

COST Action ES1406

Soil fauna: key to soil organic matter dynamics and modelling

HANDBOOK OF METHODS



Version 1

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Juan J. Jiménez & Juliane Filser (Eds.)
& The KEYSOM Team

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This Handbook of methods aim to provide the different techniques and methodologies to obtain a minimum data set of variables, from soil biodiversity assessment to SOM dynamics, including, isotope analysis, bioturbation assessment and metagenomics. With the knowledge gathered in forthcoming projects and studies, researchers (biogeochemists and soil ecologists), early career investigators (ECI) and students will gain a better understanding of the direct and indirect impacts of soil organisms on nutrient availability, carbon sequestration, greenhouse gas emissions and plant growth. This knowledge will be key to incorporate soil fauna-driven SOM dynamics in the context of global carbon cycling models.

The main objective of COST Action ES 1406 (“Soil fauna: key to soil organic matter dynamics and modelling (KEYSOM)”) at scientific/technological advancement was to provide immediate benefits to the scientific community by:

- Constructing a significant and transparent network of existing soil organic matter and soil faunal ecology experiments across Europe
- Ensuring close interactions between soil organic matter and soil fauna experimentalists and modellers
- Providing training and education of the next generation of researchers in connecting these fields
- Identifying gaps in knowledge and guiding future research, including also those related to extreme events;
- Providing a strong collaborative counterpart to global research networks of soil diversity and functionality.

Introduction

Soil harbours a multitude of different organisms – microorganisms, such as bacteria, fungi, protists, as well as larger organisms such as nematodes, springtails, insect larvae, ants, termites, earthworms and ground beetles. They thrive important ecological functions and ecosystem services like nutrient cycling and nitrogen fixation, disease and pest control, organic matter decomposition and carbon sequestration, maintenance of a good soil structure for plant growth and rainwater infiltration, and detoxification of contaminants. The decline of soil biodiversity leads to soil degradation.

Soil organic matter (SOM) is key to maintaining soil fertility, mitigating climate change, preventing land degradation, and conserving above- and below-ground biodiversity and associated soil processes and ecosystem services. In these processes soil organisms play an important role in the dynamics of SOM and nutrient cycling and the modification of soil structure. SOM quantity, quality and stability are controlled not only by the molecular structure but also by environmental and biological controls (Fontaine et al., 2007; Schmidt et al., 2011).

Soil fauna affect soil structure formation through their burrowing, consumption and excretion activities, significantly enhancing OM incorporation into the soil and contribute to macroaggregate formation. Soil animals, their biodiversity and species traits (morphological and functional aspects) are relevant for SOM turnover (Uvarov 2009; Wall et al. 2008). The important contribution of soil fauna to SOM dynamics has been considered mainly by soil ecologists who have developed their own models.

SOM modelling has thus far largely ignored soil fauna due to various reasons:

- (i) hardly existing communication between [C flow centered] biogeochemistry and [organism-centered] soil ecology (different societies, conferences, journals – one of the main reasons for this Action),
- (ii) lack of [awareness of] data on soil animals (both in the field and from laboratory experiments),
- (iii) soil ecologists have at least two rather different ways to explain processes: foodweb vs. self-organization (Barot et al. 2007).

This situation is perpetual since the new scientific achievements in soil biogeochemistry and soil ecology are weakly linked due to the lack of collaboration and interaction (Filser et al., 2016; Lavelle et al. 2016). Nowadays, the approach is being discussed and new models on SOM dynamics include the role of these organisms (Deckmyn et al., submitted; Flores et al., submitted). The inclusion of soil animal activities (plant residue consumption and bioturbation that alters the formation, depth, hydraulic properties and physical heterogeneity of soils) can fundamentally affect the predictive outcome of SOM models. Without considering the role of soil animals, models are less predictive. Consequently, there is a current need for a common view and conceptual framework to all soil ecologists to resolve this internal debate.

This manual aims to provide a set of protocols for soil organisms and soil organic matter relationships' studies, complying with the need for developing hands-on dissemination materials. It is thus produced as a feasible way of performing a Europe-wide experiment on the questions addressed during the lifetime of the Action, and partly also based on complementing existing protocols developed in other European initiatives such as Ecofinders (www.ecofinders.eu), Drilobase (<http://taxo.drilobase.org>), Betsi (<https://portail.betsi.cnrs.fr>) and on-going activities like Eudaphobase COST Action (CA18237).

We are confident that ECI and experimented researchers will take advantage of this tested handbook by selecting one or several protocols for their on-going and further research needs.

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Acknowledgements

This document is intended for Early Career Investigators (ECI) including Ms.C. and Ph.D. students but also senior researchers and stakeholders. It was prepared as part of the agreed activities approved by the Management Committee of COST Action ES1406 for the preparation of a handbook of methods on soil fauna and soil organic matter relationships for field validation and use in both scholar training and national and international projects and initiatives related to soil functionality across Europe.

COST Scientific officer: Dr. Deniz Karaca (Deniz.Karaca@cost.eu)

COST Administrative officer: Mr. Christophe Peters (Christophe.Peeters@cost.eu)

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ES 1406

Protocol KEYSOM-01

Determination of Soil Aggregate Size Distribution

Rationale

According to present knowledge, soil organic matter (SOM) is sequestered by its association with minerals in aggregates. Soil animals play a key role not only in the formation and size distribution of aggregates. Several studies have suggested that re-distribution of SOM from larger to smaller aggregates is particularly relevant for long-term sequestration, yet the quantitative contribution of soil fauna in this process is insufficiently understood. Here we include a simple methodology for a quick assessment of distribution of soil aggregates by size.

Materials

Field: Soil augers or steel cylinders (min. diameter 2 cm), plastic bags for transport, markers, shovel, scissors or knife.

Laboratory: drying oven (105 °C), smooth (ideally: glossy) paper, sieve cascade (5, 3, 2 and 1 mm mesh size), frame (8 mm high), rolling pin (or any other long cylinder), balance.

Procedure

At each sampling site, three replicates (at least 5 m apart) are taken. Remove litter layer and cut off any vegetation above the soil surface. When using an auger, collect the soil horizon-wise, when using steel cylinders, collect the soil according to the respective depth. Take a pooled sample from at least 3 different points so that at least 100 cm³ (e.g. 5 cm diameter, 4 cm deep or 3 and 15 cm, respectively) of soil for each depth or horizon¹ is collected for each replicate. In the laboratory, open the plastic bags and dry the soil at room temperature. After this, spread each sample on the smooth paper inside the frame and gently crush aggregates

→ 8 mm by moving the rolling pin over the frame. Sieve sample through the cascade by gentle shaking. Collect each size fraction in porcelain vessels, dry these at 105 °C for 24 h and weigh them.

Data

Note sampling date, coordinates, dominant vegetation, horizons sampled and person in charge. Calculate total weight of oven-dried soil and percentages for each size fraction.

Literature cited

Blume, H.-P., Stahr, K., Leinweber, P. (2011): Bodenkundliches Praktikum, Spektrum, Heidelberg

Protocol prepared by:
Juliane Filser
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¹ Within KEYSOM, these depths were established as 0-5, 5-15, 15-30, 30-60 and 60-90 cm for most protocols.



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Protocol KEYSOM-02

Sampling and preserving earthworms and macrofauna bycatch

Rationale

Earthworms can be sampled by various methods. Here we explain hand-sorting and chemical extraction, which can be used singly or in combination.

2.1. Hand-sorting in the field

Hand-sorting is a physical or passive method where the worms are directly removed from the soil by hand.

Materials

- simple wire or wooden frames (25 cm x 25 cm)
- spade (flat blade if possible)
- large plastic sheets or trays
- tweezers/forceps, labels, permanent markers
- plastic containers with tight lids (e.g. regular household containers)
- ethanol (70%)
- cool box and ice packs

Procedure

Cut vegetation from the sampling spot without standing on it, then carefully remove the clippings and place wire frame on soil surface. If there is a litter layer, transfer it onto a plastic sheet or tray and sort the earthworms and any other clearly visible macrofauna manually. Excavate a 25 cm x 25 cm x 25 cm deep soil block² (cut along edges first, do not cut inside block). Place the soil block on the plastic sheet or tray, sort through soil manually, remove all earthworms and place them in labelled plastic or glass containers (separately for earthworms and other fauna; the vessel for the latter is filled with 70% ethanol to avoid any predation); carefully check the roots for any earthworms.

To avoid mortality keep the containers cool (in the cool box) and away from sunlight until they can be processed in the laboratory. Keep each replicate sample separate throughout the sorting and identification process. If you do not proceed with chemical extraction, put the sorted soil back into soil pit and leave the spot in a tidy state



Photo 02.1. Manual extraction of soil fauna.

2.2. Chemical expelling in the field (allyl isothiocyanate = mustard oil)

This is a dynamic or behavioural method where the worms are encouraged to leave the soil so that they can be collected from the soil surface. It supplements the hand-sorting and extracts in particular anecic species. The most efficient method is pouring the

² The method was originated by Prof. P. Lavelle to quickly assess soil macrofauna numbers and composition and it was incorporated in the TSBF (Tropical Soil Biology and Fertility Program) handbook of methods (Anderson and Ingram, 1993). Now this method is under ISO23611-5:2011.

Materials

- simple wire or wooden frames (25 cm x 25 cm)
- water drums or lightweight plastic water containers (20 L)
- watering cans (with rose)
- tweezers/forceps, labels, permanent markers
- plastic containers with lids (household equipment)
- cool box and ice packs

Preparation of mustard oil solution (carefully observe safety instructions!): In the lab, mix 2 mL (= 2.04 g) allyl isothiocyanate [Aldrich 37,743-0] into 40 mL isopropanol [2-propanol] in small bottles that can be easily transported to the field (in cool boxes). Just before application in the field, add this mixture to 20 L water in drums and mix vigorously.

Procedure

Apply freshly prepared (see above) dilute mustard oil solution using the watering can to maximum saturation of sampling spot, slowly and avoiding run-off (about 5–10 L, depending on soil conditions and water infiltration). Sit next to the sampling spot and collect the expelled earthworms and any other clearly visible macrofauna with forceps from inside sampling area as they emerge (only attempt to remove worms once they have left their burrows completely). The duration is 15–20 minutes (unless earthworms are still emerging after this time). Put the collected worms and any other macrofauna to containers containing clean tap water to rinse off the irritant. Thereafter they can be transferred to separate labelled plastic or glass containers. Keep the containers away from the sunlight and cool (in the cool box) until they can be processed in the laboratory.

The rinsing and dark, cool storage is important to avoid mortality, dead worms decompose very rapidly and cannot be identified later.

Once the worms have stopped emerging, pour off water on the soil surface and, if you hand sorted the pit first, put the sorted soil back into soil pit and leave the spot in a tidy state. Keep each replicate sample separate throughout the sorting process.

2.3. Processing the collected live material in the laboratory

Materials

- tissue paper
- balance
- Petri dishes
- tweezers/forceps, labels (ideally write on paper with pencil INSIDE the liquid!), permanent markers
- test tubes or vials

Procedure

Rinse each subsample of worms with tap water, blot on paper towels and obtain the fresh weight for total biomass per replicate.

After weighing, place the worms under a fume hood in a deep Petri dish containing a 1:1 solution of 96° ethanol and 10% formalin (alternatively: 70% ethanol) for a few minutes until they stop moving. Put 1-3 earthworms one at a time (rather than a whole handful all at once) so they do not get tangled up into a big mess of earthworms. Wear gloves and avoid inhalation and skin contact of formalin.

Thereafter carefully extend every specimen onto a flat surface and after a few seconds they can be introduced in leak proof vials containing 10% formalin (Alternative: 70% ethanol). Store the labelled vials in horizontal position for at least 24 hours.

If you have preserved the earthworms in formalin at least for 24 hours, put them in a new tube with 4% formalin (10% of the commercial formaldehyde solution) for long-term storage. They can then be stored until identification, yet should be regularly checked for any loss of liquid).

If DNA barcoding analyses are intended, then keep the earthworms in ethanol 96°, replacing the initial amount of ethanol with new one, especially if many or large individuals are in the tubes as the ethanol replaces earthworm body tissue water and may result in flabby (formless, soft-bodies) specimens.

