SOIL HEALTH ASSESSMENT AND RE-EVALUATION (SHARE) PROTOCOLS HANDBOOK

SOB4ES GUIDELINES FOR STANDARDISED PHYSICAL, CHEMICAL AND BIOLOGICAL SAMPLING

SHARE Protocols

This handbook for soil monitoring is part of a series of protocols by the EU SOB4ES project. It was developed in consultation within the consortium, with relevant external stakeholders and the SoilProS project.

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SOB4ES

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1. INTRODUCTION

Healthy soils are the foundation of food production, clean water, carbon storage, and climate resilience. To keep soils healthy – or to bring degraded soils back to life – we need regular and systematic monitoring of their physical, chemical, and biological conditions. This need is now recognised in the approved EU Soil Monitoring Directive (EC, 2025).

However, monitoring soils effectively requires access to practical, standardised methods that provide reliable and comparable data. In this handbook, we present a set of protocols that, for the first time, combines all the necessary physical and chemical measurements with comprehensive methods for assessing soil biodiversity. These protocols are designed to work across different types of land use, from farms and forests to grasslands and gardens. The metrics are designed to generate detailed biodiversity baselines that can be used to determine abundances, diversities and communities that align with healthy soils, thereby promoting benchmarks and indicator selection.

The techniques included here have been carefully tested and scientifically validated along a large-scale transect across Europe. They provide robust, ecologically meaningful results while keeping sampling efforts efficient and practical. This makes them useful not only for researchers and large monitoring programmes but also for land managers, farmers, advisors, and even interested gardeners. By reducing bias and ensuring consistency, these protocols allow for a complete and trustworthy assessment of soil health and biodiversity.
Developed by integrating national, EU, and international approaches, these protocols have been successfully applied in the Horizon Europe SOB4ES project. They have been shown to work well across different land uses, farming intensities across nine European pedoclimatic regions.

Overall, these standardised monitoring protocols represent a breakthrough in soil health assessment. For the first time, they make it possible to generate interoperable datasets across diverse ecosystems, the information necessary to highlight fundamental patterns in ecosystems that span national boundaries (van der Putten et al., 2023).
They also provide robust evidence for evaluating soil condition, informing management decisions, and supporting environmental policies. Their widespread adoption will strengthen our collective understanding of soils, improve decision-making, and help protect this vital resource for generations to come.

2. PLANNING SOIL MONITORING

To effectively monitor soils, it is important to plan carefully the entire effort from the start. This means identifying sampling sites based on their soil-climate zones, land-use type, and management intensity to ensure that the data collected is representative. Planning should also consider the statistical power of the study to make sure there are enough samples (e.g. number of sites/plots relative to treatments) to detect meaningful differences without overburdening resources. Record any deviations made from the protocols such as sample size or methodology. While standard sample sizes are recommended for most biotic samples (e.g. 5 cm diameter by 5 cm depth cores), the exact number of samples for aggregates or other abiotic factors that can be based on known local variability. Ensuring these steps are considered early on creates a solid foundation for reliable soil assessments, whether for scientific research, policy monitoring, or practical management decisions.

3. SITE SELECTION AND A PRIORI CLASSES

Choosing the right sites is a critical step in any soil monitoring programme, as it determines the representativeness, reliability, and usefulness of the data collected. Site selection must ensure that sampling locations reflect the intended land-use types, management intensities, and soil-climate (pedo-climatic) zones of interest. This is essential not only for scientific robustness but also for practical decision-making, enabling results to inform management and policy in a meaningful and context-relevant way.

To achieve this, sites should be chosen depending on project goals, ensuring sufficient coverage of the full diversity of soils and land uses within the target area. This includes agricultural fields, forests, grasslands, urban green spaces, and restored or degraded lands. Within each land-use type, it is important to include sites with different management intensities – for example, conventional versus organic farming, or grazed versus ungrazed grasslands – to capture gradients in soil health and biodiversity that result from human activities.

Other a priori classifications such as pedo-climatic zones help structure site selection by grouping soils according to their regional mineralogical and climatic characteristics. These classifications can range from broad zones covering major soil-climate regions to more detailed schemes with over 100 classes, as used in various European studies. This handbook uses the broadest classification to maximise inclusivity and ensure outputs remain practical for stakeholders across countries and regions, while retaining the option to increase resolution where project aims require finer-scale analysis.

Site history is another crucial consideration. Understanding past land use, from intensive agriculture to industrial use or natural vegetation, provides context for interpreting current soil conditions, biodiversity, and restoration potential. This includes evaluating topsoil layers, such as the organic (O) horizon and mineral (A) horizon, to ensure sampling targets the biologically active zones while accounting for site-specific variation in soil depth and structure.

Ultimately, careful and structured site selection and spatial planning ensure that soil monitoring data are robust, representative, and scalable. This enables meaningful interpretation of results for local management decisions, national monitoring targets, and EU-wide assessments of soil health and biodiversity. Incorporating these considerations at the planning stage reduces sampling bias, enhances data comparability, and optimizes monitoring strategies.

4. SOIL SAMPLING

Using standardised sampling methods ensures results are reliable and comparable across sites and studies. This guide adapts established protocols such as those from the Netherlands' SoilProS programme, which integrate vegetation surveys with comprehensive physical, chemical, and biodiversity measures. Standardised approaches to data collection and storage all facilitate better data integration and allow meaningful conclusions to be drawn about soil health and biodiversity status, supporting practical management and policy needs alike. Prior to soil sampling, make sure to arrange the following particulars:

1. Ensure consistent metadata collection and sample labelling.
2. Compile field forms and data templates.
3. Prepare permits and checklists for data collection.
4. Ensure sufficient data storage, power supply and back-up data.
5. Consider logistics for sample processing/shipping.

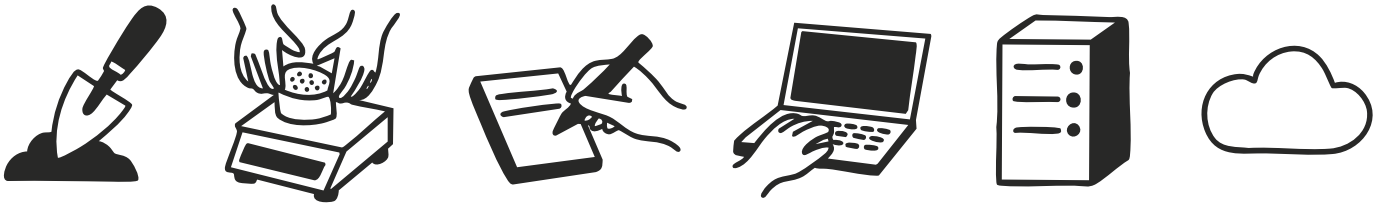


Fig. 1: Data flow: Field -> Notepad -> Server -> Cloud.

5. SITE DESCRIPTIONS AND SOIL TYPE

At each monitoring site, three 2 m x 2 m plots should be set up about 15 m apart to account for local variability while remaining practical. These plots should be chosen randomly but avoid unusual features like field edges, track marks and dung heaps. Site descriptions must include plant cover, soil temperature, and photographs. Identifying soil type based on texture, colour, and structure is crucial, as it influences key properties like nutrient availability, drainage, and biodiversity potential. Recording this information allows for better comparisons between sites with different management histories or environmental conditions.

Site description

1. Record GPS coordinates of each plot (3 per site).
2. Fill out the field form template.
3. Take photos of the canopy and soil surface from all directions (N, W, S, E).

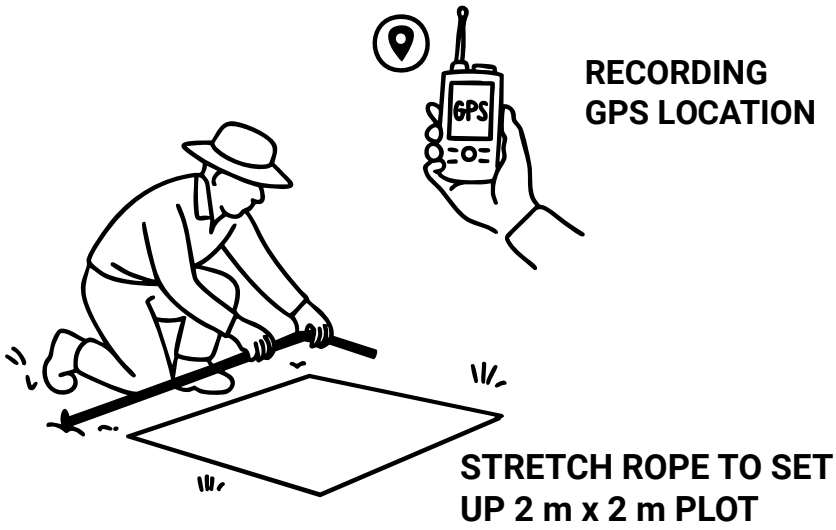
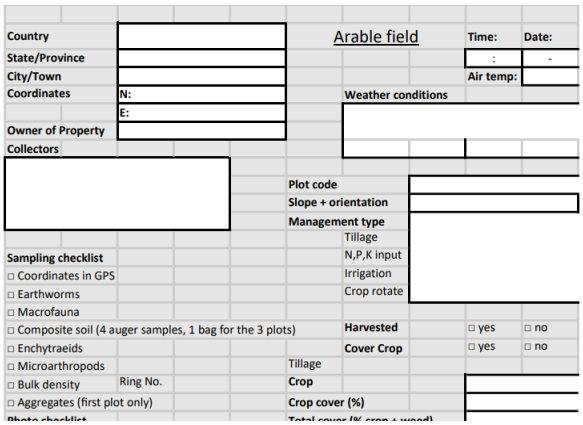


Fig. 2: Lay out a 2 m × 2 m plot and record the GPS coordinates.



Country		Arable field		Time:	Date:
State/Province				:	-
City/Town				Air temp:	
Coordinates	N:		Weather conditions		
	E:				
Owner of Property					
Collectors					
		Plot code			
		Slope + orientation			
		Management type			
		Tillage			
Sampling checklist		N,P,K input			
□ Coordinates in GPS		Irrigation			
□ Earthworms		Crop rotate			
□ Macrofauna					
□ Composite soil (4 auger samples, 1 bag for the 3 plots)		Harvested		□ yes	□ no
□ Enchytraeids		Cover Crop		□ yes	□ no
□ Microarthropods		Tillage			
□ Bulk density	Ring No.	Crop			
□ Aggregates (first plot only)		Crop cover (%)			

Fig. 3: Example of a field data collection form.

Fig. 4: Imaging plot from all directions

6. SAMPLE DEPTHS AND SOIL

Sampling depths must be consistent to allow valid comparisons. As the majority of soil biodiversity is in the organic and uppermost soil mineral layers, we recommend the top 10 cm of mineral soil to be sampled. Sampling to 30 cm is useful for more detailed soil physico-chemical profiles. In forest soils, organic layers are also sampled and described, as their depth and structure strongly affect soil biodiversity and functions. Ensuring samples are taken to the same depths across sites avoids bias and provides more accurate insights into how management or environment affects soils.

Soil humus (organic layers) description

Fig. 5: Recording descriptions and depth of organic and mineral soil layers.

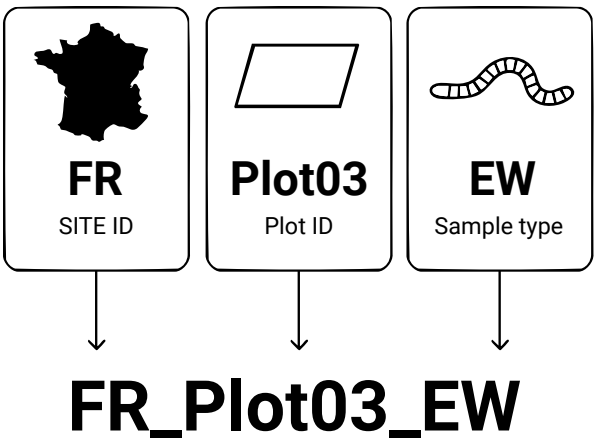


Fig. 6: Example label for an earthworm sample.

Fig. 7: Soil pit images with ruler

Soil descriptions

1. Remove any organic material covering the plot.
2. Using an auger, sample the soil (in sections) to a depth of at least 1.2 m, unless shallow bedrock is present.
3. Lay out each section on a sheet next to a ruler and image. Note changes between compacted, dense layers; texture and other features.
4. Complete the description form and identify master horizons as detailed on p.67 of the FAO (2006) guidelines.
5. Close the auger hole and tamp down the surface.

Fig. 8: Example of soil profile images.

In Forests:

1. On the walls of the macrofauna pit, observe the Organic OL+OF+OH (Organic Litter, Organic Ferment and Organic Humus) layers and describe the humus layer using the Zanella reference (ID app: TerrHum).
2. Describe the boundary between the Organic layers, mineral soils, and the A horizon structure.
3. Use the FAO protocol for description of the A horizon structure and its lower boundary.

