The following table summarizes the work performed during the full length of the project and its main achievements: in italics are the first term realization; in straight letters the second term realization.

Table A – WP's achievements

WP	Results	Achievements
WP1. Sample processing and protocol development	*Selection of DNA extraction kit; Dual fluorescent/spectrophotometric DNA measurements; PCR inhibition standard.* Development of descriptors and scores and their applications for selecting DNA extraction methods using a variety of soils.	▪ *A protocol for extracting and measuring the quantity and quality of soil DNA.* ▪ A suite a parameters, descriptors and scores for selecting methods for the extraction of DNA from environmental samples. A grading of DNA extraction methods. ▪ A short list of most efficient methods.
WP2. Sample collection and storage	*A survey of soil sampling and forensics use in Europe; design of a sampling template; comparison of DNA profiles under various storage conditions.* Guidelines for accessing soil sampling in scenes and agents, including vehicles, shoes, spades etc, and how to collect, handle, store soil forensic samples. Storage – over a short to medium term, as long as samples are not rewetted – does not affect samples. A multi-laboratory evaluation of a mock-crime scene using various analytical and geographic localization techniques, most showing the ability to compare a questioned soil to a particular crime site. Resolution of 25 m distance between samples was achieved using microbial methods.	▪ *Assessment of current procedures and interest in soil forensics in Europe.* ▪ *A soil sampling and handling procedure; Sample storage recommendations.* ▪ A "how to" set of working guidelines for the forensic practitioner. ▪ Medium term robust storage of soil samples. ▪ An in-situ, real-life demonstration of the applicability of soil microbial forensic methods with the involvement of 12 forensic and research laboratories.
WP3. Evaluation of novel technologies for soil forensic use	*Evaluation of RISA , microarray, and pyrosequencing for soil microbial profiling; evaluation of markers for soil bacterial community analysis; a comparison of soil DNA extraction method on bacterial community structure.* Sequence analysis-based evaluation of the *rpoB* and *rpoC* house-keeping genes as a marker of soil diversity concludes to dismiss it. RISA and phylochip evaluation of soil: RISA but not the phylochip approach robustly distinguishes between soil with a resolution of hundreds of meters.	▪ *RISA is fast and reliable for preliminary sample comparisons* ▪ *The 16S rRNA gene is the most effective marker but additional markers (ropB, antibiotic resistance genes) add robustness* ▪ *Soil extraction method affects community structure: all samples should be extracted using the same method*. ▪ The 16S rRNA gene-based diversity analyses are not matched by other markers. ▪ Phylochips analysis is not precise enough for forensics ▪ RISA as a fast and cheap method can be applied as a first step in soil sample analysis
WP4. Delimiting spatial and temporal boundaries of microbial soil profiling	*Comparisons of PCR protocols, primer tags, DNA dilution effects, PCR replicates, and TRFLP runs on the resolution of soil samples; A comparison of 18 statistical pipelines for analysis of TRFLP data; Comparison of marker genes between 3 closely related soils and within soils (distance-decay); A comparison of TRFLP and MPS with the 16S-rRNA gene; A scrutiny of legal aspects on soil DNA uses.* Complete analysis of a multi-methodological comparison between chemical, array, pattern-	▪ *A protocol for soil DNA analysis by TRFLP* ▪ *A protocol for TRFLP data handling and statistical analysis* ▪ *Confirmation of the 16S rRNA gene as a reliable and precise marker* ▪ *TRFLP and MPS yield comparable results* ▪ *A protocol for soil DNA analysis by TRFLP* ▪ The demonstration that soil samples can robustly be discriminated using various technological platforms, i.e. that

