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# Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil

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**National foreword**

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INTERNATIONAL STANDARD

**ISO 17601**

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# Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil

*Qualité du sol — Estimation de l'abondance de séquences de gènes microbiens par amplification par réaction de polymérisation en chaîne (PCR) quantitative à partir d'ADN directement extrait du sol*

Reference number
ISO 17601:2016(E)

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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

# Contents

Page

# 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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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

# Introduction

DNA (DNAs) is a major component of any living organisms coding for enzymes responsible for their biological activities. The study of DNA sequences from DNA sources extracted from different environmental matrices, by means of numerous molecular approaches, provides molecular markers that can be used to sharply distinguish and identify different organisms (bacteria, *archaea*, and *eucaryotes*).

Up to now, most of the studies aiming to develop microbial quality indicators applicable to complex environment such as soil were biased by the poor culturability of many microorganisms under laboratory conditions and the lack of sensitivity of traditional microbiological methods. The recent development of a large set of molecular biology methods based on amplification of soil-extracted nucleic acids have provided a pertinent alternative to classical culture-based microbiological methods providing unique insight into the composition, richness, and structure of microbial communities.[2] [3] [4] [5] [6] DNA-based approaches are now well established in soil ecology and serve as genotypic markers for determining microbial diversity. The results of molecular analyzes of soil microbial communities and/or populations rely on two main parameters: a) the extraction of DNA representative of the indigenous bacterial community composition and b) PCR bias such as the choice of primers, the concentration of amplified DNA, errors in the PCR, or even the method chosen for analysis.[7] [4] [8] [9]

Numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils.[10] Recently, ISO 11063 reporting "a method to extract nucleic acids directly from soil samples" derived from Reference [10] is opening a new window for developing standardized molecular approaches to estimate soil quality.[11]

The aim of this International Standard is to describe the procedure used to set up and perform quantitative PCR to quantify the abundance of soil microbial phyla, as well as functional groups from DNA directly extracted from soil samples. The quantification of soil microbial phyla, as well as functional groups by qPCR assays can contribute to the development of routine tools to monitor soil quality. The repeatability and the reproducibility of the procedure of the quantitative PCR were assessed in an international ring test study (see Annex B). The repeatability of this procedure was successfully evaluated for both 16S rRNA genes, as well as genes coding a functional marker of denitrifiers (the nitrite reductase gene *nirK*). The reproducibility of this procedure revealed a laboratory effect which can be overcome by interpreting the results of the quantification of the abundance of the microbial groups by comparison, either by using an external reference (DNA extracted from a control strain) in the assay or by calculating a percentage of variations between treatments to normalize the data.

INTERNATIONAL STANDARD ISO 17601:2016(E)

# Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil

## 1 Scope

This International Standard specifies the crucial steps of a quantitative real-time polymerase chain reaction (qPCR) method to measure the abundance of selected microbial gene sequences from soil DNA extract which provides an estimation of selected microbial groups.

It is noteworthy that the number of genes is not necessarily directly linked to the number of organisms that are measured. For example, the number of ribosomal operon is ranging from one copy to 20 copies in different bacterial phyla. Therefore, the number of 16S rRNA sequences quantified from soil DNA extracts does not give an exact estimate of the number of soil bacteria. Furthermore, the number of sequences is not necessarily linked to living microorganisms and can comprise sequences amplified from dead microorganisms.

## 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 11063, *Soil quality — Method to directly extract DNA from soil samples*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**soil DNA**
DNA extracted from soil of living and dead biota

EXAMPLE Microorganisms, plants, animals.

**3.2**
**polymerase chain reaction**
**PCR**
method allowing the amplification of a specific DNA sequence using a specific pair of oligonucleotide primers

**3.3**
**quantitative polymerase chain reaction**
**qPCR**
method allowing the quantification in a DNA *template* (3.4) of the number of a specific DNA sequence using a specific pair of oligonucleotide primers

**3.4**
**template**
DNA sample used to perform *PCR* (3.2) to amplify a specific DNA sequence

**3.5**
**amplicon**
PCR product obtained by *PCR* (3.2) from a *template* (3.4)

**3.6**
**cloning vector**
circular DNA molecule in which the *amplicon* (3.5) is inserted by ligation used to transform competent Escherichia coli for cloning the amplicon

**3.7**
**qPCR standard**
cloned DNA target used as *template* (3.4) for qPCR reaction to establish the standard curve relating the abundance of target sequence as a function of cycle threshold values (Ct)

**3.8**
**non-template control**
**NTC**
control, usually molecular grade water, that is used as negative control in qPCR assay to check for the absence of contaminant in the qPCR mix

**3.9**
**cycle threshold**
**Ct**
number of qPCR cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level)

Note 1 to entry: The Ct value is inversely proportional to the abundance of the target sequence.

## 4 Principle

This International Standard describes qPCR assay using fluorescent DNA binding dye as reporter. This qPCR assay has been validated by an international ring test conducted with the SYBR Green, a commonly used fluorescent DNA binding dye which binds all double–stranded DNA and can be detected by measuring the increase in fluorescence throughout the cycle.

The method aims to measure the abundance of selected microbial gene sequences from soil DNA extract. The method comprises four tasks and eight steps as summarized in Figure 1. According to Reference [1], the three critical steps to be validated for each qPCR assay are as shown in Figure 1.

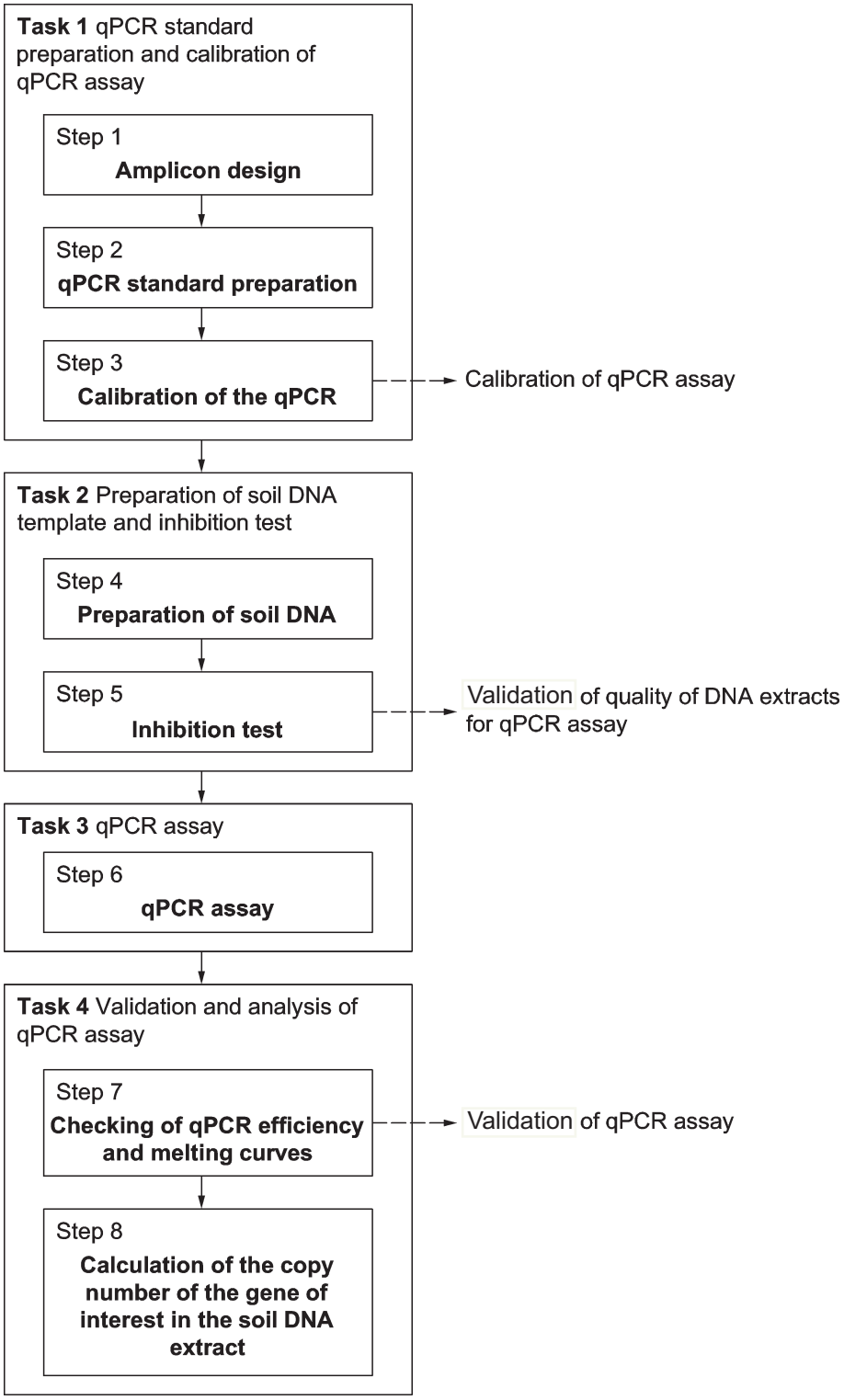


**Figure 1 — Main tasks and critical steps to estimate the abundance of selected microbial gene sequences by qPCR assay**

This International Standard describes qPCR assay based on the use of fluorescent DNA binding dye which has been validated by an international ring test using SYBR Green®[1)] qPCR. In Annex A,

1) SYBR Green is a registered trademark of Molecular Probes. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

information about TaqMan®[2)] qPCR assay not tested in the international ring test are given. The first task is made of three steps describing the design of optimal amplicon for qPCR (step one), the preparation of qPCR standards (step two), and the procedure to calibrate the qPCR assay (step three). The second task includes two additional steps describing the procedures to prepare soil DNA samples (step four) and to test for the presence of qPCR inhibitors in soil DNA samples (step five). The third task is constituted of a single step describing the protocol to perform qPCR assay (step six). Finally, the fourth task is made of two steps, one describing the procedure to validate qPCR assays (step 7) to check the quality of qPCR assay and another one describing the different options to calculate the number of sequences of the gene of interest copy from cycle threshold (Ct) obtained from the analysis of qPCR amplification plots (step 8).

## 5 Test materials

### 5.1 DNA

**5.1.1** **DNA**, extracted from pure bacterial and fungal isolates using classical extraction procedures or by using commercial kit to extract genomic DNA.