Data

Note sampling date, site coordinates (specifying projection system), dominant vegetation, horizons sampled and person in charge. Calculate total fresh weight of the earthworms collected per replicate. Identification of the specimens and of any other collected fauna optional.

Hazard information (make sure it is up-to-date):

Formaldehyde makes earthworms hard and therefore easier to handle when identifying them to species level. Working with formaldehyde should always take place under controlled laboratory conditions (fume cabinet). Despite these precautions its use is recommended in ISO standard ISO/DIS 23644-1, formalin is toxic and safety precautions must be taken. It should not be breathed, swallowed or come in contact with bare skin or eyes. If external contact does occur wash the area with large amounts of water. If ingested contact a medical center immediately. Formalin has been shown to cause cancer in laboratory animals



Allyl isothiocyanate (AITC) is a colorless to pale-yellow oily liquid with an irritating odor. It causes skin and eye irritation and was also a skin sensitizer. Intolerance and allergic reactions to mustard or radishes may be due to their AITC-content. AITC was of high acute toxicity to laboratory animals by the oral and dermal routes and there was some indication of genotoxicity in mammalian cells in culture.



Literature cited

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Protocol prepared by:

Maria J.I. Briones

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Protocol KEYSOM-03

Stable isotope composition (bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in soil profile

Rationale

Our aim is to investigate how $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of bulk SOM change with soil depth from the litter to a maximum of 90 cm depth when possible. In fact, most changes in delta values occur in the upper part of the soil profile. Stable isotope profile reflects both, development/evolution of SOM, and (likely) bioturbation rates.

To account for the small-scale heterogeneity, take three soil samples (replicates) from each site at least 5 m apart. The same soil pits/cores should be preferably used for collecting samples for total C and Stable Isotope Analysis.

Each sample should be ca. 1.5 ml in volume³ (1.5 ml Eppendorf tubes are ok). Small paper envelopes or bags can be used. Samples should be dried at 50-60°C (48+ h) soon after collection.

Materials

- spade
- soil corer or auger (optional)
- tape-line or folding rule
- knife/tweezers/forceps, labels, permanent markers
- 1.5 ml Eppendorf tubes or small paper bags
- drying oven (50-60°C)

Procedure

1. Choose three spots in a generally uniform site (e.g. within 5 × 5 m area).

³Please note that 5 mg of soil and 1 mg of litter at least is needed for performing one analysis. Large samples are unnecessary. Each sample needs to be powdered using a laboratory mill, thus they should not be too small. 1.5 ml is just a right size for soil and also for litter.

2. At each spot, collect a mixed sample of the most abundant (or typical) litter species. Cut or tear to small (5 x 5 mm) pieces and put into Eppendorf tubes or paper bags. Samples should not be packed too densely, otherwise they will not dry. If there is a thick layer of organic deposits, samples should be taken from each layer (L, F, H).
3. Make three soil pits (to a maximum depth of ca. 90 cm from the surface of mineral soil). Soil corer or auger can be used instead. The same pits/cores should be preferably sampled for total C and for stable isotope analysis.
4. From each pit (or soil core) take soil samples at the depths of 0 cm, 5 cm, 10 cm, 20 cm, 30 cm, 60 cm and 90 cm (measured from the surface of mineral soil). Each sample (ca. 1.5 cm³) is composed of several subsamples (ca. 0.5 cm³) taken at the corresponding depth. Try to avoid dead or living roots and other spots with locally high C content such as soil invertebrate structures.
5. Dry all samples (directly in the open Eppendorf tubes or in paper bags) at 50-60°C in an oven for 48 – 72 hours.

Data and Labeling

All samples should be carefully labeled (see General Nomenclature). The label should contain at least Country, Site#, Replication# and Depth. Please do not write essential information directly on the Eppendorf tubes. Instead, a small paper label should be either put inside each tube, or securely attached using duct tape or cello tape.

**Hi, I'm a small label: RUS Site-1,
Pine forest, R2, 90 cm**

Data on the stable isotope profile from different soil types (as depended on climate, vegetation, land use history and animal activity) are of interest for biogeochemical studies.

Protocol prepared by:
Alexei Tiunov
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Protocol KEYSOM-04

Soil sampling for Near Infrared Spectroscopy (NIRS) readings

Rationale

The aim is to distinguish biogenic structures and aggregates produced by soil invertebrates (earthworms, ants, termites, insect larvae) from non-ingested, non-biologically aggregated soil. The procedure explained here follows the technique originally described in Velásquez et al. (2007).

Sampling of biogenic structures and soil aggregates

First, mark a small area (e.g. 10×10 cm) adjacent to the macrofauna monolith (protocol KEYSOM-02). Collect all biogenic structures that are visible on soil surface within this area and describe them as globular (compact) or granular (loose) (Photo 1). If possible, identify the species that produced the structures.



Photo 04.1. Example of granular casts produced by *Prosellodrilus psammophilus* Qiu & Bouché, 1998 a native earthworm from the semi-arid lands of “Los Monegros” at the Ebro basin (Spain). © J.J. Jiménez.

Then, collect a block of soil with an equal area (e.g. 10x10x10 cm), gently break up the soil and sort out the macro-aggregates into categories explained below. Depending on the soil and the experience of the worker, it takes ca. 90 minutes to process one block. Most of the biologically-produced soil aggregates are found in the top 5-cm layer of soil.

- a) Biogenic aggregates (BA) are produced by macroinvertebrates (mainly earthworms, ants, termites, Coleopteran larvae, Isopoda and Diplopoda). Due to OM transformations, these aggregates have a rounded shape and a darker colour than other aggregates, but the exact size and form vary with the animal group. Isopod droppings are almost black, more or less flat and rectangular, like short cuttings of a two-wire electric cable. Diplopoda pellets look like rounded-rectangular chocolate chips (Photo 2), and earthworm casts comprise embedded round and concave structures, which follow from successive defecations of soil material into previously created macropores. Most other BA are round-shaped. Other macro-aggregates are also classified as biogenic when structures associated with large invertebrates are visible on at least one side of the aggregate. If possible, separate the aggregates by animal groups.



Photo 04.2. Pellets produced by *Glomeris marginata* L. (Diplopoda) in a *Quercus* sp. forest.

- a) Rhizosphere aggregates (RHIZ) consist of soil that is attached to roots. These aggregates have a varying shape. These aggregates are less stable than BA (Fonte et al. 2012). If some of these aggregates have been produced by soil invertebrates, they are classified as “root biogenic aggregates”.
- b) Physical aggregates (PHYS) are produced by physical processes, e.g. by drying and rewetting of the soil. These are truly physical aggregates. Dry them and weigh.
- c) Non-macroaggregated fraction (NON) includes the “other soil”, i.e. smaller soil aggregates that are difficult to identify without magnification and the soil particles and unidentified aggregates ≤ 5 mm in size.

Once you have finished sorting the soil block, dry the aggregates of different categories (60 °C, 24 h) and weigh them. The remaining material consisting of roots, leaf debris at different stages of decomposition (entire or fragmented dead leaves), shoot and woody debris, invertebrates, seeds and stones can be disregarded, or sorted out and quantified if needed for any other purposes.

Visible and Near Infrared spectral signatures

Near infrared spectroscopy (NIRS) allows identifying the origin of macroaggregates produced by soil invertebrates (Hedde et al. 2005; Velasquez et al., 2007; Zangerlé et al., 2016). Each type of biogenic structure or aggregate, collected either at soil surface or within soil samples, has a specific NIRS signature, which differs from the signature produced by the surrounding soil and which can be linked to the invertebrate that produced it as well as to soil C and N contents. To appropriately link the animals with the structures they produce may, however, require laboratory mesocosms to rear species and collect their casts directly (Zangerlé et al. 2016).

Producing NIRS readings of the sorted soil structures is fast: 10-15 g of each type of biogenic structure, biogenic aggregate and soil not biologically aggregated, is crushed by hand, passed through a 2-mm mesh sieve and scanned using a NIRS spectrophotometer with a spectral range of 400–2500 nm. The obtained spectra represent reflectance of re-emitted light as a function of wavelength and they can be statistically analysed using multivariate analyses, such as Principal Component Analysis (PCA), to allow for comparison between samples and significance tests of differences among samples' groups.

Literature

Fonte, S.; Quintero, C.; Velasquez, E.; Lavelle, P. 2012. Interactive effects of plants and earthworms on the physical stabilization of soil organic matter in aggregates. *Plant and Soil* 359, 205-214.

Hedde, M., Lavelle, P., Joffre, R., Jiménez, J.J., Decaëns, T. 2005. Specific functional signature in soil macro-invertebrate biostructures. *Functional Ecology* 19(5): 785-793.

Velasquez, E., C. Pelosi, D. Brunet, M. Grimaldi, M. Martins, A. C. Rendeiro, E. Barrios, and P. Lavelle. 2007. This ped is my ped: Visual separation and near infrared spectra allow determination of the origins of soil macroaggregates. *Pedobiologia* 51:75-87.

Zangerlé, A., Hissler, C., McKey, D., Lavelle, P. 2016. Using near infrared spectroscopy (NIRS) to identify the contribution of earthworms to soil macroaggregation in field conditions. *Applied Soil Ecology* 104, 138-143.

Protocol prepared by:

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Protocol KEYSOM-05

Soil sampling and analysis for eDNA

Rationale

The necessity of precise identification of microorganisms requires the use of techniques enabling determination of their assignation to a genus, species and/or strain. The use of DNA analysis techniques allows identification of microorganisms. Some of the molecular techniques used to identify microorganisms include rep-PCR (repetitive sequences-Polymerase Chain Reaction), PCR-RFLP (PCR-Restriction Fragment Length Polymorphism), gene sequence analysis, and DGGE (Denaturing Gradient Gel Electrophoresis).

Equipment/Materials

- Soil corer/auger with a diameter of 2-5 cm.
- Bucket
- Large plastic bags to line the bucket (e.g. trash bags)
- Shovel
- Four plastic bags with zipper
- One larger plastic bag with zipper, to accommodate 4 smaller ones
- Scissors
- Corner marks, e.g. sticks
- Bucket with screw cap and wide opening containing 1% hypochlorite solution
- Paper towels
- Labelled plastic bags with site and sample codes.

1. Procedure

Identify a homogeneous part, with respect to plant community and soil properties, of the study location and record GPS coordinates and altitude. Divide the selected study area into four plots of 4-10 m² (i.e. 2 x 2 to 3 x 3 m). Remove abundant aboveground plants with scissors from the soil core surface or push them aside. Place one large plastic bag inside the bucket. Put on new rubber gloves. Clean the soil corer from visible dirt, if any. Rinse the corer in hypochlorite solution 1% v/v and wipe with paper towels multiple times.

From each plot randomly collect 10 soil cores to a depth of 20 cm including the top organic litter layer, with a fresh weight of about 0.1 kg, and place them in the bucket lined with plastic bag. The 10 soil cores collected from each of the four plots are kept separate. The ten soil cores from the same plot are thoroughly mixed in a bucket into one large sample of approximately 1 kg. Collect 25-g sub-sample from each of the four mixed bulked soil sample and put into a labelled plastic bag. Join this one with the four small bags (four plots) into one plastic bag.



Photo 05.1. Example of the field material normally used in soil sampling studies.

Equipment should be cleaned with a 1% v/v hypochlorite solution, then rinsed with water or wiped with paper towels to remove any traces of hypochlorite (that degrades DNA!) between each plot sampling. Change rubber gloves between each plot sampling. During transportation and shipment samples must be kept cool at 0-10°C (do not freeze!). Upon arrival in the lab samples are frozen at -20 °C until DNA extraction.

Specific analyses:

Protocol 5.1. Identification of bacterial strains cultivated on microbiological media with use of rep-PCR technique

Rationale

The genomes of bacteria and fungi contain a large number of dispersed repetitive DNA sequences located at distant intergenic positions throughout the genome. The rep-PCR technique employs primers that bind to repetitive sequences, which enables generation of DNA profiles for bacterial strains and species.