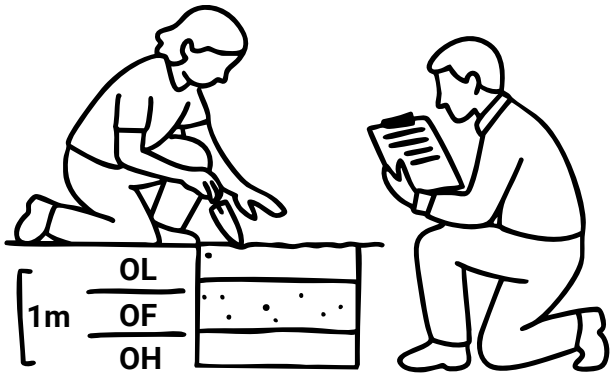


Fig. 9: Examining Organic Litter, Organic Ferment and Organic Humus soil profile layers.

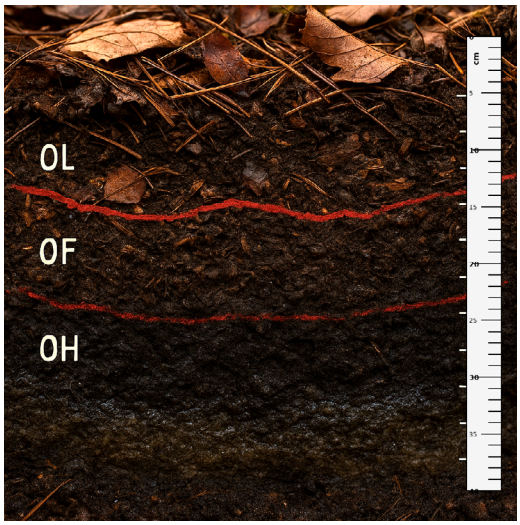


Fig. 10: Sample image of soil layers with Organic Litter, Organic Ferment and Organic Humus layers as indicated.

7. SOIL PHYSICO-CHEMICAL

Measuring physical and chemical soil properties alongside biodiversity is essential to understand how soils function. Parameters such as bulk density, organic matter content, water holding capacity, and aggregate stability provide insights into soil structure, fertility, and erosion risk, and also influence biological communities. Including these measurements ensures a more complete assessment of soil health, informing decisions that support productivity, ecosystem services, and resilience under changing management or climatic conditions.

Sampling for aggregate stability

1. Fill up a 100 ml air-tight container with undisturbed soil from the 0–10 cm layer. Either push the container into the soil, or transfer a core of the same volume into the container.
2. Ensure container is completely filled (but not compacted) to minimize disturbance during transportation.

ISO 10930:2012 - Soil aggregate test ISO

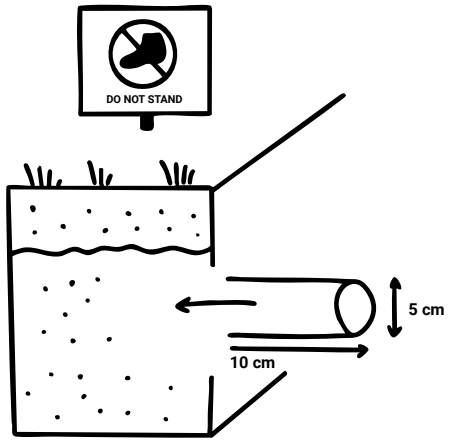


Fig. 11: Bulk density sampling using a core of known volume. Do not stand on the area above the sampling site.

Fig. 12: Gloves must be worn when handling composite samples for eDNA analysis.

Measuring bulk density and soil water content

1. Weigh the wet soil (from the core for bulk density).
2. Dry the soil in the oven at 105 °C for 24 h (or until dry).
3. Weigh the dried soil again.
4. Calculate bulk density from known volume (g/cm³), and soil moisture as % moisture.

ISO 11272:2017 - Bulk density ISO

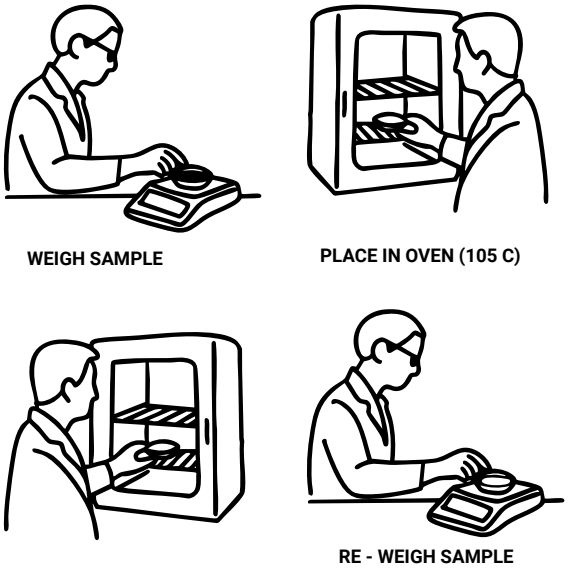


Fig. 13: Soil water content and dry bulk density processing steps: weigh wet sample, dry in oven at 105 °C overnight, re-weigh dry sample.

Measuring soil pH

1. Use the $CaCl_2$ or plain water method.
2. Weigh 10 g of soil into a beaker.
3. Add 50 ml of 0.01 M $CaCl_2$ solution (soil to 0.01 M $CaCl_2$ or H_20 at 1:5 w/v).
4. Shake for 60 min.
5. Let stand for 60 min.
6. Measure the soil pH twice and take the mean.

ISO 10390:2021 - Soil pH ISO.

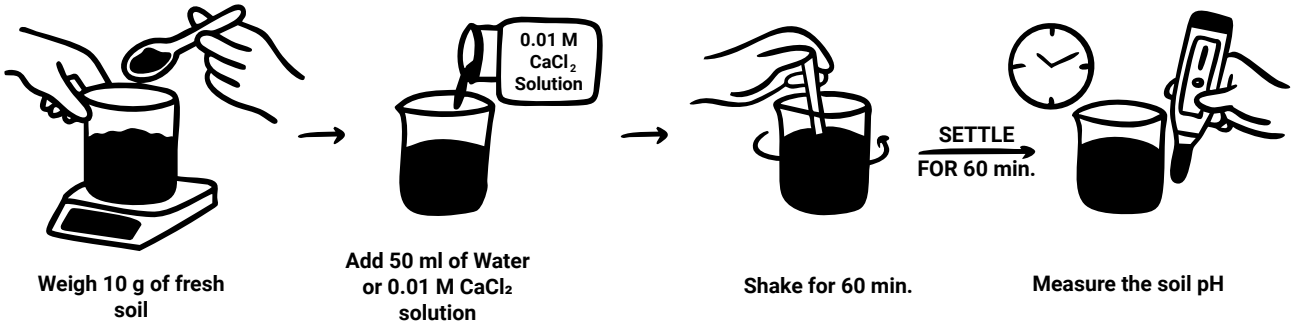


Fig. 14: Soil pH measurement: weigh sample, add $CaCl_2$ solution, shake and let settle, measure pH.

8. SOIL BIODIVERSITY SAMPLING

Soil biodiversity is the foundation of healthy ecosystems, driving key processes such as nutrient cycling, organic matter decomposition, soil structure formation, and pathogen suppression. These processes, in turn, underpin ecosystem services that are essential for human wellbeing, including food production, climate regulation, and water purification. Despite their importance, soil organisms are often overlooked in monitoring programmes or included only partially due to practical constraints or lack of standardised protocols, although are essential for determining practical benchmarks and monitoring targets (Schram et al., 2024).

This handbook provides a comprehensive approach to assessing soil biodiversity, ensuring representation across all major groups and trophic levels. By systematically monitoring soil organisms – from macrofauna to microbiomes – we can gain a full understanding of the living component of soils and how it interacts with physical and chemical properties to support soil health and resilience.

Macrofauna such as earthworms, beetles, millipedes, and spiders play vital roles in soil aeration, organic matter breakdown, and nutrient redistribution. Their presence and diversity often serve as reliable indicators of soil condition and management impacts, that are included in most existing biotic monitoring protocols.

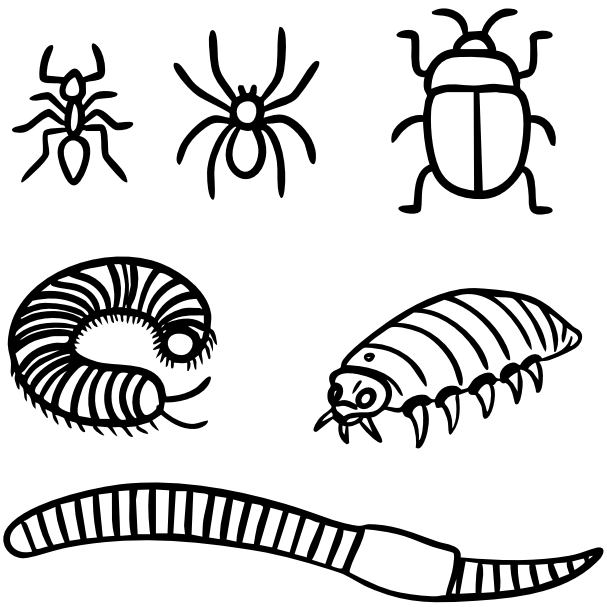


Fig. 15: Macrofauna: ants, spiders, beetles, millipedes, isopods, earthworms.

Fig. 16: An example of an earthworm pit with a 25 cm quadrat.

Fig. 17: Hand-sorting macrofauna in Alpine grasslands.

Sampling macrofauna (Earthworms and others)

1. Hand-sort the earthworms from a 25 cm x 25 cm pit of 20 cm depth.
2. Place into labelled 100 ml containers and other macrofauna (ants, spiders, beetles, millipedes, centipedes, isopods, etc. in a separate container.
3. Return the soil back to the pit.
4. All macrofauna can be stored in the fridge (4 °C) until processing within 24 h.

ISO/DIS 23611-1 - Soil invertebrate sampling ISO (2025)

Fig. 18: Sampling with hammer and pre-cut PVC soil core.

Mesofauna, including microarthropods like the Acari, known as mites, Collembola, called springtails, pseudoscorpions alongside a diverse range of lesser ecologically important groups. Mites and springtails are important decomposers that fragment organic material, regulate microbial populations, and improve soil structure through their movement and feeding activities. Large enchytraieds are more ecologically active than pseudoscorpions, pauropods and wingless Proturans and Diplurans. They fragment organic material, regulate microbial populations, and enhance soil structure through their movement and feeding activities. Assessing their diversity and abundance provides insights into soil food web complexity and ecosystem stability.

Enchytraeids, also called potworms, aid in decomposition and nutrient mineralisation, especially in acidic soils where earthworm activity is limited. Monitoring these groups helps identify changes in soil functioning, potential pest pressures, and restoration outcomes.

Fig. 19: Composite and mesofaunal sampling in temperate grasslands.

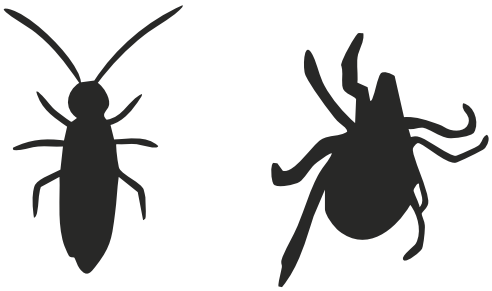


Fig. 20: Examples of mesofauna. Collembola (springtail) 2 mm long, 1 mm wide (left) and Oribatida (oribatid mite) 0.8 mm, 0.4 mm wide.

Sampling mesofauna (Mites and Collembola)

1. Using a 5 cm-diameter soil corer, sample to a depth of 10 cm.
2. Transfer the core to a labelled air-tight container. You can re-use the core for other plots/ sites, but make sure to wipe it with 70 % ethanol and a paper towel between sites.
3. Repeat 1-2 for each plot, for a total of 3 cores / bags per site.

Once back in the lab, store the samples in the fridge (4 °C) until extraction.

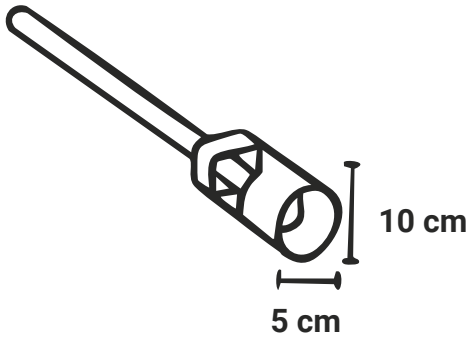


Fig. 21: Standard soil auger.

Fig. 22: Soil coring following Occupational Safety and Health (OSH) standard guidelines.

Sampling mesofauna (Enchytraeids)

1. Using a 5 cm-diameter soil corer, sample to a depth of 10 cm.
2. If the sample can be easily removed from the core, transfer it to a labelled air-tight container.
3. Re-use the core for other plots/sites, but make sure to clean with 70 % ethanol spray between sites.
4. Once you are back in the lab, store these samples in the fridge (4 °C) until extraction.

