TECHNICAL SPECIFICATION

ISO/TS 20131-1

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Soil quality — Easy laboratory assessments of soil denitrification, a process source of N_2O emissions —

Part 1: Soil denitrifying enzymes activities

Qualité du sol — Essais simples de laboratoire de caractérisation de la dénitrification dans les sols, un processus source d'émission de N_2O

Partie 1: Activités des enzymes dénitrifiantes du sol

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality,* Subcommittee SC 4, *Biological characterization*.

A list of all parts in the ISO/TS 20131 series can be found on the ISO website.

Introduction

The ISO/TS 20131 series presents some easy laboratory assessments of soil denitrification, denitrification being a process source of N_2O emissions.

— **Scientific context**

Denitrification is the main process of nitrogen returning to the atmosphere. This process corresponds to the reduction of nitrate to nitrite and then to gaseous form, successively nitric oxide, nitrous oxide and dinitrogen. Soils (natural and anthropic) are an important source for denitrification and nitrous oxide emissions. Generally, soil denitrification involves a microbial catalysis. Denitrification is a microbial process where nitrogen oxides act as acceptor of electrons during anaerobic respiration. Each step of the denitrification process is catalysed by a specific enzyme. Denitrification is known as a process linking the nitrogen and carbon cycles. During the denitrification process, soil organic compounds may act as the donors of electrons. See Figure 1.

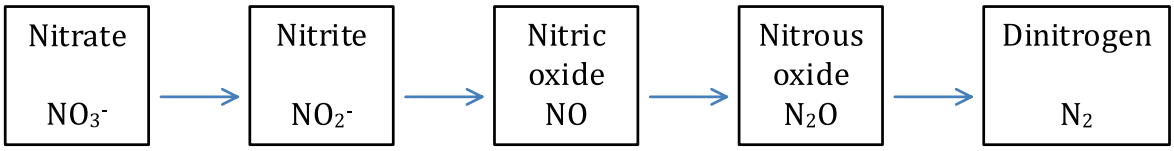

Figure 1 — Description of the denitrification process

There are different concerns in studying the denitrification process in soil at the field scale: understanding the nitrogen cycle for limiting loss of nitrogen for agricultural production, understanding the fate of contaminants of water like nitrate and nitrite, understanding the production and the fate of atmospheric pollutants like NO and N_2O. Knowledge on denitrification in soils is also necessary for global approach of the biogeochemical cycles and of global changes. Denitrification also constitutes an interesting model for microbial ecology.

The gaseous form nitrous oxide (N_2O), mainly produced during the denitrification process, is a greenhouse gas with a high radiative forcing per unit mass or molecule, estimated to 296 fold higher than this of carbon dioxide (CO_2) on a 100 years period[1]. Nitrous oxide is also involved in ozone depletion[2]. N_2O concentrations have risen from a pre-industrial value of 270 ppb to a 2016 value of 328 ppb. At the global scale, nitrous oxide is estimated to contribute to 6 % of the radiative forcing. Agricultural and natural soils appear as the main source of this greenhouse gas[3].

Soils act as both sources and sinks of N_2O. However on the global scale, the N_2O emissions dominate the sink activity. The production and consumption of N_2O in soils mainly involve biotic processes. Numerous groups of microorganisms contribute to the production and consumption of N_2O, but biological denitrification is considered as the dominant processes involved. Only the last step of denitrification is recognized as a significant biological consumptive fate for N_2O. It involves the N_2O reductase enzyme activity that is inhibited by an elevated acetylene partial pressure[4].

— **Methodological context**

Direct measurements of denitrification in soils are expensive, time-consuming, labour intensive because of the immediate dilution of the N_2 produced in the atmosphere and because of high levels of spatial and temporal variability. So far, easy laboratory experiments, even if they are not sufficient for understanding *in situ* denitrification, could be useful for best understanding soil denitrification and assessing soil nitrous oxide emissions. To find some generic use of the results of these laboratory tests, it appears essential to perform them in strictly standardized conditions.

The ISO/TS 20131 series includes two tests that had previously been published in peer reviewed journals and that are accessible to most research and analytical laboratories involved in soil sciences. As they are both performed on sieved soils, they are quite easy to be done and can be used for a wide range of soils.

