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Soil quality — Sampling of soil invertebrates —

Part 1: Hand-sorting and formalin extraction of earthworms

Qualité du sol — Prélèvement des invertébrés du sol —

Partie 1: Tri manuel et extraction au formol des vers de terre

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Foreword

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ISO 23611-1 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 free-living stages of terrestrial nematodes*

Introduction

This part of ISO 23611 has been drawn up since there is a growing need for the standardisation 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 [21], [26], [34];

— terrestrial bioindication and long-term monitoring [9], [12], [28];

— evaluation of the effects of chemicals on soil animals (ISO 11268-3).

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 standardised methods is one of the most important reasons why bio-classification and bio-assessment in terrestrial (i.e. soil) habitats has so far relatively rarely been used in comparison to aquatic sites.

Originally, the methods described here were developed for taxonomical and ecological studies, investigating the role of earthworms in various soil ecosystems. These animals are without doubt the most important soil invertebrates in temperate regions and, to a lesser extent, in tropical soils [25], [13], [15]. Since Darwin (1881), their influence on soil structure (e.g. aeration, water holding capacity) and soil functions like litter decomposition and nutrient cycling is well-known [8]. Due to their often very high biomass they are also important in many terrestrial food-webs.

Since it is neither possible nor useful to standardize methods for all soil organisms, the most important ones have been selected.

Soil quality — Sampling of soil invertebrates —

Part 1:
Hand-sorting and formalin extraction of earthworms

1 Scope

This part of ISO 23611 specifies a method for sampling and handling earthworms from 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 earthworms and their use as bioindicators in the terrestrial environment can be found in the references listed in the bibliography.

This part of ISO 23611 applies to all terrestrial biotopes in which earthworms occur. The sampling design of field studies in general is specified in ISO 10381-1 (see also Reference [38]) and guidance on the determination of effects of pollutants on earthworms in field situations is given in ISO 11268-3. These details can vary according to the national requirements or the climatic/regional conditions of the site to be sampled (see also Annex C).

This part of ISO 23611 is not applicable for semi-terrestrial soils and it can be difficult to use under extreme climatic or geographical conditions (e.g. in high mountains). Methods for some other soil organism groups, such as collembolans, are covered in other parts of ISO 23611.

This part of ISO 23611 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 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
earthworm
megadrile soil-inhabiting earthworms (length of adult individuals: few centimetres to more than 1 m) belonging to the order Oligochaeta (class Clitellata, phylum Annelida)

EXAMPLE Species of the families Lumbricidae (Holarctic), Glossoscolecidae (Latin America), Eudrilidae (Africa) or Megascolecidae [Asia, North America (Pacific Coast)].

2.2
peregrine species
earthworms occurring in many regions world-wide today, usually introduced by man

NOTE 1 Well-known examples of peregrine species are several lumbricid species like *Aporrectodea caliginosa* (originally coming from Eurasia, but now living also in the Americas and Australia) or the pan-tropical species *Pontoscolex corethrurus* (probably coming from Northern Brazil and/or the Guyanas).

NOTE 2 See Reference [15].

2.3
clitellum
ring or saddle of epidermal thickening only in mature worms which is near the anterior and eventually forms the cocoon

3 Principle

Earthworms at a certain site are sampled from the soil by using a combination of two different methods:

— hand-sorting animals from a certain area (e.g. 0,25 m^2) or volume (e.g. 50 cm × 50 cm × 20 cm);

— extraction of worms from the soil by applying formalin.

Both methods are well-known for a long time [5],[20]. After extraction, the earthworms are fixed and transported to the laboratory. There they are preserved in a way that they can be stored in a collection indefinitely (e.g. for taxonomical purposes). In addition, the determination of the biomass of earthworms is described. Finally, abundance and biomass values can be recalculated to area (usually 1 m^2) or, more rarely, volume parameters.

NOTE 1 Alternative methods can be useful under special circumstances (e.g. electrical extraction or the use of mustard as a chemical expellent), but cannot be recommended as a general procedure (see Annex A). In addition, the use of other extraction chemicals than formalin seems to have no advantages concerning extraction efficiency [11]; [33].

NOTE 2 The sampling of earthworms is often included in much broader monitoring programs, trying to cover the whole soil fauna or parts of it (e.g. the macrofauna). The design of such programs is not included in this part of ISO 23611 [1].

NOTE 3 Some hints for the taxonomy of peregrine (occurring in many regions world-wide) earthworms, mainly belonging to the family Lumbricidae, are given in Annex B.

4 Reagents

4.1 **Formalin** [formaldehyde solution 4 % (volume fraction)].

4.2 **Formalin** [formaldehyde solution 37 % (volume fraction)].

4.3 **Ethanol**, 70 % (volume fraction).

5 Apparatus

Use standard laboratory equipment and the following.

5.1 **Plastic vessels,** capacities 250 ml and 500 ml, for storing the worms.

5.2 **Plastic gloves**.

