HoliSoils
Working together for forest soils

D1.3 - Harmonised methodologies for quantification of biomass of soil organisms

Holistic management practices, modelling, and monitoring for European forest soils, HoliSoils

Project duration: 1.5.2021–31.10.2025

H2020 Grant Agreement No 101000289

Coordinator: Natural Resources Institute Finland (Luke)

Deliverable D1.3 – Harmonised methodologies for quantification of biomass of soil organisms		
In this deliverable, we review different methods for biomass quantification. Based on the results and their application across the HoliSoils study sites in Europe, we propose a harmonized methodology to be utilized across the variety of European forest soils.		
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Date of publication	30.4.2024	
Dissemination level		
PU	Public, fully open e.g. web	x
CO	Confidential, only for members of the consortium (including the Commission Services)	
CI	Classified, information as referred to in Commission Decision 2001/844/EC	
Nature of the Deliverable		
R	Document, report	x
DEM	Demonstration, pilot, prototype, plan design	
DEC	Websites, patents filing, market studies, press & media actions, videos etc.	
OTHER	Software, technical diagram etc.	
Ethics	Ethics deliverables	

This project has received funding from the European Union's H2020 research and innovation programme under grant agreement No 101000289.

Contents

1. Introduction

Estimating the biomass of different trophic groups in forest soils is crucial for understanding nutrient cycling, soil fertility, and ecosystem functioning. Different trophic groups, including bacteria, fungi, microfauna, mesofauna and macrofauna play specific roles in multiple processes including the provision of nutrients to plants, decomposition of organic matter, nutrient cycling, and creating soil structure. Quantifying their biomass helps in assessing the health and productivity of soil ecosystems, predicting responses to environmental change and forest management practices.

Various methods to assess biomass were applied in the past in forest soils, each with its specific features, some of which are discussed below.

2. Methods to study microbial biomass in soils

2.1 Fumigation-Extraction

This method involves killing of soil microbial cells by fumigation with chloroform and then quantifying the amount of this biomass by measuring its respiration. This method is widely used due to its relative simplicity and quantitative reliability. It was long considered a standard for estimating microbial biomass carbon in soil samples, but it quantifies total microbial biomass without directly distinguishing between bacterial and fungal biomass (Brookes et al., 1985; Xu et al., 2013).

2.2 Content of phospholipid fatty acids (PLFAs)

PLFAs analysis involves extracting phospholipids, whose content corresponds to the area of biological membranes and thus biomass, from soil and separating them into individual fatty acids, which are then quantified using gas chromatography. The total amount of these fatty acids can be used to estimate the total microbial biomass while the quantification of specific PLFA molecules may help to quantify specific groups of organisms (e.g., bacteria, eukaryota(fungi)). This method may provide reliable and quantitative information on bacterial and fungal biomass simultaneously, but is a labour-intensive method and requires specialized equipment (Frostegård et al., 1991; Joergensen, 2022).

2.3 Quantitative polymerase chain reaction (qPCR)

qPCR is a molecular technique used to amplify and quantify DNA, including microbial DNA, from environmental samples. In soil microbiology, it is used to estimate the abundance of microbial species or groups by targeting specific genes in general terms these groups can be also broadly defined as bacteria and fungi. This method provides high sensitivity 7 enabling the detection and quantification of even low abundance organisms, but the technique is prone to process errors (Smith and Osborn, 2009).

PLFA analysis is probably the most robust one to be used for microbial biomass estimation that covers both bacteria and fungi, based on previous comparisons with other methods (e.g.

Baldrian et al., 2013). Unlike fumigation-extraction, which gives a total biomass estimate without group distinction, or qPCR which requires specific primers for each group and can be influenced by gene copy number variations, PLFA analysis can directly reflect the microbial biomass and its composition, as bacteria and fungi have unique fatty acid profiles. Therefore, the same sample and method are used for estimating both fungal and bacterial biomass, with less variation compared to DNA-based methods (Baldrian et al., 2013; Joergensen, 2022).

