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D1.4 Harmonised methodologies for analysis of soil biodiversity, functional redundancy & community resilience in forest soils

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Deliverable D1.4: Harmonised methodologies for analysis of soil biodiversity, functional redundancy & community resilience in forest soils		
D1.4 gives guidelines for assessing the biodiversity of key groups of soil organisms, their functional redundancy, and resilience.		
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Contents

1. Introduction

The harmonised methodologies for analysis of soil biodiversity, functional redundancy & community resilience are aimed at providing precise and unified description of the biological qualities of forest soils suitable for a wide range of forest ecosystems in Europe, both natural and managed. It consists of guidelines for soil sampling, sample processing, assessment of community composition of soil biota based on environmental DNA metabarcoding and assessment of diversity, functional redundancy and resilience.

2. Soil sampling

The sampling of soil is designed so that it sufficiently captures the characteristics of local soil biota, bacteria, fungi and eukaryotic organisms while it is unbiased by the proximity of subsamples. Soil variables including soil chemistry, microbial activity and community composition vary in space (Baldrian, 2014) and multiple (sub)samples are needed to capture this variation. On the other hand, closely located soil samples show similar properties and show high similarity in the community composition of bacteria and fungi. In forest soils, samples located 2 or more meters from each other are no longer spatially autocorrelated in terms of microbial community composition and the number of subsamples required for robust characterization of a plot-level community composition 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.

Equipment requirement for microbiology:

- Notebook and pencil(s)
- GPS device or a mobile phone with GPS
- Compass
- Measuring tape (20 m)
- Plastic soil cores, 4-cm diameter, 25 cm length, 6-7 per forest stand. Such cores can be easily cut from plastic tubes used by plumbers, available in hobby markets. One end of the core is straight, the other end has an angle of approx. 30°.
- Rubber hammer(s)
- Spray for tree labelling
- Permanent marker(s)
- Paper and pen
- 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. Sometimes more than one sample plot per forest stand is required.

Record the date and time of sampling and the sample location identification code. Define the exact plot center on the ground. Hammer the first soil core (center core) into the plot center to a depth of 15 cm.

Determine the GPS coordinates of the center 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 cores, 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 cores up to the stone layer (see Figure 1).

Optional: In peatland forests where plastic tubes cannot be hammered into the soil, cut five 10 x 10 x 10 cm blocks of soil with a thin sharp knife.

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 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, % ferns, % mosses), and plant species of dominant plants, if possible.

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

Place all five cores from the plot into a single plastic bag. 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 cores using adhesive tape. During handling and transport, keep the cores in a horizontal position as much as possible.

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 processes 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).

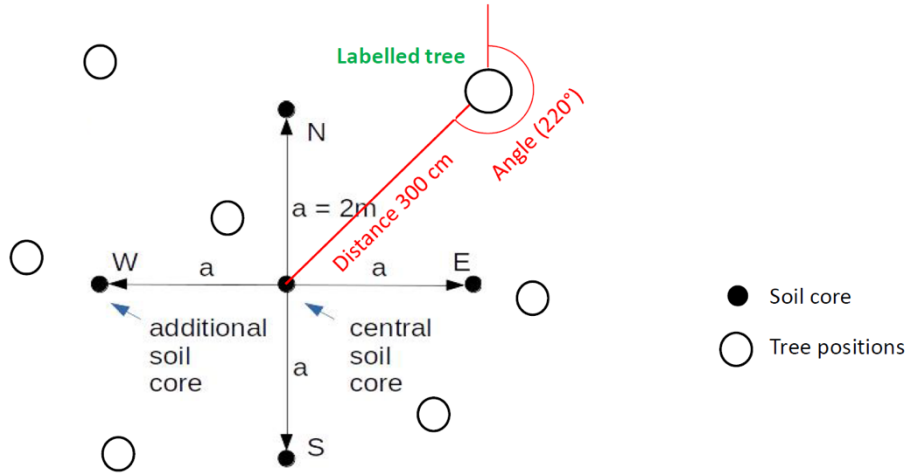

Figure 1: Illustration of soil sampling for biodiversity analysis for WP4 sites

3. Laboratory processing of soil samples

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 for pushing soil out of cores
- Sharp knife
- Laboratory spoon
- Sheet of plastic
- Ruler, 20 cm
- Permanent marker(s)
- Latex/nitrile 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 and wash it with ethanol, if available. Wait until ethanol evaporates.

