Essais inter-laboratoire pour évaluer le standard ISO 11063 'Qualité du sol – méthode pour l'extraction directe des acides nucléiques du sol'

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ECOTOX

Colloque Ecotox, Saint-Lager, 7-9 Novembre 2011

Historique du standard ISO 11063

2001

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2001, p. 2354–2359 Vol. 67, No. 5
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.5.2354–2359.2001

DNA Extraction from Soils: Old Bias for N
Diversity Analysis Methods

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Received 11 December 2000/Accepted 12 February 2001

types in soils. Additionally, our work suggests that the use of standard soil DNA extraction and PCR methods by soil microbiologists could provide a more complete understanding of the composition and diversity of soil microbial communities.

2004 Présentation à l'AFNOR du projet de standard

2005-2006 Essai inter-laboratoire Français et proposition NWI à l'ISO

2007 Adoption du NWI par l'ISO

2009-2010 Essai inter-laboratoire International et proposition de norme à l'ISO

2011 Adoption de la norme, publication, traduction (et publications scientifiques)

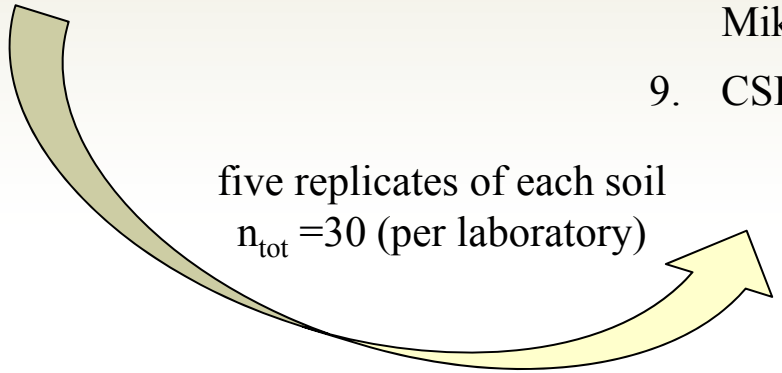
1. Organisation of the International ring test

Soils (6):

4 **agricultural soils**
(collected in Sweden and France)

1 **forest soil**
(collected in Germany)

1 **polycyclic aromatic hydrocarbons** (PAH) **contaminated**
(collected in Finland)



Participant laboratories (9):

1. LSEM (**France**, F Martin-Laurent)
2. INERIS (**France**, P Pandard)
3. IPL santé, environnement durables Est (**France**, T Chesnot)
4. University of Uppsala (**Sweden**, S Hallin)
5. GSF München (**Germany**, M Schloter)
6. JKU (**Germany**, K Smalla)
7. University of Catane (**Italy**, C Abbate)
8. University of Helsinki (**Finland**, Anu Mikkonen)
9. CSIC Grenada (**Spain**, E Romero)

Physico-chemical properties of 6 studied soils:

	soil					
	GER	BOR	EPO	FIN	MAR	SWE
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

Procedure for the direct extraction of DNA from soil

Soil sample (250 mg)

Addition of homogenization buffer

1 Mechanical and chemical lyses

Bead-beating (30s, 1600 rpm)

Incubation at 70°C for 10 min

Centrifugation (1 min, 14 000g, 4 °C)

Soil lysate

Addition of potassium acetate 3M (1/10V)

2 Protein precipitation

Incubation on ice for 10 min

Centrifugation (5 min, 14 000g, 4 °C)

Crude extract

Addition of cold isopropanol (1V)

3 Nucleic acids precipitation and washing

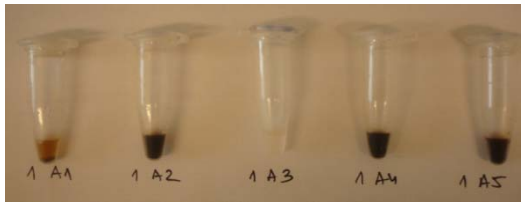
Incubation at -20°C for 15 min

Centrifugation (30 min, 14 000g, 4 °C)

Addition of cold 70° ethanol

Centrifugation (15 min, 14 000g, 4 °C)

Soil DNA

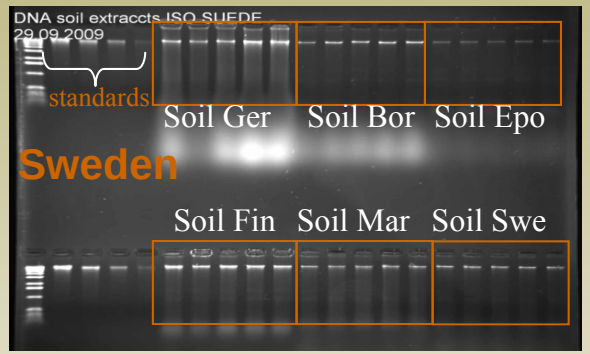


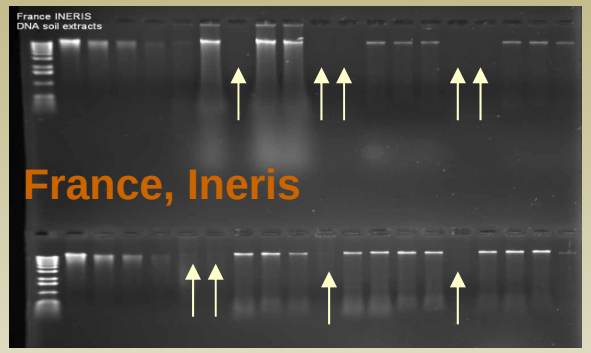
<u>Purification columns:</u>

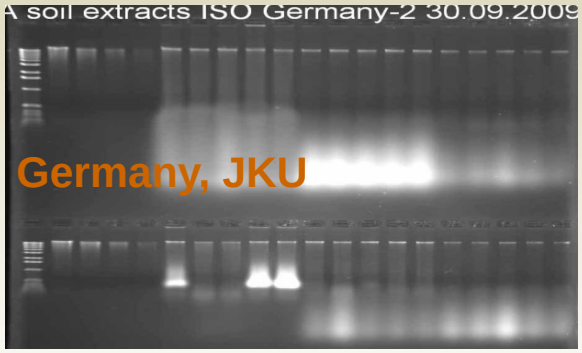
1. PVPP (Polyvinyl-polypyrrolydone)
2. Sepharose 4B

