

MICROBIAL BIODIVERSITY OF CONTAMINATED SOILS AND IDENTIFICATION OF MICROBIAL BIOINDICATORS FOR THE ASSESSMENT OF SOIL HEALTH: FROM CULTURAL METHODS TO NEXT GENERATION SEQUENCING

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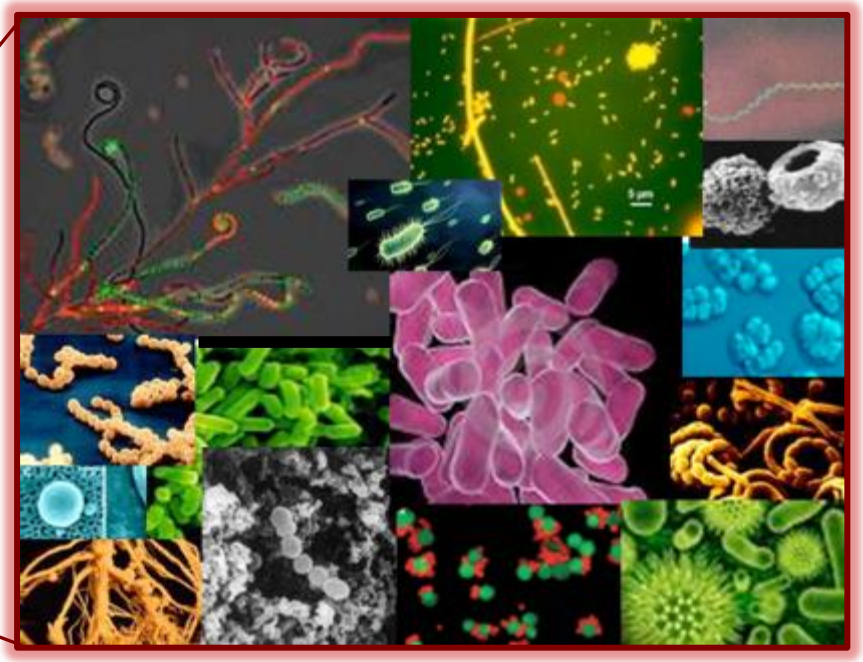
WE ARE IMMERSED IN MICROBES

They live in our bodies, in our food, and in everything that surrounds us; we cannot live without them.

Soil has the highest microbial diversity in respect to other natural habitats



SOIL BIOLOGICAL FERTILITY

The capacity of organisms living in the soil (microorganisms, fauna, and roots) to contribute to the nutritional requirements of plants and foraging animals for productivity, reproduction, and quality while maintaining biological processes that contribute positively to the physical and chemical state of the soil (Abbott and Murphy, 2007)

What happens in the soil when toxic xenobiotic compounds are released?

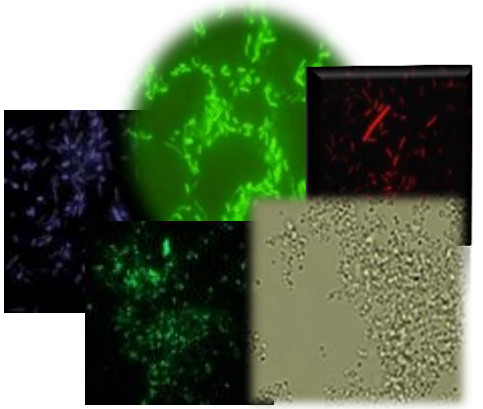
The presence of toxic xenobiotic contaminants determine the establishment of biological disequilibrium conditions in the soil, in the rhizosphere and among microbial populations.

Contaminants can be used as a carbon source by some species of microorganisms

Establishment of a new biological equilibrium by the development of new microbial community.

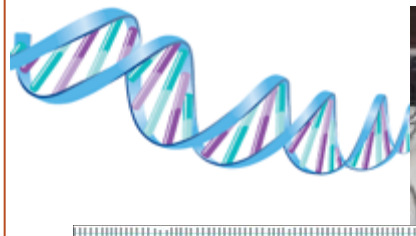
The assessment of the response of microbiota to anthropogenic pressures due to environmental pollution and to bioremediation techniques is a critical issue in soil ecology.

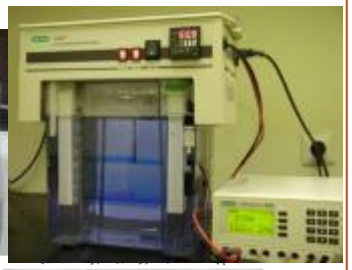
Evaluation of the microbial diversity and identification of potential microbial bioindicators for the assessment of soil heath

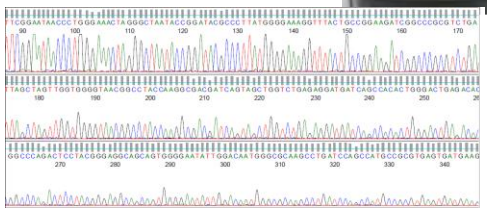


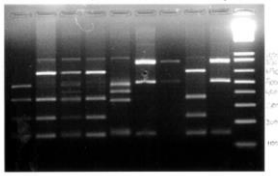
Culture-dependent methods

Culture-independent methods





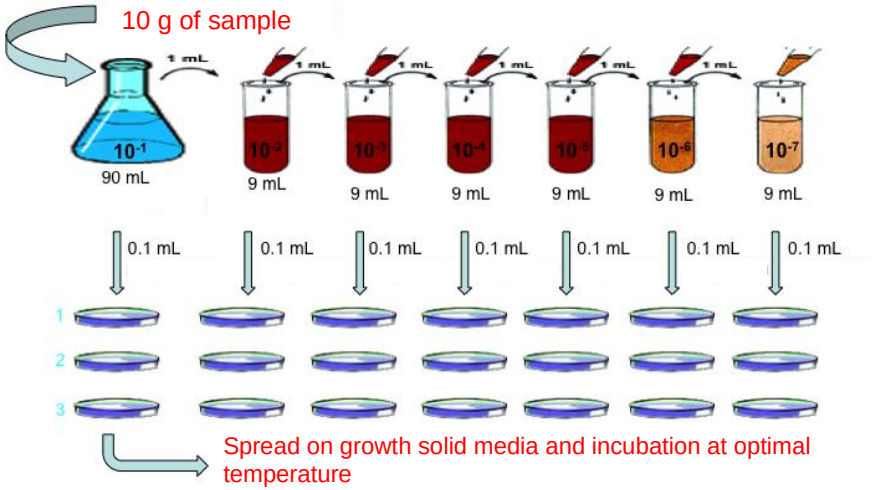


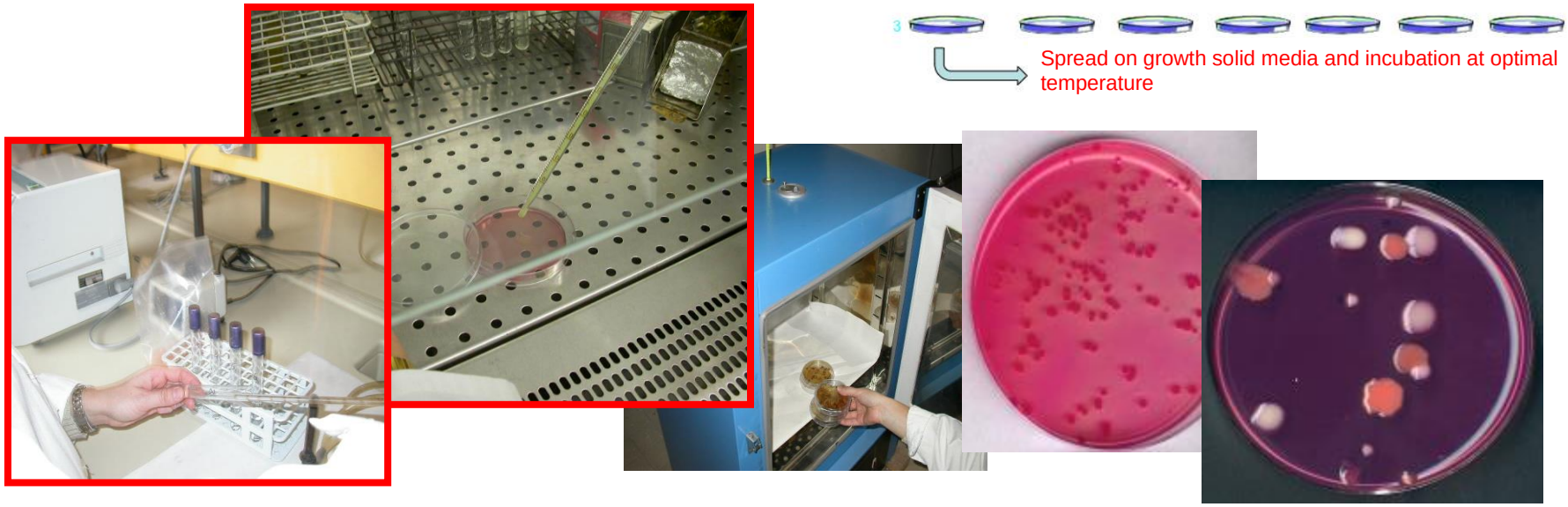


Culture-dependent approach: use of growth media for counting specific eco-physiological microbial groups