**5.1.2** **Soil DNA**, extracted from aliquots of soil according to ISO 11063.

### 5.2 Bacteria

**5.2.1** ***Escherichia coli* strain**, usually used for cloning of PCR product.

### 5.3 Plasmid

**5.3.1** **Cloning vector**, usually used for cloning of PCR product in competent *Escherichia coli.*

### 5.4 Enzyme

**5.4.1** Taq polymerase.

**5.4.2** T4 DNA ligase.

**5.4.3** T4 gene T32.

**5.4.4** Bovine serum albumin (CAS No. 9048-46-8).

### 5.5 Chemicals

**5.5.1** **Ampicilline sodium**, $C_{16}H_{18}N_3NaO_4S$ (CAS No. 69-52-3).

**5.5.2** **Boric acid**, $BH_3O_3$ (CAS No. 10043-35-3).

**5.5.3** **Deoxynucleotide solution**, dNTPs.

**5.5.4** **SYBR Safe® DNA gel stain**.

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2) TaqMan is a trademark of Roche Molecular Systems, Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

**5.5.5 Ethylenediaminetetraacetic acid disodium salt (EDTA)**, $C_{10}H_{14}N_2O_8Na_2{\cdot}2\ H_2O$ (CAS No. 6381-92 6).

**5.5.6 Glucose**, $C_6H_{12}O_6$ (CAS No. 50-99-7).

**5.5.7 Chlorhydric acid**, HCl (CAS No. 7647-01-0).

**5.5.8 IPTG, Isopropyl-Beta-D-Thiogalactopyranoside**, (CAS No. 367-93-1).

**5.5.9 Magnesium chloride**, $MgCl_2$ (CAS No. 7786-30-3).

**5.5.10 Magnesium sulfate**, $MgSO_4$ (CAS No. 7487-88-9).

**5.5.11 Molecular-biology-grade water**, $H_2O$.

**5.5.12 Potassium chloride**, KCl (CAS No. 7447-40-7).

**5.5.13 Sodium chloride**, NaCl (CAS No. 7647-14-5).

**5.5.14 Tris[hydroxymethyl]aminomethane**, $C_4H_{11}NO_3$ (CAS No. 77-86-1).

**5.5.15 X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside**, (CAS No. 7240-90-6).

## 5.6 Product for bacterial culture medium

**5.6.1 Bacto tryptone®**[3], enzymatic digest of casein.

**5.6.2 Yeast extract powder** (CAS No. 8013-01-2).

## 5.7 Buffer and reagents

**5.7.1 Ampicilline solution**, 2 g of ampicilline sodium in 4 ml of 0,22 µm filter sterilized $H_2O$. Adjust to 20 ml with sterilized $H_2O$, prepare 1 ml aliquots, and store at -20 °C.

**5.7.2 EDTA**, 0,5 $mol{\cdot}l^{-1}$, 186,10 g of EDTA in 1 000 ml of $H_2O$ adjusting with NaOH (10 $mol{\cdot}l^{-1}$) to pH 8,0.

**5.7.3 SYBR Safe™ DNA gel stain**, dilute 10,000X SYBR Safe™ gel stain in TBE buffer × 1.

**5.7.4 IPTG stock solution**, 1 g of IPTG in 8 ml of $H_2O$. After careful mixing, the solution is adjusted to 10 ml and sterilized under security microbiology post. Prepare 1 ml aliquot of IPTG and store at -20 °C.

**5.7.5 Solid LB medium**, 10 g of Bacto tryptone®, 5 g of yeast extract, 5 g of sodium chloride, and 15 g of agar in 1 000 ml of $H_2O$. After autoclaving for 20 min at 120 °C, 1 ml of ampicilline stock solution at 100 $mg{\cdot}ml^{-1}$ is added to LB medium and plated in Petri dishes (20 ml) under a security microbiology post. 100 µl of IPTG solution are plated on solid LB-ampicilline medium. When IPTG solution is entered in LB-ampicilline medium, 20 µl of X-Gal solution is plated on solid LB-ampicilline medium. Solid LB medium is stored at 4 °C until its use.

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3) Bacto tryptone is the trademark of a product supplied by Difco Laboratories. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

**5.7.6 SOC medium**, 20 g of Bacto tryptone®, 5 g of yeast extract, 0,58 g of NaCl, 0,95 g of $MgCl_2$, 2,46 g of $MgSO_4$, and 3,60 g of glucose in 1 l $H_2O$. Sterilize by 20 min autoclaving at 120 °C. Prepare 950 ml aliquots and store at -20 °C.

**5.7.7 Tris-HCl**, 1 mol·l$^{-1}$, 121,14 g of Tris in 1 000 ml of $H_2O$ adjusting with 4 mol·l$^{-1}$HCl to pH 8,0.

TBE buffer × 10, pH 8,0, 108 g of Tris base, 55 g of boric acid, and 40 ml of 0,5 mol·l$^{-1}$EDTA (pH 8,0) in 1 000 ml of $H_2O$.

**5.7.8 TBE buffer** × 1, 100 ml of TBE buffer × 10 in 900 ml of $H_2O$.

**5.7.9 TE buffer** × 10, pH 8,0, 100 ml of 1 mol·l$^{-1}$Tris-HCl pH 8,0, 20 ml of 50 mmol·l$^{-1}$EDTA pH 8,0 in 880 ml of molecular grade water.

**5.7.10 TE buffer** × 1, 100 ml of TE buffer × 10 in 900 ml of $H_2O$.

**5.7.11 X-gal solution**, 250 mg of X-Gal in 5 ml of dimethylformamide 5 ml. After careful mixing, prepare 0,5 ml aliquot and store at -20 °C.

## 6 Apparatus

Use standard laboratory equipment including pipettes, a centrifuge, fume hood cabinet, horizontal electrophoresis system and the following.

**6.1 Quantitative PCR**, allowing the real-time quantification of amplicons from various DNA templates with a theoretical detection limit of one copy of a sequence target per sample analyzed.

**6.2 Spectro-photometer**, allowing the quantification of double-strand DNA at 260 nm.

**6.3 Spectro-fluorimeter,** allowing the quantification of double-strand DNA.

NOTE Only one of these two apparatus is required to estimate DNA concentration.

## 7 Procedure

### 7.1 qPCR standard preparation and calibration of qPCR assay (task 1)

#### 7.1.1 General

qPCR assay is based on the quantification of the amplicons at the end of each PCR cycle by using a DNA dye which fluoresces when intercalated in the double strand amplicons. The purpose of this task is to describe the definition of appropriate amplicon to settle down a qPCR assay (step one), the preparation of qPCR standard (step two), and the calibration of the qPCR assay (step three).

#### 7.1.2 Amplicon design (task 1, step 1)

##### 7.1.2.1 General

The first step aims at designing oligonucleotide primer pair; it can be designed in *silico* using different programs using the sequence of the microbial gene of interest to be quantified by qPCR from soil DNA extracts. The specificity of the primers shall be checked in *silico* by comparing their sequences to known sequences available in the Genbank database (http://www.ncbi.nlm.nih.gov/genbank/). Only primers specific for the gene target shall be considered. The main parameters to be considered to design oligonucleotide primer pair for establishing qPCR assay are listed thereafter.

#### 7.1.2.2 qPCR

— Optimal amplicon length ranges between 100 bp to 250 bp.

— Optimal primer length ranges between 18 bp and 25 bp with a GC content of 50 % and melting temperature between 58 °C and 65 °C.

— The five nucleotides at the 3” end of each primer should have no more than two G and/or C bases.

— Avoid succession of identical nucleotide, especially true for guanine.

— 3” self-complementarity of the primer taken as a measure of its tendency to form a primer-dimer with itself should be checked and avoided.

— Avoid design of primers with more than four mismatches because too high degeneracy of the primer contributes to fluctuation of qPCR results.

### 7.1.3 qPCR standard preparation (task 1, step 2)

Step 2 of task 1 describes the procedure used to generate qPCR standards targeting a sequence of the microbial gene of interest from different DNA templates (pure bacterial or fungal isolate, environmental DNA, or artificial DNA). It also reports the procedure used to insert the qPCR standard in a cloning vector, transform *Escherichia coli*, and purify recombinant plasmids harboring qPCR standard for further use for qPCR assays.

### 7.1.4 Isolate DNA, environmental DNA, artificial DNA

#### 7.1.4.1 General

The first step of qPCR standard preparation relies on the extraction of DNA templates known to harbour the microbial gene of interest. This can be done starting from different materials such as the following:

a) pure cultures of microorganisms;

DNA is extracted from cells harvested from a fresh culture of microorganisms by using conventional genomic DNA extraction protocols.

b) artificial DNA.

If no biological samples are available or known to harbour the gene of interest, artificial DNA made of the sequence of the gene of interest can be synthesized.

In all cases, the quality of DNA template used for amplifying the qPCR standard by PCR shall be verified by electrophoresis on 1 % agarose gel in TBE buffer stained with appropriate staining (e.g. SYBR Safe™ staining). The concentration of DNA is measured by spectro-photometry at 260 nm. DNA template is diluted to 10 $ng \cdot \mu l^{-1}$ in a final volume of 20 µl and stored at -20 °C.

The qPCR standard sequence is amplified by PCR using a specific primer pair designed according to recommendations described in task 1 of block 1. The amplification reaction is carried out in a final 25 µl volume containing 2,5 µl of 10 × Taq polymerase buffer, 200 $\mu mol \cdot l^{-1}$ of each dNTP, 1,5 $mmol \cdot l^{-1}$ of $MgCl_2$, 0,5 $\mu mol \cdot l^{-1}$ of each primer and 0,625 U of Taq polymerase. A volume of 2,5 µl of DNA (e.g. 25 ng of DNA) is used as template for the PCR reactions. PCR is performed in a thermocycler according to the following program: one cycle of 4 min at 94 °C; 39 cycles of 1 min at 94 °C, 1 min at annealing temperature specific for the qPCR standard amplicon, 1,5 min at 72 °C, and a final extension step at 72 °C for 5 min. The expected size of the qPCR standard amplicon is verified by electrophoresis on 2 % agarose gel in TBE buffer stained with appropriate staining (e.g. SYBR Safe™ staining). Amplicons are purified either from the gel using appropriate methods or by using exclusion chromatography columns to remove primers. Purified amplicons are then quantified by spectro-photometry at 260 nm or by spectrofluorimetry.

#### 7.1.4.2 Cloning, dilution preparation of qPCR standard

##### 7.1.4.2.1 Ligation of amplicon of qPCR standard

For an optimal ligation of an amplicon into a cloning vector should a 3:1 molar ratio the mass of PCR product ($Q$ in ng of DNA) to be used for ligation can be calculated (see Formula 1):

$$Q = \frac{m_{\text{plasmid DNA}} \times n_{\text{insert}}}{n_{\text{plasmid}}} \times 3 \quad (1)$$

Q = [(amount of plasmid DNA × size of the insert (bp)/size of the plasmid (bp)] × (3/1)

where

$Q$ is the mass of PCR product, in nanograms (ng);

$m_{\text{plasmid DNA}}$ is the mass of plasmid DNA, in nanograms (ng);

$n_{\text{insert}}$ is the size of the insert, in bp;

$n_{\text{plasmid}}$ is the size of the plasmid, in bp;

Taking into account that a plasmid size of 3 000 bp, a 16S rRNA insert of 200 bp, and 50 ng of plasmid DNA per ligation reaction the amount of PCR amplicon to be used per ligation (see Formula 2) is:

$$Q = \frac{50 \times 200}{3000} \times 3 = 10 \quad (2)$$

The ligation reaction is made of the required amount of qPCR standard purified amplicon ($Q$), 50 ng of plasmid DNA, 5 µl of 2 × ligation buffer, 3 U of T4 DNA ligase, and molecular grade water to reach a final volume of 10 µl. The ligation reaction is incubated overnight at 4 °C or for adequate T4 DNA ligase, one hour at ambient temperature.

