



Food and Agriculture
Organization of the
United Nations

Standard operating procedure for soil microbial biomass (carbon)

*chloroform fumigation-extraction
method*

A decorative graphic consisting of a series of colored dots and squares arranged in a semi-circular arc, transitioning from dark brown to light yellow.

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Microbial biomass carbon chloroform fumigation-extraction method

VERSION HISTORY

N°	Date	Description of the modification	Type of modification
01	02 January 2024	All comments by RESOLANs and reviewers to the draft SOP were addressed	Finalization of the SOP
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1. Brief introduction to soil microbes

Microbial biomass is considered as an estimation of soil biological activities and the capacity to mediate soil biochemical reactions. It is the most dynamic and labile of soil organic matter fractions, generally accounting for 1 to 5 percent of soil organic matter and very sensitive to soil management. The need to quantify soil microbiota has become increasingly relevant in current times as they are responsible for many different processes like the degradation of organic matter, the stability of aggregates and most of the nutrient cycling that occurs in soils.

Determining the microbial biomass of soils is important so that soil quality, fertility and the potential ability to degrade added organic materials can be assessed. Moreover, it is essential to know the amount of microbial population so that the correct amount of organic amendments can be applied and the impact of anthropogenic activity on soil biota can be assessed (ISO, 1995). Soil microbial biomass is also used as one of the alternative indicators in the *Protocol for the assessment of Sustainable Soil Management* (FAO-ITPS, 2020).

2. Scope and field of application

Chloroform fumigation-extraction is used for the estimation of soil microbial biomass by the measurement of extractable organic biomass in soil. This method is applicable to both aerobic and anaerobic conditions over the whole range of soil pH, regardless of land use type. While chloroform fumigation also affects soil fauna, the carbon aliquot derived from these organisms is generally small (less than 5 percent) and can usually be disregarded.

3. Principle

Through the fumigation of the soil sample (using chloroform and for 24 hours), intact microbial cells are lysed and the microbial organic matter is released. The organic carbon is extracted using 0.5 mol/L potassium sulphate and the amount is determined in both fumigated and unfumigated samples, with the difference in extracted organic carbon being used to determine microbial biomass carbon. The method is usually termed fumigation-extraction (FE).

4. Apparatus

The apparatus needed for the method are as follows:

- an incubator, capable of being maintained at 25 °C ± 2 °C;
- a desiccator;
- filter paper (Whatman No. 42 [or similar]);

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- glass beakers;
- petri dishes;
- polyethylene bottles (250 mL);
- a vacuum line;
- an overhead shaker;
- a freezer, operating at -15 to -20 °C;
- anti-bumping granules;
- a 250 mL round-bottomed flask;
- a Liebig condenser;
- a 10 mL burette; and
- a 2 mL pipette.

5. Materials

The materials needed for the method are as follows:

- silicone grease;
- ethanol-free chloroform;
- potassium sulphate solution ($c[\text{K}_2\text{SO}_4] = 0.5 \text{ mol/L}$ [$p = 87.135 \text{ g/L}$]);
- soda lime;
- potassium dichromate ($c[\text{K}_2\text{Cr}_2\text{O}_7] = 0.0667 \text{ mol/L}$) (19.6125 g dry potassium dichromate/litre of water);
- phosphoric acid ($[\text{H}_3\text{PO}_4]$ [$p = 1.71 \text{ g m/L}$]);
- sulphuric acid ($[\text{H}_2\text{SO}_4]$ [$p = 1.84 \text{ g m/L}$]);
- iron(II) ammonium sulphate, titration solution ($c[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}] = 0.040 \text{ mol/L}$);
- 1, 10 phenanthroline sulphate complex solution (0.025 mol/L); and
- acid mixture (two volumes sulphuric acid mixed with one volume phosphoric acid).

6. Health and safety

Safety glasses, gloves and lab coats must be worn when performing this analysis. The unknown potential hazards from the soil chemical and biological hazards as well as toxicities from chemicals used in the microbial biomass determination should be kept in mind throughout the procedure. Therefore, it is advised to handle the chloroform fumigation inside a fume hood. Be careful when acids or acid mixtures are used even when they are not considered hazardous, as repeated contact with the skin can cause dryness or cracks. Similarly, accidental contact with the eyes can lead to redness and or swelling. The wearing of a mask particularly during sample preparation and chloroform fumigation should also be encouraged, as well as during sample disposal and cleaning.

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7. Sample preparation

The soil's water content must be higher than 30 percent to ensure uniform chloroform distribution. Dry soils (or dried and stored soils) should be remoistened over a recommended period of at least ten days of preincubation at 25 °C and 40–50 percent water-holding capacity (Franzluebbers *et al.*, 1996). If the soil's moisture is high, sample must be oven dried at 40 °C until a water-holding capacity of ≈ 40 percent is reached. The sample must also be sieved to a particle size of 2 mm.

8. Procedure

The analytical procedure of the method can be divided in three main steps:

1. Fumigation

- a. Weigh 50 g of each sample by duplicate into two glass beakers.
- b. Place them in a desiccator, with a beaker containing 50 mL of ethanol-free chloroform with ten anti-bumping granules, as well as a beaker with 5 g of soda lime. Connect the desiccators to a vacuum pump to start the fumigation until the CHCl₃ starts boiling and let them boil for two minutes.
- c. Close the desiccator tap and incubate at 25 °C for 24 hours.
- d. After fumigation, remove the beaker with the chloroform and remove the chloroform vapour from the soil (with five to seven repeated vacuum extractions). The samples are now ready for extraction.