The biological material consists of colonies of bacteria or fungi cultivated on standard microbiological growth media.

Equipment/Materials

- Commercial kits for DNA isolation from bacteria, yeast or fungi
- Centrifuge
- Thermoblock
- Laminar chamber
- Pipettes
- Gel documentation system
- Thermocycler
- Horizontal electrophoresis equipment
- Chemical reagents: polymerase DNA, dNTP set, primers, agarose, 1 kb DNA ladder

The genomes of bacteria and fungi contain a large number of dispersed repetitive DNA sequences located at distant intergenic positions throughout the genome. The rep-PCR technique employs primers that bind to repetitive sequences, which enables generation of DNA profiles for bacterial and fungal strains and species. This technique makes it possible to determine the genetic diversity of isolates of microorganisms extracted from the soil and cultivated on microbiological media. By using the rep-PCR technique, it is possible to discriminate bacteria and fungi even down to the subspecies, strain, or isolate level.

The biological material consists of colonies of bacteria or fungi cultivated on standard microbiological growth media.

Extraction of DNA from bacterial colonies is carried out by means of commercial kits for isolating DNA from bacteria, yeasts or fungi (e.g. Bacterial & Yeast Genomic DNA Purification Kit, EURx, Plant & Fungi DNA Purification Kit, EURx).

DNA concentration in the samples is measured with a spectrophotometer at a wavelength of 260 nm. For further analyses, the samples are diluted using sterile Milli-Q water to a final concentration of 10 ng/μl.

Amplification of DNA fragments of the selected isolates is carried out with the use of primers complementary to repetitive sequences in the bacterial or fungal genome: ERIC1R (5'-ATGTAA-GCTCCTGGGGATTC AC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGA GCG-3'), and BOXA1R (5'-CTACGGCAAGGCGACGCTG ACG-3').

The reaction mixture (20 μl) consists of 1 × buffer for PCR containing 1.5 mM MgCl₂, 0.2 mM MgCl₂, 0.25 mM of each nucleotide, 0.75 μM of each primer, 1.5 U of DreamTaq™ polymerase (ThermoScientific®) and 25 ng DNA. The following thermal profiles of the reactions are used: ERIC-PCR – 42 cycles (94°C × 1 min., 52°C × 1.5 min., 65°C × 8 min.); BOX-PCR – 37 cycles (94°C × 1 min., 40°C × 2 min., 72°C × 2 min.).

The products of the reactions are separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Only informative, clear and reproducible products of the reactions are analyzed.

On the basis of the data obtained with the rep-PCR technique, a genetic similarity analysis of the isolates is made using XLSTAT software (Addinsoft) based on the Jaccard coefficient. The results are used to construct dendrograms depicting the genetic similarity of the isolates.

Protocol 5.2. Identification of microorganisms cultivated on microbiological media with use of PCR-RFLP technique

Rationale

PCR - RFLP (PCR-Restriction Fragment Length Polymorphism) technique is a fast, simple, and accurate molecular tool for the identification of the main bacterial or fungal population in different environments. The use of this technique has made it possible to identify many species of rhizospheric and endophytic bacteria inhabiting plants.

Equipment/Materials

- Commercial kits for DNA isolation from bacteria, yeast or fungi
- Centrifuge
- Thermoblock
- Laminar chamber
- Pipettes
- Gel documentation system
- Thermocycler
- Horizontal electrophoresis equipment
- Chemical reagents: polymerase DNA, dNTP set, primers, agarose, DNA ladders, restriction enzymes

The biological material consists of colonies of bacteria or fungi cultivated on standard microbiological growth media.

Extraction of DNA from bacterial colonies is carried out by means of a commercial kits for isolating DNA from bacteria, yeast or fungi.

DNA concentration in the samples is measured with a spectrophotometer at a wavelength of 260 nm. For further analyses, the samples are diluted to a final concentration of 10 ng/μl.

Identification of bacteria is based on the analysis of genes such as 16S rRNA, rpoB, nifH, groEL or the bacterial ribosomal operon 16S-ITS-23S rRNA

The 16S rRNA gene is amplified using 27F (AGA GTT TGA TCM TGG CTC AG)/ 1492R (TAC GGH TAC CTT GTT ACG ACT T) primers.

Reaction mixtures (20 μl) consists of 1 × buffer for PCR, 0.2 mM of each nucleotide, 0.2 μM of each primer, 0.5 U of DreamTaq™ polymerase (ThermoScientific®) and 20 ng DNA. Amplification of the 16S rRNA gene is carried out in 35 cycles (94°C × 1 min., 55°C × 1 min., 72°C × 2 min.) and amplification of the 16S-ITS-23S rRNA operon is carried out in 25 cycles (94°C × 15 s, 64°C × 30 s, 68°C × 5 min.).

Restriction analysis of the 16S rRNA gene and the 16S-ITS-23S rRNA bacterial ribosomal operon is performed using FastDigest HaeIII, TaqI and RsaI enzymes (ThermoScientific®). Restriction digestion of the amplified fragments is carried out in 30 μl of a reaction mixture containing 17 μl deionized water, 2 μl of 10x reaction buffer, 1 μl of one restriction enzyme, and 10 μl of template DNA.

Restriction fragments are separated on 2.5% agarose gel, stained with ethidium bromide and visualized under UV light.

On the basis of the data obtained with the RFLP techniques for the 16S rRNA gene and the 16S-ITS-23S rRNA operon, a genetic similarity analysis of the isolates is made using XLSTAT software (Addinsoft 2006) based on the Jaccard coefficient. The results are used to construct dendrograms depicting the genetic similarity of the isolates.

Protocol 5.3. Identification of microorganisms cultivated on microbiological media based on the analysis of gene sequences

Rationale

This technique allows identification of species of microorganisms based on the sequence of several, usually seven, selected loci, with the analysis involving the sequences of those genes that determine the basic functions of the cell.

The data collected on the basis of sequences can be compared between laboratories.

Equipment/Materials

- Commercial kits for DNA isolation from bacteria, yeast or fungi
- Centrifuge
- Thermoblock
- Laminar chamber
- Pipettes
- Gel documentation system
- Thermocycler
- Horizontal electrophoresis equipment
- Chemical reagents: polymerase DNA, dNTP set, primers, agarose, DNA ladders

Procedure

Reactions with the use of specific primers and amplification reactions of the *rpoB*, *gyrA*, *recA*, *tuf*, genes are carried out with the with primers: *rpoBF/rpoBR* (*rpoB* gene), *gyrA47F/gyrA1066R* (*gyrA* gene), *recAF/recAR* (*recA* gene), *tufGPF/tufGPR* (*tuf* gene).

Reactions are carried out in thermal conditions as follows: *rpoB* gene - 30 cycles: 94°C×30s., 50°C×45s., 68°C × 40s., *gyrA* gene - 35 cycles: 94°C×30s., 57°C×30s., 72°C×1 min., *recA* gene - 30 cycles: 94°C 1 min., 50°C×1 min., 72°C×1 min., *tuf* gene - 35 cycles: 94°C×30s., 55°C×1 min., 72°C×min..

Identification of bacterial strains based on the sequences obtained is performed by comparing them with the data collected in the NCBI database [National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA, <http://www.ncbi.nlm.nih.gov>].

Multiple 16S rRNA gene sequence alignments are performed with MEGA 5.2 using CLUSTAL W. Phylogenetic trees are constructed using the neighbour-joining method with a bootstrap analysis based on 1,000 resamplings of the dataset.

Protocol 5.4. Assessment of fungal diversity with the use of the DGGE technique

Rationale

Only a small proportion of microorganisms living in the soil environment start growing on nutrient media in the laboratory. For this reason, molecular techniques based on the analysis of DNA extracted directly from the soil are used to study the microbial diversity of soils. These techniques include the DGGE (Denaturing Gradient Gel Electrophoresis) technique based on the separation of PCR products in acrylamide gels. The DGGE technique consists in separating single amplicons of the same length, but differing in sequence, in a polyacrylamide gel, in a chemical gradient. This technique allows assessment of microbial diversity of soils, as well as precise identification of strains of microorganisms, which can be done by extracting DNA from individual bands in the gel and obtaining DNA sequences.

Identification of bacteria by means of this technique is most often based on the analysis of the 16S rRNA gene, while the identification of fungi is based on the 18S rRNA gene or the ITS region.

Reagents:

- formamide 100%
- 40% acrylamide/Bis 37.5:1
- Urea
- TEMED
- APS (ammonium persulphate)
- Tris-Acetate-Edta buffer (TAE) 50X
- Loading buffer
- Sybr green 10 000X
- Deionized water (milliQ quality)

Equipment

- DCode System for DGGE from Bio-Rad
- Gradient former
- Syringe
- Tips for pipettes (all sterilized)
- glass plates, spacers, combs
- pipettes
- distilled water
- 50% EtOH solution

Procedure

Extraction of DNA from the soil is carried out by means of a commercial GeneMATRIX Soil DNA Purification Kit (EURx) for isolating DNA from the soil. DNA concentration in the samples is measured with a spectrophotometer at a wavelength of 260 nm. For further analyses, the samples are diluted to a final concentration of 10 ng/μl.

PCR conditions

Amplification of 18S rDNA is carried out with the use of a primer for PCR-DGGE on fungi:

NS1: GTAGTCATATGCTTGTCTC

FUNG-GC:CGCCCGCCGCGCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGT
TG (Hoshino and Morimoto 2008)

Reaction mixtures (20 μl) consist of 1 × buffer for PCR, 0.2 mM of each nucleotide, 0.2 μM of each primer, 0.5 U of AmpliTaq™ DNA Polymerase polymerase (ThermoFisher Scientific®) and 20 ng DNA. Reactions with primers NS1 / FUNG-GC are performed in 30 thermal cycles (94°C 15s, 50°C 30s, 68°C 30s). The reaction products are separated in a 7% polyacrylamide gel in a 15-40% chemical gradient, and then stained with SYBR GREEN and visualized under UV light.

Preparation of acrylamide solutions for gradient gel (7%)

Solutions should be placed in 2 different bottles (LOW 15% and HIGH 40%)

Denaturing solution 15%: 40% Bis/Acrylamide 35 ml, 50X TAE buffer 4 ml, Formamide 12.0 ml, Urea 12.6 g, dH₂O to final volume 200 ml

Denaturing solution 40%: 40% Bis/Acrylamide 35 ml, 50X TAE buffer 4 ml, Formamide 32.0 ml, Urea 33.6 g, dH₂O to final volume 200 ml

Setup of glass plates for DGGE

16x16 cm glass plates (inner and outer) should be cleansed thoroughly with 50% ethanol. The glass plates with 2 spacers should be placed into 2 blocks, aligned and placed on the caster.

Pouring the gel

15 ml each of the 2 acrylamide solutions should be placed in the HIGH and LOW beakers. 10 µl/mL of 0.1% APS and 1 µl/mL of TEMED should be added to each of the 2 beakers. The 2 solutions, High and Low, should be placed at the right place in the gradient former. The gel should be filled up to the top after placing the needle between the 2 glass plates. The gel polymerization lasts overnight.

Sample and gel preparation for electrophoresis run

The DGGE apparatus should be filled with 6.5 litres of 1XTAE and heated to 60°C 1 hr before the run by DCode heating system. After turning off the apparatus, the samples should be loaded (10 µl 2X load dye buffer and 10 µl of sample). After closing the apparatus, the temperature should be set at 60°C and the system should be turned on (200 V for 4.5 hours).

Gel visualization

The gels should be incubated in 1X SYBR GREEN solution in darkness for 30 minutes and visualized with the use of the system for gel visualization (e.g. Geldoc). Identification of microorganisms is carried out by comparing the obtained sequences with the data collected in the NCBI (National Center for Biotechnology Information) database using the BLASTN tool (<http://www.ncbi.nlm.nih.gov/blast>).

These analyses can be used also with samples collected from biogenic structures produced by soil invertebrates, i.e., casts, pellets, excrements.

Literature cited

Hoshino Y. T., Morimoto S. 2008. Comparison of 18S rDNA primers for estimating fungal diversity in agricultural soils using polymerase chain reaction-denaturing gradient gel electrophoresis. *Soil Science and Plant Nutrition* 54(5): 701-710.