Table 1. Comparison of the most used methods for microbial biomass estimation

Method	Advantages	Limitations
Fumigation-Extraction	Direct measure of microbial biomass; widely accepted	Can not discriminate bacteria and fungi; can underestimate biomass
PLFA	Quantifies specific microbial groups; high accuracy	Laborious
qPCR	High specificity; quantifies specific microbial groups	Requires prior knowledge of target sequences; potential inhibition by contaminants

3. Guidelines and Protocols

The sampling of soil is designed so that it sufficiently captures the characteristics of local soil considering its spatial heterogeneity. Soil variables including soil chemistry and microbial biomass content vary in space (Baldrian, 2014) and multiple (sub)samples are needed to get a valid representation of the study site. On the other hand, closely located soil samples show similar properties and thus in forest soils, samples located 2 m or more should be sampled to avoid spatial autocorrelation of microbial biomass content; the number of subsamples required for robust characterization on a plot-level is >4 (Štursova et al., 2016). Since >95% of soil biota biomass is located in the top 10 cm of the soil profile (Šnajdr et al., 2008), sampling up to this depth gives an exhaustive representation of soil biota.

3.1.1 Sampling – microorganisms

Equipment requirement for the sampling:

- Notebook and pencil(s)
- GPS device or a mobile phone with GPS
- Compass
- Measuring tape (20 m)
- Plastic soil corers (plastic tubes of 4-cm diameter, 25 cm length, used to extract soil cores. Such corers can be easily cut from plastic tubes used by plumbers and available in hobby markets. One end of the corer is straight, the other end has an angle of approx. 30-45°). Six to seven such corers are needed per site.
- Rubber hammer(s)
- Spray for tree labelling
- Permanent marker(s)
- Plastic bags that accommodate 5 or more soil cores
- Adhesive tape
- Optional: Camera

Within the forest stand to be analysed, locate an appropriate sample plot (a 5 m radius circle). The appropriate plot is generally representative of the forest stand to be characterised in terms of the composition of surrounding trees, ground vegetation and other properties, ideally more than 20 m from a stand edge. In some cases more than one sample plot per forest stand is required (to account for heterogeneity, depending on the area, treatments etc).

Record the date and time of sampling and the sample location identification code. Define the exact plot centre on the ground. In this protocol, the cores are first collected from all the plots, and then processed in the laboratory. Five cores per plot are collected, resulting in one composite sample.

Hammer the first soil corer (the centre corer) into the plot centre to a depth of 15 cm.

Determine the GPS coordinates of the centre core.

In order to make repeated measurements of the same location possible, if this is required, find the nearest healthy living tree and label it with a spray, e.g., using two horizontal lines visible from all sides of the tree.

Using a measuring tape, measure the distance between the labelled tree and the central core. Using a compass, measure the angle between north and the direction from the tree to the central core. Please note that compass applications in mobile phones are not very precise.

Hammer in four additional corers, located 2 m south, 2 m north, 2 m east and 2 m west from the central core. In stony places, locate cores into less stony patches. If stones are underground at <15 cm depth, hammer the corers up to the stone layer (see Figure 1).

Optional: If possible, take defined photos of the site: (a) forest stand, (b) forest ground (optimally showing the area between and around all five cores), (c) canopy.

Optional: Record tree species of trees in the circle defined by the soil cores and all closest trees around the perimeter. If possible, record also distance of each tree to the central core and its breast height diameter (DBH).

Optional: Record numbers and species of juvenile trees / seedlings in the circle defined by the soil cores.

Optional: If possible, record ground vegetation (% of area barren litter/soil, % grasses and herbs, % ericoid shrubs), and plant species of dominant plants.

Pull out soil corers by twisting them gently left and right to prevent soil from falling out.

Place all five corers containing the soil cores from the plot into a single plastic bag (these will be later combined into a composite sample). Write the sampling plot identification code on a piece of paper and insert it into the bag. Wrap the bag tightly and fix it wrapped around the corers using adhesive tape. During handling and transport, keep the corers in a horizontal position as much as possible, to minimize disturbing the soil profile.

Write the sampling plot identification code onto the bag using a permanent marker.

Transport the samples into the laboratory for processing the same day and refrigerate them upon arrival to the laboratory (4°C). The soil cores should be processed in the laboratory the first day following sampling or at latest the second day after sampling.

Optional: When re-sampling the same plot next time, move the position of the central core each re-sampling time at least 20 cm from the location of the previous sampling location(s).

