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Soil quality — Method to directly extract DNA from soil samples

Qualité du sol — Méthode pour extraire directement l'ADN d'échantillons de sol

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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11063 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

DNA (deoxyribonucleic acid) is an essential component of any living organism coding for enzymes responsible for any biological activities. The study of DNA sequences from DNA sources extracted from different matrixes, 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 soil quality indicators applicable to complex environments, such as soil, were biased by the unculturability of many microorganisms and the lack of sensitivity of traditional microbiological methods [16]. The recent development of numerous molecular biology methods based primarily 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 [15], [18], [26], [27], [36]. DNA-based approaches are now well-established in soil ecology and serve as genotypic (= molecular genetic) markers for determining microbial diversity.

The results of molecular analyses of soil microbial communities and/or populations rely on two main parameters:

a) the extraction of DNA representative of the indigenous bacterial community composition;

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 [23], [26], [38], [40]. Recently, numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils [20].

The aim of this International Standard is to describe the procedure used to extract DNA directly from soil samples. The reproducibility of this soil DNA extraction procedure was assessed in an international ring-test study (Annex A). The reproducibility of this soil DNA extraction procedure was successfully evaluated on both quantitative (q-PCR) and qualitative (A-RISA) approaches.

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Soil quality — Method to directly extract DNA from soil samples

1 Scope

This International Standard specifies a method for direct extraction of DNA from soil samples to analyse the global structure and the abundance of soil bacterial communities using PCR-based technologies. This method is mainly dedicated to agricultural and forest soils. This method can possibly not be suitable for soils rich in organic matter (e.g. peat soils) or soils heavily polluted with organic pollutants or heavy metals.

The direct extraction of DNA from soil samples provides unique insight into the richness and structure of microbial communities which are key parameters to estimate the biodiversity of soil microbiota. Molecular approaches based on PCR (polymerase chain reaction) amplification of soil DNA constitute a promising domain and can contribute in the near future to the development of routine tools to monitor the microbiota of soil environments.

Users of the method ought to be aware that although soil submitted to the DNA extraction procedure is sieved thoroughly (2 mm mesh, procedure described in 5.1), plant residues can still remain in soil samples and, as a result, traces of plant DNA can contaminate the soil DNA extract.

2 Normative references

The following referenced documents are indispensable for the application 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 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*

3 Terms and definitions

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

3.1
soil DNA
DNA extracted from soil-living microorganisms and remaining DNA from dead microorganisms

4 Principle

DNA is directly extracted from 0,25 g soil samples using the following extraction procedure. This method reliably allowed analysing the global structure of bacterial and archeal communities and could be adapted (extraction from a 1 g soil sample) to assess the global structure of fungal communities[32]. Soil samples added with an extraction buffer are submitted to mechanical and chemical lyses. The lysis step, e.g. by bead beating, is a crucial step to also extract DNA from microbes that are difficult to lyse. After a brief centrifugation, soil debris are removed and proteins are precipitated with potassium acetate. After centrifugation, the supernatant is recovered and nucleic acids are precipitated with ice-cold isopropanol. After centrifugation, the nucleic acids pellet is washed with 70 % ethanol and suspended in sterile ultra-pure water. DNA quality is then checked by electrophoresis on an agarose gel and the DNA quantity is estimated using a spectro-fluorimeter. A schematic overview of the procedure is given in Figure 1.

5 Test materials

5.1 Soil

Soil samples should be collected and sieved (2 mm mesh). If samples are not immediately processed, they should be stored for up to two years at −20 °C or up to 10 years at −80 °C or in liquid nitrogen (−180 °C) as specified in ISO 10381-6. If soil samples are frozen, they may be thawed only once. Some of these storage conditions are currently under testing.

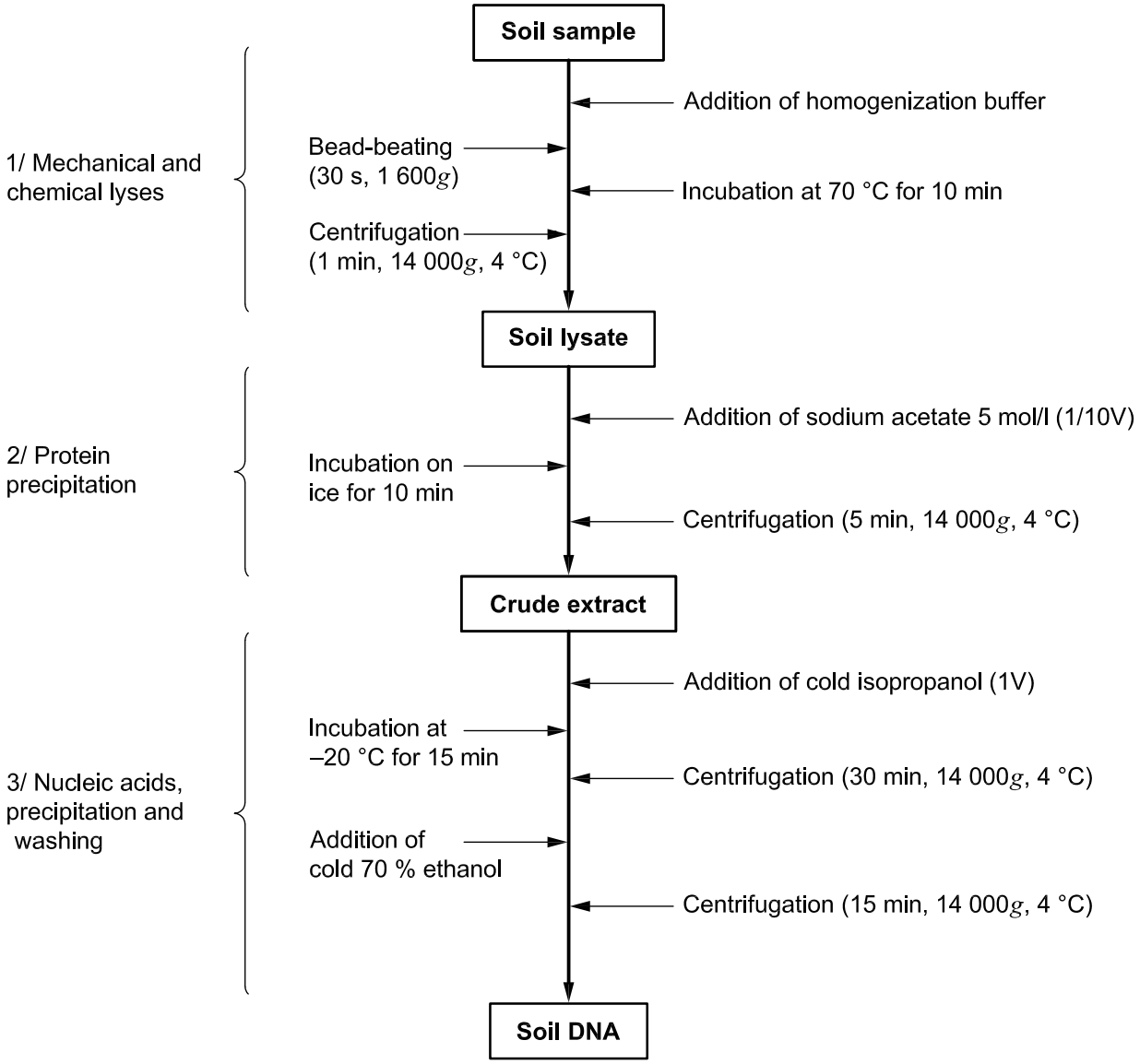

Figure 1 — Schematic overview of soil DNA extraction procedure

5.2 Chemicals

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

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

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

5.2.4 Sodium dodecyl sulfate (SDS), $CH_3(CH_2)_{11}OSO_3Na$ (CAS No. 151-21-3).

5.2.5 Polyvinylpyrrolidone (PVP), $[C_6H_9NO]_n$ (CAS No. 9003-39-8).

5.2.6 Sodium acetate, CH_3COONa (CAS No. 6131-90-4).

5.2.7 Acetic acid or glacial acetic acid, CH_3COOH (CAS No. 64-19-7).

5.2.8. Isopropanol, $CH_3CHOHCH_3$ (CAS No. 67-63-0).

5.2.9 Ethanol, CH_3CH_2OH (CAS No. 64-17-5).

5.2.10 Molecular-biology-grade water, H_2O.

5.3 Buffers and reagents

Buffers and reagents (except intercalent molecules) used for soil DNA extraction are sterilized (120 °C for 20 min) and stored at room temperature. Ethanol and isopropanol are stored at −20 °C.