Protocol prepared by:
Paul Henning Krogh, Maarja Öpik, Beat Frey
Anna Lisek, Lidia Sas-Paszt, Paweł Trzciński
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ES 1406

Protocol KEYSOM-06

Soil profile and humus form description

Rationale

Soil profile descriptions are a valuable source of information for field-based soil science. The description of the different layers (soil horizons) of a profile gives a good idea of what is going on in a profile in terms of soil genesis, leaching, bioturbation and water table dynamics. Soil profiles are best studied in a soil profile pit, but if it is not possible to dig one, basic information can be obtained with a soil augur or soil corer. Standardized guidelines for describing soils have been published by FAO (2006).

For studies relating natural habitats, the characteristics of the litter layer are a very informative addition to the soil profile description. They reflect the equilibrium between litter production, litter decomposition and interaction with the mineral soil. The "litter layer" refers to all the dead organic debris (leaves, needles, branches, twigs, etc., either still recognizable or significantly altered) that is found on top of the mineral soil surface. The "A horizons" are the mineral topsoil horizons containing organic matter, less than 20% organic carbon by mass in the fine (≤ 2 mm fraction) are not considered to be part of the litter layer. Zanella et al. (2011) proposed a new description and classification system for European soils.

The data from soil profile and litter layer descriptions are very useful when interpreting soil fauna data, to understand the general context of the environment these organisms live in. When applying this protocol, the complementary excel data sheets by using terms, definitions and symbols as proposed in the FAO and Zanella guidelines should be filled out. A soil sampling method is also specified, to allow soil geochemical analysis in a later stage of the experiment.

Location of profile pit or auguring/coring points

Ideally, a profile pit is dug at each sampling site. Dig the pit as closely as possible to the sampling points without disturbing them. If there is the possibility of choosing between an upslope (plateau) situation, slope or downslope position, the plateau profile will be the best developed and most informative. A profile pit has a depth of 1.5 – 2 m deep (unless there is a shallow bedrock), ca. 2m wide and a few meters long to allow easy access.

Define one wall of the pit for describing (e.g. depending of the slope (description side at the upslope side), the position of the sun (to avoid strong contrast between shadow and sun), and make a very clean cut when excavating this side, disturbing the litter layer and horizons as little as possible. Do not walk or stand on the edge of the profile pit, and certainly avoid any soil material falling inside. The opposite side of the pit ideally has some steps, to be able to get in and out of the pit. If the pit is left open unattended, please take safety precautions to avoid people or animals getting injured. Allow for an 'escape route' (stairs, planks, ...) so smaller fauna can get out of the pit.

If it is not practical or not allowed to dig a profile pit, augers are used. This method is much faster but only a limited set of features can be described. A small section of soil is brought up to the surface by using an Edelman augur or soil corer of at least 1,5 m. Even if you dig a pit, it is recommended to do some coring in the surrounding area to check soil heterogeneity. Again use the soil auger as closely as possible to the sampling site without disturbing it, to a depth of 1.5 m if possible. If possible, do at least three repetitions.

During coring, lay out the different subsamples taken along a depth profile on a piece of black or gray plastic, next to a tape or ruler. When using an Edelman auger, check the depth frequently when auguring and laying out the samples taken.

The choice between an Edelmann auger or a corer can be based on what is available or what is more practical: the Edelmann auger is an all-rounder, easy to carry around and to use. Soil corer is more practical when taking samples at a certain depth, but it is generally heavier (a hammer is also needed) and can be difficult to dig it into the soil or extract it, e.g. if the soil is too hard or dry. It is advisable to take small incremental depth cores if that is the case. Also, soil corers can be easily damaged when there are stones in the soil profile. Watch the corer carefully and stop if it jumps back upon impact by the hammer.

The soil should not be too dry or it will not be retained inside the auger and it will be too hard to dig a profile pit. If a shallow water table is present, ideally wait for a drier period: soaked soil will also fall out of augers/corers and it is unpractical to have to scoop the water out with a bucket or pump all the time.

Equipment/Materials

- A spade with a straight and sharp blade of min. 25 cm length.
- A shovel (when digging a profile pit)
- Auger or soil corer (1.5 m long) and hammer
- Guidelines (FAO, Zanella), field forms, notebook
- pencil
- Knife with a sturdy (blunt) blade of 10-15 cm
- Meter of 2m long, readable when photographed. If that is not the case, paint every other 10 cm in the tape measurement with a marker pen.
- Bottle of water (demineralized)
- Spraying bottle with demineralized water
- Munsell soil color book (There are also smartphone apps)
- Trowel, palette knife or small hoe
- Small bottle for acid solution (HCl, 1M)
- Set of transparent polyethylene bags (1 L)
- Marker pen (permanent), labels
- Pocket eye magnifying glass
- Digital camera, if possible with a wide-angled lens; spare battery and memory card
- Garden shear or scissors

The following equipment is needed in the laboratory:

- Recipients to allow samples to air-dry
- 2 mm sieve

Frequency of description/sampling

Description and sampling should be done once for every plot.

Procedure

- Clean the soil wall of the profile pit to be described with a knife/trowel. Ideally, one half of the profile wall should be scraped flat and one half should be prepared for soil structure. Starting from the top and working from left to right, stick the knife a few cm into the soil and move the handle down, so the soil breaks naturally showing the aggregate surfaces. Take care to remove the knife marks of the previous row when working on a new, deeper row. Place a tape measurement at one side of the profile wall, starting from the mineral soil surface. Try not to disturb the top or the litter layer. If it is a hot day, spray the surface with water and then gently with a spray bottle with demineralised water.
- Take a picture of the entire profile with the tape measurement next to it, holding the camera straight and perpendicular to the profile wall (here a wide-angle lens would be handy). Take a picture of particular details if necessary, indicating at what depth they occur. Also take a picture of the landscape surrounding the pit for later reference. If it is very sunny, dark shades may significantly reduce the quality of the picture. Using an umbrella to create a shade can be a solution.
- Take some time to 'get to know' the particular features of the profile. Probe the wall with a knife to check if the soil e.g. becomes more difficult to probe with depth. This can indicate the presence of illuvial horizons that can be difficult to spot with based on colour.
- Identify the main soil horizons. On page 67 of the FAO guidelines there is a section on how to identify and name the main soil horizons (referred to as master horizons)
- Fill out the KEYSOM soil profile description form (Annex 1) for profile pits while using the FAO guidelines. Do this for every master horizon. For easy reference, the most important table numbers are stated above their respective columns in the form.
- For estimating field texture, refer to this video: <https://youtu.be/GWZwbVJCNeC>. Although the soil texture triangles of USDA and FAO are not exactly the same, the differences are too small to be relevant for the field method.
- Close the profile pit, taking care to respect as much as possible the sequence of horizons.



Photo 06.1. Testing the reaction to HCl to detect carbonates in soil under field conditions.

Litter layer description

- Carefully observe the litter layer and fill out the KEYSOM humus description form, using the Zanella reference guidelines.
- When using augers for the soil profile description, dig a 'micro-pit', typically 30-50 cm wide x 20 cm deep, for describing the boundary between litter layer and mineral soil, and the structure of the A-horizon. Make a straight, clean cut with the spade at one side of the profile, disturbing soil and litter layer as little as possible.
- Place a tape measurement in the micro-pit and take a photograph.
- Fill the second section of the KEYSOM humus description form. For consistency, use the FAO protocol to describe the structure of the A horizon and its lower boundary. Use a magnifying glass to examine the zoogenic pellets.

Sample collection and storage

Sampling of the mineral soil

In general, there are two possibilities for taking soil samples for geochemical analysis: you can sample either by horizon, or by depth. To be consistent with the other protocols in the KEYSOM handbook, a sampling by depth was chosen.

Take a bulk soil sample of ca. 200 g at 0-5, 5-10 and 15-30 cm depth and store it in a labelled plastic bag. Note that marker pens can rub off when the bags are dusty, so take care that the labelling is permanent.

When auguring, make composite samples of at least 3 auguring points, by combining the 0-5 cm sample of the tree augers into one bag. Same for the 5-10 and 15-30 cm samples. When digging a pit, take a 'mixed' sample by combining material along the width of the profile wall, from at least 3 random points.



Sampling of the litter layer

Lay out 3 quadrats of 25 x 25 cm on the litter layer. If ground vegetation is present, cut it off just above the top soil. Carefully remove the litter layer within each quadrat and introduce it in labeled bags. Sample the OL, OF and OH layers, but not the mineral soil.

Sample storage

Transport the samples back to the laboratory and air-dry them as quickly as possible (max 40°C) until a constant weight is reached.

Sieve the soil samples to 2 mm (fine soil fraction). Aggregates that can be crushed between fingers are considered to be soil and hence, they should be passed through the sieve too.

Store in a cool, dry and dark place for future chemical analysis, if required



Photo 06.3. Example of basic soil characteristics determination in the lab in front of several students.

Literature cited

FAO, 2006. Guidelines for soil description. Fourth edition. Food and Agriculture Organization of the United Nations, Rome. Available online at: ftp://ftp.fao.org/agl/agll/docs/guidel_soil_descr.pdf

Zanella, A., Jabiol, B., Ponge, J.F., Sartorid, G., De Waal, R., Van Delfte, B., Graefe, U., Cools, N., Katzensteiner, K., Hager, H., Englisch, M., Brethes, A., Broll, G., Gobat, J.M., Brun, J.J., Milbert, G., Kolb, E., Wolf, U., Frizzera, L., Galvan, P., Kolli, R., Baritz, R., Kemmerse, R., Vacca, A., Serra, G., Banas, D., Garlato, A., Chersich, S., Klimo, E., Langohr, R. 2011. European humus forms reference base. Available online at: https://hal.archives-ouvertes.fr/file/index/docid/561795/filename/Humus_Forms_ERB_31_01_2011.pdf

Protocol prepared by:
Karen Vancampenhout
Revised by the KEYSOM team

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ES 1406

Protocol KEYSOM-07

Sampling of undisturbed soil cores for X-ray cross-tomography

Rationale

Soil fauna is characterized by the production and construction of structures, like galleries, burrows, nests and other biogenic structures which are important for many soil-related process, like water infiltration, soil aggregation and others. The use of X-ray cross tomography allows visualizing some of these structures under a range of different field conditions.

Materials

The following material is needed in addition to the set provided [2 cylinders, four lids, 1 plate, 1 shearing tin] (photos 1/1 and 1/2):

Spade, little shovel, long knife, small knife, scissors, folding rule, plastic foil (from plastic bag), tissue paper.

Procedure

Two replicate soil cores are taken per site with the cylinders. It is recommended that soils should be near field capacity (pF 2.8), i.e., take the soil cores after several days of rain⁴.

- Place set on the ground, add a long knife and scissors (photo 07.1), and mark with a spade a 20 x 20 cm square, ca. 10 cm deep (photo 07.2),
- Excavate with a small shovel a cuboid with a conic shape (broader near the ground) and 14 cm height (photo 07.3),
- Deepen it to a depth of 16 cm from the soil surface (photo 07.4)
- Cut off the vegetation with scissors (photo 07.5),
- Place one cylinder on top of the cuboid (photo 07.6),
- Cut the sward around the cylinder to a depth of 2-3 cm with the long knife (photo 07.7),
- Place the lid on top of the cylinder, adjust it and press the cylinder smoothly into the ground (photo 07.8) until it is completely filled with soil (photo 07.9),
- Remove the soil around the cylinder with the long knife (photo 07.10)
- When ca. 3 cm of soil are taken away, clean the cylinder with tissue paper, place the plastic foil on top of the column, and adjust the yellow lid (photo 07.11),
- Go with the spade ca. 10 cm under the lower edge of the cylinder, and gently lift the column, turn the column upside down,
- Remove parts of the soil with the shear plate (from set) (photo 07.12),
- Then carefully remove the soil exceeding the plastic cylinder by cutting it with the metal piece (photo 07.13)
- Cut the roots off with the scissors if necessary (photo 07.14)
- Remove the soil core completely with the metal piece (photo 07.15)
- Place plastic foil on the lower edge of the cylinder and add the yellow lid (photo 07.16)
- Turn the column, number it on top and on the side. Pack the undisturbed column(s) without the other sampling materials (lid, metal piece, shearing, etc.) carefully and tight with additional filling material (07.17).

