Spade or shovel.

6.6 Dissecting microscope, with low magnification (10× to 40×).

6.7 Balance (0,01 g to 200 g).

6.8 Water-can, (preferably 20 l) with water (20 l per sampling plot).

6.9 Pencil, note book, water resistant marker, labels.

6.10 Thermometer, e.g. for measuring air temperature.

6.11 Drying cabinet, for soil water content determination.

7 Procedure

7.1 Sampling of earthworm populations

Sampling of earthworms is done by a combination of two different methods: hand-sorting and formalin extraction. Based on several comparative studies, this combination is clearly recommended in the various reviews on earthworm ecology (e.g. [9], [10], [13]). For details of extraction procedures, see ISO 23611-1.

Sampling should be done at times of the year where the animals are not forced by the environmental conditions (i.e. low soil water content and/or extremely high or low temperatures) into diapause (i.e. are not reacting to formalin).

Due to the individual size of the worms, a large plot shall be identified: a square of 50 cm × 50 cm is often sufficient in the Holarctic where most adult earthworms have a length approximately between

1) Derosal is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

1 cm and 20 cm. However, at places with a low density of earthworms [e.g. soils with low pH (<4,5) or which are anthropogenically used like crop sites], larger plots (i.e. 1 m^2) are recommended. On the other hand, at sites with a high earthworm density (e.g. many meadows in temperate regions), a smaller plot of 0,125 m^2 is sufficient.[12] The individual samples are taken randomly over the test plot.

NOTE 1 If in special situations, such as in Southern Europe, anecic species do not occur, sampling by hand-sorting up to a soil depth of 30 cm is sufficient. When sampling conditions are not optimal, e.g. at extremely low soil water content or dense root layer, hand-sorting can even be the only effective extraction method.

In case in a behavioural sampling method formalin alone is used, an additional check by hand-sorting is necessary and it should be performed as described in this International Standard. Hand-sorting combined with the formalin method usually yields satisfying results. If a combination of hand-sorting and an extraction method is used, no additional efficiency check is necessary.

7.2 Preservation

Preservation shall be carried out according to ISO 23611-1.

7.3 Determination of biomass

Determination of biomass shall be carried out according to ISO 23611-1.

8 Preparation for the test

8.1 Test site

8.1.1 Selection and description

In general, the test site should be as homogenous as possible to improve the statistical power of the test. Gradients in environmental conditions should be avoided (e.g. adjacent ditches), canopy influences as woodland borders, or compacted tractor tracks on the site. The site should be on level ground and should have the same cropping and soil characteristics throughout.

Grassland is the preferred study site for testing effects of substances on earthworms. In grassland, earthworm density and diversity are generally higher and more stable than on arable land, which makes it easier to detect significant effects on earthworm populations. If effects on earthworms are observed on a grassland site, a refined risk assessment should include specific scenarios (crops and regions) covering the intended use patterns of the test substance (e.g. pesticide). Orchards are not recommended for testing because of the heterogeneity of the site due to tree rows and strips without trees. If an orchard is used, it shall be ensured that the higher variability is compensated by taking more samples or restricting sampling to specific areas. A suitable grassland test area should have an earthworm density of at least 100 individuals per square metre. With lower population densities, more samples should be taken than recommended in 8.1.3.

If information on effects on bare soils is required, then arable plots may be used, provided that there are at least 60 earthworms per square metre present at the start of the test according to the results of the pre-sampling.

The experimental plots should support a mixed population of earthworms[11] which are generally representative of the type of environment selected. In agricultural areas, for example, important anecic and endogeic species should be present at a sufficiently high density (at least 10 % of the population for each group) that plots can be taken as representative. Care should be taken not to select plots where uncharacteristic species predominate.

NOTE Due to natural reasons, a certain ecological group possibly does not occur in some regions (e.g. anecic worms in parts of the Mediterranean). In such cases, expert knowledge is required in order to identify the ecologically most important species of that region.

In order to satisfy these requirements, samples should be taken from prospective plots before the start of the study for preliminary investigation of species distribution.

Extreme soil types, e.g. very sandy, clay, or moory soils, should be avoided when selecting the test site.

A description of the test site should contain the following physicochemical and biological information:

— particle-size distribution (as specified in ISO 11277);

— organic-carbon content (as specified in ISO 10694);

— pH-value (as specified in ISO 10390);

— water-holding capacity, WHC_{max} (in the A-horizon, as specified in ISO 11274);

— description of vegetation.

Determination of these characteristics should be made using standard methods.