The efficiency of the ligation is verified by electrophoresis by loading 1 µl ligated plasmid and open plasmid (i.e. 5 ng of plasmid) on 1 % agarose gel in TBE buffer stained with appropriate staining. Ligated plasmid is characterized by a shorter migration in the agarose gel.

##### 7.1.4.2.2 Transformation of competent *Escherichia coli*

Competent *E. coli* are transformed by heat shock as described below. Competent cells ($10^8$ cfu·µg$^{-1}$ of DNA) freshly thawed out are incubated for 5 min on ice. Then 1 µl of the ligation reaction is added to cells, smoothly mixed, and incubated for 20 min on ice. Bacterial cells are heat shock treated for 50 s incubation at 42 °C and immediately placed on ice and incubated for 2 min. Then 950 µl of SOC medium are added and the bacterial cells are incubated at 37 °C under agitation at 150 min$^{-1}$ for 1 h. 100 µl bacterial cells aliquots are plated onto LB/Amp/IPTG/X-Gal solid medium. Petri dishes are then incubated at 37 °C overnight.

##### 7.1.4.2.3 Screening for recombinant clone

Plates are placed at 4 °C for several hours to accentuate coloration of bacterial colonies. White colonies are picked, plated onto LB/Amp/IPTG/X-Gal solid medium, and incubated overnight at 37 °C. Several white colonies were picked and put in 100 µl molecular grade water. PCR is carried out to confirm the presence of the insert in the recombinant clone. The insert is amplified by PCR using SP6 (5'-ATT TAG GTG ACA CTA TAG –3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG –3') primers. The amplification reaction is carried out in a final 25 µl volume containing 2,5 µl of 10 × Taq polymerase buffer, 200 µmol·l$^{-1}$ of each dNTP, 1,5 mmol·l$^{-1}$ of $MgCl_2$, 0,5 µmol·l$^{-1}$ of each primer and 0,625 U of Taq polymerase. A volume of 2,5 µl of bacterial suspension is used as template for the PCR reactions. PCR is performed in a thermocycler according to the following program: one cycle of 4 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 55 °C,

1,5 min at 72 °C, and a final extension step at 72 °C for 5 min. The size of the expected qPCR amplicon is verified by electrophoresis on 2 % agarose gel in TBE buffer stained with appropriate staining.

#### 7.1.4.2.4 Purification and linearization of recombinant plasmid

Recombinant clones, white in colour confirmed by PCR are inoculated to 10 ml LB/Amp liquid medium incubated at 37 °C under agitation (150 $min^{-1}$) overnight. Plasmid is purified from 2 ml cell suspension using conventional mini-preparation. Plasmid DNA is then quantified by spectro-photometry at 260 nm and aliquots are prepared and stored at –20 °C until use.

Plasmid is linearized with a restriction enzyme presenting a single restriction site in the sequence of the plasmid. User shall make sure that the chosen restriction enzyme is not also cutting the insert. Digestion of the plasmid is performed overnight at 37 °C in a final volume of 10 µl containing 250 ng of recombinant plasmid, 0,5 U of restriction enzyme, 1 µl of 10 × restriction enzyme buffer, and molecular grade water. The efficiency of the restriction of the plasmid is verified by electrophoresis on 1 % agarose gel. Linearized plasmid is stored at -20 °C and is used as stock solution to prepare serial dilution of qPCR standard used to calibrate qPCR assay.

The concentration in DNA of linearized plasmid is measured by spectrophotometry at 260 nm or by spectrofluorimetry in order to determine the plasmid copies number. This operation can be facilitated by using an online calculator such as oligo calc (http://www.basic.northwestern.edu/biotools/oligocalc.html). From this stock solution, an initial solution containing 0,5 × $10^8$ copies of the qPCR standard per µl is prepared in 100 µl of molecular grade water. Tenfold serial dilutions are then prepared to reach 0,5 × $10^1$ copies of the plasmid per µl. Additional intermediary dilutions can also be prepared depending on the range where copy numbers are expected.

### 7.1.5 Calibration of the qPCR (task 1, step 3)

#### 7.1.5.1 General

The procedure used to generate the calibration curve and evaluate the efficiency of the qPCR assay is described thereafter.

#### 7.1.5.2 qPCR assay

qPCR calibration assay is performed on serial dilution of the cloned standard (ranging from $10^8$ to $10^1$ copies per µl) using a primer pair specifically targeting the gene of interest. The amplification reaction is carried out in a final 15 µl volume containing 2 µl of plasmid standard, 1 $\mu mol \cdot l^{-1}$ of each primer, 7,5 µl of 2 × Taq master mix or 1,5 µl of 10 × Taq master mix containing a fluorescent DNA binding dye, dNTPs, $MgCl_2$, and Taq polymerase and molecular grade water. qPCR reaction is performed in a real-time thermocycler according to the following program: one cycle of 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C, 30 s at 80 °C where the fluorescence is collected, and a final dissociation stage by increasing the temperature from 80 °C to 95 °C. qPCR calibration is performed in triplicate and three NTC are also included.

#### 7.1.5.3 Establishment of the calibration curve and calculation of qPCR efficiency

At the end of qPCR assay, results are analyzed using the automatic option. Validation of the qPCR required the observation of: a) no amplification for NTC, b) a single dissociation peak for each dilution of qPCR standard, and c) a linear calibration curve with $r^2$ equal or superior to 98 %. qPCR calibration curve is giving the number of Ct as a function of the amount of the log of the number of copy of standard sequences.

The efficiency of the qPCR assay is estimated as given in Formula 3 from values determined from the calibration curve formula.

$$\mathrm{Ct} = a \cdot q + c \tag{3}$$

where

Ct is measured cycle threshold;

$q$ is the copy number of qPCR standard;

$a$ slope of the calibration curve;

$c$ ordinate at the origin (Ct for 1 copy of qPCR standard).

$$E = 10^{\left(-1/a\right)} - 1 \tag{4}$$

where

$E$ is the efficiency of the calibration assay;

$a$ slope of the calibration curve.

It shall be noticed that calibration curve having a slope equal to −3,32 is 100 % efficient. A twofold- or a 10-fold-dilution of a given DNA template gives a Ct difference of 1 or of 3,3 respectively (i.e. $10^8$ Ct = 10 and $10^7$ Ct = 13,3) for qPCR assay efficient at 100 %.

## 7.2 Preparation of soil DNA template and inhibition test (task 2)

### 7.2.1 General

This task describes the procedure used to prepare DNA template and to check for the presence of Taq polymerase inhibitors in DNA extracts used as template for qPCR assay. This test is an obligatory prerequisite to validate the quality of DNA extracts and allow their use as template for conducting qPCR assays.

### 7.2.2 Soil DNA preparation (task 2, step 4)

Step 4 of task 2 describes the procedure used to extract DNA from soil samples. Soil samples shall be collected, handled, and stored according to ISO 10381-6. Soil DNA extraction shall be done according to ISO 11063. Soil DNA samples are diluted to 1 ng·µl$^{-1}$ and 0,1 ng·µl$^{-1}$ and stored at −20 °C until their use.

### 7.2.3 Inhibition test (task 2, step 5)

The procedure used to test for the presence of Taq polymerase inhibitors in soil DNA extracts is described thereafter. This step is prerequisite that shall be done prior to perform qPCR assay from soil DNA extracts. Indeed, Taq polymerase inhibitors such as humic acid substances often co-extracted with soil DNA and only soil DNA extract free of inhibitors can be subjected to qPCR analysis to estimate the abundance of selected microbial gene sequences in soil. Two inhibition tests are described thereafter.

#### 7.2.3.1 Spiking of exogenic DNA in soil DNA extract

Search for inhibitors can be done by quantifying the abundance of exogenic DNA spiked in known amount to soil DNA. The protocol proposed below describes the analysis done after spiking plasmid DNA to soil DNA extract. This procedure can be adapted to any exogenic DNA sources by performing the qPCR with appropriate primer pair specific for the sequence of spiked DNA.

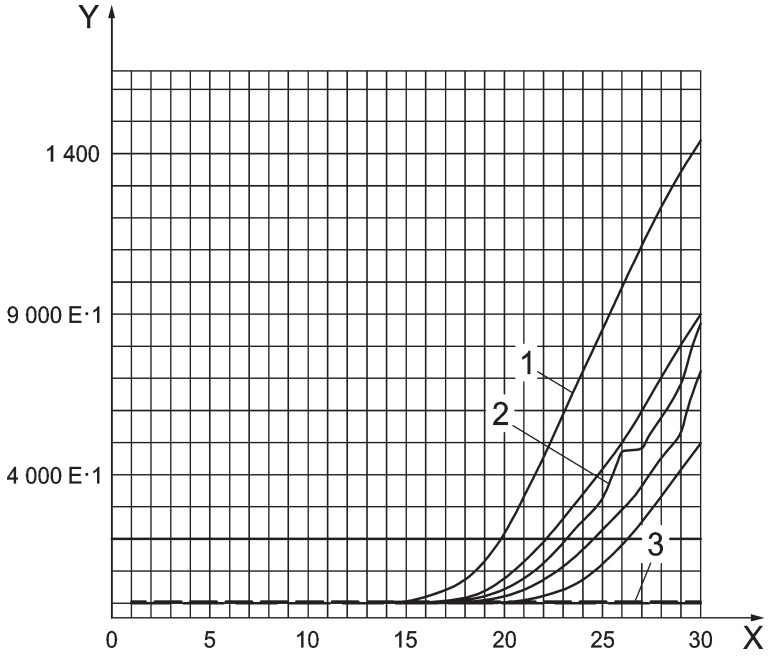
qPCR is performed using SP6 and T7 primers specific from the plasmid. The amplification reaction is carried out in a final 15 µl volume containing 2 µl of plasmid DNA, 2 µl of soil DNA (using the two dilutions 1 and 0,1 ng·µl$^{-1}$), 1,0 µmol·l$^{-1}$of each primer, 7,5 µl of 2 × Taq master mix or 1,5 µl of 10 × Taq master mix containing fluorescent DNA binding dye dNTPs, $MgCl_2$, and Taq polymerase and molecular grade water. qPCR reaction is performed in a real-time thermocycler according to the following program: one cycle of 15 min at 95 °C; 30 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. SP6-T7 qPCR inhibition test is performed for each dilution of soil DNA extract to be tested, three positive controls containing only plasmid DNA and three NTC.

The inhibition test is validated by observing the following:

a) no amplicon in NTC control;

b) similar Ct values in qPCR performed from soil DNA extract and plasmid DNA (Figure 2).