	based, and sequencing approaches, and alternative marker, applied to soil sample discrimination. All methods besides the array-based method and the alternative marker significantly distinguished between soil samples some with high spatial resolution. Analysis of a soil manipulation experiment trying to re-created seasonal changes. It showed that although the potential exists, further research is required.	the measurements truly represent factual differences. ▪ Showing that seasonal mitigation may be achieved but needs further research.
WP5. Bioinformatics tools	*Establishing a pipeline for T-RFLP data analysis; Software requirements for metagenomics data analysis.* Finishing the development and launching a metagenomics bioinformatics workbench. The application of the workbench in the mock-crime scene scenario showing its fitness to the task. The workbench includes sequence-based and TRFLP-based analysis tools. Inclusion of various web-available geographic tools to a forensic biogeographic application to exclude and include evidence from crime-related scenes.	▪ *Standard operating procedures (SOP) for trace signal processing and visualisation* ▪ *Specification of MPS software solution* ▪ *Specifications for the geoforensic database* ▪ Initial prototype interface to spatial database ▪ Initial testing of creation of spatial database for Israel study area ▪ A fully-fledged, integrated bioinformatics platform for the analysis of soil and other environmental samples. ▪ The implementation of multiple parameters as mapping tools at various scales and their integration.
WP6. Evaluation and validation of protocols and material, legal support	Establishing a pipeline for T-RFLP data analysis; ISO-level protocols for soil sampling, storage, and analysis. Legal context analysis of the use of novel technologies in soil forensics	▪ Standard operating procedures (SOP) for trace signal processing and visualisation ▪ Review of application of soil microbial approach in both civil and common law systems.

WP01: Sample processing and protocol development

Table 1: DNA extraction methods evaluated in WP1.

Code	Designation	Abbreviation	Manufacturer
K01	FastDNA Spin kit for Soil	FastSpin	MP Biomedicals
K02	UltraClean Soil DNA Isolation Kit	UltraClean	MoBio Laboratories
K03	PowerLyser PowerSoil DNA Isolation kit	PowerLyser	MoBio Laboratories
K04	SoilMaster DNA Extraction kit	SoilMaster	Epicentre Technologies
K05	ZR Soil Microbe DNA MiniPrep	ZRSoil	Zymo Research
K06	SurePrep Soil DNA Isolation Kit	SurePrep	Fisher BioReagents
K07	NucleoSpin Soil	NucleoSpin	Macherey-Nagel
K08	EZNA soil DNA isolation kit	EZNA	Omega Bio-tek
K09	Soil DNA Isolation Kit	Norgen	Norgen
TM01	JHI modified Griffith method	GriffithMod	-

Table 2: Normalization of descriptors

Descriptor	Normalization
DNA Yield (µg/g of soil)	For a given soil, the highest recorded yield among extraction methods takes the maximum value of 20
260/280	A 260/280 ratio of 2.0 takes the maximum value of 20
260/230	A 260/230 ratio of 2.0 takes the maximum value of 20
16S undil	For a given soil, the highest amount of amplicon (ng) among extraction methods takes the maximum value of 20
16S 1/10 dil	For a given soil, the highest amount of amplicon (ng) among extraction methods takes the maximum value of 20
SAC undil	For a given soil, the maximum amplification yield of SacB gene of 100% takes the maximum value of 20
SAC 1/10 dil	For a given soil, the maximum amplification yield of SacB gene of 100% takes the maximum value of 20

Table 3: Soils evaluated in the full evaluation of DNA extraction methods.

Soil	Supplementary data
Rothamsted	2 mm sieved
Rathen 891520/2	Brown earth imperfectly drained
Drumlithie 891403/2	Humus Iron Podzol freely drained
Ardallie 891509/02	Peaty Gley Poorly drained

Table 4. Scoring index for the evaluated DNA extraction methods

		SCORES				
		0	1	2	3	4
Descriptor	Yield	[0 ; 2]	]2 ; 10]	]10 ; 20]	]20 ; 40]	>40
	260/280	[0 ; 1,2[	[1.2 ; 1.6[	[1.6 ; ...[		
	260/230	[0 ; 1,0[	[1.0 ; 1.5[	[1.5 ; ...[		
	SAC undil	[0 ; 10[	[10 ; 25[	[25 ; 50[	[50 ; 75[	[75 ; 100]
	16S undil	0	]0 ; 100]	]100 ; 200]	]200 ; 400]	>400
	16S 1/10 dil	0	]0 ; 100]	]100 ; 250]	]250 ; 500]	>500

Table 5. Scored results of nine extraction methods on four soil types. For each Method x Soil condition, the score is given. For each method, the average score is calculated on the four soils.