5.3.1 Tris-HCl, **1 mol/l**, 121,14 g of tris in 1 000 ml of H_2O, adjusting with 4 mol/l HCl to pH 8,0.

5.3.2 EDTA, **0,5 mol/l**, 186,10 g of EDTA in 1 000 ml of H_2O, adjusting with NaOH (10 mol/l) to pH 8,0.

5.3.3 NaCl, **1 mol/l**, 58,44 g of NaCl in 1 000 ml of H_2O.

5.3.4 PVP 40, **20 %**, 200 g of PVP in 1 000 ml of H_2O.

5.3.5 SDS, **20 %**, 200 g of SDS in 1 000 ml of H_2O.

5.3.6 Homogenization buffer (newly prepared just before being used), 100 ml of 1 mol/l tris-HCl (pH 8,0), 200 ml of 0,5 mol/l EDTA (pH 8,0), 100 ml of 1 mol/l NaCl, 50 ml of 20 % PVP 40, 100 ml of 20 % SDS in 450 ml of H_2O.

5.3.7 Sodium acetate, 5 mol/l (pH 5,5), 410,15 g of CH_3COONa in 800 ml of H_2O. Add 120 ml of acetic acid and then adjust the pH to 5,5 with glacial acetic acid. Add water to make up to 1 000 ml.

5.3.8 Ethanol, 70 %, 700 ml of pure ethanol in 300 ml of H_2O.

5.3.9 TE buffer, pH 8,0, 10 mmol/l tris-HCl, 1 mmol/l EDTA.

5.3.10 Glass beads (106 µm).

5.3.11 Glass beads (2 mm).

5.3.12 Ethidium bromide, 5 mg of ethidium bromide in 1 000 ml of H_2O.

5.3.13 Fluorescent nucleic acid stain, excitation at 480 nm and emission at 520 nm.

5.3.14 Pure DNA (100 ng/µl)

5.3.15 TBE buffer × **10**, pH 8,0, 108 g of tris base, 55 g of boric acid, 40 ml of 0,5 mol/l EDTA (pH 8,0) in 1 000 ml of H_2O.

5.3.16 TBE buffer × 1, 100 ml of TBE buffer × 10 in 900 ml of H_2O.

6 Apparatus

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

6.1 Mini-bead beating apparatus, with a beating frequency varying from, for example, 100 min^{-1} to 2 600 min^{-1} and a 16 mm amplitude of agitation.

6.2 Spectro-fluorimeter, allowing the quantification of double-strand DNA at 520 nm with a fluorescent nucleic acid stain excited at 480 nm.

7 Procedures

7.1 Preparation of soil samples

Weigh 0,25 g of soil (equivalent dry mass) in 2 ml micro-tubes just before extracting, or immediately freeze the soil sample in liquid nitrogen and keep it frozen at −80 °C until its use.

7.2 Mechanical and chemical lyses

Add 0,5 g of 106 µm glass beads (wear a mask for protection) and two glass beads (2 mm diameter) to the soil sample. Add 1 ml of homogenization buffer (composition given in 5.3.6). Agitate the soil samples 1 600g for 30 s (16 mm of amplitude) using a bead-beating system (tube support previously placed at −20 °C). Incubate at 70 °C for 10 min. Centrifuge for 1 min at 14 000g (4 °C). Carefully recover the supernatant and transfer it to a new 2 ml microtube.

7.3 Protein precipitation

To the supernatant obtained in 7.2, add 5 mol/l sodium acetate (pH 5,5) (composition given in 5.3.7) of an amount that is 1/10 of the volume of the supernatant. Mix by vortexing and incubate on ice for 10 min. Centrifuge for 5 min at 14 000g (4 °C). Carefully recover the supernatant and transfer it to a new 1,5 ml microtube.

7.4 Nucleic acid precipitation and washing

Perform all these steps below a fume hood because of dangerous isopropanol vapours. Liquid and solid wastes shall be evacuated as chemical waste.

To the supernatant obtained in 7.3, add cold isopropanol (-20 °C) of an amount that is 1/1 of the volume of the supernatant. Incubate the samples at −20 °C for 15 min. Centrifuge for 30 min at 14 000g (4 °C). Carefully eliminate the supernatant. Wash the nucleic acids pellet with cold 70 % ethanol (do not resuspend the pellet). Centrifuge for 15 min at 14 000g (4 °C). Eliminate any traces of ethanol and let the nucleic acid pellet dry for 15 min at 37 °C. Suspend the pellet in 100 µl of ultra-pure water or TE buffer (pH 8) (composition given in 5.3.9).

7.5 Nucleic acid storage

Aliquot the soil DNA (4 × 25 µl) and store the DNA samples at −20 °C until their use. Repeated freezing and thawing of the DNA extracts should be omitted.

8 Estimation of soil DNA quality and quantity

8.1 Soil DNA quality and purity

The quality and the size of the soil DNA are checked by electrophoresis on 1 % agarose gels in TBE buffer. Gels are stained with appropriate staining (e.g. ethidium bromide, 5 mg/l). The purity of the soil DNA is assessed by spectrophotometry at 260 nm for the DNA analysis and at 400 nm for humic acid substances [11].

The step of chemical and mechanical lysis is critical, and it should be adequate to lyse a representative portion of microbes but avoid fragmentation of the DNA [39].

DNA extracts which are still slightly brownish need a further DNA purification.

8.2 Soil DNA quantity

The soil DNA content is determined using a fluorescent nucleic acid stain (5.3.13) which fluoresces when intercalated within the double helix of DNA. A calibration curve relating the amount of standard DNA (5 ng, 10 ng, 20 ng, 50 ng, 100 ng, 150 ng and 200 ng of pure DNA) to the amount of fluorescence quantified is established and used to estimate the amount of DNA extracted from the soil. Measurements are performed using a spectro-fluorimeter (6.2). The analysis is carried out by relevant software.

Alternatively, the soil DNA content can be determined by resolving soil DNA extracts by electrophoresis in a 1 % agarose gel, stained with ethidium bromide and photographed under a camera. Dilutions of pure DNA were included in each gel and a standard curve of DNA concentration (1 000 ng, 500 ng, 250 ng, 125 ng, 62,5 ng to 31,25 ng). The ethidium bromide intensity was integrated to establish a standard curve used for estimating soil DNA concentration as described previously by Reference [32].

Alternatively, the soil DNA content can be determined by spectrophotometry at 260 nm when soil DNA is lowly contaminated with humic acid substances (400 nm) and proteins (A260/A280 averaging 1,6).

9 Validation of the extraction procedure

The laboratory can validate the procedure of soil DNA extraction by processing the reference soil and comparing the obtained yield of soil DNA extraction to the expected one.

