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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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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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).

An immediate fixation in 4 % formalin (volume fraction) (5.4) 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 (e.g. 30 °C to 40 °C) tap water. The earthworms relax in the water, and after that they can be transferred to ethanol.

7.1.3 AITC extraction

The same plot, from which the top soil has been removed for hand-sorting, is used for AITC extraction. A sufficient amount of water shall be transported (5 l to 10 l per sampling plot) beforehand to the plots using large water-cans (6.8). To prepare the AITC solution, an amount of 1 g of AITC is dissolved in 50 ml of isopropanol, and the resulting solution is made up with water to a total volume of 10 l. The density of AITC is 1,013 kg/l; i.e. using a volume fraction, the concentration would be given as 98,7 ml/l. The diluted AITC solution is carefully and evenly applied into the plot from which the top soil has been removed for hand-sorting. The solution should be applied in several portions (usually 2 to 3) according to the seepage capacity of the soil until 5 l to 10 l of AITC-solution have been added, depending on the soil properties (in cases where the sampling plot is larger than 0,25 m^2, the amount of AITC-solution shall be increased accordingly). 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.

AITC readily decays in water, and also in the stock solution in isopropanol. Decay is enhanced by sunlight and warmth. Thus, the final solution shall be prepared immediately before use.

Large earthworms should be collected by hand using rubber gloves (6.2) and small earthworms by forceps (6.3). The repellent AITC produces rapid earthworm emergence from 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) (5.3) using the 250 ml or 500 ml plastic vessels (6.1) 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 (6.10).

At sites where giant earthworms are occurring (South America, South East Asia, and Australia), and where hand-sorting is not appropriate to get them, AITC-solution should be applied on an area of 4 m^2 (its amount shall be increased accordingly) 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 AITC 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 AITC shall be taken on a case-by-case basis.

NOTE 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 AITC and/or the emergence of deep burrowing worms. In such conditions, the smeared burrow openings can be opened before the AITC application (e.g. using a knife).

WARNING — Appropriate precautions (i.e. gloves and protective clothing) shall be taken when dealing with AITC to avoid danger from inhalation or skin exposure. According to the "Material Safety Data Sheet" for AITC as published by producing companies, the compound is irritating to the skin and to the eyes and toxic to aquatic organisms. It is legally notified in industrialised countries for commercial (e.g. as a biofumigant) and scientific use.

7.2 Preservation

Two methods are possible.

a) After fixation, the animals can be kept in 4 % formalin (volume fraction) (5.4) 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) (5.3).

b) An alternative method is to fix the worms immediately after collection in a mixture of 70 % ethanol (volume fraction) (5.3) and 4 % formalin (volume fraction) (5.4) 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 not recommended[15]. Instead, the worms shall fixed and stored in ethanol (95 %).

7.3 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 (6.7). Afterwards the worms are stored again in 70 % ethanol (volume fraction) (5.3) 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 Reference[42] the loss during fixation varies between 10 % to 25 %, while the gut content is highly variable, but values around 20 % to 29 % are cited[43]. The loss of mass during fixation is approximately the same as the mass of the gut content[23]. Therefore, no compensation is necessary. Afterwards, the measured fresh mass can be converted to dry mass by multiplying by a factor of 0,15[23]. However, this factor has been determined on the basis of mineral dwellers from grassland sites[23] 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).

8 Data assessment

The following measurement end points can 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 AITC 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 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 (6.6).

9 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 23611-1:2018;

b) a full description of the test 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, distinguishing between methods (i.e. hand-sorting, AITC extraction);

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

h) if appropriate, biomass of caught worms;

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

j) all information, including all measured raw data, developed during all phases of the test;

k) discussion of the results;

l) all details not specified in this document or which are optional, as well as any incident which may have affected the results.