	Ardallie	Drumlithie	Rathen	Rothamsted	Average Score
NS	18 (3|22|434)	14 (1|21|443)	16 (1|22|434)	17 (2|22|434)	**16,25**
NO	12 (2|10|333)	14 (1|20|443)	11 (2|20|434)	17 (4|20|434)	**13,50**
PL	4 (0|10|012)	14 (1|11|434)	10 (3|22|003)	12 (4|22|004)	**10,00**
EZ	9 (3|20|004)	6 (1|10|013)	13 (1|20|334)	11 (3|21|203)	**9,75**
ZR	11 (1|10|243)	6 (1|00|410)	14 (1|10|444)	6 (2|10|003)	**9,25**
UC	3 (2|10|000)	6 (1|11|003)	8 (2|21|003)	10 (3|22|003)	**6,75**
FS	4 (3|10|000)	7 (2|10|004)	9 (3|20|004)	7 (4|10|002)	**6,75**
SP	3 (2|10|000)	5 (1|10|003)	3 (1|00|002)	2 (1|10|000)	**3,25**
SM	4 (0|01|210)	1 (0|00|001)	3 (0|02|001)	2 (0|10|001)	**2,50**

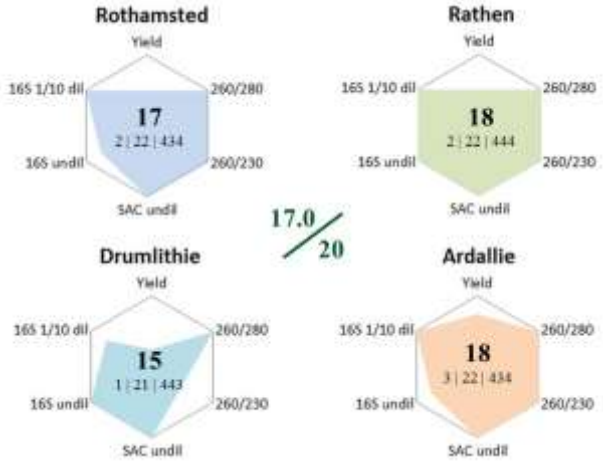

Fig. 1. Multi-parameter, weighed representation of the DNA extraction performance of the Nucleo-Spin kit on the four tested soils.

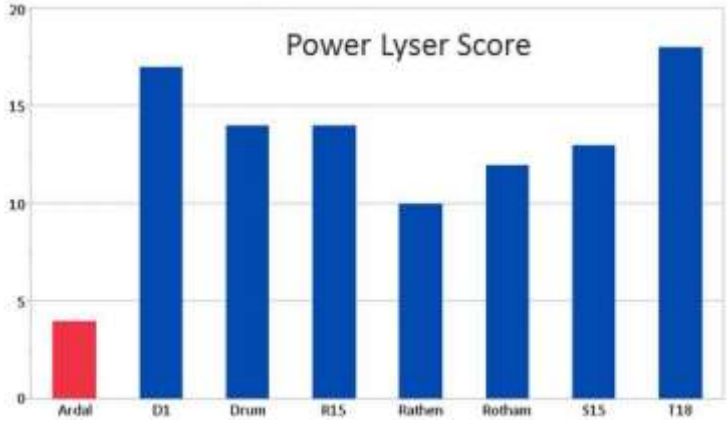

Fig. 2. Score results of DNA extraction using the PowerLyser PowerSoil kit on the eight reference soils