10 International ring test

This method for extracting soil DNA was evaluated through an international ring test involving nine different laboratories working on six different European soils. The report of this ring test is provided in Annex A.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard: ISO 11063:2012;

b) soil collection, including date and place (GPS coordinates) of collection;

c) treatment and storage of soil sample (e.g. sieving method, conditions and length of storage);

d) physical and chemical characteristics of the soil;

e) quantity of soil used for DNA extraction;

f) date(s) of extraction;

g) duration of nucleic acids storage (if appropriate);

h) tables of results including concentration of soil DNA extracts and amount of DNA extracted per gram of soil (dry weight equivalent);

i) 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)

International ring test for evaluating soil DNA extraction procedure

A.1 Introduction

Up to now, most of the microbial diversity studies conducted in complex ecosystems, such as soil, has been biased essentially by the unculturability of many microorganisms and the lack of sensitivity of traditional microbiological methods. In the past decade, applications of numerous molecular biology methods based primarily on amplification of indirect or direct soil-extracted nucleic acids (DNA or DNA/RNA) have provided a pertinent alternative to classical culture-based microbiological methods, providing unique insight into the composition, richness and structure of microbial communities [15], [18], [26], [27], [36]. DNA-based approaches are now well-established in soil ecology and serve as genotypic (= molecular genetic) markers for determining microbial diversity.

However, the results of molecular analysis of microbial communities rely mostly on two main parameters:

a) the extraction of DNAs representative of the indigenous bacterial community composition;

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. Recently, numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils.

Although comparative studies have been performed to analyse the efficiency of methods for extraction and purification of soil DNA recovered, the reproducibility of soil DNA extraction within different laboratories has not yet been assessed. Therefore, in this context, the aim of this study was to evaluate the reproducibility of a soil DNA extraction method developed by Reference [20].

A.2 Materials and methods

A.2.1 Laboratories involved in the study

Nine different laboratories from six European countries (France, Finland, Germany, Spain, Italy and Sweden) were involved in the international ring test with the following organization. Laboratory in charge of the program: INRA/Université de Bourgogne, Laboratoire de Microbiologie du Sol et de l'Environnement, (Dijon, France). The participant laboratories are listed below: Institut National de l'Environnement Industriel et des Risques, INERIS (Verneuil-en-Halatte, France); IPL santé, environnement durables Est, Laboratoire Etudes et Expertises (Nancy, France); Swedish University of Agricultural Sciences (Uppsala, Sweden); GSF Munich (Munich, Germany); Julius Kühn-Institut Bundesforschungsinsinstitut für Kulturpflanzen, JKI (Brauschweig, Germany); Universita di Catania, DACPA-Sezione Scienze Agrochimiche (Catania, Italy); CSIC, Estacion Experimental del Zaidin (Grenada, Spain); University of Helsinki (Helsinki, Finland).

A working group constituted of each responsible scientist of each research laboratory was established. The main objectives of the working group were to:

— design the set-up of the inter-laboratories assay,

— analyse and discuss the results produced in this study.

The procedure used for the international ring test study is described below. The working group decided that this assay should only concern the evaluation of the reproducibility of soil DNA extraction. To do so, the working group decided that each laboratory should extract DNA from different soil samples following the same protocol. Further work (DNA purification and DNA analyses) was only done by the laboratory leading this

project (Laboratoire de Microbiologie du Sol et de l'Environnement, Dijon, France) in order to avoid adding several biases due to these two additional steps.

Therefore, after soil DNA extraction, each laboratory involved in this project sent their samples to "Laboratoire de Microbiologie du Sol et de l'Environnement" for further analyses. Upon their arrival, soil DNA samples were purified as described in A.2.4. Soil DNA extracts were quantified on agarose gel stained with ethidium bromide. Following purification, extracted soil DNA was further analysed using PCR:

a) 16S rDNA quantitative PCR assay for estimating the total bacterial community abundance in studied soils;

b) *pcaH* and *narG* quantitative PCR assay for estimating the *narG* and *pcaH* microbial community abundances in studied soils;

c) A-RISA method (automatic ribosomal intergenic spacer analysis) for studying the structure of the global bacterial community.

A.2.2 Soil physico-chemical properties and preparation

The working group chose six different soils named soil A, B, C, F, M and soil S (see Table A.1) collected in different European countries. One soil was PAH-contaminated (collected in Finland), four were agricultural soils (collected in Sweden and France) whilst one was a forest soil (collected in Germany).

Fresh soil samples were sieved (2 mm mesh) and 250 mg aliquots were prepared in 2 ml tubes. These aliquots were immediately frozen in liquid nitrogen and stored at −80 °C until their shipment. Five aliquots of each soil were sent in dry ice to each laboratory where they were stored at −80 °C until soil DNA extraction were carried out.

Table A.1 — Physico-chemical characteristics of the studied soils

	Soil					
	A Forest soil	B Agricultural soil	C Agricultural soil	F PAH-contaminated soil	M Agricultural soil	S Agricultural soil
Clay %	17,6	nd	43,2	15,5	43,2	47,8
Silt %	26,9	nd	50,3	26,2	23,7	26,8
Sand %	55,5	nd	6,5	58,3	33,1	25,4
Organic C %	7,55	1,45	1,29	1,12	3,27	1,53
Total N %	0,46	0,12	0,14	0,33	0,36	0,16
C/N	16,5	11,7	9,21	33,5	9,11	9,7
Organic matter %	13,1	2,51	3,25	19,3	5,65	2,66
pH	3.76	6,41	7,50	6,22	7,88	7,99
CEC Metson ($cmol^+/kg$)	17,8	8,09	nd	10,7	19,9	12,6
P_2O_5 Olsen %	nd	0,09	0,03	nd	0,26	nd
nd = not determined CEC: Cation exchange capacity						

A.2.3 Soil DNA extraction

The procedure used for soil DNA extraction follows this International Standard. Briefly, 1 ml of a solution containing 100 mmol/l tris (pH 8,0), 100 mmol/l EDTA, 100 mmol/l NaCl, 1 % (mass fraction) polyvinylpyrrolidone, and 2 % (mass fraction) sodium dodecyl sulfate was added to 250 mg of soil in a 2 ml mini-bead-beater tube containing 0,5 g of 106-mm and two 2-mm-diameter glass beads. Samples were then homogenized for 30 s at 1 600g in a mini-bead-beater cell disruptor (6.1), after which the samples were centrifuged at 14 000g for 1 min at 4 °C. The collected supernatants were incubated for 10 min on ice with 1/10 volume of 5 mol/l sodium acetate

and centrifuged at 14 000g for 5 min. After precipitation with one volume of ice-cold isopropanol, the nucleic acids were added with 70 % ethanol. Soil DNA extracts were then shipped to Laboratoire de Microbiologie du Sol et de l'Environnement (Dijon, France), in dry ice.

It has to be noted that all the soil DNA extractions were done with two identical mini-bead beaters which were shared between the different laboratories involved in this project.

A.2.4 Soil DNA purification

The procedure used for soil DNA purification consists of two steps:

a) an affinity column constituted of polyvinylpyrrolidone (PVPP) which specifically bounds humic acid substances;

b) an exclusion column constituted of Sepharose 4B[1)] as described in Reference [20]. Briefly, PVPP-columns were prepared by placing 92 mg to 95 mg (i.e. 1,2 cm) of PVPP powder into micro-spin chromatography columns[2)] after which 400 µl of H_2O were added and the tubes were centrifuged (2 min at 1 000g). The procedure was repeated twice. For the Sepharose-columns, 1 ml of Sepharose 4B was placed in micro-spin chromatography columns and centrifuged (2 min, 1 100g) after which the columns were washed by the addition of 500 µl of TE buffer (tris-EDTA, pH 8) and centrifugation of the columns (2 min at 1 100g). After preparation of the columns, soil DNA extracts were purified by centrifugation of the sample first through the PVPP-column (1 000g for 4 min at 10 °C). The eluate was collected and was then passed through a Sepharose-column by centrifugation at 1 500g for 4 min (10 °C).