Soil DNA dilution showing no inhibition is chosen as template to perform qPCR assay.




**Key**

X ΔRn
Y cycle
1 pGEM-T
2 partial inhibition
3 inhibition

**Figure 2 — Results of inhibition test carried out by qPCR assay targeting SP6-T7 region of pGEM-T plasmid spiked in known amount to soil DNA extracts**

If a full or partial inhibition is observed (i.e. no amplicon or drift in Ct value, respectively), soil DNA extract shall be diluted to remove the inhibition and submitted again to a new inhibition test. If this does not yield in the improvement of the qPCR inhibition test, then the DNA extracts shall be further purified as recommended in ISO 11063 and submitted again to the inhibition test. When acceptable results are obtained for the inhibition test, soil DNA samples can be used to run qPCR assay.

It shall be noticed that qPCR assay performed from soil DNA extract can be improved by using carriers that favour the activity of Taq polymerase. Among most popular serum albumin bovine used at 400 ng·µl$^{-1}$of qPCR, reaction is proved to eradicate inhibition. Alternatively, T4 gene 32 protein at 30 ng·µl$^{-1}$of qPCR reaction is giving similar results.

#### 7.2.4 Dilution of DNA template

Alternatively, the presence of Taq polymerase inhibitors in soil DNA extract can be searched by performing qPCR assay targeting the gene of interest using as template 10 times dilution of soil DNA extracts (10 ng·µl$^{-1}$, 1 ng·µl$^{-1}$, 0,1 ng·µl$^{-1}$). The qPCR assay is performed following the procedure described above. qPCR inhibition test is performed for each dilution of soil DNA extract to be tested including three positive controls containing only standard DNA and three NTC. The inhibition test is validated by observing the following:

a) no amplicon in NTC control;

b) amplification in positive control;

c) for each sample test, Ct values difference of 3,32 between 10 times dilution.

Soil DNA extracts showing inhibition shall be treated as suggested in 7.2.1.

### 7.3 qPCR assay (task 3)

#### 7.3.1 General

Step 6 of task 3 describes the procedure to quantify the abundance of a microbial gene (called thereafter gene of interest) from soil DNA extract. qPCR reactions are performed using as template a) the dilution of soil DNA extract chosen in step 5, task 2 (inhibition step), b) duplicate each dilution of the calibration curve (from 0,5 × $10^8$ to 0,5 × $10^8$ copies of standard DNA per µl), and c) three non-template controls made of molecular grade water only.

#### 7.3.2 qPCR

qPCR assay targeting the gene of interest is performed on duplicate of each soil DNA templates at the dilution showing no inhibition of Taq polymerase on duplicate of each plasmid standard dilution (ranging from $10^8$ to $10^1$ copies per µl) and on triplicate of NTC. Primer pair specific for gene of interest are used. The amplification reaction is carried out in a final 15 µl volume containing 2 µl of DNA template, 1 µmol·l$^{-1}$ of each primer, 7,5 µl of 2 × Taq master mix or 1,5 µl of 10 × Taq master mix containing fluorescent DNA binding dye dNTPs, $MgCl_2$, and Taq polymerase and molecular grade water. qPCR reaction is performed in a real-time thermocycler according to the following program: one cycle of 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at appropriate annealing temperature, 30 s at 72 °C, and 30 s at 80 °C where the fluorescence is collected and a final dissociation stage by increasing the temperature from 80 °C to 95 °C.

### 7.4 Validation and analysis of qPCR assay (task 4)

#### 7.4.1 General

Step 7 of task 4 describes the procedure used for checking the validity of the qPCR assays and processing qPCR crude results. Step 8 of task 4 proposes two modes of calculation to estimate the abundance of the gene of interest expressed either as sequence copy number per ng of soil DNA or per g of soil.

#### 7.4.2 Validation of the qPCR assay

##### 7.4.2.1 General

At the end of the qPCR reaction, results are analyzed using the automatic option. The parameters to be considered to validate the qPCR assay are detailed thereafter.

#### 7.4.2.2 qPCR assay

Validation of the qPCR requires the observation of the following:

a) no amplification for NTC;

b) a linear calibration curve with $r^2$ equal or superior to 98 %;

c) a dissociation curve showing a single peak at the expected melting temperature specific for each gene target attesting for the specificity of the amplification.

It is noteworthy that if degenerated primer pair is used, divergent sequences are amplified from soil DNA extract and as a result, dissociation curve cannot form a single sharp peak as observed for the standard, but a smooth peak.

### 7.4.3 Calculation of the copy number of the gene of interest in the soil DNA extract

#### 7.4.3.1 General

For each qPCR assay, the equation of the calibration curve shall be calculated, as well as qPCR efficiency. These two parameters shall be recorded with the estimated number of copies of the gene of interest. The abundance of the gene of interested estimated qPCR assay can further be exploited to express the results as sequence copy number per ng of soil DNA or per g of soil.

#### 7.4.3.2 Estimation of the number of sequences of the gene of interest per ng of soil DNA

Formula 5 shall be used to estimate the number of sequences of the gene of interest per ng of soil DNA.

$$n\left[\text{gene of interest}\right]_1 = q \Big/ \left(2 \times c\left[DNA\right]\right) \tag{5}$$

where

$q$ is the number of sequences of the gene of interest measured in the assay;

2 is the volume of soil DNA used as template for the qPCR assay, in µl;

$c[DNA]$ is the concentration of soil DNA extract used to run qPCR assay, in $ng \cdot \mu l^{-1}$;

$n[\text{gene of interest}]_1$ is the number of sequences of the gene of interest per ng of soil DNA.

#### 7.4.3.3 Estimation of the number of sequences of the gene of interest per g of soil

Formula 6 shall be used to estimate the number of sequences of the gene of interest per g of soil.

$$n\left[\text{gene of interest}\right]_2 = \left(n\left[\text{Gene of interest}\right]_1 \times y\right) \Big/ z \tag{6}$$

where

$n[\text{gene of interest}]_1$ is the number of sequences of the gene of interest per ng of soil DNA;

$y$ is the amount of soil DNA extracted from $z$ g of soil sample, in ng;

$z$ is the amount of soil sample from which DNA is extracted, in g of dry mass equivalent;

$n[\text{gene of interest}]_2$ is the number of sequences of the gene of interest per g of soil (dry mass equivalent).

## 8 Examination of the critical steps of the qPCR assay

For each qPCR assay, the laboratory shall examine the following values obtained for three critical steps shown in Figure 1 in order to validate the qPCR assay and process with further analysis of qPCR data:

a) verifying the absence of Taq polymerase inhibitors;

b) estimating the efficiency of the qPCR assay from the calibration curve;

c) checking the quality of the amplification by analysing the dissociation curve (for fluorescent DNA binding dye qPCR assay only) and the absence of contaminant in NTC samples.

## 9 Expression of the results of the qPCR assay

The results of the qPCR assay shall be presented as tables of results including the abundance of selected microbial gene sequences estimated by qPCR from DNA directly extracted from soil expressed in sequence copy number of gene of interest per ng of soil DNA and/or in sequence copy number of gene of interest per gram of soil (dry mass equivalent). The results should also present the equation of the standard curve, as well as the values of non-template control.

## 10 International ring test

This method for quantifying the abundance of microbial groups was evaluated through an international ring test involving six different laboratories on DNA samples extracted directly from six different soils. The ring test was composed of two parts aiming at estimating the effect of qPCR kit and of T4Gp32 on the quantification of the abundance of microbial groups and evaluating the repeatability and the reproducibility of the quantification of the abundance of microbial groups. The reports of the two parts of the ring test are provided in Annexes A and B, respectively.

## 11 Test report

The test report shall include the following information:

a) a reference to this International Standard, i.e. ISO 17601:2016;

b) soil DNA extract information including name of the sample, date of extraction, duration, and conditions of nucleic acids storage (if appropriate);

c) thermal conditions used to conduct qPCR assay (cycle program, thermal ramping) and method used to analyse crude qPCR results;

d) tables of results including the abundance of selected microbial gene sequences estimated by quantitative real-time PCR from DNA directly extracted from soil expressed in sequence copy number of gene of interest per ng of soil DNA and/or in sequence copy number of gene of interest per gram of soil (dry mass equivalent);

e) any details not specified in this International Standard or which are optional, as well as any effect which may have affected the results.

# Annex A
(informative)

# Description of principal steps of TaqMan® qPCR assay

## A.1 General

Additional information is given in this annex to describe only parts of the protocol of TaqMan® qPCR assay diverging from that described in Figure 1 of this International Standard.

## A.2 qPCR standard preparation and calibration (task 1)

TaqMan® qPCR assay is based on the release of a fluorescent dye following the hydrolysis of an internal probe as a result of the 5′–3′ exonuclease activity of Taq polymerase is estimated at the end of each PCR cycle. The purpose of this task is to describe the definition of appropriate amplicon to settle down a qPCR assay (step one), the preparation of qPCR standard (step two), and the calibration of the qPCR assay (step three). In the case of TaqMan® qPCR assay, steps two and three are identical to those described in this International Standard for fluorescent DNA binding dye qPCR assay. However, due to technical specifications, step one is different.

### A.2.1 Amplicon design(task 1, step 1)

First, the TaqMan® probe shall be designed and then the primers chosen to surround as close as possible to the probe without overlapping. Amplicon size shall be not larger than 250 bases. The main parameters to be considered to design the internal probe are listed thereafter:

— Size of the probe shall be kept in the range of 20 bases and should be no more than 80 bases away from the 5′end of the amplicon;

— GC content shall be kept in the 20 % to 80 % range;

— the melting temperature ($T_m$) should be between 58 °C and 65 °C (similar to that of the primer pair used in the TaqMan® assay);

— avoid succession of identical nucleotide, especially true for guanine,

— do not put a G on the 5′ end of the TaqMan® probe,

— select the strand with more Cs than Gs.

The recommendations listed for qPCR to choose the primer pair shall also be fulfilled to design primers for TaqMan® qPCR. However, users shall keep in mind that the shortest amplicon length is the best, but without overlap with TaqMan® probe.

### A.2.2 Calibration of the qPCR assay (task1, step 3)

qPCR calibration assay is performed on serial dilution of the cloned standard (ranging from $10^8$ to $10^1$ copies per µl) using a primer pair and an internal probe specifically targeting the gene of interest as well. The amplification reaction is carried out in a final 15 µl volume containing 2 µl of plasmid standard, 1 µmol·l$^{-1}$ of each primer, 250 nmol·l$^{-1}$ of TaqMan® probe, 7,5 µl of 2 × TaqMan® master mix or 1,5 µl of 10 × TaqMan® master mix, dNTPs, $MgCl_2$, and Taq polymerase and molecular grade water. qPCR reaction is performed in a real-time thermocycler according to the following program: one cycle of 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 30 s at annealing temperature, 30 s at 60 °C. qPCR calibration is performed in triplicate and three NTC are also included.