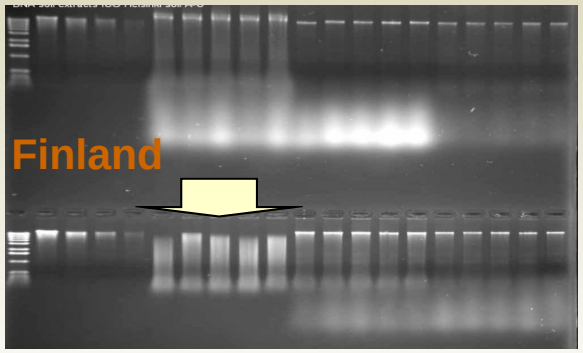
Extracted soil DNA

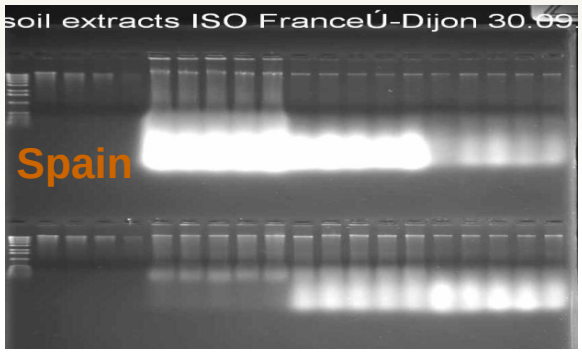
Quantification on 1 % agarose gels with tymus DNA included as standards points (ImageQuaNT software)

