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Soil quality — Sampling of soil invertebrates —

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

Qualité du sol — Prélèvement des invertébrés du sol —

Partie 4: Prélèvement, extraction et identification des nématodes du sol

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Contents

Page

Foreword

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality,* Subcommittee SC 4, *Biological characterization,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 23611-4:2007), which has been technically revised. The main changes are as follows:

— examples of the use of nematodes in soil monitoring programmes have been added (including presentation of their results) as an informative annex (see Annex D).

A list of all parts in the ISO 23611 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document 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 necessary for the following purposes:

— biological classification of soils including soil quality assessment [37],[42],[57];

— terrestrial bio-indication and long-term monitoring [25],[28],[31],[50];

— evaluation of the effects of chemicals on soil animals in the field (see ISO 11268-3[4]).

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 bio-classification and bio-assessment in terrestrial (i.e. soil) habitats has so far been relatively rarely used in comparison to aquatic sites.

Nematodes are an important and major part of the soil fauna. Some authors estimate that this group is probably the most dominant one of the multicellular organisms (Metazoa) on earth[52]. Nematodes occur from the Antarctic to the tropics and from deep sea sediments to mountain regions. They are active in every place with sufficient water and organic material. The species diversity and functional variety are impressive[14]. Nematodes are commonly known as parasites of animals and plants, but the major part of the nematode fauna participates in decomposition processes by feeding on bacteria and fungi.

Nematodes occur in high numbers ($0{,}2 \times 10^6$ m^{-2} to 9×10^6 m^{-2}) and with a high (10 to 100 species) diversity in almost every soil sample[12]. Moreover, there is a broad ecological spectrum of feeding types and food web relations among the nematodes such as bacterivores, fungivores, herbivores, predators and omnivores[57],[58]. These factors make the group highly suitable as indicators for ecological soil quality[56], but standardization of methods is urgently needed for comparison and combination of results.

In the past 100 years, nematology has developed strongly from the viewpoint of agriculture, advisory sampling and phytosanitary regulations because some terrestrial nematodes cause a lot of damage in crops. With respect to methods, there are several “schools” in different parts of the world with their own history, practical advantages and disadvantages. A comprehensive overview is given by Oostenbrink[14] and Southey[48],[49]. The more recently described methods (or variants) are often developed with special interest to certain plant parasitic species. Within the past 20 years new methods have evolved that allow a DNA-based taxonomic identification of nematode species[21],[34],[54]. This opens the taxonomic analysis of nematodes to a broader community of non-specialists.

Since Bongers[16] introduced the Maturity Index, the use of nematodes in bio-indication for soil quality has increased rapidly[56]. Nematodes are now used for ecological soil research and monitoring in several countries all over the world. Monitoring activities make special demands on methodology, for instance, that a large number of soil samples is processed on a routine basis against reasonable costs. Some of the methods originally developed for advisory sampling in agriculture are very suitable for ecological research. They form the basis for specific variants described in this document.

The nematodes that are characterized by the proposed procedure are all the free-living forms of nematodes found in soil. They include non-plant-feeding nematodes as well as ectoparasitic plant-feeding nematodes and free-living stage of endoparasitic nematodes. The quantification of obligate plant-feeding nematodes in roots requires specific methods. Basic information on the ecology of nematodes and their use as bio-indicators can be found in the bibliography.

Soil quality — Sampling of soil invertebrates —

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

1 Scope

This document specifies a method for sampling and handling free-living nematodes from terrestrial field soils as a prerequisite for using them as bio-indicators (e.g. to assess the quality of a soil as a habitat for organisms).

This document applies to all terrestrial biotopes in which nematodes occur. The sampling design of field studies in general is specified in ISO 18400-101.

This document is not applicable to aquatic nematodes because of differences in the sample matrix (e.g. water column). Methods for some other soil organism groups such as earthworms, collembolans enchytraeids or macro-invertebrates are covered in ISO 23611-1, ISO 23611-2, ISO 23611-3 and ISO 23611-5.

This document does not cover the pedological characterization of the site which is highly recommendable when sampling soil invertebrates. ISO 10390, ISO 10694, ISO 11272, ISO 11274, ISO 11277, ISO 11461 and ISO 11465 include suitable procedures for measuring pH, particle size distribution, C/N ratio, organic carbon content and water-holding capacity.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp/

— IEC Electropedia: available at https://www.electropedia.org/

3.1
nematode
small, non-segmented free-living worm (up to a few millimetres in length) belonging to the class Nematoda

Note 1 to entry: Nematodes without a soil-inhabiting stage are not included in this context.