A.2.5 Soil DNA quantification

The quantity of the extracted soil DNA was determined by resolved samples in 1 % agarose gels. After electrophoresis, gels were stained with ethidium bromide. Dilutions of calf tymus DNA[3)] were included in each gel and a standard curve of DNA concentrations (60 ng, 30 ng, 15 ng, 7,5 ng and 3,25 ng) was used to estimate the quantity of DNA in the soil extracts. Obtained pictures of the gels were analysed using ImageQuaNT[4)] software. The quantity of DNA was determined using the standard curve relating the intensity of DNA band to the amount of DNA in ng. Yield of DNA extraction was calculated from the values as follows:

$$[\text{yield of DNA, µg/g of soil}] = \text{DNA (ng/µl)} \times 0{,}4.$$

Data were analysed using the non-parametric Kruskal-Wallis test ($p < 0{,}05$).

A.2.6 Inhibition test

The presence of PCR inhibitors in the soil extracts was tested by the qPCR assay by mixing DNA extracts with control plasmid DNA (pGEM-T Easy eVector[5)]). The test was performed on the purified soil DNA extracts diluted at four different concentrations: 1 ng/µl, 0,5 ng/µl, 0,1 ng/µl and 0,01 ng/µl. The control plasmid was amplified using universal primers Sp6 and T7 in accordance with the procedure described previously [13].

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1) Sepharose 4B is the trade name of a product supplied by GE Healthcare Europe GmbH. This information is given for the convenience of users of this International Standard 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.

2) Micro-spin chromatography columns are the trade name of a product supplied by Bio-Rad, USA. This information is given for the convenience of users of this International Standard 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.

3) Calf tymus DNA is the trade name of a product supplied by Bio-Rad, USA. This information is given for the convenience of users of this International Standard 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.

4) ImageQuaNT is the trade name of a product supplied by Molecular Dynamics, Ca, USA. This information is given for the convenience of users of this International Standard 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) pGEM-T Easy Vector is the trade name of a product supplied by Promega, France. This information is given for the convenience of users of this International Standard 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.

A.2.7 Quantitative PCR

The abundance of the total bacterial community was estimated by quantifying the amount of 16S rDNA sequences with universal primers specific for eubacteria: 341f (5'-CCT ACG GGA GGC AGC AG-3') and 534r (5'-ATT ACC GCG GCT GCT GGC A-3'). Thermocycling conditions were as follows: 15 min at 95 °C; 30 cycles consisting of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and 30 s at 80 °C.

Abundance of *narG* and *pcaH* bacterial communities was estimated by quantifying the amount of *narG* and *pcaH* sequences, respectively. For *narG* amplification, degenerated primers narGf (5'-TCG CCS ATY CCG GCS ATG TC-3') and narGr (5'-GAG TTG TAC CAG TCR GCS GAY TCS G-3') were used [30]. Thermocycling conditions were as follows: 15 min at 95 °C; 6 cycles consisting of 15 s at 95 °C, 30 s at 63 °C, with a touchdown of 1 °C by cycle, and 30 s at 72 °C; 40 cycles consisting of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and 30 s at 80 °C. For the amplification of *pcaH* sequences, degenerated primers pcaHf (5'-GAG RTS TGG CAR GCS AAY-3') and pcaHr (5'-CCG YSS AGC ACG ATG TC-3') were used with the following thermocycling conditions: 15 min at 95 °C; 8 cycles consisting of 15 s at 95 °C, 30 s at 64 °C, with a touchdown of 0,5 °C by cycle, and 30 s at 72 °C; 30 cycles consisting of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and 30 s at 80 °C [9].

All qPCR assays were carried out in a total of 15 µl reaction volume containing SYBR green PCR Master Mix (Absolute QPCR SYBR Green Rox[6]) 100 ng of T4 gp32[7], 2 µl of template DNA (0,01 ng/µl) and 2 µmol/l of each primer, except for the *pcaH* assay in which primers were used in different concentrations (pcaHf 2 µmol/l and pcaHr 1,2 µmol/l). qPCR assays were performed for each replicate ($n = 5$, $n_{tot} = 270$). No-template controls were also included in all the assays. Standard curves for all the assays were obtained using tenfold serial dilutions of a linearized plasmid pGEM-T (102 to 107 copies) containing 16S rRNA or *narG* sequence originating from *Pseudomonas aeruginosa* PAO1 or, for the *pcaH* assay, *pcaH* sequence originating from the environmental clone. Melting curves were generated after amplification by increasing the temperature from 80 °C to 95 °C.

The qPCR data were analysed using

a) multiple comparison parametric Tukey-Kramer test,

b) non-parametric Kruskal-Wallis test, and

c) Mantel test of correlation.

A.2.8 Automated RISA fingerprinting

Automated Ribosomal Intergenic Spacer Analysis (A-RISA) was used for studying the structure of bacterial communities in the six soils under investigation in the international ring test. A-RISA was conducted on the purified soil DNA diluted at 0,01 ng/µl. Targeted 16S–23S intergenic spacer of the bacterial rDNA was amplified in a final volume of 25 µl with 0,5 µmol/l of A-RISA-1552f (5'-TCG GGC TGG ATG ACC TCC TT-3') and A-RISA-132r (5'-CCG GGT TTC CCC ATT CGC -3') universal primers, 2,5 U of Taq DNA polymerase[8] and 2 µl of template DNA. The primer A-RISA-1552f was labelled in the 5' position with IRD 800 dye fluorochrome[9]. PCR amplification was carried out in a gradient thermocycler VentiTM[10] with the following conditions: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C; plus an additional cycle for 15 min at 72 °C.

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6) Absolute QPCR SYBR Green Rox is the trade name of a product supplied by ABgene, France. This information is given for the convenience of users of this International Standard 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.

7) T4 gp32 is the trade name of a product supplied by QBiogene, France. This information is given for the convenience of users of this International Standard 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.

8) Taq DNA polymerase is the trade name of a product supplied by Appligene Oncor, France. This information is given for the convenience of users of this International Standard 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.

9) IRD 800 dye fluorochrome is the trade name of a product supplied by MWG SA Biotech, Ebersberg, Germany. This information is given for the convenience of users of this International Standard 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.

10) VentiTM is the trade name of a product supplied by Applied Biosystems, USA. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named.

The quality of the A-RISA PCR products was checked by electrophoresis on 1 % agarose gel with Smart Ladder[11] DNA marker, after which products were loaded on the 6,5 % polyacrylamide gels, (25 cm in length) and run on a LiCor 4 300 DNA Analysis System[12]. Before loading on polyacrylamide gels samples were denatured at 90 °C for 3 min. Separation of the A-RISA fragments were done during a 4 h run with electrophoresis conditions as follows: 2 000 V, 25 mA and 45 W.

The data were analysed using the 1D-Scan software[13]. The software converted fluorescence data into electrophoregrams where peaks represented PCR fragments. The height of the peaks was calculated in conjunction with the median filter option and the Gaussian integration in 1D-Scan, and represented the relative proportion of the fragments in the total products. Lengths (in base pairs) were calculated by using a size standard with 15 bands ranging from 206 to 1 119 bp. Data from the 1D-Scan were converted into a matrix summarizing the bands presence (i.e. peak) and intensity (i.e. height of peak) using PrepRISA program (http://pbil.univ-lyon1.fr/ADE-4/microb/). Principal component analysis (PCA) on an A-RISA covariance matrix was performed on the data using ADE-4 software (http://pbil.univ-lyon1.fr/ADE-4/home.php).

A.3 Results

A.3.1 Soil DNA extraction yields

The yield of DNA extracted by each of the nine participant laboratories from the six studied soils is presented in Table A.2.