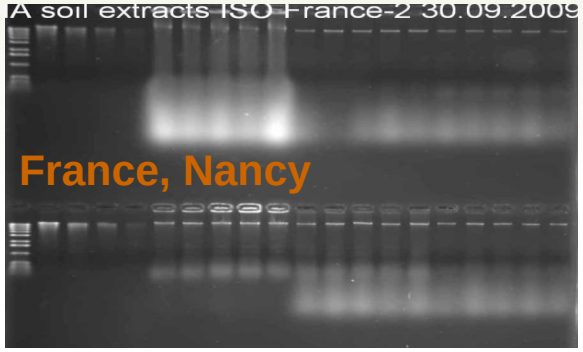


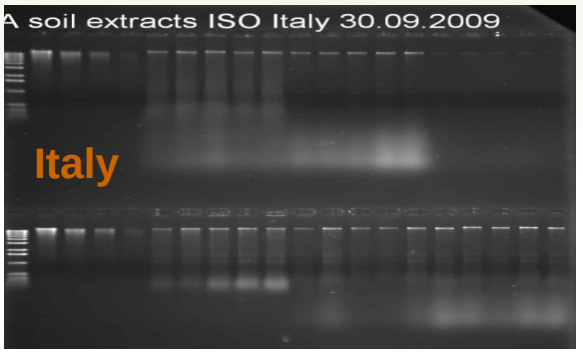




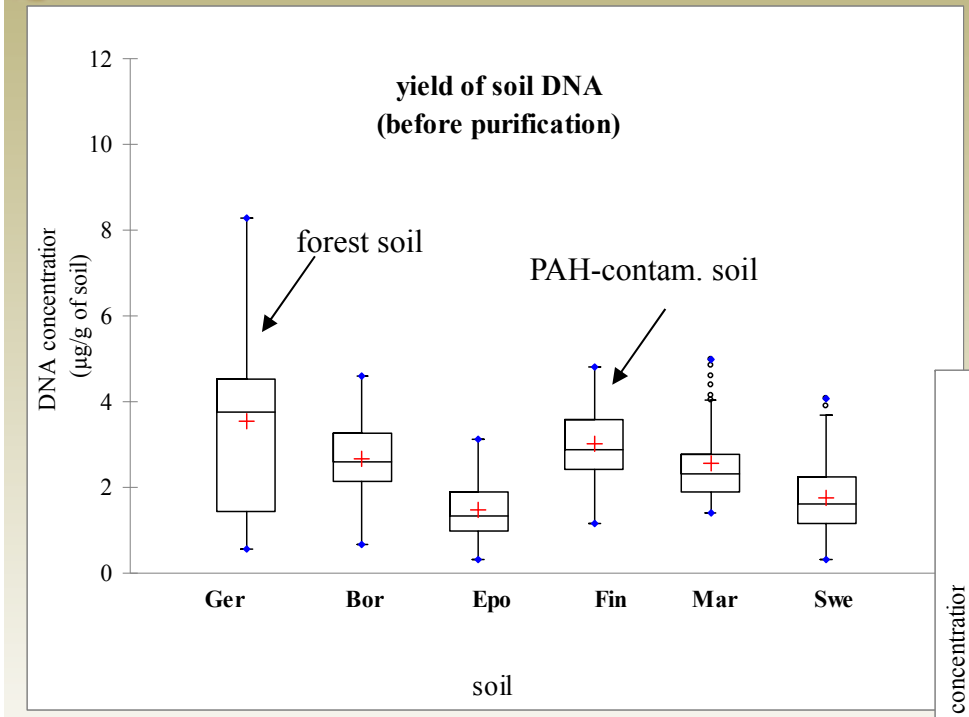






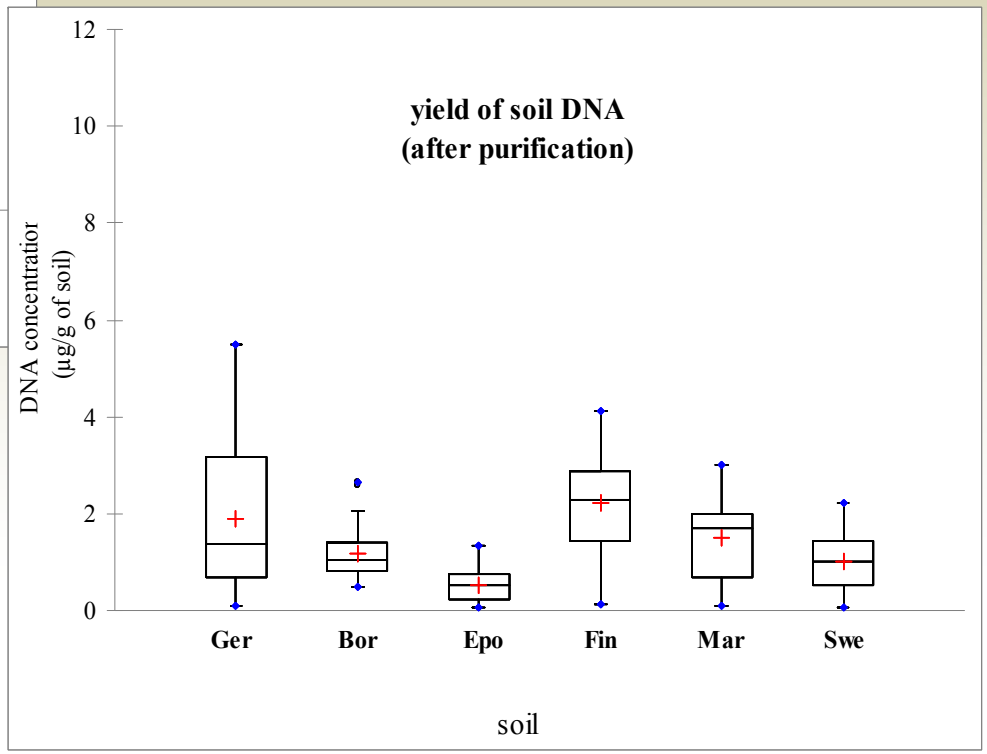


Amount of the soil DNA extracted using proposed method



- ✓ DNA quantities range: **0.44 - 6.75 µg/g**
- ✓ highest quantity (mean value):
  soil Ger (3.54 µg/g); soil Fin (3.01 µg/g)
- ✓ lowest quantity (mean value):
  soil Epo (1.46 µg/g); soil Swe (1.74 µg/g)
- ✓ soil Bor and soil Mar: 2.6 µg/g

losses of the originally extracted DNA



Kruskal-Wallis statistical analysis ($p<0.05$)

Laboratory	Soil Ger	Soil Bor	Soil Epo	Soil Fin	Soil Mar	Soil Swe
Sweden	ab*	ab	a	b	a	b
France Ineris	ab	a	abc	ab	ab	ab
Spain	a	ab	ab	ab	a	ab
Finland	ab	b	c		ab	ab
France Nancy	ab	ab	abc	ab	ab	ab
France Dijon	b	ab	bc	ab	b	ab
Italy	ab	ab	ab	a	a	a
Germany JKU	ab	b	bc	ab	ab	ab
Germany Munchen	a	ab	abc	ab	ab	ab

*Letters (a, b, c) assigned to each value represents groups appointed by the statistical analysis. Values in the same group are not significantly different between each other

- interlaboratory difference for the given soil (grouping mostly A & B)
- difference could not be appointed to any specific participant laboratory
- values in same order of magnitude

Conclusion 1

- method is successful to extract DNA from **different types of soils**
  - forest, agricultural, contaminated
  - soil rich in organic matter, clay material (up to 48 %) and soils with different acidity (pH 3.8 to pH 8)
- yield of DNA extraction depends on the soil matrix
- **good reproducibility** was shown between laboratories
- variability inside given soil **could not be appointed to any specific participant laboratory**

✓ co-extraction of contaminants (brownish color of the extracts) → additional purification steps

Quality of the soil DNA extracted using proposed method

Impact on further analyses based on amplification of extracted DNA by polymerase chain reaction ?

Molecular methods:

Quantitative analyses

Quantitative PCR (qPCR)

✓ targeting 16S rDNA, *pcaH* and *narG* gene sequences

Abundance of soil bacterial communities

Qualitative analysis

Automated Ribosomal Intergenic Spacer Analysis, A-RISA

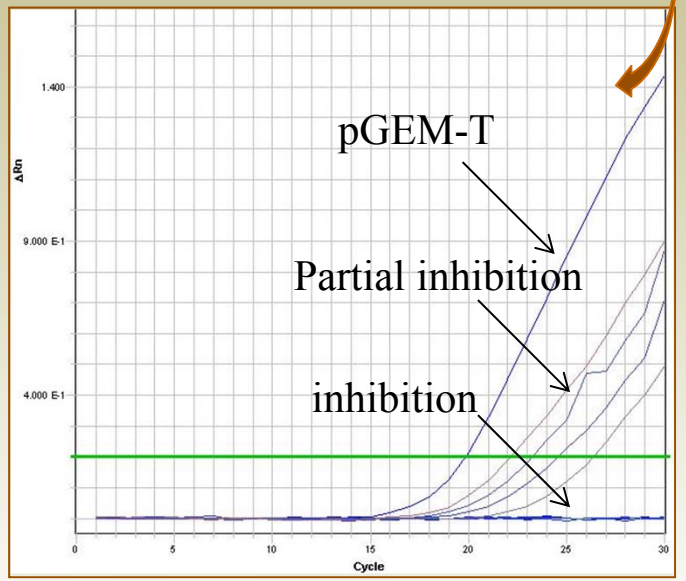
✓ targeting 16S–23S intergenic spacer of the bacterial rDNA