Annex A
(informative)

Other methods for sampling

A.1 Electrical extraction method

Thielemann[35] developed the Octet method as an alternative to hand-sorting and earthworm extraction with chemical expellants, using a ring of 8 electrodes (diameter: 52 cm) to generate an electrical field by which the worms are driven out of the soil. The advantages of this method are: no use of potentially toxic chemicals, no need of water and less laborious and faster than other methods. The disadvantages of the method include the heaviness and high price of the equipment. More importantly, the success rate of this method seems to depend quite strongly on the actual soil conditions (especially water content). Various methodical comparisons did not show clear advantages of electrical compared with chemical methods (e.g. Reference [38]). Therefore, it can only be recommended when several closely-related plots are investigated, e.g. when assessing the effects of a chemical, or in protected areas where hand-sorting is not allowed.

A.2 Extraction with mustard

Gunn[13] proposed to replace potentially toxic substances like formalin by mustard flour for extraction purposes. Mustard was selected since it also causes an avoidance reaction by earthworms. For example, 60 g of mustard flour are mixed in 10 l of water, which are applied to a certain surface area just like other expellents. Studies comparing different methods gave variable results which are difficult to assess, e.g. Reference [17] but, in general, they show a lower extraction efficiency of the mustard method compared to formalin, e.g. Reference [38]. As in the case of other behavioural methods, mustard extraction should be combined with hand-sorting since otherwise the population density of earthworms (in particular endogeic species) can be underestimated[21].

Annex B
(informative)

Species determination in earthworms

Identification of lumbricid earthworms sampled in most parts of Europe, as well as the eastern parts of Canada and the United States of America, follows the keys prepared in References [5], [12] or [32]. Reference [34] provides some dichotomic keys to also determine the juveniles of the most common lumbricids. An excellent introduction in earthworm species determination is given in Reference [19]. Up to now, no comprehensive keys on earthworms from other parts of the world are available. They are lacking especially for tropical families like Glossoscolecidae (Latin America), Eudrilidae (Africa) or Megascolecidae [Asia, North America (Pacific Coast)]. However, a very detailed description of the peregrine earthworms of the world has been compiled, covering 101 species (36 megascolecids, 34 lumbricids and 31 species from other families), see Reference [4].

All important, partly internal, partly external, features can easily be seen by means of a dissecting microscope. In case the earthworms are to be determined alive, the determination process can be facilitated by using small glass tubes in which the living earthworm is put[36]. Due to its inability to move, the external properties are easily detectable.

Annex C
(informative)

The modified TSBF method

The main features of the method proposed in this document are similar to the one known as the "modified TSBF method" (Tropical Soil Biology and Fertility[1]), i.e. both are based on a combination of hand-sorting and extraction with a chemical expellant. However, the scope of this method is broader, covering the sampling of the whole macrofauna. Originally developed for tropical regions, the modified TSBF method is also applicable in temperate regions. It is performed in three steps.

a) When litter is present, it is collected and put into resistant and hermetic plastic bags to avoid loss of litter fauna. During this process, visible fauna can also be picked up manually and stored in plastic vessels.

b) Soil blocks (monoliths) of 25 cm × 25 cm × 15 cm size are put into plastic bags and transported to the laboratory where they are hand-sorted.

c) In the remaining pit, formalin solution [0,2 % (volume fraction)] is sprayed at 10 min intervals in order to expel all "active" fauna remaining in the soil.

It is not clear whether and when the TSBF method will be modified regarding the use of formalin. For the time being it is assumed that AITC does work as part of the TSBF approach, meaning that an exchange of the extraction fluid is recommended.

Usually, three plots per sample area (e.g. a specific land use) are used. In each plot, 8 to 10 soil blocks are taken, each separated by a distance of at least 7 m. The sampling of these blocks is performed in transects, which are divided in parallel fragments if the whole plot is too small for one large transect.

Annex D
(normative)

Determination of maximum water-holding capacity

D.1 General

The following method has been found to be appropriate for determining the water-holding-capacity (WHC) of the soil from the study site.