Fig. 23: Enchytraeidae: potworm microdrile oligochaetes , < 30 mm long, < 2 mm wide.

Microorganism communities – bacteria, fungi, and protozoa – are the smallest yet most abundant and functionally diverse components of soil biodiversity. They drive decomposition, nutrient cycling, carbon sequestration, and disease suppression. Advances in environmental DNA (eDNA) techniques now allow high-resolution characterisation of these communities, revealing their composition, diversity, and functional potential across sites, land uses, and management intensities.

Fig. 24: Nematoda: Free-living roundworms, ~ 1 mm long.

Sampling microbial communities and Nematodes

(Composite sampling)

1. Make a composite sample from 4 soil cores taken from around the macrofauna pit to a depth of 10 cm.
2. Combine the 4 samples in a labelled air-tight container.
3. Collect at least 400 g of soil in total over the site.
4. Store the samples in the fridge (4 °C) until further processing (preferably on the same day).
5. Clean all sampling equipment with 70 % Ethanol between sampling sites.

Fig. 25: Sampling composites in Mediterranean South wetlands.

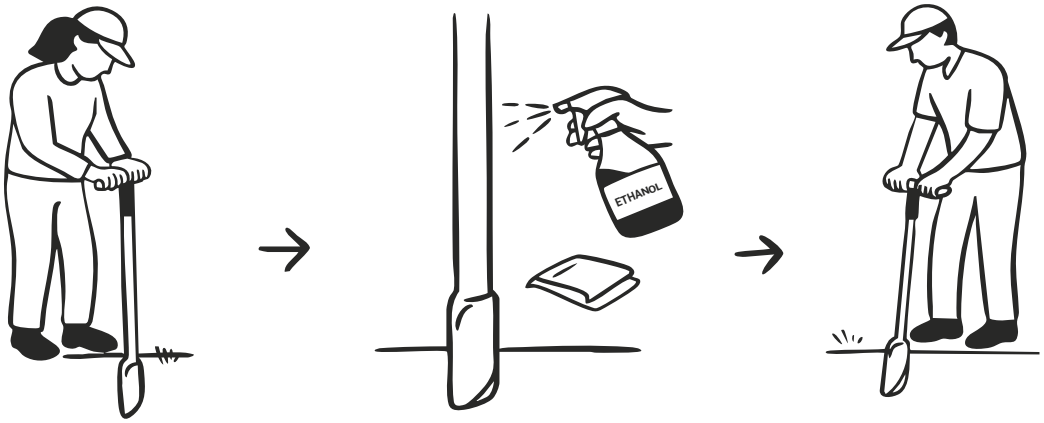


Fig. 26: To avoid contamination between sites, sterilize corer with 70 % ethanol and wipe dry.

Together, assessing these groups provides comprehensive data on soil abundance, diversity, and community structure. This enables identification of ecologically important taxa, evaluation of soil health status, and tracking of changes due to management or environmental pressures. Incorporating soil biodiversity into monitoring frameworks is crucial for generating robust, holistic evidence to inform land management decisions, develop effective restoration strategies, and achieve national and EU targets for soil protection and ecosystem service delivery. Furthermore, widespread adoption of these protocols will enhance interoperability of data, allowing comparisons across regions and projects thus building the evidence base needed to protect and restore soil health at scale.

9. SOIL BIODIVERSITY PROCESSING

Samples must be processed via alternate approaches for best results. These combine both hand-sorting, wet and dry extraction techniques that are optimized based on the biology of the organisms.

Processing macrofauna (Earthworms)

1. Rinse earthworms with water.
2. Dry to remove excess moisture.
3. Record the weight (to 0.001 g, if possible) of live earthworms per sample.
4. Transfer the live earthworms into a labelled air-tight container. Fix the worms with 4X worm volume of 4 % formalin or 70 % ethanol solution.

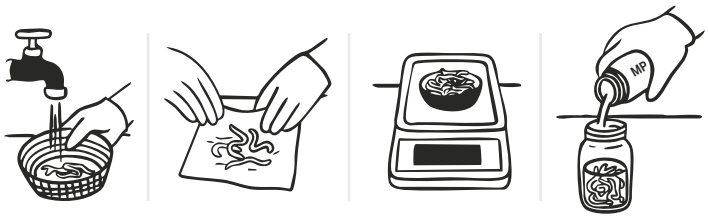


Fig. 27: Panels left to right: Rinsing, Drying, Weighing, Fixing.

Fig. 29: Macrofauna: Centipede.

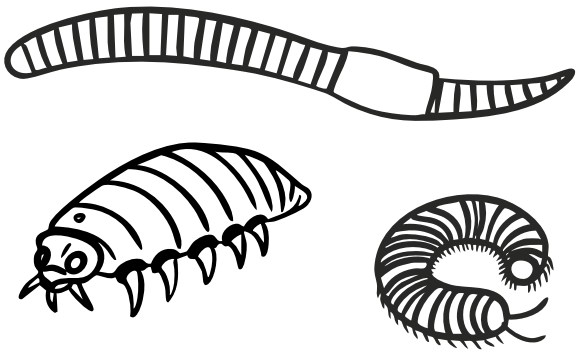


Fig. 28: Macrofauna: earthworms, beetles, millipedes.

Fig. 30: Macrofauna: Isopoda

Processing mesofauna (Mites and Collembola)

1. Dry extraction by placing soil cores in an inverted Berlese-Tullgren rack (upper soil layer facing down).
2. Label and place collection containers with 70 % Ethanol under funnels.
3. Turn on the heating mats < 40 °C.
4. Extract the samples over 1 - 3 weeks, or until dry. Do not disturb extraction equipment for the duration for cleanest samples.
5. Replace lids on containers. Store at room temperature.

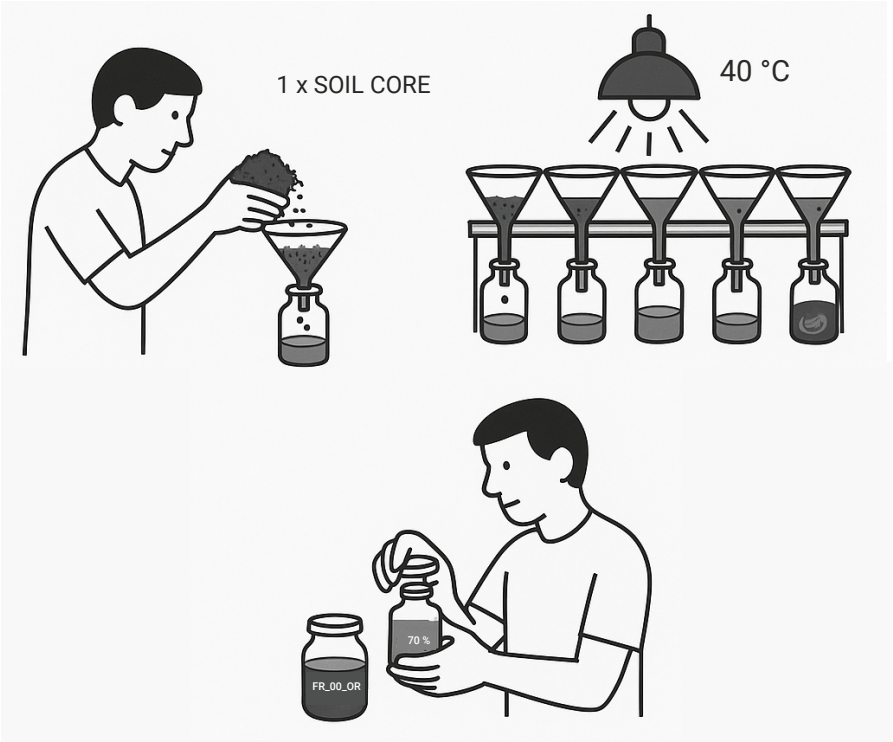


Fig. 31: Dry extraction of mesofauna (Mites and Collembola). Load fresh soil cores onto mesh above Berlese-Tullgren funnels under a heat source. Allow live fauna to migrate into labelled containers with 70 % ethanol. Replace caps after at least a week or until dry.

Fig. 32: Sample pre-cut with bevelled, sharpened edge.

Fig. 33: Mesofauna: *Collembola Neanurinae* sp., *Oribatida Galumnidae* sp.

Processing microfauna (Nematodes and Enchytraeids)

1. Wet extraction using Baermann funnels or equivalent. Place weighed soil cores on a mesh with tissue paper or muslin cloth.
2. Fill the funnel with tap water until the sample is fully soaked but not completely submerged.
3. Turn on the heat source, < 40°C.
4. After 4 hours, collect the enchytraeid-containing liquid in a labelled 100 ml jar.

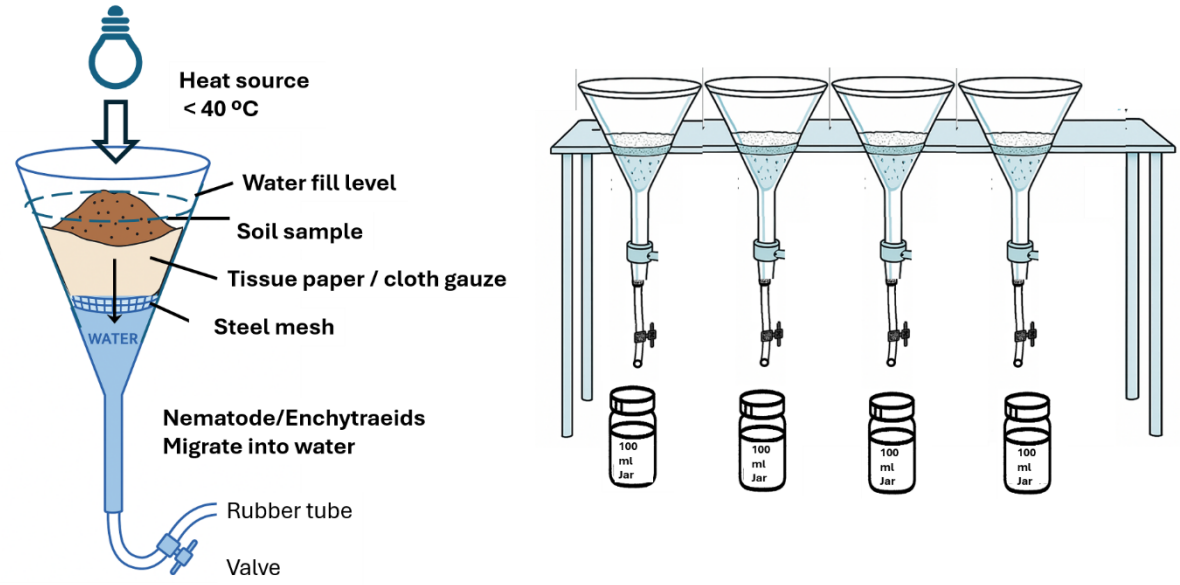


Fig. 34: Wet extraction of microfauna.

Fig. 35: Microfauna: enchytraeid (left), nematode (right).

Processing composite samples

1. Collect soil from samples within plot/site.
2. Mix-up the samples.
3. Before sieving, sub-sample 100 g for nematode extractions.
4. Sieve with 4 mm mesh and remove stones.
5. Sub-sample 100 g for physico-chemical tests.
6. Sub-sample 10 g for eDNA sequencing.

ISO 11063:2020 - Soil Quality, Direct extraction of DNA ISO

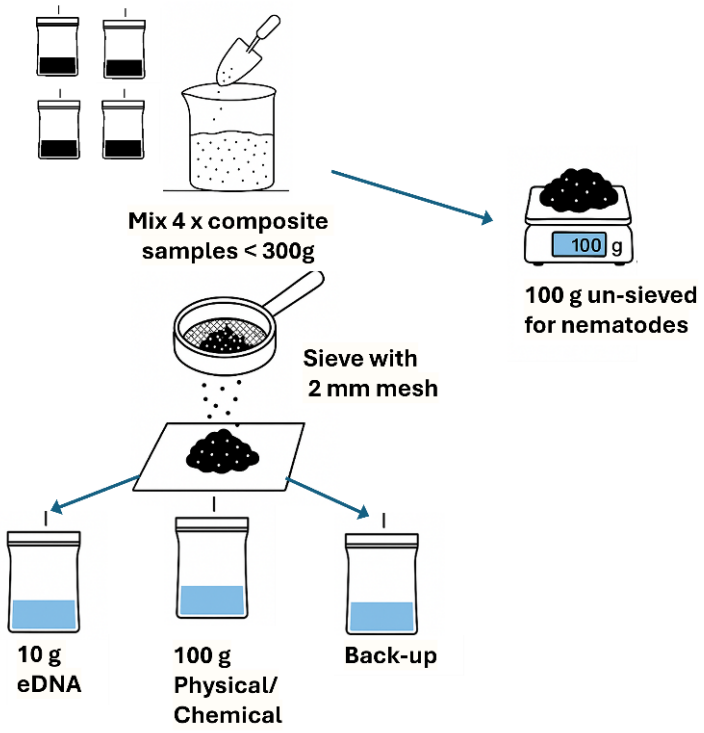


Fig. 36: Flow Diagram of composite sub-sample processing.

