BS EN ISO 23611-3:2011

BSI Standards Publication

Soil quality — Sampling of soil invertebrates

Part 3: Sampling and soil extraction of enchytraeids (ISO 23611-3:2007)

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National foreword

This British Standard is the UK implementation of EN ISO 23611-3:2011. It is identical to ISO 23611-3:2007.

The UK participation in its preparation was entrusted to Technical Committee EH/4, Soil quality.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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This European Standard was approved by CEN on 17 June 2011.

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Foreword

The text of ISO 23611-3:2007 has been prepared by Technical Committee ISO/TC 190 “Soil quality” of the International Organization for Standardization (ISO) and has been taken over as EN ISO 23611-3:2011 by Technical Committee CEN/TC 345 “Characterization of soils” the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by January 2012, and conflicting national standards shall be withdrawn at the latest by January 2012.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

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Endorsement notice

The text of ISO 23611-3:2007 has been approved by CEN as a EN ISO 23611-3:2011 without any modification.

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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.

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ISO 23611-3 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

ISO 23611 consists of the following parts, under the general title *Soil quality — Sampling of soil invertebrates*:

— *Part 1: Hand-sorting and formalin extraction of earthworms*

— *Part 2: Sampling and extraction of micro-arthropods (Collembola and Acarina)*

— *Part 3: Sampling and soil extraction of enchytraeids*

— *Part 4: Sampling, extraction and identification of soil-inhabiting nematodes*

Introduction

This part of ISO 23611 has been drawn up since there is a growing need for the standardization of terrestrial zoological field methods. Such methods, mainly covering the sampling, extraction and handling of soil invertebrates, are needed for the following purposes:

— biological classification of soils including soil quality assessment (e.g. References [21], [25], [27]);

— terrestrial bioindication and long-term monitoring (e.g. References [13], [26]);

— evaluation of the effects of chemicals on soil animals (References [15], [22]).

Data for these purposes are gained by standardized methods since they can form the basis for far-reaching decisions (e.g. whether a given site should be remediated or not). In fact, the lack of such standardized methods is one of the most important reasons why biological classification concepts in terrestrial (i.e. soil) habitats have so far been relatively rarely used in comparison to aquatic sites.

Originally, the methods described here were developed for taxonomical and ecological studies, investigating the role of enchytraeids in various soil ecosystems. These animals without doubt belong to the most important soil invertebrates in temperate regions (mainly in acidic soils [5]). Their influence on soil functions like litter decomposition and nutrient cycling is well-known [14], [19]. Due to their number which is often very high (and to their population biomass), they are also important in many terrestrial food-webs [4]. Some species have unintentionally been distributed by man in many soils of the world.

Since it is neither possible nor useful to standardize methods for all soil organisms, the most important ones have been selected. [Microbiological parameters are already covered by existing ISO guidelines (e.g. ISO 10381-6 [29], ISO 14240-1 [37] and ISO 14240-2 [38])].

INTERNATIONAL STANDARD **ISO 23611-3:2007(E)**

Soil quality — Sampling of soil invertebrates —

Part 3:
Sampling and soil extraction of enchytraeids

1 Scope

This part of ISO 23611 specifies a method for sampling, handling and extracting enchytraeids from terrestrial field soils as a prerequisite for using these animals as bioindicators (e.g. to assess the quality of a soil as a habitat for organisms).

Basic information on the ecology of enchytraeids and their use as bioindicators in the terrestrial environment are included in the Bibliography.

This part of ISO 23611 applies to all terrestrial biotopes in which enchytraeids occur. The sampling design of field studies in general is specified in ISO 10381-1. These details can vary according to the climatic/regional conditions of the site to be sampled and an overview on the determination of effects of pollutants on enchytraeids in field situations is given in Reference [6].

Methods for some other soil organism groups such as earthworms or micro-arthropods are specified in ISO 23611-1 and ISO 23611-2.

