Annex 1:

NITROGEN MINERALIZATION PROTOCOL

by Ghent University

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1. Introduction

This report contains description of the protocol on how to assess nitrogen mineralization from organic materials. The described protocol is the official protocol used by research institutes in Flanders, and in this report they are translated in English from their original source.

The nitrogen mineralization protocol is provided by VITO (Flemish institute for technological research) and titled as *VITO, 2010. Bodem-Bepaling van snel vrijkomende organische stikstof.*

In the protocol attempt is made to mimic field conditions as much as possible. Therefore, tested materials are always applied in fresh form, as it is done in practice by farmers. The incubation temperature in nitrogen protocol respresents the average soil temperature in Flanders.

2. Nitrogen mineralization protocol

2.1.1. Principle

The organic material to be assessed is incubated in a reference soil under controlled conditions of temperature, moisture content and density. At regular intervals, samples are taken to determine the amount of mineral N in the soil. The mineralization (possibly immobilization) of N from the organic material is then calculated based on the mineral N data obtained in the time series.

2.1.2. Method

2.1.2.1. Pre-treatment of soil

The reference soil[1] is air dried and sieved through a 2 mm screen. The soil is then placed in a container, stacked to a density of 1.4 Mg m^{-3} and wetted to a moisture content of 35% of water filled pore volume[2]. This mixture is allowed to incubate for one week at a temperature of 15 ± 2 °C.

2.1.2.2. Organic matter addition

After the pre-incubation, the organic material to be assessed is then administered to the soil. The organic material must be in fresh condition, as will be used in practice. Ideally, the material should consist of mostly fine particles and well homogenized. Coarse organic materials (e.g. plants), must be finely chopped into particles with a size of 0.25 to 0.5 cm^2.

The desired, predetermined amount of the organic material is then intensively mixed with a known amount of reference soil (enough to fill one incubation container). The

[1] The reference soil has a content of initially present mineral N <20 mg $N\text{-}NO_3^-$/kg and a low mineralization potential, so that the mineralization from the added organic material is easy to follow. The texture of the reference soil must be loamy sand, light sandy loam, sandy loam or loam with a pH-KCl between 5 and 7.5, and an organic carbon content of less than 1.5%.

[2] The pore volume is calculated as 1 - (dry soil density / 2.65). In this case 1 - (1.4 / 2.65) = 47.2%. A moisture content of 35% with water filled pore volume corresponds to 16.5 volume % moisture per volume unit of dry soil. Taking into account the density of dry soil, this is 118 ml of water add to 1 kg of dry soil.

application rate as used in practice typically forms the guideline for determining the amount of organic material to be administered.

2.1.2.3. Incubation

PVC cylinders with a length of 0.18 m and inside diameter of 0.046 m and sealed bottoms are used as incubation containers. The incubation containers are filled to a height of 10 cm with the mixture of the organic material and soil. The bulk density of the soil in the container is brought to a predetermined value by pressing the mixture. This pressing must be done evenly during the filling of the container, so that one homogeneous density is obtained over the entire length of the container. Care must be taken to ensure that the soil surface is not compacted when adjusting the density, since that can negatively influence mineralization.

For a filling height of 10 cm and a bulk density of 1.4 Mg m^{-3}, approximately 233 g of dry reference soil can be weighed in the container. The moisture content of the soil after the pre-incubation must, of course, be considered before weighing the soil. After filling, the moisture content of the mixture in the incubation containers is adjusted to 50% of water filled pore volume, taking into account the moisture content that is already present in the organic material and in the soil. The containers are then closed with a layer of parafilm, which minimizes the moisture losses during the incubation. A few pin holes are made on the parafilm to ensure gas exchange. The weight of the filled and sealed containers is then determined. This weight is noted and monitored regularly during the incubation to see if there any excessive moisture losses occur.