Fig. 37: Mixing and sub-sampling nematodes. Sieve remaining sample through 2 mm mesh and sub-sample for physico-chemical and eDNA samples.

Processing micro/mesofauna (Nematodes and Enchytraeids)

1. Wet extraction by placing the weighed soil core on top of the cheesecloth/funnel apparatus.
2. Fill the funnel with tap water until the sample is fully soaked but not completely submerged.
3. Turn on the heat source, < 40 °C.
4. After 4 hours, collect the microfauna-containing liquid in a labelled 100 ml jar.
5. Concentrate nematodes and enchytraeids by leaving to settle overnight. Remove water and transfer 10 ml into a vial.
6. Fix the sample by removing water to leave 2.3 ml of sample. Add 7.7 ml of 96% Ethanol for a final solution of 70% Ethanol (v/v).

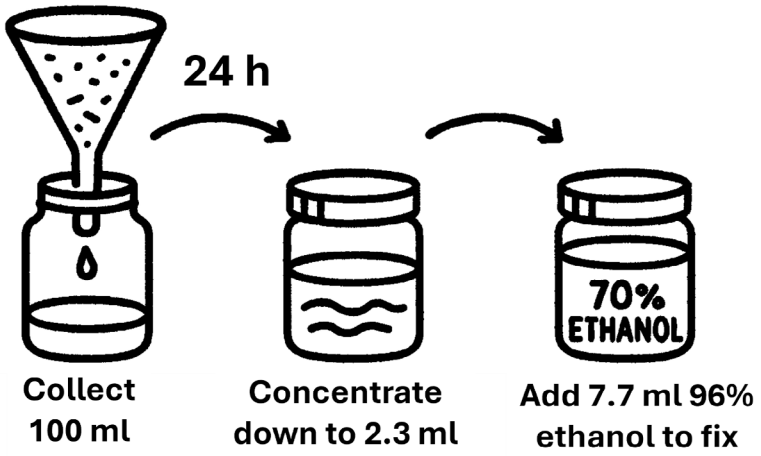


Fig. 38: Enchytraeid and simple nematode extraction followed by sample concentration and fixing. Leave overnight to settle. Remove water and transfer to 10 ml vial. Fix sample by adding 7.7 ml 96 % ethanol to the 2.3 ml sample for a final concentration of 70 % ethanol.

Fig. 39: Concentrating and counting nematode and enchytraeid samples.

Optional extraction of nematodes with Oosterbrink funnel for clean samples of living nematodes from fresh samples.

1. Wet extraction with < 100 g of fresh soil from composite sample.
2. Transfer the weighed soil to an Oosterbrink funnel and ensure all sample is entered into the chamber (optional pre-soaking step).
3. Follow the standard protocol (for specific setup) for extraction.
4. Wash the funnel run-off through 1 × 75 µm, 2 × 45 µm sieves into a basin.
5. Let the soil particles settle for 10 min.
6. Pour the filtrate onto 3 x milk filters (1 x Universal Hygia FavoritII filter from NIFA, Leeuwarden, NL) + 2 x Type S475-30 filters from Lekko B.V., Veenendaal, NL). Clamp the filters in a dish with 80 ml water. Leave for 48 h to allow nematodes to migrate into the water.
7. Transfer the nematode-containing water for concentration and storage in 70 % Ethanol.

ISO 23611-4:2022 Nematode Extraction ISO

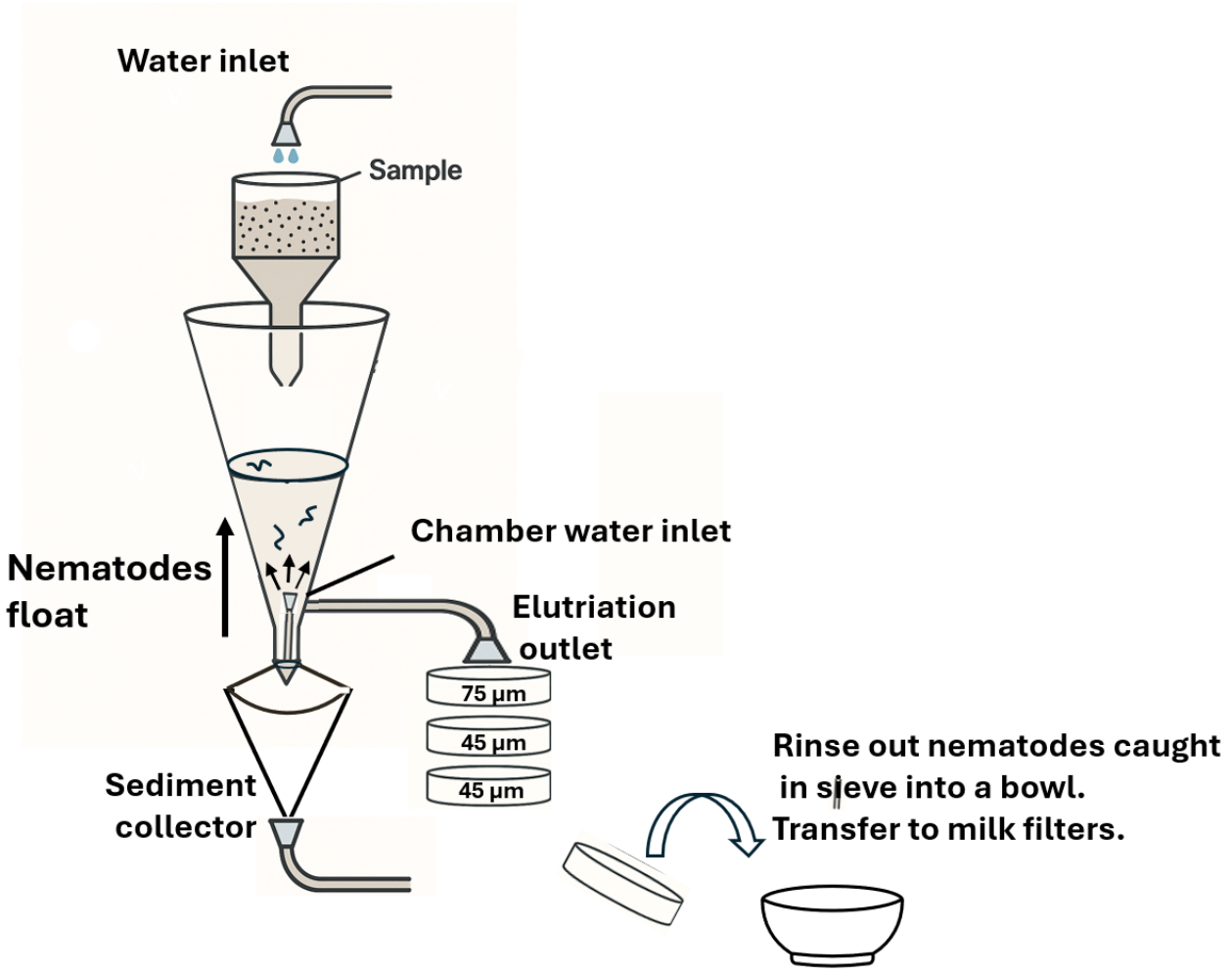


Fig. 40: Nematode extractions with an Oosterbrink funnel apparatus for clean samples. Place 100 g sample at top and rinse with water into funnel. Reduce water pressure inlet from 1.0 to 0.8 bar from chamber water inlet to aid nematode flotation.

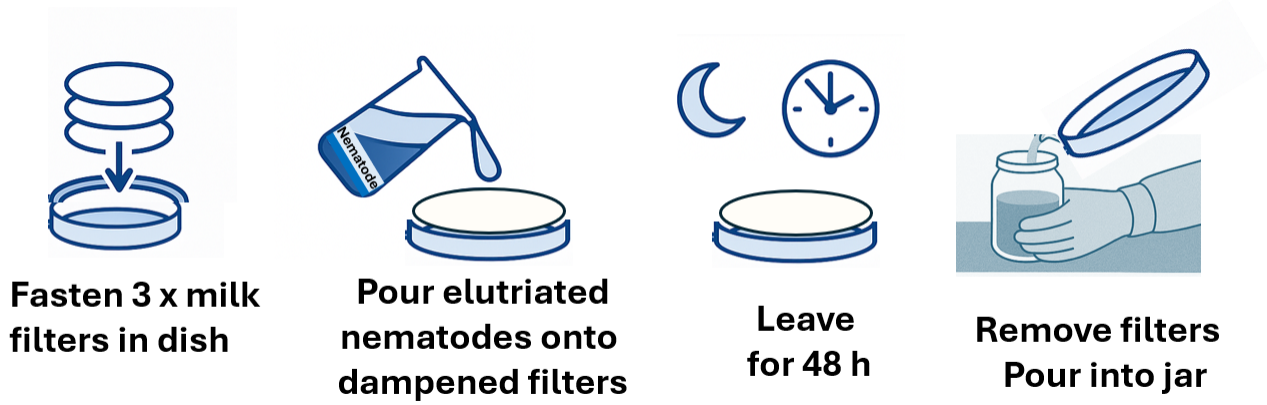


Fig. 41: Prepare milk filters in clamp, pour nematode elutrient onto damp filters in dish, leave for 48 h, remove filters and pour dish water into jar for concentration and fixing.

10. MEASURING SOIL BIODIVERSITY

Gathering information on abundances, densities and diversities based on morphological identification of soil faunal specimens is the final and most important step of the biodiversity monitoring process. Ensuring reliable and repeatable figures requires accurate counting using standard approaches. For earthworms and macrofauna, this is the simple step of converting raw counts to densities per m², or weights to biomass measurements. For the smaller nematodes, enchytraeids and microarthropods (mites and Collembola), densities per square meter of land are acquired by: i) plating samples onto a dish with embedded counting graticule. Simplified identification charts are provided for: i) main macrofaunal groups, ii) main soil mite orders, iii) main Collembola orders, iv) main microfaunal groups, and v) Nematode trophic guilds.

In highly dense samples, counting a quarter of a sample in a dish with graticule can reduce counting time. Images using high resolution microscopy is also an option for later analysis using machine learning software. Molecular sequencing allows for the relative abundances and proportions of microbial bacterial and fungal communities to be assessed. Verification of eukaryotic sequence outputs with morphologically identified specimens that have been matched using curated sequences can indicate communities of soil faunal taxa. Microbial diversity can be acquired following standardised protocols for the acquisition of eDNA samples using 16S and ITS rRNA markers (ISO, 2020).

Soil pathogens are an important aspect of natural ecosystems and can belong to i) fungal e.g. root rot *Fusarium sp*., Late blight *Phytophthora infestans, P. cinnamomi* , ii) bacterial e.g. *Ralstonia spp.* ,and iii) herbivorous root knot nematodes e.g. *Meloidogyne sp.*. Whilst soil pathogens can constrain the positive benefits of biodiversity, they are not suggested as subjects for regular monitoring.

Measuring biodiversity (all groups)

1. Plate samples into a Petri dish with graticule.
2. Count all appropriately identified individual specimens per group.
3. Replace sample back into container.
4. Convert counts to ind. / 100 g. These can then be converted to density per area or biomass using the formulae below.

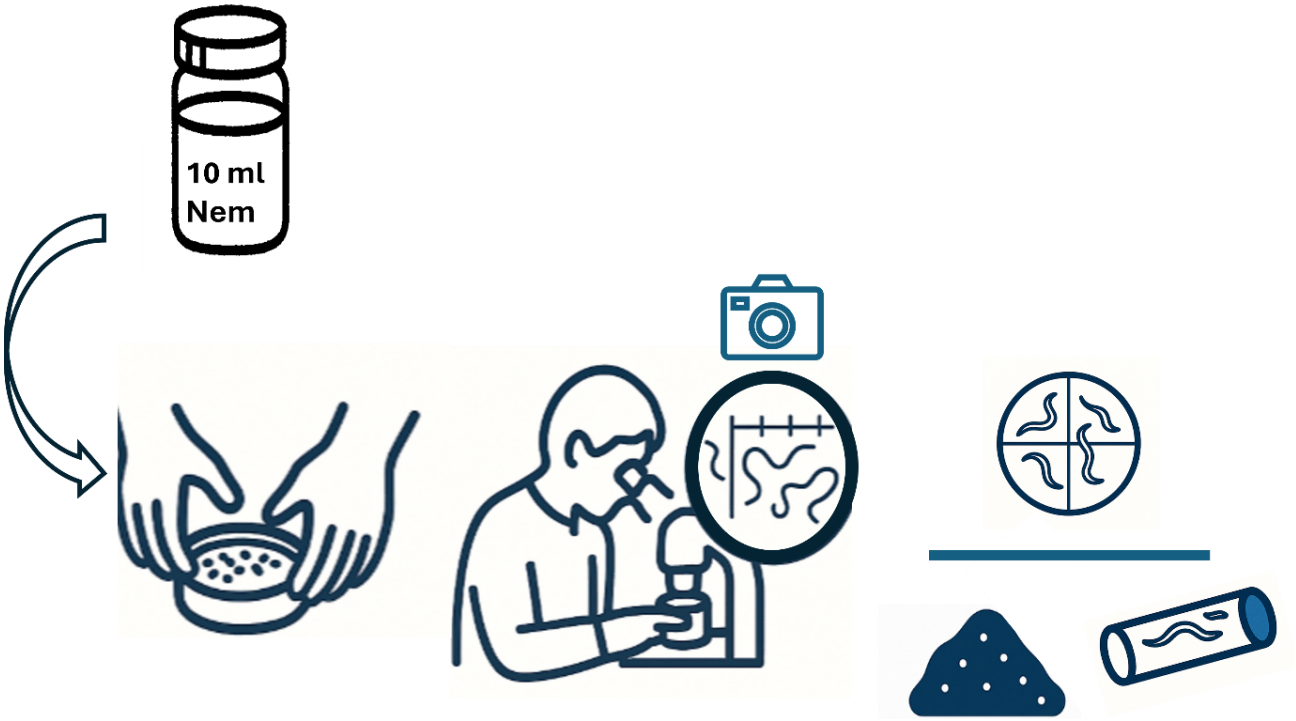


Fig. 42: Plating, counting/imaging, converting to density.