Microclimate measurements (soil and air temperature, soil water content, rainfall quantity, sunshine duration) are particularly important for the period of chemical application and temperature, and rainfall quantity should be recorded over the year.

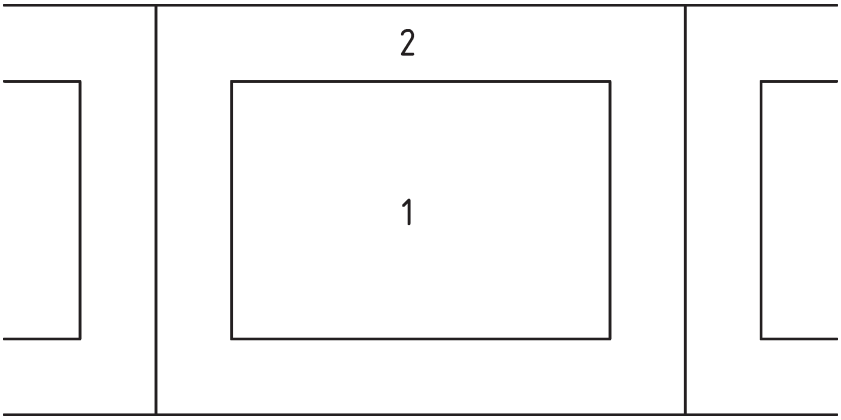
The history of the test site should be known (e.g. applications of pesticides, mineral fertilizers, sewage sludge, tillage).

8.1.2 Design of experiment

The experimental design depends on the objectives of the study and the amount and quality of information available from the study site. Usually (i.e. in the case of testing a chemical substance), a negative control (i.e. plots sprayed just with water) and a positive control (i.e. plots sprayed with a reference substance, e.g. a substance known to be toxic to earthworms) are sprayed. In general, it should be taken into consideration that a dose-response design clearly facilitates environmental risk assessment as compared to single-dose studies. In any case, the reasons for the selected test design shall be explained in the study report.

The test should be designed as a randomized complete block. The number of the treatment groups and planned sampling dates determine the number of plots and, therefore, the surface area of the field site.

However, the size of the individual study plots should be at least 100 m^2 (10 m × 10 m). The samples are taken exclusively from the central area of the plots so that around the sampling area, there is a 1 m to 2 m wide edge strip which is also treated (see Figure 1). The sampling area of the samples taken per treatment, plot (replicate), and date depends on the earthworm density and distribution of the selected experimental field (see 7.1) and can range between 1 m^2 and 0,125 m^2, but most often, 0,25 m^2 is appropriate.



Key

1 sampling area

2 edge strip

Figure 1 — Schematic view of a test plot

Samples taken on the same date should be at least 2 m apart and sampled areas should not be used for sampling at subsequent sampling dates.

The required number of random samples depends, among other things, on the density and distribution of the earthworm population over the test area.[14]

For each test variant (control, reference substance, test substance), at least four replicates should be used and four random samples taken per replicate (i.e. 16 individual samples per test variant).

On grassland, a sampling area of 0,25 m^2 per individual sample is sufficient. Use of a metal or plastic enclosure with a size of 50 cm × 50 cm (square) or a diameter of 56 cm and a height of 10 cm to 15 cm is recommended. On arable land, the sample area shall usually be increased to 1 m^2 due to low population density or non-homogeneous distribution of the worms.

On grassland, the vegetation at the sampling area should be cut carefully before sampling so that all earthworms appearing on the surface can be seen and collected.

Care should be taken that the entries of earthworm holes are not blocked and, therefore, operators should avoid walking on sampling areas.

NOTE Guidance concerning application rates of pesticides is given in Annex A.

8.1.3 Maintenance of test fields

Grassland fields should be mulched regularly (two times to six times per year) using grass clippings of a mulching lawn mower in order to keep the grass cover short. Mulching should be carried out one week to two weeks before the application of the test substance to ensure that the grass on the surface, which acts as a food source for some earthworms, has been in contact with the test material. The last mulch before application of the test substance may only remain on the field, provided that it does not create a coherent grass mat. In the case of mulching over the course of the year, the mulch should remain on the field as it serves as food for some earthworm species.

If a test is carried out on arable land, usual agricultural practice should be used. However, ploughing and other soil treatment measures should be avoided as much as possible during the experiment.

Pesticides should not be used on the test area, but if an application is unavoidable, then the chemical chosen should be non-toxic to earthworms (the selection should be based on the risk assessment for the respective compound as published in EFSA dossiers). For herbicides to be tested, arable land or grassland should be used which is treated with another herbicide (which is not the test substance),