Enumeration of microorganisms on growth media

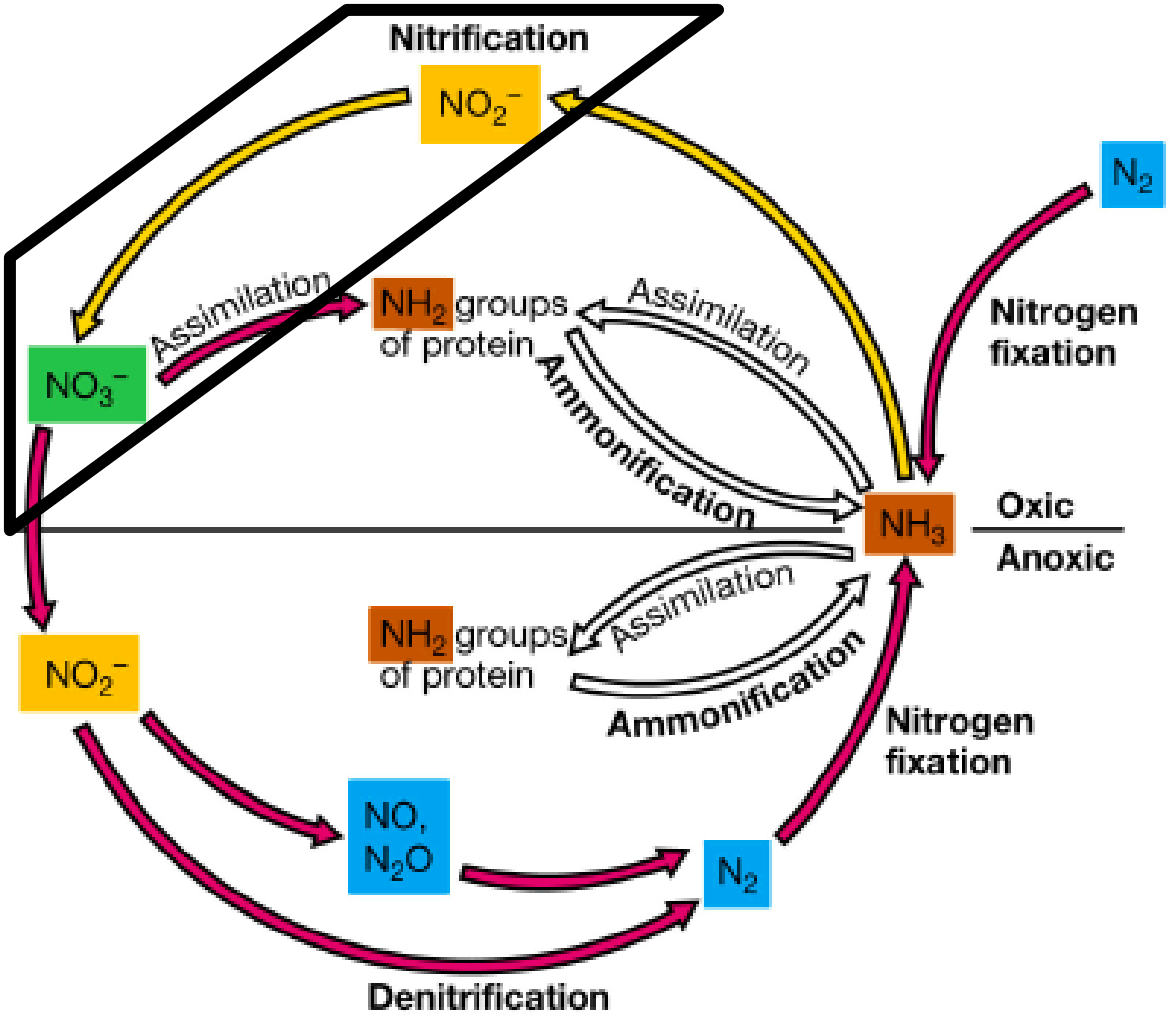
- Sample preparation;
- ten-fold serial dilutions;
- inoculation on growth media;
- microbial counting (CFU or MPN/g).





Although cultural-dependent methods are less effective in the study of microbial ecology, they are fundamental in the search for microbial strains for specific functional activities.

Specific microbial populations, such as those involved in the nitrogen cycle have been found to be particularly sensitive to Potentially Toxic Elements (PTE) and in particular the nitrification process is considered one of the most sensitive microbial assays.



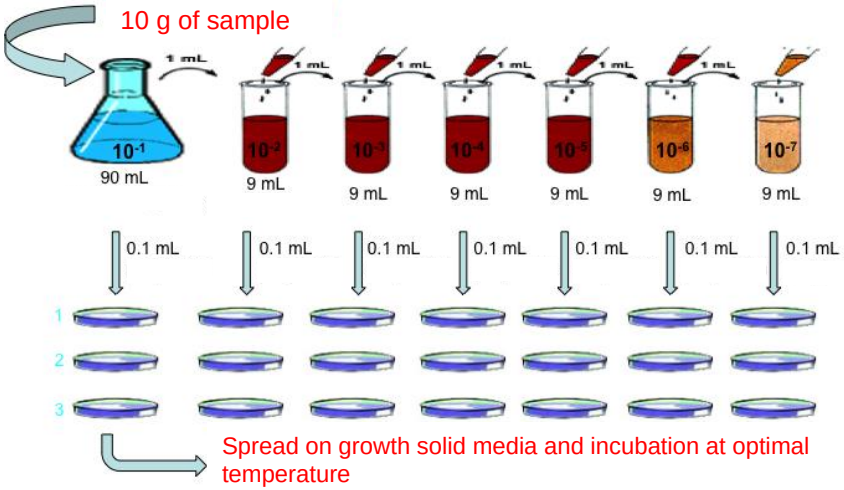
Culture-dependent approach: case study

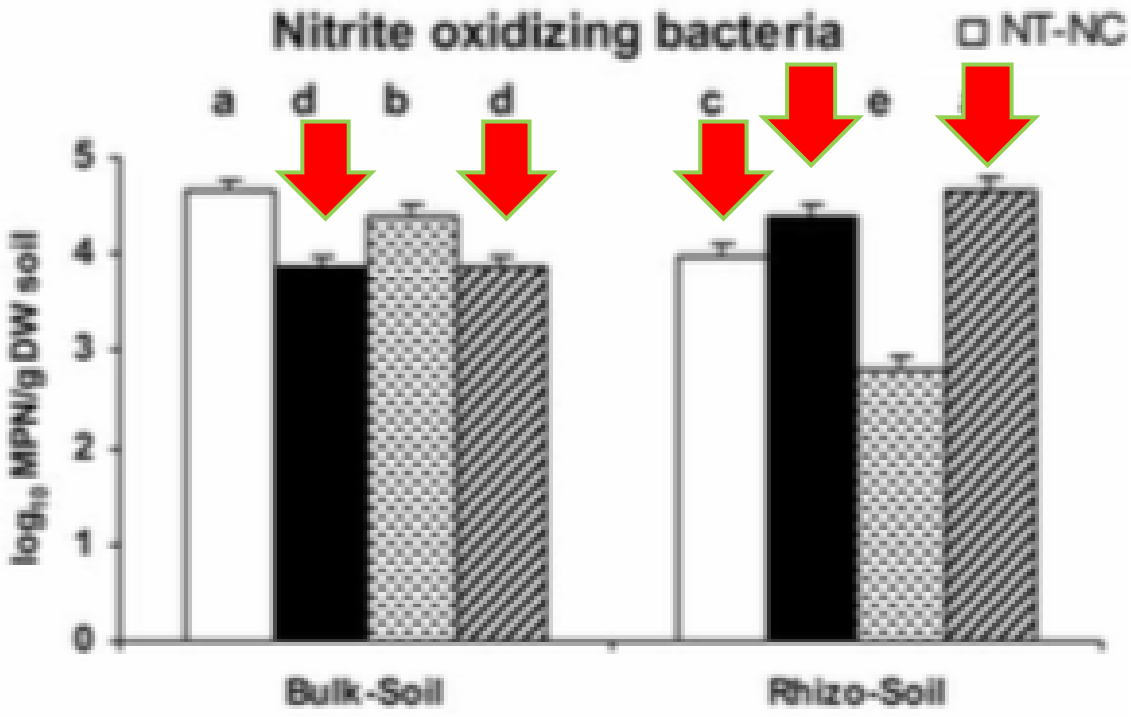
Italian Journal of Agronomy 2013; volume 8:e29

Assisted phytoextraction of heavy metals: compost and *Trichoderma* effects on giant reed (*Arundo donax* L.) uptake and soil N-cycle microflora

Nunzio Fiorentino, Massimo Fagnano, Paola Adamo, Adriana Impagliazzo, Mauro Mori, Olimpia Pepe, Valeria Ventorino, Astolfo Zoina

- Cadmium contamination.
- Assisted phytoextraction by *Arundo donax* L. with compost and *Trichoderma.*
- Evaluation of the abundance of specific microbial functional groups following assisted phytoextraction.

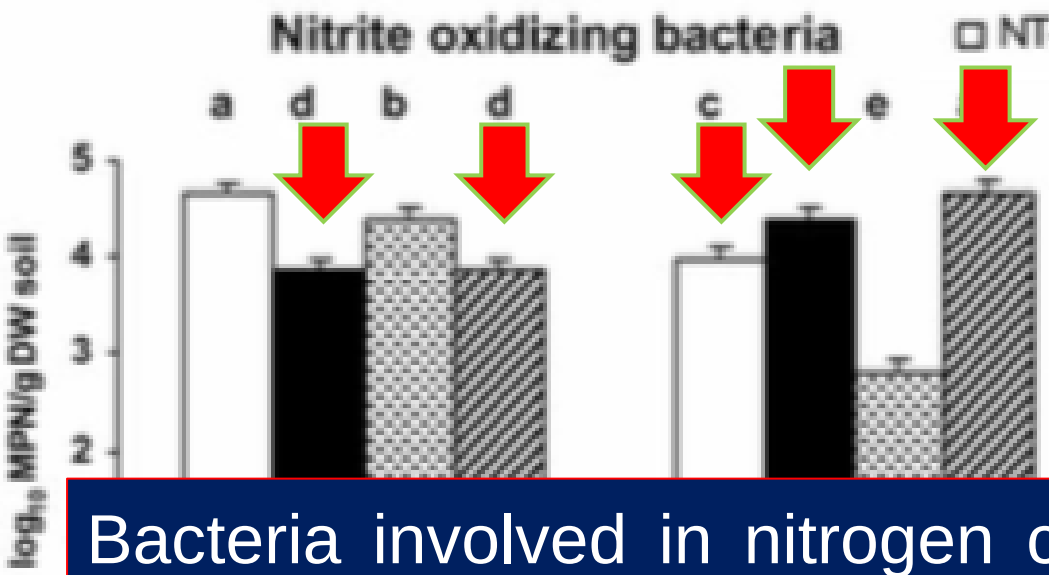




NT, no *Trichoderma*;
NC, no compost;
C, compost;
T, *Trichoderma*.