Table A.2 — Yield of soil DNA extracted from the six studied soils (µg of DNA/g of soil)

Laboratory	DNA concentration (µg/g soil)					
	A	B	C	F	M	S
Sweden	3,53 ab	2,09 ab	0,44 a	4,21 b	1,75 a	1,48 b
France, Ineris	4,70 ab	1,23 a	1,09 abc	2,75 ab	3,47 ab	3,18 ab
Spain	1,01 a	2,45 ab	1,07 ab	2,88 ab	1,88 a	0,68 ab
Finland, Helsinki	3,98 ab	3,92 b	2,75 c	nd	2,66ab	1,81 ab
France, Nancy	3,63 ab	2,46 ab	1,50 abc	3,05 ab	2,34 ab	1,39 ab
France, Dijon	6,75 b	2,47 ab	2,12 bc	3,17 ab	4,53 b	2,55 ab
Italy	3,21 ab	2,78 ab	0,95 ab	2,00 a	1,83 a	2,31 a
Germany, JKI	4,04 ab	3,53 b	2,00 bc	2,40 ab	2,51 ab	1,37 ab
Germany, Munich	0,98 a	2,94 ab	1,22 abc	3,59 ab	2,15 ab	0,93 ab

Letters (a, b, c) assigned to each value represent groups appointed by the Kruskal-Wallis statistical analysis ($p < 0,05$). Values in the same group are not significantly different between each other.

nd = not determined

As can be seen from Table A.2, all nine laboratories were successful in extracting DNA from all studied soils, likewise agricultural, forest and PAH-contaminated soil. Depending on the soil, results showed that DNA quantities ranged in different samples from 0,44 µg (soil C extracted by the Swedish laboratory) to 6,75 μg of DNA per gram of soil (soil A extracted by the Dijon laboratory). Mean values determined for each of the six

Equivalent products may be used if they can be shown to lead to the same results.

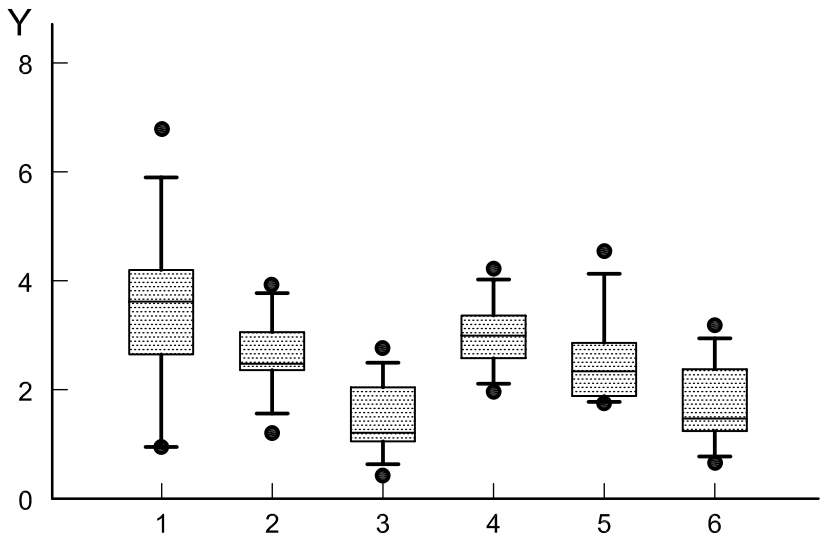
11) Smart Ladder is the trade name of a product supplied by Eurogentec, Belgium. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

12) LiCor 4 300 DNA Analysis System is the trade name of a product supplied by Biosciences, USA. This information is given for the convenience of users of this International Standard 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.

13) 1D-Scan software is the trade name of a product supplied by Science Tec, France. This information is given for the convenience of users of this International Standard 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.

studied soils showed that the highest amounts of DNA were extracted from the soil A and F (3,54 µg/g and 3,01 µg/g of soil, respectively), whilst the lowest were gained from the soil C and S (1,46 µg/g and 1,74 µg/g of soil, respectively). For the remaining soil B and soil M, the amount of 2,6 µg of DNA per gram of soil was determined in the soil DNA extracts. One could underline that the highest amounts of DNA were extracted from soils chosen for their complexity (soil A, rich in organic matter and acidic, pH 3,7; soil F, contaminated with PAH).

For each soil, statistical analysis (Kruskal-Wallis, $p < 0,05$) showed good reproducibility among the yields of DNA extracted by the different laboratories (see Table A.2). Only a few significant differences were observed between laboratories. However, observed variability for each soil could not be appointed to any specific participant laboratory. Furthermore, despite the observed differences between the obtained data, it could be seen that extracted DNA quantities are in the same order of magnitude, no matter the soil or the laboratory considered (see Figure A.1).



Key

1 Soil A
2 Soil B
3 Soil C
4 Soil F
5 Soil M
6 Soil S
Y DNA concentration µg/g of soil

Box = Tukey box with mean value (line), upper hinge of the box indicating 75th percentile of the data set, lower indicating 25th percentile and standard deviation (whiskers); dot = outliers, 5 % and 95 % percentiles of the data set.

Figure A.1 — Range of variation of the yield of DNA extracted from the six studied soils

One could conclude that the proposed method was successful to extract DNA from all the soils studied in the international ring test including the forest soil and the PAH-contaminated soil rich in organic matter (13 % and 19 % of organic matter, respectively). In addition, this method was also successful to extract DNA from soil rich in clay material (up to 48 % in soil S). Soil DNA extraction was proven to work for soils presenting different acidity (from pH 3,8 to pH 8). Although DNA could be extracted from the different soils, its amounts differed between soils, ranging from 0,44 µg to 6,75 µg of DNA per gram of soil. For each soil, good reproducibility was shown between laboratories. Together with DNA, contaminants were co-extracted as shown by the brownish colour of the extracts. Additional steps of purification are required to use these DNA matrices for molecular studies. Estimation of the yield of DNA extraction revealed that the observed variability inside given soil could not be appointed to any specific participant laboratory.

A.3.2 Quantitative PCR analyses

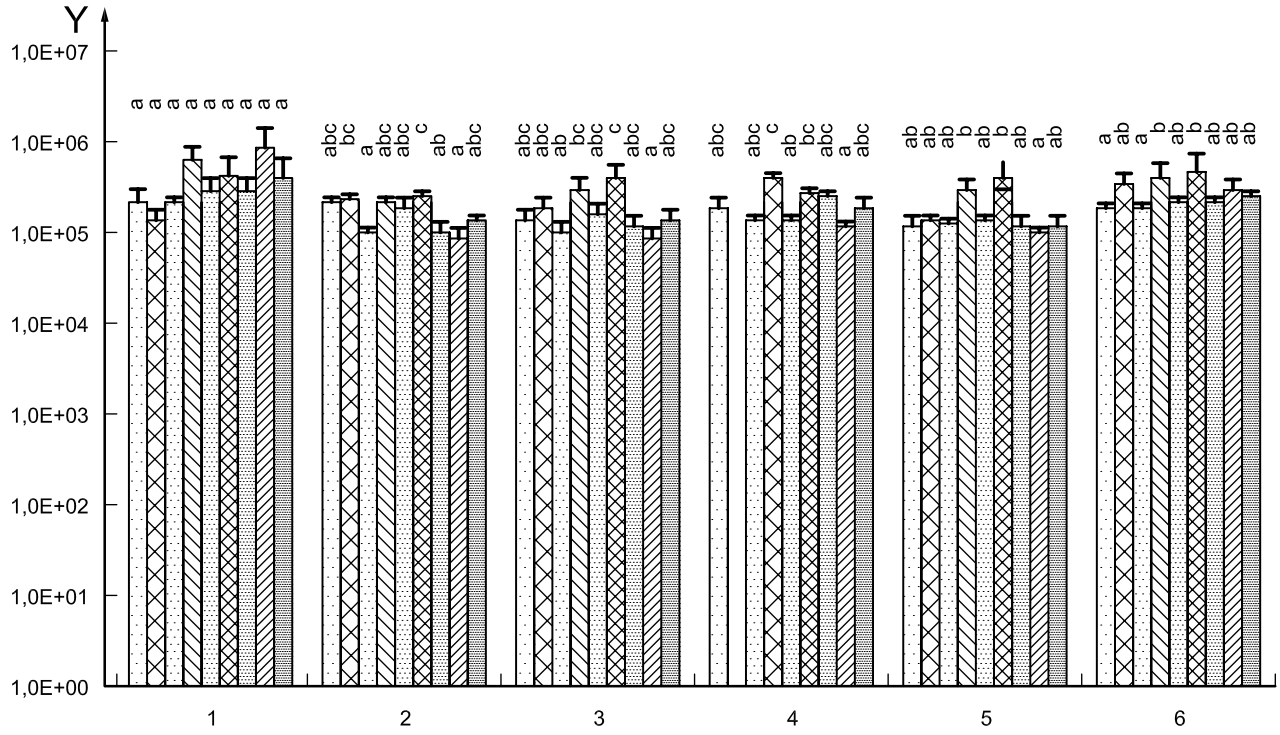
Results of the inhibition test showed that, although the proposed method led to co-extraction of contaminating substances, such as humic acid substances, the purification step was efficient to remove most of these compounds known to inhibit PCR analyses. Indeed, inhibition of qPCR assays was entirely eradicated by diluting purified DNA extract at 0,01 ng/µl. For further molecular analyses, samples were diluted to 0,01 ng/µl.