## A.3 qPCR assay (task 3)

TaqMan® qPCR assay targeting the gene of interest is performed on duplicate of each soil DNA templates at the dilution showing no inhibition of Taq polymerase, on duplicate of each plasmid standard dilution (ranging from $10^8$ to $10^1$ copies per µl) and on triplicate of NTC. Primer pair and TaqMan® probe specific for gene of interest are used. The amplification reaction is carried out in a final 15 µl volume containing 2 µl of plasmid standard, 1 µmol·l$^{-1}$ of each primer, 250 nmol·l$^{-1}$ of TaqMan® probe, 7,5 µl of 2 × TaqMan® master mix or 1,5 µl of 10 × TaqMan® master mix, dNTPs, $MgCl_2$, and Taq polymerase and molecular grade water. qPCR reaction is performed in a real-time thermocycler according to the following program: one cycle of 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at annealing temperature, and 30 s at 60 °C. qPCR calibration is performed in triplicate and three NTC are also included.

## A.4 Validation and analysis of qPCR assay (task 4)

Validation of the TaqMan® qPCR assay required the observation of the following:

a) no amplification for NTC;

b) a linear calibration curve with $r^2$ equal or superior to 98 %.

# Annex B
(informative)

# International ring-test for evaluating qPCR to quantify the abundance of selected microbial gene sequences from DNA directly extracted from soil

## B.1 Laboratories involved in the ring-test

Six laboratories participated in the international ring test: qPCR machines, qPCR kits, and used of T4Gp32 by the different partners are presented in the Table B.1.

**Table B.1 — List of qPCR machine, qPCR kit and use of T4Gp32 used by each laboratory involved in the ISO 17601 international ring-test**

| Laboratory | qPCR machine | qPCR kit | T4Gp32 |
|---|---|---|---|
| A | BioRad CFX96 | Sybr® Select Master Mix, Applied Biosystems | yes |
| B | Via 7, Applied Biosystems | Absolute Sybr Green low rox mix, Thermo Scientific | yes |
| C | 7500 Fast, Applied Biosystems | Power SYBR Green® PCR Master Mix, Applied Biosystems | yes |
| D | 7300, Applied Biosystems | Power SYBR Green® PCR Master Mix, Applied Biosystems | yes |
| E | Mx3005P, Stratagene | KAPA SYBR® FAST Universal qPCR kit | no, replaced by BSA |
| F | 7300, Applied Biosystems | Power SYBR Green® PCR Master Mix, Applied Biosystems | yes |

## B.2 Organization of the ring-test

The ring test protocol was describing the objectives of the ring test, the materials to be used (soil DNA, qPCR primers), the qPCR assay method to be applied, and the procedure of the delivery of the results. The organizer (INRA) of the ring-test was in charge of extracting, purifying, and quantifying soil DNA according to ISO 11063. It was in charge of preparing soil DNA dilutions and of testing by qPCR assay (as described in ISO/DIS 17601) soil DNA extracts for the presence of Taq polymerase inhibitors. When all these different steps were accomplished and validated, it was also in charge of sending all the materials required to conduct the ring-test to all the partners including soil DNA, qPCR primers, qPCR standards, and qPCR protocol. All the materials were sent in dry ice package using appropriate transporter.

All the partners were in charge to conduct qPCR assays targeting both *16S rRNA* and *nirK* (nitrite reductase) gene sequences using their own facilities (qPCR machine and qPCR kit, see Table B.1) following the protocol provided by the organizer. In particular, each partner has to repeat three times each qPCR reaction on a same plate in order to estimate the *variability* of the quantification and each qPCR assay on different plates to test the *repeatability* of the quantification. After having performed qPCR assays, all partners were asked for filling provided excel sheets with results obtained and for sending their results to the organizer of the ring test.

Finally, the organizer was in charge of collecting, analyzing, and synthesizing the results of the ring-test in a report.

## B.3 Material and methods

### B.3.1 Soils

Six different soils have been used to extract DNA following the procedure described by ISO 11063. Soil physicochemical characteristics are provided in Table B.2 shown thereafter. Soil samples were collected from surface horizon (0 - 20 cm), sieved to 2 mm, and kept frozen at -20° C until used.

**Table B.2 — Physicochemical characteristics of soils used for the ISO 17601 ring-test**

| Location | Gargalianoi | Thessaloniki | La Piacenza | Viseu | Limagne | Pierrelaye |
|---|---|---|---|---|---|---|
| Country | Greece | Greece | Italy | Portugal | France | France |
| Soil textural class | Sandy loam | Loam | Sandy loam | Sandy clay loam[1] | Silty clay loam | Sandy loam |
| Sand (%) | 71 | 25,9 | 82,3 | 55,2 | 23 | 75,0 |
| Silt (%) | 19 | 47,6 | 13,5 | 13,6 | 39 | 17,0 |
| Clay (%) | 10 | 26,5 | 4,2 | 31,2 | 38 | 8,0 |
| pH (a.u.) | 5,5 | 8,6 | 7,33 | 5,20 | 7,8 | 7,4 |
| Organic carbon (%) | 1,23 | 0,59 | 1,52 | nd | 1,9 | 1,5 |
| Soil DNA ($ng.\mu L^{-1}$) | 2,4 ± 0,1 | 4,9 ± 0,4 | 3,9 ± 0,4 | 2,7 ± 1,1 | 2,0 ± 0,18 | 4,47 ± 0,7 |

### B.3.2 Soil DNA extraction, purification, quantification and inhibition test

DNA was extracted directly from soil samples and purified according to ISO 11063 procedure derived from Reference [11]. For each soil, three different replicates were extracted ($n_{tot} = 18$). Size and quality of genomic DNA were checked by electrophoresis on 1 % agarose gel. DNA was quantified by fluorescence's measurement of the Picogreen dye using the Quant-iT™ dsDNA Assay Kit (Invitrogen, USA).

Soil DNA concentration was comprised between 2,0 ± 0,2 and 4,9 ± 0,4 $ng.\mu l^{-1}$ for the Limagne (France) and Thessaloniki (Greece) soils, respectively. The highest variability in the concentration of DNA extracts was observed for the soil of Viseu (Portugal).

The presence of PCR inhibitors in the soil DNA extracts was verified by mixing a known amount of control plasmid DNA (pGEM-T Easy Vector, Promega, France) either with water or extracted soil DNAs. The control plasmid was then quantified in all samples by qPCR using a Via-7 (Applied Biosystems, USA) and universal primers SP6 and T7 (sequences of primers are given in Table B.3) in accordance with the procedure described by Henry et al. (2006). Inhibition tests were performed on DNA samples diluted at 1, 0,5, and 0,1 $ng.\mu l^{-1}$. No inhibition of SP6-T7 qPCR amplification was recorded for samples of soil DNA diluted at 0,1 $ng.\mu l^{-1}$ (data not shown). Accordingly, soil DNA extracts diluted to 0,1 $ng.\mu l^{-1}$ in molecular grade ultrapure water, were kept at −0,20 °C until use for the ring-test.

### B.3.3 Quantitative PCR assay

#### B.3.3.1 Primer pairs

Primer pairs used to target pGEM-T, *16S rRNA*, and *nirK* sequences are given in Table B.3.

**Table B.3 — Primer pairs used to amplify pGEM-T, *16S rRNA*, and *nirK* sequences by qPCR**

| Target | Forward primer 5' - > 3' | Reverse primer 5' - > 3' | Tm (°C) | Reference |
|---|---|---|---|---|
| Pgemt | SP6 GGTGACTATTTAACTATAG | T7 AATACGACTCACTATAG | 55 | Promega |
| 16SrRNA | 341F GGACCTACGGGCAGCAG | 543 ATTACCGCGGCTGCTGGCA | 60 | Lopez-Gutierrez et al., 2004 |
| nirK | 876F ATYGGCGGVCAYGGCGA | 1040 GCCTCGATCAGRTTRTGGTT | 58 | Henry et al., 2004 |

#### B.3.3.2 *16S rRNA* and *nirK* qPCR assays

The abundances of the global bacterial community and of the denitrifiers were estimated by qPCR assays using 341F-534R primer pair targeting *16S rRNA* sequences and 876F/1040R primer pair targeting *nirK* sequences, respectively. Quantitative PCR assays were carried out in accordance to furnisher recommendations. Standard curves for all the assays were obtained using tenfold serial dilutions of a linearized plasmid pGEM-T Easy Vector ($10^2$ to $10^7$ copies) containing the appropriate standard sequence. Melting curves were generated after amplification by increasing the temperature from 80 °C to 95 °C. qPCR assays were conducted in triplicate on all samples ($n_{tot}$ = 54) and the results were calculated as mean values of these assays. Variances are indicated as standard deviation (SD) of all measurements.

For each gene target, each partner has to carry out on the same plate qPCR reactions in triplicate on the 18 soil DNA extracts to assess the variability of qPCR assay and to repeat this three times in order to assess the repeatability of qPCR assay (i.e. 6 soils × 3 different extractions × 3 repeats per plate × 3 plates = 162 samples analysed per partner and per gene target). For the *16S rRNA* qPCR, the results of the six partners ($n_{tot\_16S}$ = 972) were taken into consideration for conducting statistical analyses. For the *nirK* qPCR assay, the results of five of six partners were analyzed ($n_{tot\_nirK}$ = 810).

### B.3.4 Statistical analyses

Several statistical analyses have been done using XLSTAT©[4] 2009 (Addinsoft) such as Student's *t* test, Fischer's F test, ANOVA test, and Kruskal-Wallis test ($\alpha$ = 0,05) in order to estimate the effect of the different parameters tested on the quantification of the abundance of microbial sequences from soil DNA extracts. The size of statistical effect was estimated by calculating the d index of Cohen as follows: $d = (m_1-m_2)/[(e_1+e_2)/2]$ where $m_x$ and $e_x$ are mean and standard deviation for x (Cohen, 1992).

## B.4 Results

### B.4.1 *16S rRNA* and *nirK* qPCR standard curves analyses

For each gene target (*16S rRNA* and *nirK* genes), a standard curve was generated starting from dilution $10^6$ to $10^2$ copies per µl. qPCR assays were performed in triplicate on three different plates by each partner. In total for each gene target, 18 standard curves were generated by the six partners involved in the ring-test for ISO/DIS 17601. The parameters of the standard curves (slope, Y-intercept and $R^2$ of standard curves) as well as the qPCR efficiencies of *16S rRNA* and *nirK* qPCR assays are shown in the Figures B.1 and B.2.

4) XLSTAT is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product.