The significant increase of nitrite-oxidizing bacteria in the rhizosphere of giant reed following compost addition could be due to the increase of metals availability and their consequent plant uptake.

Whereas, the reduction of this bacterial group in bulk-soil could resulted from the increased metal availability (without plant uptake).



NT, no *Trichoderma*;
NC, no compost;
C, compost;
T, *Trichoderma*

Bacteria involved in nitrogen cycle were seen to be useful biomonitors to assess soil quality and PTE bioavailability, representing a viable complementary technique to the common soil chemical analyses.

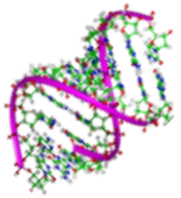
T… …e rhizosphere of giant reed following compost addition could be due to the increase of metals availability and their consequent plant uptake.

Whereas, the reduction of this bacterial group in bulk-soil could resulted from the increased metal availability (without plant uptake).

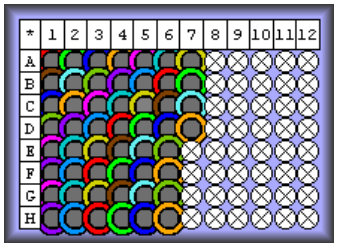
Culture-independent approach: quantitative real-time polymerase chain reaction (qPCR)

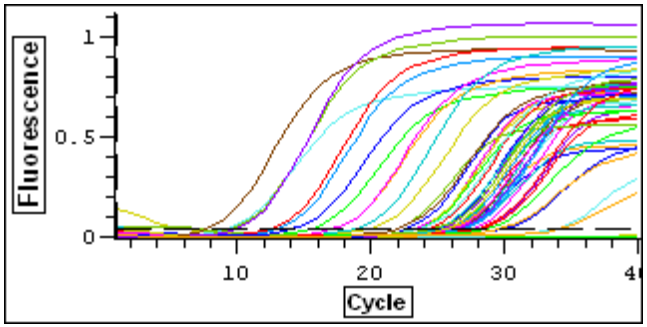
qPCR is a sensitive and suitable approach for determining abundance of functional genes from soil-derived NA

✓ **Total genomic DNA extraction (directly from soil);**

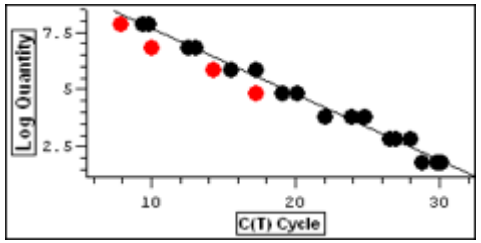


✓ **amplification of bacterial functional genes using a qPCR mix, contains dNTPs, Taq DNA Polymerase, $MgCl_2$, SYBR® Green, enhancers, stabilizers, fluorescein;**

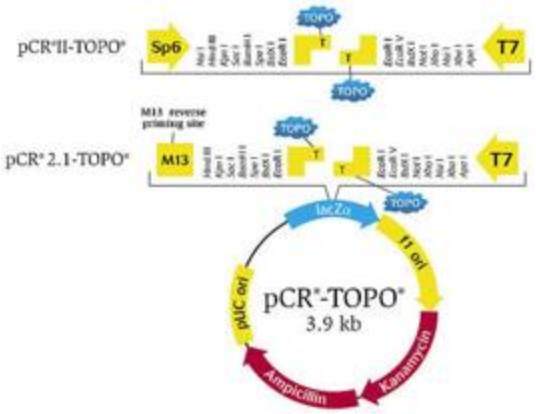




✓ **quantification of the abundance of bacterial functional genes (copies g^{-1} of soil) comparing the copynumber with a standard curve.**



➢ standard curve generation:



1. Each target **gene is amplified** with specific primers from a type strain;

2. amplicons are purified and cloned in *Escherichia coli*-competent using a **cloning** kit promoter;

3. plasmid DNA containing a single copy of the target gene is **purified and quantified**;

4. the **copy number µl^{-1} of each cloned gene is calculated** using the following formula: **(A × B) / (C × D)**

   where A is the DNA concentration (ng µl^{-1}), B is the Avogadro number (6.023 × 10^{23} copies mol^{-1}), C is the average molecular weight of a DNA base pair (6.6 × 10^{11} ng mol^{-1}), and D is the DNA size (bp);

5. tenfold serial dilutions, ranging from 10^{1} to 10^{8} gene copies µl^{-1}, of each plasmid DNA sample, are used to **generate a standard curve** for each target gene.

qPCR: case study

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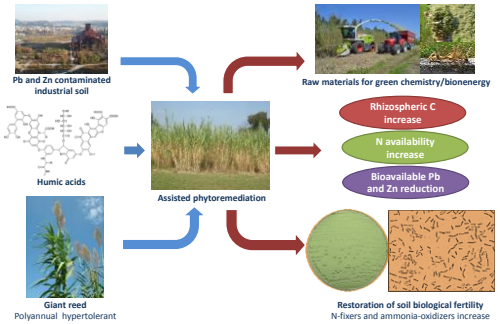
journal homepage: www.elsevier.com/locate/scitotenv

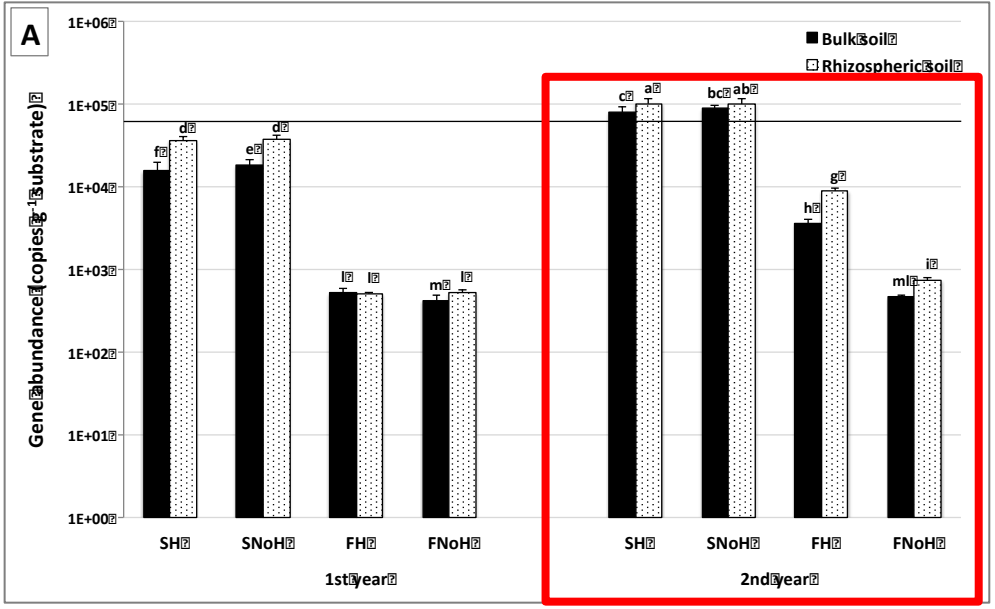
Giant reed growth and effects on soil biological fertility in assisted phytoremediation of an industrial polluted soil

N. Fiorentino *, V. Ventorino, C. Rocco, V. Cenvinzo, D. Agrelli, L. Gioia, I. Di Mola, P. Adamo, O. Pepe, M. Fagnano

- **Assisted phytoremediation (*Arundo donax* L.) of industrial polluted soils treated with humic acids.**
- **Lead (Pb) and zinc (Zn) contamination.**
- **Evaluation of microbial populations following assisted phytoremediation.**

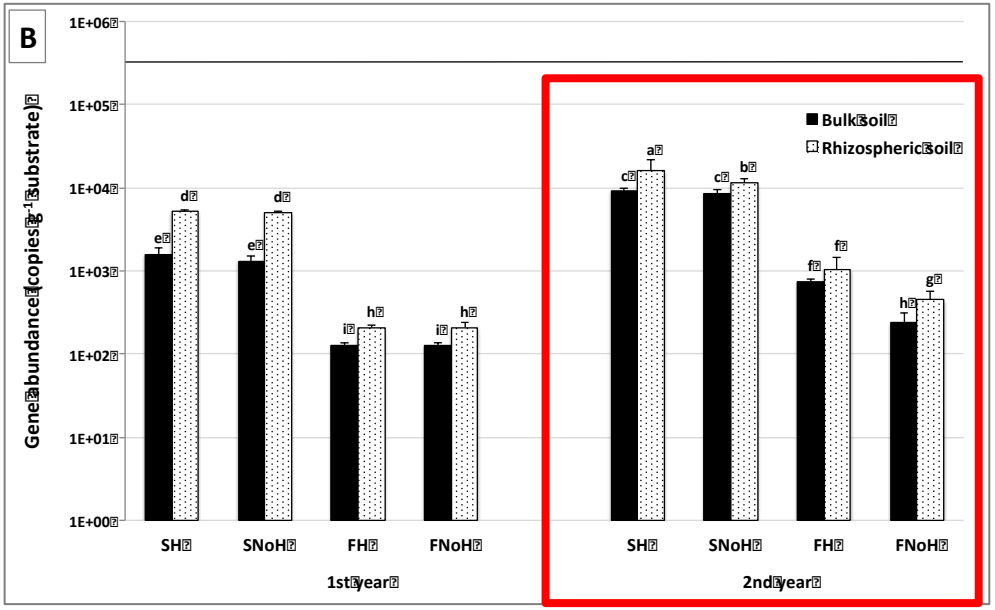


- ✓ **Total genomic DNA extraction from soil samples (bulk soil and rhizosphere);**
- ✓ **qPCR of bacterial functional genes:**
  - ➢ ***nif*H, encoding for nitrogenase reductase;**
  - ➢ ***amo*A, encoding for ammonia monooxygenase;**
- ✓ **estimation of the abundance of functional genes at the end of each cropping cycle to evaluate the influence of plant growth and humic acid treatment on the microflora related to the N cycle.**



A marked increase in nitrogen fixers was observed at the end of the 2nd year regardless of treatments.