- **WP02: Sample collection and storage**

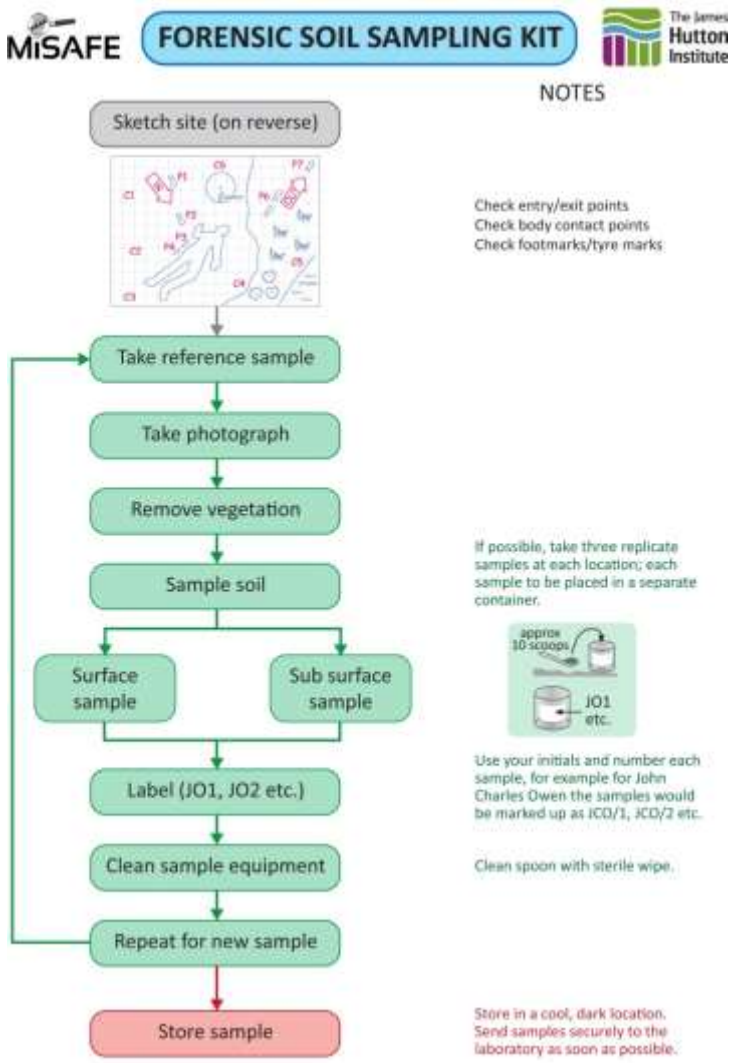

Fig. 3 Flow chart of a guidance protocol for field soil sampling.

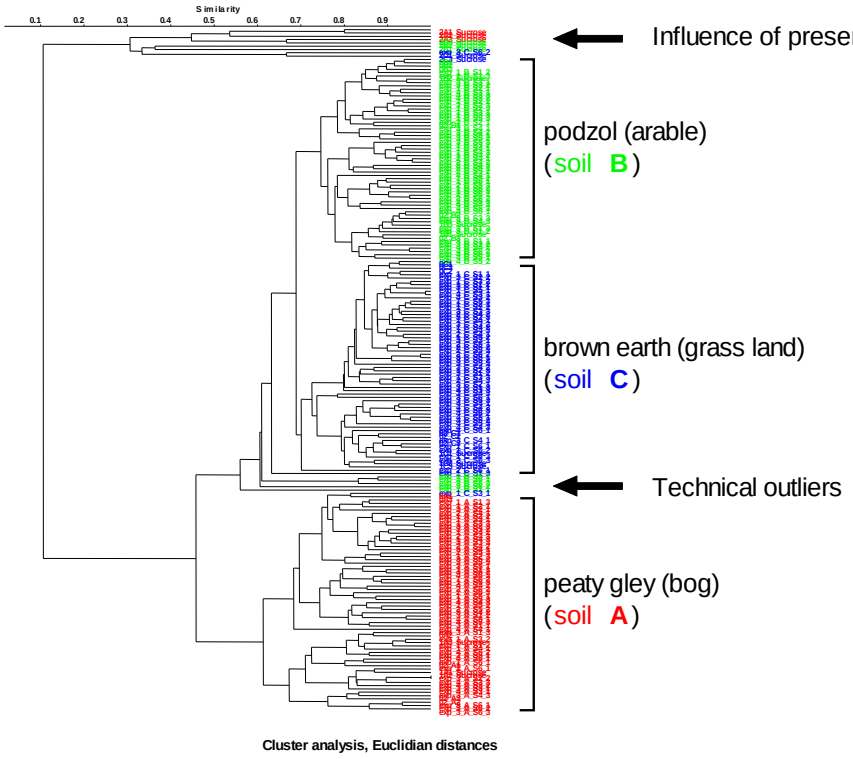

Fig. 4. Grouping of three soil types with all storage treatments combined.