5.3 **Forceps**.

5.4 **Piece of thick plastic sheeting**, 1 m^2 to 2 m^2.

5.5 **Spade** or **shovel**.

5.6 **Dissecting microscope,** with low magnification (10 to 40 times).

5.7 **Balance,** able to weigh from 0,01 g to 200 g.

5.8 **Water-can,** preferably 20 l, with water (20 l per sampling plot).

5.9 Watering can.

5.10 Pencil, **notebook**, **water resistant marker**, **labels** that go in the vessel.

5.11 Thermometer, e.g. for measuring air temperature.

5.12 Drying cabinet, for soil moisture determination.

6 Procedure

6.1 Sampling of the earthworms

6.1.1 General

Sampling of earthworms is done by a combination of two different methods: hand-sorting and formalin extraction. Based on several comparative studies, this combination is clearly recommended in the various reviews on earthworm ecology (e.g. References [7], [8], [15]).

Sampling should be done at times of the year where the animals are not forced by the environmental conditions (i.e. low soil moisture and/or high temperatures) into diapause (i.e. are not reacting to formalin). In temperate regions, such unfavourable sampling times are winter and, in particular, midsummer periods [15]. Earthworms sampled from the same plot, but sampled under the two different methods, should be stored in individual plastic vessels. After the end of the sampling process, the excavated and examined soil is returned to the original sampling plot. In some cases, it is appropriate to use only one of the two methods; e.g. when no deep-burrowing animals are occurring at a given site, formalin extraction is not necessary. On the other hand, at sites where giant earthworms are living (parts of South America, South East Asia and Australia), hand-sorting is not useful [22]. A very similar method, known as modified TSBF method, is particularly suitable for tropical regions (see Annex C).

NOTE Usually the earthworms are determined after preservation, but if the species spectrum of a sampling site is well known, worms can also be determined alive [30]; see also Annex B.

In the case collected earthworms are to be used for further analysis or testing, e.g. for biomarker measurements or for use in bioassays, storage or incubation of the worms in a small portion of soil from the sampling site is recommended. In the case of formalin extraction, rinsing the worms in tap water is needed before incubation in soil.

6.1.2 Hand-sorting

Due to the individual size of the worms, a large plot shall be identified. A square of 50 cm × 50 cm is often sufficient in the Holarctic where most adult earthworms have approximately a length between 1 cm and 20 cm. However, at places with a low density of earthworms [e.g. soils with low pH (< 4,5) or which are anthropogenically used like crop sites], larger plots (i.e. 1 m^2) are recommended (ISO 11268-3). On the other hand, at sites with a high earthworm density (e.g. many meadows in temperate regions), a smaller plot of 1/8 m^2 is sufficient [24]. Even smaller sample sizes (e.g. 1/16 m^2 ; [36]) can lead to very low, and thus variable, individual worm numbers per sample, which in turn leads to an increase in sample numbers (i.e. 16 replicates).

In any case, the soil is removed by means of a spade or shovel (5.5) up to a depth of 20 cm from this plot (20 cm are suitable for many temperate sites, but the depth also depends on the site properties). The excavated soil is spread out on a piece of plastic (5.4); this can be done in the field but, especially in periods of bad weather, the whole procedure can also be performed in the laboratory or greenhouse. Afterwards, the soil is searched cautiously for earthworms. Big earthworms are collected by hand using plastic hand gloves (5.2) and small ones by using forceps (5.3). To avoid autotomy and further damage of the worms, the animals should only be touched at the anterior part of the body. If worms are cut by the spade used to dig out the soil, both parts are collected in order to measure the correct biomass, whereas only front parts are counted when determining the number of individuals.

NOTE 1 With a naked eye, the front end of adult worms can be identified by the position of the clitellum: it is always located closer to the head than to the tail.

The collected earthworms should immediately be fixed in 70 % ethanol (4.3) using the 250 ml or 500 ml plastic vessels (5.1) for at least half an hour, but not longer than 24 h. The vessels shall be labelled and observations (e.g. whether worms have been in a quiescence stage) should be recorded in the notebook (5.10).

An immediate fixation in 4% formalin (volume fraction) (4.1) is possible, but not recommended due to the fact that the handling of this compound should be minimised as much as possible (in particular under field conditions).

NOTE 2 In order to avoid morphological changes (e.g. an inversion of the prostomium) due to immediate fixation in ethanol, the individual worms can be put briefly (about one minute) into warm tap water. The earthworms relax in the water, and after that they can be transferred to ethanol.

6.1.3 Formalin extraction

During the hand-sorting, the same plot from which the top soil has been removed is used for formalin extraction. A sufficient amount of water shall be transported (20 l per sampling plot) beforehand to the plots using large water-cans (5.8). The formalin solution (about 0,5 % volume fraction) is prepared by diluting 25 ml of 37 % formalin (volume fraction) (4.2) in approximately 5 l water using a watering can (5.9). Diluting 25 ml of 37 % formalin in 5 l water produces a solution of about 0,2 %. The diluted formalin solution is carefully and evenly applied into the plot from which the top soil has been removed for hand-sorting. This step shall be repeated until 20 l of formalin-solution are added; the amount of formalin solution can be adapted depending on the soil properties. During the application, the plot shall be observed in order to collect all earthworms appearing on the soil surface of the sampling plot. The sampling is finished 30 min after the application of the last watering can.