An overall positive effect of plant on soil microbial growth was recorded in industrial soil, with a significant increase in ammonia oxidizers over the years. This is a key point to evaluate the efficiency of a phytoremediation technique.

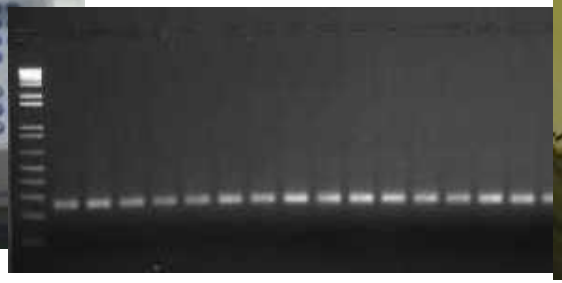


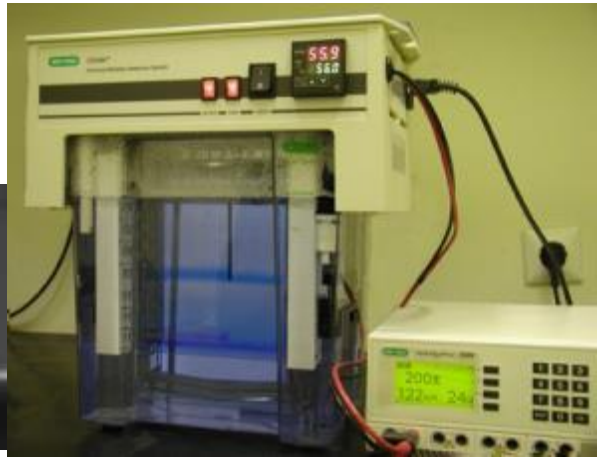
A significant increase in ammonia oxidizers was observed at the end of the 2nd cropping cycle in all conditions.

Culture-independent approach (qualitative method): Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

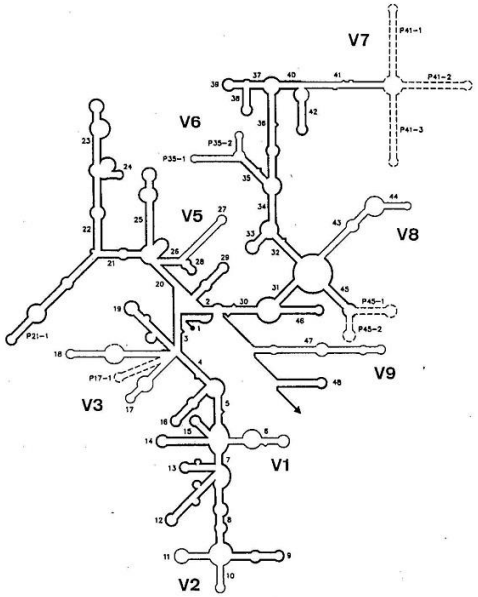
DGGE is a molecular method that allow to separate PCR products, having similar size (bp), on the basis of differences in nucleotide sequence that result in differential DNA denaturation characteristics.

- ✓ **Total genomic DNA extraction (directly from soil);**
- ✓ **touchdown-PCR of hypervariable region of rRNA genes;**
- ✓ **check on agarose gel;**
- ✓ **electrophoresis in polyacrylamide gels characterized by a denaturant gradient.**



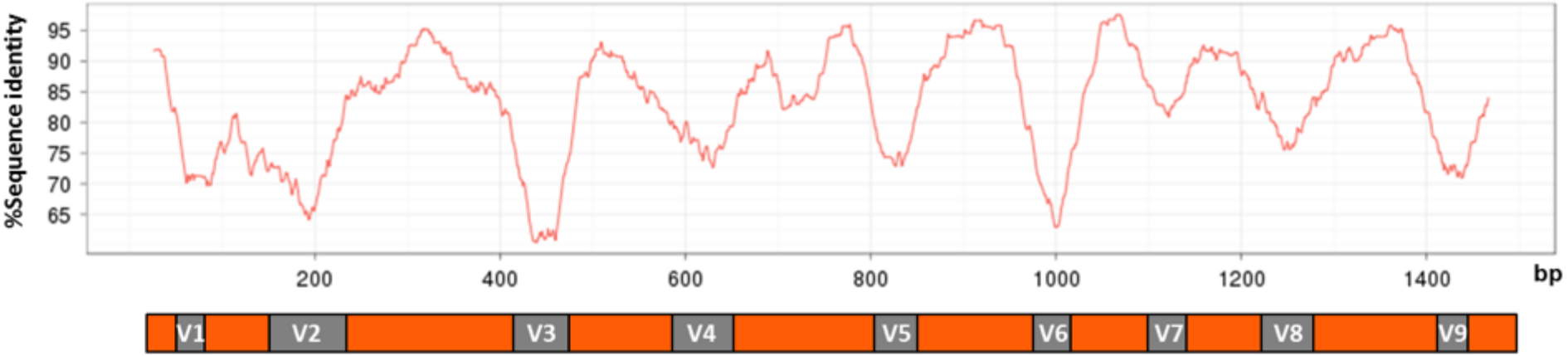


Why rRNA?



- High genetic stability
- It includes both conserved and hypervariable regions
- Database avaiability

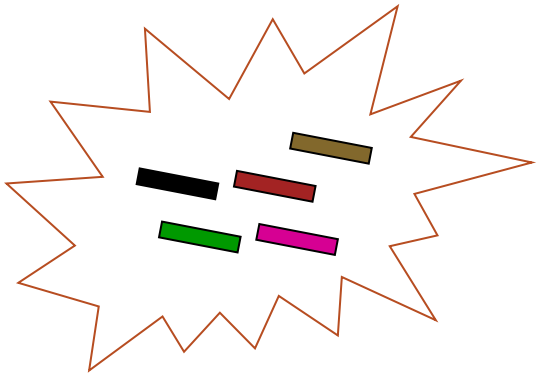
16S rRNA gene

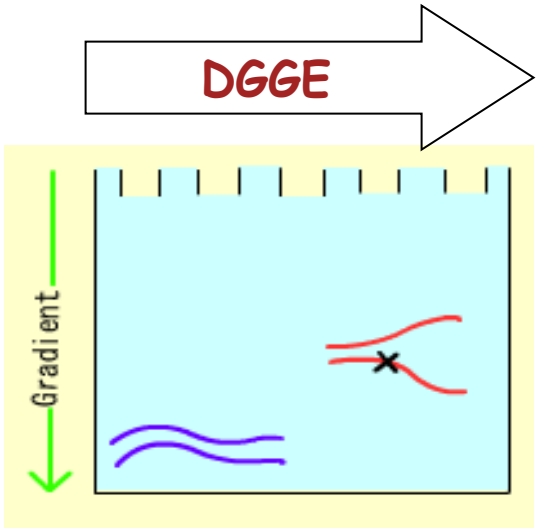


Culture-independent approach: PCR-DGGE

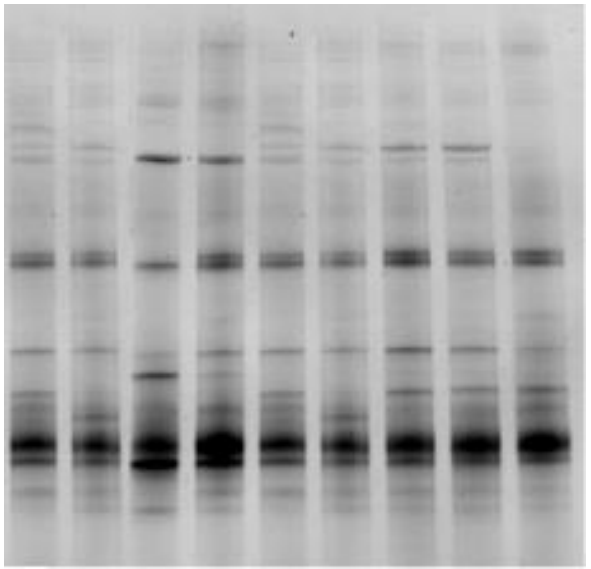
PCR products encounter increasingly higher concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Different sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands.

