TECHNICAL SPECIFICATION

ISO/TS 20131-2

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

Part 2: Assessment of the capacity of soils to reduce N_2O

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

Partie 2: Évaluation de la capacité des sols à réduire le N_2O

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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.

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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.

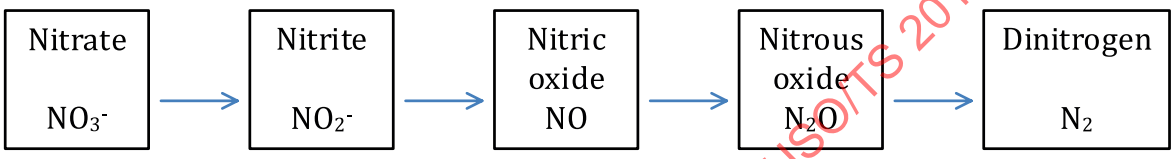


Figure 1 — Description of the denitrification process

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.

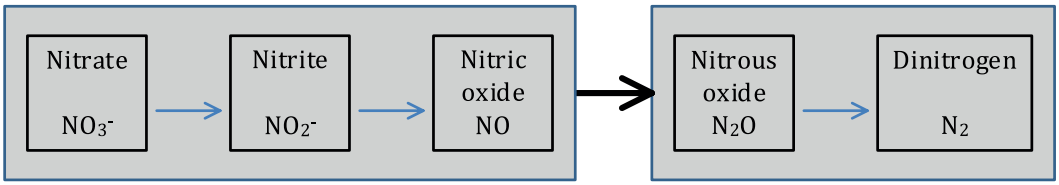


Figure 2 — Focus of the step of the denitrification process mainly investigated during the DEA test

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].

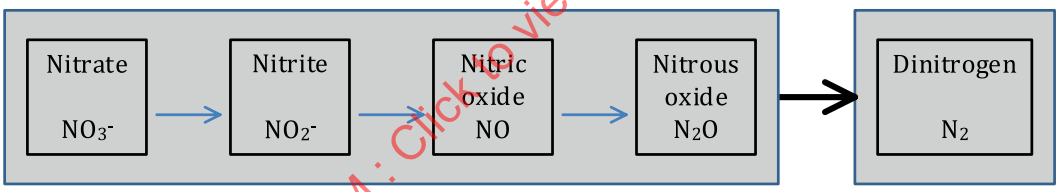


Figure 3 — Focus of the step of the denitrification process mainly investigated during the study of soils' capacity to reduce N_2O

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 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 2: Assessment of the capacity of soils to reduce N_2O

1 Scope

This document specifies a laboratory test for characterizing the ability (or inability) of soils to reduce the greenhouse gas N_2O into N_2 as it was previously shown that soils with a low ability to reduce N_2O into N_2 constitute situations with a risk of large emission of N_2O[6], higher than those basically estimated by the use at the plot scale of the equations proposed in the IPCC guidelines for National Greenhouse Gas Inventories[10].

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 except in very exceptional and extreme conditions of dryness. Results obtained are stable over time for situations that do not receive neither organic nor lime amendments.

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/

4 Symbols and abbreviated terms (except chemicals and reagents)

GC gas chromatograph

NOE nitrous oxide emission (model of)

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

TCD thermal conductivity detector

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

5 Principle

The release of N_2O by soil slurries placed under anaerobic conditions is measured over a period of seven days in the presence and without acetylene. Results obtained are indicative of the ability of soils to reduce N_2O. Indeed, soils exhibiting a low capacity to reduce N_2O are especially suspected to emit very large levels of this gas [6][9].

Results of this test can be synthetized through two indicators, r_{max} and index[6]. r_{max} is the maximum ratio of the accumulated N_2O during incubation. It is one of the biological parameters of the model of soils nitrous oxide emission, NOE[9]. The index combines information on the level and on the time of accumulation of N_2O during incubations (Annex A).

