
**Soil quality — Sampling of soil
invertebrates —**

**Part 3:
Sampling and extraction of
enchytraeids**

*Qualité du sol — Prélèvement des invertébrés du sol —
Partie 3: Prélèvement et extraction des enchytréides*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, 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 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 23611-3:2007), which has been technically revised. The main changes to the previous edition are as follows:

- addition of examples of enchytraeid monitoring programmes (including presentation of their results) as an informative annex.

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

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

Introduction

This document has been developed to address a growing need for the standardization of terrestrial zoological field methods. Such methods, mainly covering the sampling, extraction and handling of soil invertebrates, are needed for the following purposes:

- biological classification of soils including soil quality assessment (e.g. References [4], [25], [27], [31], [36]);
- terrestrial bioindication and long-term monitoring (e.g. References [4], [30]);
- evaluation of the effects of chemicals on soil animals (References [18], [26], [28]).

Data for these purposes are gained by standardized methods since they can form the basis for far-reaching decisions (e.g. whether a given site should be remediated or not). In fact, the lack of such standardized methods is one of the most important reasons why biological classification concepts in terrestrial (i.e. soil) habitats have so far been relatively rarely used in comparison with aquatic sites.

Originally, the methods described here were developed for taxonomical and ecological studies, investigating the role of enchytraeids in various soil ecosystems. These animals without doubt belong to the most important soil invertebrates in temperate regions (mainly in acidic soils^[7]). Their influence on soil functions like litter decomposition and nutrient cycling is well known^{[17][23]}. Due to their often very high numbers, and their population biomass, they are also important in many terrestrial food-webs^[6]. Some species have unintentionally been distributed by humans in many soils of the world.

Since it is neither possible nor useful to standardize methods for all soil organisms, the most important ones have been selected. Microbiological parameters are already covered by existing ISO standards (e.g. ISO 14240-1, ISO 14240-2, ISO 17601, ISO/TS 29843-1 and ISO/TS 29843-2).

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Soil quality — Sampling of soil invertebrates —

Part 3: Sampling and extraction of enchytraeids

1 Scope

This document specifies a method for sampling, handling and extracting enchytraeids from terrestrial 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 enchytraeids and their use as bioindicators in the terrestrial environment is included in the Bibliography.

This document applies to all terrestrial biotopes in which enchytraeids occur. The sampling design of field studies in general is given in ISO 18400-101. These details can vary according to the climatic/regional conditions of the site to be sampled and an overview on the determination of effects of pollutants on enchytraeids in field situations is given in Reference [6].

Methods for some other soil organism groups such as earthworms or arthropods are given in ISO 23611-1, ISO 23611-2, ISO 23611-4 and ISO 23611-5.

This document is not applicable for very wet or flooded soils and might be difficult to use under extreme climatic or geographical conditions (e.g. in high mountains).

When sampling soil invertebrates, it is highly recommendable to characterize the site (e.g. concerning soil properties, climate and land use). However, such a characterization is not covered by this document. 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 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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

- ISO Online browsing platform: available at <https://www.iso.org/obp/>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

enchytraeid

small soil-inhabiting worm (a few millimetres to several centimetres in length) belonging to the family Enchytraeidae, class Oligochaeta, superclass Clitellata, phylum Annelida

Note 1 to entry: The common name for enchytraeid is potworm^[35].

EXAMPLE Species of the genera *Enchytraeus*, *Fridericia* or *Cognettia*.

4 Principle

Enchytraeids at a certain site are sampled from the soil by using a split soil corer (diameter usually 3 cm to 6 cm) (6.1). After sampling, the soil samples containing the enchytraeids are transported to the laboratory. Then the enchytraeids are extracted from soil by means of a wet extraction method. (This approach has been well known for a long time[12][20][24]) After extraction, the enchytraeids are identified alive and, if required, preserved in such a way that they can be stored in a collection indefinitely (e.g. for taxonomical purposes).

The determination of the biomass of enchytraeids is also described in this document. The abundance and biomass values can be recalculated to the area of the soil corer or, more rarely, volume parameters.

