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Soil quality — Sampling of soil invertebrates —

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

Qualité du sol — Prélèvement des invertébrés du sol —

Partie 2: Prélèvement et extraction des micro-arthropodes (Collembola et Acarina)

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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 23611-2:2006), which has been technically revised.

The main changes are as follows:

— an additional Note was added in 7.3.2.1 with the description of an alternative method to the classic pre-heating techniques for specimen preparation for Collembola taxonomic identification;

— the bibliographic references list was revised and updated.

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 was prepared in response to a growing need for the standardization of sampling and extraction methods of soil micro-arthropods. These methods are needed for the following purposes:

— biological classification of soils including soil quality assessment (e.g. References [19], [24], [27], [30], [36], [40], [41]);

— terrestrial bioindication and long-term monitoring (e.g. References [3], [12], [14], [19], [31], [34], [37]).

Data collected by standardized methods can be more accurately evaluated, allowing more reliable comparisons between sites (e.g. polluted versus non-polluted sites, changes in land-use practices).

From the several micro-arthropod groups, Collembola and Acarina are the most studied in soil ecology. Their relevance for the soil system comes from their high abundance and diversity, and also from their role in key biological processes. Collembola and Oribatid mites act mainly as catalysts in organic matter decomposition,[6],[21] whereas predacious mites can act as webmasters in soil food webs.[21],[26] These characteristics, allied to a widespread taxonomic knowledge, allow their use as study organisms in several research programmes dealing with the impacts of forest practices (e.g. References [8], [16], [17], [18], [22], [23], [24], [28], [29], [32], [33], [35], [42]) or crop management practices (e.g. [2], [7], [10], [13], [20], [25], [43], [44].). These features make them suitable organisms to be used as bio-indicators of changes in soil quality, especially due to land-use practices and pollution[38].

For the sampling design of field studies in general, see ISO 18400-104[45] for general guidance on the development of site investigation strategies and detailed guidance on the development of sampling strategies.

Methods for other soil organism groups, such as earthworms, enchytraeids, nematodes and macro-invertebrates are covered in ISO 23611-1[52], ISO 23611-3[53], ISO 23611-4[54] and ISO 23611-5[55], respectively.

Soil quality — Sampling of soil invertebrates —

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

1 Scope

This document specifies a method for sampling, extracting and preserving collembolans and mites from field soils as a prerequisite for using these animals as bio-indicators (e.g. to assess the quality of a soil as a habitat for organisms).

The sampling and extraction methods of this document are applicable to almost all types of soils. Exceptions can be soils from extreme climatic conditions (hard, frozen or flooded soils) and other matrices than soil, e.g. tree trunks, plants or lichens.

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 terminology 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
micro-arthropod
group which is defined by its small size (range size from 100 µm to a few millimetres) making up a significant part of the below-ground food web in many terrestrial ecosystems

Note 1 to entry: This group is mainly composed by mites (Acarina), springtails (Collembola), Protura, Diplura, garden centipedes (Symphyla), Pauropoda, small centipedes and millipedes, small arachnids (spiders and pseudoscorpions), and insects and their larvae from several orders (Hymenoptera, Diptera, Coleoptera, etc.).

4 Principle

Soil samples are collected in the field using a split corer. Soil cores are placed in plastic tubes (or plastic bags) and transported to the laboratory. Afterwards, Collembola and Acarina are rapidly (within a few days) extracted by behavioural methods, using a MacFadyen apparatus, and preserved for future identifications. [12],[34] In addition, preparation techniques are also described. Finally, abundance values can be recalculated related to area (usually 1 m^2), volume or weight (usually 1 kg).

NOTE Alternative methods for extraction can be used under special circumstances. Flotation methods (e.g. the heptane flotation method) can be used in clay or loamy soils and a Kempson extractor (6.18) can be used in the case litter is sampled[34].

