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Soil quality — Effects of pollutants on insect larvae (*Oxythyrea funesta*) — Determination of acute toxicity

*Qualité du sol — Effets des polluants vis-à-vis des larves d'insectes (*Oxythyrea funesta*) — Détermination de la toxicité aiguë*

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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 2.

The main task of technical committees is to prepare International Standards. 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.

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.

ISO 20963 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

This International Standard describes a method for the determination of the acute toxicity of contaminated soils and chemicals to the larvae of *Oxythyrea funesta*, a phytophagous coleopteran (*Scarabaeidae*, *Cetoniinae*) with wide geographic distribution (Europe, North Africa and the Middle East).

Oxythyrea funesta has many characteristics which make it suitable for soil quality monitoring or testing effects of chemicals:

— ecological relevance: this type of organism contributes in many ways to soil structure by stimulating soil aeration and drainage;

— the first stages of development, i.e. incubation of eggs, larval cycle and pupation, are underground;

— the larvae of *Oxythyrea funesta* are tolerant to modifications of the test substrate granulometry;

— this species can be bred under controlled conditions.

Soil quality — Effects of pollutants on insect larvae (*Oxythyrea funesta*) — Determination of acute toxicity

1 Scope

This International Standard describes a method for the determination of the effects of contaminated soils and substances on the survival of the larvae of *Oxythyrea funesta*. The larvae are exposed to the pollutants by cuticular and alimentary uptake.

For contaminated soils, the effects on the survival are determined in the test soil and in a control soil. Depending on the objectives of the study, the control and dilution substrates (dilution series of contaminated soil) are either uncontaminated soil comparable to the soil sample to be tested or artificial soil substrate. Effects of substances are assessed using a defined artificial soil substrate.

This International Standard is not applicable to volatile substances, i.e. substances for which Henry's constant or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,001 33 Pa at 25 °C.

NOTE This method does not take into account the possible degradation of the substances or pollutants during the test.

2 Normative references

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

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 11268-1, *Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) — Part 1: Determination of acute toxicity using artificial soil substrate*

ISO 11269-2:—[1)], *Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of chemicals on the emergence and growth of higher plants*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

1) To be published. (Revision of ISO 11269-2:1995)

3.1
lethal concentration 50
LC 50
median lethal concentration of test substance or percent dilution of contaminated soil, which kills 50 % of the test organism within the test period

3.2
lethal concentration x
LCx
concentration of the test substance or percent dilution of contaminated soil which kills x % of the test organism within the test period

NOTE x is the percentage (10, 20, 25) of this effect.

3.3
Lowest Observed Effect Concentration
LOEC
lowest tested concentration of test substance or contaminated soil at which a statistically significant effect is observed compared with the control

NOTE All test concentrations above the LOEC have a harmful effect equal or greater than those observed at the LOEC.

3.4
No Observed Effect Concentration
NOEC
highest tested concentration of test substance or contaminated soil at which no statistically significant effect is observed compared with the control

NOTE The NOEC is the test concentration immediately below the LOEC.

3.5
test mixture
mixture of the test substance with the test substrate, mixture of contaminated soil with the test substrate or mixture of contaminated soil with an uncontaminated soil comparable to the soil sample to be tested

3.6
diapause
interruption of metabolism during egg, larva, pupa or imago development

4 Principle

Larvae of *Cetoniinae* (species *Oxythyrea funesta*) are exposed to a dilution range of contaminated soil or to a range of concentrations of test substance. The mortality of larvae is determined after 10 days. Test mixtures are prepared at the start of the test and are not renewed within the test period.

The results obtained from the test are compared with a control and are used to determine the concentration which causes mortality of 50 % of the larvae ($LC\ 50_{10days}$).

The test is conducted in two steps:

— a preliminary test to determine appropriate dilution/concentration range in the final test;

— the definitive test to determine the dilutions/concentrations causing between 10 % and 90 % mortality, which yields the test result.

It may also be possible to determine the effects of contaminated soils or substances on the growth of larvae (optional). The increase in mass within the test period allows this criterion to be considered as complementary to mortality, in order to assess the effects of contaminated soils or substances.