Converting counts to density and biomass

Counts per unit soil

Determining soil faunal abundance is a primary source of biodiversity information. Raw counts made using ID keys can be the basis for density measurements of individuals per 100 g wet weight of soil. For greater comparability, these can then be converted into densities per unit of dry weight of soil using the following formulae.

For microarthropods such as oribatid mites and Collembola that were collected from soil cores, this can be scaled up to make a simple unit of density per square meter. Densities can also be converted to biomass for comparison with metrics of aboveground plant biomass.

Converting counts to densities per square metre (m²) collected with soil cores

For samples taken with a 5 cm diameter × 10 cm soil corer (mites and Collembola)

Core cross-sectional area $A = \pi \times (d/2)^2 = \pi \times (2.5\ cm)^2 \approx 19.635\ cm^2$

Scaling factor to m² over 10 cm depth:

$S = 10{,}000\ cm^2 / A \approx 10{,}000 / 19.635 \approx 509.4$

Therefore, to convert counts to densities per square metre, multiply counts per core by 509.4.

Converting counts to densities per 100 g dry soil

For wet extracted nematode and enchytraeids that were expressed as individuals per 100 g wet weight, counts can be converted. to density per 100 g dry soil. For this, bulk density and soil water content must be included in the calculations

Finally, counts can be converted to individuals per square metre, to further integrate with microarthropod samples.

Inputs Required

- Fauna count per 100 g wet soil
- Soil water content (%)
- Bulk density (g/cm³)
- Soil depth (cm)

Step-by-Step calculations

1. Convert soil water content to dry soil fraction:
   Dry fraction = 1 - (Soil water content (%) / 100)
2. Convert count to per gram dry soil:
   Fauna per gram dry soil = (Count per 100 g wet soil / 100) ÷ Dry fraction
3. Estimate dry soil mass per m²:
   Dry soil mass per m² (g) = Bulk density (g/cm³) × 10,000 × Depth (cm)
4. Calculate fauna density per m²:
   Fauna per m² = Fauna per gram dry soil × Dry soil mass per m²
5. Densities may be converted into biomass estimations by multiplying the densities by the mean biomass per unit density for specific biotic groups.

Measuring Microbial communities (Bacteria, Fungi, Protists, eDNA)

Soil microbiomes can be revealed using molecular approaches that unravel the genetic code found in all microscopic organisms. Information can be acquired to reveal microbiome characteristics such as the proportion of pH tolerant bacterial phyla associated with nitrification or carbon sequestration fungal functional groups. Target genes can be amplified to provide rapid and affordable biodiversity data, making DNA sequencing the cheapest part of the DNA workflow (Hebert et al., 2025). Primer selection can be made to target the most ecologically important microbial groups of Bacteria, Fungi, Protists. eDNA can also be targeted with 18S rRNA that can indicate a wide diversity of arthropod and other macrofauna (Köninger et al., 2023).

Table 1: Suggested DNA markers for broad taxonomic coverage of bacteria, fungi and eukaryotic species from microbial gDNA/eDNA with regions, basepair sizes and primer sequences

Gene	Target Group	Primer Set F/R	Region	Size	Sequence(5'-3')
16S rRNA	Bacteria / Archaea	515F / 806R	V4	~250 bp	F:GTGCCAGCM CGCGGTAA R:GGACTACHVG GTWTCTAAT
		341F / 785R	V3–V4	~460 bp	F:CCTACGGNG CWGCAG R:GACTACHVGTATC AATCC
ITS2	Fungi	ITS3 / ITS4	ITS2 region	~300–400 bp	F:GCATCGATGAGAA GCAGC R:TCCTCCGCTTATG TATGC
		fITS7 / ITS4	ITS2 region	~250–300 bp	F:GTGARTCAGAATCTTTG R:TCCTCCGCTTATG TATGC
18S rRNA	Eukaryotes (Protists, Fungi, Microfauna)	528F / 706R	Broad 18S	~450 bp	F: GCGGTAATTCCAGCTCCAA R:AATCCRAGAATTTCACCTCT
		TAReuk 454FWD1 / TAReuk REV3	V4 region	~380 bp	F: CCAGCASCYGCG TAATTCC R:ACTTTCGTTCTTGA TYRA
		Euk528 / EukB	18S rRNA	~400–500 bp	F: CGGTAATTCCA CTCCAA R:TGATCCTTCTGCAGGT CACCTAC

Table 2: Suggested DNA markers for mesofaunal and microfaunal with regions, basepair sizes and primer sequences.

Gene	Target Group	Primer Set F/R	Region	Amplicon size	Sequence(5‘ - 3‘)
COI (Cytochrome Oxidase I)	Mesofauna (mites, collembola); Microfauna (nematodes, rotifers)	**LCO1490 / HCO2198**	Folmer region	~658 bp	F:GGTCAACAAATC TAAAGATATTGG R:TAAACTTCAGG TGACCAAAAAATCA
COI Mini-barcode	Degraded or small-bodied taxa	**mlCOIintF / jgHCO2198**	Internal COI	~313 bp	F:GGWACWG WTGAACWGTWTA CYCC R:TAAACTTCAGG TGACCAAAAAAYCA
16S rRNA Gene (Animal)	Mesofauna & Microfauna (mitochondrial)	**16Sar-L / 16Sbr-H**	Mitochondrial 16S	(420 - 480, taxon - specific)	F:CGCCTGTTTA CAAAAACAT R:CCGGTCTGAAC CAGATCACGT
28S rRNA Gene	Mesofauna & Microfauna (nuclear ribosomal marker)	**fITS7 / ITS4**	ITS2 region Includes D3 domain	~800–900 bp	F:GTGARTC GAATCTTTG R:TCCTCCGCTTATG TATGC
18S rRNA	Eukaryotes (Protists, Fungi, Microfauna)	**D1/D2 Region:28SF / 28SR**	Nuclear 18S rRNA	~800–900 bp	F:ACCCGCTGAAT TAAGCAT R:GACTCC TGGTCCGTGTTTCA AGAC
18S rRNA	Nematoda	**NF1 / 18Sr2b**	V7-V8	380 bp (447 bp, + HTS adapters)	F: GGTGGTGCATGG CGTTCTTAGTT R: TACAAAGGGCAG GACGTAAT
MtDNA 12S	Enchytraeidae	**12S-a / 12SC**	Mitochondrial 12s	~80 bp	F: GCTGCACTT GACTTGAC R: AGCC GTGTACTGCTGTC

In order to find out the diversity of bacteria, fungi and pathogens in your soil, it will be necessary to process the eDNA samples using the following simple steps that turn the raw genomic DNA into detailed relative abundances of all the microbes in the sample (Fig. 43).

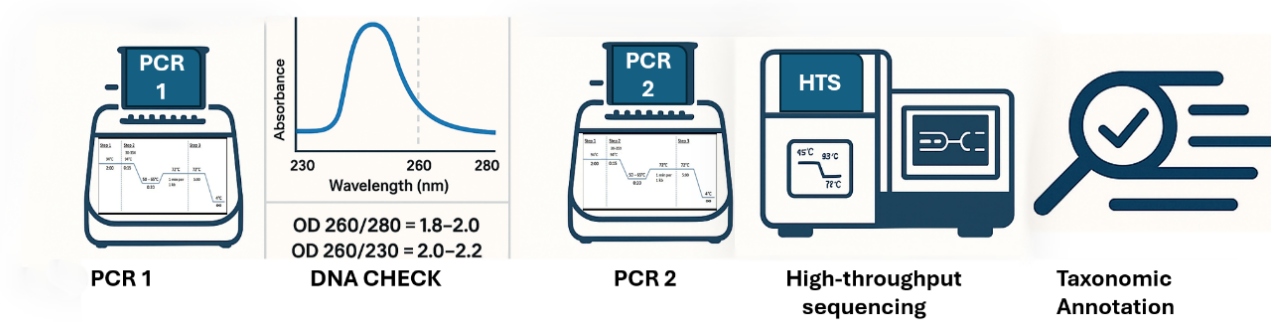


Fig. 43: Steps to generate microbial and other molecular-based community data. From left to right: (i) amplify marker with first PCR step, (ii) check DNA quality and OD, (iii) amplify with second PCR step to attach adapters and barcodes, (iv) perform High-throughput sequencing (HTS), and (v) determine species ID using sequence libraries.

DNA extraction from fresh soil samples

1. Air-dry 10 g of composite sample and place in 1.5 ml Eppendorf tubes.
2. Extract genomic gDNA from 0.25 g fresh soil with appropriate commercial extraction kit (e.g. DNeasy PowerSoil), or personal lysozyme solution. Elute into > 50 µl RNAse-free Water. DNA Extraction ISO 11063:2020
3. Check DNA Quality with a microvolume Spectrophotometer(e.g. NanoDrop or Qubit) for Optical Density, OD 260/230 = 2 - 2.2 (Salts and other residuals) 260/280 = 1.8 – 2.0 (Phenol/Protein or RNA residuals). Adjust concentration to 10 ng/µl. Minimum quantity for sequencing > 200 ng.
4. PCR amplification to concentrate DNA in low-yield samples and and run an agarose gel to visualise presence of target DNA markers.

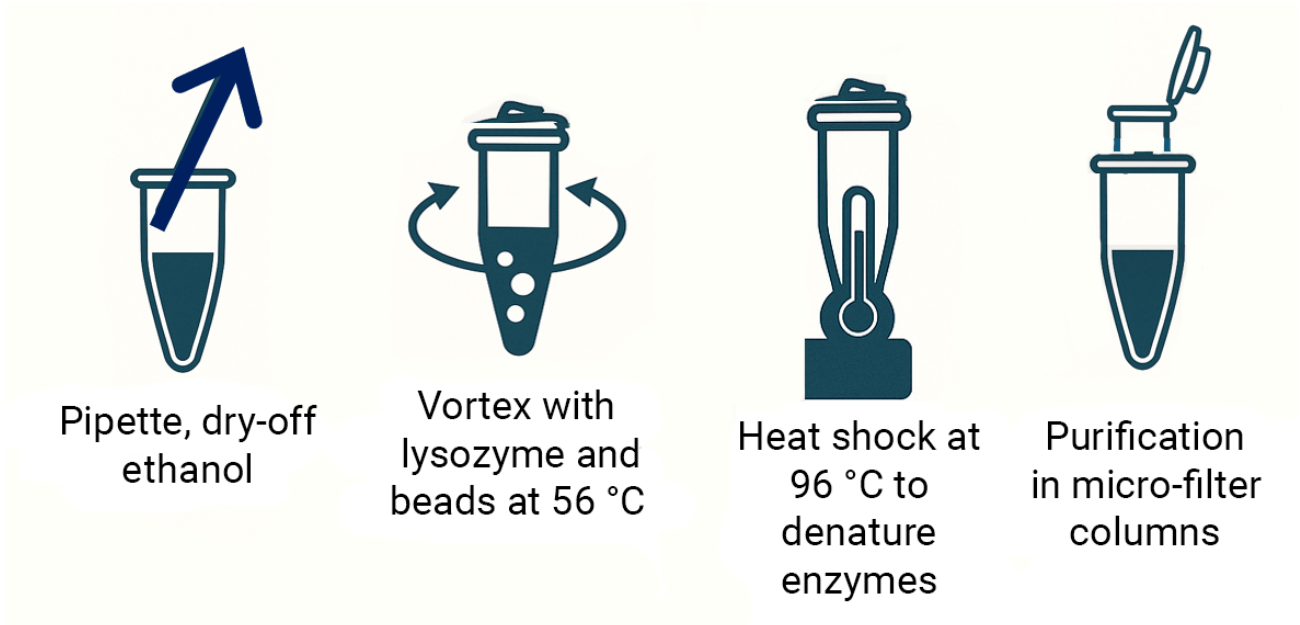


Fig. 44: DNA Extraction from microfaunal and mesofaunal samples: i) Remove ethanol, leave to dry, ii) Vortex with lysozyme and beads at 56 °C, iii) heat shock at 96 °C to denature enzymes, iv) final purification in micro-filter columns.