NOTE 1 The sampling of enchytraeids is often included in much broader monitoring programmes which try to cover the whole soil fauna or parts of it (e.g. the mesofauna). Examples of the use of soil invertebrates are given in [Annex B](#). The design of such programmes is not included in this document (but see e.g. Reference [3]).

NOTE 2 Some hints for the taxonomy of enchytraeids are given in the Bibliography.

5 Reagents

5.1 **Tap water** (without toxic properties, e.g. due to copper contamination).

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

5.3 **Bengalred**, 4,5,6,7-Tetrachloro-2',4',5',7'-tetraiodofluorescein formulated as a staining agent.

5.4 **Bouin's fixative**, buffered solution of formaldehyde, acetic acid and picric acid.

5.5 **Paracarmin**, staining agent, prepared as a mixture of carmine acid, aluminium chloride and calcium chloride solved in ethanol.

5.6 **Canada-balm**, natural yellowish viscous fluid containing 13 % to 14 % (volume fraction) Canadin acid ($C_{20}H_{38}O_2$), 48 % to 50 % (volume fraction) α - and β -Canadinol acid ($C_{19}H_{30}O_2$) and 5 % (volume fraction) Canadoesen ($C_{21}H_{40}O$).

6 Apparatus

6.1 **Split soil corer** (e.g. diameter 3 cm to 6 cm; extracted core length 10 cm to 30 cm); length in total variable (depending whether or not a handle is used) and a plastic or wooden impact-absorbing hammer.

6.2 **Plastic bags** (e.g. 1-l freezer bags); general store.

6.3 **Temperature recorder** or a **minimum/maximum-thermometer**.

6.4 **Plastic bowls**, diameter approximately 20 cm, height approximately 10 cm; general store.

6.5 **Plastic sieves**, diameter approximately 15 cm, mesh width approximately 1,0 mm; general store.

6.6 **60 W bulbs** as a heating device; general store.

6.7 **Glassware**, for example petri dishes (square format) with a size of 8 cm × 8 cm or small glass vessels (e.g. 50 ml).

6.8 Large, sharp knife.

6.9 Refrigerator.

6.10 Dissecting microscope with low magnification (10 times to 40 times).

6.11 Microscope with high magnification (60 times to 400 times) and equipped with an interference lighting device.

6.12 Spring steel pincers (flat).

6.13 Transfer tool, pasteur pipette, soft steel forceps or a hooked needle.

7 Procedure

7.1 Soil sampling

The soil samples to be used for the investigation of the enchytraeid community are taken destructively by means of a split soil corer (6.1). The corer is carefully pressed into the soil. The depth depends on the land use and soil type, but usually varies from 10 cm (e.g. forests) up to 30 cm (e.g. crop sites), i.e. those layers in which the bulk of the enchytraeids are living. In rare cases, for example if thick roots are present, a plastic or wooden impact-absorbing hammer can be used to take the samples. After removing the soil corer, its valve is opened and the soil core is carefully taken out by hand. The core is divided into cylinders (e.g. 3 cm to 4 cm in height) with a knife (6.8). These soil cylinders may be stored in small plastic bags (6.2) in a refrigerator (6.9) at approximately 4 °C to 6 °C for a period of preferably not longer than one week to two weeks (storage should not exceed one month in any case^[9]). The soil corer is cleaned with water afterwards.

7.2 Extraction of the enchytraeids

In principle, the extraction of the worms from the soil is caused by their active movement through the water-saturated sample into the water-filled bowl (6.4).