D.2 Apparatus

D.2.1 **Glass tube**, approximately 20 mm to 50 mm diameter and at least 100 mm in length.

D.2.2 **Water bath**, at room temperature.

D.2.3 **Filter paper**.

D.2.4 **Drying oven**, set to (105 ± 5) °C.

D.2.5 **Balance**, capable of weighing with an accuracy of ±0,1 g.

D.3 Method

Plug the bottom of the tube with filter paper, and after filling with the artificial soil substrate to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil but below the upper edge of the tube. Leave the substrate sample in the water for about 3 h.

As not all water absorbed by the substrate by capillary can be retained, the tube containing the sample should be placed for a period of 2 h on very wet finely ground quartz sand for draining. The same quartz sand as is used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at 105 °C and reweigh it.

D.4 Calculation of the water-holding capacity (WHC)

$$WHC = \frac{m_S - m_T - m_D}{m_D} \times 100 \quad \text{(D.1)}$$

where

WHC is the water-holding capacity in percentage of dry mass, %;

m_S is the mass of the water-saturated substrate plus the mass of the tube plus the mass of the filter paper;

m_T is the tare (mass of tube plus mass of filter paper);

m_D is the dry mass of substrate.

Annex E
(informative)

Examples of earthworm monitoring programmes (including presentation of their results)

E.1 General

In order to improve the performance and, in particular, the presentation and interpretation of earthworm monitoring studies some examples are given in this annex. It should be noted that the information provided here is to be understood as an offer how monitoring data could be used.

In recent years essential contributions to a biological soil assessment were made in several European countries. Often, a "battery-approach" using several invertebrate groups as well as microbial parameters for the assessment of soil quality has been proposed (Griffiths et al. 2016)[48]. There is also a general agreement that monitoring results should be assessed using previously defined reference values [also called Normal Operating Ranges (NORs), which are defined for individual species (and thus ultimately communities)]. In order to operationalize the assessment of biodiversity, site-specific reference systems have been developed. In the Netherland, farms located on different soil types and different land-use intensities were sampled (e.g. Rutgers et al. 2008)[52]. In Germany, sites were classified into habitat types (Riecken et al. 2006)[50], based on soil and site parameters, while in France sites were classified according to their land-use (Cluzeau et al. 2012)[46]. In each case, species richness and total abundance were determined for each of these site groups (partly also a list of species expected to occur or to be absent was established). Significant deviations from these reference values are considered as an indication for an impacted habitat function of the soil (e.g. Jaensch et al. 2013). In France, the classification was established according to the soils textural classes used in the Database of Hydraulic Properties of European Soils (HYPRES).

NOTE While this information aims to be applicable globally for all terrestrial sites that are inhabited by soil invertebrates, the existing information refers mostly to Europe. However, theoretical considerations allow the conclusion that the information provided here are useful for other regions as well valid, especially in tropical Latin America (e.g. Gonzalez et al. 1996[47]; Römbke et al. 2009[51]).

E.2 Presentation of the results of a monitoring programme for earthworms

Earthworms are by far the best studied soil invertebrate group. The most recent compilation and meta-analysis of earthworm monitoring programmes has been done by Rutgers et al. (2016)[53] who collected data from all over Europe, but mainly from France, The Netherlands, Great Britain, Ireland and Germany. Smaller data sets were also provided by other countries such as Spain and Denmark. Based on this work it was possible to prepare earthworm maps of Europe, for example on total abundance or the number of species per site (Figure E.1). In addition, the occurrence of individual species could be mapped, e.g. for the ecologically important deep-burrowing species *Lumbricus terrestris*) (Figure E.2).