Mesofaunal and microfaunal DNA Sequencing

Extracting DNA from soft-tissue taxa (Nematodes, Enchytraeids, Collembola).

1. Decant ethanol preservative and dry-off at 70 °C heat-block for 10 min.
2. Extract using commercially available DNA extraction from animal tissue kits as per manufacturer's instructions. Alternatively, apply 100 µl lysozyme (20 mg/ml) and 100 ml of 5 % chelex resinous beads. Vortex for 30 min at 56 °C.
3. Apply heat-shock at 96 °C for 15 min to de-activate enzymes prior to sequencing.
4. Centrifuge at 30 k RPM for 15 min. Decant supernatant gDNA.
5. Purify with magnetic beads or silica column.
6. Check DNA Quality. Adjust concentrations to 10 ng/µl for each biotic type.

*Sclerotized taxa such as oribatid mites: additional pre-lysis micro-pestle grinding with liquid Nitrogen (-196 °C), or shaking with 1 mm diameter steel ball-bearing at 5 k RPM for 2 min.

In order to determine the presence of the desired barcode marker, Polymerase Chain Reactions (PCR) amplify the section (with specific primers) to be used for high-throughput sequencing and biodiversity assessment.

Perform Polymerase Chain Reactions (PCR) with following reaction ingredients:

1. < 10 ng template DNA – NOTE: Excess gDNA can inhibit reactions.
2. 1 Unit High-fidelity Taq Polymerase enzyme per 50 µL reaction
3. 1 Unit Polymerase buffer + 2.5 mM $MgCl_2$ (Optimizes reaction conditions: pH, ionic strength and stabilizers).
4. 0.2 µM of Forward and Reverse primers.
5. dNTP mix (Deoxynucleotide Triphosphates) 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP).

Component	Stock Conc.Group	Final Conc.	Volume (µL)
10X PCR buffer	10X	1X	5.0
$MgCl_2$	25 mM	2.0 mM	4.0
dNTP mix	10 mM each	200 µM each	1.0
Forward primer	10 µM	0.4 µM	2.0
DNA template	-	1–10 ng	1.0
Taq polymerase	5 U/µL	1 U	0.2
BSA (optional)	1 mg/mL	0.5 µg/µL	1.0
Water	-	-	up to 50.0

Table 3: Standard PCR mix with stock concentrations, final concentrations, and total volume per reaction.

PCR reactions can be performed using the standard thermal cycling steps.

1. Initial Denaturation Step at 98 ºC for 1 min
2. 30 x Denaturation Steps at 98 ºC for 10 s
3. Annealing at 50 - 60 ºC for 30 s
4. Elongation at 72 ºC for 30 s
5. Final Extension at 72 ºC for 5 min
6. Hold at 4 ºC

Table 4: Steps for primary PCR of marker sequences.

Step	Temperature (°C)	Time	Notes
Initial Denaturation	95	3 min	Fully denature template DNA
Denaturation	95	30 s	Separates strands
Annealing	50 - 60	30 s	~3–5 °C below primer Tm
Extension	72	30 s	30 s for ~500 bp; 300 bp usually sufficient
Repeat	-	25 - 35 cycles	
Final Extension	72	5 min	Completes any unfinished products
Hold	4	∞	Store products before transfer to -20 °C

DNA visualization of PCR amplification products with Agarose gel electropheresis

1. Prepare with 1.5 % Agarose gel in 100 ml 1 x TAE/TBE buffer solution with SYBR Safe or other DNA Stain.
2. Load 1 µL DNA product with 5X loading buffer alongside 1 Kb DNA Ladder.
3. Run in 1X TAE/TBE solution tank at 100 V for 30 min. or until DNA has migrated 3/4 of the gel.
4. Image under UV or blue-light transilluminator.

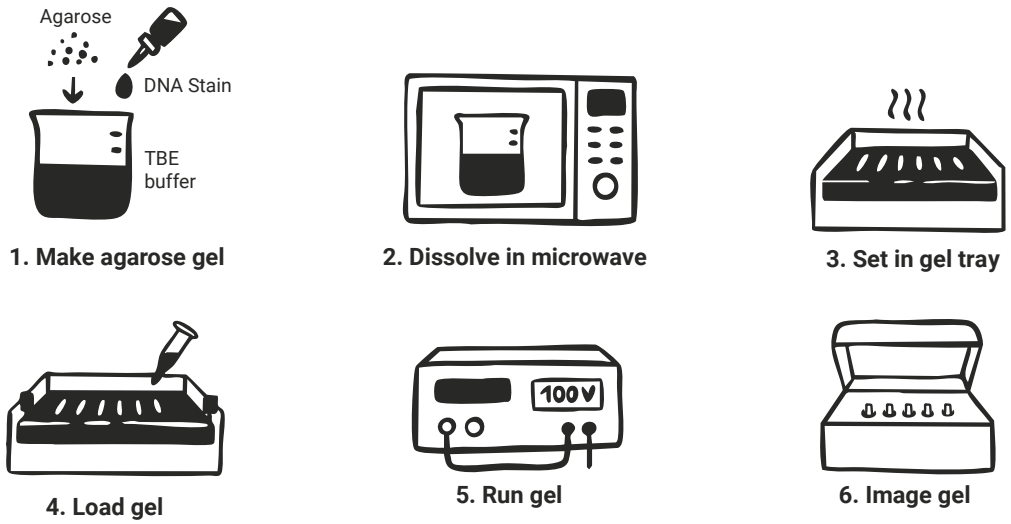


Fig. 45: Main steps in visualising amplified PCR product using electrophoresis on agarose gel.

A secondary PCR step is required for high-throughput sequencing to prepare samples with the addition of barcodes and adapters required for multiplexing.

Fewer cycles for Library prep (8–12 vs. 25–35). This minimizes amplification bias.

Table 5: Steps for secondary PCR for barcoding sequences.

Step	Temperature (°C)	Time	Notes
Initial Denaturation	95	3 min	Activate polymerase
Denaturation	95	30 s	Strand separation
Annealing	55 - 60	30 - 60 s	Slightly lower than standard PCR
Extension	72	30 s	Depends on amplicon length (300–600 bp)
Repeat	-	8 - 12 cycles	
Final Extension	72	5 min	Completes library fragments
Hold	4	∞	

High-throughput sequencing can be performed at a sequencing facility or with a platform that provides sufficient output size (> 50 k reads 5 Gb/Sample).

1. Pool gDNA or amplicon products according to extraction or biomass calibrations.
2. Library Preparation
    - Use a ligation-based library preparation kit compatible with single-molecule, long-read nanopore technology or other high-throughput sequencing platforms.
    - Attach sequencing adapters and barcode indices (for multiplexing).
    - Perform quality check (QC) with a fragment analyzer or TapeStation.
3. Sequencing
    - Load prepared libraries onto flow cells of a single-molecule nanopore-based sequencer.
    - Run sequencing in real-time until desired depth is reached (>50 k reads per sample).
    - For hybrid sequencing, parallel Illumina (short-read) sequencing can be done for error correction.
4. Data Processing

Basecalling: Convert raw electrical signals to nucleotide sequences using open-source basecalling software.

Processing sequence outputs

Soil microbiomes can be revealed using molecular approaches that unravel the genetic code found in all microscopic organisms. Information can be gained on the proportion of pH tolerant bacterial phyla associated with nitrification or carbon sequestration fungal functional groups. Target genes can be amplified to provide rapid and affordable biodiversity data, making DNA sequencing the cheapest part of the bioinformatics workflow (Hebert et al., 2025). Primer selection can be customized to the ecologically important microbial groups of bacteria, fungi and protists.

1. Assign reads to samples using barcodes, trim adapters and primers.
2. Merge paired-end reads with FLASH (Magoč & Salzberg, 2011).
3. Quality filter with fastp software (Bokulich et al., 2013), remove chimeras using vsearch (Edgar et al., 2011).
4. Denoise with DADA2 in QIIME2 to generate ASVs (Amplicon Sequence Variants) or OTUs (Operational Taxonomic Units).
5. Taxonomic annotation: Bacterial 16S/ Eukaryotic 18S: SILVA, Fungal ITS: UNITE
6. Generate ASV/OTU tables, Shannon diversity indices.
7. Deposit raw sequences in the GBIF or other Open Access Archive.
8. If processing genetic information from foreign sites, ensure compliance with the UN Nagoya protocols to ensure sharing of benefits derived from national genetic information.

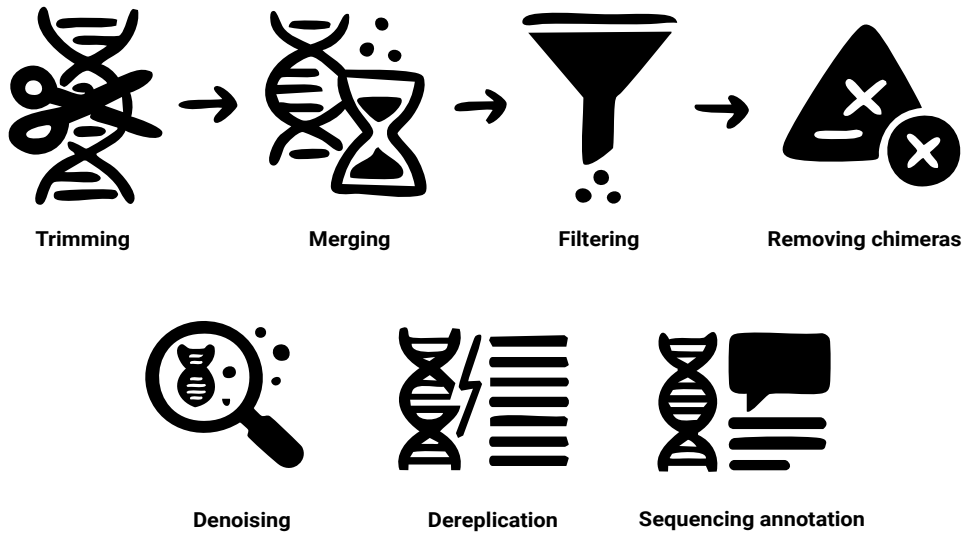


Fig. 46: Flow chart of bioinformatics pipeline from raw reads to Species annotating. Trimming, Merging, Filtering, Removing chimeras, Denoising, Dereplication, Sequence annotation.

Computer vision: Machine learning assisted counting and biomass estimation

1. **Define Output:** Count per image (optionally, classification by Order, trophic group or developmental stage).

2. **Data Collection**

   a. Image Sources: Collect brightfield or stereo microscope images of nematodes, enchytraeids, mites or collembola in preservative medium (70 % Ethanol). Ideally include variation: specimen diversity, different levels of debris. Optimize lighting and other physical settings. Set pixel size to the smallest feature at ≥ 2–3 px, lock white balance/exposure, prefer low ISO and 12–16-bit RAW/TIFF.

   b. Data Quantity Initial training with 100 -200 for initial model, scaling up to >1,000 for robustness with < 100 images, and scaling up to produce > 95 % self-validation efficiency within 100 epochs.

   c. Open Dataset

   - Release the dataset on platforms like Zenodo or Kaggle for community contributions.

3. **Annotation Strategy:** Train and apply filters based on size, sphericity, opacity or other factors to avoid artefact detection e.g. root hyphae, soil particles. Tools such as Labellmg, Roboflow or CVAT can be used for segmentation (counting).

4. **Preprocessing:** Standardise image magnification, lighting direction and intensity, background, Region-of-interest sub-sampling and colourization (B&W, Gray-scale, Colour). Applying noise reduction can reduce issues with focal plane by creating silhouette and contrast adjustment to visualize opaque specimens.

5. **Algorithm Selection**

   - Phase 1: Counting
     - Use an object detection model (e.g. YOLOv8, Detectron2, EfficientDet) trained to detect nematodes, enchytraeids or microarthropods.
     - Output: Bounding boxes -> Count = number of detections.
   - Phase 2: Segmentation (Optional)
     - For finer analysis (e.g. length measurement), use instance segmentation (Mask R-CNN).
     - Thresholds for classing objects can be defined by identifying the value that best separates groups using ROC analysis, percentile cut-offs, or clustering methods such as k-means or Gaussian mixtures). The level of size confounding for biomass estimation can be determined by plotting values for sphericity vs. surface area.