The extraction should commence as soon as possible after the sampling (see 7.1). The bowls (6.4) are carefully filled up with tap water (5.1). The samples (i.e. soil cylinders) are put in the sieves, and are, if necessary (e.g. in cases of heavy loam soils), carefully broken apart by hand (see Figure 1). The samples in the sieve shall be completely submerged and the bottoms of the sieves should not reach the bottom of the bowls. To ensure an extraction efficiency of Enchytraeidae from the samples of more than 90 %, the extraction of soil should last for 2 d to 7 d and extraction of litter for 0,5 d to 2 d at $(12 \pm 2) ^\circ\text{C}$ (water temperature). The duration depends mainly on the organic content of the sample. These times can be modified according to organizational requirements and the number of individuals in a sample. However, the worms quickly die if an oxygen deficiency occurs. In order to avoid this problem, the water should be changed after 18 h to 24 h, and again after 48 h (if extraction period exceeds 2 d). For that purpose the sieve with the sample should be carefully transferred to a bowl with fresh water. An acceleration of the extraction using a heat source [e.g. a 60 W bulb (6.6)] placed above the sample can be helpful, but should be carefully used (i.e. slow temperature increase over at least 3 h), since otherwise — species-specifically — many animals, especially juveniles and fragmentation stages, remain in the soil (see Annex A).

NOTE 1 In order to reduce the amount of debris at the bottom of the extraction bowls, a fine wiping cloth (mesh size 1 mm) can be put in the sieve before the soil sample is put in.

At the end of the extraction procedure, the sieves are removed. The requirements for the disposal of the soil are given in the appropriate national regulations. The water is slowly and carefully decanted from the bowl. The finest fraction of soil at the bottom of the bowls should not be disturbed (see Figure 2). A small amount of water (up to a height of 5 mm to 10 mm) shall remain in the bowls. Subsequently, the

finest fraction of soil is suspended in the overlying water, placed in a petri dish (6.7) and briefly stored until soil particles have settled and the water becomes clear. Since the whitish worms are heavier than water, but are rarely able to hide themselves in the narrow soil layer, they can easily be collected out of the petri dish under a dissecting microscope (6.10). For this transfer, a soft steel forceps, a Pasteur pipette or a hooked needle (6.13) can be used, but in any case, damaging of the worms shall be avoided. The most convenient way of counting the total number is to divide the surface of the petri dish into parallel rows which are checked one after another. Due to their white colour, the worms are clearly visible against the usually brownish soil particles. The animals are transferred to small plastic or glass vessels (e.g. 20 ml).

The number of samples which can be extracted simultaneously is theoretically unlimited. However, due to the size of the water bowls, space limitations can occur. Since they (i.e. at least the water) shall be cooled, usually only up to 40 to 50 samples can be processed at one time. These limitations can be overcome by carrying out the procedure in a cool room, for example a cellar.

NOTE 2 In rare cases, the enchytraeids can be confused with Diptera larvae (which very often possess brownish or black head capsules) or nematodes (non-peristaltic movement; usually smaller and faster moving than oligochaetes). Additionally, fungal hyphae or fine root material can be mistaken for enchytraeids, since they can possess the same length and colour. However, they always lack the segmentation of oligochaete worms.

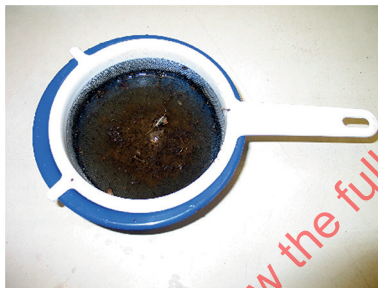


Figure 1 — Extraction bowl with soil sample

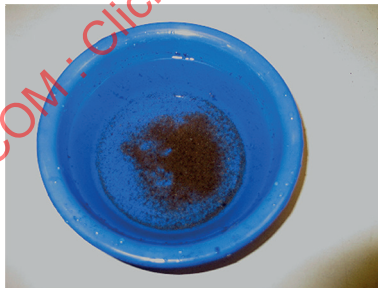


Figure 2 — Sediment layer (including enchytraeids)

7.3 Microscopic identification

The microscopic identification should be done as soon as possible, because the animals die in water after some days, even if stored in a refrigerator (6.9). A soft steel forceps, a Pasteur pipette or a hooked needle (6.13) can be used carefully to transfer the animals with a drop of water to a slide. If the worms are moving too fast on the slide, they can be anaesthetised with CO₂ (e.g. by using a drop of mineral water with gas, but it should be used with care, otherwise the worms are killed).