This part of ISO 23611 is not applicable for semi-terrestrial (i.e. living in or close to the pure water) soils and might be difficult to use under extreme climatic or geographical conditions (e.g. in high mountains).

When sampling soil invertebrates, it is highly recommendable to characterize the site (e.g. concerning climate and land use). However, such a characterization is not covered by this part of ISO 23611. ISO 10390, ISO 10694, ISO 11272, ISO 11274, ISO 11277, ISO 11461 and ISO 11465 are more suitable for measuring pH, particle size distribution, C/N ratio, organic carbon content and water holding capacity.

2 Terms and definitions

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

2.1
enchytraeids
small soil-inhabiting worms (a few millimetres to several centimetres in length) belonging to the family Enchytraeidae, order Oligochaeta (class Clitellata, phylum Annelida)

EXAMPLE Species of the genera *Enchytraeus, Fridericia* or *Cognettia*.

3 Principle

Enchytraeids at a certain site are sampled from the soil by using a split corer (diameter usually 3 cm to 6 cm). After sampling, the soil samples containing the enchytraeids are transported to the laboratory. Then the enchytraeids are extracted from soil by means of a wet extraction method. (This approach has been well-known for a long time [11], [17], [20].) After extraction, the enchytraeids are identified alive and, if required, preserved in such a way that they can be stored in a collection indefinitely (e.g. for taxonomical purposes).

The determination of the biomass of enchytraeids is also described in this part of ISO 23611. The abundance and biomass values can be recalculated to the area of the soil corer or, more rarely, volume parameters.

NOTE 1 The sampling of enchytraeids is often included in much broader monitoring programmes which try to cover the whole soil fauna or parts of it (e.g. the mesofauna). The design of such programmes is not included in this part of ISO 23611 (but see e.g. Reference [3]).

NOTE 2 Some hints for the taxonomy of enchytraeids are given in Annex A.

4 Reagents

4.1 Tap water (without toxic properties, e.g. due to copper contamination).

4.2 Ethanol, 70 % (volume fraction).

4.3 Bengalred, 4,5,6,7-Tetrachloro-2',4',5',7'-tetraiodofluorescein formulated as a staining agent.

4.4 Bouin's fixative, buffered solution of formaldehyde, acetic acid and picric acid.

4.5 Paracarmin, staining agent, prepared as a mixture of carmine acid, aluminium chloride and calcium chloride solved in ethanol.

4.6 Canada-balm, natural yellowish viscous fluid containing 13 % to 14 % (volume fraction) Canadin acid ($C_{20}H_{38}O_2$), 48 % to 50 % (volume fraction) α- and β-Canadinol acid ($C_{19}H_{30}O_2$) and 5 % (volume fraction) Canadoesen ($C_{21}H_{40}O$).

5 Apparatus

5.1 Split soil corer (e.g. diameter 3 cm to 6 cm; extracted core length 10 cm to 30 cm); length in total variable (depending whether or not a handle is used).

5.2 Plastic bags (e.g. 1-l freezer bags); general store.

5.3 Temperature recorder or a **minimum/maximum-thermometer**.

5.4 Plastic bowls, diameter approximately 20 cm, height approximately 10 cm; general store.

5.5 Plastic sieves, diameter approximately 15 cm, mesh width approximately 0,5 mm; general store.

5.6 60-W bulbs as a heating device; general store.