The first part of the ISO/TS 20131 series (this document) presents a generic method for assessing denitrifying enzyme activities in soils[5]. It globally characterizes the transformation of the nitrate form to the nitrous oxide and dinitrogen forms. This method was first proposed by Reference [5] with the acronym DEA for Denitrifying Enzyme Activities. It mainly focuses on the black arrow of Figure 2.

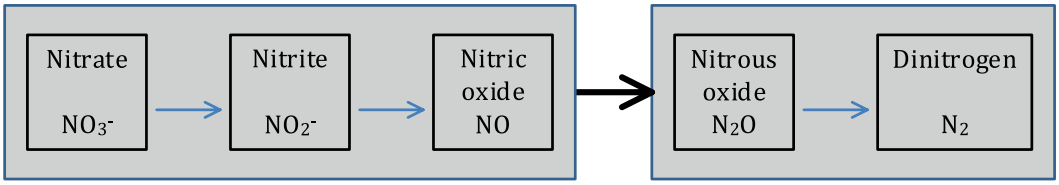

Figure 2 — Focus of the step of the denitrification process mainly investigated during the DEA test

DEA estimates the process of denitrification of fresh soil samples incubated under optimal conditions of substrates (nitrate and carbon sources) and environment (anaerobiosis, controlled temperature) for the denitrification process. The *de novo* enzyme synthesis is blocked by the use of chloramphenicol. DEA is believed to represent the size of the denitrifying enzyme pool present in the soil sample at the time of sample collection. It is a standardized technique used in numerous scientific studies.

The second part of the ISO/TS 20131 series presents a test revealing soils capacities to reduce N_2O, the last step of the denitrification process (i.e. the reduction of N_2O produced through the nitrate denitrification to the dinitrogen form). It mainly focuses on the black arrow of Figure 3. This test allows determining the transient accumulation of N_2O during the denitrification process. It derives from a study proposed by Reference [5]. Methodological adaptations and new interpretations of the results had been explained in Reference [6].

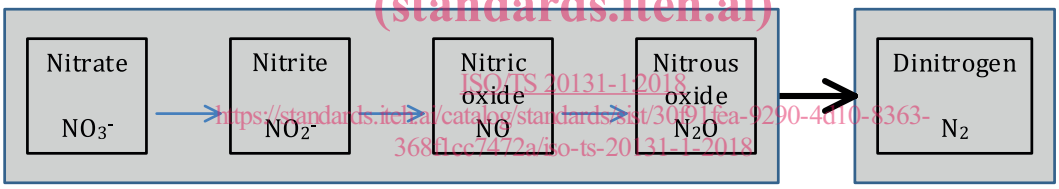

Figure 3 — Focus of the step of the denitrification process mainly investigated during the study of soils' capacity to reduce N_2O

The principles of the two parts of the ISO/TS 20131 series are summarized in Table 1.

Table 1 — Summary of the two parts of the ISO/TS 20131 series

	Part one: Soil denitrifying enzymes activities[5]	Part two: Soil capacity to reduce N_2O[6]
Principles of the methodology	Anaerobiosis to optimize the denitrification process	
	Use of acetylene to inhibit the N_2O reductase	
	Substrate addition — Nitrate — Carbon	Substrate addition — Nitrate — N_2O (optionally)
	Chloramphenicol addition	

Table 1 *(continued)*

	Part one: Soil denitrifying enzymes activities[5]	**Part two: Soil capacity to reduce N_2O[6]**
Ability to assess field denitrification	The test reveals the concentration of functional denitrifying enzymes in sample at the time of sample collection[5] [7]. In certain cases, correlations had been observed between DEA and annual denitrification in soils[8]	
Ability to assess N_2O emission	No evidence	Results could be used — by themselves to discriminate soils with potentially high levels of N_2O emission on the field scale[6] — combined in the NOE model[9] to calculate soil N_2O emission
Number (n) of publications in which the test has been used	$n > 100$	$10 > n > 100$

Soil quality — Easy laboratory assessments of soil denitrification, a process source of N_2O emissions —

Part 1: Soil denitrifying enzymes activities

1 Scope

This document specifies a laboratory test for characterizing the denitrifying enzyme activities in soils[5]. It globally characterizes the transformation of the nitrate form to the nitrous oxide and dinitrogen forms. This method was first proposed by Reference [5] with the acronym DEA for Denitrifying Enzyme Activities. It is a standardized technique used in numerous scientific studies.

