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Soil quality — Sampling of soil invertebrates —

Part 1: Hand-sorting and extraction of earthworms

Qualité du sol — Prélèvement des invertébrés du sol —

Partie 1: Tri manuel et extraction des vers de terre

Reference number
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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization.*

This second edition cancels and replaces the first edition (ISO 23611-1:2006), which has been technically revised. The main changes are:

— the use of a new chemical extraction compound, AITC (allyl-isothiocyanate), instead of formalin;

— the addition of examples of earthworm monitoring programmes (including presentation of their results) as an informative Annex E.

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

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[25][31][39];

— terrestrial bio-indication and long-term monitoring[11][14][33];

— 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.

Since it is neither possible nor useful to standardize methods for all soil organisms, the most important ones have been selected. In this document sampling of earthworms is described.

Originally, the methods described in this document 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 boreal and tropical soils[30][16][18]. Since Darwin (1881) (see Reference [8]), their influence on soil structure (e.g. aeration, water holding capacity) and soil functions like litter decomposition and nutrient cycling is well-known[10]. Due to their often very high biomass they are also important in many terrestrial food-webs.

In the previous version of this document the chemical formalin was recommended as extraction fluid. However, within the last years evidence increased that formalin does have critical properties, mainly in terms of human toxicity. In December 2012, the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) stated that there is sufficient scientific evidence to classify this chemical as "probably carcinogenic for humans (Category Ib). In addition, negative effects on non-target organisms (including soil microorganisms, mesofauna and plants) have been reported (e.g. see Reference [7]). Therefore, this substance has been replaced.

Due to the growing reservations against the use of formalin, several alternatives have been studied. In Reference [40] allyl isothiocyanate (AITC) was tested for its effectiveness as a chemical expellant for sampling earthworms. AITC is a natural breakdown product of glucosinolates in many Cruciferae, i.e. it is the component imparting the sharp taste of mustard. According to the European Chemical Agency (ECHA), there is no concern regarding its use under outdoor conditions.

Over the last years, some studies have been performed in which the extraction efficiency of formalin and AITC were compared at the same sites and dates. According to Reference [22] no differences were found in numbers or biomass of earthworms extracted at crop sites when using either formalin or AITC as extractant. In a recent unpublished review (see Reference [28]) no significant differences were reported in earthworm numbers/biomass when comparing the efficiency of the two extraction chemicals. Also, no interaction was found on the sampling sites between the extractant and the site, indicating that no site-specific differences were observed in extraction efficiency of the extractants. When plotting the correlation between worm numbers extracted with AITC versus formalin in a Bland-Altman graph (a common way to compare a gold-standard method to an alternative method in the medical sciences), no significant bias of the AITC method as compared to the formalin method was found, indicating the similarity / exchangeability of the two methods.

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.

Soil quality — Sampling of soil invertebrates —

Part 1:
Hand-sorting and extraction of earthworms

1 Scope

This document 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).

This document applies to all terrestrial biotopes in which earthworms occur. The sampling design of field studies in general is given in ISO 18400-101 and guidance on the determination of effects of pollutants on earthworms in field situations is given in ISO 11268-3. These aspects can vary according to the national requirements or the climatic/regional conditions of the site to be sampled (see also Annex C).

This document 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.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11260, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

ISO 11277, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

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 http://www.electropedia.org/

3.1
earthworms
megadrile soil-inhabiting earthworms belonging to the order Oligochaeta (class Clitellata, phylum Annelida)

Note 1 to entry: The length of adult individuals can vary from a few centimetres to more than 1 m.

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

3.2
peregrine species
earthworms occurring in many regions world-wide today, usually introduced by man

Note 1 to entry: 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 to entry: See Reference [18].

3.3
clitellum
ring or saddle shaped epidermal thickening only in mature worms which is near the anterior and eventually forms the cocoon

4 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) of varying depth, depending on land use (e.g. at crop sites: 20 cm), soil properties and the scope of the sampling;

— extraction of worms from the soil by applying AITC.

The first method is known for about 100 years while the second method using the new extraction fluid was proposed about 15 years ago[7][22][40]. 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), but cannot be recommended as a general procedure (see Annex A).

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 programmes is not included in this document.

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.

5 Reagents

5.1 Allyl-isothiocyanate (AITC), synthetic grade (about 94 % to 97 % (volume fraction).

5.2 Isopropanol, 100 % (volume fraction).

5.3 Ethanol, 70 % (volume fraction).

5.4 Formalin, formaldehyde solution 4 % (volume fraction), for storage purposes only.

5.5 Ethanol, 95 % (volume fraction), for storage purposes when using genetic methods such as barcoding.

6 Apparatus

Use standard laboratory equipment and the following.

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

6.2 Rubber gloves.

6.3 Forceps.

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

6.5 Spade or shovel.

6.6 Dissecting microscope, with low magnification (×10 to ×40).

6.7 Balance, weigh range from 0,01 g to 200 g.

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

6.9 Watering can.

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

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

6.12 Drying cabinet, for soil moisture determination.

7 Procedure

7.1 Sampling of the earthworms

7.1.1 General

Sampling of earthworms is done by a combination of two different methods: hand-sorting and AITC extraction. Based on several comparative studies, the combination of a physical and a chemical method is clearly recommended in the various reviews on earthworm ecology, independent from the type of chemical expellant (e.g. References [9], [10] and [18]).

Sampling should be done at times of the year when 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 AITC). In temperate regions, such unfavourable sampling times are winter and, in particular, midsummer periods[18]. 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, AITC 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[26]. 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[35] (see also Annex B).

In case the 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 AITC extraction, rinsing the worms in tap water is needed before incubation in soil.

For the interpretation of test results, the following characteristics shall be determined for the field site to be studied:

a) pH in accordance with ISO 10390;

b) texture (sand, loam, silt) in accordance with ISO 11277;

c) water content in accordance with ISO 11465;

d) water holding capacity as specified in Annex D;

e) cationic exchange capacity in accordance with ISO 11260;

f) organic carbon in accordance with ISO 10694.

7.1.2 Hand-sorting

The size of the sample plot should be chosen according to the expected mean size and density of the worms. 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 (see 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[29]. Even smaller sample sizes (e.g. 1/16 m^2[41]) can lead to very low, and thus variable, individual worm numbers per sample, which in turn leads to an increase in sample numbers (e.g. 16 replicates).

In any case, the soil is removed by means of a spade or shovel (6.5) 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 (6.4). 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 rubber gloves (6.2) and small ones by using forceps (6.3). 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 (5.3) using the 250 ml or 500 ml plastic vessels (6.1) for at least 0,5 h, but not longer than 24 h. In case the ethanol solution is diluted by body fluids and/or contains soil particles, the ethanol solution shall be exchanged after 24 h to 48 h. The vessels shall be labelled and observations (e.g. whether worms have been in a quiescence stage) should be recorded in the notebook (6.10).