For control, place three non-fumigated control samples (with the same mass as the fumigated samples) in polyethylene bottles with 200 mL of potassium sulphate.

2. Extraction

- a. Transfer 30 g of each sample (both fumigated samples and non-fumigated control samples) into separate polyethylene bottles, adding 200 mL of potassium sulphate to each sample, and shake in an overhead shaker at 200 rpm for 30 minutes.
- b. Filter the extracts through filter paper.

If not analysed immediately, store samples in a freezer at a temperature between –15 °C and –20 °C.

3. Determination of organic carbon in the extracts

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- a. Add 2 mL of potassium dichromate solution and 15 mL of the acid mixture to an aliquot of 8 mL of the extract, in 250 mL volumetric flasks.
- b. Gently shake the mixture for 30 minutes and dilute with 20 mL of water.
- c. Digest duplicate blanks (refluxed blanks) containing 8 mL of potassium sulphate solution in the same way.
- d. Titrate with iron ammonium sulphate solution, using three or four drops of 1, 10-phenanthroline-iron (II) sulphate complex as indicator.

9. Calculation

Microbial biomass is calculated as shown in the following equations:

$$C = \left[\frac{(V_H - V_S)}{V_C} \right] \times M \times P_D \times E \times \frac{1000}{P_S}$$

where:

C = the extractable organic carbon in $\mu\text{g/mL}$;

V_H = the volume titrant consumed by the refluxed blank (mL);

V_S = the volume titrant consumed by the sample (mL);

V_C = the volume titrant consumed by the unrefluxed blank (mL);

M = c($\text{K}_2\text{Cr}_2\text{O}_7$) in moles/L;

P_D = the added volume of $\text{K}_2\text{Cr}_2\text{O}_7$ solution in mL;

E = conversion of organic C to CO_2 ; and

P_S = the added volume of sample, in mL.

$$C \left(\frac{\mu\text{g}}{\text{g dry soil}} \right) = C \left(\frac{\mu\text{g}}{\text{ml}} \right) \times \left(\frac{P_k}{D_w + S_w} \right)$$

where:

P_k = the mass of extractant in g;

D_w = the dry mass of sample in g; and

S_w = the soil water (g of water/g of dry soil).

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Finally, the microbial biomass carbon (B_c) is calculated as:

$$B_c = \left(\frac{E_c}{k_{EC}} \right)$$

where:

E_c = the difference between the mass of organic C extracted from fumigated soils and the mass of organic C extracted from unfumigated soils; and

K_{EC} = efficiency coefficient of the extraction = 0.38 (since there is no global consensus on the value of this coefficient, please always indicate the used value).

Results should be reported in $\mu\text{g/g}$ soil, to two decimal places.

10. Quality assurance and quality control

10.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 2. If not, the root cause should be identified, and corrective and preventive actions developed.

10.2. Precision test

A replicate analysis should be performed every 10 to 20 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2} \right)} \times 100\%$$

where:

M_1 = result of sample; and

M_2 = result of sample's duplicate.

10.3. Laboratory control sample

The measurement of a sufficiently available sample and of a known moisture content value of can be analysed per batch of analysis, to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference sample or master sample.

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10.4. Control chart

A chart needs to be produced to register the RPD values of each batch test. The chart should include the limit RPD value established by the laboratory (a maximum 15 percent of RPD is recommended).

A chart for the laboratory control sample can also be used as quality control, with the limit values or a limit RPD if a duplicate of this sample is also evaluated per batch of analysis.

10.5. Calibration

Instrument calibration should be verified on a regular basis.

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Appendix I

GLOSOLAN would like to thank Nicolas Yusep Bulla Marin and Diana Maria Delgado Londoño from Colombia for leading the harmonization of this SOP and the members of the working group that served as leaders for their regions and contributed to the writing of this SOP (Appendix II).

GLOSOLAN would also like to thank the experts who were part of the review panel and the members of the GLOSOLAN technical committee who ensured the finalization of the SOP (Appendix II), and all the laboratories that provided inputs for the harmonization of this method (Appendix III).

GLOSOLAN would also like to thank all the experts from the International Network on Soil Biodiversity (NETSOB) for their kind support in the review of this SOP.

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- Marisa Phiomtan De Bels, Loei Rajabhat University, **Thailand**

Appendix III– Contributing laboratories

GLOSOLAN would like to thank the following laboratories for completing the GLOSOLAN form on the method and providing information on their SOP for microbial biomass carbon by chloroform fumigation-extraction. This information was used as a baseline for the global harmonization.

From the African region:

- Soil Research Institute Analytical Services Laboratory, **Ghana**
- National Semi Arid Resources Research Institute (NaSARRI), **Uganda**

From the Asian region:

- Soil Resource Development Institute, **Bangladesh**
- Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences (CAAS), **China**
- Division of Soil Biotechnology - Land Development Department, **Thailand**

From the European region:

- Central Institute for Supervising and Testing in Agriculture, **Czechia**
- The Institute of Microbiology and Biotechnology, **Republic of Moldova**
- Soil and Fertilizer Research Laboratory (SOFLER-TR), **Türkiye**

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From the Latin American region:

- Dirección de Laboratorio e Innovación Ambiental - CAR, **Colombia**

From the Near East and North African (NENA) region:

- KIMIA AB environmental and agricultural consulting laboratory, **Islamic Republic of Iran**

From the Pacific region:

- Fiji Agricultural Chemistry Laboratory, **Fiji**
- Landcare Research/ Palmerston North, **New Zealand**
- The University of the South Pacific, **Samoa**

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