5 Test materials

5.1 Biological material

Collembola (springtails) are small wingless hexapods (from 150 µm up to 9 mm in length), having a distinctive head with a pair of antennae, without true compound eyes, with six abdominal segments and three pre-genital appendages in the abdomen. In the first segment, there is the ventral tube (or collophore) that is used for adhering to smooth surfaces. The name Collembola comes from this structure (from Greek *colla* = glue and *embolon* = bar). In the third segment, there is the *tenaculum*, which holds the jumping apparatus on its normal position. This jumping appendage, the *furcula* (or spring), is located in the fourth segment, when present. Springtails live in litter and soil, and have very distinctive life forms. They belong to the class Collembola and can be separated into 33 families[4].

Soil mites are small chelicerate arthropods related to spiders (length from 150 µm up to < 5 mm), living in soil and litter and presenting very distinctive life forms. They belong to the class Arachnida, subclass Acarina, and can be separated into four groups: Cryptostigmata (Oribatida), Mesostigmata (Gamasida), Prostigmata (Trombidiformes) and Astigmata.

NOTE Some hints for the taxonomy of springtails and mites are given in Annex A.

5.2 Reagents

Unless otherwise specified, use only reagents of good quality and distilled water.

5.2.1 Propan-2-ol, 80 % (volume fraction).

5.2.2 Formalin [formaldehyde solution 40 % (volume fraction)].

5.2.3 Acetic acid.

5.2.4 Phenol, C_6H_5OH, crystalline (carbolic acid).

5.2.5 Hydrogen chloride, *c*(HCl) from 8 mol/l to 10 mol/l.

5.2.6 2,2,2-Trichloro-1,1-ethanediol (chloral hydrate).

5.2.7 1,2,3-Trihydroxypropane (glycerine).

5.2.8 von Törne fixative, used to preserve the extracted animals and composed by Propan-2-ol (80 %), formalin (40 %) and glacial acetic acid (a volume fraction 10:0,3:0,03).

5.2.9 Nesbitt clearing medium, used to clear mite specimens composed of chloral hydrate (80 g), distilled water (50 ml) and concentrated hydrogen chloride (5 ml).

5.2.10 Lactophenol solution, used to clear mite specimens composed of lactic acid (10 ml), crystals of phenol (3,6 g) and distilled water (5 ml).

5.2.11 2-Hydroxypropanoic acid (lactic acid), to clear and observe micro-arthropod specimens, especially oribatid mites under the microscope.

5.2.12 Ethanol, 70 % to 75 % (volume fraction), used for fixation and preservation (in this case, also in combination with glycerine, 10:1).

5.2.13 Hoyer's medium, used to mount Collembola specimens composed of distilled water (50 ml), gum-arabic (30 g), chloral hydrate (200 g) and glycerine (20 ml).

5.2.14 DNA extraction buffer (SNET buffer solution), used to clear collembolans.

5.2.15 Protease K solution, used to clear collembolans.

5.2.16 Ethanol 35 % (volume fraction), used for preservation of the specimens.

5.2.17 Formol 3 %, used for preservation of the specimens.

5.2.18 Marc André 2 medium, to clear and provide the best optical properties to the specimens for identification.

6 Apparatus

Use standard laboratory equipment and the following.

6.1 Measuring tape.

6.2 Collecting flasks.

6.3 Wash bottle.

6.4 Forceps, **pipette**, **fine painting brush**, **fine needles**.

6.5 Petri dishes.

6.6 Stereomicroscope.

6.7 Microscope, with phase or interference contrast is preferable.

6.8 Microscopic slides, with excavated area in the centre, and **lamellae**.

6.9 Electrical heating plate.

6.10 Plastic vials.

6.11 Ceramic heating elements.

6.12 Pencil, **notebook**, **water resistant marker**, **labels**.

6.13 Split corer

Sampling device made of stainless steel or aluminium (40 cm long and e.g. 5,6 cm diameter may be used; the length and diameter should not differ considerably from these numbers, in order to maintain comparable conditions), used to collect soil cores (samples). It can be composed of two independent parts that fit together along the corer main axis or it can consist of one tube. On the top, it has a handle and, on the bottom, a cutting edge.

6.14 Glass vials.

6.15 Drying oven.

6.16 MacFadyen apparatus

High-gradient (multiple) device used to extract micro-arthropods from soil samples. The principle is to create an artificial temperature gradient between the canister where the sample is placed (hot) and the collecting device below (cold), inducing a negative thermotactic (at the same time, a positive hygrotactic, negative phototactic, and positive skototactic) behaviour on the animals that, by this way, leave the soil sample.