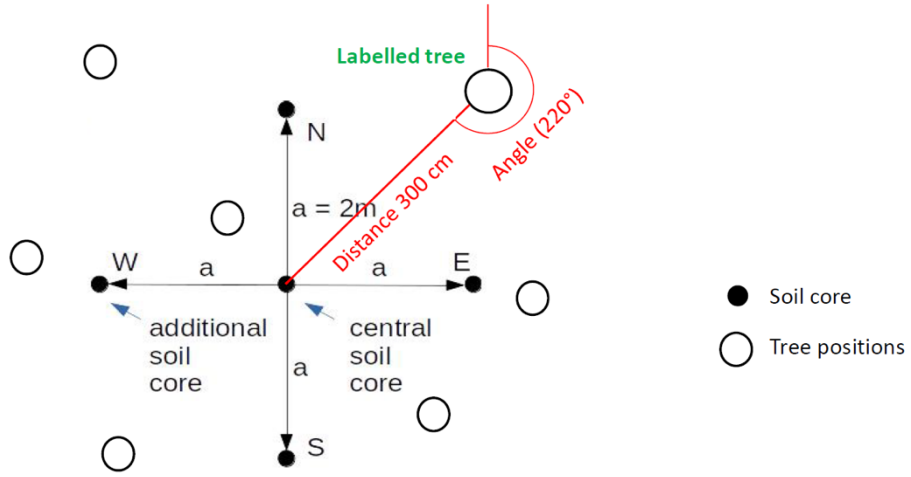

Figure 1: Illustration of soil sampling for biodiversity analysis for WP4 sites

3.1.2 Processing of soil samples (microorganisms)

Equipment requirement:

- Notebook and pencil(s)
- Balance
- Sheets of plastics (large plastic bags, transparent or light color, if possible)
- Sieve for soil with 5-mm mesh size
- Plastic 50-ml plastic tubes Alternatively, plastic ziplock bags with 50 ml volume.
- Wooden rod, approx. 3 cm diameter
- Sharp knife
- Laboratory spoon
- Ruler, 20 cm
- Permanent marker(s)
- Latex gloves
- Scissors
- Cellulose tissues
- Optional: Tweezers
- Optional: Ethanol

Prepare weighted sterile 50 mL plastic tubes for all samples and describe them with sampling plot code both on a cap and on the tube.

Measure the mass of each empty tube + lid (resolution 0.01 g) and record it.

Prepare a sheet of plastic where to collect the content of soil cores. Wipe it free of soil (if reused) and wash it with ethanol, if available. Wait until ethanol evaporates.

Wear Latex gloves when working with soil cores, if possible.

Push the content of the first soil cores out of the tubes with a wooden stick such as to cause minimal disturbance of the soil profiles. From the core surface, collect all aboveground litter (fallen plant material, such as leaves, needles, twigs etc.) and discard it.

After litter removal, measure 10 cm of the soil profile with a ruler, cut the extra soil from the bottom of the core aside with a knife and throw it away.

Remove all stones and plant roots (including dead roots) and pass the remaining soil through a 5-mm mesh sieve.

Remove any small roots that passed the sieve using tweezers.

Mix the soil combined from all five cores from the same plot thoroughly in a plastic bag and fill the 50-mL plastic tube up to 40 mL.

Record the wet mass of the tube and soil and place the tube into freezer (-18°C or less). When fully frozen, freeze-dry the content of the tube and record the dry mass of the tube and soil. Calculate the dry mass content as: (dry mass of soil and tube – mass of empty tube) / (wet mass of soil and tube – mass of empty tube).

Store tubes with freeze-dried soil samples at -18°C until PLFAs (phospholipid fatty acids) extraction. The samples stored this way should be stable for >5 years.