Amplicon mix from a single sample



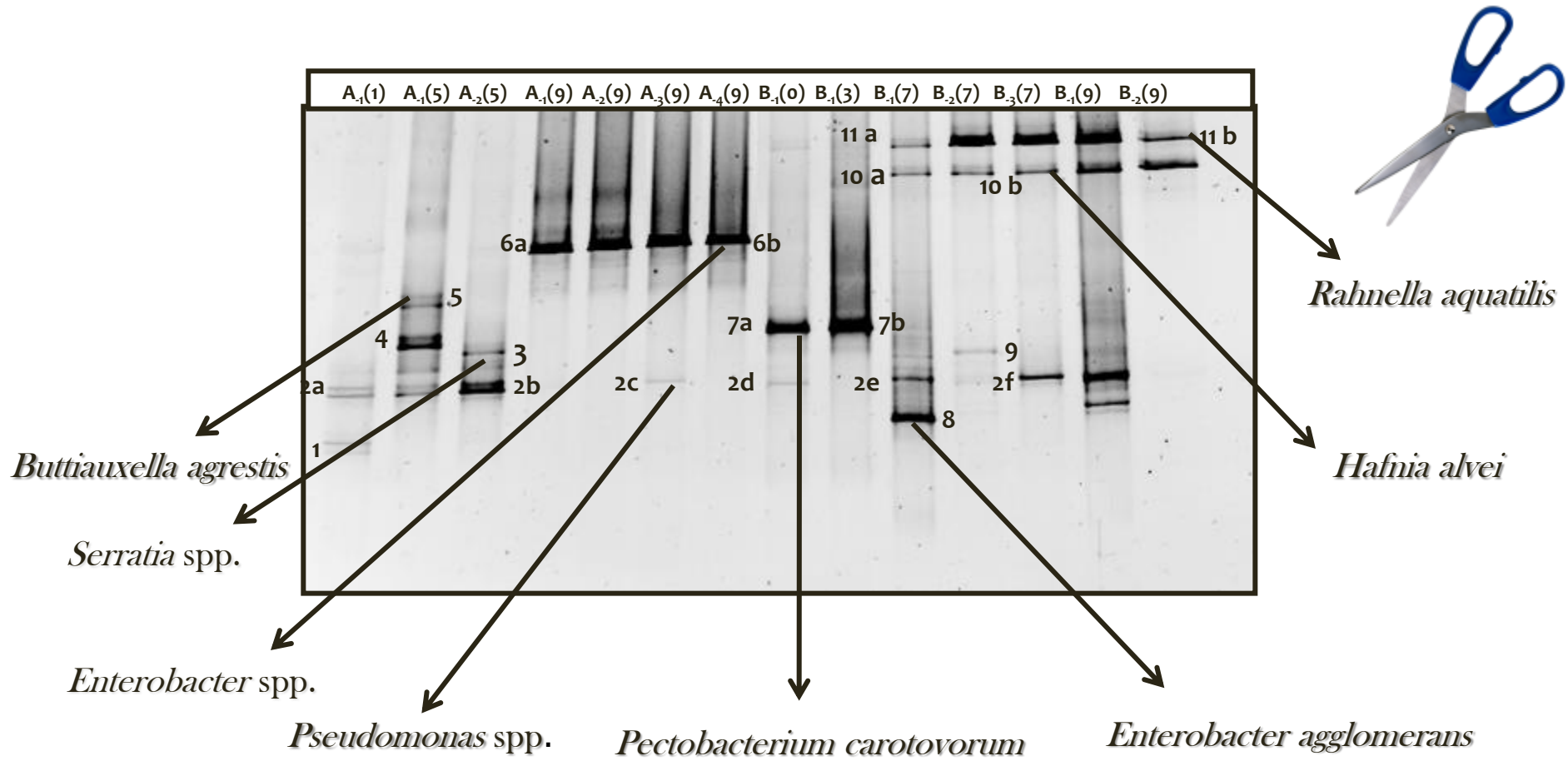


DNA fingerprinting



Culture-independent approach: PCR-DGGE

Each band theoretically represents a different bacterial species present in the community. The different bands can be cut, purified, sequenced and uploaded into databases to identify the microbial species present in the sample.



PCR-DGGE: case study

pagepress
Italian Journal of Agronomy 2018; volume 13(s1)

Responses of bacterial community structure and diversity to soil eco-friendly bioremediation treatments of two multi-contaminated fields

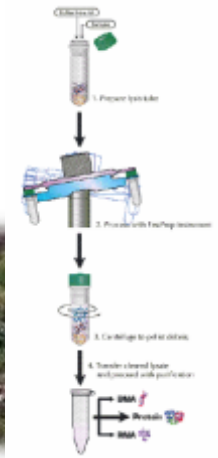
Valeria Ventorino,[1,2] Vincenza Faraco,[2,3] Ida Romano,[1] Olimpia Pepe[1,2]

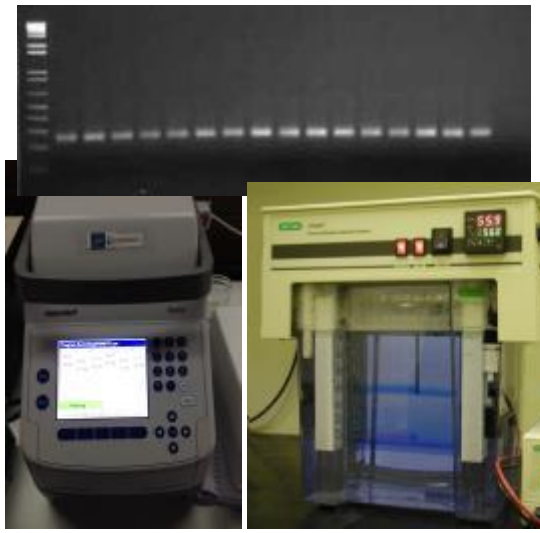
AIM

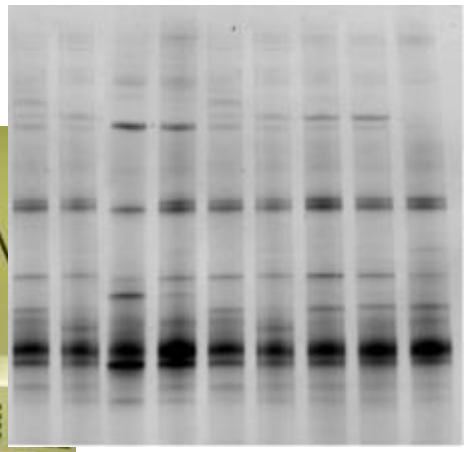
Impact of contamination as well as the use of environmentally compatible techniques for soil remediation on diversity of bacterial communities in soil samples collected from two multi-contaminated fields of the area of the Litorale Domitio Agro Aversano (Giugliano and Trentola Ducenta), used as pilot fields in the LIFE-Ecoremed project.

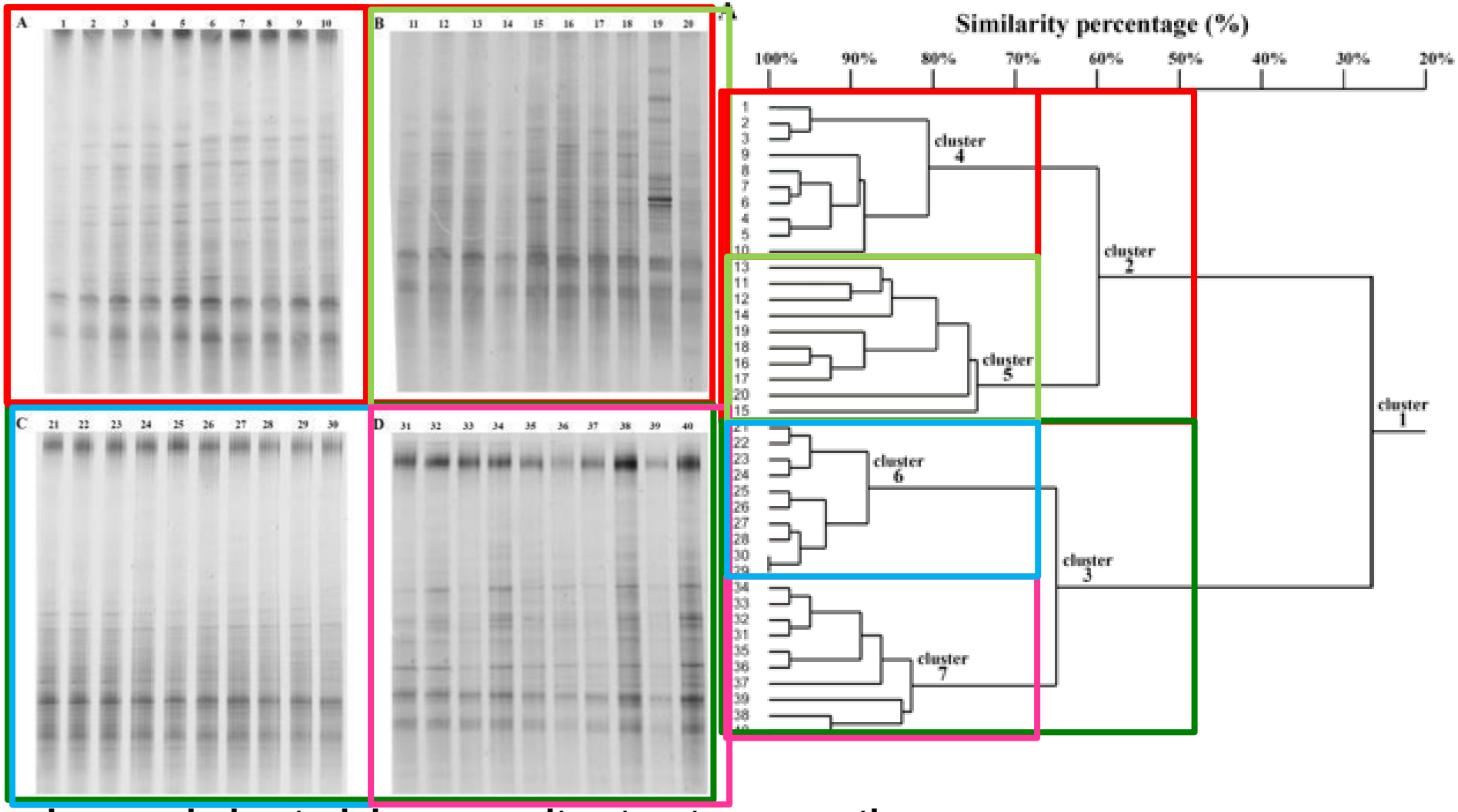
PROGETTO LIFE11 ENV/IT/275
ECOREMED

- ✓ **Soil samples (contaminated both organically and with PTEs) collection at four sampling times:**
  1. **T0, after waste removal and before any remediation practices;**
  2. **TC, after compost addition;**
  3. **TIa, after the first inoculation of microbial consortium;**
  4. **TIb, after the second inoculation of microbial consortium.**
- ✓ **total genomic DNA extraction;**
- ✓ **touchdown-PCR of V3 hypervariable region of 16S rRNA gene;**
- ✓ **DGGE;**
- ✓ **cluster analysis.**









- changes in bacterial community structure over time;
- increase in the number of bands after inoculation treatments;
- two main groups: cluster 2 (samples collected before any remediation practices and after compost addition) and cluster 3 (samples after the first and second inoculum);
- the subgroupings within each of the major clusters associated to sampling time.