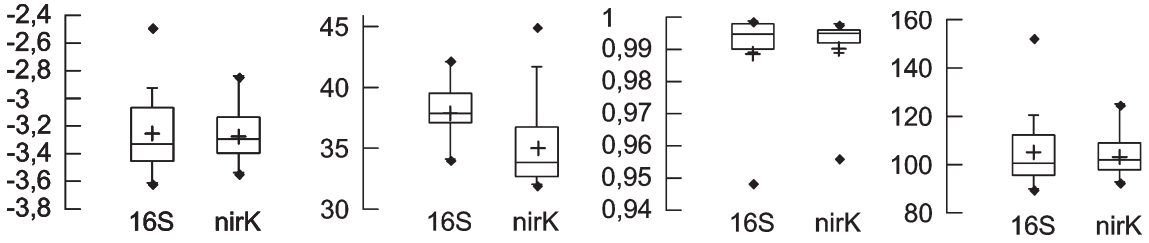



**Figure B.1 — Boxes and whisker plots showing distribution of standard curves parameters (slope, Y-intercept and $R^2$) and qPCR assay efficiency measured by the six laboratories involved in the ring-test for *16S rRNA* (16S) and *nirK* (nirK) qPCR assays**

One could observe that slope values measured for *16S rRNA* qPCR were comprised between −3,623 < $slope_{16S}$ < −2,483 and for *nirK* between −3,543 < $slope_{nirK}$ < −2,845 (see Figure 1). Mean slope values for *16S rRNA* and *nirK* qPCR were −3,24 ± 0,29 and −3,27 ± 0,17, respectively. Y-intercept measured for *16S rRNA* were comprised between 34,10 < $Y\text{-intercept}_{16S}$ < 42,21 and for *nirK* between 31,94 < $Y\text{-intercept}_{nirK}$ < 45,147. Mean Y-Intercept for *16S rRNA* and *nirK* qPCR assays were 38,01 ± 2,37 and 35,08 ± 3,85, respectively. Correlation coefficients of the standard curve ($R^2$) for *16S rRNA* qPCR were comprised between 0,948 < $r^2_{16S}$ < 0,999 and for *nirK* between 0,956 < $r^2_{nirK}$ < 0,998. Mean $R^2$ for *16S rRNA* and *nirK* qPCR assays were 0,989 ± 0,016 and 0,990 ± 0,011, respectively. Finally, it is noteworthy that calculated *16S rRNA* qPCR efficiencies were comprised between 88,80 < $qPCR\ efficiency_{16S}$ < 152,76 and for *nirK* between 99,91 < $qPCR\ efficiency_{nirK}$ < 101,48. Mean qPCR efficiencies for *16S rRNA* and *nirK* qPCR assays were 104,82 ± 15,32 and 102,77 ± 8,12, respectively. Besides, a few number of extreme values the variations of the parameters estimated from the standard curves and of the qPCR efficiency of *16S rRNA* and *nirK* qPCR assays are acceptable.

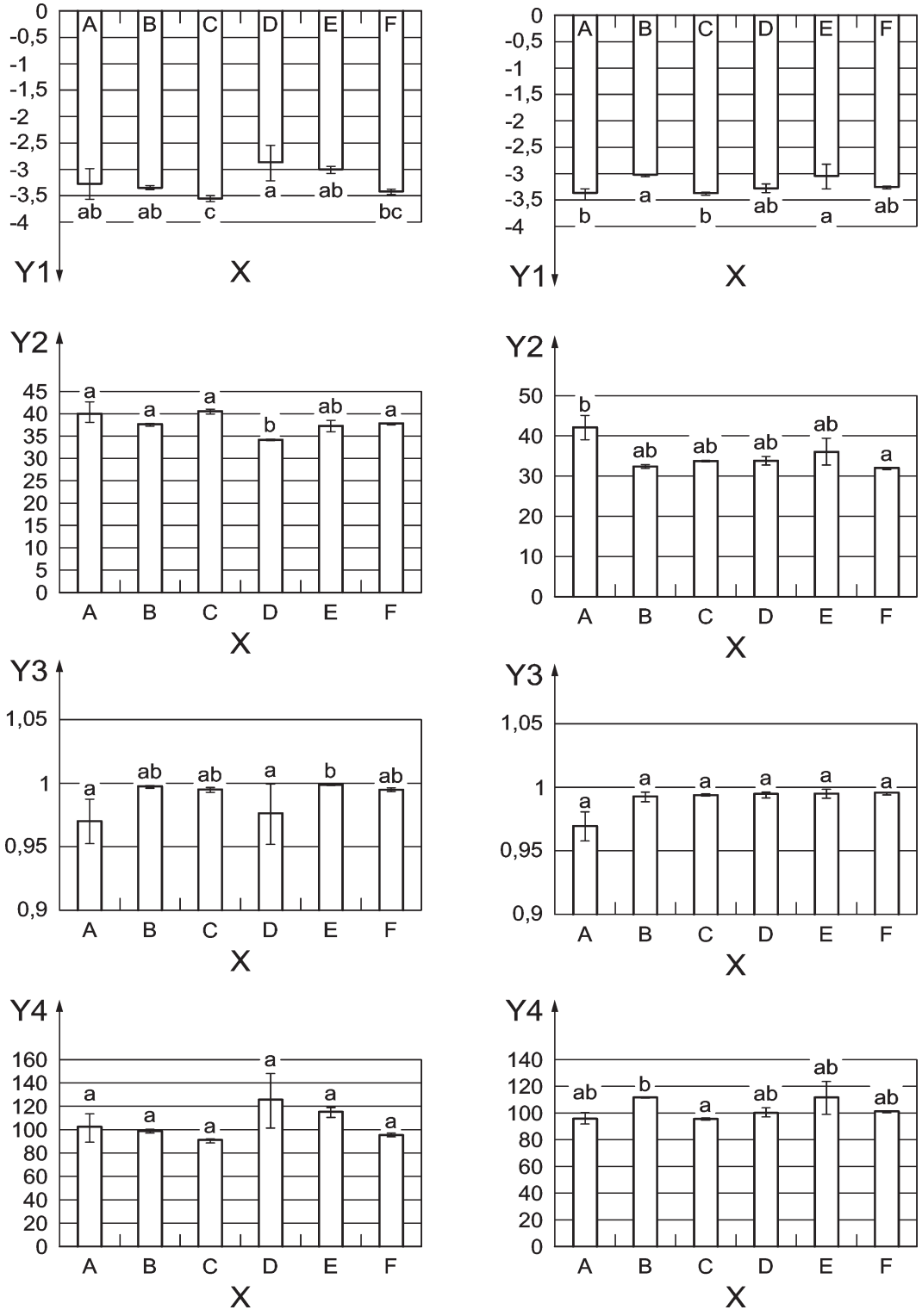



**Figure B.2 — Mean values of standard curves parameters (slope, Y-intercept and $R^2$) and qPCR assay efficiency measured by the six laboratories involved in the ring test for *16S rRNA* (blue) and *nirK* (brown) qPCR assays**

1) Bars indicate standard deviation.

2) Bars identified by different letters significantly differ.

Statistical analyses were performed using non-parametric statistics by applying the procedure of Dunn used a posteriori of Kruskal-Wallis test (significance of the test $\alpha = 0,05$). The results of these analyses

are shown in Figure B.2. Although some significant differences can be observed between different laboratories for several parameters of standard curves, it did not impact significantly the efficiency of *16S rRNA* qPCR assay. This was not the case for *nirK* qPCR assays which showed statistically different efficiencies (B > A, D, E, *F* > C).

Conclusions: In any of the cases, mean efficiencies for both qPCR assays were comprised within an acceptable range (85 % < qPCR efficiency < 115 %) thereby, validating the values presented in the ring-test.

### B.4.2 Repeatability of qPCR assays

The repeatability of both *16S rRNA* and *nirK* qPCR assays was addressed by comparing the mean sequence copy numbers obtained by repeating three times each qPCR assays on three different plates. The mean sequence copy number is calculated from the values obtained for the six different soil DNA extracts.

#### B.4.2.1 *16S rRNA* qPCR assay

The three independent plates obtained by B, C, and F laboratories showed an excellent repeatability of *16S rRNA* quantification. Indeed, they were found to be clustered in the same statistical group: laboratory B, group I, laboratory C, group D, and laboratory F, group H (Table B.4). In addition, the three independent repeats obtained by the three laboratories (A, D, and E) showing a weaker repeatability of *16S rRNA* quantification were clustered in different statistical groups: laboratory A, groups F (5,1), G (5,1), and I (4,6); laboratory D, groups C (5,6), D (5,5), and E (5,3); laboratory E, groups A (5,8), B (5,7), and D (5,5). However, it has to be noticed that except for laboratory A for which one repeat was significantly lower than the two others that were almost identical (plate#1 = 4,5 < plate#2 = plate#3 = 5,1), results obtained by laboratories D and E are rather similar (i.e. 5,3 < laboratory D < 5,6 and 5.5 < laboratory E < 5,8).

**Table B.4 — Statistical analyses of the repeatability of *16S rRNA* qPCR assays (no matter of the soil DNA considered) carried out by ANOVA (α = 0,05) on the mean values of the log (*16S rRNA* copy number per 0,2 ng of soil DNA)**

| Plate#laboratory | Mean log 16SrRNA copy number | Statistical group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Plate#-3*Lab-E | 5,824 | A | | | | | | | | |
| Plate#-2*Lab-E | 5,689 | | B | | | | | | | |
| Plate#-3*Lab-D | 5,613 | | | C | | | | | | |
| Plate#-2*Lab-C | 5,502 | | | | D | | | | | |
| Plate#-1*Lab-E | 5,488 | | | | D | | | | | |
| Plate#-2*Lab-D | 5,477 | | | | D | | | | | |
| Plate#-3*Lab-C | 5,474 | | | | D | | | | | |
| Plate#-1*Lab-C | 5,446 | | | | D | | | | | |
| Plate#-1*Lab-D | 5,274 | | | | | E | | | | |
| Plate#-3*Lab-A | 5,145 | | | | | | F | | | |
| Plate#-2*Lab-A | 5,069 | | | | | | | G | | |
| Plate#-3*Lab-F | 4,861 | | | | | | | | H | |
| Plate#-1*Lab-F | 4,853 | | | | | | | | H | |
| Plate#-2*Lab-F | 4,844 | | | | | | | | H | |
| Plate#-1*Lab-B | 4,581 | | | | | | | | | I |
| Plate#-1*Lab-A | 4,573 | | | | | | | | | I |
| Plate#-3*Lab-B | 4,546 | | | | | | | | | I |
| Plate#-2*Lab-B | 4,541 | | | | | | | | | I |

### B.4.2.2 *nirK* qPCR assay

Results of the quantification of the abundances of *nirK* sequences are shown in Figure B.4. *nirK* sequence copy numbers quantified in all the different soils were comprised between 2,6 and 3,6 log of *nirK* copy number per 0,2 ng of soil DNA. The results obtained by laboratory E were not taken into account because they were higher than those obtained by the other laboratories (i.e. plate#1 = 5,4, plate#2 = 5,7, and plate#3 = 5,8 log of *nirK* copy number) and also out of the range of the *nirK* abundances recorded in the literature (Bru et al., 2011). Besides, it is well known that *nirK* denitrifiers represent a few percent of the total bacterial community while the *nirK* abundance recorded by lab E was in the range of that of the *16S rRNA* (i.e. plate#1 = 5,5, plate #2 = 5,7 and plate#3 = 5,8 log of 16S *rRNA* copy number. This was most likely due to non-specific amplification which can be seen from melting curve and from electrophoresis of *nirK* amplicons which were giving a smear. This non-specific amplification might be due to technical limitation of the Stratagene qPCR used by laboratory E.