6.17 Plastic tubes, with caps (5 cm diameter, 5 cm long), or **plastic bags**, for storing the soil samples.

6.18 Kempson extractor, in the case litter is sampled.

6.19 Sample frame, 25 cm × 25 cm × 15 cm, made of stainless steel and with sharpened edges, to sample animals from the litter layer.

NOTE For details concerning the equipment in 6.13, and 6.16 to 6.19, see References [12] and [34].

7 Procedure

7.1 Collecting the soil samples

At each sampling point (previously defined according to sampling design rules), a soil sample is collected using a split corer (6.13); for flooded soils the same corer may be employed, but an auger tip should be present to retain the soil after extraction.

NOTE In addition to the general characterization of the site, it is useful to determine the actual moisture of the soil to be sampled.

After the sample is taken, the corer is opened (a picture of the soil core profile can complement the site characterization) and the soil core is separated into litter layer (including the humus horizon) and the upper 10 cm of the mineral soil. Generally, 5 cm layers are used for the upper part of the mineral horizon, but if a finer analysis is required, thinner layers can be defined. The depth of the litter layer should be registered. After this procedure, each layer is conditioned in plastic tubes; these are sealed with caps, labelled, and stored for transportation to the laboratory. Plastic bags can be used as substitutes of the plastic tubes (6.17), but special care shall be taken during conditioning to avoid disturbing the core structure and compaction of the soil material, that can lead to the death of animals. The time lapse between sampling and extraction should be recorded and should not exceed 5 days (if the samples remain at 20 °C ± 2 °C and the soil is kept moist), in order to avoid undesirable side effects due to confinement and shifts in micro populations.

If sampling of animals is restricted to the litter layer, a sample frame (6.19) is used instead. The frame is pressed into the litter by hand. Directly afterwards, the litter inside the frame is collected and the litter samples are placed in plastic bags (6.17), labelled, and stored.

When sampling in soil, the site should be physico-chemically characterized. In particular, pH, particle size distribution, C/N ratio, organic carbon content and water-holding capacity should be measured using ISO 10390[46], ISO 10694[47], ISO 11274[48], ISO 11277[49], ISO 11461[50], ISO 11465[51].

7.2 Extracting Collembola and Acarina from soil samples

In the laboratory, animals are extracted by behavioural methods, e.g. using a MacFadyen high-gradient extractor (6.16). Each sample core is placed inverted into the canister having a plastic or metal net (2 mm mesh size) on the bottom. This is connected to a funnel attached to a collecting flask (6.2) with 25 ml of "von Törne-fixative" (5.2.8).

Alternatively, a saturated solution of picric acid, a 50 % ethylene glycol solution (plus some drops of a detergent) or even 75 % ethanol (5.2.12) may be used as fixative.

A temperature gradient is created between the upper part (where the samples are placed) and the lower part of the system (where the collecting flasks are placed). Heat can be provided by ceramic heating elements (6.11), giving approximately 10 W per sample. The collecting flasks are immersed in a cooling

water bath. In some commercial apparatus, the temperature gradient is obtained by circulating heated air in the canister area and cooled air on the collecting area.

The temperature difference between the upper and lower parts should be around 30 °C to 35 °C, with the upper part being heated at 45 °C to 50 °C and the lower part being cooled usually at 10 °C. Special care shall be taken to avoid a fast increase in temperature in the upper part, which can cause the rapid desiccation of the sample and the death of the animals. Therefore, the temperature of the upper part should increase gradually, starting with approximately 5 °C above field temperature during the first three days, and intensifying the gradient for the next six to seven days.

The extraction procedure takes nine to 10 days. Afterwards, animal samples are labelled and ready to be stored until processing (sorting and identification). Extraction should start as soon as possible (i.e. the day of sampling). In case storage is necessary, the soil samples should be kept at 4 °C.

NOTE 1 The method described is only efficient for active live stages, with an average extraction efficiency of 75 % to 80 %.[5],[33],[36] Eggs, other quiescent stages, and animals enclosed in plant debris are not extracted by this method; alternatively, the heptane flotation method[39] can be used (see Annex B).