6 Materials

6.1 Test materials

6.1.1 Pre-evacuated flasks (<10 ml) with butyl septa and crimp capsules.

6.1.2 Needles, syringes.

6.1.3 Rubber lids and screw-caps for reagent bottles.

6.2 Apparatus

Usual laboratory equipment:

6.2.1 Reagent bottles with an around 500 ml capacity.

6.2.2 Fume cupboard.

6.2.3 Rotating or end-to-end shaker (150 r/min).

6.2.4 Laboratory balance (accuracy 0,1 g).

6.2.5 Vacuum pump.

6.2.6 Gas chromatograph.

6.2.7 TCD detector.

6.2.8 Capillary or filled Porapak Q column.

6.3 Reagents

6.3.1 Chemicals.

6.3.1.1 Potassium nitrate, KNO_3.

6.3.1.2 Nitrogen, N_2.

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

6.3.1.3 Acetone-free Acetylene, C_2H_2.

6.3.1.4 **Nitrous oxide**, N_2O.

6.3.1.5 **Krypton**, Kr, optional.

6.3.2 **Solution S2**.

Solution S2 is constituted by KNO_3 (7 mmol·l^{-1}).

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 (see NOTE 1) allowing to obtain around 1 kg of fresh soil. In order to obtain a result relevant on a long period, avoid collecting samples neither in exceptional and extreme climatic conditions for a specific localization nor during the two weeks following a fertilisation. Make a soil composite by sieving (2 mm) (see NOTE 2) altogether the 10 samples.

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

NOTE 2 A larger sieving (up to 5 mm) is accepted as a 2 mm sieving is not possible for all 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

Prepare six reagent bottles each with 50 g of sieved fresh soil. Add 50 ml of the S2 solution (6.3.2) to each bottle.

Close the reagent bottles with an airtight rubber lid. Evacuate the bottle gas atmosphere and refill with an inert gas (N_2) (6.3.1.2) 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.

Then separate the bottles into two groups of three bottles.

For the first group of three bottles, remove with a syringe 10 ml of the gas atmosphere and add 10 ml of C_2H_2 (6.3.1.3).

Homogenize the gas atmosphere with a syringe.

Sample the gas atmosphere of each flask of both groups either with a syringe or directly in a pre-evacuated flask with a double needle.

Set the samples on the rotating or end-to-end shaker (150 r/min) for seven days at (20 ± 2) °C. Sample the gas atmosphere after 24 h, 48 h, 72 h, 96 h and 168 h.

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

NOTE Optionally, this experiment can be performed with addition of 5 ml of N_2O instead of addition of nitrate to test how much your soil can reduce N_2O added at a high level. These treatments are not further used for the calculation of the index.

7.3 Gas samples analysis

Gas samples are analysed on a calibrated GC fitted with a TCD detector (temperature 60 °C), a Porapak Q column (60 °C) and Helium as carrier gas.

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

8 Control of procedure (optional)

The inert gas Kr could be used to verify that no leaks occur during the experiment. Kr (1 ml) is added into the anaerobic atmosphere of the flasks before they are set on the shaker. The Kr concentration should remain constant over time. A decrease of the Kr concentration over time reveals a leak in the bottle or in the flask and results obtained from this sample cannot be further used.

9 Data presentation and interpretation

Plot the graph of the quantity of N_2O produced in bottles (mean of the three replicates) over time for the two treatments, i.e. with acetylene and without acetylene. The quantity of produced N_2O is expressed in µg N_2O-N·g^{-1} soil and is calculated according to Formula (1).

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

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 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 bottle;

K is the solubility constant in water of N_2O; K = 0,48·10^{-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.

Define r_{max}, the maximum ratio of the accumulated N_2O (N_2O produced during incubation without acetylene) to the denitrified nitrate (N_2O produced during incubation with acetylene). r_{max} is limited to 1,2. r_{max} could be used for predicting *in situ* N_2O emission by soil using the NOE algorithm[9].

Define the time t over which N_2O accumulates in the bottle during the incubation without acetylene, i.e. the production rate is higher to the consumption one, leading to an increase in N_2O concentration in flasks.

Calculate the index by multiplying r_{max} and t. If the index is higher than 50, the soil poorly reduces N_2O, and emissions of N_2O in this situation are especially suspected to be very large.

Examples of data presentation and interpretation are shown in Annex A.

10 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO/TS 20131-2:2018;

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

f) the results expressed in accordance with Clause 9.