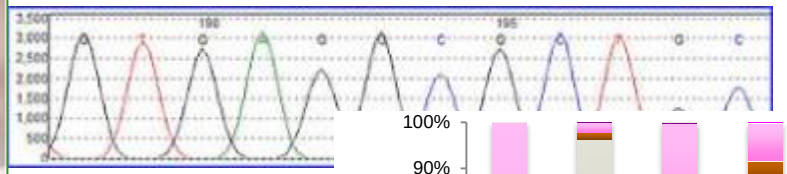
PROGETTO LIFE11 ENV/IT/275

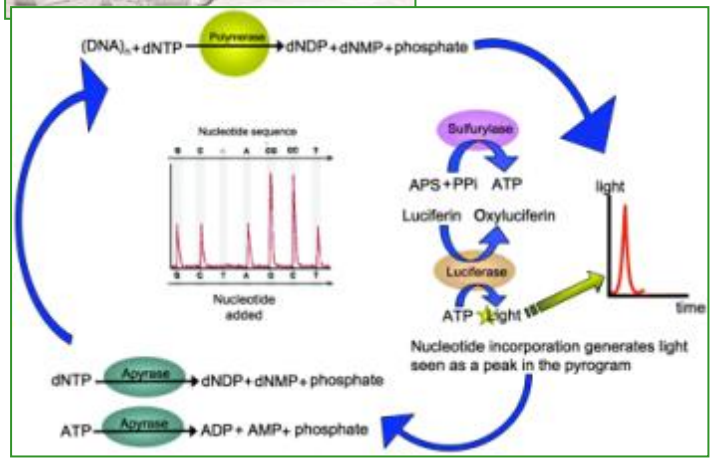
ECOREMED

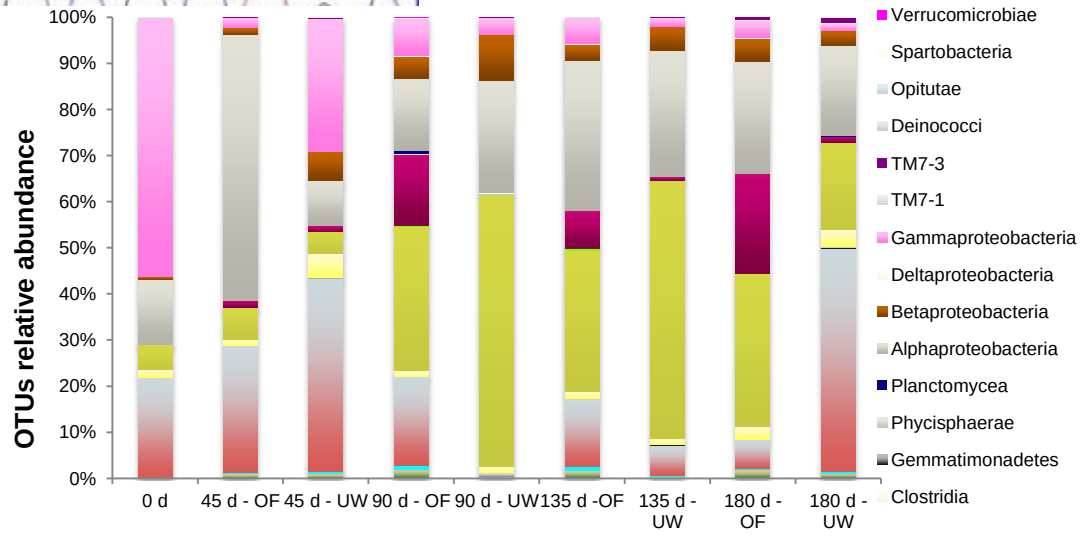
Depletion of pollutants after remediation treatments caused an improvement in the biodiversity of the bacterial populations and a redistribution of the bacterial specimen in the soil interpreted as the recovery of the resilience of the matrix

Culture-independent approach (quantitative method): HIGH-THROUGHPUT SEQUENCING (HTS)

HTS technologies allow to estimate the concentration and the different microbial populations at different taxonomic levels (phylum, class, order, family, genus, species) by bioinformatic analysis of sequencing data.







Metagenomics-High-throughput sequencing = Bioinformatics

The result of HTS is a huge amount of sequences to be analyzed using bioinformatics tools in order to obtain the structure of the microbiota (amplicon based sequencing).

- **Clustering;**
- **Taxonomy assignment;**
- **Phylogenetic distances;**
- **Stats**

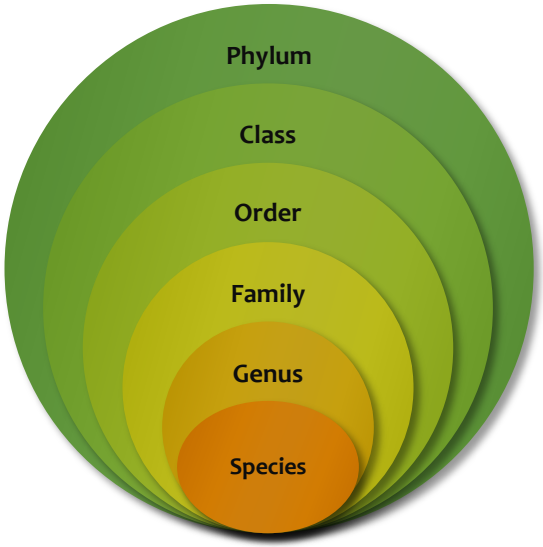
KEGG: Kyoto Encyclopedia of Genes and Genomes

MOTHUR

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

DATA ANALYSIS

- **Qualitative and quantitative composition of the microbiota in each sample.**
- **Operational Taxonomic Units (OTUs) are defined at different taxonomic levels (phylum, class, order, family, genus, species).**



- In simple systems or when looking for specific ecological process/activity it is important to identify the OTUs at genus/species level.

- In very complex ecosystems usually the differences among the samples are determined at higher taxonomic levels.

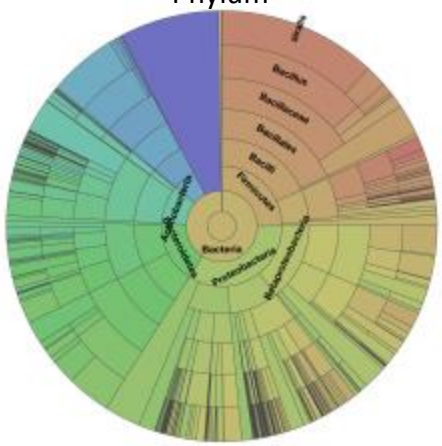
DATA ANALYSIS

Total reads: 361,003

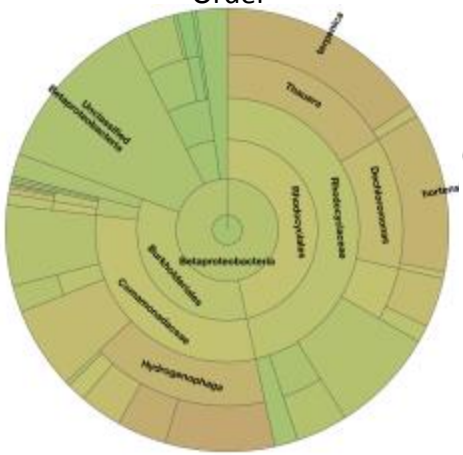
Shannon diversity index: 3.4

Taxonomic Level	Reads Classified to Taxonomic Level	% Total Reads Classified to Taxonomic Level
Kingdom	360,240	99.79 %
Phylum	353,597	99.79 %
Order	349,224	96.74 %
Class	339,792	94.12 %
Family	332,137	92.00 %
Genus	308,687	85.51 %
Species	216,378	59.94 %

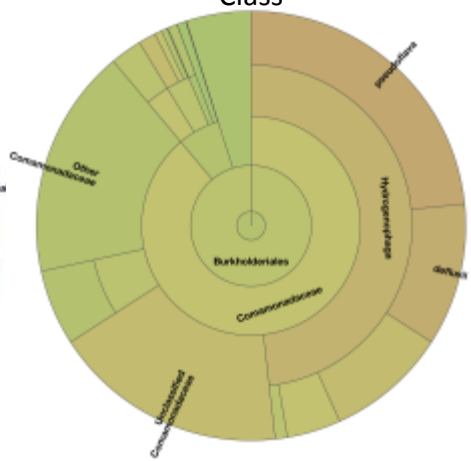
Phylum



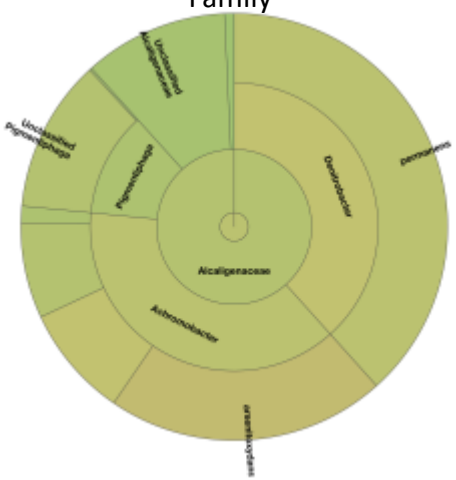
Order



Class



Family



Data analysis: Alpha diversity

Diversity within an ecosystem (sample):