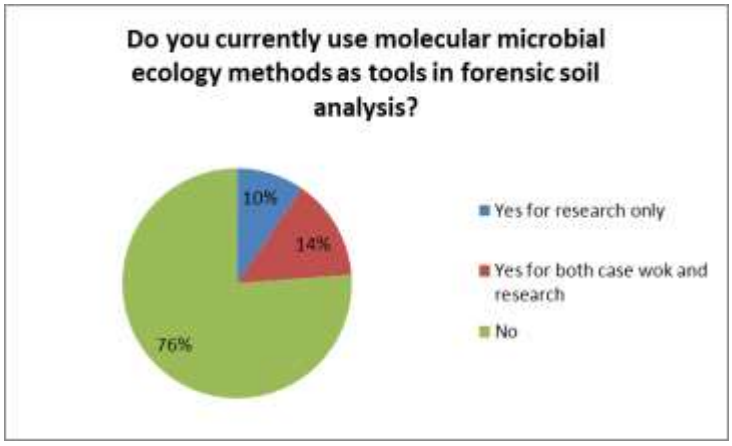

Fig. 5. Chart presenting the extent of use of molecular microbial methods in forensic science.

WP03: Evaluation of novel technologies for soil forensic use

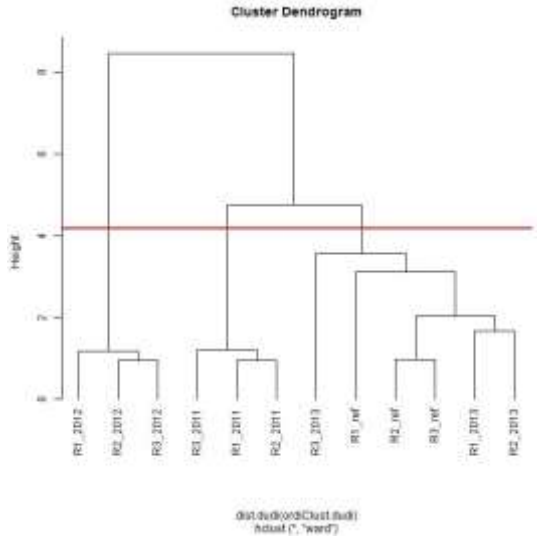

Fig. 6. Cluster analysis of various Rothamsted soil samples stored under different conditions.

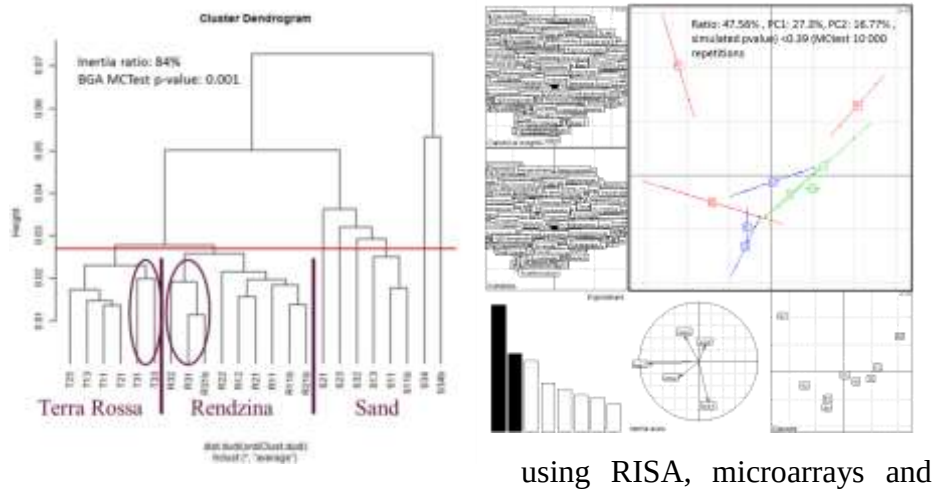

Fig. 7. RISA (left) and phylogenetic microarray analysis of three soils from Israel.

As part of the mock-crime scene partnership, analyses were performed within the frame of WP3, using RISA, microarrays and Illumina MiSeq high throughput sequencing. The results obtained matched those described above in the WP4: RISA and MiSeq are effective methods for soil spatial comparison – microarrays are not.