A.3.2.1 Estimation of the size of the global bacterial community

The analysis of the qPCR results showed that the abundances of 16S rDNA ranged in different soils from $2{,}5 \times 10^5$ to $2{,}7 \times 10^6$ sequences per gram of soil (Figure A.2).

Statistical analysis showed no significant differences between abundances of 16S rDNA sequences obtained by any of the participating laboratories for the soil A (Kruskal-Wallis test, $p < 0{,}05$). For the other soils, slight differences could be observed on 16S rDNA abundances. The analysis done for the soil B revealed that only three laboratories differed significantly forming two groups: group A included Nancy, France and Sweden ($2{,}5 \times 10^5$ 16S rDNA sequences per ng of DNA) and group C of Germany, Munich (9×10^5 16S rDNA sequences per ng of DNA). For the soil C, two laboratories differed significantly Nancy ($2{,}5 \times 10^5$ sequences per ng of DNA) on one side and Germany, Munich on the other one ($1{,}1 \times 10^6$ sequences per ng of DNA). In the soil C, only two laboratories differed from the others, Nancy, France ($3{,}4 \times 10^5$ sequences per ng of DNA) and Spain ($1{,}1 \times 10^6$ sequences per ng of DNA). Analysis for the soil M and soil S revealed that only three laboratories in each soil differed significantly, grouping into two groups. For the soil M, group A was made of Nancy, France ($2{,}6 \times 10^5$ sequences per ng of DNA) and for the soil S from Ineris, France and Sweden (5×10^5 sequences per ng of DNA) whilst the group B was for both soils comprised from Germany, Munich and Spain laboratories (average of $1{,}2 \times 10^6$ and 1×10^6 sequences per ng of DNA).

One could conclude that DNA extracted using the proposed method could be successfully used for conducting qPCR analyses allowing the estimation of the abundance of global bacterial community. For each soil, the abundance of the global bacterial community can differ significantly between laboratories but this difference remained rather low (comprised between 8 % and 70 % depending on the soil considered) considering known variations of this parameter in different soils differing in their physico-chemical composition or submitted to different stresses.



Key

	France, Ineris	1	Soil A
	Finland, Helsinki	2	Soil B
	Sweden	3	Soil C
	Spain	4	Soil F
	Italy	5	Soil M
	Germany, Munich	6	Soil S
	Germany, JKI	Y	16 S rDNA (copy number ng-1 DNA)
	France, Nancy		
	France, Dijon		

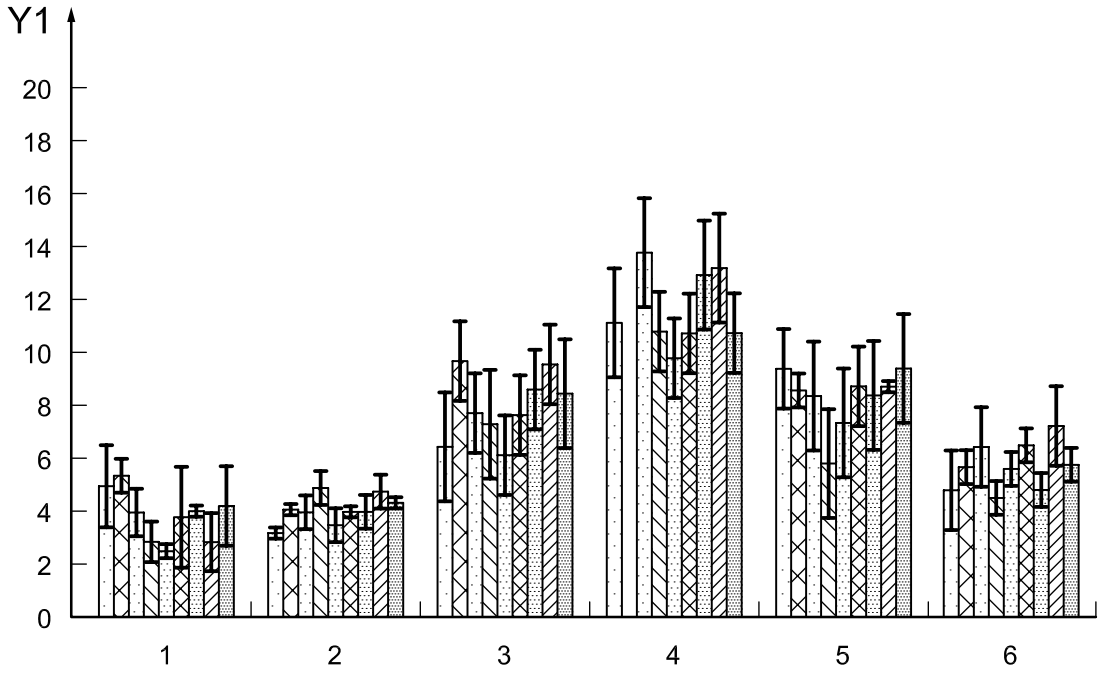
Letters (a, b, c) assigned to each value represent groups appointed by the Kruskal-Wallis statistical analysis ($p < 0,05$). Values in the same group are not significantly different between each other

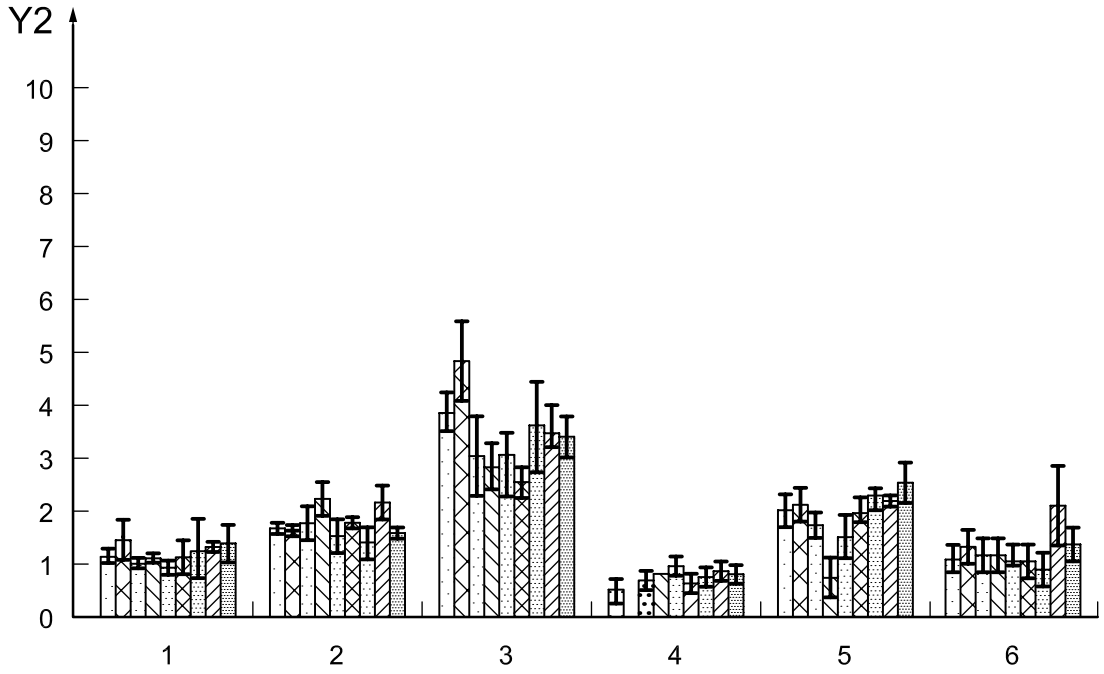
Figure A.2 — Abundance of global bacterial communities measured in the six studied soils