Structure of soil bacterial communities

Inhibition test

presence of PCR inhibitors in soil DNA extracts → quantitative PCR assay with control plasmid pGEM-T Easy by *(Henry et al., 2006)*

- ✓ non diluted DNA extract were shown to inhibit PCR assays
- ✓ inhibition was more correlated with soil properties than with the laboratory
- ➢ heavy colored soil DNA extracts (soil Ger and Bor) inhibited most PCR assays



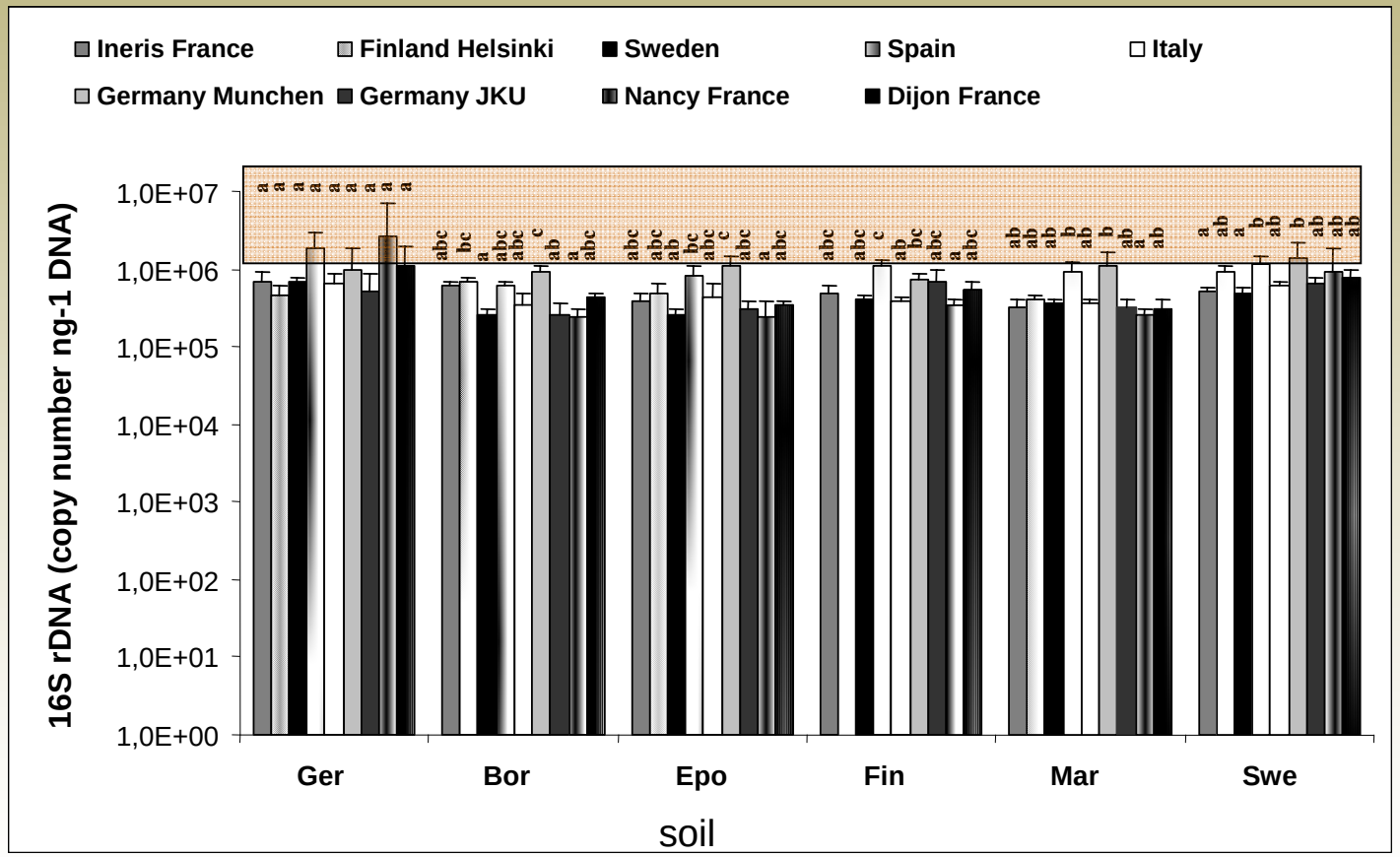
Proposed extraction method led to co-extraction of contaminating substances:

- ✓ purification step efficiently removed most of the inhibitors
- ✓ inhibition was entirely eradicated by appropriate dilution of the extracts

Henry, S., D. Bru, B. Stres, S. Hallet, and L. Philippot. 2006. *Appl. Environ. Microbiol*. 72:5181–5189.