Wear Latex/nitrile 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 litter 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 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 DNA (deoxyribonucleic acid) extraction. The samples stored this way should be stable for >5 years.

4. Extraction of DNA, amplification of molecular markers and high-throughput sequencing

4.1 DNA extraction and purification

Extract DNA from two separate subsamples (200 mg each) of each freeze-dried sample using the modified method of Miller (Sagova-Mareckova et al., 2008). This method is recommended for use since it was proven efficient across a wide variety of soils with differing properties including sand / silt / clay content, organic matter content, pH and salinity (Sagova-Mareckova et al., 2008).

Pool the extracts and purify the pooled DNA extract using GeneClean Turbo Kit (Biogenic) following the manufacturer´s instructions. Purified DNA can be stored at -18°C or less for >5 years.

4.2 Polymerase chain reaction (PCR), library preparation, and sequencing

Polymerase chain reaction (PCR) is used to amplify DNA regions that serve as taxon-specific barcodes and can distinguish individual species of organisms.

For bacteria, partial sequence of 16S rRNA gene is amplified using the primers 515F/806R (Table 1) (Caporaso et al., 2012). This primer combination provides good coverage of the bacterial tree of life and is the most widely used so far in bacterial community analyses in forest soils (Větrovský et al., 2020).

For fungi, sequence of internal transcribed spacer 2 (ITS2) of the ribosomal DNA cassette is amplified using the primers gITS7/ITS4 (Table 1) (Ihrmark et al., 2012). This primer

combination provides good coverage of the fungal tree of life with minimum bias in representation of fungal taxa when compared to alternative molecular markers and is among the most widely used so far in fungal community analyses (Větrovský et al., 2020).

For eukaryotes, partial sequence of the 18S rRNA gene is amplified using the primers 1389F/1510R (Table 1) (Amaral-Zettler et al., 2009). This primer combination provides good coverage of the eukaryotic tree of life although its coverage and resolution varies among eukaryotic taxa and its sensitivity for fungi is inferior to the gITS7/ITS4 primer combination (Amaral-Zettler et al., 2009).

Primer ID	Primer sequence	Target, reference
515F	5'-GTGCCAGCMGCCGCGGTAA-3'	bacteria (Caporaso et al., 2012)
806R	5'-GGACTACHVGGGTWTCTAAT-3'	bacteria (Caporaso et al., 2012)
1389F	5'-TTGTACACACCGCCC-3'	Eukaryotes (Amaral-Zettler et al., 2009)
1510R	5'-CCTTCYGCAGGTTCACCTAC-3'	Eukaryotes (Amaral-Zettler et al., 2009)
gITS7	5'-GTGARTCATCGARTCTTTG-3'	fungi (Ihrmark et al., 2012)
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	fungi (Ihrmark et al., 2012)

Table 1. PCR primers for metabarcoding

Both forward and reverse primers need to be barcoded to provide specific barcode combination for each sample to be amplified and analysed in the same sequencing run.

Perform PCR reaction for each sample in triplicate: combine the Mastermix of 2.5 µL of 10× DyNAzyme buffer; 0.75 µL DyNAzyme II DNA polymerase (2 U μL^{-1}); 1.5 µL BSA (10 mg mL^{-1}); 0.5 µL PCR Nucleotide Mix (10 mM); 0.5 µL forward primer (10 µM); 0.5 µL of reverse primer (10 µM); 1.0 µL of template DNA (5–50 ng μL^{-1}) and sterile ddH_2O up to a total volume of 25 µL.

Conditions of the PCR (bacteria) include an initial denaturation step at 94 °C for 4 min, followed by 25 cycles of amplification and a final extension of 72 °C for 10 min. Each cycle includes a denaturation step at 94 °C for 45 s, an annealing step at 62 °C for 60 s, and an extension step at 72 °C for 60 s.