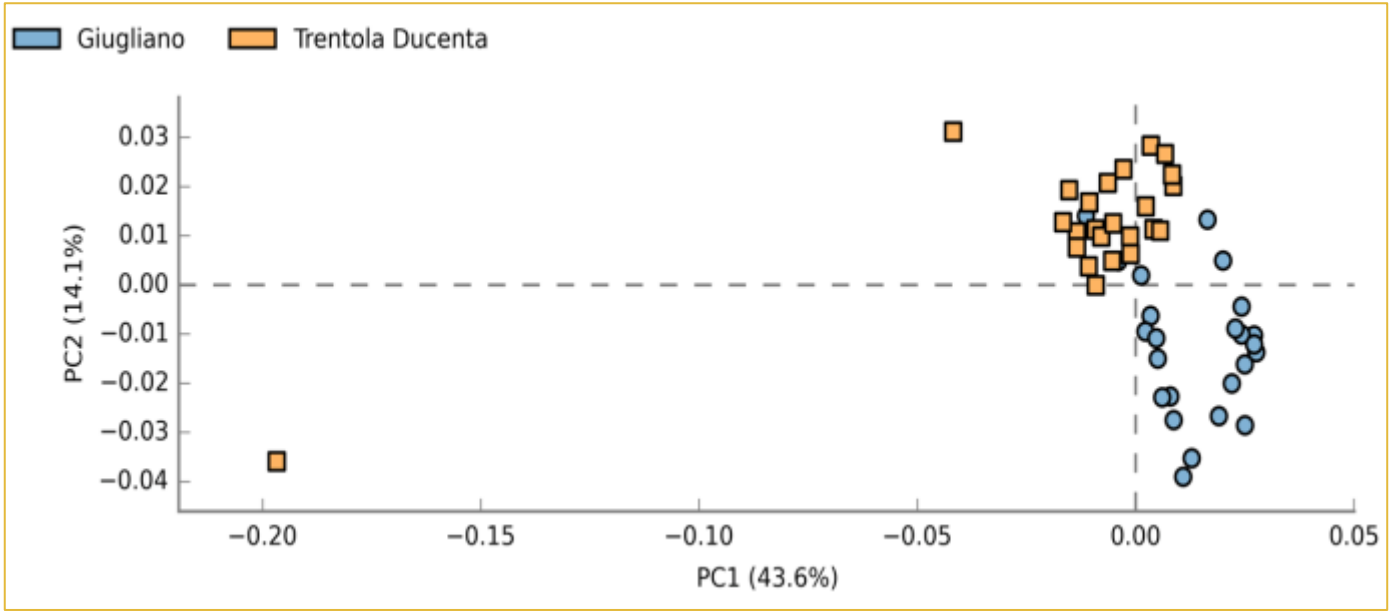
- **richness in OTUs or genus;**
- **Expressed with several indices (Chao, Shannon, Good/ESC, etc).**

…it could give information on the sample coverage as a function of its richness in microbial species.

Data analysis: Beta diversity

Diversity among the samples:

- **Use of phylogenetic information to compare environmental samples (index of dissimilarity between samples).**



SCIENTIFIC REPORTS

HTS: case study

Comparative assessment of autochthonous bacterial and fungal communities and microbial biomarkers of polluted agricultural soils of the Terra dei Fuochi

Valeria Ventorino[1,5], Alberto Pascale[1], Paola Adamo[2], Claudia Rocco[2], Nunzio Fiorentino[3], Mauro Mori[3], Vincenza Faraco[4,5], Olimpia Pepe[1,5] & Massimo Fagnano[3]

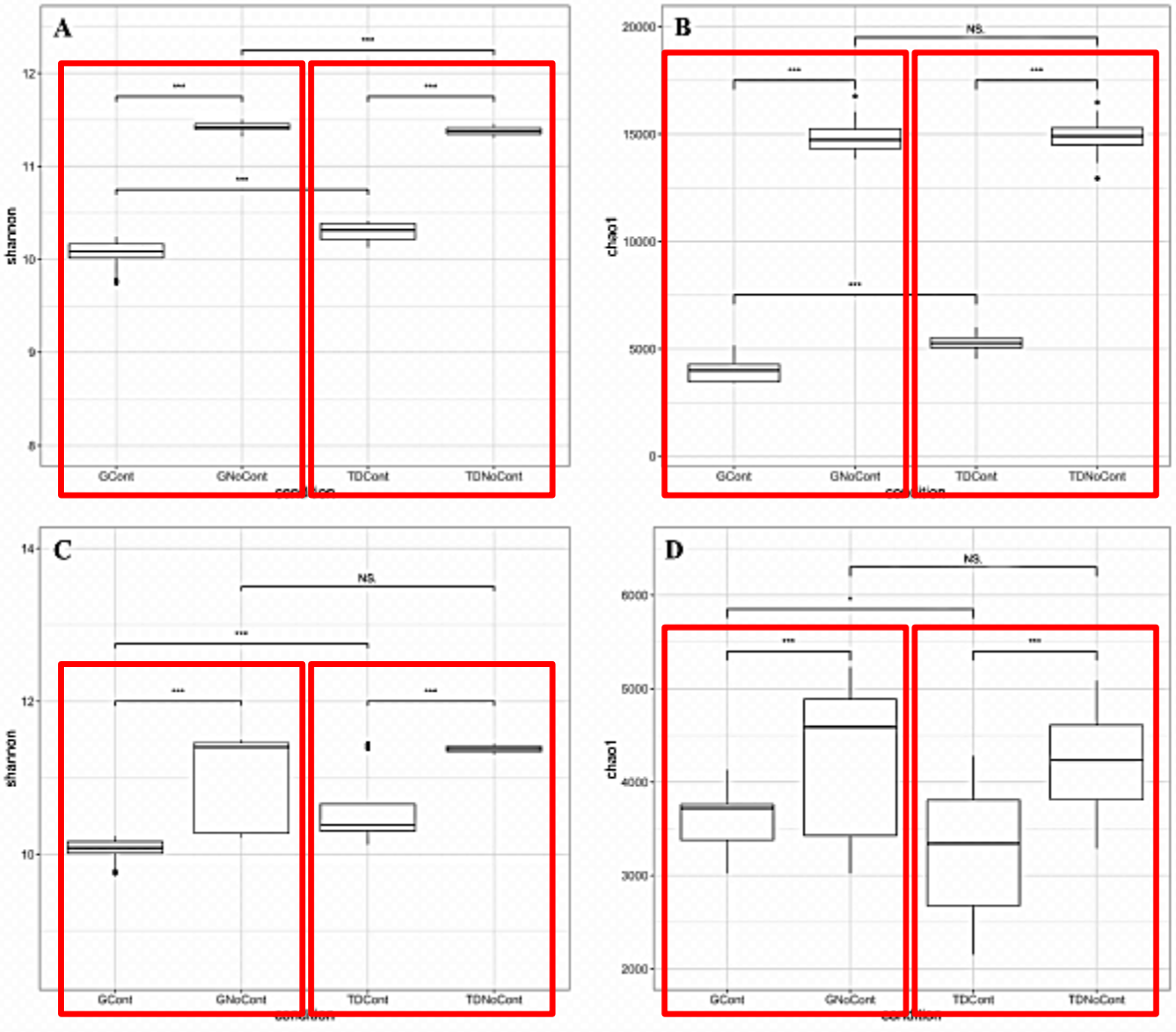
- The study sites were two fallow rural fields that were used in the past for illegal waste disposal and dumping: Giugliano-NA and Trentola Ducenta-CE.

- The aim was to determine and to describe the native microbiota and the impact of anthropogenic pollution (mainly by heavy hydrocarbons but in some cases also by copper and zinc) on the diversity and richness of prokaryotic and eukaryotic communities.

- ✓ Total genomic DNA extraction from contaminated and noncontaminated soil samples;
- ✓ amplicon-based metagenomic sequencing for bacteria and fungi:
- ✓ Bioinformatic analysis.

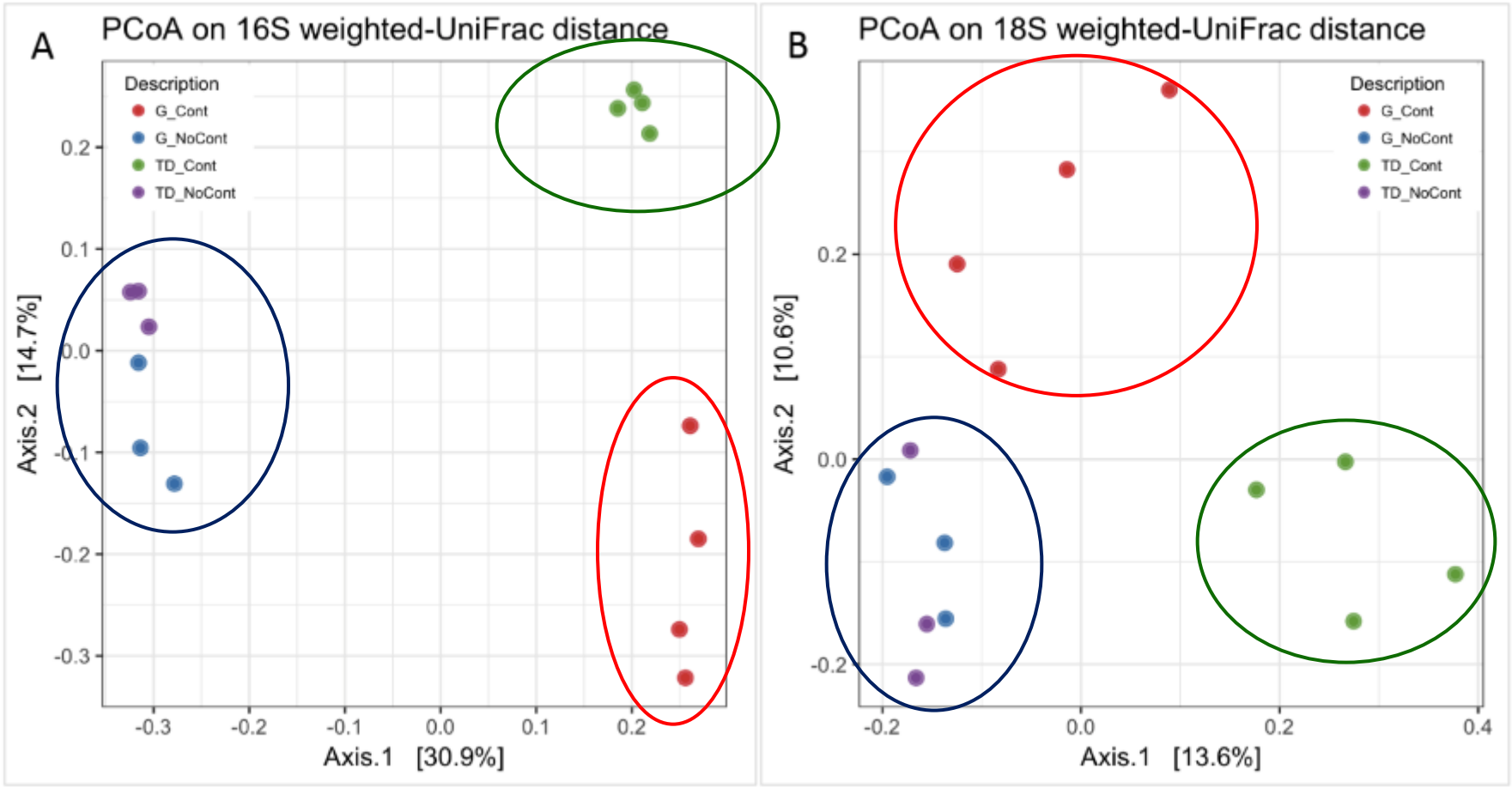
Alpha diversity

Box plots showing Shannon diversity and Chao1 richness indices based on prokaryotic (A,B) and eukaryotic (C,D) communities in the soil samples.