Quantitative analyses
Estimation of the total bacterial community abundance

- copy number of 16S rDNA sequences (qPCR assay, 341f/534r universal primers)



16S rDNA abundance range:

2.5×10^5 - 2.7×10^6 sequences per g of soil

Conclusion 2

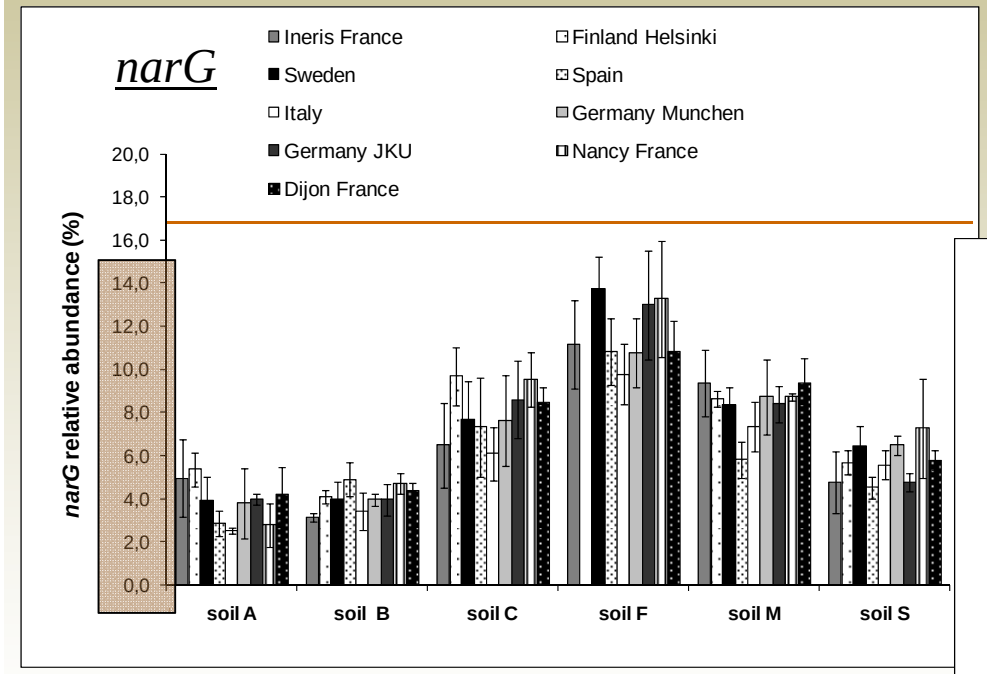
- soil DNA extracted using the proposed method was shown to be successfully used to determine the size of the total soil bacterial community
- statistic analysis showed differences between abundances obtained for given soil → difference remained rather low (between 8% and 70%)

  (this parameter differs in different soils with different physico-chemical composition or submitted to different stresses)
- variability due to the extraction of soil DNA by the different laboratories did not compromise the quantification of the abundance of total community

Estimation of the abundance of nitrate-reducing and protocatechuate-degrading bacterial community

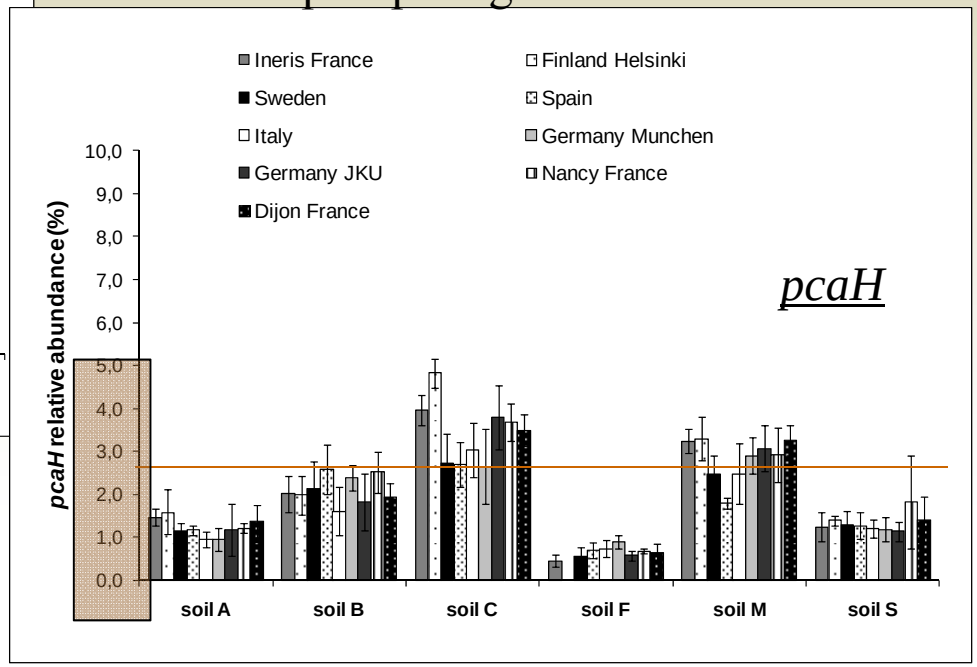
➢ Copy number of *narG* and *pcaH* gene sequences (qPCR assays , primers narGr/narGf; pcaHr/pcaHf *(Philippot et al. 2002, Bru et al. 2007)*

→ specific richness (%) of targeted communities determined by comparison to the global bacterial community (16SrDNA)



$9.7x10^3$ - $1.1x10^5$
copies per ng of DNA

$2.1x10^3$ - $5.6x10^4$
copies per ng of DNA



Bru D, Sarr A, Philippot L. 2007. *Appl Environ Microbiol*. Sep; 73(18): 5971-5974
Philippot L., Piutti S., Martin-Laurent F., Hallet S., Germon JC 2002. *Appl. Environ. Microbiol.* 68 : 6121-6128

Kruskal-Wallis statistical analysis (p<0.05)