3.2
location
study area or plot that is characterized based on the composition of (among others) the nematode fauna

3.3
bulk-sample
composite soil sample made out of many small soil cores to get an impression of the average nematode composition

3.4
soil sampler
tool to collect soil material in a quick and standardized way

3.5
mass slide
microscopic slide on which 300 to 400 nematodes are mounted for species *identification* (3.7)

3.6
identification
determination of the species, genus or family of an individual based on morphological characteristics (mouth parts, sexual organs, body ratios) with an identification key

3.7
colonizer–persister (cp) scale
ecological classification of nematodes

Note 1 to entry: Proposed by Bongers[16],[17].

Note 2 to entry: The principle is analogous to the r-K life strategies during succession, distinguished in fundamental ecology. Non-plant-feeding nematode families are classified to one of the five cp-groups. This is also the basis for the calculation of the Maturity Index.

4 Principle

Nematodes are collected in soil samples with a small cylindrical core (diameter: approximately 2 cm; length: 10 cm or 15 cm) or an auger (see Figure A.2). For monitoring purposes, the soil samples are combined in a bulk-sample from a homogeneous area. The total number of samples to be taken depends on the investigated surface area and its homogeneity (e.g. pedology, land use, crop). The individual samples can be gathered in the field in a standard plastic bag or plastic bucket. The combined bulk-sample is too large for direct examination and therefore it is mixed and subsampled. In the field and during transport to the laboratory, the soil samples shall be protected against strong fluctuations in temperature, water-loss and heavy mechanical disturbance. They can be stored for at most four weeks at 4 °C.

NOTE 1 The sampling method described above is derived from "the Dutch Method"[49] for determining the infestation of a field with potato-cyst nematodes, and has been used for many years in several European countries.

The Oostenbrink funnel method is recommended for routine extractions of soil samples, for instance in a monitoring network. The Oostenbrink method is not the simplest one that can be used under any circumstance. However, it has several advantages: it is highly standardized and constant in extraction efficiency. The Oostenbrink wet funnel method combines three basic means that can be used for the separation of nematodes from soils: washing, sieving, active movement. Therefore, it obtains better results than any one of the basic methods individually. Further advantages are given below:

— relatively large soil samples of any soil type can be treated at once (100 g to 500 g);

— clean nematode suspensions;

— isolation of most living and active nematodes;

— there are many years of experience with enormous amounts of routine soil extractions;

— it is used in many places around the world.

After sampling, the nematodes are extracted from the soil using the Oostenbrink elutriator[1)] (model III) (see Figure A.3 and Annex B). In this technique, an upward current of water separates the nematodes from soil particles and holds them in suspension while the heavier particles sink[10],[33],[44],[49]. This suspension of nematodes and small particles passes through three sieves (mesh width: 45 µm). The catch is washed from the sieves onto a cotton-wool filter (milk filter). The cotton-wool filter is mounted on a supporting sieve and is placed in a dish with 100 ml of tap water. For three days, through their active downwards movement, the nematodes separate themselves from the debris on the filter. Thus, the living nematodes actively crawl through the filter in a dish with tap water.

After extraction, the nematodes are counted in 2 times 10 ml of the 100 ml suspension, then concentrated, preserved and mounted on mass slides. Finally, at least 150 individuals or a fixed percentage of the total number in the sample is identified under the microscope.

Mature nematodes can be identified to species level. However, populations in the soil are often dominated by juveniles and the genera level of taxonomy is a practical (but less sensitive) way of distinction.

Alternative extraction methods such as the Seinhorst elutriator[44], Baermann funnel (see Annex C) can be useful under special circumstances, but are not recommended as general procedures because the Oostenbrink elutriator is robust, easy to operate and usually quantitatively superior to most other techniques. For preserved samples containing non-living organisms, the Oostenbrink elutriator method is not suitable. Here, flotation/centrifugation methods using colloidal silica are recommended as they allow the extraction of all the forms of nematodes[26].

NOTE 2 This document is not applicable for aquatic nematodes because these nematodes do not pass through the filter. Special centrifugation techniques are available for sediment samples.

NOTE 3 Identification with a light microscope is based on morphological characteristics. In some cases, it is not possible to recognize the specimen on species or even genus level, e.g. juveniles. New techniques, such as DNA barcoding or metabarcoding,[40],[54] offer a morphology-independent taxonomy, so that also juveniles can be identified to genus or species level.