5.7 Glassware, e.g. petri dishes (square format) with a size of 8 cm × 8 cm or small glass vessels (e.g. 50 ml).

5.8 Sharp, large knife.

5.9 Refrigerator.

5.10 Dissecting microscope with low magnification (10 to 40 times).

5.11 Microscope with high magnification (60 to 400 times).

5.12 Spring steel pincers (flat).

5.13 Eppendorf pipette, a soft steel forceps or a hooked needle.

6 Procedure

6.1 Soil sampling

The soil samples to be used for the investigation of the enchytraeid community are taken destructively by means of a soil corer (5.1). The corer is carefully pressed into the soil. The depth depends on the soil type, but usually varies between 10 cm (e.g. forests) and up to 30 cm (e.g. crop sites), i.e. those layers in which the bulk of the enchytraeids are living. In rare cases, e.g. if thick roots are present, a plastic or wooden hammer can be used to take the samples. After removing the soil corer, its valve is opened and the soil core is carefully taken out by hand. The core is divided into cylinders (e.g. 3 cm to 4 cm height) with a knife (5.8). These soil cylinders may be stored in small plastic bags (5.2) in a refrigerator (5.9) at approximately 4 °C to 6 °C for a period of preferably not longer than one to two weeks (storage should not exceed one month in any case [7]). The soil corer is cleaned with water afterwards.

6.2 Extraction of the enchytraeids

In principle, the extraction of the worms from the soil is caused by their active movement in the water-saturated sample.

The extraction should commence as soon as possible after the sampling (see 6.1). The bowls (5.4) are carefully filled up with tap water (4.1) until the empty sieves (5.5) are completely covered by the water. The samples (i.e. soil cylinders) are put in the sieves, and are, if necessary (e.g. in cases of heavy loam soils), carefully broken apart by hand (see Figure 1). The bottom of the sieves should not reach the bottom of the bowls. To ensure an extraction efficiency of Enchytraeidae from the samples of more than 90 %, the extraction of soil should last for 4 d to 7 d and of litter for 0,5 d to 2 d at (12 ± 2) °C (water temperature). The duration depends mainly on the organic content of the sample. These times can be modified according to organizational requirements and the number of individuals in a sample. However, the worms quickly die if an oxygen deficiency occurs (in order to avoid this problem the water can be changed after 24 h or 48 h). An acceleration of the extraction using a heat source [e.g. a 60-W bulb (5.6)] placed above the sample can be helpful, but should be carefully used (i.e. slow increase over at least 3 h), since otherwise — species-specifically — many animals, especially juveniles and fragmentation stages, remain in the soil (see Annex B).

NOTE 1 In order to reduce the amount of debris at the bottom of the extraction bowls, a fine wiping cloth (0,5 mm) can be put in the mesh before the soil sample is put in [23].

At the end of the extraction procedure, the sieves are removed, the soil is discarded and disposed according to local waste regulations. The water is slowly and carefully decanted from the bowl. The finest fraction of soil at the bottom of the bowls should not be disturbed (see Figure 2). A small amount of water (up to a height of 5 mm to 10 mm) shall remain in the bowls. Subsequently, the finest fraction of soil is suspended in the overlying water, placed in a petri dish (5.7) and briefly stored until soil particles have settled and the water becomes clear. Since the whitish worms are heavier than water, but are rarely able to hide themselves in the narrow soil layer, they can easily be collected out of the petri dish under a dissecting microscope (5.10). For this transfer, a soft steel forceps, an Eppendorf pipette or a hooked needle (5.13) can be used, but in any case damaging of the worms shall be avoided. The most convenient way of counting the total number is to divide the surface of the petri dish in parallel rows which are checked one after another. Due to their white colour, the worms are clearly visible against the usually brownish soil particles. The animals are transferred to small plastic or glass vessels (e.g. 20 ml).

The number of samples which can be extracted simultaneously is theoretically unlimited. However, due to the size of the water bowls, space limitations can occur. Since they (i.e. at least the water) shall be cooled, usually only up to 40 to 50 samples can be processed at one time. These limitations can be overcome by carrying out the procedure in a cool room, e.g. in a cellar.

NOTE 2 In rare cases, the enchytraeids can be confused with diptera larvae (which very often possess brownish or black head capsules) or nematodes (usually smaller and faster moving than oligochaetes). Additionally, fungal hyphae or fine root material can be mistaken for enchytraeids, since they can possess the same length and colour. However, they always lack the segmentation of oligochaete worms.