Conditions of the PCR (fungi) include an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of amplification and a final extension of 72 °C for 10 min. Each cycle includes a denaturation step at 94 °C for 45 s, an annealing step at 62 °C for 60 s, and an extension step at 72 °C for 60 s.

Conditions of the PCR (eukaryotes) include an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of amplification and a final extension of 72 °C for 10 min. Each cycle includes a denaturation step at 94 °C for 15 s, an annealing step at 62 °C for 30 s, and an extension step at 68 °C for 60 s.

Combine amplicons of the same sample, purify the combined amplicon using MinElute Kit (Qiagen), and quantify DNA with a Qubit™ dsDNA BR Assay kit (Thermo Fisher Scientific). Check the amplicon for expected size on an agarose gel.

Prepare libraries after pooling amplicons in equimolar concentrations using the TruSeq DNA PCR-Free Kit (Illumina) and sequence it on an Illumina MiSeq sequencer (2 × 250-base reads or 2 × 300-base reads).

Note: Sequencing should deliver, as a minimum, 14 000 sequences of bacteria and eukaryotes or 7 000 sequences of fungi per sample so that at least 10 000 sequences of bacteria and eukaryotes or 5 000 sequences of fungi are retained after bioinformatic data processing and taxonomy assignment step.

4.3 Bioinformatic data processing and assignment of taxonomy and lifestyle to community members

Merge raw reads obtained from Illumina MiSeq sequencing to generate paired end-joint single reads using fastq-join (Aronesty, 2013).

Exclude resulting sequences of inferior quality using, for example, SEED 2 software (version 2.1.2) (Větrovský et al., 2018), removing the sequences with mean quality Phred scores Q<30 and sequences with mismatches in barcodes. Cut off barcoded primers from the sequences.

For **bacterial sequences**, remove sequences shorter than 230 bases or longer than 280 bases. Construct operational taxonomic units (OTUs, molecular species of bacteria) by clustering sequences at 97% sequence similarity using the UPARSE algorithm in USEARCH version 8.1.1861 (Edgar, 2013) removing all sequences identified as chimeric in this process. For taxonomic identification, select the most abundant sequence as a representative for each OTU and find the closest matching sequence using the BLASTn algorithm against SILVA SSU reference database, latest release (Quast et al., 2013). Discard all sequences with no hit to database or with a hit to a taxon other than bacteria. Based on the phylum level classification of the best hit sequence, classify each OTU into a bacterial phylum.

For **fungal sequences**, extract the complete fungal ITS2 regions from the sequences using the ITSx tool (version 1.0.11) (Bengtsson-Palme et al., 2013). Remove any resulting sequences shorter than 40 bases. Construct operational taxonomic units (OTUs, molecular species of fungi) by clustering sequences at 97% sequence similarity using the UPARSE algorithm in

USEARCH version 8.1.1861 (Edgar, 2013) removing all sequences identified as chimeric in this process. For taxonomic identification, select the most abundant sequence as a representative for each OTU and find the closest matching sequence using the BLASTn algorithm against UNITE reference database, latest release (Abarenkov et al., 2023). Discard all sequences with no hit to database or with a hit to a taxon other than fungi. Based on the genus level classification of the best hit sequence, assign primary lifestyle to each OTU based on FungalTraits database (Põlme et al., 2020). Lifestyles: animal endosymbiont, animal parasite, arbuscular mycorrhizal, bacterivorous, dung saprotroph, ectomycorrhizal, epiphyte, foliar endophyte, lichen parasite, lichenized, litter saprotroph, moss symbiont, mycoparasite, plant pathogen, pollen saprotroph, protistan parasite, root endophyte, soil saprotroph, sooty mold, unspecified saprotroph, unspecified symbiotroph, wood saprotroph; all other lifestyles or taxa with no primary lifestyle information classified as “other”.