Large earthworms should be collected by hand using plastic gloves (5.2) and small earthworms by forceps (5.3). The repellent formalin produces rapid earthworm emergence of the soil; worms should only be collected when the largest (preferably whole) portion of the body becomes visible, otherwise damage or retraction back into the soil occurs. To avoid autotomy and damage of the worms, the animals should only be touched at the anterior part of the body, usually in front of the clitellum. The collected earthworms should immediately be fixed in 70 % ethanol (volume fraction) (4.3) using the 250 ml or 500 ml plastic vessels (5.1) for at least 30 min but not longer than 24 h. The vessels shall be labelled and any observations should be recorded in the notebook (5.10).

At sites where giant earthworms are occurring (South America, South East Asia, and Australia), and where hand-sorting is not appropriate to get them, formalin should be applied on an area of 4 m^2. Before doing so, herbs and litter shall be removed from the soil surface. In all other respects, the sampling process is the same.

It is not necessary to perform the formalin extraction if no vertical burrowers (especially *Lumbricus terrestris* or *Aporrectodea longa*) are living at a given site (e.g. in very acid soils). The occurrence of these large worms is indicated by surface casts and collected litter at their burrow openings (diameter approximately 0,5 cm) which are easily detectable before excavation of the soil used for hand-sorting. Therefore, any decision about the use of formalin shall be taken on a case-by-case basis.

NOTE 1 The use of formalin at low concentrations, as proposed here, does not cause problems for other soil organisms like arthropods and does not have long-term negative effects on microbial parameters [17]. The compound is microbiologically degraded within some days to several weeks. It can delay the development of certain plant species by the same order of magnitude [7].

NOTE 2 In soils with high clay content, the digging of the sampling pit can close tightly many earthworm burrows at the bottom of the pit, thus preventing the infiltration of formalin and/or the emergence of deep burrowing worms. In such conditions, the smeared burrow openings can be opened before the formalin application (e.g. using a knife).

WARNING — Appropriate precautions (i.e. gloves) 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.

6.2 Preservation

Two methods are possible:

a) after fixation, the animals can be kept in 4 % formalin (volume fraction) (4.1) for four days at minimum, but preferably one or two weeks. Afterwards, the worms can be stored for an unlimited period in 70 % ethanol (volume fraction) (4.3);

b) an alternative method is to fix the worms immediately after collection in a mixture of 70 % ethanol (volume fraction) (4.3) and 4 % formalin (volume fraction) (4.1) in a ratio of 98 % to 2 %. This preservation liquid is replaced by fresh preservation liquid on the day after sampling at the latest.

Preservation in pure ethanol should be avoided since the outer surface of the worms sometimes becomes very soft, so that important features are not visible any more.

In the case that earthworm tissue shall be preserved for biochemical or genetical studies, preservation in formalin is recommended [37].

6.3 Determination of biomass

Determination of biomass is performed using the preserved material. The animals are washed in water for 5 min, quickly dried on a piece of paper and subsequently the mass is determined using a suitable balance (5.7). Afterwards the worms are stored again in 70 % ethanol (volume fraction) (4.3) or in the ethanol/formalin mixture. Due to the change of mass during preservation and the soil content in the gut, the measurements can be corrected by using factors published in the literature to determine the biomass of the animals. According to the literature (e.g. References [7] and [15]), worms seem to lose about 10 % to 20 % of their mass during fixation. This is approximately the same mass as the mass of the gut content. Therefore, no compensation is necessary. Afterwards, the measured fresh mass can be converted to dry mass by multiplying by a factor of 0,15 [19]. However, this factor has been determined on the basis of mineral dwellers from grassland sites [19] and, site-specifically, it can vary considerably depending on the land-use form (e.g. in litter dwellers, it is smaller than in mineral dwellers).

NOTE If the earthworms have been identified alive, they can be directly weighed (either individually or in groups, according to species and age stage).

7 Data assessment

The following measurement endpoints 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 or volume);

— biomass (fresh or dry mass of the earthworms 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. the adult/juvenile ratio);

— morphological alterations in individuals.

Firstly, the number of worms is counted and expressed as individuals per sample (separately for hand-sorting and formalin samples). Secondly, both values are added in order to determine the total abundance of earthworms. This number is then multiplied by a factor in order to achieve the number of worms per square meter [the factor is four in the case 0,25 m^2 is used (usually 50 cm × 50 cm samples)]. Additionally, the age structure (juveniles and adults are differentiated by the presence of a clitellum) can be determined with the help of the dissecting microscope (5.6).