Decreases in the moisture content with more than 1% (absolute) in the course of the incubation can be adjusted by adding distilled water. The prepared PVC tubes, with a mixture of soil and material, are incubated at a constant temperature of 15 °C. Control samples are also incubated with only the soil, i.e. without added organic material, which is necessary to determine the net N mineralization (= blank samples). The total duration of the incubation is 4 months.

2.1.2.4. Sampling

Nine samples are advised to be taken during the incubation. This number is needed to overcome the strong variability that is inevitable when working with fresh organic

material. At predetermined times, a number of incubation containers are sampled (each time containers with and without added organic material) for a destructive determination of the mineral N content in the soil. Sampling is done on day 0 and then every 15 days until the end of the incubation (day 15, 30, 45, 60, 75, 90, 105, and 120). The sampling is done at least in triplicate, i.e. at least three containers with and three containers without added organic material must be analyzed per sampling time.

2.1.2.5. Analysis

Based on the recommended dose of the organic material expressed in tonnes per hectare, a mass to area ratio equivalent is calculated to determine the application rate for the incubation. The moisture content is determined both in the reference soil and in the sample in order to adjust the moisture content in the incubation container to 50% of the water filled pore volume.

A total N analysis is first performed on the sample and this forms the basis for determining the added quantity of sample per incubation container. At the start of the incubation, at 7 intermediate times, and after 4 months, an analysis of mineral N (NO_3^--N + NO_2^--N + NH_4^+-N) in the mixtures and in the control is performed. To do this, the incubation containers are emptied, and their contents are intensively mixed. Then a fresh sub-sample (30 grams) is immediately extracted with a KCl solution (150 ml, 1 M) for the determination of the amount of mineral N (NO_3^--N + NO_2^--N + NH_4^+-N). Another sub-sample (30 grams) is used to determine the moisture content of the soil (drying at 105 °C to constant weight).

The following data points can be calculated on the basis of the analyzed extracts at any sampling time:

a. [NO_3, soil]: nitrate content in reference soil expressed in mg N-NO_3^- per kg soil
b. [NO_4, soil]: ammonium content in reference soil expressed in mg N-NH_4^+ per kg soil
c. [NO_3, mixture]: nitrate content in mixture expressed in mg N-NO_3^- per kg soil
d. [NO_4, mixture]: ammonium content in mixture expressed in mg N-NH_4^+ per kg soil

A summary of the analyses to be performed are as follows:

Before incubation

a. Total-N in samples
b. Mineral-N in samples and soil
c. Moisture content in sample and soil (before and after pre-incubation)

During incubation (9 times)

a. 54 extractions with KCl
b. 54 determinations of NO_3^--N + NO_2^--N
c. 54 determinations of NH_4^+-N
d. 9 determinations of the moisture content

2.1.3. Calculations

Both the net N release ($N_{rel,net}$) from the added products and the net N mineralization ($N_{min,net}$) are calculated. The Nrel,net is the difference between the mineral N measured in the amended soil minus the mineral N measured in the blank (i.e. unamended soil), calculated according to De Neve & Hofman (1996), as follows:

$$N_{rel,net}(\%) = \frac{([NO_3^- - N, treatment] - [NO_3^- - N, control]) + ([NH_4^+ - N, treatment] - [NH_4^+ - N, control])}{N_{total} applied} \times 100$$

At t = 0, the $N_{rel,net}$ (%) equals the product $N_{mineral}/N_{total}$ ratio x 100. $N_{min,net}$ (%) is the N mineralized from the organic fraction of the product (expressed as a percentage of total N in the product), and is calculated by subtracting the amount of mineral N already present in the products at t = 0, as follows (Sigurnjak et al., 2017):

$N_{min,net}$(t; %total N) = $N_{rel;net}$(t) – $N_{rel;net}$(t = 0)

$N_{min,net}$ can also be expressed as percentage of organic N added.

A positive $N_{min,net}$ value indicates net mineralization, whereas a negative $N_{min,net}$ value indicates net N immobilization.

3. Appendix (N protocol in Dutch)

Available at: https://esites.vito.be/sites/reflabos/2010/Online%20documenten/BAM_deel1_12.pdf