NOTE 4 The sampling of nematodes is often included in much broader monitoring programmes which try to cover the whole soil fauna or parts of it (e.g. the mesofauna). Examples of the use of soil invertebrates are given in Annex D. The design of such programmes is not included in this document.

5 Reagents

5.1 Formalin [formaldehyde solution, 60 ml/l].

5.2 Paraffin, with melting point near 60 °C.

6 Apparatus

Use standard laboratory equipment and the following.

6.1 Sampling

6.1.1 Soil sampler, of an open, closed or split-tube type.

EXAMPLE Grass plot sampler (diameter: 23 mm) or soil auger (see Figure A.2); commercially available.

6.1.2 Plastic bucket (collection of soil samples in the field).

1) Oostenbrink elutriator is the trade name of a product supplied e.g. by MEKU Erich Pollähne GmbH (https://www.meku-pollaehne.de/Nematologie/Oostenbrink-Elutriator/oostenbrink-elutriator.html). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6.1.3 Plastic container, for mixing of the bulk-sample.

6.1.4 Sieve, with 8 mm apertures.

6.1.5 Coated bags or **plastic bags** or **glass vessels** (transport and storage).

6.1.6 Permanent marker or **pre-printed labels**.

6.2 Extraction

6.2.1 Beaker, of capacity 100 ml to 250 ml.

6.2.2 Balance, able to weigh 1 kg to 25 kg, for weighing the total sample mass.

6.2.3 Oostenbrink elutriator [1] (see also Figure A.3, Annex B), metal funnel with an upward water flow to separate nematodes from larger soil particles.

6.2.4 Three sieves, with 45 µm apertures and 30 cm diameters.

6.2.5 One sieve, with 250 µm apertures and a 10 cm diameter.

6.2.6 Plastic bowl, of capacity approximately 2 l.

6.2.7 Clamping ring.

6.2.8 Extraction sieve, with 1 000 µm apertures and 16 cm diameter.

6.2.9 Milk- or **cotton-wool filters**.

6.2.10 Shallow trays (Petri dishes) or **special extraction dishes**.

6.2.11 Glass vessel, of capacity 100 ml, with a screw-cap.

6.3 Counting

6.3.1 Dissecting microscope, 10× to 50× magnification.

6.3.2 Small counting dish with grid or glass slide with grid.

NOTE Counting dishes in several sizes and different grids are available from the manufacturers of laboratory equipment. They can also be made out of small plastic Petri dishes by scratching a grid on the bottom with a needle.

6.3.3 Simple hand counting device.

6.3.4 Aquarium pump, for mixing nematode suspensions.

6.3.5 Pipette (drop glass), with adjustable volume.

6.3.6 Handling needle.

6.3.7 Bottle, of volume 100 ml.

6.4 Fixation and preparation of mass slides

6.4.1 Water jet pump, for concentration of suspension.

6.4.2 Glass slides, 50 mm × 76 mm.

6.4.3 Cover glasses, 45 mm × 45 mm.

6.4.4 Electric heating plate.

6.4.5 Metal stamp, 40 mm × 40 mm, for paraffin seal on glass slides.

6.5 Identification

6.5.1 Microscope, magnification 400× to 1 000×.

6.5.2 Ocular micrometer indicator.

6.5.3 Identification keys[15].

6.5.4 Standard form, to list the identification results.

7 Procedure

7.1 General

For quality assurance, each sample shall be given a unique code from the moment it is taken in the field. This code (label) shall stay with the sample during all the processing and analysis steps. Standard (electronic) form(s) should be used to follow the routing of the samples and collection of analysis results. These basic data may be combined in a spreadsheet or database file for further calculations and statistical testing.

7.2 Sampling

While the density and diversity of soil nematodes are the highest in the top 10 cm of the mineral soil, a grass plot sampler (6.1.1) with a 10 cm or 15 cm long sampling-tube is appropriate for most biomonitoring purposes. It is recommended to use a closed tube with a fixed length and diameter.

EXAMPLE 1 A grass plot sampler consists of a stainless-steel gouge auger (available in different dimensions) consisting of a steel auger pipe, a collecting bucket (6.1.2) and a stick with a steel handle. Because of the conical shape of the pipe, the sample is easily pushed toward the collecting bucket when the next sample is taken. The sample depth is constant and soil cores can be collected easily over a large area (see Figure A.1). This device can be used in many situations.

