Soil BON Earthworm - A global initiative on earthworm distribution, traits, and spatiotemporal diversity patterns

Supplementary Material to SOIL ORGANISMS 96(1) 2024 Pierre Ganault et al.  
<https://doi.org/10.25674/362>

SI A - Sampling protocol

After decades of earthworm research, sampling techniques are still diverse, from soil hand sorting, to chemical extraction (formalin, AITC, mustard and other) and electric extraction, each with advantages and limitations. Moreover, those techniques are based on sampling units (replicates) of varying dimensions (area and depth), which are organized through varying sampling designs (i.e. sample number and spatial disposition). One of the goals of Soil BON Earthworm is to define a standardized sampling protocol and sampling design for earthworm communities assessment worldwide feeding a standardized template for community data. The protocol builds on the on-going global initiative Soil BON and Soil BON Foodweb (Guerra *et al.* 2021b; Potapov *et al.* 2022) to promote synergies with other global soil biota monitoring schemes. Here, we develop the different steps of earthworm sampling and specify the similarities and differences with Soil BON Foodweb.

### Overall procedure

Sampling sites for earthworm assessment can be or not, part of the pre-selected set of sites from Soil BON Foodweb, planned in communication with the respective researchers involved in the core Soil BON Foodweb network. Soil BON Earthworm does not require the sampling to occur in paired sites outside and inside areas that have a ‘protection’ status (e.g. nature conservation reserves) to evaluate the effect of present conservation strategies on soil biota (Guerra *et al.* 2021a)**.**

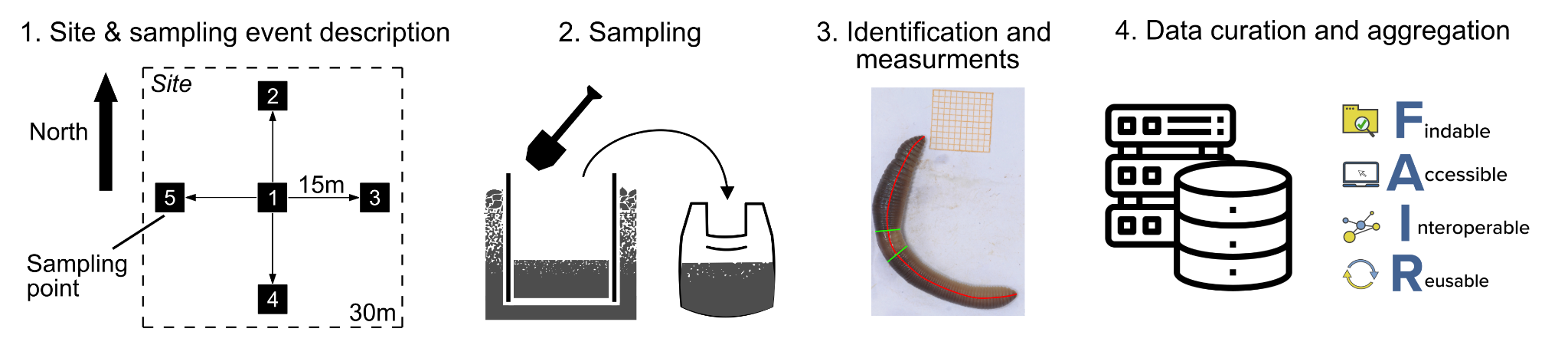
The overall procedure follows exactly Soil BON Foodweb with:

1) site and sampling event description;

2) sampling of soil communities;

3) lab identification, imaging and trait measurement;

4) data curation and collation to global databases



**Figure 1 - Workflow for the global monitoring of earthworm communities**. Local teams collect the samples in the field and perform the identification and the optional measurement and weighing of individuals. The produced data is then entered in a template (see Supp Mat SI1) and collated in a global database following FAIR principles.

