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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

Reference number
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Contents

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 11268-3 was prepared by Technical Committee ISO/TC 190, *Soil quality,* Subcommittee SC 4, *Biological methods.*

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

— *Part 1: Determination of acute toxicity using artificial soil substrate*

— *Part 2: Determination of effects on reproduction*

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

Introduction

This document provides guidance on the evaluation of the effects of substances on earthworm species under field conditions. Due to the complex character of field tests, modifications will often be necessary depending on conditions prevailing at specific sites.

These guidelines have been drawn up taking into consideration the results and recommendations of an international workshop entitled "Ecotoxicology of Earthworms" held in Sheffield, UK, in April 1991 (see Bibliography).

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 describes 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.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 11268. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 11268 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 10390:1994, *Soil quality — Determination of pH.*

ISO 10694:1995, *Soil quality — Determination of organic and total carbon after dry combustion (elemental analysis).*

ISO 11274:1998, *Soil quality — Determination of the water-retention characteristic — Laboratory methods.*

ISO 11277:1998, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation.*

3 Units

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

4 Principle

Species and numbers of earthworms collected by sampling plots treated with a test substance are compared with those collected from treated control and reference plots. The duration of the study depends on the characteristics of the test substance, but will normally be of one year's duration. Sampling dates are chosen to lie within the periods of activity of the earthworms.

Sampling provides relative numbers of earthworms, and it is not necessary to attempt to define absolute numbers. The test is of a completely randomized block design with four replicates. Statistical analysis of numbers of each species collected at each sampling occasion is used to determine the effects of treatments.

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

5 Sampling of earthworm populations

5.1 General

Due to the need to take a large number of samples within the shortest period of time, mechanical extraction methods such as hand selection, washing out or screen flotation are generally too labour intensive. Mechanical extraction methods, however, allow samples to be taken when the worms are not active as a result of the weather.

For the purpose of the test described here, mainly formaldehyde extraction (Raw 1959) and the electrical extraction or Oktett method (Thielemann 1986, Cuendet *et al.* 1991) may be used. These extraction methods can only be used when the earthworms are active. To increase the effectiveness of the methods, they may be combined with hand selection (Lee 1985).

The individual samples are taken in the form of a random distribution over the test plot.

5.2 Formaldehyde extraction method

A solution of formaldehyde (0,2 %) is applied uniformly at a rate of 5 litres/0,25 m^2 to 10 litres/0,25 m^2. The formaldehyde solution is poured onto the test site in 2 to 3 portions according to seepage capacity. The total duration of extraction is 30 min.

All earthworms coming to the soil surface within the sampling area are collected and put into a preservation liquid (5 % formol or 70 % alcohol) (see 8.1). After an extraction period of 30 min, the soil surface and the grass cover are thoroughly searched in order to collect worms so far overlooked (usually small young worms and the slender species *Aporrectodea rosea*).

5.3 Mustard extraction method

On the day before the extraction, 60 g of mustard powder are mixed with 1 litre of distilled water. Just before use, this mustard emulsion is added to 9 litres of distilled water and applied in the same way as the formaldehyde solution. The remaining procedure is similar to the formaldehyde extraction method described in 5.2.

5.4 Electrical extraction method

In general, electrodes are inserted into the soil at the corners of a test plot, and worms rise to the surface as the current is applied. There are a number of different devices for electrical extraction, operating under different conditions (direct current or alternating current, electrode penetration depth, segmentation of electrodes, etc.). The specific equipment and conditions used should thus be reported. The total duration of the extraction is 30 min. After the 30 min extraction period, the soil surface should be intensively searched for overlooked worms.

6 Preparation for the test

6.1 Test site

6.1.1 Selection and description

The test should be conducted on a site similar to that where the test substance would normally be applied, or where a spillage or discharge could occur. The site should be on level ground and should have the same cropping and soil characteristics throughout.

Grassland and orchards are generally better suited to field studies with earthworms than arable land as they generally support higher earthworm populations. However, if information on effects on bare soils is required then arable plots may be used providing that there are at least 20 earthworms per square metre and a reasonable species diversity is evident.

A suitable grassland test area should have an earthworm density of a least 100 individuals per square metre. With lower population densities, more samples should be taken than recommended in 6.1.3.

The experimental plots should support a mixed population of organisms (Bouché 1977) which are generally representative of the type of environment selected. In agricultural areas for example, important species such as *Lumbricus terrestris* and *Aporrectodea caliginosa* should be present at a sufficiently high density (at least 10 % of the population) that plots can be taken as representative. Care should be taken not to select plots where uncharacteristic species predominate.

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 physico-chemical and biological information:

— soil profile;

— particle-size distribution (ISO 11277);

— organic-carbon content (ISO 10694:1995);

— pH-value (ISO 10390);

— field capacity (in the A-horizon);

— description of vegetation.