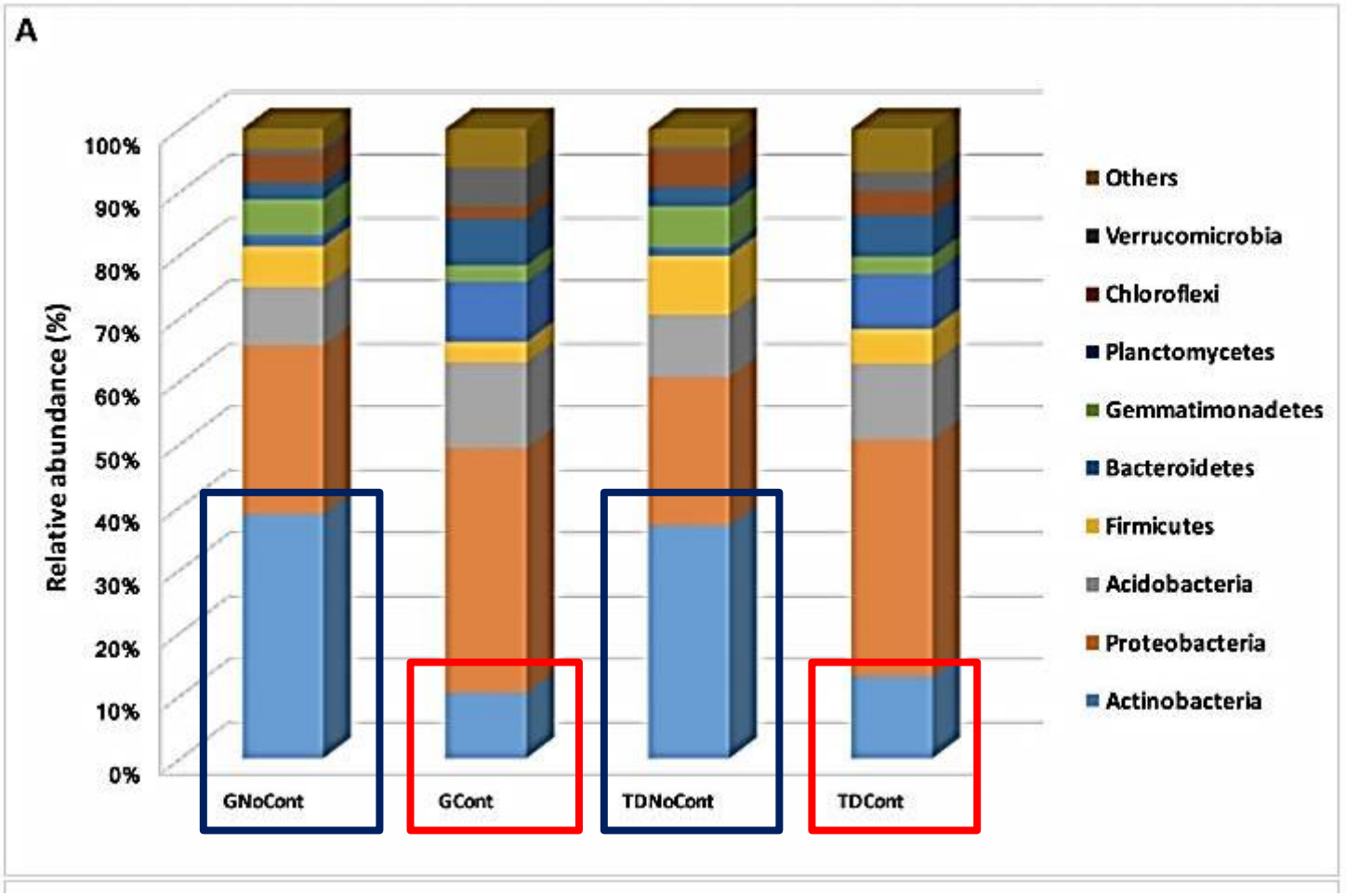
- **Strong differences in prokaryotic and eukaryotic diversity were found between noncontaminated and contaminated soils;**
- **the native microbiota was similar between the G and TD sites.**

Beta diversity: Principal Coordinates Analysis of weighted UniFrac distances for 16S (A) and 18S (B) rRNA gene sequence data of Giugliano and Trentola Ducenta soil samples.



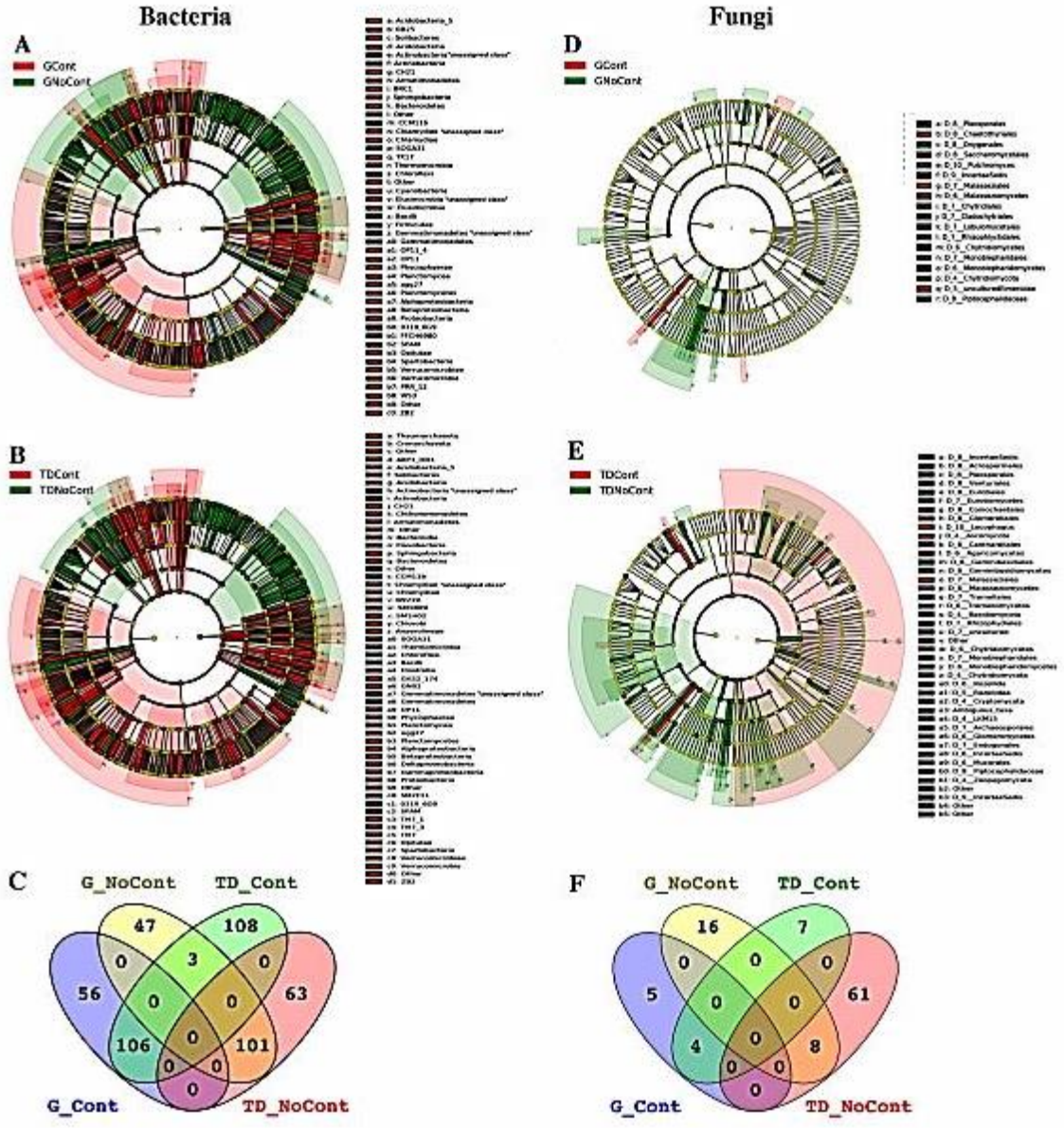
- **Marked difference between the microbiota of noncontaminated and contaminated samples;**
- **Difference between contaminated samples of the two sites (TD and G)**

Microbial taxonomic composition



- **The abundance of the main taxa depended on the presence of pollutants, regardless of site origin.**
- **Actinobacteria strongly decreased in contaminated soils**

Microbial biomarkers



Among the several biomarkers descovered, Actinobacteria represents a sensitive biomarker for assessing soil pollution and therefore could provide general information on the health status of the environment.

CONCLUSIONS

- **Microbial ecology is fundamental to understand the dynamic of a microbiological process and to identify genetic and enzymatic potential sources finalized to wide applications in agricultural, environmental and industrial fields;**

- **Investigating the microbiota of multicontaminated agricultural soils could represent a good opportunity to clarify microbial adaptation with important applications in the field of bioremediation and/or biostimulation and to understand the capacity of microbial populations to colonize the soil ecosystem to recover natural biofertility.**



THANKS for your attention