NOTE 2 The size of canisters can vary according to the type of apparatus. Commonly, plastic or metal canisters of 200 cm^3 (2,5 cm radius and 10 cm high) are used. Some commercial MacFadyen apparatus, however, use larger canisters of about 800 cm^3.

NOTE 3 Other types of apparatus using the same principle (e.g. Berlese-Tullgren funnel) can be used to extract the animals.

NOTE 4 Semi-permanent slide mounts can be obtained by directly mounting ethanol-fixed specimens (5.2.12) into a mixture of phenol (5.2.4), chloral hydrate (5.2.6) and lactic acid (5.2.11), then preserving the preparation from desiccation by successive deposits of nail varnish or similar resins.

WARNING — Appropriate precautions shall be taken when dealing with formalin to avoid danger from inhalation or skin exposure. According to the "Material Safety Data Sheet" for Formaldehyde 37 % solution as published by producing companies, the compound is a skin sensitizer and is considered to be carcinogen (humans: limited evidence; animals: sufficient evidence). It is legally notified in industrialised countries for scientific use.

7.3 Sorting, preserving and identifying Collembola and Acarina

7.3.1 Sorting and preserving

After extraction, animals shall be sorted into groups. This procedure is done under a stereomicroscope (6.6) using Petri dishes (6.5) to place the samples. Animals can be manipulated with a pipette or a fine brush (6.4) and transferred to plastic vials (6.10) containing ethanol at 70 % to 75 % (5.2.12) for further identification.

7.3.2 Identification

7.3.2.1 Collembola

For taxonomic identification, specimens are temporarily mounted on a cavity slide (6.8) in lactic acid (5.2.11) or in a permanent mount. After placing the cover glass (covering half of the slide) and adjusting the specimens, the slide is heated in an electric plate (6.9) until the body of the animal is totally cleared. After this, the specimen can be identified under a microscope (6.7) and the size can be measured, if required. The slide mount done by this process is not permanent and allows a better observation of the animal in all angles. A comprehensive analysis of the type of characters used for Collembola identification is given in Reference [11].

NOTE 1 Lactic acid (5.2.11) can be replaced by a mixture of lactic acid, glycerine (5.2.7) and formalin (5.2.2) (volume fraction of 5:1:5) during the heating process of clearing the specimen.

NOTE 2 Permanent slide mounts, using Hoyer's medium (5.2.13) as mounting solution, can also be made for identification purposes, after the heating process to clear the specimen.

NOTE 3 DNA extraction buffers (SNET; 5.2.14) can be used in clear Collembola specimens as an alternative to the classic pre-heating techniques. This procedure efficiently dissolves the inner body parts while keeping the cuticle of micro-arthropods in good conditions for subsequent taxonomic determination.[9] The clearing technique developed for DNA extraction, involves the addition of 200 µl of SNET buffer solution (5.2.14)[15] plus 10 µl of proteinase K solution (5.2.15) to micro-arthropod specimens. The specimens are formerly placed in small glass vials (6.14) and the residual ethanol can be removed with a pipette (6.4).[9] The clearing mixture is kept at 55 °C for 3 h to 5 h in a lab drying oven (6.15) and then 500 µl ethanol 35 % (5.2.16) and 1,5 µl formol 3 % (5.2.17) can be added directly to the mixture.[9] Finally, the samples can be stored at room temperature during 3 days (minimum) in order to remove the remaining buffer salts from the body of micro-arthropod specimens.[9] After this clearing procedure, the specimens can be mounted on microscope slides in a drop of Marc André 2 medium (5.2.18), which is the solution that provides the best clearing and optical properties especially after incubation of the specimens in DNA extraction solution (SNET)[9].

7.3.2.2 Acarina

Prior to taxonomic identification, heavy sclerotized or old specimens shall be cleared. This can be done by immersing the specimens in Nesbitt medium (5.2.9) for one to seven days, depending on the degree of sclerotization. For the Astigmata, no clearing is required in most cases; however, if this procedure is necessary, a shorter immersion time is needed. In the case of Oribatida, clearing is usually achieved by immersing the specimens in lactic acid (5.2.11). When dealing with extremely sclerotized specimens, additional heating can help. In the clearing process of Oribatids, the duration of the immersion in lactic acid depends on the degree of sclerotization. Immersion periods ranging from several hours to few days are used.