Annex A
(informative)

Results obtained on two agricultural soils

A.1 Levels of *in situ* N_2O emission of these two soils[5]

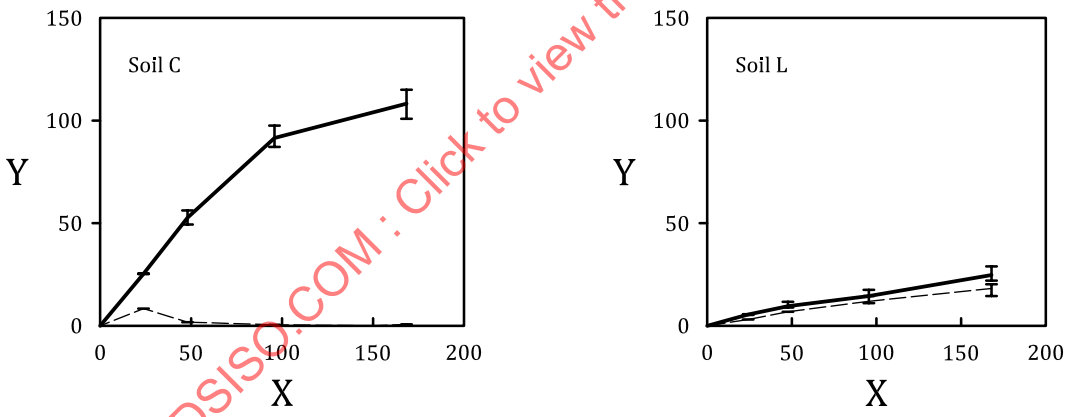
This annex presents the results obtained by applying this test on two agricultural soils (soil C and soil L) located in the North East Part of France.

In situ N_2O emissions were measured using the classical static chambers method over a five months period on both soils C and L that were submitted to equivalent agricultural and climatic conditions.

In situ N_2O emissions were always less than 5 g $N{\cdot}ha^{-1}{\cdot}d^{-1}$ for the C soil while they occasionally reach values higher than 50 g $N{\cdot}ha^{-1}{\cdot}d^{-1}$ on the L soil. While the IPCC guidelines for National Greenhouse Gas Inventories[10] consider a mean Fertilised Induced Emission as N_2O of 1 %, FIE was higher than 2 % on the L situation.

A.2 Data presentation and interpretation

Figure A.1 presents the evolution of the quantity of N_2O produced by soils in the flasks, obtained for soils C and L respectively.



Key

X incubation time (h)

Y N_2O (µg $N{\cdot}g^{-1}$ soil)

– – - without acetylene

—— - with acetylene

Figure A.1 — Kinetics of denitrification of soil C and soil L placed in anaerobic conditions with nitrate addition, with and without acetylene

r_{max} is the maximum ratio of the accumulated N_2O by (1) calculating at each time the ratio between N_2O produced without acetylene and N_2O produced in presence of acetylene and (2) selecting the highest one.

$(r_{max})_{soil\ c} = 0,1$ $(r_{max})_{soil\ L} = 0,9$

The time t is the time over which N_2O accumulate in the bottles during the incubation without acetylene, i.e. the production rate is higher to the consumption one.

$(t)_{soil\ c} = 24$ $(t)_{soil\ L} = 168$

The index (i) by multiplying r_{max} and t

$(i)_{soil\ c} = 2,4$ $(i)_{soil\ L} = 151$

While both soils are potent sources of N_2O, soil L with an index value higher than 50 is specially suspected to emit very large levels of N_2O. This is in accordance with the level of emissions observed *in situ* for situations C and L.

Annex B
(informative)

International ring test

The ring test was conceptually organized into 3 steps:

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

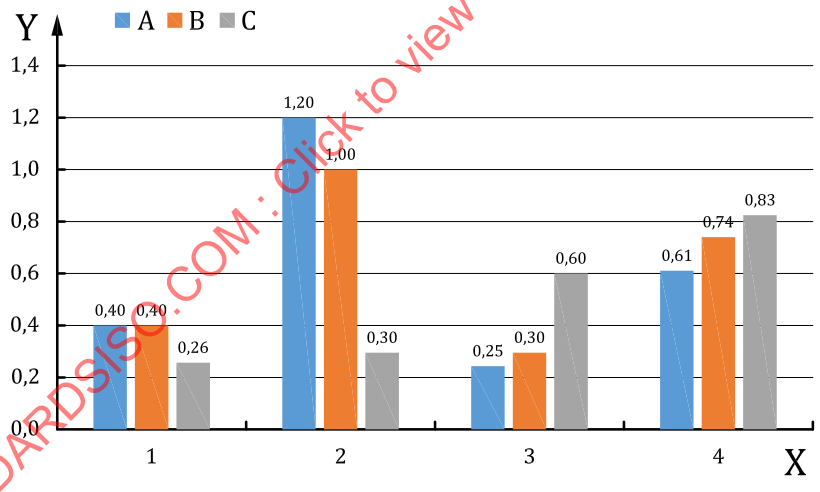
b) a second step during which the possible identified problems are addressed;

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

The first step was performed during the first half of 2016 involving 3 laboratories named A, B, C. These laboratories were located in Europe, irrespectively in France, Spain and Finland.

4 soil samples (identified 1, 2, 3 and 4) were collected in a 100 km area around Orleans (France) 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. Soil samples were then placed in laboratories at (20 ± 2) °C 2 days before the beginning of the experiments. Experiments started on week 11.

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

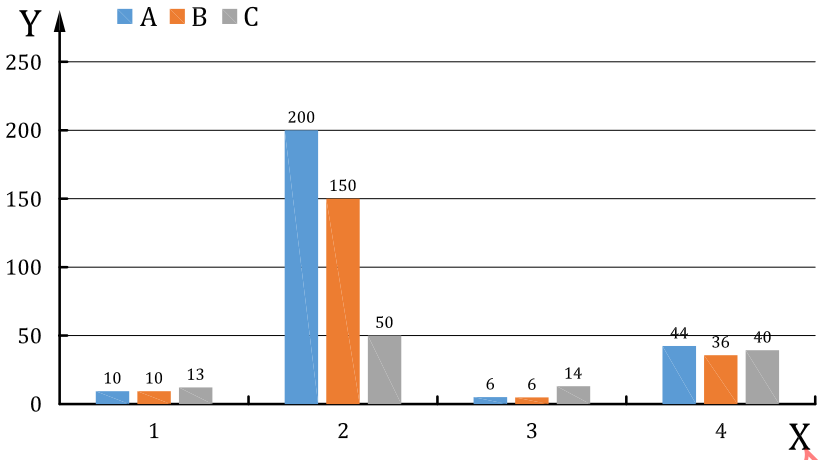


Key

X soils' number

Y r_{max} (dimensionless)

Figure B.1 — Results obtained for r_{max} during the first step of the ring test for the four soils



Key

X soils' number

Y index (dimensionless)

Figure B.2 — Results obtained for Index during the first step of the ring test for the four soils

Except that for the soil 2 where the results were markedly different for the laboratory C compared to the others, results obtained were consistent. Without the value obtained on the soil 2 by the lab C, non-parametric statistics reveal that some significant differences exist between soils, especially the soil 2 has the lowest capacity to reduce N_2O. During this experiment, laboratory C observed the speed of the shaker to be too fast and not secure for flasks. Some have been broken and the number of replicates was then reduced. This could have affected the results and could explain why the results of laboratory C during this first step were not be totally consistent with others.

During the second step of the ring test, we decided to change the speed of the shaker into the protocol. Initially defined at 180 r/min, it was finally defined at 150 r/min to avoid breaking flasks during the shaking incubation. Moreover, during the first step and despite globally consistent observations, we detected some discrepancies between laboratories concerning N_2O analysis by GC. At the end, these discrepancies did not affect final results that are ratio of N_2O concentrations, but we were all interested to test our calibrations. We decided to compare results of N_2O analysis of standard gases. Laboratories involved in this test were located in Czech Republic, France, Germany and Finland. They were irrespectively called G, H, I, J, with the laboratories in the group [H,J] being irrespectively in the group of laboratories [A,C] involved in the first and third steps of this ring test. Certified gas samples were sent during august 2016 to the different laboratories for analysis. Figure B.3 presents the obtained results. It reveals that corrections of gas analysis are required for at least the H laboratory to improve absolute values of N_2O concentrations.

Laboratory H obtained a lower signal than the other laboratories.