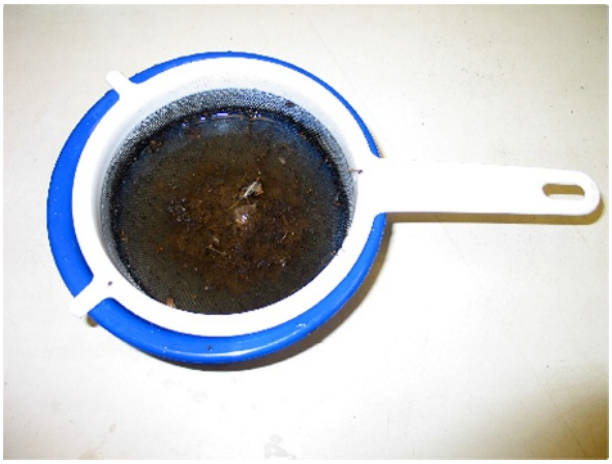

Figure 1 — Extraction bowl with soil sample

Figure 2 — Sediment layer (including enchytraeids)

6.3 Microscopic identification

The microscopic identification should be done as soon as possible, because the animals die in water after some days, even if stored in a refrigerator (5.9). A soft steel forceps, an Eppendorf pipette or a hooked needle (5.13) can be used carefully to transfer the animals with a drop of water to a slide. If the worms are moving too fast on the slide, they can be anaesthetised with CO_2 (e.g. by using a drop of mineral water with gas, but it should be used with care, otherwise the worms are killed).

NOTE Identification of the enchytraeids is difficult. Therefore, in many cases just the number of animals is determined. Otherwise the key of Reference [16] is used as well as later publications (compilation in References [8], [12], [24] and, in particular, Reference [23]). A compromise can be the use of a site-specific key since usually only 3 to 25 species occur at any given site (in this case, often worms fixed in ethanol can be identified to the species level). An overview over the information (parameters, drawings, etc.) needed for the identification of a certain species is given in Reference [9].

6.4 Preservation of Enchytraeidae

Enchytraeidae can be preserved for further investigations (e.g. species descriptions) in 70 % (volume fraction) ethanol (4.2). However, the preservation is accompanied by a loss of visible morphological details. Animals difficult to identify or those selected as reference specimen may also be identified after a fixing in Bouin (4.4) respective colouring in Paracarmin (4.5) and storing in Canada-balm (4.6) (which can be relatively elaborate). For the species identification of fixed specimen, the use of an interference microscope (5.11) is strongly recommended.

6.5 Validity of the extraction process

Extraction efficiency can be checked by fixing soil samples with ethanol (96 %), which are taken in parallel to other field samples. The soil is spread in a thin layer on the bottom of a flat plastic vessel (e.g. Bellaplast[1)]: 16 cm × 11 cm) and then the ethanol is added. Afterwards, some drops of Bengalred (4.3) is applied to the ethanol. After one day, the brightly red coloured worms can easily be counted. However, this procedure is only necessary when using samples from an unknown site for the first time. Additionally, this check shall be done with six to eight replicates since the variability of enchytraeid numbers can be quite high.

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

6.6 Determination of biomass

For an estimation of the ecological role (e.g. in the soil food-web) of enchytraeids at a certain site, the determination of their biomass is necessary. Since direct weighting is difficult due to the low individual mass of most species, the potential corruption by changing gut content and especially the quick desiccation, the biomass may also be indirectly estimated by the following methods:

— creation of specific species calibration curves to the ratio of length and mass [1], [2], then measurment of the length of the animals in a sample;

— automatic calculation of the mass by computer-assisted measuring of the length-mass-ratio of embedded individuals and following computation with the largely constant density of enchytraeids [18].

7 Data assessment

The following measurement endpoints may be used for the bio-classification of a soil, including bio-indication or bio-monitoring (e.g. anthropogenic stress like chemicals or land use changes):

— abundance (number of individuals per area or volume);

— biomass (fresh or dry mass of the population per area or volume);

— number of species or other taxonomically or ecologically defined groups;

— dominance ratio (in percentage of the population);

— age structure of the population (e.g. adult/juvenile ratio), either all species together or individual species;

— distribution in the soil (e.g. the vertical distribution within the soil core);

— morphological, physiological or biochemical alterations in individuals (e.g. open wounds).