The three independent repeats obtained by laboratory F showed an excellent repeatability of *nirK* qPCR assay (Table B.5). They were found to be clustered in the same statistical group laboratory F, group D. In addition, the three independent repeats obtained by the four other laboratories (A, B, C, and D) showing a poor repeatability of *nirK* qPCR assay were clustered in different statistical groups: laboratory A, groups F (2,9) and G (2,7); laboratory B, groups F (2.,9; 2,8), and G (2.,7); laboratory C, groups E (3,1; 3,0) and G (2,7); laboratory D, groups A (4,2), B (3,9), and C (3,8). It is noteworthy that missing values in plate#1 of laboratory A do not allow taking it into consideration in the statistical analysis. However, it has to be noticed that for each laboratory, the range of variation of mean *nirK* sequence number between different plates was relatively low (i.e. 2,7 < laboratory A < 2,9; 2,7 < laboratory B < 2,9; 2,7 < laboratory C < 3,1; and 4,2 < laboratory D < 3,8).

**Table B.5 — Statistical analyses of the repeatability of *nirK* qPCR assays (no matter of the soil DNA considered) carried out by ANOVA (α = 0,05) on the mean values of the log (*nirK* copy number per 0,2 ng of soil DNA)**

| Plate#-laboratory | Mean log nirK copy number | | | | | | |
|---|---|---|---|---|---|---|---|
| Plate#-3*Lab-D | 4,225 | A | | | | | |
| Plate#-2*Lab-D | 3,990 | | B | | | | |
| Plate#-1*Lab-D | 3;811 | | | C | | | |
| Plate#-3*Lab-F | 3,375 | | | | D | | |
| Plate#-1*Lab-F | 3,313 | | | | D | | |
| Plate#-2*Lab-F | 3,308 | | | | D | | |
| Plate#-1*Lab-C | 3,130 | | | | | E | |
| Plate#-2*Lab-C | 3,054 | | | | | E | |
| Plate#-2*Lab-B | 2,936 | | | | | F | |
| Plate#-2*Lab-A | 2,893 | | | | | F | |
| Plate#-3*Lab-B | 2,860 | | | | | F | |
| Plate#-1*Lab-B | 2,723 | | | | | | G |
| Plate#-3*Lab-C | 2,718 | | | | | | G |
| Plate#-3*Lab-A | 2,706 | | | | | | G |
| Plate#-1*Lab-A | | | | | | | |

### B.4.2.3 Conclusion on the estimation of *16S rRNA* and *nirK* qPCR repeatability

One could conclude that the overall repeatability of *16S rRNA* qPCR assay was higher than that of *nirK* qPCR assay most likely because of the nature of primer pairs used (high degeneracy of *nirK* primer) and the higher abundance of *16S rRNA* sequences in soil DNA extracts makes it easier to amplify while the *nirK* sequences representing a few percent of the overall bacterial community.

Over the six laboratories, three (B, C, and F) demonstrated excellent repeatability for the *16S rRNA* qPCR assay, the mean *16S rRNA* sequence numbers obtained for the three independent plates being identical. The other three laboratories (A, D, and E) had an acceptable repeatability (laboratory A Δplate = 0,6; laboratory D Δplate = 0,3; laboratory E Δplate = 0,3). It is noteworthy that the lowest repeatability recorded for laboratory A was due to one of the plates for which significantly less *16S rRNA* sequences were amplified than in the two others (plate#1 = 4,5 < plate#2 = plate#3 = 5,1).

Laboratory F was the only one to obtain an excellent repeatability in the *nirK* qPCR assay, the mean *nirK* sequence numbers obtained for the three independent plates being identical. Even if the four other laboratories had variation in the quantification of *nirK* sequence copy numbers between the independent plates, the repeatability was acceptable (laboratories A and B Δplate = 0,2; laboratories C and D Δplate = 0,4).

### B.4.3 Reproducibility of qPCR assays

The reproducibility of both *16S rRNA* and *nirK* qPCR assays was addressed by comparing the mean sequence copy numbers obtained by each laboratory on each soil. Data set was analysed statistically to search for a laboratory effect and for a soil effect.

#### B.4.3.1 *16S rRNA* qPCR assay

One could observe that no matter of the soil considered the laboratory B quantified significantly less *16S rRNA* than the other laboratories (see Figure B.3). On the other hand, laboratory E was quantifying significantly more *16S rRNA* than all other laboratories. Only laboratories C and D were quantifying similar amount of *16S rRNA* sequences in all soil DNA extracts considered. The estimation of the size of the effect by the calculation of the index d of Cohen confirmed the statistics in most of the cases (except for differences recorded between laboratories F/A and B/F for the soils of La Piacenza and Pierrelaye, respectively). Overall, the ranking of laboratories according to the amount of *16S rRNA* amplified from almost all the soils considered was lab B < lab A = lab *F* < lab C = lab D < lab E. It has to be noticed that laboratories F and A yield in significantly different values for the soils of Viseu, Limagne, Pierrelaye, and Gargalianoi.

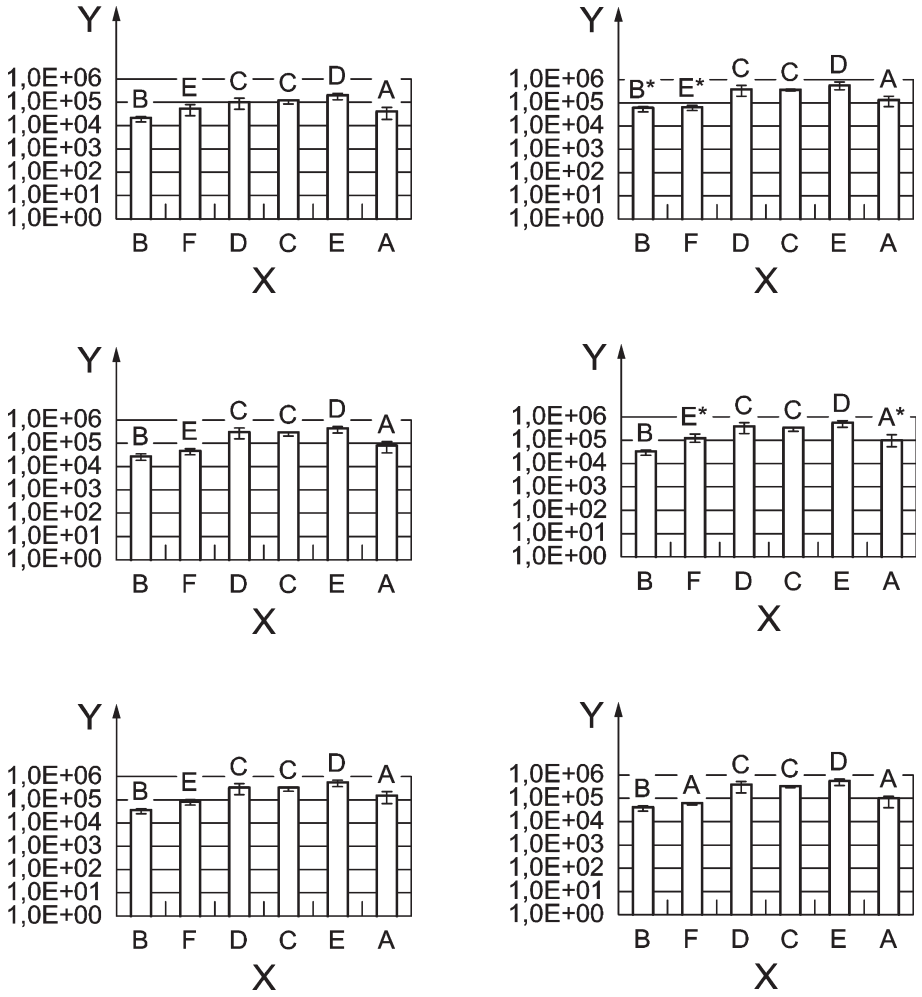



**Figure B.3 — Quantification of *16S rRNA* copy number in the six studied soils (Viseu, Gargalianoi, La Piacenza, Limagne, Pierrelaye, and Thessaloniki) by the six laboratories (A to F)**

1) Values are given as mean number of copies of *16S rRNA* per 0,2 ng of soil DNA.

2) Standard deviation is given on each bar. Values of bars indicated with different letters are significantly different ($P < 0,05$).

3) Indicates values that are not differing although belonging to different statistical classes according to the calculation of the size of the effect (Cohen, 1992).

### B.4.3.2 *nirK* qPCR assay

One could observe that for most of soil, DNA extracts considered the laboratory B quantified significantly less *nirK* sequence copy number than the other laboratories (see Figure B.4). On the other hand, laboratory D was quantifying significantly more *nirK* sequence copy number than all other laboratories.

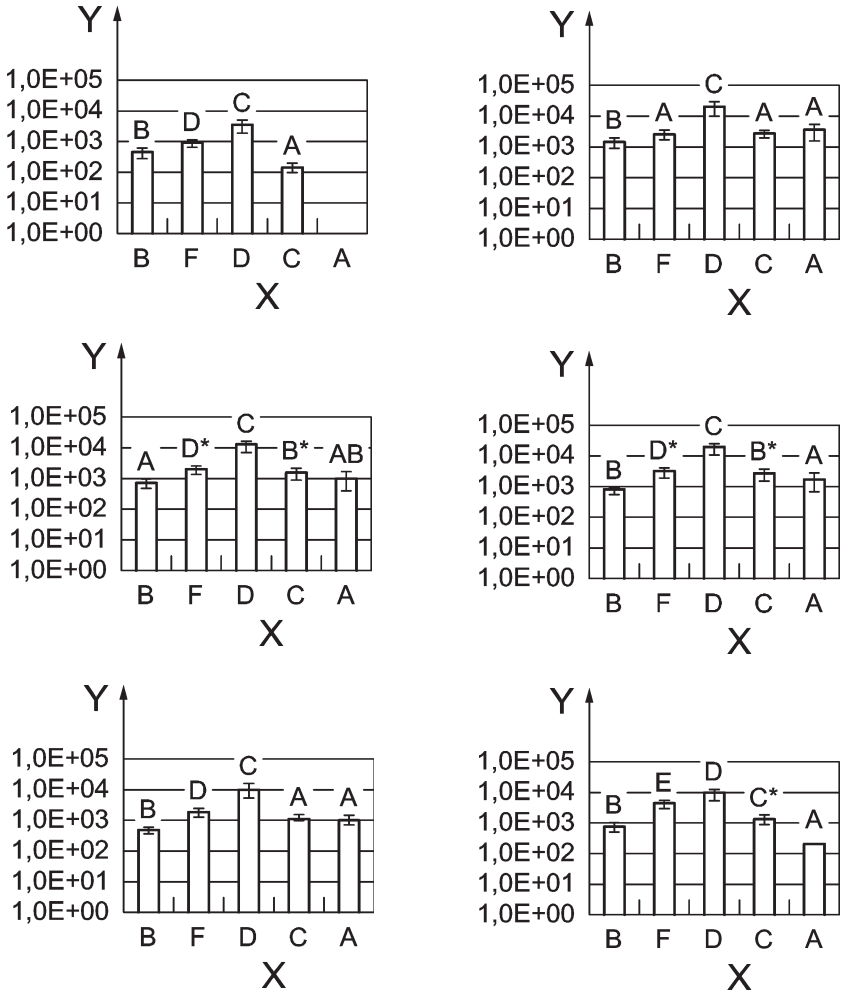



**Figure B.4 — Quantification of *nirK* sequence copy number in the six studied soils (Viseu, Gargalianoi, La Piacenza, Limagne, Pierrelaye, and Thessaloniki) by the six laboratories (A to F)**

1) Values are given as mean number of copies of *nirK rRNA* per 0,2 ng of soil DNA.