Determination of these characteristics should be made using standard methods.

Micro-climate measurements (soil and air temperature, soil moisture, 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, etc.).

NOTE When a specific chemical is being tested, the site should not have been exposed to a similar chemical during the last 3 years.

6.1.2 Maintenance of test fields

Grassland fields should be mulched regularly (two to six times per year) in order to keep the grass cover short. Mulching should be carried out one to two weeks before the application of the test substance to ensure that the grass on the surface, which will act 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 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, normal field practice should be used. However, the soil should not be treated during the test as far as possible.

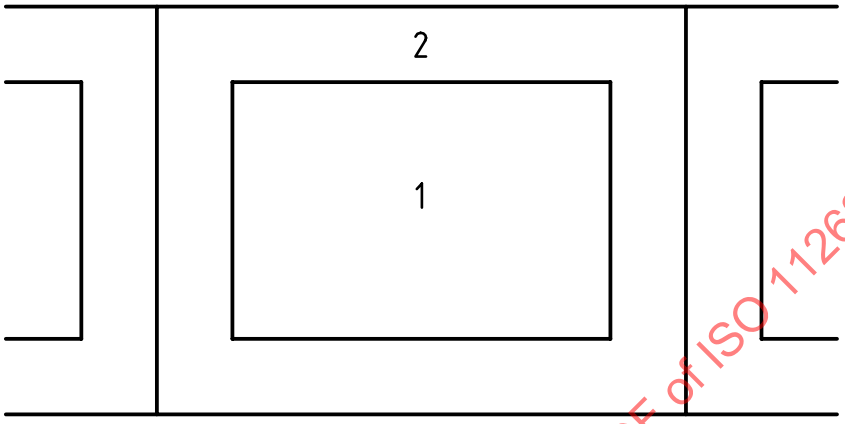
Pesticides should not be used on the test area, but if an application is unavoidable then the chemical chosen should be non-toxic to earthworms. The same substance should also be applied to the control plots. With respect to the interpretation of the test results, it has to be kept in mind that, even if this chemical does not affect earthworms, interactions between residues of the non-toxic chemical and the test substance might occur.

In individual cases, artificial overhead irrigation of the test field may be useful as earthworms will only become active and rise to the surface when sufficient soil moisture is present. Irrigation after application of the test substance can increase the direct exposure of the worms to the test chemicals. The irrigation given will depend on local conditions and hence firm recommendations cannot be made. Irrigation one to two weeks before sampling may facilitate or even be necessary to allow sampling, since this depends on the worms' activity.

6.1.3 Design of experiment

The test should be designed as a randomized plot test. The number of planned sampling dates will determine the surface area of the test plot.

However, the plots (= replicates) 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).



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 (Daniel & Bieri 1988).

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 diameter of 56 cm and a height of 10 cm to 15 cm to give wind protection is recommended. On arable land, the sample area will normally have to 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.

6.2 Check on efficiency of extraction method

The efficiency of the chosen extraction method should be checked at the beginning of the sampling period. To this end, the earth underneath the sampled area is excavated, after sampling, to a depth of 30 cm to 50 cm (depending on the earthworm distribution in the various soil horizons) and is placed on a plastic sheet or in boxes. Then the soil is carefully reduced in size by hand and searched for earthworms. Diapause or quiescence stages should be noted. The chosen extraction method should isolate at least 60 % of the earthworms collected as described above. Excavations should be made from at least three sample sites before actual sampling is continued.

When sampling is carried out over several days, efficiency checks on random samples should be carried out at suitable intervals to take account of weather changes or the soil drying out.

7 Procedure

7.1 Application of the test substance

The test substance and reference substance should be fully identified in the test report, and information on physical and chemical properties included if this will be helpful in the interpretation of the test results.

When chemicals are designed for soil application (e.g. agricultural chemicals), then application rates, formulations and modes of application will be specified by suppliers and should be followed. Ideally, in such cases, application in the test should be carried out using application equipment similar to that used in practice (e.g. when testing pesticides, application should be carried out using appropriate agricultural spraying equipment designed to deliver equivalent volumes in the same manner). All equipment should be adjusted prior to use to deliver the chemical at a rate equal to the maximum which would reasonably be used in practice.

NOTE With pesticides which are applied in water, a water application rate of 200 litres/ha to 300 litres/ha should be used on arable land. On grassland, 400 litres/ha to 800 litres/ha should be applied to ensure penetration. If several applications are planned, they should be carried out at intervals corresponding to normal application procedures.

When the effects of accidental spillages or leaching of chemicals are under study, then application should aim to mimic realistic circumstances as closely as possible, but with regard to sampling constraints (e.g. even distribution over plots). As earthworms in temperate countries are most active in spring and autumn, it is recommended that the test should begin in spring.