6. **Training Pipeline**
    - Framework: PyTorch or TensorFlow (both open-source, well-supported).
    - Training: Split data: 70% train / 20% validation / 10% test. Use pretrained weights (COCO) -> fine-tune to reduce dataset size requirement. Loss function: combination of classification + localization loss. Evaluation metrics: Mean Average Precision (mAP), F1-score, counting accuracy (MAE).

7. **Deployment:** Package as a streamlit or Gradio web app and drag-and-drop images for counting. Containerize with Docker for reproducibility. Host on Hugging Face Spaces for free, interactive use.

8. **Community Involvement:** Open GitHub repo with Dataset, Model weights, Training code. Invite labs to contribute more images (active learning: model highlights uncertain detections for review).

9. **Continuous Improvement:** Periodic re-training with new data (semi-supervised or weakly-supervised learning). Multiple class counting (e.g. Mite Orders, Nematode trophic guilds).

10. **Biomass conversion:** Counts and surface area (S.A.) can be converted to biomass based on estimated density of soil biota accounting for volume of sample.

11. IDENTIFICATION KEYS

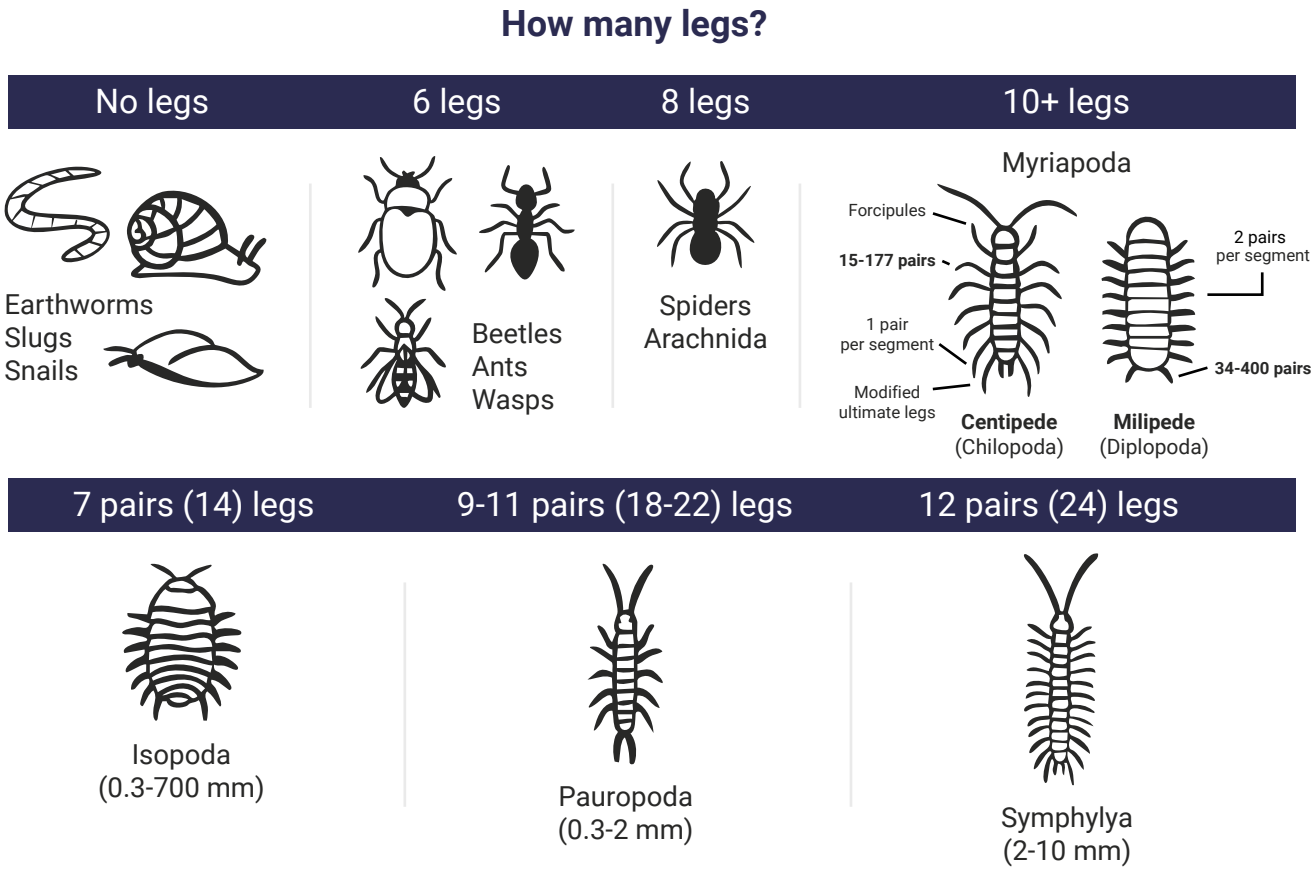


Fig. 47: Identification key of main macrofaunal taxonomic groups.

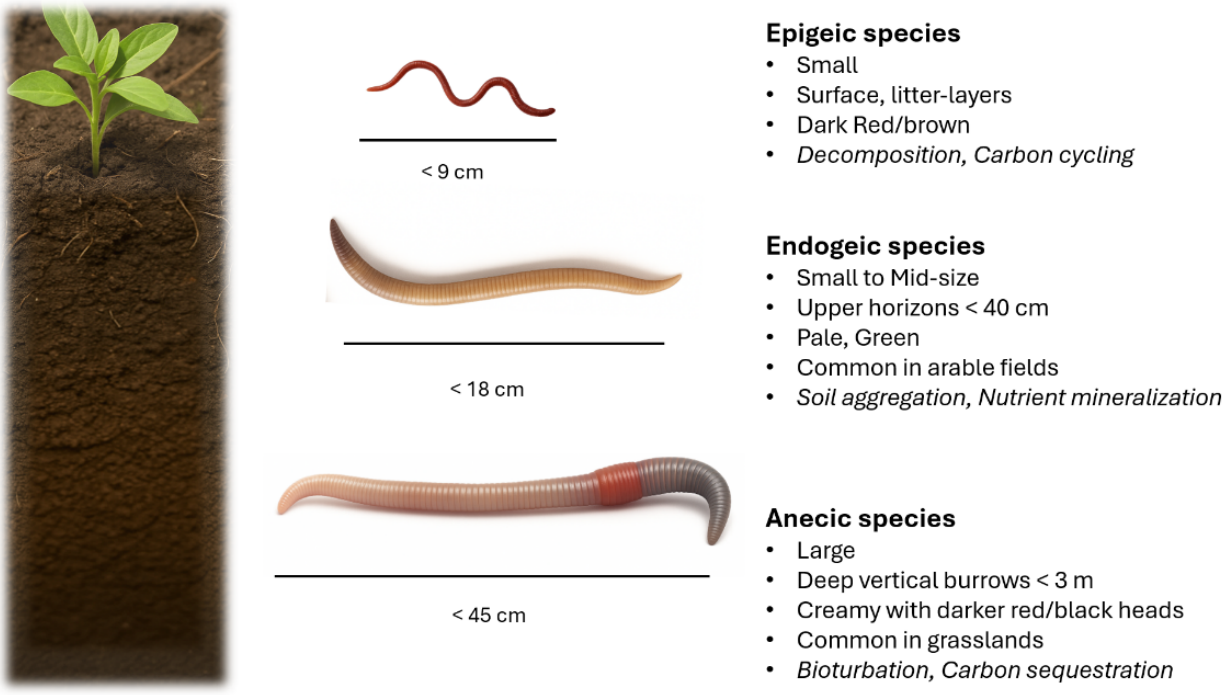


Fig. 48: Identification key of main earthworm ecological groups, including size, soil stratification depth, colour, habitat, and putative ecological roles.

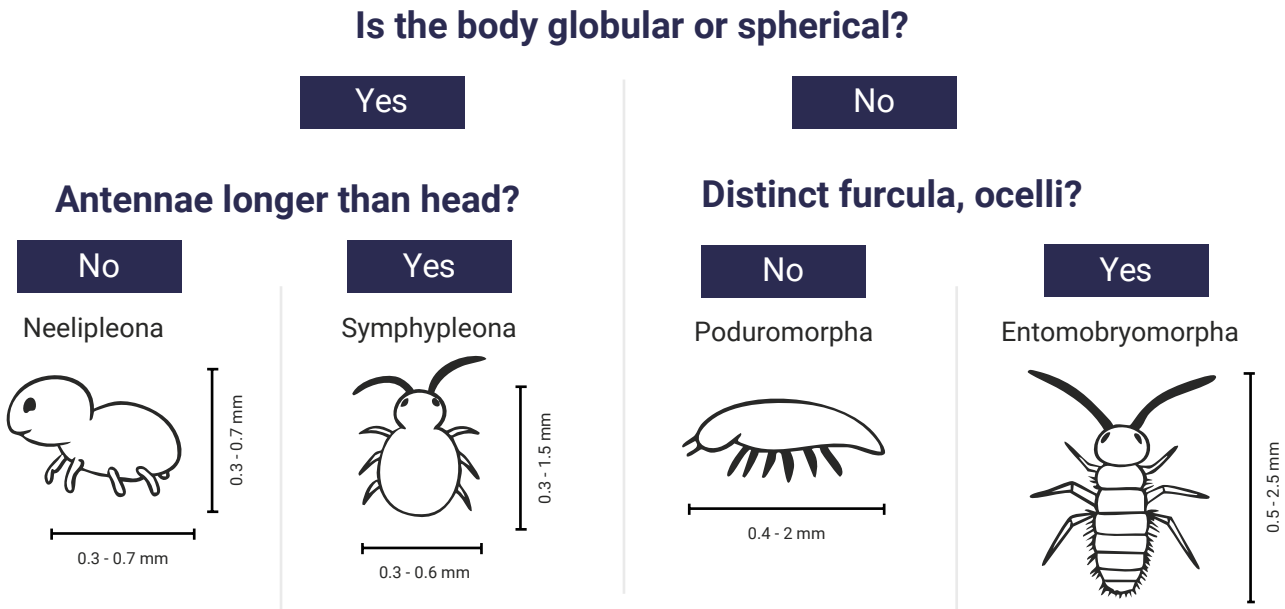


Fig. 49: Identification key of main Collembola orders.

Fig. 50: Examples of four Collembola orders, clockwise from top left: *Entomobryomorpha sp.*, Neelipleona – *Neelides sp.*, Symphypleona – *Katianna sp.*, Poduromorpha – *Holocanthella sp.*

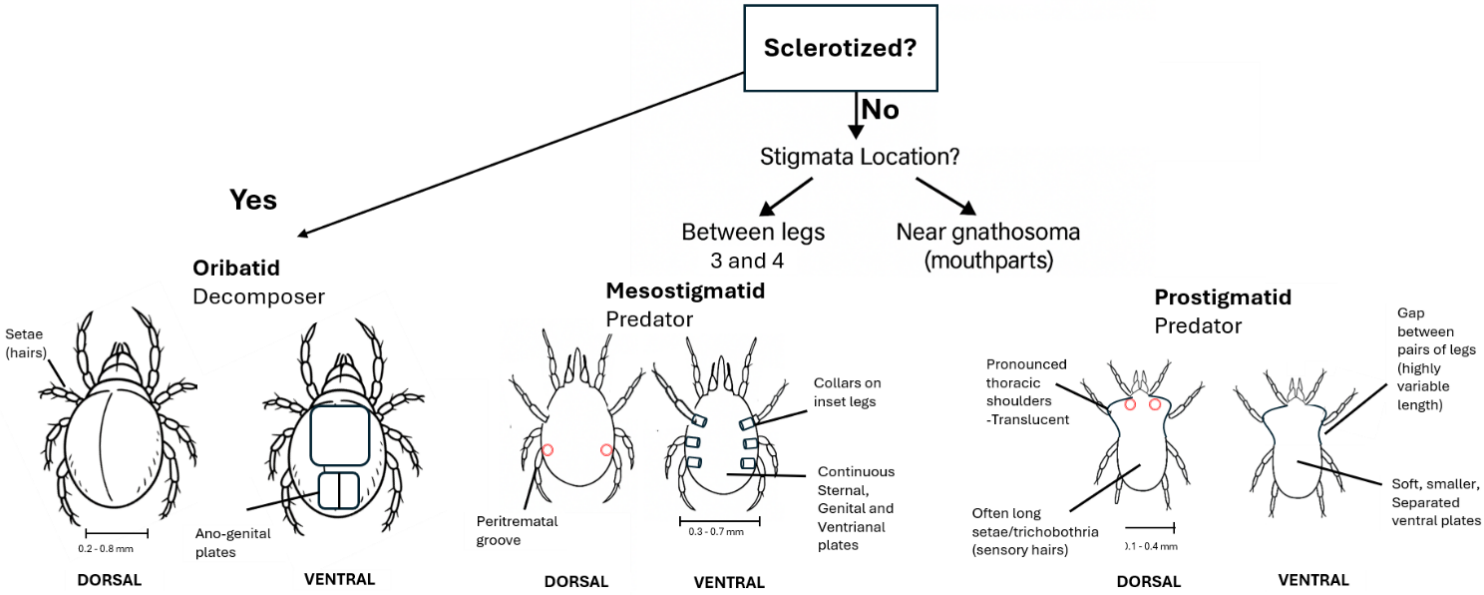


Fig. 51: Identification key of main soil mite orders: Oribatida, Mesostigmata and Prostigmata.