For all sites we collect information on the sampling event, i.e. date, collector identity, location, vegetation characteristics and history. On each site, five sampling points are assessed: one at the georeferenced center of the site, and four at sampling points 15 m in each of the four directions, N, S, W, E from the center. All samples at each sampling point are taken randomly within one square meter area. In total, the following materials and information are collected from each site:

* Site and sampling event description
* 3x5 vials with hand-sorted earthworms from 3 different layers
* 5 plastic bags with litter samples (if present) for weighing
* 5 photographs of topsoil profiles

### Sampling protocol

Compared to Soil BON Foodseb protocol, Soil BON Earthworm makes the sampling of the 10 to 20 cm layer mandatory, and makes the sampling of remaining macrofauna by hand sorting of the same samples or micro and mesofauna extraction optional. Soil BON Earthworm adds optional steps to perform chemical extraction when suited to the ecosystem investigated, and to increase the number of sampling points to 9 by adding the 4 corners of the 30 x 30 m plot. At each of the 5 sampling points, a 25 x 25 cm square is used to collect the litter layer and the two underlying 10 cm-depth soil layers. The litter layer limit is defined as in Soil BON Foodweb (Potapov *et al.* 2022). All earthworms are hand-sorted using tweezers, briefly washed in water and placed in a vial with 70% ethanol, or 99% ethanol if barcoding is planned. Hand sorting ends when the entire sample is checked. Use a headtorch in limited light conditions. The animal collection must be done outside of the site to avoid disturbance and can be done in the laboratory/field station, if litter and soil can be safely transported there within a day. After sampling, the site should be left as it was found at arrival, that is sorted soil has to be put back into the hole.

**Detailed protocol:**

**A) Site description**

**1.** Take a picture of the plot

**2.** Place a label on site to find it back if possible

**3.** Write site information using Soil BON Foodseb chart available in Supplementary Material of [(Potapov *et al.* 2022)](https://www.zotero.org/google-docs/?3CTgwE)

**B) Earthworm sampling**

Put the 25 x 25 cm² frame on the ground and press it down to fix. Cut and remove the ground vegetation within the frame; keep mosses+lichens.

**1.** Collect litter (+fauna) inside the frame with your hands and place it in a plastic bag (step B1, Fig. 2). Wear gloves if dangerous animals are present in the area.

**2.** Excavate the underlying soil (0-10 cm) with a spade and put it in another bag (step B2, Fig. 2). If soil is not deep enough, note the actual depth of the layer.

**3.** Excavate the underlying soil (10-20 cm) with a spade and put it in another bag (step B3, Fig. 2). If soil is not deep enough, note the actual depth of the layer.

**4.** Sort separately the earthworms from the litter and the two soil layers collected by placing small amounts of litter/soil from the bag into sorting trays and separating the leaves or breaking soil aggregates carefully. Wash specimens if necessary and fix them in vials with 70% ethanol, or 99% ethanol if barcoding is planned, and ensure that the volume of earthworm does not exceed half the volume of ethanol. Do not forget to put a label inside the three vials. EW for earthworms, OL for litter layer, S1 for first topsoil layer (0-10 cm), S2 for second topsoil layer (10-20 cm). Also write the sample code on the vial.

**5.** Place the litter back in the plastic bag after all earthworms are captured. Close the bag, put the corresponding label inside and write the sample code on the bag. Collect a sub-sample of soil excluding rock, roots, and branches (~200 g) from each layer in separate plastic bags (step B6, Fig. 2) for further lab analyses (soil water content, pH, CN, texture). Label the bag with a sticker and write the sample code on it.

**C.** **Soil profile picture**

From the soil pit, select one of the sides with the clear soil profile and where eventual horizons are well visible. Take a picture with

Put the remaining soil back inside the hole and leave the site minimally disturbed. Transport and submit the vials and litter and soil samples to the local lab. You should have a total of 15 bags and 15 vials.