Data

Note sampling date, coordinates, dominant vegetation and person in charge.

⁴When this is not possible, ensure that the soil at the required depth is soaked by repeated watering with moderate amounts of water. In case this cannot be achieved during several hours, the procedure will have to be repeated on the following day or wait for rain. The more sandy your soil is the higher is the likelihood that the aggregate structure collapses during transport of the dry soil.



Photo 07.1



Photo 07.2



Photo 07.3



Photo 07.4



Photo 07.5



Photo 07.6



Photo 07.7



Photo 07.8



Photo 07.9



Photo 07.10



Photo 07.11



Photo 07.12



Photo 07.13



Photo 07.14



Photo 07.15



Photo 07.16



Photo 07.17

Literature cited

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Protocol prepared by:
Monika Joschko, Helmut Rogasik
Juliane Filser, Monika Joschko
Revised by the KEYSOM team

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ES 1406

Protocol KEYSOM-08 Bioturbation mesh bag experiment

Rationale

Mesh bags with and without access for macrofauna are buried into the soil at the study site and filled with a mixture of sand and kaolin. The sand/clay mixture loosely follows OECD (1984) artificial soil; instead of peat, natural litter is being used as carbon source (plus in-growing roots etc.). The pH will quickly adjust to the surroundings upon watering and subsequent rainfall. Glass beads are spread onto the surface and covered with litter of the site. After one year, the cores are excavated, subdivided into layers and samples distributed to various participating labs for the analysis of bioturbation and aggregate formation.



Photo 08.1. Material and installation of mesh-bags in the field.

Materials

Laboratory supplies:

- 1 mesh bag without holes (see “field equipment” below; for testing of required mass per core)



Photo 08.2. Close-up of the mesh-bag before folding the upper part in the field.

- 13 plastic bags or bottles with screw caps (one extra in case you lose something)
- oven for drying at 105 °C
- sand (industrial quartz sand, predominantly fine sand with more than 50% of the particles between 50 and 200 microns, e.g. “quartz sand, washed, 0.1–0.5 mm grade”: https://ie.vwr.com/store/catalog/product.jsp?product_id=8611518)
- kaolin clay (kaolinite content preferably above 30%, e.g. “Technical grade, washed”: <https://ie.vwr.com/store/product/2347663/bole-white-kaolin-technical-washed>).
- Dust mask and gloves for handling kaolin

About 0.6 kg of material will be required per core [about 0.4 L volume with 1.5 kg L⁻¹ density or 450 g sand + 150 g kaolin], i.e. 6 kg per site (about 6 kg sand and 2 kg kaolin, to be on the safe side).

Field equipment:

- Soil auger or steel cylinder (diameter ca. 5 cm, at least 20 cm long) and/or small shovels
- long knife
- scissors
- 5 mesh cylinders 5 cm diameter, 20 cm deep – made of fine mesh (1 mm)
- 5 mesh cylinders 5 cm diameter, 20 cm deep – made of fine mesh (1 mm) with holes for macrofauna access.
- long spoon or spatula
- Two containers (ca. 0.5 and 1 L) with lids for collecting removed litter and soil
- 20-30 cm long tube (e.g. escape pipe) with about 4 cm diameter and a matching (powder) funnel fixed or glued on top.
- 10 plastic bags/bottles filled with the sand + clay mixture for each core
- 10 capped vials with glass micro-beads (see protocol KEYSOM-09).
- 10 (plus a few extra) pegs or U-shaped pieces of solid wire (ca. 10 cm long; you may also use a large paper clip bent accordingly)
- bamboo sticks or the like for marking
- if no fix points (see below) are available: GPS pegs, big stone or the like.



Note: The procedure was filmed on video and is available here:

<https://www.youtube.com/watch?v=b40mrkkRFw&feature=youtu.be>

Procedure

Step 1 – Field installation

Laboratory: Oven-dry sand and kaolin (105 °C) over night before use. Mix 75% sand with 25% kaolin by weight (specifications see above). Use dust mask and gloves when handling kaolin. Test the required mass of your mixture required for a mesh cylinder (about 450 g sand and 150 g kaolin). Prepare the appropriate mass of the mixture for each core individually to ensure equal proportions of sand and clay and mix it thoroughly. Fill them into plastic bags or bottles for transport.

A short video is available here:
<https://youtu.be/CD4spdgZDBM>

Field: Mark for each core a 5 cm diameter spot, remove litter and/or cut off vegetation and collect it in a plastic box. Cut any vegetation and scrape off surface litter of the excavated core, sort out manually any macrofauna (let go free) and retain the plant matter and surface litter (no drying required). Excavate 5 * 2 holes (5 cm diameter, 20 cm deep – better a bit more), using metal cylinders, auger and/or shovel/knife, arranged in pairs in close proximity (with and without macrofauna access). The five pairs of cylinders should be at least 5 m apart from each other and located randomly in your site. Discard the uppermost rooted horizon and collect the rest of the soil (remove larger roots) in a box (for filling any remaining gaps between cylinder and surrounding soil).

Mark 20 cm with a permanent pen on mesh bags without holes. Insert one mesh bag into one of the paired holes and one bag with holes in the other paired hole. Use the plastic tube for inserting to ensure contact with the underlying soil. Slowly and carefully fill in the sand + clay mixture through the funnel/tube, making sure that no material gets lost, especially from the bags with holes, and that the mesh cylinder is in good contact with the surrounding soil. This is achieved best via the funnel and tube, which is placed inside the mesh bag and slowly pulled upward while filling in the sand/clay.

Add the glass beads onto the sand/clay surface (the content of one glass-beads vial per core), spreading them as evenly as possible. Apply the plant matter and surface litter from each soil core on top of the sand-clay core before closing the bags.

Add the glass beads onto the sand/clay surface (the content of one glass-beads vial per core), spreading them as evenly as possible. Apply the plant matter and surface litter from each soil core on top of the sand-clay core before closing the bags.

The mesh from each bag is longer than 20 cm. To close them, bend it horizontally to the side, fold it twice at the end (double-fold about 2 cm long), fix it at the surface with a peg or wire and water the cores after preparation with ~100 mL tap water. Fill any gaps between cylinder and surrounding soil with part of the excavated soil. Cover everything with surrounding litter (invisibility is crucial to avoid damage by curious birds etc!). Mark location of the core AND additionally make a detailed drawing of the locations and distances to permanent fix points such as trees or large stones (keeping in mind that you have to find them one year later).

Step 2 – Final sampling (1 year after burial)

- Additional material: Spade, ruler, long knife, big spoon, tray (at least 30x30 cm), robust plastic sheet (at least 30x30 cm), solid scissors, plastic containers with lids or plastic bags [and cooling box] for transportation, water-proof pen for labelling, paper and pen, optional: camera.

Attention: make sure that the soil is wet before excavating the cores – else you might lose most kaolin and sand and probably part of biogenic structures as well! Ideally do this right after ample rain, otherwise you might have to thoroughly water your cores the day before you dig them up (to allow for sufficient downward water transport).

Locate your mesh core and remove any litter (not the living aboveground vegetation which should be roughly recorded ("few grasses, one other seedling" or the like) before cutting it off) on top. Cut with a long knife a ring around the core at a few cm distance from the mesh. With a spade excavate adjacent to both sides of this ring a soil block (about 20x20x20 cm) so you can easily access the core with its surrounding soil "mantle". Then cut off surrounding roots, insert the spade well underneath → 20 cm, then carefully lift this core-mantle block and place it inside a tray. Carefully remove the mantle soil, cut off any roots directly at the mesh surface using the knife and put the cleaned core onto the plastic sheet.

Cut off the top part of the mesh and then cut open the mesh vertically (along the core from top to the bottom) and spread the mesh to both sides. Measure the length of the organic horizon on top and take pictures / note peculiarities if you see anything interesting (e.g. when most litter apparently has been moved outside, which we encountered once in a bag with holes). Ignore any white/clay that has fallen outside the mesh bags. Subdivide the core into 0-5, 5-10, 10-20 cm layers, put these separately into labelled plastic containers (or plastic bags, but making sure the cores keep their structure during transport) and store them in a cool box (or at least in a cool place) until you have collected the rest. The wet and largely unmodified 10-20cm rest is more easily lifted (lower loss) when using your hands rather than a shovel.



Photo 08.3. Close-up of the mesh-bag after being retrieved from the field (a large bioturbation and soil mixture is observed).

In the lab:

- Hand-sort the uppermost sample (0-5 cm) for ingrown roots and living plants, earthworms and any other larger fauna, take down root categories (0: none – 1: few – 2: many visible roots), earthworm numbers, earthworm fresh mass and other fauna numbers. Extract mesofauna from remaining soil using Berlese or Macfadyen funnels (detailed procedures may vary according to equipment, soil type and your experience). Typically this should last about a week, increasing the soil surface temperature stepwise from room temperature to 50 or 60 °C.
- Other core layers (5-10, 10-20 cm): Try to separate larger biogenic structures. Carefully break the rest roughly apart and spread for drying (at room temperature). Check root in-growth and larger fauna (if present) as above.
- After air-drying / mesofauna extraction: Separate biogenic structures (i.e. aggregates and soil including any organic traces, not the pure white clay/sand mixture which can be disposed of) for each layer. Separate aggregates either manually (visual appearance) to the required size classes or according to protocol KEYSOM-01. Dry biogenic structures in a desiccator until mass constancy and record dry mass.
- For counting the glass beads from the dried biogenic structures see protocol KEYSOM-09.



Photo 08.4. Close-up of the soil retrieved from the mesh-bag and packed in different containers for subsequent analyses.

Additional Data

Note sampling date, coordinates, dominant vegetation, any peculiarities and person in charge.

Literature cited

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Protocol prepared by:
Juliane Filser, Olaf Schmidt, Pascal Querner
Revised by the KEYSOM team

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ES 1406

Protocol KEYSOM-09

Bioturbation experiment.

Information on glass micro-beads

Rationale

Micro-beads (about 300–355 μm diameter) made from clear glass are used as a new physical marker to estimate bioturbation. A known mass of beads is applied to the sand surface of cores at the start of the experiment (see protocol KEYSOM-08), and beads will be retrieved and counted from sand layers at the end of the experiment.

Materials provided

Dry, clean beads are provided in very small batches (0.5 g each) in Eppendorf tubes, ready for application to individual cores in the field. No weighing, dividing or any other preparatory step is required.

Field application

Apply the content of one Eppendorf tube (0.5 g) to one core. Use a system that ensures that each of the 10 cores at a site receives one batch of beads.

Apply to the surface of the sand/clay mixture after filling / packing each core with the mix, but before local litter is replaced. Sprinkle the beads carefully and slowly and as evenly as possible over the sand surface. Note that the dry beads flow freely and fast! Try to tap the tube very gently. Replace the surface litter afterwards.

The empty tubes are for disposal (non-hazardous).

Counting glass beads using X-ray Computed Tomography (CT) – See protocol KEYSOM 06.

When preparing samples for X-ray CT scanning, first weight the received biogenic structures, including the bag in which they arrived, so no material is accidentally lost. With this weight the total bead concentration can be calculated from the total amount of collected biogenic material.

The X-ray CT scanner obtains the highest resolution scans when the sample is as close as possible to the X-ray gun, and the detector is near the sample as well. In other words, try to position the sample in such a way that it takes up a large proportion of the available detector. In order to do so, it may be necessary to fold the bag a couple of times to make it more compact.

When making scans, it is essential that the mounted sample is very stable. The sample will be rotated 360° and any slight movement will make it impossible to construct a reliable 3D volume from the scans. In case of the KEYSOM samples, the folded bags were fixed with tape, to prevent movement of loose corners of the bags.

The actual scan parameters will depend on the conditions of each sample and will need to be altered by the scan operator.

After obtaining the scan, a 3D volume needs to be constructed which can be exported as an image stack of .tiff files for further downstream analysis.