Laboratory	**Ineris FR**	**Nancy FR**	**Dijon FR**	**Germany Munchen**	**Germany JKU**	**Spain**	**Sweden**	**Finland**	**Italy**
Ineris FR				Epo, Fin*		Epo, Mar	Epo		
Nancy FR	Bor, Swe			Swe	Swe	Mar			
Dijon FR						Mar		Epo	
Germany Munchen						Mar		Ger, Epo	
Germany JKU		Swe				Mar			
Spain	Bor, Mar	Mar, Swe	Mar	Mar	Mar			Epo, Mar	
Sweden	Bor					Mar		Epo	
Finland						Mar			Ger, Epo
Italy		Bor, Epo					Fin	Epo	

*Statistically different values between two laboratories considered are labelled

pcaH data set

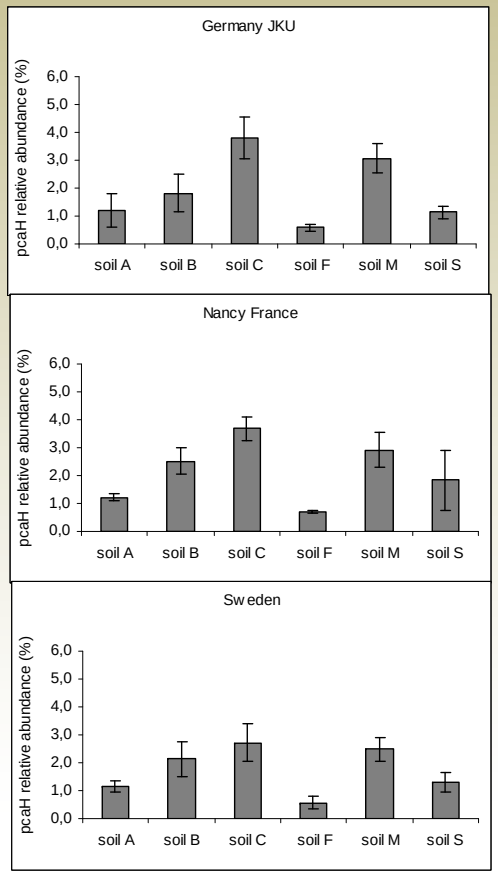
narG data set

good reproducibility among values → most of the *narG* or *pcaH* relative abundance values not differing significantly among each other.

Good reproducibility

→ **specific pattern** formed by the targeted communities abundances obtained by participant laboratories (Mantel & Kruskal-Wallis statistic tests, $p<0,05$)

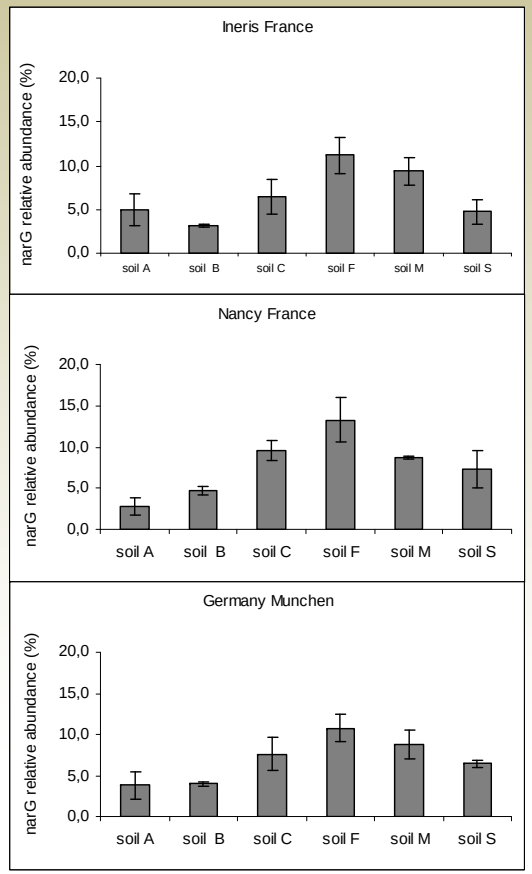
pcaH



Soil Fin<soil Ger<soil Swe<soil Bor<soil Mar<soil Epo

Soil Fin>soil Mar~soil Epo~soil Swe~soil Ger~soil Bor

narG



Conclusion 3

- soil DNA extracted by the proposed method was shown to be successfully used to quantify also less numerous functional bacterial communities in soils
- pattern for the two molecular markers studied was conserved between all laboratories involved in the ring test → robustness, efficiency and reproducibility of quantitative analyses performed on soil DNA extracts
- 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

International ring test on direct soil DNA extraction reveled:

1. proposed method was efficient in extracting DNA from six different soils showing contrasting physico-chemical properties
2. event though method was mainly dedicated to the agricultural and forest soils, results revealed that this method is also efficient for extracting soil DNA from complex soils (heavily contaminated with PAHs) and soils rich a in organic matter and clay and for soils presenting different acidity
3. soil DNA extracted from the six soils can successfully be used for both quantitative (qPCR assays) and qualitative (A-RISA fingerprinting).
4. allowing the estimation of the abundance of the global bacterial community as well as the estimation of the abundance of less numerous functional microbial communities
5. allowing the estimation of the structure of the global bacterial community
6. It can be concluded that the impact of the manipulator using the proposed method did not impair further molecular analyses performed on the extracted DNA

ISO 11063 publication

- ISO 11063 was unanimously adopted by the 21 countries member of ISO.
- It was published in July 2011 and traduced to French in October 2011 to be published by the French standardization agency (AFNOR).