NOTE Identification of the enchytraeids is difficult. Therefore, in many cases only the number of animals is determined. Otherwise, keys for European enchytraeid species^[20] and for species of the genus *Fridericia*^[32], as well as other publications, are available. A compromise can be the use of a site-specific key, since usually only three to 25 species occur at any given site (in this case, often worms fixed in ethanol can be identified to the species level). An overview of the information (e.g. parameters, drawings) needed for the identification of a certain species is given in Reference [11].

In the case of high individual numbers (>100) a sample partitioning can be carried out. Partitioning shall guarantee that all species (whether big or small) are parted to a known degree. Therefore, this process shall be described in detail.

7.4 Preservation of Enchytraeidae

Enchytraeidae can be preserved for further investigations (e.g. species descriptions) in 70 % (volume fraction) ethanol (5.2)^[33]. However, the preservation is accompanied by a loss of visible morphological details. Animals difficult to identify or those selected as reference specimen may also be identified after a fixing in Bouin (5.4), respective colouring in Paracarmin (5.5) and storing in Canada-balm (5.6) (which can be relatively elaborate). For species identification, the use of a microscope (6.11) equipped with differential interference contrast (DIC) is strongly recommended.

7.5 Validity of the extraction process

Extraction efficiency can be checked by fixing soil samples with ethanol (96 %), which are taken in parallel to other field samples. The soil is spread in a thin layer on the bottom of a flat plastic vessel (e.g. Bellaplast¹⁾ 16 cm × 11 cm) and then the ethanol is added. Afterwards, some drops of Bengalred (5.3) are applied to the ethanol. After one day, the bright-red coloured worms can easily be counted. However, this procedure is only necessary when using samples from an unknown site for the first time. Additionally, this check shall be done with six to eight replicates since the variability of enchytraeid numbers can be quite high.

7.6 Determination of biomass

For an estimation of the ecological role (e.g. in the soil food-web) of enchytraeids at a certain site, the determination of their biomass is necessary. Since direct weighing is difficult due to the low individual mass of most species, potential corruption from changing gut content and, in particular, quick desiccation, the biomass may also be indirectly estimated by the following methods:

- creation of specific species calibration curves to the ratio of length and mass^{[1][2]} then measurement of the length of the animals in a sample;
- automatic calculation of the mass by computer-assisted measuring of the length-mass-ratio of embedded individuals and following computation with the largely constant density of enchytraeids^[23].

8 Data assessment

The following measurement end points may be used for the bio-classification of a soil, including bio-indication or bio-monitoring (e.g. anthropogenic stress-like chemicals or land-use changes):

- abundance (number of individuals per area);
- biomass (fresh or dry mass of the population per area);
- number of species or other taxonomically or ecologically defined groups;
- dominance ratio (in percentage of the population);
- age structure of the population (e.g. adult/juvenile ratio), either all species together or individual species;
- distribution in the soil (e.g. the vertical distribution within the soil core);
- morphological, physiological or biochemical alterations in individuals (e.g. open wounds).

1) Bellaplast is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Usually, the total number of worms is counted and expressed as individuals per sample. This number is then multiplied by a factor determined by the diameter of the soil corer (6.1) in order to get the number of worms per square meter. Additionally, the age structure (juveniles and adults differentiated by the presence of a clitellum) and the vertical distribution can be determined with the help of the dissecting microscope (6.10) (i.e. no species determination is done). For the determination of all other end points, a detailed microscopic examination is necessary, since then the species have to be identified.

9 Test report

The test report shall contain a summary of the results obtained along with the methods and variables used during the study. It shall provide the following information:

- a) a reference to this document, i.e. ISO 23611-3;
- b) a full description of the study 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;
- g) details of fixation and preservation of the biological material;
- h) recalculated values to 1 m² or another standard size, if necessary;
- i) all information, including all measured raw data and all problems which might have occurred, developed during the study;
- j) discussion of the results.

Annex A (informative)

Quick extraction of enchytraeids

In specific cases, the extraction method described in 7.2 may not be optimal. This is, for example, true for samples which have been taken in organic-rich coniferous forest soils, in particular in northern temperate regions like Scandinavia. In these soils (including the litter layer), often fragmenting species like *Cognettia sphagnetorum* are highly dominant^{[1][2]}. During the proposed extraction period of several days, asexual species could fragment or die. Thus, the number of individuals could change considerably.

