TECHNICAL SPECIFICATION

ISO/TS 20131-1

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2018-07

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

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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.

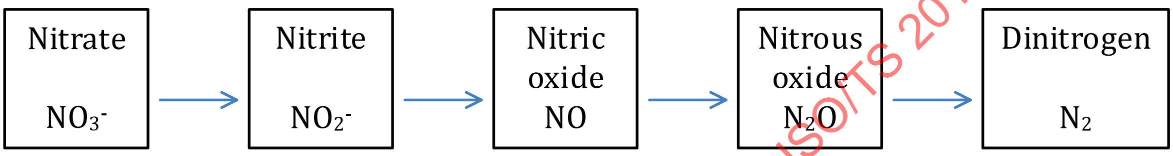

Figure 1 — Description of the denitrification process

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.

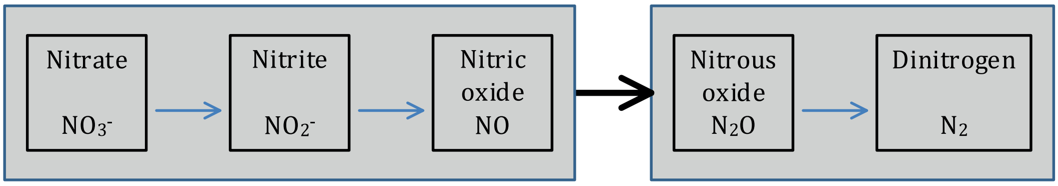

Figure 2 — Focus of the step of the denitrification process mainly investigated during the DEA test

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].

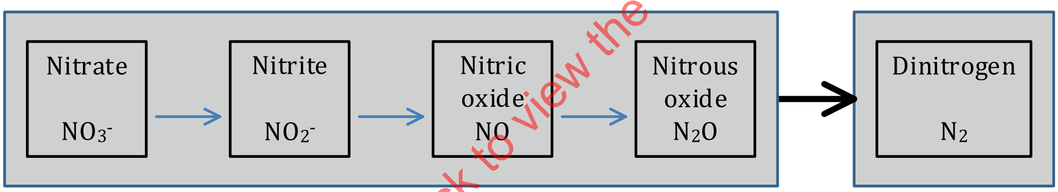

Figure 3 — Focus of the step of the denitrification process mainly investigated during the study of soils' capacity to reduce N_2O

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/

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[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.

7.2 Soil slurries incubation

In a laboratory maintained at (20 ± 2) °C, prepare three flasks each with 25 g of sieved fresh soil. Add 25 ml of the solution S1 (6.4.2.1) to each flask.

Close the reagent bottles with an airtight rubber lid. Evacuate the flask gas atmosphere and refill with the inert gas (N_2) to a slight overpressure (ca +50 kPa) to speed up anaerobiosis. Repeat this operation four times for soil slurries to be in anaerobic conditions. At the end of the evacuation/refill cycles, the overpressure is released by creating for few seconds a little leak with a syringe (1 ml) partially filled with water. Remove the syringe when the water in this syringe stops to bubble.

Remove 10 ml of gas atmosphere. Add 10 ml of C_2H_2.

Set the samples on the shaker (180 r/min) for 2 h at (20 ± 2) °C. Using a syringe, sample the gas atmosphere after 0 h - 0,5 h - 1 h and 2 h. Each gas sample is either immediately analysed or placed in a pre-evacuated flask (6.1.1).

Due to the short time of incubation, the temperature of the laboratory for preparing soil slurries and the temperature of incubation have to be the same. In tropical and equatorial conditions, this temperature could be higher than 20 °C and then is indicated in the report.

Gas samples could be stored for three weeks in airtight flasks closed with butyl septa before analysis.

7.3 Gas samples analysis

Gas samples are analysed on a calibrated GC fitted with an ECD detector (temperature 320 °C), a Porapak Q column (temperature 60 °C) and Argon Methane (90/10) as carrier gas for N_2O analysis.