A.3.2.2 Estimation of the size of *narG* and *pcaH* bacterial communities

The abundances of the nitrate-reducing and protocatechuate-degrading bacterial communities were estimated by qPCR assays targeting *narG* gram negative and *pcaH* gene, respectively. In all six studied soils, *narG* abundances were higher than those of *pcaH* (data not shown). Indeed, *narG* abundances ranged in different samples from $9,7 \times 10^3$ to $1,1 \times 10^5$ copies per ng of DNA, whilst it ranged from $2,1 \times 10^3$ to $5,6 \times 10^4$ copies per ng of DNA for *pcaH*.

Relative abundances of the targeted communities were determined for each of the six studied soils. They were calculated as a percentage (%) of either *narG* or *pcaH* sequence copy numbers per copy number of 16S rDNA sequences (see Figure A.3).





Key

	France, Ineris	1	Soil A
	Finland, Helsinki	2	Soil B
	Sweden	3	Soil C
	Spain	4	Soil F
	Italy	5	Soil M
	Germany, Munich	6	Soil S
	Germany, JKI	Y1	narG relative abundance (%)
	France, Nancy	Y2	pcaH relative abundance (%)
	France, Dijon		

Figure A.3 — Relative abundances of narG (A) and pcaH (B) communities in the six studied soils

The highest relative abundances of *narG* bacterial community (mean value 11,6 %) were determined in soil F (PAH-contaminated), whilst the lowest were measured in soils A (forest soil) and B (vineyard agricultural soil) (mean value 3,8 % and 4,1 %, respectively). *narG* relative abundance determined in the other soils S, C, and M were shown to present 5,7 % to 8,3 % of the global bacterial community. Contrary to the *narG* community, the lowest relative abundances of *pcaH* community were measured in soil F (mean value 0,7 %), whilst the lowest abundance of this community was present in soil C (mean value 3,4 %). *pcaH* community determined in the rest of the studied soils (soils A, M and S) were shown to present 1,2 % to 2,8 % of the global bacterial community.

Statistical analyses (Tukey-Kramer test, $p < 0,05$) performed on the data sets obtained for both targeted communities are presented in Table A.3. Results revealed good reproducibility among relative abundances determined in nine laboratories participating in the international ring test.

Table A.3 — Statistical differences among relative abundances of narG or pcaH community determined in the six studied soil

Laboratory	Ineris France	Nancy France	Dijon France	Germany Munich	Germany JKI	Spain	Sweden	Finland	Italy
Ineris France				C, F		C, M	C		
Nancy France	B, S			S	S	M			
Dijon France						M		C	
Germany Munich						M		A, C	
Germany JKI		S				M			
Spain	B, M	M, S	M	M	M			C, M	
Sweden	B					M		C	
Finland						M			A, C
Italy		B, C					F	C	

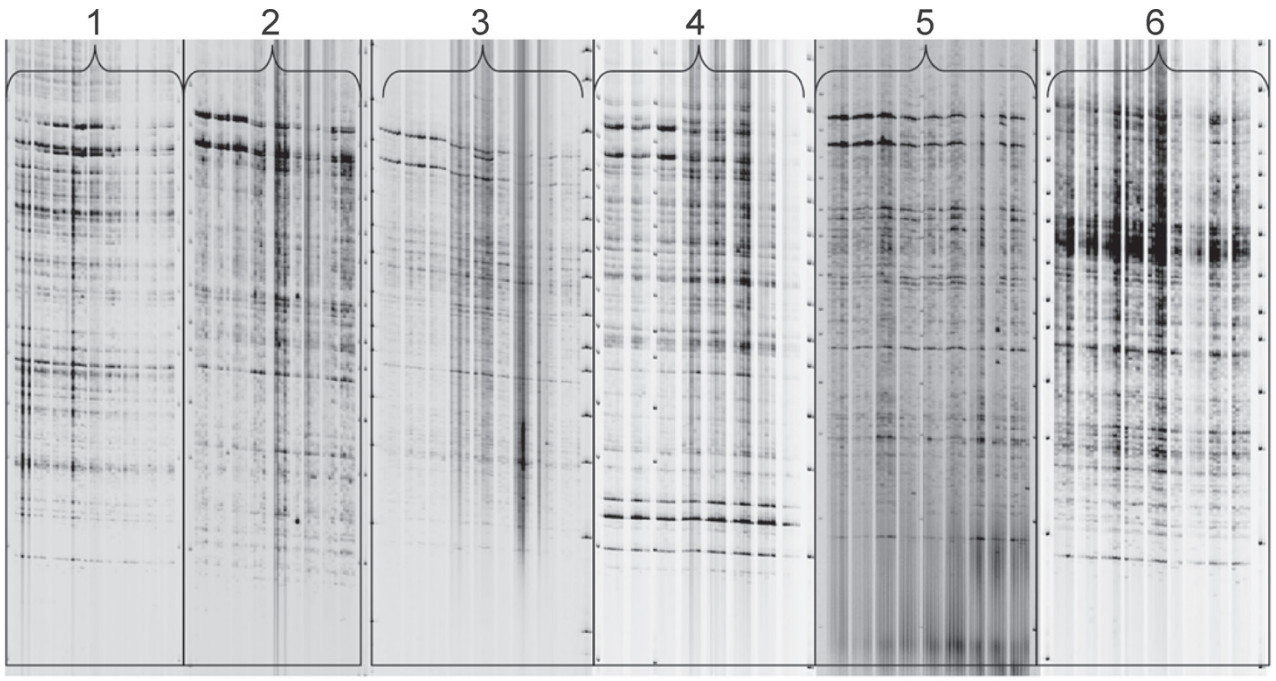
The lower half of the table (in white) reports the results of the statistical analysis of the narG data set and upper half (in grey) of the *pcaH* data set. Statistically different values are labelled by the capital letter corresponding to the six studied soils (A, B, C, F, M and S). The label means that the values differed between the two laboratories considered.

For each of the nine participating laboratories, the pattern formed by the *pcaH* community abundances determined in six studied soils was determined (Kruskal-Wallis test, $p < 0,05$). No matter the laboratory considered, values of the *pcaH* abundances ranked in studied soils as follows: soilF<soilA<soilS<soilB<soilM<soilC. A small discrepancy could be seen only for the Spanish laboratory (soilF<soilA<soilS<soilM<soilB<soilC). *narG* community data were analysed in the same manner. *narG* abundances determined in six soils followed the same pattern in almost all laboratories: soil F>soilM~soilC~soilS~soilA~soilB>soilA. Slight differences in the pattern was observed only for the Spanish and Italian laboratories (soil F>soilM~soilC~soilS~soilA~soilA>soilB). However, the Mantel test ($p < 0,05$) proved there were no significant differences in *pcaH* or *narG* patterns ($p < 0,000\ 1$).

One could conclude that the soil DNA extracted by the proposed method can be successfully used to quantify functional microbial communities, those less numerous than the global bacterial community but playing a key role in soil ecosystemic services. *narG* and *pcaH* qPCR reproducibility was good between laboratories. For each soil, only a few values differed significantly from one laboratory to another one, but the range of variation was rather low (approximately 50 %). In addition, the abundance pattern for the two molecular markers studied was conserved between the nine laboratories involved. Therefore, the variability due to the extraction of soil DNA by the different laboratories did not compromise the quantification of the abundance of functional communities in the studied soils.