Fig. 52: Mesofauna (L-R) Oribatida - Euphthiracaridae sp., Mesostigmata Laelapidae sp., Prostigmata - Trombidiidae sp..

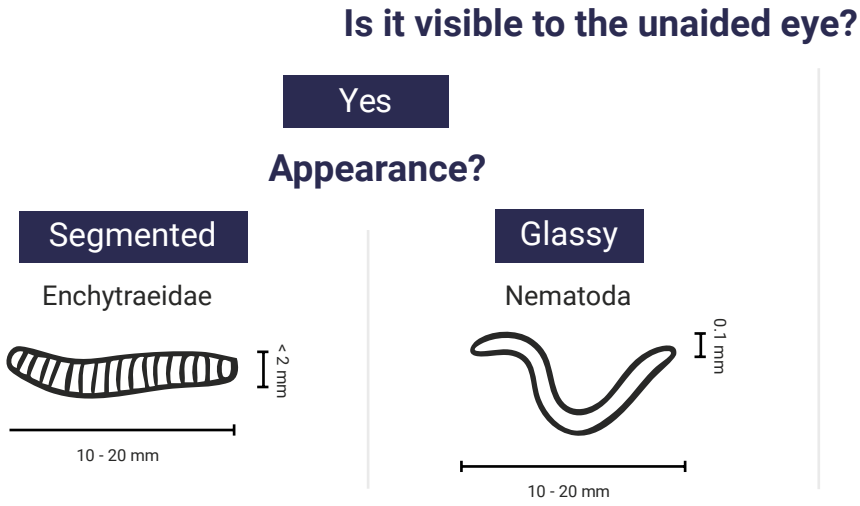


Fig. 53: Identification key of main microfaunal groups.

1. **Bacterivore:** Funnel shaped stoma, Large basal bulb
2. **Fungivore:** Thin stylet, Small basal bulb
3. **Predator:** Large tooth, Defined musculature
4. **Omnivore:** Hollow stylet, No knobs
5. **Herbivore:** Stylet of various thickness, oesophageal knobs

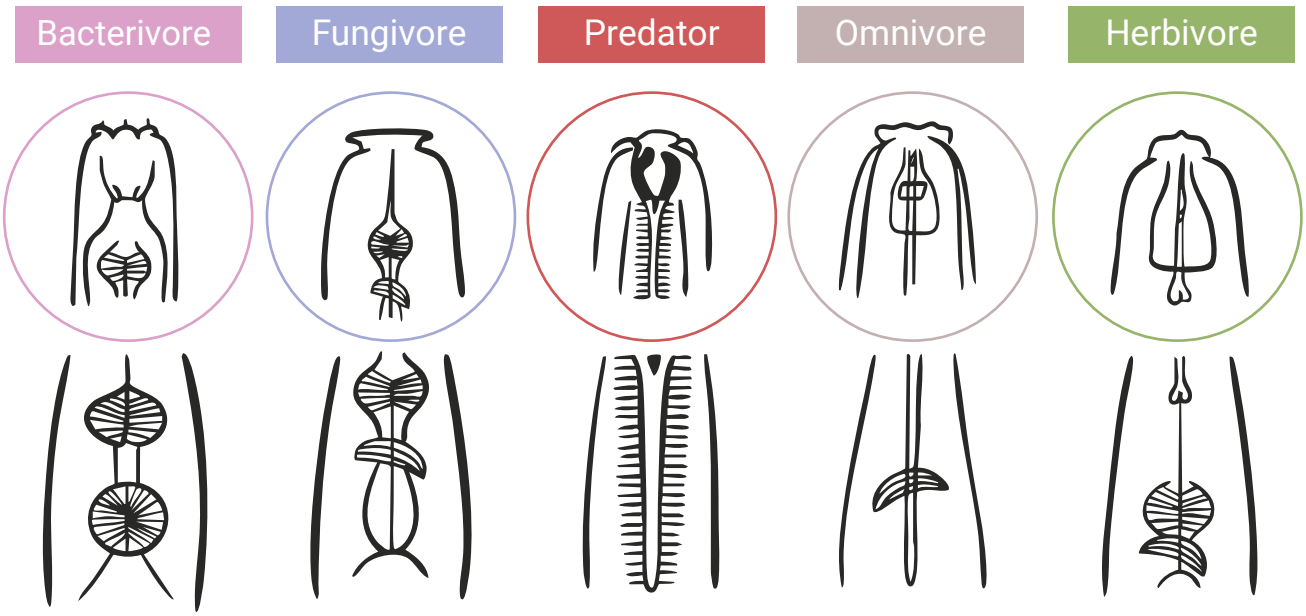


Fig. 54: Mouthparts and main features of nematode trophic groups.

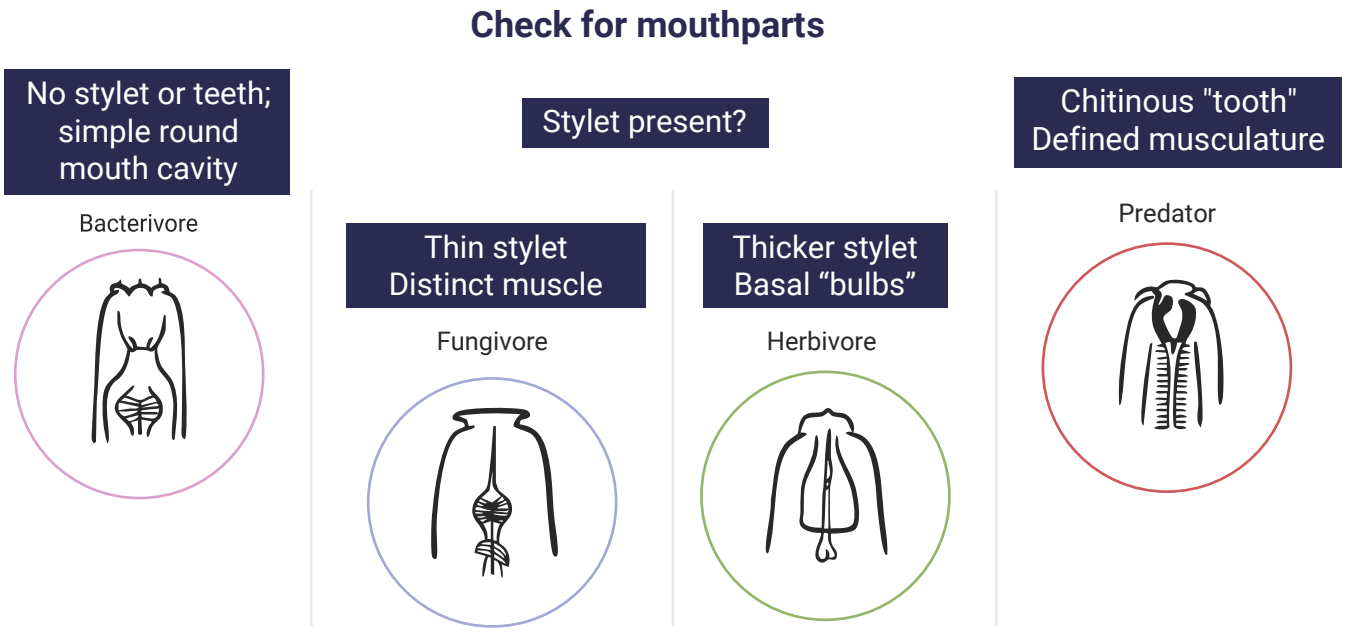


Fig. 55: Identification key of main nematode trophic groups.

Main Soil Fungal Families and Ecological Functions

Table 6: International Organization for Standardization (ISO) manuals, ISOs for main sampling steps, planning, sampling, biotic sampling in ISO and European ISO numbers and release years.

Topic / Parameter	ISO (number:year)	EN ISO (number:year)
Sampling framework (plans)	ISO 18400-101:2017	EN ISO 18400-101:2017
Soil invertebrates — Study design / programmes	ISO 23611-6:2012	EN ISO 23611-6:2012
Select & apply sampling techniques	ISO 18400-102:2017	EN ISO 18400-102:2017
Safety in sampling	ISO 18400-103:2017	EN ISO 18400-103:2017
Sampling strategies / site investigation	ISO 18400-104:2018	EN ISO 18400-104:2018
Packaging, transport, storage & preservation	ISO 18400-105:2017	EN ISO 18400-105:2017*
Bulk density (dry)	ISO 11272:2017	EN ISO 11272:2017
Soil pH	ISO 10390:2021	EN ISO 10390:2022
Water-dispersible aggregate stability	ISO 10930:2012	EN ISO 10930:2013
Water content — mass basis (gravimetric)	ISO 11465:1993	— (EN ISO Under revision)
Soil for biological tests (collection/ handling/storage)	ISO 18400-206:2018	EN ISO18400 206:2018
Soil invertebrates — Macro-invertebrates (excl. earthworms)	ISO 23611-5:2024	EN ISO 23611-5:2024
Soil invertebrates — Earthworms	ISO 23611-1:2018	EN ISO 23611-1:2018
Soil invertebrates — Microarthropods (mites & collembola)	ISO/DIS 23611-1 -	RELEASE 2025
Soil invertebrates — Enchytraeids	ISO 23611-3:2019	EN ISO 23611-3:2019
Soil invertebrates — Nematodes	ISO 23611-4:2022	EN ISO 23611-4:2022

Fig. 56: Imaging nematodes with high-resolution microscope.

12. RECOMMENDED USE

These protocols are designed to be as inclusive as possible and should not be constrained by the availability of specific apparatus. The aim is to encourage citizen scientists to generate valuable soil data, which is essential for enhancing our understanding of what makes soils healthy. Flexibility is intended in the number of sites and plots that are dependent on the monitoring aims. However, all directions provided in these protocols are intended to be applied as written to maximise interoperabiity.Any deviations from the protocols should be clearly noted in the metadata. Implementing these monitoring protocols enables the capture of soil biodiversity across all trophic levels and across different land-use types. Paying particular attention to organic layers in forests increases the accuracy of measuring biodiversity in these systems, given the significant proportion of biota that reside in the organic layers rather than the mineral soil.

Standardising the assessment of biodiversity is an important step towards monitoring the densities of different trophic levels in soils and the irreplaceable services each provides. Wide-scale adoption by farmers, land managers, researchers, and gardeners is recommended, as it can generate reliable, interoperable data. Such data can guide everyday management, inform restoration efforts, and evaluate progress towards environmental targets. Consistent, large-scale monitoring will build the robust, evidence-based database urgently needed to understand the complex responses of soils to different uses and practices. Ultimately, this will support more sustainable decisions that protect soil biodiversity, enhance productivity, and strengthen climate resilience.The protocols presented in this handbook lay the foundation for a standardised, repeatable approach to monitoring soil health across time and space. Whether applied in a farm setting, a citizen science garden, or a national-scale monitoring program, these guidelines combine essential physical, chemical, and biological parameters to offer a robust and holistic assessment of soil condition. All comments and suggestions can be emailed to SOB4ESprotocols@nioo.knaw.nl.

13. FUTURE DIRECTIONS

The protocols presented here lay the foundation for a standardised, repeatable approach to monitoring soil physico-chemical properties alongside detailed soil biodiversity across time and space. Whether applied in a farm setting, a citizens garden, or a national-scale monitoring program, these guidelines combine the essential parameters required to give a robust and holistic assessment of soil condition.

Looking ahead, accelerating developments in global soil biodiversity mapping, remote sensing, and biomonitoring technologies offer powerful opportunities to expand and refine soil monitoring efforts. Tools such as high-throughput sequencing, electrochemical, lab-on-a-chip systems, AI-assisted imaging, and biosensors are rapidly becoming more applicable. These innovations have the potential to make soil biodiversity assessment faster, less expensive, and more scalable — especially when used alongside or verified with traditional taxonomic methods (Ross et al., 2022).

A critical role of the protocols described here is to provide a framework for validating these emerging approaches. Ground-truthed, morphologically verified data remains essential for ensuring the reliability of novel approaches such as eDNA analysis, NDVI-based remote sensing, and acoustic soil monitoring. By doing so, we can build confidence in rapid screening tools while ensuring ecological relevance.

Linking soil biodiversity with the functions it supports—such as nutrient cycling, water retention, and carbon storage—will be key to identifying meaningful bioindicators across land-use types. These can guide management decisions and support international efforts like the EU Soil Mission and IUCN Red List expansion for invertebrate taxa.

Ultimately, we can simplify biodiversity assessments with targeted, cost-effective assessment. This will help translate soil health science into practical management strategies that contribute to ecosystem productivity and sustainability.

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