**A. Earthworm specimens**

**1.** Identify earthworms to the finest taxonomic resolution possible.

**2.** Use the community data template especially developed, available here: <https://zenodo.org/records/10284283>.

**3.** Ensure to use taxonomic names from the last updated worldwide checklist of earthworm species names available here <https://zenodo.org/records/8348860>.

**4.** If you plan to perform any trait, refer to the trait section of the main manuscript and use the trait template available here <https://zenodo.org/records/10302872>.

**B.** **Litter samples**  
**1.** Weigh each litter sample with at least 0.1 g precision (=fresh weight).

**2.** Oven dry all five litter samples at 40°C for 48 h.

**3.** Weigh each litter sample again (=dry weight).

**4.** Record both fresh and dry weight of each sample.

**C.** **Soil samples**

**1.** Weigh a small portion of ca. 10 g of each of the 5 soil samples to 0.1 g precision (=fresh weight).

**3.** Place the remaining soil to dry in the oven at 40°C for 48 h to prepare for chemical and physical analysis.

**3.** Oven dry the 10 soil samples at 105°C for 24 h.

**4.** Weigh each soil sample again (=dry weight) to obtain fresh and dry weight to calculate soil moisture.

**5.** Record both fresh and dry weight of each sample.

### Optional steps

#### Microhabitat sampling

If the study involves a biodiversity focus (including species richness assessment), microhabitats should be investigated for 1 h per site with two people. This must be done after soil samples are excavated. We recommend exploring any microhabitat available on the plot, by actively searching for earthworms in spots with accumulating litter, rotting logs, under stones, moss, or animal dungs; next to water bodies such as streams, rivers, lakes, ponds, or marshes; inside bromeliad leaf axils or other large epiphytes, inside banana or other large plant axils; inside, under, or next to termite mounds. Additionally, where there is evidence of large surface casts, dig a larger hole (50 x 50 cm to 1 m²), and deeper (up to 50 cm if possible) in order to collect larger species that may not be collected in the smaller monoliths or with the mustard extraction. If the species is not spotted by excavation only, pour mustard at the bottom of this large hole in order to attempt to bring it up to the surface. If sampling in a cultivated field, at least look under surface litter (if present) and dig a big hole. Put specimens in one vial per microhabitat, labeling the type of microhabitats under which it was found.

#### Additional samples

Depending on ecosystem type, for example in tropical areas, more than five samples might be necessary to capture earthworm diversity at the site. In that case, and if sufficient manpower is available four additional samples should be taken in the four corners of the 30 x 30 m plot, as performed for Soil BON. The same procedure must be then followed for each of those additional samples, leading to a total of 9 litter samples and 27 earthworm vials (9 points x 3 layers). Note down for which site additional sampling was performed.

#### Chemical extraction

Chemical extraction uses the expellant properties of certain solutions to force earthworms to emerge at the soil surface. Different chemicals can be used (formalin, mustard, allyl isothiocyanate) with different efficiency (Pelosi et al. 2009). Among these chemicals, mustard is the most cost effective, easy to get, and environmental and user friendly product. Mustard expellant power is linked to allyl isothiocyanate (AITC) concentration and an optimal value of 100 g AITC per liter must be reached (Zaborski 2003). AITC power fades with time before expiry and to a lesser extent between batches (Pelosi *et al.* 2014). Hence, sampling should be done with mustard powder as far as possible from expiry date, and be harmonized across sampling events.

Depending on the mustard powder available on your country, adapt the quantity of powder and water to reach the recommended concentration of 100 g AITC per liter of water (Zaborski 2003). Shake mustard-water mixture for 3 min. Pour the mustard solution into each 25 x 25 cm pits. The solution may need to be applied in several portions depending on the soil properties (i.e., drainage time). Remove any earthworms that appear, and fix them in ethanol (separate vials for each sample), the label should mention “M” as “mustard” (e.g. M\_EW\_1 to 5 or 9 depending on the number of samples taken). Sampling is finished 30 min after the application of the last portion of mustard solution.

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