The downstream analysis of the KEYSOM samples was performed using ImageJ, which is freely available open source software. In this software, the contrast between the soil matrix and beads (which have similar grey scales) can be increased compared to the surrounding pore space. From this image stack, beads can be manually counted. Methods to automatically identify beads from the scans are still being developed.

Alternatively, received biogenic material can be subsampled and divided over multiple Eppendorf tubes which will allow scans of a higher resolution.

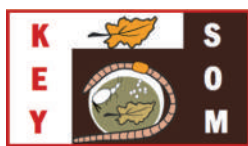
At the end of the study, use 10 g of dried biogenic structures and count glass beads using a stereomicroscope. The beads are easily distinguished from sand grains by their perfect shape and iridescent properties.

Literature cited

OECD (Organisation for Economic Co-operation and Development) (1984). Guidelines for testing of chemicals No. 207. Earthworm, acute toxicity test. OECD, Paris.

Protocol prepared by:
Olaf Schmidt, Saorsie Tracy, Oskar Franken
Revised by the KEYSOM team

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ES 1406

Protocol KEYSOM-10

Functional diversity indices of soil bacterial community estimated by Biolog EcoPlates™ assay

Rationale

Microorganisms are present virtually in all ecosystems, and due to their rapid responses to physical and chemical changes, they can be used as bioindicators of environmental quality. The Community-Level Physiological Profiling (CLPP) is a rapid and relatively inexpensive technique to relate microbial functional diversity over space and time to changes in the environment, e.g. due to soil management and ecotoxicological studies (Muñiz et al. 2014).

Biolog® EcoPlates™ (Biolog Inc.; Hayward, CA, USA) were developed to analyze the functional diversity of soil bacterial communities by means of measuring their ability to oxidize carbon substrates. An EcoPlate is a 96-well microplate that contains 31 common carbon sources from altogether five compound groups – i.e. carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers – plus a blank well as a control, all these replicated thrice to control variation in inoculum densities (Fig. 1).

Each EcoPlate is filled with a dilution of one soil suspension, thus representing one soil sample. The utilization rates of carbon compounds in the wells are quantified spectrophotometrically by following the reduction of water-soluble colorless triphenyl tetrazolium chloride to purple triphenyl formazan. For the measurements of optical density (OD), two filters are used: 1) 590 nm (absorbance peak of tetrazolium) to evaluate color development and 2) 750 nm to measure turbidity values.

Every bacterial community has a characteristic reaction pattern with different OD values for different carbon compounds, called a 'metabolic fingerprint'. However, the inoculated bacterial density significantly affects the rate of color development in the wells and therefore, inoculum densities have to be standardized for different soil samples using a plate count culture-based method.

Culture-based plate count method

This analysis must be conducted under sterile conditions in a laminar-flow hood using single-use sterile plastic material and autoclaved solutions and glassware.

Materials

- Sterile flasks and tubes
- Sterile spatula
- Sterile pipettes
- P1000 and P100 micropipettes with sterile tips
- Petri dishes (size 90 mm, polystyrene, γ -irradiated)
- Sterile hockey stick (disposable cell spreaders)

Apparatus

- Laminar flow hood
- Autoclave
- Incubator
- Water bath
- Magnetic stirrer
- Ultrasonic bath

Reagents

- Tryptic Soy Agar (TSA)
- Cycloheximide (to inhibit fungal growth)
- 25% sterile Ringer solution (NaCl 2.25 g l^{-1} , KCl 0.105 g l^{-1} , CaCl_2 0.045 g l^{-1} and NaHCO_3 0.05 g l^{-1})
- 1.8% (w/v) sterile sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 18 g/L or 18 mg/mL) solution (to disperse soil colloids)

Procedure

Making and plating TSA

- add 3 g of TSA powder to 1 l of distilled water in a 2-l glass bottle
- sterilize (autoclave) at 121 °C for 20 min
- cool at 50 °C
- add 100 μg cycloheximide ml^{-1} and mix thoroughly
- pour 20 ml in each Petri dish

Plate counting technique

- add 5 g (dry weight equivalent) of fresh soil to 45 ml of sterile sodium pyrophosphate-Ringer solution (40.5 ml of 25% Ringer solution + 4.5 ml of 1.8% sodium pyrophosphate)
- sonicate for 2 min
- settle soil particles at 4 °C for 15 min
- make ten-fold serial dilutions of the supernatant up to 10^{-7} in sterile Ringer solution

- spread a 100 μL -aliquot of each dilution onto a TSA plate (3-5 replicates per dilution)
- incubate at 28 °C for 72 h
- for microplate incubations, choose the dilution that leads to ca. 104 colony forming units (CFUs) mL^{-1} solution.

Microplate incubation

Materials

- Multichannel pipet and sterile tips
- Sterile plastic multichannel reservoir
- Biolog® EcoPlates™ (Biolog Inc., Hayward, CA, USA)

Apparatus

- Laminar flow hood
- Incubator
- Biolog® Microplate Reader™ equipped with 750-nm and 590-nm filters

Preparing the microplates

- pour 10 ml of the dilution that was chosen in plate counting (2.4.2.) into a sterile reservoir of an 8-channel pipet (be careful there are no bubbles in the dilution) and inoculate 120 μL of the dilution into each well of a microplate
- place the microplate in its bag to avoid desiccation and incubate at 25 °C in dark, continuously shaking to obtain a uniform distribution of color
- take spectrophotometric readings at both 590 ($\text{OD}_{590\text{nm}}$) and 750 nm ($\text{OD}_{750\text{nm}}$) at time 0 and daily, up to a 167-h incubation

Selection of the optimal incubation time for microplate analyses

Follow this pattern for each incubation time (as an example, measuring times at 0 h and 24 h are given here):

- calculate a color value for each substrate well i and the blank (water) well b for each incubation time by subtracting the $\text{OD}_{750\text{nm}}$ value from the $\text{OD}_{590\text{nm}}$ value:

$$0 \text{ h: } i_{0h} = \text{OD}_{590\text{nm}} - \text{OD}_{750\text{nm}} \quad \text{and} \quad b_{0h} = \text{OD}_{590\text{nm}} - \text{OD}_{750\text{nm}}$$

$$24 \text{ h: } i_{24h} = \text{OD}_{590\text{nm}} - \text{OD}_{750\text{nm}} \quad \text{and} \quad b_{24h} = \text{OD}_{590\text{nm}} - \text{OD}_{750\text{nm}}$$

- subtract the blank well OD reading from the OD value of each substrate well to obtain a blank-corrected value (i_{bc}) for each well:

$$0 \text{ h: } i_{bc0h} = i_{0h} - b_{0h}$$

$$24 \text{ h: } i_{bc24h} = i_{24h} - b_{24h}$$

- subtract the blank-corrected OD reading at time 0 from subsequent blank-corrected daily readings to obtain color development values (c_i) for each well for each incubation time:

$$\text{e.g. } c_{i24h} = i_{bc24h} - i_{bc0h}, \text{ and set negative values to } 0$$

- calculate the average well color development (AWCD) for all incubation times separately using the equation:

$$AWCD = \sum \frac{c_i}{93}$$

and for the best discrimination of bacterial communities, choose the shortest incubation time, at which 90% of substrates show an OD reading $\rightarrow 0.25$

Utilizing the AWCD and c_i values

The AWCD calculated above is an estimate of the total capacity of a bacterial community to use different carbon compounds.

Using the c_i values of the chosen incubation time, you can further calculate indices of bacterial functional diversity, such as:

- a) Shannon's diversity index (H'), which is related to the number of carbon substrates the bacterial community is able to degrade as well as the evenness of c_i values across the carbon substrates,

$$H' = -\sum p_i (\ln p_i)$$

- b) and Shannon's evenness index (E), which particularly focuses on the evenness of c_i values across all utilized substrates,

$$E = \frac{H'}{\ln S}$$

where p_i is c_i divided by the sum of all c_i values and S is the number of utilized carbon substrates (i.e. where $c_i \rightarrow 0$).

Other diversity indices can be used, like Simpson's λ diversity index.

For a more detailed analysis, the carbon substrates can eventually be divided into eight classes of compounds (polysaccharides and complex compounds, cellulose, hemicellulose, chitin, phosphorylated compounds, organic acids, amino acids, and biogenic amines) and the AWCD and diversity values calculated for each group separately.

Literature cited

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Protocol prepared by:
Patrizia Ricciuti and Adriano Sofo
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ES 1406

Protocol KEYSOM-11

Soil samples for microbial analyses (classical)

Rationale

For classical soil microbial analysis⁵, collect the approximately 50-100 g of soil from 3-4 replicates per plot (1 ha aprox.), mix them to prepare a pooled sample. Place each pooled sample in a sterile plastic bag, or similar container. Sample should be transported in a cool box (4-8°C) to the laboratory where samples should be preserved at 4°C until being processed (do not exceed 1 month of storage).

Estimation of the number of microorganisms (classical method)

Suspend 10 g of each pooled soil sample in sterile distilled water at a ratio of approximately 1:9 (w/w) (if necessary use higher volume of distilled water). Homogenize the suspension using a laboratory shaker for 45 minutes at the speed of 150 rates per minute. The best will be a reciprocating movement shaker, but other equipment can be used i.e. vortex (if no equipment is available shake the suspended samples by hand). Use the homogenized suspension to prepare serial decimal dilution (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}), which will be used to inoculate the growing media (use 3 replicates per dilution). Depending on the parameter to be estimated use the following media (see below), incubation time and temperature:

- Copiotroph microorganisms - TSA medium, 2-5 days 28°C
- Oligotrophic bacteria on 100 fold diluted TSA medium, 10-14 days at 28°C
- Spore forming bacteria on 10 fold diluted TSA medium, 10-14 days at 28°C
- Pseudomonas bacteria (including fluorescent Pseudomonads) on S1 medium, 72 hours at 30°C
- Actinomycetes on colloidal chitin agar, 7 days at 28°C
- Diazotrophs on Burk medium, 72 hours at 30°C
- Microscopic fungi on Rose Bengal agar medium with chloramphenicol, 5-7 days at 25°C

⁵ Many soil microorganisms are not cultured in the lab with classical techniques, so metagenomics analysis is recommended instead.

Growth media

trypticase soy agar (TSA)

Ingredients	grams/litre
Casein peptone (pancreatic)	15.0
Soya peptone (papainic)	5.0
Sodium chloride	5.0
Agar	15.0
Final pH	7.3 +/- 0.2 at 25°C

ten-fold diluted trypticase soy agar (TSA) (Ghyselinck et al. 2013)

Ingredients	grams/litre
Casein peptone (pancreatic)	1.5
Soya peptone (papainic)	0.5
Sodium chloride	0.5
Agar	15.0
Final pH	7.3 +/- 0.2 at 25°C

one hundred-fold diluted trypticase soy agar (TSA) (Ghyselinck et al. 2013)

Ingredients	grams/litre
Casein peptone (pancreatic)	0.15
Soya peptone (papainic)	0.05
Sodium chloride	0.05
Agar	15.0
Final pH	7.3 +/- 0.2 at 25°C

Rose Bengal Chloramphenicol Agar (according to BTL formula, cat. number. P-0117)

Ingredients	grams/litre
Peptone	5.0
Dextrose	10.0
K ₂ HPO ₄	1.0
Rose bengal	0.05
Chloramphenicol	0.1
Agar	15.0
Final pH	5.6 +/- 0.2 at 25°C

colloidal chitin agar (Hsu and Lockwood 1975)

Ingredients	grams/litre
dry colloidal chitin	4
K ₂ HPO ₄	0,7
K ₂ HPO ₄	0.3
MgSO ₄ x 5 H ₂ O	0.5
FeSO ₄ x 7 H ₂ O	0.01
ZnSO ₄	0.001
MnCl ₂	0.001
agar	20
final pH	8.0 +/- 0.2 at 25°C

Burk's medium (Kennedy et al. 2005)

Ingredients	grams/litre
K ₂ HPO ₄	0.64
K ₂ HPO ₄	0.2
MgSO ₄ × 7H ₂ O	0.2
NaCl	0.2
CaSO ₄ × 2H ₂ O	0.05
N _{a2} MoO ₄	0.001
FeSO ₄	0.003
mannitol	20
agar	12
Final pH	7.3 +/- 0.2 at 25°C

S1 medium (Gould et al. 1984)

Ingredients	grams/litre
sucrose	10
glycerol	12.6
casamino acids	5
NaHCO ₃	1
MgSO ₄ × 7H ₂ O	1
K ₂ HPO ₄	2.3
Sodium lauroyl sarcosinate	1.2
agar	18
Final pH	7.3 +/- 0.2 at 25°C
trimethoprim 20 mg,	

Remarks

Add antibiotics to medium after sterilization in sterile conditions, mix it carefully and pour into Petri dishes.

