COST Action ES1406

Soil fauna: key to soil organic matter dynamics and modelling

HANDBOOK OF METHODS

Version 1

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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.

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KEYSOM protocols

In the study of soil fauna – SOM relationships the protocols used for field sampling, lab analysis, and experimentation are central.

Here we describe the protocols that were used in a common field experiment (Figure 1) performed along a gradient of climate in Europe during the lifetime of the COST Action ES1406. The results of such experimentation are being analysed for several forthcoming publications in different specialized documents and journals, including open access journals.

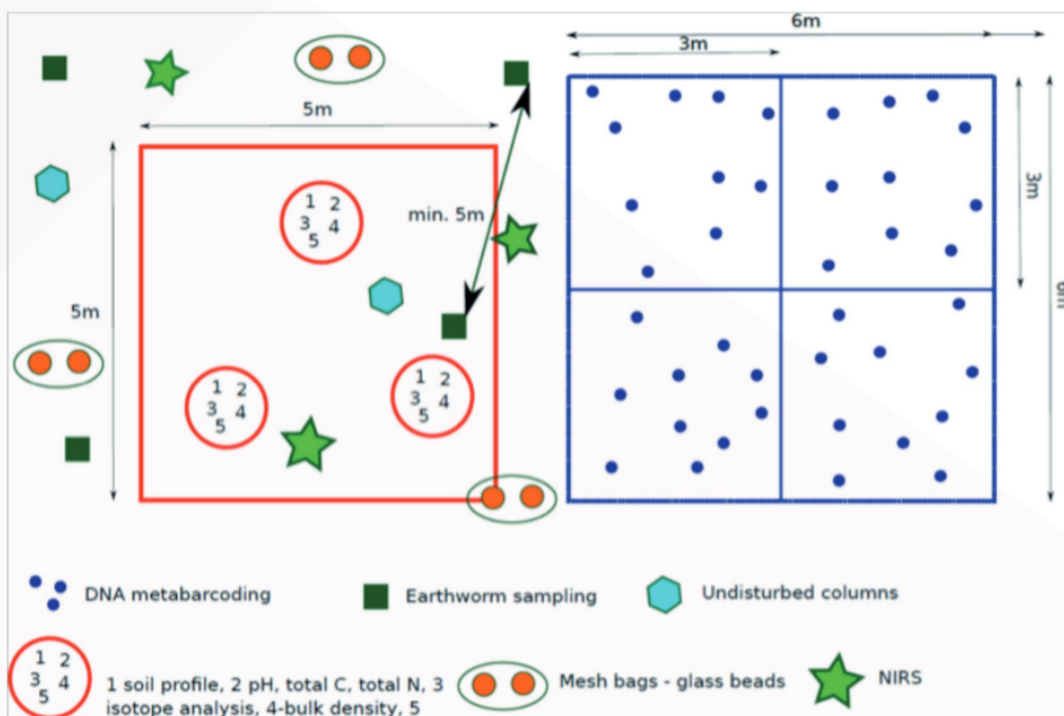


Figure 1. Sampling scheme used in the common experimentation protocol.

KEYSOM common experimentation protocols:

1. Protocol KEYSOM-01. Soil aggregation.

2. Protocol KEYSOM-02. Sampling and preserving earthworms and macrofauna bycatch.

3. Protocol KEYSOM-03. Stable isotope composition (bulk δ13C and δ15N) in soil profile.

4. Protocol KEYSOM-04. Soil sampling for Near Infrared Spectroscopy (NIRS) readings.

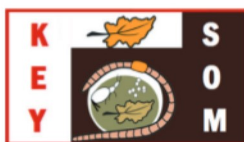
5. Protocol KEYSOM-05. Soil sampling and analysis for eDNA.

6. Protocol KEYSOM-06. Soil profile and humus form description and sampling field protocol.

7. Protocol KEYSOM-07. Sampling of undisturbed soil cores for X-ray cross-tomography.

8. Protocol KEYSOM-08. Bioturbation mesh bag experiment I.

9. Protocol KEYSOM-09. Bioturbation mesh bag experiment II. Information on glass micro-beads.



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}, CaCl2 0.045 g l^{-1} and $NaHCO_3$ 0.05 g l^{-1})
- 1.8% (w/v) sterile sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$, 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 ReaderTM 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 (OD_{590nm}) and 750 nm (OD_{750nm}) 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 OD750nm value from the OD590nm value:

0 h: $i_{0h} = OD_{590nm} - OD_{750nm}$ **and** $b_{0h} = OD_{590\,nm} - OD_{750nm}$

24 h: $i_{24h} = OD_{590nm} - OD_{750nm}$ **and** $b_{24h} = OD_{590\,nm} - OD_{750nm}$

- subtract the blank well OD reading from the OD value of each substrate well to obtain a blank-corrected value (ibc) for each well:
  - **0 h:** $i_{bc0h} = i_{0h} - b_{0h}$
  - **24 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 (ci) for each well for each incubation time: **e.g.** $c_{i24h} = i_{bc24h} - i_{bc0h}$, 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 → 0.25

Utilizing the AWCD and ci values

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

Using the ci 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 ci 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 ci values across all utilized substrates,

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

where p_i is ci 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.

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

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