WP04: Delimiting spatial and temporal boundaries of microbial soil profiling

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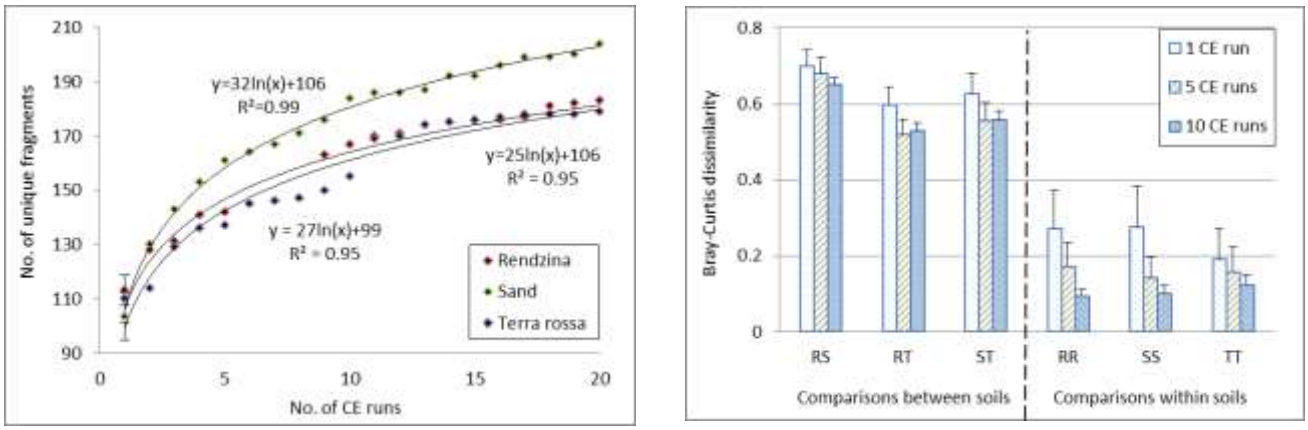


Fig. 8. Effect of the number of capillary electrophoresis (CE) runs (from the same PCR amplification) on TRFLP efficiency. Left, number of unique terminal restriction fragments in three soil types (values at 1 CE run are mean±SD of all 20 runs); Right, Bray-Curtis dissimilarity between and within soil types (R - rendzina, S – sand, T - terra rossa). Higher Bray-Curtis dissimilarity values denote less similarity between bacterial communities. Values are mean±SD.

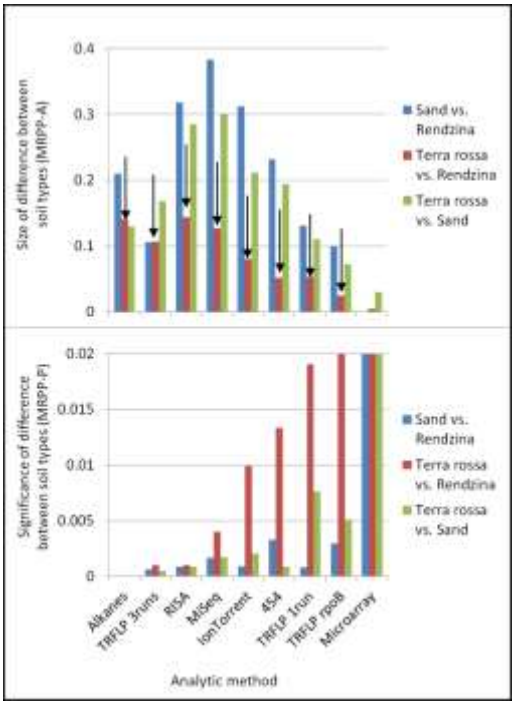
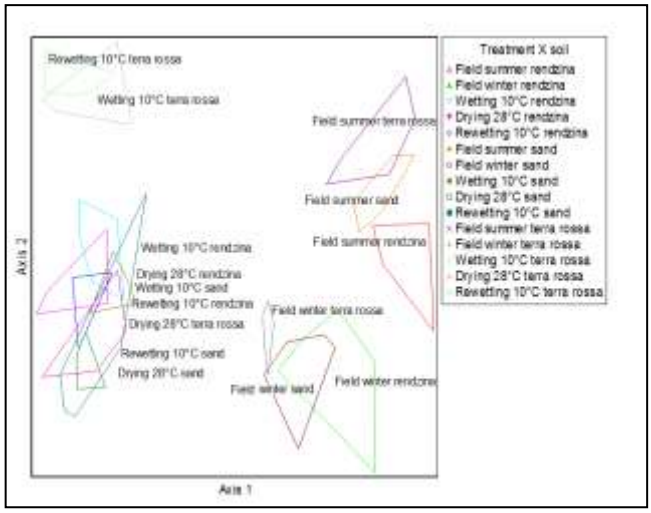
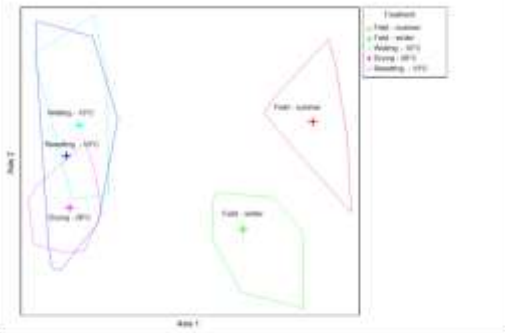