NOTE Some other GC specifications (detector and column temperatures, carrier gas…) can be used by laboratories.

8 Validity criteria

The production of N_2O over time shall be linear.

9 Additional suggestions

9.1 Soil slurries incubation

The experiment could also be performed on three other reagent bottles carried in the same conditions with the only difference that acetylene is not added during soil incubation (in this case, do not remove 10 ml of the anaerobic atmosphere of the flasks). The ratio of the results obtained without acetylene to the results obtained with acetylene provides some information about the relative production of N_2O and N_2 during soil denitrification.

9.2 Control of procedure

The inert gas Kr could be used to verify that no leaks occur during the experiment. Kr (1 ml) is added just after the acetylene addition into the anaerobic atmosphere of the flasks. The Kr concentration should remain constant over time. A decrease of the Kr concentration over time reveals a leak in a flask and results obtained from this flask cannot be further used.

9.3 Gas samples analysis

Kr, CO_2 and C_2H_2 could be analysed on a calibrated GC with a TCD detector (temperature 60 °C), a porapak Q column (60 °C) and Helium as carrier gas.

10 Data presentation and interpretation

10.1 Denitrifying Enzyme Activity (DEA)

Plot the graph of the change of the quantity of N_2O in each flask having received acetylene, over time. The quantity of produced N_2O is expressed in µg N_2O-N·g^{-1} soil and is calculated according to Formula (1). Calculate DEA as the slope of the plot [Formula (2)]. DEA is expressed in µg N_2O-N·g^{-1} soil·h^{-1}.

$$Q_{N_2O} = \left\{ \left[N_2O\right] \times \frac{V_{gaz}}{V_{MOL}} + n_{water} \times \left[N_2O\right] \times K \times P_{atm} \right\} \times 28 / m_{drysoil} \times 10^6 \tag{1}$$

$$DEA = \frac{\Delta Q_{N_2O}}{\Delta t} \tag{2}$$

where

Q_{N_2O}	is the quantity of N_2O produced by soil unit, in µg N_2O-N·g^{-1} dry soil;
$[N_2O]$	is the N_2O concentration in flask atmosphere, in l·l^{-1};
V_{gaz}	is the gaseous volume in reagent bottles, in l;
V_{MOL}	is the molar volume (l) at 20 °C and atmospheric pressure;
n_{water}	is the number of moles of water in each flask;
K	is the solubility constant in water of N_2O; $K = 0{,}48 \cdot 10^{-6}$ mol· mol^{-1} ·hPa^{-1} at 20 °C;
P_{atm}	is the atmospheric pressure, in hPa;
$m_{drysoil}$	is the mass of dry soil, in g;
t	is the time, in h.

Calculate the mean and the standard deviation for the three replicates.

10.2 Additional suggestion: relative production of N_2O during denitrification (*r*)

Perform the same calculation for data obtained during incubations without acetylene. The relative production of N_2O during denitrification (*r*), is

$$r = \frac{\left[\Delta Q_{N_2O}\right]_{without\ acetylene}}{\left[\Delta Q_{N_2O}\right]_{with\ acetylene}} \tag{3}$$

where

$\left[\Delta Q_{N_2O}\right]_{without\ acetylene}$	is the variation of the quantity of N_2O produced by soil unit, in µg N_2O-N·g^{-1} dry soil, in the flasks without acetylene;
$\left[\Delta Q_{N_2O}\right]_{with\ acetylene}$	is the variation of the quantity of N_2O produced by soil unit, in µg N_2O-N·g^{-1} dry soil, in the flasks with acetylene.

11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO/TS 20131-1:2018;

b) the origin of the field soil used in accordance with ISO 18400-206;

c) the date of sampling, the date of incubation and conditions of soil conservation when necessary also in accordance with ISO 18400-206;

d) the water content of the tested soil;

e) temperature conditions if different from this document;

f) all details not specified in this document or considered as optional, as well as any effect which may have affected the results;

g) the results expressed in accordance with Clause 10.

