INTERNATIONAL STANDARD

ISO 20963

First edition
2005-04-01

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*

Reference number
ISO 20963:2005(E)

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Published in Switzerland

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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.