Fig. 9. Comparative analysis of nine soil analysis procedures. Upper figure: Bray-Curtis differences (MRPP-A); lower figure: significance of difference (MRPP-P).





Sample manipulation has the potential to mitigate soil seasonal fluctuations but cannot currently be applied in soil forensics.

Fig.10. MNDS plot of all treatments. Season-based soils are all different (MRPP test, P≤0.05). Similarly all manipulated samples are different from the natural samples(MRPP test, P≤0.05) (left). All samples analysed together (right).

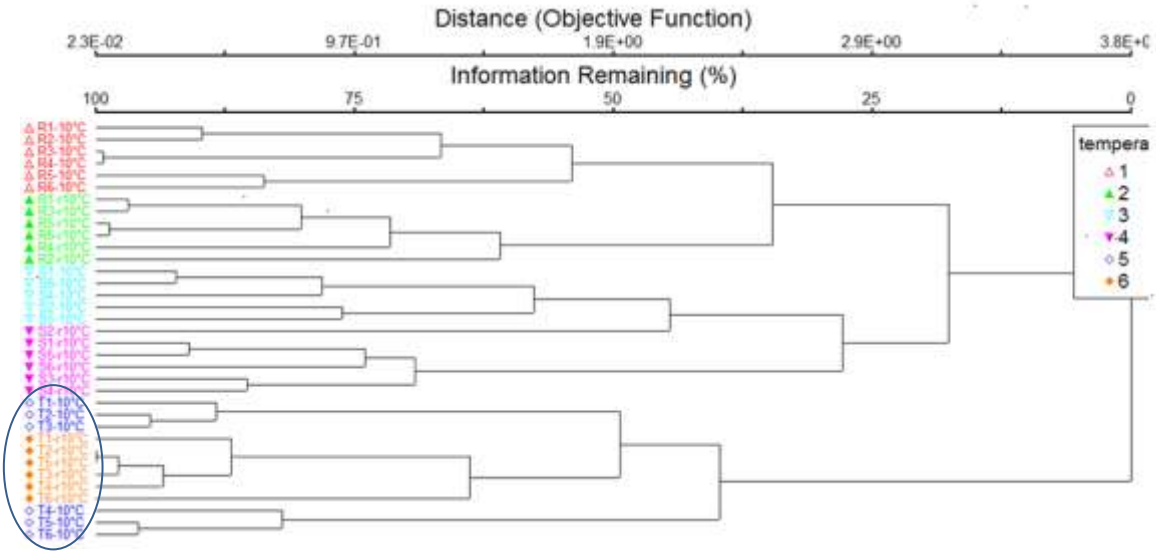


Fig. 11. Cluster analysis of the wetted and rewetted treatments.