For **eukaryotic sequences**, remove sequences shorter than 100 bases or longer than 140 bases. Construct operational taxonomic units (OTUs, molecular species of eukaryota) by clustering sequences at 97% sequence similarity using the UPARSE algorithm in USEARCH version 8.1.1861 (Edgar, 2013) removing all sequences identified as chimeric in this process. For taxonomic identification, select the most abundant sequence as a representative for each OTU and find the closest matching sequence using the BLASTn algorithm against SILVA SSU reference database, latest release (Quast et al., 2013). Discard all sequences with no hit to database or with a hit to a taxon other than eukaryota. Based on the phylum level classification of the best hit sequence, classify each OTU into an eukaryotic phylum.

5. Assessment of biodiversity, functional redundancy and community resilience

5.1 Assessment of biodiversity

To assess biodiversity, randomly select the same number of sequences from each sample. For bacteria or eukaryotes, select at least 10 000 sequences per sample, for fungi, select at least 5 000 sequences per sample. With this subset of sequences, calculate an estimate of total diversity using the Chao-1 metric.

$$\text{Chao-1} = So + a1(a1 - 1)/(2 \times a2 + 1)$$

Where **So** is the number of observed OTUs, **a1** is the number of OTUs present as single sequence and **a2** is the number of OTUs present as two sequences. Chao-1 provides an estimate of total diversity that accounts for the non-observed species in the community.

Chao-1 can be conveniently calculated using, for example, SEED 2 software (version 2.1.2) (Větrovský et al., 2018).

5.2 Assessment of functional redundancy

To assess functional redundancy, calculate the diversity of the functionally important guilds of the community considering the number of species. The higher is the number of predicted molecular species (OTUs) in each of the functional guilds, the more taxa with similar functions are present and the more functionally redundant the community is.

For **bacteria**, phylum-level taxa often harbour species that are functionally similar. In each sample, select randomly 10 000 sequences and calculate Chao-1 estimates for OTUs separately for each phylum, considering ten most abundant phyla. For each phylum, the functional redundancy of the phylum corresponds to the respective Chao-1 estimate, the total functional redundancy can be expressed as the sum of all Chao-1 estimates summed across the top ten phyla.

Use the same method for the assessment of functional redundancy of **eukaryotes**.

For **fungi**, functional redundancy should be calculated for the most important functional groups based on primary lifestyle. In each sample, select randomly 5 000 sequences and calculate Chao-1 estimates for OTUs separately for the following groups by primary lifestyle: animal parasite, ectomycorrhizal, litter saprotroph, mycoparasite, root endophyte, soil saprotroph, wood saprotroph. For each functional group, the functional redundancy of the phylum corresponds to the respective Chao-1 estimate, the total functional redundancy is the sum of all Chao-1 estimates summed across the functional groups.

Chao-1 can be conveniently calculated using, for example, SEED 2 software (version 2.1.2) (Větrovský et al., 2018).

5.3 Assessment of community resilience

To assess community resilience, calculate the diversity of the functionally important guilds of the community considering the number of observed species and the evenness of their communities. The community is more resilient the more molecular species (OTUs) are observed but also the more similar is their relative abundance (evenness). In communities with low species numbers or those with unbalanced abundances, the probability of species loss due to disturbance is higher than in species-rich communities with equitable abundances of species. The latter are thus more resilient.

For **bacteria**, phylum-level taxa often harbour species that are functionally similar. In each sample, select randomly 10 000 sequences and calculate the Shannon Diversity Index for OTUs separately for each phylum, considering ten most abundant phyla.

$$\text{Shannon Index (H)} = -\sum_{i=1}^{s} p_i \ln p_i$$

The p_i represents the relative abundance of the i-th community member (OTU) and **s** is the total number of observed species.

For each phylum, the resilience corresponds to the respective Shannon Diversity Index estimate, the total resilience can be expressed as the sum of all Shannon Diversity Indices summed across the top ten phyla.

Use the same method for the assessment of community resilience of **eukaryotes**.

Use the same method for the assessment of community resilience of **fungi**, except for randomly selecting 5 000 sequences instead of 10 000 sequences and considering the following functional groups based on primary lifestyle: animal parasite, ectomycorrhizal, litter saprotroph, mycoparasite, root endophyte, soil saprotroph, wood saprotroph.

Shannon Diversity Index can be conveniently calculated using, for example, SEED 2 software (version 2.1.2) (Větrovský et al., 2018).

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