5 Test environment

Tests shall be performed at a temperature of (26 ± 1) °C in complete darkness.

6 Reagents

6.1 Biological material

The species used in the test is *Oxythyrea funesta* (*Scarabaeidae*, *Cetoniinae*). Third-instar larvae with a fresh mass within the range 100 mg to 200 mg are required to perform the test. The larvae shall be healthy, without any bites or other visible injuries.

NOTE Depending on the breeding conditions, described in Annex A, larvae approximately two weeks old are suitable for the test.

Larvae of similar size shall be selected. The difference in mass between the smallest and the largest larva within a single test container shall not exceed 50 mg.

Eliminate the particles of breeding substrate stuck to the integument using, for example, a soft brush before weighing the larvae. It is also possible to leave the animals to move along on slightly moist paper in order to eliminate the breeding substrate stuck to the integument.

Synchronisation of breeding is necessary. An example of breeding technique for *Oxythyrea funesta* is given in Annex A.

6.2 Test substrate

The mass of substrate used per glass container (7.1) shall be equivalent to 300 g (dry mass).

The substrate, called artificial soil, shall have the following composition (in accordance with ISO 11268-1):

— sphagnum peat, air-dried, finely ground and with no visible plant remains: 10 % (expressed on a dry mass basis);

— kaolinite clay containing not less than 30 % kaolinite: 20 % (expressed on a dry mass basis);

— industrial quartz sand (dominant fine sand with more than 50 % particle size between 0,05 mm and 0,20 mm): 70 % (expressed on a dry mass basis).

Add pulverised calcium carbonate ($CaCO_3$), of recognised analytical grade, as necessary to bring the pH of the wetted substrate to 6,0 ± 0,5 (commonly between 0,5 % and 1 % of the mass of the dry ingredients).

Prepare the artificial soil by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. The amount of calcium carbonate required can vary, depending on properties of the individual batch of sphagnum peat, and should be determined by weighing subsamples immediately before the test.

Store the mixed artificial soil at room temperature. To determine pH and the maximum water-holding capacity, pre-moisten the dry artificial soil at least two days before starting the test by adding deionized water to obtain half of the required final water content of 50 % of the maximum water-holding capacity.

Determine the water-holding capacity in accordance with ISO 11269-2:—, Annex A, and pH in accordance with ISO 10390. If the measured pH is not within the required range, add a sufficient amount of $CaCO_3$ or prepare a new batch of artificial soil.

6.3 Larvae food, i.e. dried and finely ground cow-dung, no piece larger than 1 mm.

The cow-dung shall come from healthy animals which have not received any treatment (antibiotics, growth promoters) during a two-week period preceding the date of sampling. It should be verified especially that animals have not recently been treated against intestinal worms.

6.4 Reference substance, i.e. 2,4,5-trichlorophenol of recognised analytical grade.

7 Apparatus

Use usual laboratory equipment and the following materials.

7.1 Glass containers, of capacity about 0,5 l to 1 l, covered with a polyethylene membrane (7.5) to allow exchange between the medium and the atmosphere.

7.2 Crusher, or any other apparatus to obtain food as described in 6.3.

7.3 Large scale laboratory mixer for the preparation of the test substrate (6.2).

7.4 Precision balance with an accuracy of at least 1 mg.

7.5 Polyethylene membrane, perforated with small holes to allow exchange between the medium and the atmosphere.

8 Procedure

8.1 Test design

8.1.1 Preliminary test

Carry out this test over a large range of dilutions/concentrations (e.g. contaminated soils: 0 %, 1 %, 5 %, 25 %, 50 %, 75 %, 100 %; substances: 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg, 1 000 mg/kg).

Substances are usually not tested at concentrations higher than 1 000 mg/kg (dry mass of test substrate).

Use preliminary test results to select the range of dilutions/concentrations for the final test. In order to establish this range, the highest concentration at which no lethal effect is observed and the lowest concentration at which 100 % mortality is observed should be determined.

Conduct the preliminary test without replication.