WP05: Bioinformatic tools

No added figures or tables

WP06: Evaluation and validation of protocols and material, legal support

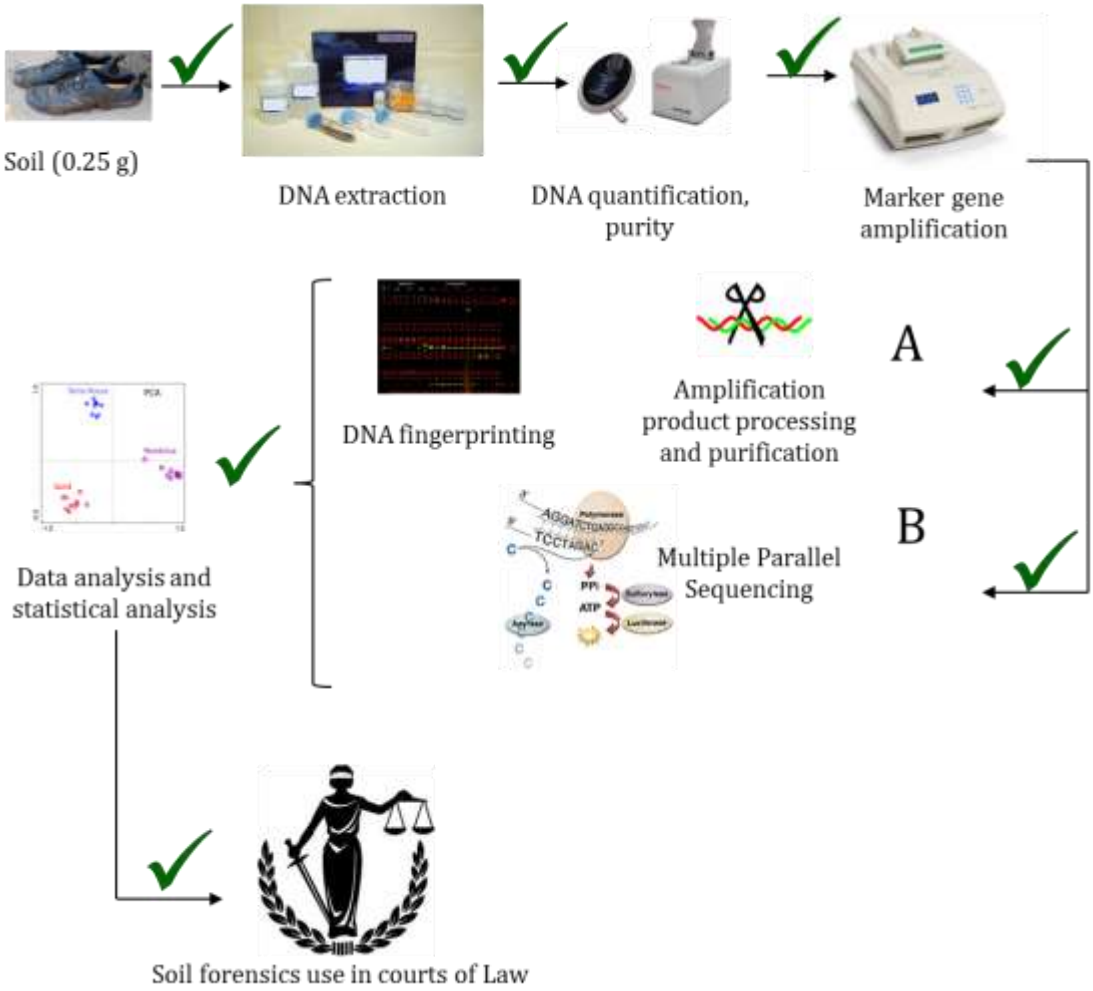


Fig. 12. The process for bacterial soil DNA profiling of forensics use. Green "V" signals an accomplishment of MiSAFE.

Figure 12 depicts the full chain of events from the site to the court as may happen to a soil sample. Each of the various steps was evaluated, tested, and implemented successfully.

Soil investigations are not limited to personal crimes. Soil bacterial profiling may be applied to help decipher environmental crimes; to link agricultural performance to soil biological properties; to map sites for metagenomics analyses, and more.

Dissemination

Dissemination of the outputs of the MiSAFE research was carried out under WP8. A website was established (Fig. 13) and there were over 60 related events (talks, publications, interviews for example). The international impact was considerable with successful collaboration with European and global networks. The details of the dissemination section are provided in the tables attached.

Fig. 13. The MiSAFE web site at http://forensicmisafe.wix.com/misafe