Usually, the total number of worms is counted and expressed as individuals per sample. This number is then multiplied by a factor determined by the diameter of the soil corer (5.1) in order to get the number of worms per square meter. Additionally, the age structure (juveniles and adults differentiated by the presence of a clitellum) and the vertical distribution can be determined with the help of the dissecting microscope (5.10) (i.e. no species determination is done). For the determination of all other endpoints, a detailed microscopic examination is necessary, since then the species have to be identified.

8 Test report

The test report shall contain a summary of the results obtained along with the methods and variables used during the study. It shall provide the following information:

a) a reference to this part of ISO 23611, i.e. ISO 23611-3;

b) a full description of the study design and procedures;

c) characterization of the test site (especially soil properties);

d) sampling method;

e) description of the sampling conditions, including date and duration of sampling in the field and climatic parameters like air temperature;

f) number of worms caught;

g) details of fixation and preservation of the biological material;

h) recalculated values to 1 m^2 or another standard size, if necessary;

i) all information, including all measured raw data and all problems which might have occurred, developed during the study;

j) discussion of the results.

Annex A
(informative)

Species identification in enchytraeids

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Annex B
(informative)

Quick extraction of enchytraeids

In specific cases, the extraction method described in 6.2 may not be optimal. This is, for example, true for samples which have been taken in organic-rich coniferous forest soils, in particular in Northern temperate regions like Scandinavia. In these soils (including the litter layer), often fragmenting species like *Cognettia sphagnetorum* are highly dominant [1], [5]. During the proposed extraction period of several days, asexual species could fragment or die. Thus, the number of individuals could change considerably.

For this reason, a modified extraction method as already described by Reference [17] can be used. The performance of the method is summarized as follows.

The sampling is done by using a soil corer (5.1) in accordance with 6.1.

The extraction should commence as soon as possible after the sampling. Each extraction unit consists of a plastic funnel (diameter of about 10 cm to 12 cm) fixed in a hole (e.g. a wooden board). A sieve (mesh-size 0,5 mm) with a slightly smaller diameter than the funnel is put into it. The sample placed on the sieve should be completely covered by water. The bottom of the funnel is closed by a screw clip on a piece of rubber tubing. The lower end of this tube ends in a small vessel (e.g. 20 ml) in which the enchytraeids are collected. Heat is supplied from a 60-W bulb (5.6) enclosed in a light metal cylinder (e.g. 11 cm diameter and 18 cm height). The bottom of this cylinder is about 10 cm to 12 cm above the funnel. The heating, preferably of several extraction units in parallel, is controlled by means of a variable resistance. During the extraction process, the heat is increased gradually, so that the water surface reaches a temperature of approximately 45 °C after 150 min to 250 min, when extraction is complete. Then, the worms are run out from the bottom of the funnel into the small vessel for counting and identification.

Afterwards, the enchytraeids are handled in accordance with 6.3.

An example of an apparatus is given in Figure B.1.

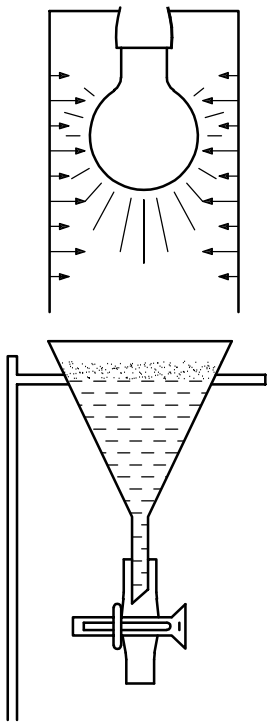

Figure B.1 — Schematic overview of the method
(from Reference [10])

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