Identification can be done by temporary mountings done with lactic acid in cavity slides (6.8). In some specimens, dissection with fine needles (6.4) is necessary to observe fine structures.

Nesbitt media can be replaced by a lactophenol solution (5.2.10) or pure lactic acid (5.2.11). However, special care shall be taken to avoid the collapse of the animals. Specimens shall be immersed in a grade of water-alcohol solution before being transferred to the mounting media.

NOTE For permanent preparations, Hoyer's medium (5.2.13) or gum-arabic can be used as mounting media.

8 Assessment of results

The following measurement end points may be used for the bioclassification of a soil, including bioindication or biomonitoring (e.g. anthropogenic stress like chemicals or land use changes):

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

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

— diversity indices (alpha, beta and gamma diversity).

Firstly, the number of individuals (total number or by species or group) is counted and expressed as individuals per sample. If the soil core has been divided into several vertical fractions, add up these values. Secondly, the total abundance of individuals is multiplied by a factor (509 in the case of a 5 cm diameter split corer) to obtain the number of individuals per square meter.

To convert the abundance per area to abundance per volume, the abundance of the sample is divided by the volume of the core; and the result is the number of individuals per cubic centimetre. If the soil core has been divided into several layers, the total depth of the core is the sum of all the depths.

To obtain the number of individuals per unit mass (generally by kilogram), the abundance of the sample is divided by the dry mass of the sample (obtained by weighing the sample after drying it in an oven at 105 °C for 15 h to 20 h after the extraction). If the soil core has been divided into several layers, the total mass of the core is the sum of all individual masses.

9 Study report

The study report shall include the following information:

a) a reference to this document, i.e. ISO 23611-2:2024;

b) a full description of the study design and procedures, including any deviations from the procedures;

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

d) sampling method;

e) description of the sampling conditions, including date and duration of sampling in the field and weather parameters, such as air temperature and humidity, rain or snow;

f) details of the extraction procedure of the biological material;

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

h) a summary of the results obtained;

i) discussion of the results;

j) all information, including all measured raw data and all problems which have occurred, developed during all phases of the study.

Annex A
(informative)

Species determination in collembolans and mites

For Collembola, approximately 9 000 species are identified worldwide, but extrapolation to the actual number is difficult. New species are discovered yearly and the cumulative plot of new species per year shows no signs of stabilization.[1],[11] Collembola are distributed in every habitat of the world and recent checklists of species for country and biogeographical regions are given in Reference [4].

Regarding mites, several thousand species are known to science. However, this represents a fraction of the true number, since local faunas of many countries/regions are still not studied.

Some identification keys for Collembola and Acarina are the references [56] to [85]. A comprehensive list of identification keys for mites (mainly Oribatida) is also given in Reference [3].

Annex B
(informative)

Alternative method for sampling of micro-arthropods

For those soil types where the behavioural method described is not so efficient, the heptane flotation method can be used.[39] This method takes the advantage of the arthropod cuticle to adhere to heptane. It is performed in several steps.

a) Pour the sample into a graduated 1 l cylinder and add 50 % ethanol (volume fraction) to 1 l line.

b) Add about 10 ml of heptane (the heptane layer is approximately 2 cm thick).

c) Plug the flask with a rubber stopper and carefully invert the cylinder and allow the heptane to rise to the other end. When most of the heptane has risen to the top, invert the cylinder again and wait for the heptane to rise. Never shake and repeat this procedure twice.

d) Allow the cylinder to stand until fine soil particles settle to the bottom (minimum 4 h).

e) With minimal disturbance of sediment, pour heptane layer through a fine sieve, turning the cylinder as it is poured, in order to rinse all sides. Stop before the particles from the sediment enter the sieve.

f) Rinse the sieve thoroughly with 95 % ethanol (volume fraction) in order to remove all heptane, and finally rinse the contents of the sieve into a sample container.

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