Annex A
(informative)

International ring test

The ring test was conceptually organized into 3 steps:

a) a first step during which all the participants performed the hereby protocol on four French soils to obtain first results and identify potential difficulties;

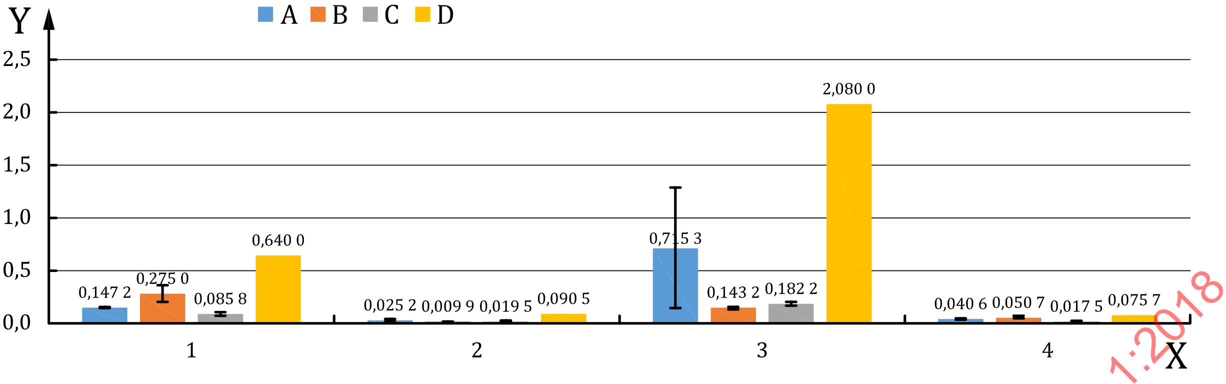
b) a second step during which the possible identified problems are addressed;

c) a last step during which all the involved laboratories are going to perform the test after possible initial problems have been solved.

The first step was performed during the first half of 2016 involving 4 laboratories named A, B, C and D. These laboratories were located in France, Czech Republic, Spain and Finland.

4 soil samples (identified 1, 2, 3 and 4) were collected in a 100-km area around Orleans on week 9 in 2016. They were all sieved within 2 days after sampling. They were sent to the participants on week 10. Laboratories received the samples 2 or 3 days after and kept them fresh at 4°C until Friday March 10th. They were then placed in laboratories at (20 ± 2) °C two days before the beginning of the experiment. Experiments started on week 11.

The following results were obtained. See Figure A.1 and Figure A.2.

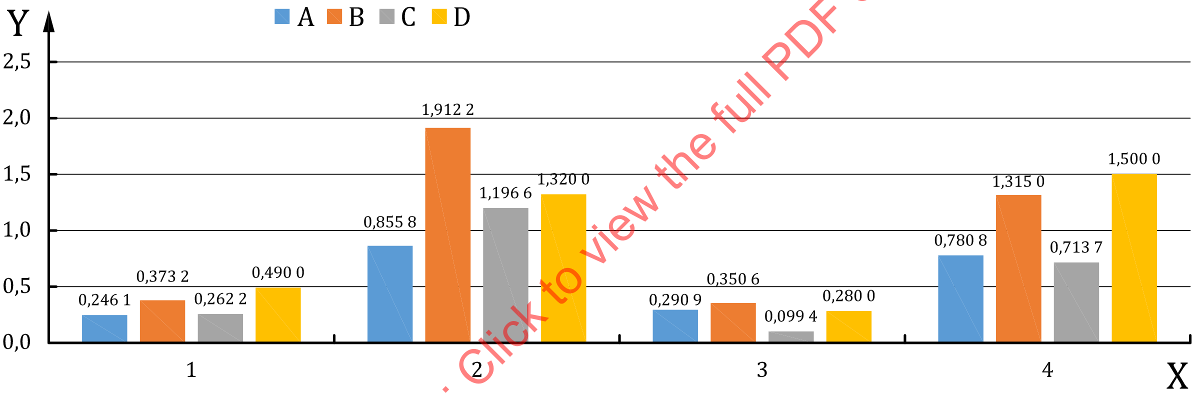


Key

X soils' number

Y DEA (µg N_2O-N·g^{-1} soil·h^{-1})