With herbicide tests, all the vegetation on the plots may be destroyed. Since this may influence the earthworm population at the test site, such tests are normally best carried out on bare soil sites. Freshly ploughed grassland which has been subsequently sown may also be used.

7.2 Sampling dates

After application of the test substance, at least three sampling dates should be planned, which must fall in periods of earthworm activity:

First sampling: about 1 month after application;

Second sampling: about 4 to 6 months after application;

Third sampling: about 12 months after application.

The test duration depends on the properties of the test chemical. Any further sampling that may be necessary should be carried out at half-year intervals during periods of earthworm activity.

7.3 Reference substance

The simultaneous testing of a reference substance (toxic standard) is necessary to obtain information on the effect of a test substance under the specific experimental site conditions.

The active ingredients Benomyl or Carbendazim, which are toxic to earthworms, are suitable for this purpose. (Niklas & Kennel 1978, Edwards & Brown 1982, Heimbach 1990). A field application rate of 2 kg to 4 kg of active ingredient per hectare is considered suitable to obtain the desired effect (reduction of population by 40 % to 80 %). Since the effect may also be dependent on the formulation concerned, preliminary tests should be carried out or more than one application rate should be selected.

8 Test evaluation

8.1 Identification of earthworm species

Identification down to species level is made by means of the relevant identification literature (Graff 1953, Sims & Gerard 1985), and the nomenclature used should be in accordance with Easton (1983).

The earthworms collected may be fixed in 5 % formaldehyde solution and kept there until identification. Alcohol (70 %) may also be used as a fixation and preservation liquid. However, alcohol has the disadvantage of bleaching the worms, which makes identification more difficult. Identification of living earthworms in the field is also possible but requires that operators have the skills necessary to identify species.

Adults and young worms of a species are counted separately. With young worms which are difficult to identify, a distinction between *Tanylobes* and *Epilobes* should at least be made.

NOTE To facilitate the taxonomic identification of juvenile earthworms, it is helpful and sufficient to differentiate between *Epilobous* and *Tanylobous* species. The first body segment, the peristomium, surrounds the mouth and dorsally carries a forwardly directed fleshy lobe, the prostomium. When the worm is at rest, the prostomium acts as a flap and seals the entrance to the mouth or buccal cavity, but otherwise it is employed as a tactile and chemo-sensory probe. In *Lumbricus spp.*, it is additionally prehensile and used to draw grasses and leaves into the burrow. The prostomium may be continuous posteriorly with the peristomium (*Zygolobous*), have a simple demarcation (*Prolobous*), have a short posterior tongue-like projection (*Epilobous*) or have the tongue-like projection extend back to the first furrow and divide the peristomium dorsally (*Tanylobous*).

8.2 Weighing

Before weighing, the fixed worms are put onto filter paper to remove any adhering liquid. The mass of the worms is recorded on the basis of species and age.

9 Calculation and expression of results

For each species collected on each sampling occasion, determine the number of adults and juveniles and their mass. Comparisons between treatments and controls are made using suitable statistical methods. Statistical testing and inference depends on whether the replicate values are normally distributed and are homogeneous with regard to their variance.

In order to test for normality and variance homogeneity, use Kolmogoroff-Smirnov's test procedure and Bartlett's test procedure, respectively. With normally distributed and homogeneous data, multiple *t*-tests such as Dunnett's or William's test ($\alpha = 0{,}05$, one-sided) should be performed. Otherwise, a multiple U-test, e.g. the Bonferroni U-test in accordance with Holm (1979), is recommended. If only one treatment has been performed and the prerequisites (normality, homogeneity) of parametric test procedures are fulfilled, use the pairwise Student's *t*-test, or otherwise the Mann-Whitney U-test procedure.

NOTE It should be noted that certain species may be over- or under-represented when activity-related extraction methods are used. For example, adult worms of the *Lumbricus terrestris* species which are deep in the soil are not covered representatively when electrical extraction methods are used (Cuendet *et al.* 1991). Similarly, smaller endogeneous species of worms in the soil may be killed as a result of formol extraction, and may thus be under-represented (Raw 1959).

10 Test report

The test report should include the following information:

a) a reference to this part of ISO 11268;

b) the results, expressed as in clause 9;

c) a detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test results;

d) the characteristics of the test site (see 6.1.1);

e) the weather conditions during the test period;

f) a detailed description of the test design and the management of the test site (size of test plots, number of replicates, number of samples);

g) the extraction method used for sampling;

h) the overall abundance and mass of the earthworms collected for all sampling dates together;

i) tables showing the percentage change per test plot, treatment and date compared to the control;

j) the overall abundance and mass of each species for all sampling dates together;

k) tables showing the numbers and mass per sample and date for each species;

l) a graphical representation of the abundance and mass change for each individual species during the test period;

m) the results obtained with the reference substance;

n) any operational details not specified in this part of ISO 11268, and any incidents liable to have affected the results.