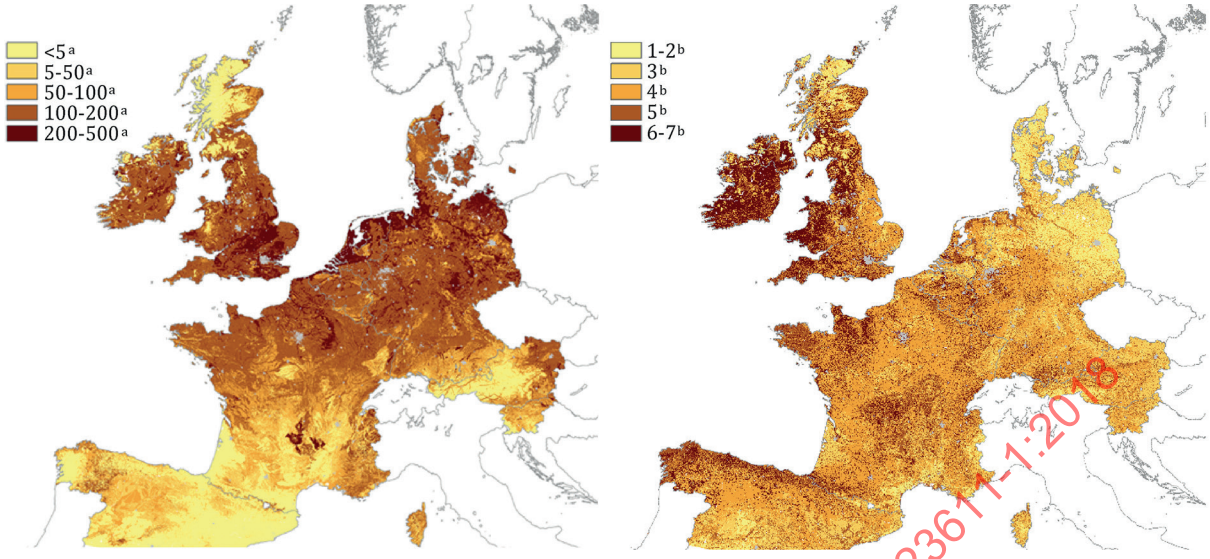

Figure E.1 — Predicted abundance (left) and predicted richness (right) of earthworm communities in Europe (Rutgers et al. 2016)[53]