DEA estimates the process of denitrification of fresh soil samples incubated under optimal conditions of substrates (nitrate and carbon sources) and environment (anaerobiosis, controlled temperature) for the denitrification process. The *de novo* enzyme synthesis is blocked by the use of chloramphenicol. DEA is believed to represent the size of the denitrifying enzyme pool present in the soil sample at the time of sample collection.

This test is performed in laboratory on a composite of sieved samples collected at the plot scale. It can be performed on all types of soils sampled all over the year.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

ISO 18400-206[1)], *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

1) Under preparation. Stage at the time of publication: ISO/FDIS 18400-206:2017.

4 Symbols and abbreviated terms (except chemical and reagents)

DEA denitrifying enzyme activity (µg N_2O-N·g $^{-1}$ soil·h^{-1})

ECD electron capture detector

GC gas chromatograph

SWC soil water content (g water·g^{-1} dry soil)

TCD thermal conductivity detector

5 Principle

The method is based on two principles:

— optimizing all requirements for denitrifying enzymatic activity, saturation with nitrate and an electron donor, no oxygen and no diffusion limitation, so that the rate of N_2O production is proportional to denitrifying enzyme content;

— measuring the N_2O release of soil slurries in these optimized conditions as soon as possible after soil sampling to reveal the activity of pre-existing denitrifying enzymes in the soil microflora.

Therefore, soil slurries are placed under anaerobic conditions with addition of nitrate, carbon source and chloramphenicol over a period of 2 h in the presence of acetylene. N_2O release in these conditions is measured and reveals the concentration of functional denitrifying enzymes in sample at the time of sample collection[6][9].

6 Materials

6.1 Test materials

6.1.1 Pre-evacuated flasks (<5 ml) with butyl septa and crimp capsules.

6.1.2 Needles, syringes.

6.1.3 Rubber lids and screw-caps with a hole for reagent bottles.

6.2 Apparatus

Usual laboratory equipment.

6.2.1 Reagent bottles with an around 125 ml capacity.

6.2.2 Fume cupboard.

6.2.3 Rotating or end-to-end shaker (180 r/min).

6.2.4 Laboratory balance (accuracy 0,1 g).

6.2.5 Vacuum pump.

6.2.6 Gas chromatographs.

6.2.6.1 ECD detector, Capillary or filled Porapak Q column.

6.2.6.2 TCD detector, Capillary or filled Porapak Q column (optional).

6.3 Test soil

The test shall be performed on fresh samples, directly after sampling if possible.

6.4 Reagents

6.4.1 Chemicals.

6.4.1.1 Potassium nitrate, KNO_3.

6.4.1.2 Glucose, $C_6H_{12}O_6$.

6.4.1.3 Chloramphenicol, $C_{11}H_{12}Cl_2N_2O_5$.

6.4.1.4 Nitrogen, N_2.

NOTE Helium or Argon could also be used as inert gas.

6.4.1.5 Acetone-free Acetylene, C_2H_2.

6.4.1.6 Krypton, Kr, optional.

6.4.2 Solutions.

6.4.2.1 Solution S1, constituted by KNO_3 (1 mmol·l^{-1}), $C_6H_{12}O_6$ (1 mmol·l^{-1}), $C_{11}H_{12}Cl_2N_2O_5$ (3 mmol·l^{-1}). The solution S1 should be freshly prepared.

7 Procedures

7.1 Soil sampling and preparation

Collect at least 10 soil samples on the 0 cm to 20 cm of a total surface of around 1 000 m^2 of a soil plot (NOTE 1) in order to obtain around 1 kg of fresh soil. Avoid sampling the two weeks following a fertilisation event. Make a soil composite by sieving (2 mm) altogether the 10 samples (NOTE 2).

NOTE 1 Adaptable to the purpose of the study or the situation.

NOTE 2 A larger sieving (up to 5 mm) is accepted as a 2 mm sieving could be not possible for certain fresh soils.

Start incubation as soon as possible after sampling. In exceptional cases of impossibility to perform the measure rapidly after sampling, kept the soil samples according to ISO 18400-206, i.e. at (4 ± 2) °C with free access of air, no more than three months.

Determine the sieved soil water content (SWC) (g water·g^{-1} dry soil) according to ISO 11465, when starting incubation.