Figure A.1 — Results obtained for DEA during the first step of the ring test



Key

X soils' number

Y *r* (dimensionless)

Figure A.2 — Results obtained for *r* during the first step of the ring test

Non parametric statistics (pairwise comparison according to the Dunn procedure, XLstat®[2]) reveal that some significant differences exist between soils, S2 having the lowest DEA and the highest *r* value and S3 having the highest DEA activity and the lowest *r* value. This is consistent with the soils' pH. See Table A.1.

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[2] XLstat® is a registered trademark of Addinsoft. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

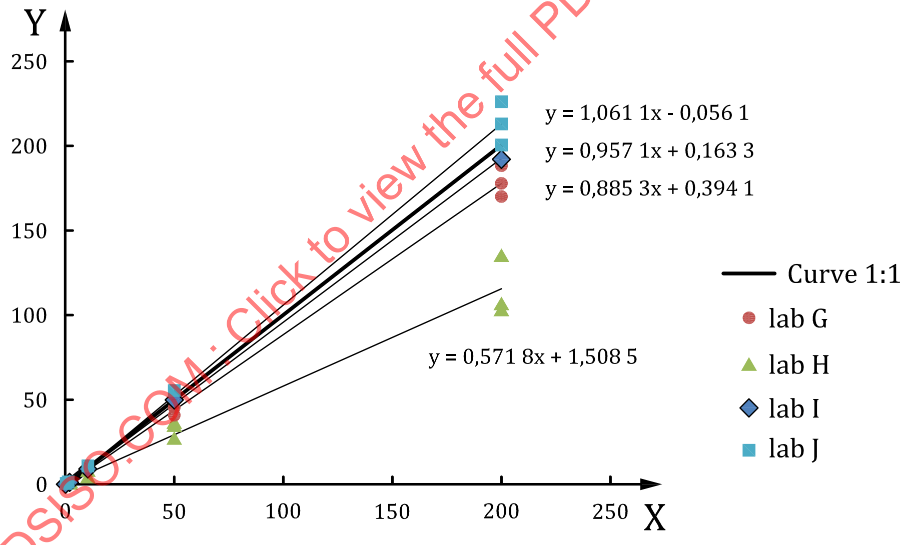
Table A.1 — Results of the statistical analysis applied to the results obtained during the first step of the ring test

Soil	Mean DEA	Statistical group	Mean *r*	Statistical group
1	0,287	AB	0,343	AB
2	0,036	B	1,321	A
3	0,78	A	0,255	B
4	0,046	AB	1,077	AB

Further to this consistent presentation of obtained results, we nevertheless observed some discrepancies between laboratories concerning N_2O analysis by GC.

We then decided for the second step of this ring test to compare results of N_2O analysis of standard gases obtained by the different laboratories irrespectively located in Czech Republic, France, Germany and Finland. Laboratories involved in this test were called G, H, I, J, with the laboratories in the group [G,H,J] being irrespectively in the group of laboratories [A,C,D] involved in the first and third steps. Certified gas samples were sent during August 2016 to the different laboratories for analysis. Figure A.3 presents the obtained results. It reveals that corrections of gas analysis are required for the next step and that the use of common standards is therefore required.

Laboratory H obtained a lower signal than the other laboratories.



Key

X	certified N_2O concentration ($\mu l \cdot l^{-1}$)
Y	detected N_2O concentration ($\mu l \cdot l^{-1}$)

Figure A.3 — Results of analysis of certified N_2O samples by different European laboratories

Concerning the third step of the ring test, soil samples were collected and prepared during week 41, sent to the different laboratories on week 42 together with certified standards and tests were performed during week 43. The following results were obtained See Figure A.4 and Figure A.5.