When no effects are observed, even at a concentration of 1 000 mg/kg, the definitive test can be designed as a limit test.

8.1.2 Definitive test

Select a range of at least five dilutions/concentrations of the contaminated soil or the test substance, providing a geometric progression between the highest dilution/concentration causing no mortality and the lowest dilution/concentration causing 100 % mortality in the preliminary test. The ratio between two consecutive concentrations should not exceed two.

If the ratio exceeds two, two concentrations are required for which the produced effect is between 10 % and 90 %.

For the final test, perform three replicates per concentration.

8.2 Preparation of test mixture

8.2.1 Soils

Pass the contaminated soils to be tested through a sieve of mesh 4 mm square to remove coarse fragments. Before the test, store soils in accordance with ISO 10381-6.

For each soil, the following characteristics should be determined:

— pH in accordance with ISO 10390;

— water content in accordance with ISO 11465;

— water-holding capacity in accordance with ISO 11269-2:—, Annex A;

— cationic exchange capacity in accordance with ISO 11260;

— organic matter content in accordance with ISO 10694.

Depending on the objective of the study, the control and dilution substrate (dilution series of contaminated soil) should be either an uncontaminated soil comparable to the soil sample to be tested or the artificial soil substrate.

If a field soil is used as control and dilution soil, it should be treated and characterised as described above.

Mix the test soil with the field soil or the test substrate (6.2), depending on the selected dilution range. The total mass of the soil and the test substrate (6.2) (or field soil) shall be equal to 300 g (dry mass) in each test container (7.1). Wet the test mixture with deionized water to reach 50 % of the total water-holding capacity determined in accordance with ISO 11269-2:—, Annex A (the total amount of water is equivalent to the water necessary to wet the mass of test substrate of the mixture + the water necessary to wet the mass of soil of the mixture). Mix thoroughly.

Determine the pH for each test mixture (one container per concentration) in accordance with ISO 10390.

8.2.2 Water-soluble substances

Dissolve the quantity of test substance required to obtain the desired concentration in deionized water (water used to wet the test substrate). Mix it thoroughly with the partly moistened test substrate.

Wet the test mixture with deionized water to reach 50 % of the total water-holding capacity determined in accordance with ISO 11269-2:—, Annex A.

Determine the pH for each test mixture (one container per concentration) in accordance with ISO 10390.

8.2.3 Substances insoluble in water but soluble in organic solvents

Dissolve in a volatile solvent (such as methanol or acetone) the quantity of test substance required to obtain the desired concentration, and mix it thoroughly with a portion (10 g to 50 g) of the quartz sand required (see 6.2). Evaporate the solvent by placing the container under a fume hood.

After evaporation of the solvent, mix the sand thoroughly with the test substrate (6.2) and the deionized water to reach 50 % of the total water-holding capacity of the test substrate.

Determine the pH for each test mixture (one container per concentration) in accordance with ISO 10390.

8.2.4 Substances insoluble in water and organic solvents

For a substance insoluble in a volatile solvent, prepare a mixture of industrial quartz sand (0 g to 50 g) and the quantity of the test substance required to obtain the desired concentration. Transfer this mixture to a glass container filled with the test substrate (6.2) and mix thoroughly.

Add deionized water to reach 50 % of the total water-holding capacity of the test substrate and mix thoroughly.

Determine the pH for each test mixture (one container per concentration) in accordance with ISO 10390.

8.3 Preparation of control container

The control container contains the test substrate wetted with deionized water to reach 50 % of the total water-holding capacity (determined in accordance with ISO 11269-2:—, Annex A).

Prepare one control container for the preliminary test and three control containers for the final test.

If the preparation of the test mixtures requires the use of a solvent, prepare an additional control containing the same amount of solvent as used for introducing the test substance. Prepare the control container(s) in accordance with 8.2.3 without addition of the test substance.

Measure the pH of the control and the control with solvent (if appropriate) in accordance with ISO 10390.

8.4 Food addition

Before addition of the larvae, mix 3 g of finely ground air-dried cow-dung (6.3) with the test mixture (8.2). Proceed in the same way for control containers.