3.1.3 Laboratory work - microorganisms

PLFA extraction

The samples (approx. 2 g of freeze-dried soil) are extracted by a mixture of chloroform–methanol–phosphate buffer (1:2:0.8), according to Bligh and Dyer (1959) and Šnajdr et al. (2008). Immediately before the extraction, each sample is spiked with the mixture of internal standards, containing 1,2-dinonadecanoyl (C19:0)-sn-glycero-3- and heneicosanoic acid (C21:0), dissolved in chloroform. Samples are then incubated at room temperature overnight upon mild horizontal shaking. Following incubation, the samples are centrifuged, decanted and citrate buffer is added, followed by additional centrifugation to attain phase separation. The lower (chloroform) fraction is collected and filtered through hydrophobic syringe filters, transferred to glass vials and evaporated to dryness under stream of nitrogen gas. Further, these total lipid extracts are fractionated using columns as described by Welc et al. (2012) to obtain neutral, glyco- and phospholipid fractions. The latter (phospholipid) fraction eluted with methanol is evaporated to dryness and transmethylated using the rapid trimethylchlorosilane-methanol derivarization approach (Konvalinková et al., 2017; Welc et al., 2012).

PLFA analysis

The abundance of the individual phospholipid fatty acid (PLFA) methylesters (dissolved in hexane and filtered through the hydrophobic filters) are analysed by gas chromatography in temperature gradient from 60 °C to 240 °C as reported previously (Šnajdr et al., 2008) .

Different fatty acids are identified according to comparison of compound retention times with fatty acid standards. Abundance of the different compounds in the original samples is calculated using the C19:0 abundances in each sample (this effectively integrates all losses during extraction, derivatization and split injection).

The assignment of different signature PLFA follows previous records (e.g. Frostegård, Bååth and Tunlio 1993; Joergensen 2022) and references therein. Specifically, the amount of 18:2ω6,9 fatty acid in the samples is used as a proxy of fungal biomass (PLFAF), whereas the sum of the amounts of fatty acids i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 serves as a proxy of bacterial biomass (PLFAB). The content of all PLFA molecules (PLFAT) is used as a proxy of total microbial biomass. The biomass content is expressed in µg PLFA per g soil dry mass.

3.2 Biomass estimation of other trophic groups

3.2.1 Sampling – nematodes and mesofauna

Equipment requirement:

- Metal soil corer, 5-cm diameter, 10 cm length
- Rubber hammer(s)
- Plastic bags that accommodate 4 or more soil cores
- Permanent marker
- Isothermal box
- Ice blocks

Within each of the experimental plots, sample 4 soil cores (10 cm, 5 cm diameter) within 3 m diameter from the permanent marked plot (it corresponds to microbial sampling plot). Introduce the intact 4 soil cores in a plastic bag (already marked with the date, site and corresponding plot), close it and put it in an isothermal box with ice blocks. Once in the lab, keep the samples at 4 ˚C in a fridge.

3.2.2 Sampling – macrofauna

Equipment requirement:

- Small shovel
- Measuring tape
- Plastic containers
- 96% ethanol
- falcon to store collected individuals
- tweezers

Macrofauna (i.e. large invertebrates >3 mm in body length including earthworms) is collected up by hand sorting directly in the field from an excavated soil monolith per plot (25 cm × 25 cm to a 15 cm depth) following the Tropical Soil Biology and Fertility method (TSBF, Anderson and Ingram 1993). Individuals of macrofauna are stored in 96% ethanol.

3.2.3 Processing of soil samples – nematodes, mesofauna and macrofauna

Once in the lab, each soil sample from nematodes and mesofauna sampling is divided in two: around 200-250 g of fresh soil for nematode extraction and between 300-500 g of fresh soil for mesofauna extraction. Samples are always kept in plastic bags in the fridge at 4˚C until extraction.

3.2.4 Nematode extraction and identification

Equipment requirement:

- 1 plastic beaker of 1 L
- 2 plastic jars of 2.5 L
- 1 plastic jar of 5 L
- Coarse sieve of 2 mm
- Fine sieve of 40 µm
- Stirring paddle
- Wash-bottle with tap water
- Beakers 200 mL
- Beakers 80 mL
- Labelling tape
- 10-ml pipette
- Stereomicroscope
- Petri dish with grid
- Microscope
- Microscope slide and cover

Extraction

Weigh around 200 g of fresh soil and put it in the 1L plastic beaker (Figure 2, step1). Label each beaker, add water and leave the soil in water for 24 h. At the same time weigh 25 g of fresh soil, put it in the oven and weigh it again after 24 h at 70 °C in the oven (to calculate gravimetric soil water content).

Take the first sample, put it in the 2.5 L jar and fill it with water. Transfer the sample from one jar to another 10 times, vigorously and making sure that the entire soil changes jar each time (Figure 2, step 2).