J Soils Sediments (2010) 10:1344–1345
DOI 10.1007/s11368-010-0265-8

ANNOUNCEMENT

Soil microbial diversity: an ISO … DNA extraction

Laurent Philippot · Cristina Abbate · Antonio Bispo · Th
Philippe Lemanceau · Kristina Lindström · Pascal Pand
Michael Schloter · Pascal Simonet · Kornelia Smalla · Bo
Imes Petric · Fabrice Martin-Laurent

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

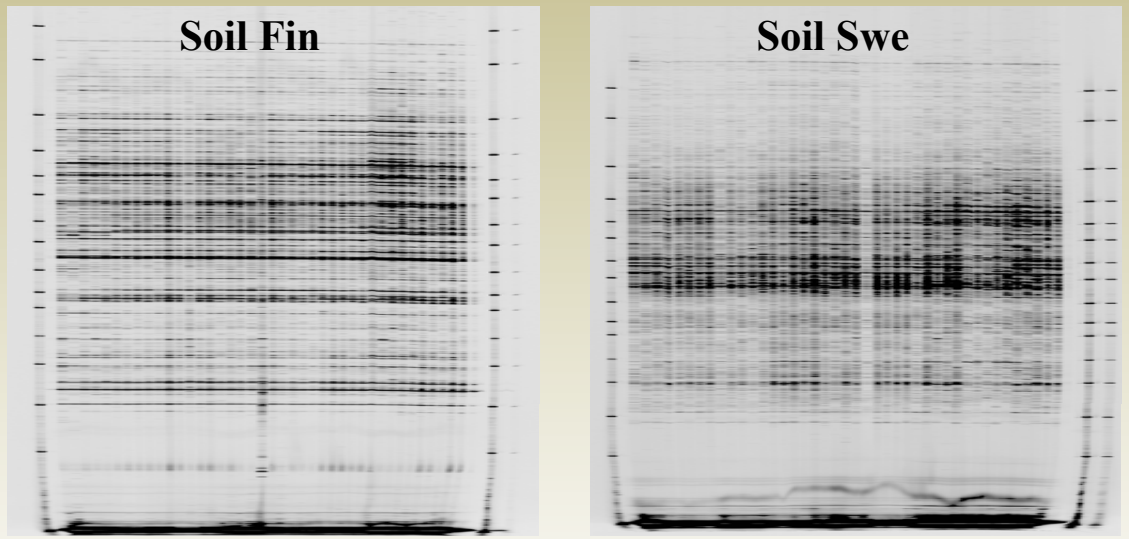
Inter-laboratory evaluation of the ISO standard 11063 "Soil quality — Method to directly extract DNA from soil samples"

I. Petric [a,1], L. Philippot [b], C. Abbate [c], A. Bispo [d], T. Chesnot [e], S. Hallin [f], K. Laval [g], T. Lebeau [h], P. Lemanceau [b], C. Leyval [i], K. Lindström [j], P. Pandard [k], E. Romero [l], A. Sarr [b], M. Schloter [m], P. Simonet [n], K. Smalla [o], B.-M. Wilke [p], F. Martin-Laurent [a,b,*]

Merci pour votre attention

Qualitative analysis
Estimation of the global structure of bacterial community

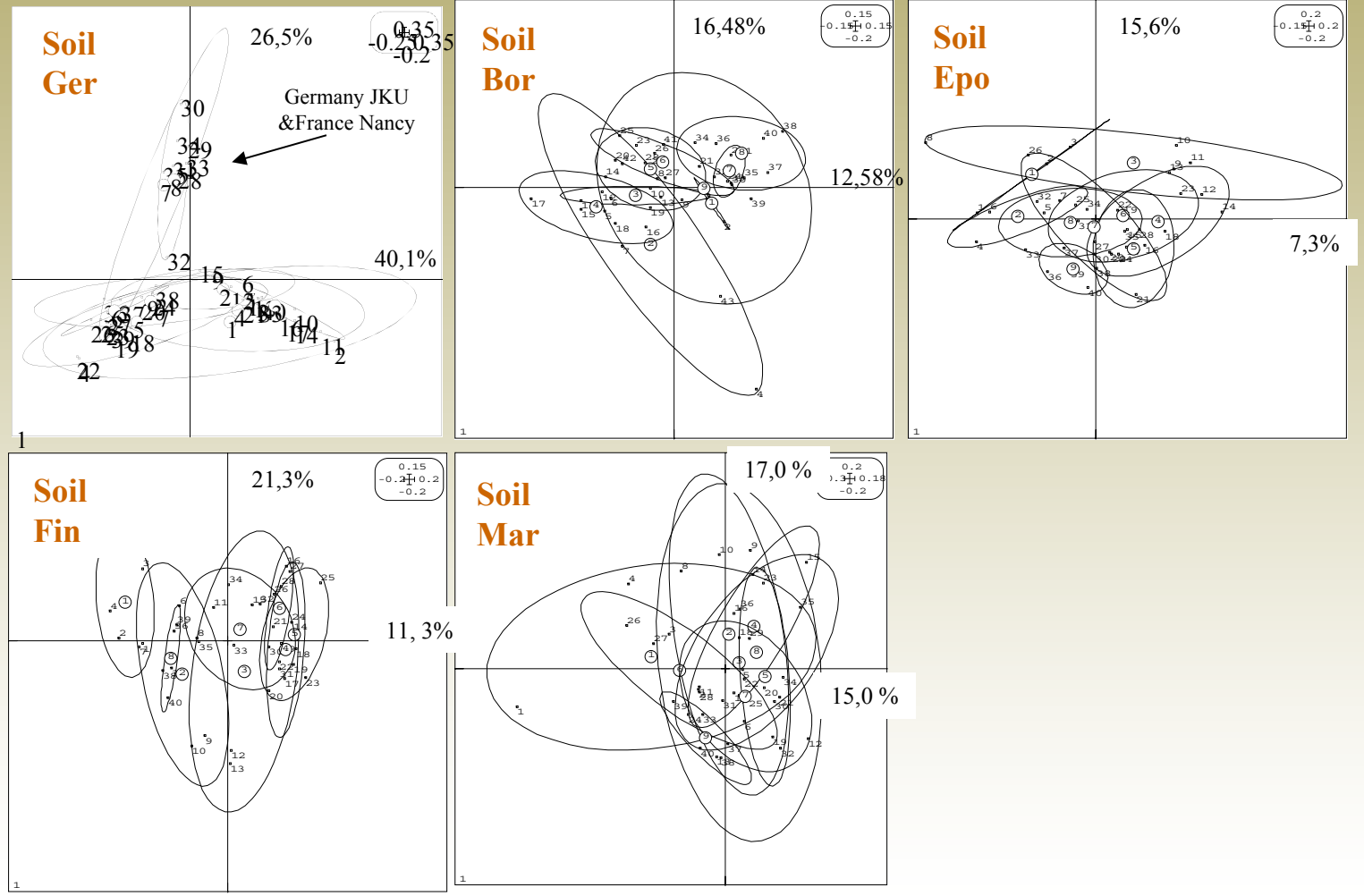
- fingerprinting method based on length polymorphism of the 16S-23S intergenic spacer of the bacterial ribosomal operon (PCR assay, primers ARISA-1552f/132r)