Also add food during the test in order to allow larval development. Add 2 g to 3 g of finely ground air-dried cow-dung (6.3) on the surface at day 3 and day 7 (only if the previous addition has been consumed).

8.5 Introduction of the biological material

Weigh 10 larvae individually and introduce them in each container. Cover it with a polyethylene membrane (7.5) perforated with small holes in order to allow exchange between the medium and the atmosphere and to avoid escape of larvae from the test container.

8.6 Test conditions and measurements

Place the containers in the test enclosure (Clause 5) for 10 days.

After 10 days, determine for each container the total number and the mass of living larvae (individual mass optional). A larva is considered to be dead if it displays no reaction to a pinprick applied to its anterior side.

Note the symptoms observed on the animals.

At the end of the test, measure the pH in one control container and in one container per test mixture in accordance with ISO 10390.

8.7 Reference substance

In order to check the biological material used, conduct at regular intervals a test with 2,4,5-trichlorophenol (6.4) in accordance with the procedure described in 8.2.2, 8.3, 8.4 and 8.5.

The LC 50_{10days} of the 2,4,5-trichlorophenol should be between 60 mg/kg and 180 mg/kg.

9 Expression of results

9.1 Calculation

9.1.1 Mortality

Present the data in tabular form, indicating the number of living larvae in each container. Calculate the percentage of mortality for each concentration.

Use any suitable statistical procedures to calculate the $LC\ 50_{10days}$ with confidence limits (P = 0,95).

When two consecutive concentrations at a ratio less than or equal to two (for example 10, 20) give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC 50 falls.

When the data are adequate, determine the NOEC by a suitable statistical procedure (for example ISO/TS 20281[4]).

9.1.2 Growth (optional)

It may also be possible to determine the effects of contaminated soils or substances on the growth of larvae.

Present the data in tabular form indicating the growth rate for each container and the percentage of growth inhibition for each concentration.

For each container, calculate the growth rate according to Equation (1):

$$R_G = \frac{(m_t - m_{t0})}{m_{t0}} \times 100 \quad (1)$$

where

R_G is the growth rate;

m_t is the mean fresh mass of the larvae per replicate at the end of the test, in milligrams;

m_{t0} is the mean fresh mass of the larvae per replicate at time 0, in milligrams.

For each concentration, calculate the mean percentage of growth inhibition according to Equation (2):

$$I_m = \frac{(m_{0t} - m_{0t0}) - (m_t - m_{t0})}{(m_{0t} - m_{0t0})} \times 100 \quad (2)$$

where

I_m is the mean percentage of growth inhibition;

m_{0t} is the mean fresh mass of the larvae at the end of the test in the control containers, in milligrams;

m_{0t0} is the mean fresh mass of the larvae at time 0 in the control containers, in milligrams;

m_t is the mean fresh mass of the larvae per concentration at the end of the test, in milligrams;

m_{t0} is the mean fresh mass of the larvae per concentration at time 0, in milligrams.

9.2 Expression of results

The LC 50, LCx and NOEC are expressed in milligrams of test substance per kilogram of test substrate (dry mass). For contaminated soils, the LC 50 and the NOEC are expressed in % (dry mass).

10 Validity of the test

The results are considered to be valid if:

— the mortality observed in the control containers does not exceed 10 % at the end of the test;

— the increase in biomass of the larvae in the control containers is higher than 80 %.

11 Test report

The test report shall refer to this International Standard and include the following information:

a) the complete description of the biological material used (species, age, mass range, breeding conditions, supplier);

b) the method of preparation of the test substrate, including the solvent used for a water-insoluble substance;

c) the origin of the contaminated soil and the type of pollutants (if they have been identified), and at least pH, water-holding capacity and water content;

d) the origin of the field soil used as control and dilution soil (if appropriate);

e) any soil treatment prior to the test;

f) the conditions of the test environment;

g) the results obtained with the reference substance;

h) a table giving the percentage of mortality obtained for each container, for each concentration or soil dilution and for the control;

i) a table giving the mass of living larvae in the control container at the end of the test and the biomass growth rate;

j) (optional) a table indicating the growth rate in each container and the percentage of growth inhibition (and calculation of EC_x and NOEC if appropriate);

k) the results expressed as in 9.2;

l) the method used for calculation of LC 50 or LC_x;

m) the highest concentration causing no observed effect, NOEC (if available);

n) a description of obvious or pathological symptoms or distinct changes in behaviour observed in the biological material;

o) all operating details not specified in this International Standard, and any occurrences likely to have affected the results;

p) a plot of the concentration/response curve.