A.3.3 Analysis of the global structure of bacterial communities (A-RISA)

Differences between the global structure of the bacterial community present in soils A, B, C, F, M and soil S were studied by A-RISA analysis. The obtained A-RISA fingerprints revealed the complexity of the communities (more than 100 bands per lane) present in the six soils studied (Figure A.4). Furthermore, visual observation revealed that each soil was characterized by a specific pattern.



Key

1 Soil A
2 Soil B
3 Soil C
4 Soil F
5 Soil M
6 Soil S

Figure A.4 — A-RISA fingerprints of the six studied soils

For further analysis, A-RISA fingerprints were digitized and used to establish a matrix in which the number of bands, as well as their size and intensity was stored. For each fingerprint, 100 bands were considered.

Even if not clearly seen on the A-RISA profiles, PCA analysis revealed slight differences in the structure of the global bacterial community determined for each studied soil by the nine participating laboratories (data not shown). The genetic structure of the global bacterial communities from A, B and C soils formed two distinct groups: one being constituted of Sweden, Germany, JKI and Germany, Munich laboratories and the other one of the six remaining laboratories. Similar results were obtained for the soil M, with all laboratories grouping close to each other, except for Sweden and Germany JKI laboratories, and in a lesser way, Nancy France laboratory. For the soil F, two groups which differed between each other comprised from three French laboratories (Nancy, Dijon and Ineris) on one side and the rest of the participating laboratories on the other. The PCA analysis obtained for the soil S showed similarities between fingerprints obtained from all nine participating laboratories.

However, when all A-RISA fingerprints were plotted onto the same PCA, one could easily discriminate grouping of data into three clusters, and differences between laboratories became less evident (see Figure A.5). As can be seen, the genetic structure of the soil F bacterial community (comprised in the 1st group) significantly differs from the other soils. This PAH-contaminated soil, rich in organic matter, was chosen to determine the limit of the soil DNA extraction method tested here. Bacterial communities found in soils A, M and soil S (2nd group) were grouped to have a similar genetic structure and differ from the last group of data (3rd group) representing

the bacterial community of soil B and soil C. These results tend to indicate that the variability due to the extraction of soil DNA by the different laboratories (intra-variability) was inferior to the variability of the profiles due to soil physicochemical properties (inter-variability).

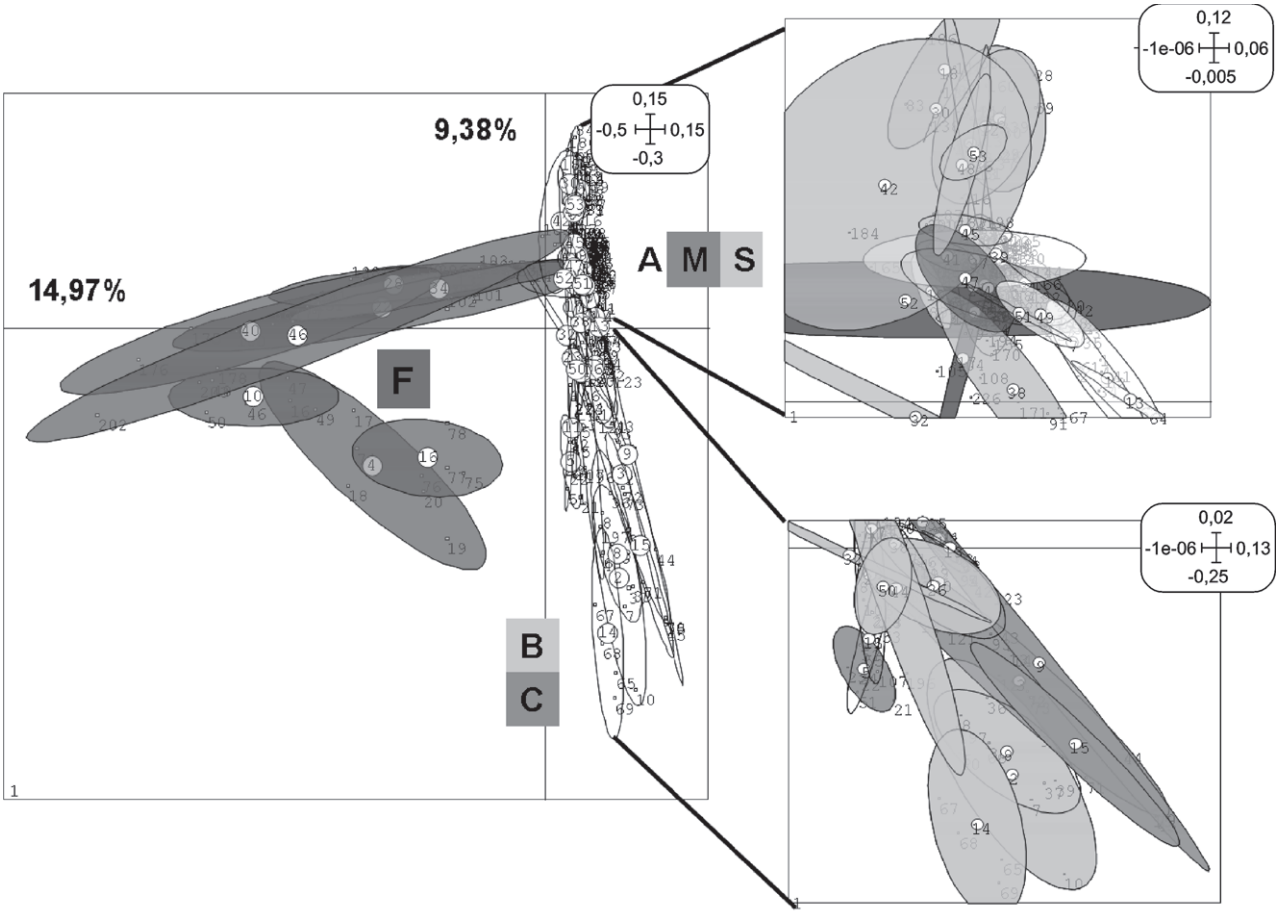


Figure A.5 — PCA of A-RISA profiles obtained from the six studied soils

One could conclude that soil DNA extracted by the proposed method can be used for bacterial community structure analysis based on A-RISA. DNA fingerprints generated from the soil DNA extracts were complex being made of more than one hundred bands. Visual observation revealed that these fingerprints

a) were different between soils, but

b) for each soil, similar between laboratories.

Fine analyses of the DNA fingerprints revealed a slight variability among laboratories for each studied soil. However, these differences observed among laboratories did not interfere with the discrimination of the soils based on A-RISA profiling, revealing the structure of the global soil bacterial community. It can be concluded that the variability due to the extraction of soil DNA by the different laboratories did not compromise the discrimination of the studied soils based on the analysis of global bacterial community structure.

A.4 Conclusions and perspectives

It can be concluded from the international ring test that the proposed method was efficient for extracting DNA directly from six soils showing contrasting physico-chemical properties. Even though the method proposed was mainly dedicated to the agricultural and forest soils, results of the ring test revealed that this method is also efficient for extracting DNA from complex soils, such as those heavily contaminated with PAHs. In addition,

this method was also shown to be suitable for soils rich in organic matter and clay and was proven to work for soils presenting different acidity.

Furthermore, it was clearly shown that soil DNA extracted from the six soils can successfully be used for both quantitative (qPCR assays) and qualitative (A-RISA fingerprinting). DNA extracted using the proposed method could be used for the estimation of the abundance of the global bacterial community, as well as the estimation of the abundance of less numerous functional microbial communities playing a key role in soil ecosystemic services.

DNA extracted by the proposed method was shown to be successfully analysed by A-RISA, thereby allowing the estimation of the structure of the global bacterial community.

Based on the ring test, one could conclude that the impact of the manipulator using the proposed method did not impair further molecular analyses performed on the extracted DNA.

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