For this reason, a modified extraction method as already described by Reference [21] can be used. The performance of the method is summarized as follows.

The sampling is done by using a soil corer (6.1) in accordance with 7.1.

The extraction should commence as soon as possible after the sampling. Each extraction unit consists of a plastic funnel (diameter of about 10 cm to 12 cm) fixed in a hole (e.g. a wooden board). A sieve (mesh-size 1 mm) with a slightly smaller diameter than the funnel is put into it. The sample placed on the sieve should be completely covered by water. The bottom of the funnel is closed by a screw clip on a piece of rubber tubing. The lower end of this tube ends in a small vessel (e.g. 20 ml) in which the enchytraeids are collected. Heat is supplied from a 60 W bulb (6.6) enclosed in a light metal cylinder (e.g. 11 cm diameter and 18 cm height). The bottom of this cylinder is about 10 cm to 12 cm above the funnel. The heating, preferably of several extraction units in parallel, is controlled by means of a variable resistance. During the extraction process, the heat is increased gradually, so that the water surface reaches a temperature of approximately 45 °C after 150 min to 250 min, when extraction is complete. Then the worms are run out from the bottom of the funnel into the small vessel for counting and identification.

Afterwards, the enchytraeids are handled in accordance with 7.3.

An example of an apparatus is given in Figure A.1.

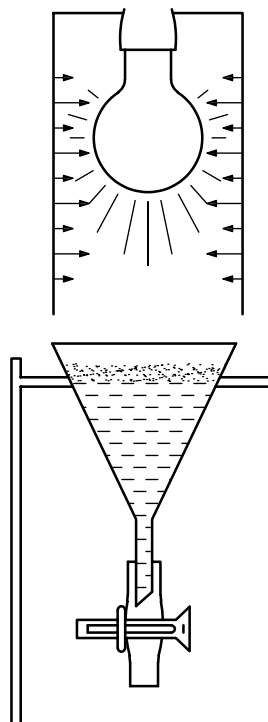


Figure A.1 — Schematic overview of the method (from Reference [12])

Annex B (informative)

Examples of the use of soil invertebrates in soil monitoring programmes (including presentation of their results)

B.1 General

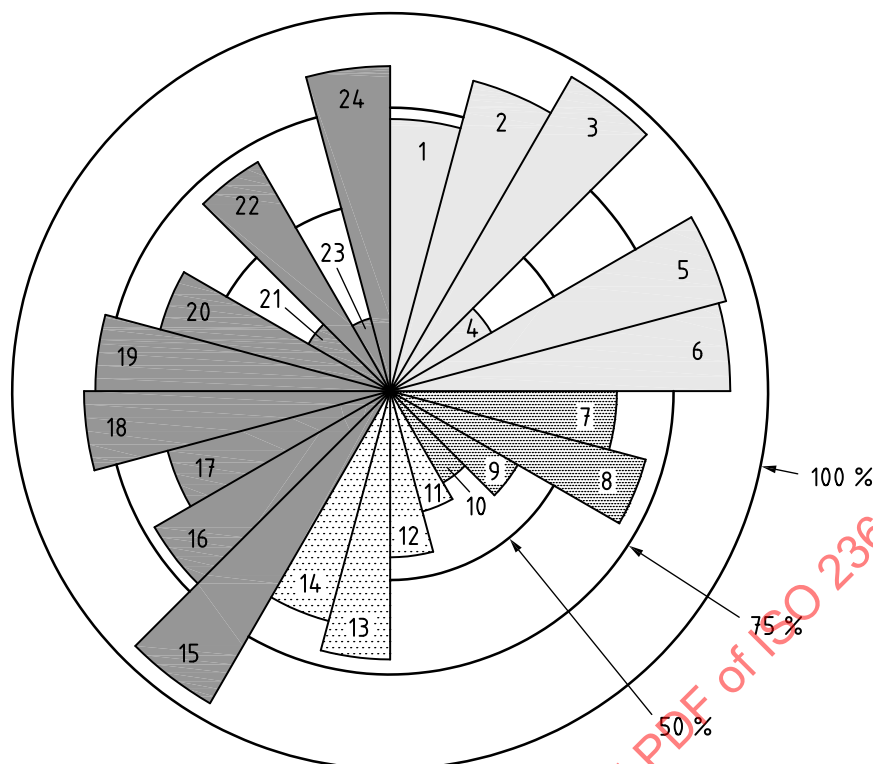
In order to improve the performance and, in particular, the presentation and interpretation of soil invertebrate monitoring studies, some examples are given in this annex. It should be noted that the information provided here is to be understood as an example of how monitoring data could be used.