For calculating of the number of microorganisms, use only the plates on which the number of colonies fell within the range of 30-300. The results shall be converted to colony-forming units per 1 gram of soil in a dry weight basis (cfu x g⁻¹ DW).

In order to express the number of microorganisms in terms of per 1 g of soil DW, determine the soil dry weight by drying about 1g of soil at 105°C for at least 18h and take note of the dry weight (use several replicates and use the average dry weight).

Assuming that we use decimal dilution rate of suspended sample

$$x = \frac{a * 10^{-b}}{c}$$

a – average number of colonies

b – dilution rate from where the number of colonies were counted

c - volume (in milliliters) of sample used for inoculation of petri plate i.e. 1 ml = 1; 50 µl = 0.05

Example: suspend 10 g of sample in 90 g of distilled water, homogenize it, prepare serial decimal dilution from it, inoculate it the agar plates with 50 µl each and after incubation period the number of obtained on plates from dilution rate 10⁻⁴ were 74, 69, 76 colonies.

a – average colonies count – (74+69+76)/3 = 73

b – dilution rate used – 10⁻⁴

c - volume (in milliliters) of sample used for inoculation of petri plate, i.e., 50 µl = 0.05 ml

$$x = \frac{\frac{74 + 69 + 76}{3} * 10^4}{0.05}$$

$$x = \frac{73 * 10000}{0.05}$$

$$x = 14600000$$

$$x = 146 * 10^5$$

Estimated number of cultivable microorganisms is 146 * 10⁵ per one gram of soil. This is than to be converted into soil DW. The final figure is presented as „colony forming units” per one gram of dry soil.

If the sample needs more solvent to be suspended property we have to use modified version of formula above

$$x = \frac{a * 10^{(-b)-1}}{c * d}$$

a – average colonies count

b – dilution rate used

c - sample first dilution rate i.e. 5 g of sample diluted in 50 g of solvent - 5/55 = 0.09

d - volume (in milliliters) of sample used for inoculation of petri plate i.e. 1 ml = 1, 50 µl = 0.05 ml

Example: suspend 9.43 g of soil in 174.2 g of distilled water, homogenize it, prepare serial decimal dilutions from it, inoculate them the agar plates with 100 µl each and after incubation period the number of colonies obtained in rate 10⁻⁴ dilution were 74, 69, 76. We treat suspended sample as a dilution 10⁻¹ and serial ten-fold dilution prepared from suspended sample as 10⁻², 10⁻³, 10⁻⁴ etc.

a – average colonies count – (74+69+76)/3 = 73

b – dilution rate used i.e. 10⁻⁴

c - sample first dilution rate i.e. 9.43 g of sample diluted in 174.2 g of solvent – 9.43/183.63 = 0.0513

d - volume (in milliliters) of sample used for inoculation of petri plate - 100 µl = 0.1 ml

$$x = \frac{\frac{74 + 69 + 76}{3} * 10^{(-(-4)-1)}}{0.0513 * 0.1}$$

$$x = \frac{73 * 10^3}{0.0513 * 0.1}$$

$$x = \frac{73000}{0.00513}$$

$$x = 14230019.49$$

$$x = 142.3 * 10^5$$

Estimated number of cultivable microorganisms is $142.3 * 10^5$ per one gram of soil. This is than to be converted into soil DW. The final figure is presented as colony forming units” per one gram of dry soil.

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ES 1406

Protocol KEYSOM-12

Determination of enzymatic activity (dehydrogenase)

Overview

The activity of dehydrogenases as a group of intracellular enzymes provides information on the biologically active population of microorganisms in soil and can be used to assess the state of the environment. Determination of soil dehydrogenase activity is based on the measurement of the amount of triphenylformase (TFF) produced from triphenyltetrazolium chloride (TTC). Dehydrogenase activity display in the process of dehydrogenation of the substrate - glucose and transfer of hydrogen to the colourless biologically active compound TTC, which is reduced to TFF with a red colour. The glucose dehydrogenation by dehydrogenase occurs during the incubation of deoxidized samples at a given time, temperature and reaction. The intensity of the colour depends on the amount of formazan produced and is proportional to the enzymatic activity of microorganisms. TFF is extracted from the sample with alcohol.

Field equipment:

See Protocol KEYSOM – 05 Soil DNA

120 ml plastic containers

Laboratory Equipment and materials:

Water bath or incubator (30oC)

Spectrophotometer

Stirrer

Sieve (Ø 2mm)

Scales

50 ml Erlenmeyer flasks

Distilled water

Triphenyl tetrazolium chloride (TTC)

Ethanol, methanol or butanol

Formazan (TTF)

CaCO₃

Quantitative Filter Papers - Grade 390

Soil sampling

See procedure Protocol KEYSOM – 05 Soil DNA. Collect additionally a 100 gram sub-sample of the four mixed bulked soil sample and put into sterile 120 ml plastic container. Store samples at 4°C until the enzyme activity is determined.

Determination of dehydrogenase activity

Use field-moist, sieved (Ø 2mm) soil.

1. Weigh 3 g of soil into a 50 ml Erlenmeyer flask - in triplicate (samples).
2. Weigh 1 g of autoclaved soil into a 50 ml Erlenmeyer flasks, prepare 2 replicates (controls).
3. Add 30 mg CaCO₃; 1 ml of 1% (w/v) aqueous solution of 3% TTC; 1,5 – 3,0 ml of distilled water to each sample.
4. For the controls, 1 ml of distilled water should be added instead of TTC.
5. Mix the content thoroughly and incubate the stoppered flasks at 30°C for 24 h in the dark.

(ATTENTION: approx. 1,5 – 3,0 ml of distilled water should so saturate the soil that a layer of liquid should appear over it, about 2 mm H₂O above the surface of the soil, organic soils even 5mm).

6. After the incubation, add 10 ml of methanol (or butanol, ethanol) to the samples and again, close the flasks. Shake vigorously for 40 minutes. Alcohol will extract the formazan insoluble in water.
7. Filter the contents of the flasks (use hard filter e.g. Filtrak No. 390).
8. Immediately afterwards, measure the absorbance ($\lambda = 490 \text{ nm}$) against methanol. Samples prepared in this way can be stored up to 1 hour.

Enzymatic activity was quantified by reference to a calibration curve constructed with data obtained by incubating TTC standards under the same conditions described above, and was expressed in $\mu\text{gTPF-g-dry soil h}^{-1}$.

The more the colour is red, the higher the activity (the intensity of the red colour increases after some time during extraction, while the greenish colour occurs in more organic soils. Blank test (methanol) - no colour.

Standard curve

1. 100 mg formazan, dissolve in 200 ml of methanol (in a volumetric flask) and heat in a water bath [100 mg/200 ml = 0.5 mg/ml]
2. Place 50 ml into a 500 ml flask and make up to the mark with methanol
 $50 \text{ ml} \times 0.5 \text{ mg/ml} = 25 \text{ mg in } 500 \text{ ml} = 50 \text{ mg in } 1000 \text{ ml} = 0.05 \text{ mg/ml} = 50 \text{ } \mu\text{g/ml}$
3. Subsequently, transfer the determined volumes into a 100 ml flask and make up with methanol:

[mg/ml]

1 ml \rightarrow 0.05 mg = 50 μg	8 $\text{cm}^3 \rightarrow$ 0.4 mg = 400 μg
2 ml \rightarrow 0.1 mg = 100 μg	9 ml \rightarrow 0.45 mg = 450 μg
3 ml \rightarrow 0.15 mg = 150 μg	10 ml \rightarrow 0.5 mg = 500 μg
4 ml \rightarrow 0.2 mg = 200 μg	11 ml \rightarrow 0.55 mg = 550 μg
5 ml \rightarrow 0.25 mg = 250 μg	12 ml \rightarrow 0.6 mg = 600 μg
6 ml \rightarrow 0.3 mg = 300 μg	13 ml \rightarrow 0.65 mg = 650 μg
7 ml \rightarrow 0.35 mg = 350 μg	14 ml \rightarrow 0.7 mg = 700 μg

Then recompose the formazan content to 1 g of absolutely dry soil. Amount of formazan = degree of dehydrogenase activity. Unit of dehydrogenase activity is $\mu\text{gTPF} \cdot \text{g}^{-1} \cdot \text{dry soil h}^{-1}$.

Literature cited

Casida L.E., Klein D.A., and Santoro T., 1964. Soil dehydrogenase activity. Soil Sci., 98, 371-376.

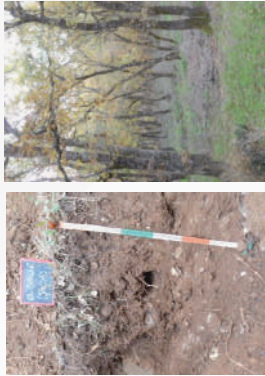
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Protocol prepared by:
Urszula Jankiewicz, Lidia Oktaba

Date and name of last revision:
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Annexes

Annex 1 – KEYSOM soil profile description form



Date	18 November 2016	Orientation	Orientation
Author	Xavier DOMENE, Stefania MATTANA	Weather	PC
Profile nr	R1	Climate	WC 1
Place	El Boalar, Jaca, Spain	Landform (table 4)	LS
Coordinates	N 42°33'15" E 0°37'53"	Slope (Table 6, 7)	5
Land-use	Oak forest	WRB-2014 Classification (optional)	pending (waiting for the lab analysis)
Parent material (geological formation or sediment)	Sandstone and lutite colluvia (Formation Belsué-Batarés)	Depth groundwater at the time of description	2 - 0,5 m

Sample -Nr	Horizon (p. 67)	Depth (cm)	Boundary Table 24)	Color Matrix (Munsell) (moist)	Color Mottles	Mottles Table 3233-34-35)	Field Texture	Carbonates (HCL, Table 38)	Structure Table 47-48)	Rock fragments Table 26-27)	Coatings	Stickiness Table 55)	Plasticity Table 56)	Consistence when moist Table 54)	Pores Table 61, 62, 63)	Roots + bio (Table 79-80)	Bioturbation Table 81-82)	Observations nsA10
	A1	0-8	GS	10YR 3/3			SL	MO	St/G	F//F		nS	sPI	Lo	I//F/M	F//C	C//type E	
	A2	8-17	GS	10YR3/4	10YR3/2	C//F/D/D	SL	ST	Mo//SAB	C//F		sS	PI	Fr	I//F/M	F//C	C//type E	
	A/B	17-33	GS	10YR4/6	10YR3/2	C//F/D/D	SL	EX	Mo//SAB	A//F		sS	vPI	Fr	I//N//C	C//F	F-C//type E-B	
	BC	33-62	AS	10YR4/6			SL	EX	We//SAB	D//F		sS	vPI	vFR	I//N//F	C//F		

Structure AB=angular blocky; SAB subangular bl; G = granular; Pl = platy; Pr = prismatic; Cr = Crumb

Litter layer description (Zanella et al., 2011)