Pour the supernatant into the 5 L jar through the 2-mm sieve, keep it and repeat the operation (fill with water the 2.5 L jar where the remaining soil is and repeat step 2), mixing the two supernatants into the 5 L jar (Figure 2, step 3).

Shake the content of the large jar until you are sure that all the substrate is in suspension. Let it rest and once the water movement has almost stopped, sift the supernatant through the 40 µm sieve.

Collect the substrate that remains on the sieve with the help of the washing bottle (Figure 2, step 4). Transfer to a 200 mL beaker with a funnel and label the beaker (Figure 2, step 5).

Once all the samples have been extracted, prepare the Baermann funnels: fill each funnel with fresh water and place a coarse sieve on top (Figure 2, steps 6 and 7). Cover each sample beaker with a thin piece of folded paper and close with a rubber band, making sure that no breaks have occurred in the paper (Figure 2, step 8). Shake the glass to put the sample in suspension and place it upside down on top of each funnel (Figure 2, step 9).

Let the sample in the funnel for at least 24 hours (during this time, nematodes will percolate through the paper and deposit at the bottom of the funnel). Then, open the clamp of the funnel to take approx. 50 mL of clean water with nematodes in the 80 mL beaker (Figure 2, step 10).

Counting and identification

Make sure that there are no suspended nematodes in the 80 ml beaker (wait at least 2 h since the extraction).

Remove the supernatant from the beaker with a 10-mL pipette until approx. 20 mL of water. Transfer to a gridded petri dish and count nematodes under a stereomicroscope.

After counting, transfer to a test tube (and transfer the label from the beaker). Adjust the weights of the tubes with tap water and centrifuge for 3 min at 1500 rpm.

Remove the supernatant, resuspend the nematodes and place a drop on the slide. Cover with large covers and identify to the level of species, genus or family (the first 100 individuals). The purpose of identification is to assign morphometric relations needed for biomass estimation.

Figure 2. Steps for nematode extraction. Pictures from Sara Sánchez Moreno.

3.2.5 Mesofauna extraction

Mesofauna is extracted using the Berlese-Tullgren funnel method (dry extraction, Berlese 1905) for 10 days. Individuals of mesofauna are stored in 96% ethanol.

Individuals of mesofauna and macrofauna stored in 96% ethanol are counted, identified and classified into a trophic group (microbivore, detritivore, predator, omnivore or herbivore) using a binocular loop following Potapov et al. (2022).

3.2.6 Biomass estimation of nematodes

Determining the weight of nematodes directly is challenging because of their small size. However, their typical vermiform shape (i.e., generally elongate and cylindrical morphology), and the standardised morphometric characteristics used in their description, enable assessment of body volume and weight (Ferris, 2010). The formula developed by Andrássy in 1956 is commonly used for this purpose:

$$W = \frac{L \times D^2}{1.6 \times 10^6}$$

In this formula, W represents the mass per individual (as fresh weight µg), L is the nematode length (µm), D is the greatest body diameter (µm), and 1.6 is an empirically determined constant.

The Nemaplex website provides morphometric data for various nematodes, which is essential for applying this formula, allowing assignment of average body mass of nematodes in soil at the level of species, genus or family. For practical applications, the NINJA system automates the calculation of nematode body mass. The input to the system is a nematode abundance table filled with the numbers of individuals of each taxon in a sample (taxa are nematode families, genera or species), where columns correspond to different taxa and rows represent treatments or sampling locations and the output is the corresponding body mass to each treatment. The output is the estimated body mass for each group, expressed per unit area, soil volume, or mass. More details about the NINJA system are available in the work of Sieriebriennikov et al. (2014).

3.2.7 Mesofauna and macrofauna biomass estimation

Fifty individuals per taxonomic group are randomly selected for length and biomass measurements. The length of meso- and macrofauna individuals is measured to the nearest 0.01 mm using a stereomicroscope with a camera connected to the ToupView software (ToupTek Photonics, China). Dry mass of mesofauna individuals is estimated through length-mass regressions from the literature (Schwarz et al. 2017), while macrofauna individuals are freeze-dried and weighed. The total biomass of each taxonomic group is determined by multiplying the mean individual biomass by their total abundance.

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