2) Standard deviation is given on each bar.

3) Values of bars indicated with different letters are significantly different ($P < 0, 05$).

4) Indicate values that are not differing although belonging to different statistical classes according to the calculation of the size of the effect (Cohen, 1992).

One could observe that for three soils (Limagne, La Piacenza, and Pierrelaye) laboratories C and F quantified similar amounts of *nirK* sequence numbers. For the soil of Pierrelaye, three laboratories (A, C, and F) quantified similar amount of *nirK* sequence numbers (except for differences recorded between laboratories F/C for the soils of La Piacenza and Limagne). Overall, the ranking of laboratories according to the amount of *nirK* amplified observed for most of the soils was lab B < lab A = lab C < lab *F* < lab D. It has to be noticed that laboratories A and C yield in significantly different *nirK* values for the soil of Thessaloniki, laboratory A had significant lower *nirK* values than laboratory B for Viseu and Thessaloniki soils, and laboratory C had significant lower *nirK* values than laboratory D.

### B.4.3.3 Conclusions on the estimation of *16S rRNA* and *nirK* qPCR reproducibility

The analysis of the reproducibility of *16S rRNA* and *nirK* qPCR assays revealed that for each soil, the sequence copy number quantified by both qPCR assays is significantly differing from one laboratory to another. Depending on the soil, *16S rRNA* sequence copy numbers were varying by a factor of 9 to 18

and *nirK* sequence copy number by a factor comprised between 13 and 50. This is in agreement with the better repeatability of *16S rRNA* qPCR than that of *nirK* qPCR assay. Statistical analysis showed that no matter of the soil considered, the laboratories can be ranked according to the amount of *16S rRNA* and *nirK* sequence copy number quantified. Interestingly, one could observe that laboratories C and D equipped with similar qPCR machines (ABI 7300 and ABI 7500) and using identical qPCR kit (Power SYBR Green® PCR Master Mix) quantified similar amounts of *16S rRNA* sequence copy number. This observation is in line with the first part of the ring test showing a clear effect of the qPCR kit on the amount of *16S rRNA* quantified. In addition, one could observe that laboratory E, the only one not using T4-Gp32 in qPCR mix, did not get acceptable results for *nirK* quantification. This is also in line with the first part of the ring-test showing that T4-Gp32 favoured the quantification of both *16S rRNA* and *nirK* sequence copy numbers. Altogether, it further suggests that the differences observed between the laboratories might result from differences in the equipment and chemical (qPCR kit and T4Gp32) used to carry out qPCR assays. However, this cannot be the only explanation because these two laboratories did not quantified the same *nirK* sequence copy number and laboratory F equipped with the same qPCR machine and using the same kit (see Table B.2) obtained significantly different *16S rRNA* and *nirK* sequence copy numbers than laboratories C and D. This last observation suggests the existence of a manipulator effect.

Despite the fact that all the laboratories worked with the same soil DNA extracts, PCR primers and qPCR standards, we observed for each of the six soils significant differences in the quantification of *16S rRNA* and *nirK* sequence copy number between the different laboratories. These differences are resulting from a range of parameters that cannot be dissociated (i.e. manipulator, qPCR machine, and chemical).

### B.4.4 Comparison of *16S rRNA* and *nirK* sequence copy number quantified in the different soils

We compared the results obtained by the different laboratories to range the different soils according to the number of *16S rRNA* and *nirK* sequence copy numbers which are known to vary from one soil to another depending on their physicochemical properties.

#### B.4.4.1 *16S rRNA* qPCR assays

One could observe that the range of variation of the *16S rRNA* abundances quantified in the different soils is comprised between a factor of 2 and 4 depending on the laboratory. Therefore, the variation due to the "laboratory effect" is much higher than the "soil effect". However, for all the laboratories with the exception of laboratory F, the lowest abundances of the bacterial community were found in the soil of Viseu, a sandy clay loam poor in organic matter. In addition, on the other extreme, most of the laboratories (except F) reported the highest abundances of the bacterial community for the soil of Pierrelaye, a sandy neoluvisoil amended for almost 100 years with waste water rich in organic matter. The classification of the soils according to the abundance of the bacterial community obtained by the different laboratories further confirms these observations (Table B.6). These observations tend to indicate that although we showed a clear "laboratory effect" on the estimation of the amount of *16S rRNA* sequence copy number, the interpretation of the results generated by each laboratory allowed classifying the soils in a similar manner according to the abundance of bacterial community. However, this conclusion has to be taken with care since only six soils were considered and also because one laboratory (F) was slightly diverging from the five others.

**Table B.6 — Ranking of soil according to *16S rRNA* abundances [from the lowest (top-) to the highest (down-table) values] obtained for the different laboratories (A to F). The letter indicated into brackets gives the statistical group**

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Viseu (A) | Viseu (A) | Viseu (A) | Viseu (A) | Viseu (B) | Limagne (A) |
| Limagne (D) | Limagne (B) | Limagne (B) | Limagne (B) | Limagne (A) | Viseu (AB) |
| Thessaloniki (D) | Piacenza (B) | Gargalianoi (BC) | Thessaloniki (B) | Gargalianoi (A) | Pierrelaye (BC) |
| Piacenza (C) | Gargalianoi (BC) | Piacenza (C) | Gargalianoi (B) | Piacenza (A) | Thessaloniki (C) |

**Table B.6** *(continued)*

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Pierrelaye (BC) | Thessaloniki (C) | Thessaloniki (BC) | Piacenza (B) | Thessaloniki (A) | Gargalianoi (D) |
| Gargalianoi (B) | Pierrelaye (D) | Pierrelaye (C) | Pierrelaye (B) | Pierrelaye (A) | Piacenza (E) |

### B.4.4.2 *nirK* qPCR assays

The quantification of the abundance of the denitrifying bacterial community estimated by *nirK* qPCR done by each laboratory in the six different soils are shown in Figure B.4. One could observe that the range of variation of the *nirK* abundances quantified in the different soils is comprised between a factor 3 and 19 depending on the laboratory. Therefore, the variation due to the "soil effect" is much lower than the one caused by the "laboratory effect". However, it can be seen from Figure B.4 that all the laboratories recorded the lowest abundances of denitrifiers in the soil of Viseu which is in accordance with the abundance of the total bacterial community. In addition, on the other extreme, most of the laboratories (except F) reported the highest abundances for the bacterial denitrifiers in the soil of Pierrelaye in line with the quantification of the abundance of the bacterial community. The classification of the soils according to the abundance of the bacterial denitrifiers obtained by the different laboratories further confirms these observations (Table B.7).

**Table B.7 — Ranking of soil according to *nirK* abundances [from the lowest (top-) to the highest (down-table) values] obtained for the different laboratories (A to F). The letter indicated into brackets gives the statistical group; different letters indicate significant differences**

| A | B | C | D | F |
|---|---|---|---|---|
| Viseu (nd) | Viseu (A) | Viseu (A) | Viseu (A) | Viseu (A) |
| Thessaloniki (A) | Gargalianoi (B) | Gargalianoi (B) | Gargalianoi (B) | Gargalianoi (B) |
| Limagne (B) | Limagne (B) | Thessaloniki (B) | Thessaloniki (B) | Limagne (B) |
| Gargalianoi (B) | Piacenza (B) | Limagne (B) | Limagne (B) | Pierrelaye (BC) |
| Piacenza (C) | Thessaloniki (B) | Piacenza (B) | Piacenza (C) | Piacenza (CD) |
| Pierrelaye (D) | Pierrelaye (C) | Pierrelaye (C) | Pierrelaye (CD) | Thessaloniki (D) |

### B.4.4.3 Conclusions

It is well known that the soil type is affecting the abundances of bacterial community and of denitrifiers. The analysis of the results produced by the six laboratories allowed classifying the six different soils in a similar manner according to *16S rRNA* and *nirK* abundances. These observations tend to indicate that although a clear "laboratory effect" can be seen on the estimation of the amount of both *16S rRNA* and *nirK* sequence copy number, the interpretation of the results generated by each laboratory allowed classifying the soils in a similar manner according to the abundance of bacterial denitrifiers. However, this conclusion has to be taken with care since only six soils were considered and also because one laboratory (F) was slightly diverging from the five others.

## B.5 Conclusions and recommendations

### B.5.1 Conclusions

According to the analysis of the standard curves of both *16S rRNA* and *nirK* qPCR assays, the values obtained by the different laboratories involved in the ring-test were validated. From this ring-test, the following can be concluded:

— repeatability of both *16S rRNA* qPCR and *nirK* qPCR assays was good. *nirK* qPCR assay was showing a lower repeatability than that of 16S rRNA qPCR which could be due to the high degeneracy of *nirK* primers or to the lower abundance of *nirK* –carrying bacteria in soil;

— reproducibility of both *16S rRNA* qPCR and *nirK* qPCR assays revealed a marked “laboratory effect” on the sequence copy number measured in the different soil DNA extracts;

— analysis of the ”soil effect” on the abundances of the bacterial community (*16S rRNA* qPCR) and of the denitrifiers (*nirK* qPCR) showed that soils can be ranked in a similar manner by most of the laboratories.

### B.5.2 Recommendations

On the basis of the results of the international ring-test, one could recommend the users of ISO 17601 to

— evaluate the repeatability of their qPCR assay (as described in the ring-test),

— interpret the results of the quantification of the abundance of the microbial groups by comparison either by using an external reference (DNA extracted from a control strain) in the assay or by calculating a percentage of variations between treatments to normalize the data, and

— avoid comparing sequence copy numbers measured in different laboratories if data are not normalized (see above).

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