Is there a litter layer present?	Yes	No			
is there an OL layer present (layer characterised by the accumulation of leaves and needles. Although possibly discolored and slightly fragmented, the leaves	Yes	No	Discontinuous		
average thickness (cm)	1				
Is it an OL _n layer (age < 1 year)	Yes	No	Discontinuous		
Is it an OL _v layer (leaves or needles are slightly altered: i.e. slightly discolored, bleached, matted, skeletonized, ...or slightly fragmented)	Yes	No	Discontinuous		
is there an OF layer present (layer characterised by the accumulation of partly decomposed litter, i.e. clearly fragmented and bleached, skeletonized... leaves or needles)	Yes	No	Discontinuous		
average thickness (cm)	-				
Is the material mainly fragmented by soil fauna (OF _{zo} - e.g. Mite pellets visible) or by fungi (OF _{noz} - layer is matted and permeated by hyphae)	OF _{noz}	OF _{zo}	intermediate		
is there an OH layer present (layer characterised by black, grey-brown or reddish-brown, well decomposed litter. Individual leaves or needles are no longer visible). Note that this layer should still be ABOVE the surface of the mineral soil	Yes	No	Discontinuous		
average thickness (cm)					
Dig a micro-pit ca 20 cm deep (or do observations in the profile pit if available) and note the transition of the litter layer to the mineral soil. Is this transition	very sharp (<3mm)	sharp (<5mm)	not sharp (>5mm)		
what is the structure of the mineral A horizon? (Table 47-50 of the FAO guidelines for profile description)	Grade: St	Type: G	Size: VF		
How is the transition between the mineral A horizon and the underlying soil (E or B horizon)? (Table 24)	Distinctness: D	Topography: B			
Are there any droppings of earthworms identifiable in the A horizon?	Yes	No	I don't know		
Are there any droppings of other soil fauna (enchytraeids and arthropods) identifiable in the A-horizon?	Yes	No	I don't know		
Zanella classification (overview on p. 24-25-26; optional)	MULL	AMPHI	MODER	MOR	TANGEL
	Eumull	leptoamphi, eumacroamphi, eumesoamphi, pachyamphi	hihemimoder, eumoder, dysmoder	hemior, humimor, eumor	eutangel, dystangel

Annex 2– KEYSOM common field experiment

Information on glass micro-beads

PRINCIPLE

Micro-beads (about 300–355 μ m diameter) made from clear glass will be used as a new physical marker to estimate bioturbation. A known mass of beads is applied to the sand surface of cores at the start of the experiment, and beads will be retrieved and counted from sand layers at the end of the experiment.

MATERIALS PROVIDED

Dry, clean beads are provided in very small batches (0.5 g each) in Eppendorf tubes, ready for application to individual cores in the field. No weighing, dividing or any other preparatory step is required.

FIELD APPLICATION

Apply the content of one Eppendorf tube (0.5 g) to one core. Use a system that ensures that each of the 10 cores at a site receives one batch of beads.

Apply to the surface of the sand after filling / packing each core with sand, but before local litter is replaced. Sprinkle the beads carefully and slowly and **as evenly as possible** over the sand surface. Note that the dry beads flow freely and fast! Try to tap the tube very gently. Replace the surface litter afterwards.

The empty tubes are for disposal (non-hazardous).

CONTACT FOR QUERIES

Olaf Schmidt [←olaf.schmidt@ucd.ie→](mailto:olaf.schmidt@ucd.ie)

List of contributors* and affiliations

Prof. Sébastien Barot, Directeur de Recherche IRD, IEES-P, Sorbonne Université, 4 place Jussieu, 75252 Paris, FRANCE.
Email: sebastien.barot@ird.fr

Prof. Matty Berg, Vrije Universiteit Amsterdam, Department of Ecological Science, 1081 HV Amsterdam, THE NETHERLANDS.

Email: m.p.berg@vu.nl

Prof. M.J.I. Briones, Dept. Ecología y Biología Animal, Facultad de Biología, Universidad de Vigo, 36310 Vigo, SPAIN.

Email: mbriones@uvigo.es

*Dr. Guillermo Bueno, Institute of Ecology and Earth Sciences, Department of Botany, University of Tartu, Tartu, ESTONIA.

Email: cgbuengog@gmail.com

Dr. Jorge Curiel Yuste, BC3-Basque Centre for Climate Change, Scientific Campus of the University of the Basque Country, 48940 Leioa, SPAIN; Email: jorge.curriel@bc3research.org

*Prof. Oleg Chertov, University of Applied Sciences Bingen, Berlinstr. 109, 55411 Bingen, GERMANY.

Email: oleg_chertov@hotmail.com

*Prof. Thibaud Decaëns, Université de Montpellier, Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175 CNRS, Montpellier, France.

Email:

thibaud.DECAENS@cefe.cnrs.fr

Dr. Gaby Deckmyn, Department of Biology, University of Antwerp, 2610 Wilrijk, BELGIUM.

Email:

gaby.deckmyn@uantwerpen.be

Dr. Xavier Domene, Ecology Unit, Faculty of Science and Biosciences, Autonomous University of Barcelona, SPAIN.

Email: x.domene@creaf.uab.es

Dr. Jack Faber, Wageningen Environmental Research (Alterra), 6700 AA Wageningen, THE NETHERLANDS.

Email: jack.faber@wur.nl

Prof. Juliane Filser, University of Bremen, UFT, Department of General and Theoretical Ecology, D-28359 Bremen, GERMANY.

Email: filser@uni-bremen.de

*Omar Flores, Department of Biogeography and Global Change, National Museum of Natural Sciences, CSIC, E-28006. SPAIN. Email: omarf@mncn.csic.es

*Dr. Oscar Franken, Dept. of Animal Ecology, Vrije Universiteit Amsterdam, THE NETHERLANDS. Email: oscarfranken@gmail.com

Dr. Beat Frey, Swiss Federal Institute for Forest, Snow and Landscape Research (WSL, ETH domain), 8903 Birmensdorf, SWITZERLAND.

Email: beat.frey@wsl.ch

Dr. Aline Frossard, Swiss Federal Institute for Forest, Snow and Landscape Research (WSL, ETH domain), 8903 Birmensdorf, SWITZERLAND.

Email: aline.frossard@wsl.ch

Dr. Jan Frouz, Institute for Environmental Studies, Charles University in Prague, Faculty of Science, Praha, CZECH REPUBLIC.

Email: frouz@natur.cuni.cz

Prof. Geog Guggenberger, Institute of Soil Science, Leibniz Universität Hannover, GERMANY.

Email: guggenberger@ifbk.uni-hannover.de

Dr. Davorka Hackenberger, Department of Biology, J. J. Strossmayer University of Osijek, Cara Hadrijana 8A, HR-31000 Osijek, CROATIA. Email: davorka@biologija.unios.hr

*Dr. Stefan Hattenschwiler, Department of Functional Ecology, Centre d'Ecologie Fonctionnelle et Evolutive, Montpellier (CEFE), FRANCE.

Email: stephan.hattenschwiler@cefe.cnrs.fr

*Dr. Petr Hedenec, Department of Geosciences and Natural Resource Management, Faculty of Science, University of Copenhagen, 1958 Frederiksberg C, DENMARK.

Email: peh@ign.ku.dk

Dr. Maria Iamandei, Institute of Plant Protection Research and Development, ROMANIA.

Email: maria_iamandei@yahoo.com

Dr. Mari Ivask, Tallinn University of Technology, School of Engineering, Tartu College, ESTONIA.

Email: mari.ivask@ttu.ee

Dr. Juan J. Jiménez, ARAID, Department of Biodiversity Conservation and Ecosystem Restoration, Pyrenean Institute of Ecology, CSIC, Jaca, SPAIN.

Email: jjimenez@ipe.csic.es

Dr. David Jones, School of Environment, Natural Resources & Geography, Bangor University, Environment Centre Wales, UNITED KINGDOM.

Email: d.jones@bangor.ac.uk

Dr. M. Joschko, Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, GERMANY. Email: mjoschko@zalf.de

Dr. Paul H. Krogh, Department of Bioscience, Science and Technology, Aarhus University, DENMARK.

Email: phk@bios.au.dk

Prof. P. Lavelle, Centre IRD Ile de France, UMR BIOEMCO 7618, F-93143, Bondy, FRANCE.

Email: patrick.lavelle@ird.fr

*Dr. Lorenzo Menichetti, Ecosystem Modelling, Science Department, SLU, SWEDEN.

Email: Lorenzo.Menichetti@slu.se

Dr. Juha Mikola, Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Lahti, FINLAND.

Email: juha.mikola@helsinki.fi

*Dr. Lena Neuenkamp, Plant Ecology Lab., Department of Botany, University of Tartu, ESTONIA.

Email: lena.neuenkamp@ut.ee

Dr. Visa Nuutinen, Natural Resources Institute Finland (Luke), FI-00790 Helsinki, FINLAND.

Email: visa.nuutinen@luke.fi

Dr. Lidia Oktaba, Warsaw University of Life Sciences – SGGW, Faculty of Agriculture and Biology, Department of Soil Environment Sciences 02-776, Varsovia, POLAND.

Email: lidia_oktaba@sggw.pl

Prof. Maarja Õpik, Department of Botany, Institute of Ecology and Earth Sciences, University of Tartu, 40 Lai Str., 51005 Tartu, ESTONIA; Email: maarja.opik@ut.ee

*Dr. Alberto Orgiazzi, European Commission, JRC, I-21027 Ispra, ITALY. Email: alberto.orgiazzi@ec.europa.eu

Dr. Pascal Querner, University of Natural Resources and Life Sciences, Department of Integrated Biology and Biodiversity, Research, Institute of Zoology, 1180, Vienna, AUSTRIA. Email: pascal.querer@boku.ac.at

Dr. Patrizia Ricciuti, Department of Soil, Plant and Food Sciences, (DiSSPA), Università degli Studi di Bari 'Aldo Moro', 70126 Bari, ITALY. E-mail: patrizia.ricciuti@uniba.it

*Dr. David J. Russell, Dept. Soil Zoology; Section Mesofauna, Senckenberg Museum of Natural History Görlitz, GERMANY.

Email: david.russell@senckenberg.de

Dr. Lidia Sas Paszt, Rhizosphere Laboratory, Research Institute of Horticulture, 96-100 Skierniewice, POLAND. Email: lidia.sas@inhort.pl

Dr. Olaf Schmidt, University College Dublin, School of Agriculture and Food Science, Agriculture and Food Science Belfield Dublin, IRELAND. Email: olaf.schmidt@ucd.ie

Dr. Adriano Sofo, Department of European and Mediterranean Cultures: Architecture, Environment, Cultural Heritage (DiCEM), Università degli Studi della Basilicata, 75100 Matera, ITALY.

Email: adriano.sofo@unibas.it

Prof. J.-P. Sousa, Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Coimbra, PORTUGAL.

Email: jps@zoo.uc.pt

Dr. Alexei Tiunov, Laboratory of Soil Zoology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninsky pr. 33 119071 Moscow, RUSSIA.

Email: a_tiunov@mail.ru

Dr. Adam O'Toole, NIBIO – Norwegian Institute for Bioeconomy Research, Department of Soil Quality and Climate, Division for Environment and Natural Resources, NORWAY.

Email: adam.otoole@nibio.no

*Dr. Saoirse Tracy, University College Dublin, Agriculture and Food Science Centre, Belfield Campus, IRELAND. Email: saoirse.tracy@ucd.ie

Dr. Karen Vancampenhout, KU Leuven, Campus Geel, Cluster for BioEngineering Technology, 2440 Geel, BELGIUM.

Email:

karen.vancampenhout@ees.kuleuven.be

Dr. E. Velásquez, Universidad Nacional de Colombia, Carrera 32 Chapinero, vía Candelaria, Palmira, COLOMBIA.

Email: evelasquezi@unal.edu.co

*Prof. Diana H. Wall, School of Global Environmental Sustainability & Dept. Biology, Colorado State University, Fort Collins, CO 80523-1036, USA.

Email: Diana.Wall@colostate.edu

Dr. Anne Zangerlé, Administration des services techniques de l'agriculture, Service agri-environnement, BP 1904, L-1470, LUXEMBOURG.

Email: anne.zangerle@asta.etat.lu

Dr. Chaoseng Zhang, School of Geography and Archaeology & Ryan Institute, National University of Ireland, Galway, IRELAND.

Email:

chaosheng.zhang@nuigalway.ie.

* Here we include also those invited speakers who attended one of the three Training Courses held during the lifetime of this Action.



COST Association

Avenue Louise 149 | 1050 Brussels, Belgium

T +32 (0)2 533 3800 | F +32 (0)2 533 3890

office@cost.eu

www.cost.eu



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