Annex A
(informative)

Example of breeding technique for *Oxythyrea funesta*

A.1 General

Oxythyrea funesta is a phytophagous coleopteran (*Scarabaeidae*, *Cetoniinae*) with wide geographic distribution (Europe, North Africa, and the Middle East). Measuring from 8 mm to 10 mm, adults appear in spring when they are sexually mature. The saprophagous larva lives in mould and mud. It goes through three different stages of development. Underground pupation occurs when larvae reach a fresh mass of 600 mg to 650 mg. Imago emergence takes place at the end of summer. Sexual maturity is obtained after a diapause period during the winter.

In laboratory conditions, the life cycle of *Oxythyrea funesta* can be summarized as follows:

Table A.1 — Life cycle of *Oxythyrea funesta*

	Mean duration days	**Temperature**
Egg incubation	12	(26 ± 1) °C
Larval development	56	(26 ± 1) °C
Pupation	56	(26 ± 1) °C
Adults Imaginal diapause	70	(7 ± 1) °C
Adults (without activity)	30	(23 ± 1) °C
Adults (egg-laying)	140	(23 ± 1) °C

A.2 Composition and preparation of breeding medium

The breeding medium has the following composition:

— cow-dung (one part, dry mass);

— horticultural mould (four parts, dry mass).

The cow-dung comes from healthy animals which have not received any treatment during a two weeks period preceding the date of sampling. It should be verified especially that animals have not recently been treated against intestinal worms.

The cow-dung and the horticultural mould are dried, individually or after mixing, at room temperature or in a drying cabinet at a temperature lower than 60 °C.

After drying, crush the cow-dung and the horticultural mould. The particle size should not be more than 1 mm. The breeding medium can be used immediately, or can be stored in sealed bags in a cool dry area for several months, or can be frozen at – 20 °C in sealed bags for several years.

Before use, add water to the breeding medium to obtain approximately 70 % of water content (mass fraction).

A.3 Adults

Adults are kept under 1 500 lx illumination (light/dark cycle: 16 h/8 h). 15 to 20 pairs are introduced in each breeding vessel (capacity of 0,5 l with a diameter of 90 mm, filled with breeding medium as described in A.2). A glass or polyethylene terephthalate cylinder (150 mm high) is placed above the vessel. The top of the cylinder is covered with a small grid in order to avoid escape of the adults.

Breeding is carried out in a climatic chamber, free from toxic vapours, at (23 ± 1) °C.

Adults are fed with pollen frequently renewed (about 1 cm^3 in a small container, renewed usually every two days).

Every week, eggs are removed from the breeding substrate and placed in the same medium at (26 ± 1) °C in the dark. Eggs can be easily collected by passing the medium through a sieve.

A.4 Incubation of eggs — Larval development

Eggs are incubated in 2-litre containers. These containers are covered with lids perforated with small holes to allow exchange between the medium and the atmosphere. Containers are filled with breeding medium as described in A.2. During larval development, the substrate is frequently renewed. Containers are checked regularly in order to avoid the development of mould.

Larvae hatch from eggs after about 12 days. Suitable larvae for experiments are obtained approximately two weeks after hatching. A part of the larvae is kept in order to ensure the breeding continuity.

Pupation occurs when larvae reach about 600 mg to 650 mg. The duration of this stage is usually 8 weeks. Sexual maturity of imago is obtained after a diapause period of 8 to 12 weeks at 7 °C to 8 °C. Young adults are maintained during this period in containers filled up with dry breeding medium (up to 50 adults per container)

After this diapause period, adults are kept according to A.3.