EXAMPLE 2 Alternatively, a soil auger can be used as a simple, cheap and quick working device. Augers are available in different diameters. Soil samples collected with an auger are less compressed. The disadvantage is that soil material can be lost more easily (see Figure A.2).

EXAMPLE 3 When accurate separation of soil layers is required, a split-tube sampler can be used. This sampling device needs more handling time and is less suited for large numbers of samples and large areas (see Figure A.2).

Samples from deeper layers can be taken with an auger to avoid excessive soil compression, or special split-tube samplers (see Figure A.2). Organic or litter material can be included in the samples, but it increases the numbers of nematodes found, sometimes considerably. Organic layers may be sampled independently. In this case, a wider split-tube corer (5 cm to 10 cm) is preferred in order to separate

the organic horizons from the mineral material. Small amounts of litter can also be treated in an Oostenbrink elutriator (6.2.3) to extract the nematodes. Extraction efficiency can be enhanced by soaking and blending the organic parts[41],[43].

When a representative sample is required from a specific type of ecosystem, a typical area of at least 5 000 m^2, and preferably 10 000 m^2, shall be selected. It is recommended to select an area which is (more or less) homogeneous in terms of soil properties, vegetation and soil-use. The studied surface is reported as part of the location information. As a rule of thumb, 100 soil cores shall be combined from 10 000 m^2. For smaller areas (e.g. 100 m^2), approximately 25 cores are sufficient to get an impression of the average nematode composition and to collect enough soil material. A denser sampling pattern results in a higher accuracy in the estimation of nematode abundance and species composition. However, there is a trade-off with the amount of subsample that is finally analysed from the homogenized bulk soil sample. So, a very large bulk-sample does not give more information because only a small part is analysed and homogenization cannot be completely perfect. Three hundred samples with a grass plot sampler (diameter 23 mm) are recommended as a maximum for a composite bulk-sample. In a biomonitoring programme, the sample density per surface area should preferably be equal in all locations. The mass of the bulk-sample and the number of soil cores in it need to be known.

The sampling plan may be regular (grid), according to a pattern or random. For larger locations, it is most practical to walk a zigzag pattern and take arbitrary samples along this route. In the case that a location consists of different parcels, the number of soil samples shall be distributed over the parcels based on their area. Samples from very atypical parts of the location such as ditches, tracks or pathways shall be avoided.

The collected bulk-sample is homogenized in a plastic container (6.1.3). This can be done in the field or laboratory, depending on the most practical way of working and transport. Mixing starts with crumbling of the cores through an 8 mm aperture sieve (6.1.4). Subsequently, the soil is gently mixed until the mass is uniform in colour and consistency. Mixing and preparation of the sample can take more than an hour for bulk-samples from clay soils or densely rooted top soils. However, this step is essential to all the analyses that are based on it and should be given enough attention. Coarse organic material, roots and stones shall be removed. The final choice for details of working shall be specified in the field sampling protocol, and again shall be uniform for the entire biomonitoring programme.

When the bulk-sample is homogenized, approximately one litre is taken out for further examination. This can be done by several spoonful from different parts of the bulk to obtain a representative subsample. Put the sample in a labelled plastic bag or glass vessel (6.1.5). At this point, more subsamples can be taken from the bulk for other biotic and abiotic analyses. Soil samples for nematode analysis shall be stored at 4 °C prior to extraction. The storage period should be kept as short as possible, four weeks at the maximum. This temperature may not be optimal for all nematodes (e.g. Aphelenchoididae, Anguinidae; see Reference [13]). In any case, the appropriate temperature shall be checked beforehand when sampling outside of the holarctic region (e.g. in the tropics).

7.3 Extraction

Mix the soil (sub)sample from the field again before extraction and fill a beaker (6.2.1) with 100 ml to 250 ml of soil. Weigh the sample in order to convert the results to a fresh-weight basis. In another sub-part of the same soil sample, measure the soil humidity to express final density as a unit of soil dry-weight. Store the remaining sample for abiotic analysis, unless material was collected separately in the field from the same bulk-sample.

Prepare the Oostenbrink elutriator (6.2.3). Put the sample in the top sieve and wash the soil in the elutriator. Specific water flow speeds depend on the type of funnel used. A detailed description of the apparatus and the way to use it is given in References [10] and [49]. The latest version of the Oostenbrink funnel has larger dimensions and is fully automated (see Figure A.5). It is suited for routine extraction of large numbers of samples. The soil sample is washed into the funnel through the top sieve. When