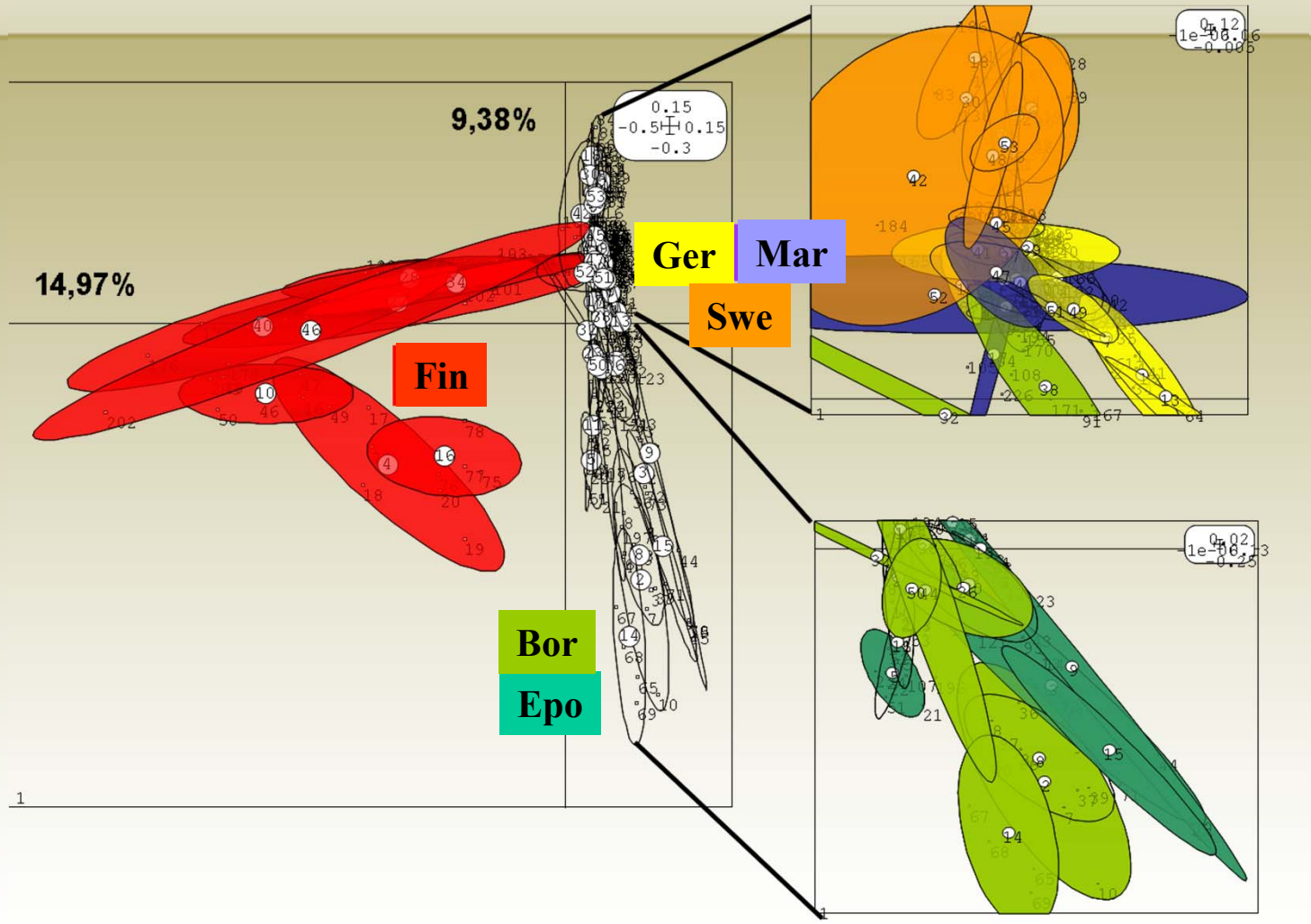
- complexity of the A-RISA fingerprints → bacterial community structure (> 100 bands per lane)
- specific soil community pattern given by each soils
- similarity in fingerprints for given soil → good reproducibility between different laboratories

Principal component analysis of the A-RISA fingerprints

- 1-D Scan software → PrepRISA program → ADE-4 software



- ✓ the structure of the bacterial community was not affected by the laboratory



✓clear discrimination between studied soils

Conclusion 4

- soil DNA extracted by the proposed method was shown to be successfully used to analyzed structure of the global soil bacterial community
- methodological biases due to the extraction and amplification 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-RISA is relevant and enough sensitive for studying bacterial communities in soil environments.