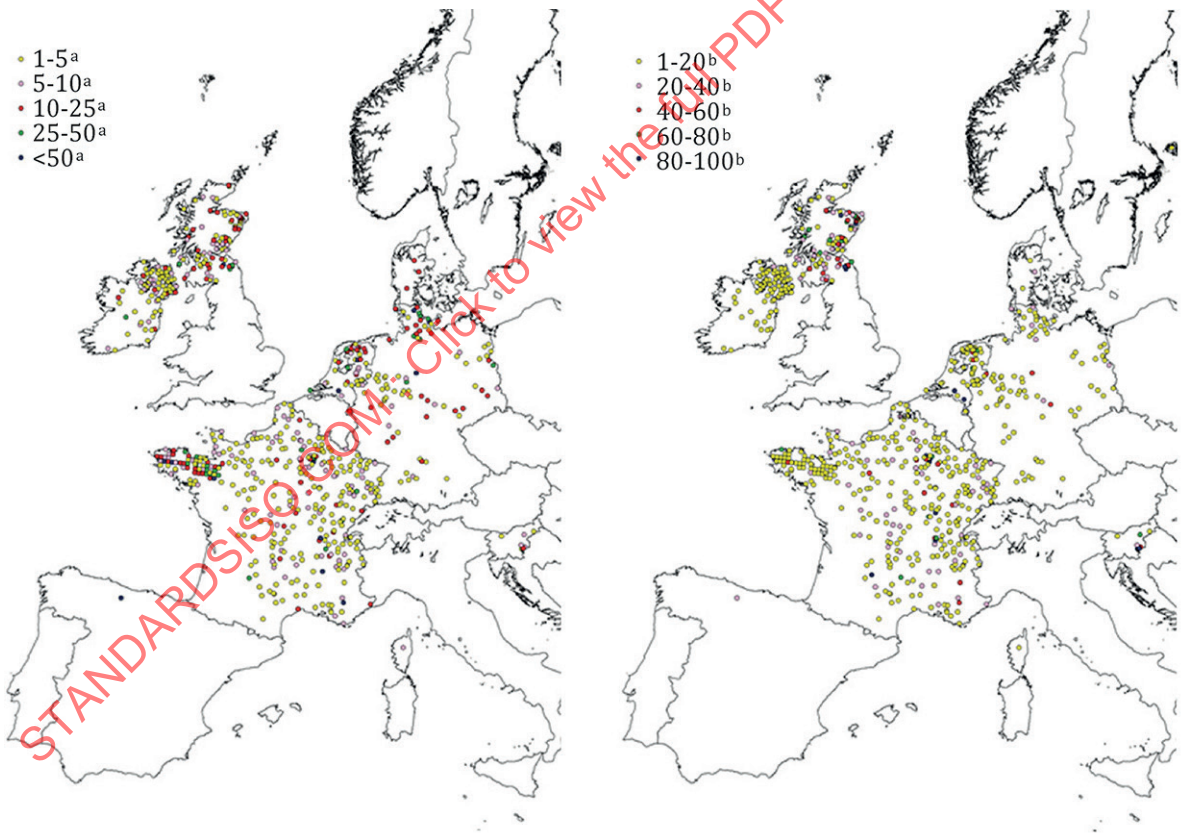

Figure E.2 — Occurrence and relative abundance of the anecic earthworm species *Lumbricus terrestris* (Rutgers et al. 2016)[53]

E.3 Assessment of the biological quality of soils

In a first step it shall be clarified whether the reference values for the same type of habitat (indicated by similar soil properties and climatic conditions) are comparable in different studies made in different European countries, but belonging to the same biogeographic region. In Table E.1, reference values derived in different ways but referring to sampling data gained with standard methods are compared. Earthworm abundance and species number are used as an example since data availability for this group is much better than for other soil organisms. Such approaches were also made in France (Cluzeau et al. 2012)[46] and Germany (e.g. Beylich and Graefe 2009[44]). Values are not easily comparable as not only the soil type is important to interpret the number and diversity of species but also the practices and the time of sampling.

Table E.1 — Comparison of mean values for earthworm abundance (Ind/m²) (left half of the table) and species number (right half of the table) for four habitat types in the Netherlands (RIVM) and Germany (UBA) and France (RMQS/INRA/University of Rennes)

Habitat classes	Dutch Reference Ind/m²	German Reference Ind/m²	French Reference Ind/m²	Dutch Reference Ind/m²	German Reference Ind/m²	French Reference Ind/m²
	Mean abundance Ind/m²			Mean species number		
Crop sites on clayey soils	200	102	259	4,2	4,4	3,2
Crop sites on loamy/silt soils	—	—	224	—	—	3,2
Crop sites on sandy soil	77	32	88	2,8	3,3	3,6
Grassland on clayey soils	793	42	418	8,3	9,1	8,0
Grassland on loamy/silt soils	—	—	383	—	—	8,7
Grassland on sandy soils	64	104	595	4,8	5,1	8,5

However, such information is difficult to compare and assess. The same is true when it comes to the use of diversity indices, since much information is lost in case all information gained in a monitoring study is put into one or few values. Therefore, a graphical way (called the Amoebae) of visualizing the information from many different organism groups and/or end points has been proposed in The Netherlands (Figure E.3) (Breure et al. 2003)[45]. It represents the distance between the actual measured value for a certain measurement end point (e.g. earthworm abundance). In this example these values were determined at a conventional fam site. The optimal area is defined in the outer white circle (it is based on data from a reference site; here a non-conventional farm) and it is set at 100 %, an intermediary quality is given by the grey circle (75 % of the optimum), and a low quality is displayed by the black circle in the middle (50 % of the optimum) (Breure et al., 2003)[45]. However, this deviation cannot directly be transformed into an ecological decision. For example: Is half the number of nematodes of equal importance as half the amount of enchytraeids?