In recent years essential contributions to a biological soil assessment have been 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^[16]. 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)^[4]. In order to operationalize the assessment of biodiversity, site-specific reference systems have been developed. In the Netherlands, farms located on different soil types and different land-use intensities were sampled (e.g. Reference ^[30]). In Germany, sites were classified into habitat types^[22], based on soil and site parameters. 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 to be an indication of an impacted habitat function of the soil (e.g. References ^[27] and ^[28]).

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. Although theoretical considerations allow the conclusion that the information provided here is useful for other regions as well, no experience regarding the use of enchytraeids in monitoring networks and the definition of reference values is available from outside Europe.

B.2 Assessment of the biological quality of soils

The first step is to clarify 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. 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 when all the information gained from a monitoring study is put into one or a few values. Therefore, a graphical way of visualizing the information from many different organism groups and/or end points (the Amoeba) has been proposed in the Netherlands (see [Figure B.1](#))^[5]. It represents the distance between the actual measured value for a certain measurement end point (e.g. enchytraeid abundance) and a previously defined reference value. In this example, the measured values were determined at a conventional farm site. The optimal area is defined in the outer circle [based on data from a reference site; here a non-conventional (organic) farm] and is set at 100 %. An intermediary quality is given by the medium circle (75 % of the optimum) and a low quality is displayed by the inner circle in the middle (50 % of the optimum)^[5]. In this example, enchytraeid biomass and the number of species of the genus *Fridericia* are sensitive indicators, while total enchytraeid abundance and number of taxa are less reactive (No. 11 to No. 14 in [Figure B.1](#)). However, this deviation cannot directly be transformed into a science-based decision. For example, 'Is half the number of nematodes of equal importance as half the amount of enchytraeids?'. Such questions have to be answered on a case-by-case basis, usually referring to a specific protection goal^[13].



Key

Microorganisms

- 1 heterotrophic bacteria (72 %)
- 2 colony-forming units (85 %)
- 3 nitrification cap (96 %)
- 4 biomass bacteria (31 %)
- 5 leucine incorporation (92 %)
- 6 thymidine incorporation (90 %)

Earthworms

- 7 epigeic worms (60 %)
8 endogeic worms (70 %)
9 biomass of earthworms (39 %)
10 abundance earthworms (28 %)

Enchytraeids

- 11 number of Friderica species (33 %)
- 12 biomass of Enchytraeids (44 %)

- 13 abundance of Enchytraeids (71 %)
14 number of taxa Enchytraeids (63 %)

Nematodes

- 15 trophic index nematodes (95 %)
16 number of taxa nematodes (72 %)
17 abundance of nematodes (61 %)
18 maturity index (81 %)
19 diverse functional group (78 %)
20 diverse plant-feeding nematodes (63 %)
21 diverse omnivorous nematodes (25 %)
22 diverse hyphal-feeding nematodes (70 %)
23 diverse carnivorous nematodes (20 %)
24 diverse bacterial-feeding nematodes (86 %)

NOTE All results of the reference site (an organic farm) have been scaled as 100 % (the outer circle). The grey and black areas represent 75 % and 50 % of the reference. The monitoring results of the conventional farms are represented in the individual bars, different for each end point[5].

Figure B.1 — Presentation of the monitoring results of Dutch farms in an Amoeba figure

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