# PRELIMINARY ASSESSMENT OF MICROBIAL COMMUNITY RECOVERY AFTER AN ACCIDENTAL OIL SPILL BY MOLECULAR ANALYSIS

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## SUMMARY

In this work, we tried a method based on molecular analysis to assess the natural microbial remediation in an unsaturated soil, accidentally contaminated by a petroleum spill-out during tank transportation. Total petroleum hydrocarbon (TPH) in soil was quantified by GC/MS. The residue amount in the contaminated soil after 1 and 13 months from the oil spill out was analyzed to be 327 and 215 mg $kg^{-1}$, respectively. DNA isolated from the soil was amplified by PCR. Amplified ribosomal DNA restriction analysis (ARDRA-PCR) was used for assessing the effect of petroleum contamination on soil microbial community. The analysis of 16S ribosomal DNA gene evidenced an important recovery of the microbial community after 1 year from the contamination accident.

**KEYWORDS:**
Petroleum contamination, soil, natural microbial recovery, amplified ribosomal DNA restriction analysis, 16S rDNA gene.

## INTRODUCTION

Petroleum crude oil and its by-products are the most widespread pollutants of various ecosystems. Spills or leaks of petroleum can lead to the contamination of terrestrial and aquatic environments, subsequent to oil deposition on soils and into surface and groundwater. The fate of petroleum products released into the soil will be particularly varied because these products generally consist of complex mixtures of hydrocarbons with greatly differing in vapour pressure and water solubility. Petroleum by-products can be retained into the soil either by entrapment into soil capillaries and pores, or by sorption on particles' surface. Physical and chemical soil properties, including hydration status, particles' size and organic matter content, can control the degree of hydrocarbon entrapment and sorption [1].

The severity of oil plunge on plant and microbial life depends on the type of hydrocarbons, amount of oil involved, habitat, degree of weathering, sensitivity of affected organisms, and land topography [2]. Natural attenuation of hydrocarbons in soil and groundwater may take place involving a number of processes, such as biodegradation by naturally occurring microorganisms (natural bioremediation), sorption, dispersion, dissolution and volatilization [3].

In unsaturated soil, native hydrocarbon-oxidizing bacteria can remedy petroleum pollution; however, the process is slow, especially in cold climates. Microorganisms can metabolize petroleum hydrocarbons producing environmental by-products, such as carbon dioxide and water [4], or small molecules and intermediates that can be incorporated into microbial pathways [5, 6]. Bioremediation agents have been applied to clean-up efforts after oil spills [6, 7]. Hydrocarbon biodegradation by microbial communities represents the most important alternative to chemical and biological decontamination, and an economical method for the environment preservation and to restore environmental quality in contaminated sites [5, 8, 9]. In particular, the hydrocarbons distributed in the topsoil are degraded by aerobic processes and hydrocarbon compounds are the electron donors for microorganisms, whereas the electron acceptor is the molecular oxygen. Indeed, many bacterial strains, which are able to use hydrocarbons as organic carbon source, are known to be ubiquitous in nature [10].

In the last years, many authors have applied molecular techniques to assess and evaluate the activity of soil microbial community. Direct DNA- or RNA-based tech-

niques allow a thorough investigation of soil microbial community structure [11]. Nowadays, analysis of microbial nucleic acids extracted from soil samples could represent an important assessment tool in investigations on soil recovery after an adverse ecological event [12].

In this paper, the natural soil microbial remediation of a petroleum-contaminated area in an unsaturated zone has been studied using GC-MS analyses and molecular techniques based on polymerase chain reaction (PCR) of the microbial community DNA extracted from soil, and the amplified ribosomal DNA restriction analysis (ARDRA). ARDRA is a DNA fingerprinting technique based on PCR amplification of 16S ribosomal DNA using primers for conserved regions, followed by restriction enzyme digestions and agarose gel electrophoresis [13]. The main target of the work is to evaluate a method based on molecular analysis for assessing the effect of oil contamination on the microbial community in soil [14].

## MATERIALS AND METHODS

### Soil and climate

During summer 2001, 1 month after the accidental spillage, and also after one year (summer 2002), soil was sampled from a field (about half a hectare) accidentally contaminated with about 1000 kg of crude oil during the transportation of a petroleum tank. During each sampling period, 10 polluted samples were collected from the same soil sites located along two transepts, one marked out on the line of maximum slope and the other traced perpendicularly to it (0-40 cm layer). To obtain a soil sample representative of the area, collected samples were mixed together by 30 min continuously stirring and sieving (2 mm) them. A true uncontaminated soil was also sampled from the same area in a boundary site in summer 2001, and another in summer 2002, and they were used as control.

The polluted countryside is situated in the proximity of Viggiano (40° 20' N, 15° 54' E), a little country town s in the Agri Valley (Southern Italy). After the accident, the polluted area was restricted to the public use and nobody else was permitted to enter it. The geographic area of lower Agri Valley has a typical Mediterranean climate characterized by dry summers (rainfall less than 1000 mm and mean temperatures >23 °C) with humid and milder winters (*http://www.ncl.ac.uk/ medaction/WEB/agri.htm*). During the experimental period, the annual rainfall was 838 mm (66% of precipitation during autumn-winter, 19% in spring, and 15% in summer).

### Sample preparation and analysis

Reference samples were prepared mixing different quantities of the same crude oil spilled out in the truck accident (API gravity 20°, high quality high density petroleum) (*http://www.adb.basilicata.it/adb/pubblicazioni/ attivitapetrolifera.asp*) to the non-contaminated soil and keeping them at 120 °C for two days. Reference, control and contaminated soil samples (3 g) were placed in screw-capped vials (40 ml), equilibrated at room temperature, and exposed for 20 min at 80 °C in the presence of 30 μm PDMS (polydimethylsiloxane) SPME (solid phase micro extraction) cartridges. SPME fibres were purchased from SUPELCO (Bellefonte, PA, USA) and used to eliminate sample matrix interference. The SPME fibres, containing the soil extracted volatile and semi-volatile fractions of hydrocarbons, were put on column set and thermally desorbed for 15 s in the GC injection port maintained at 250 °C. Total petroleum extracted hydrocarbons (TPH) were detected using a Hewlett-Packard HP6890 plus gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a HP5973 mass selective detector. The column used was a Phenomenex Zebron ZB-5 MS (30 m long, 0.25 mm id, 0.25 μm film thickness). The carrier gas was helium maintained at 40 psi and fluxed at 0.8 ml $min^{-1}$. Temperature ramping was adjusted from 50 °C (3 min hold) to 250 °C at 10 °C $min^{-1}$ with a final 15 min hold. All mass chromatograms were acquired in the electron impact (EI) mode at 70 eV. The electron multiplier was set at 1490 V.

### Soil DNA extraction

Total community DNA was extracted from 100 g of soil using direct lysis with glass beads and sodium dodecyl sulphate (SDS), followed by polyethylene glycol precipitation, potassium acetate precipitation, and phenol extraction and isopropanol precipitation. The crude extract was used in PCR analysis, according to Yeates *et al*. [15].

### Amplification of 16S-23S rDNA spacer region

Amplification of 16S-23S intergenic spacer region was applied using the primers G1 (5′-GAAGTCGTAACAAGG-3′) and L1 (5′-CAAGGCATCCACCGT-3′) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on DNA of the microbial community as described by Jensen et al. [16]. Images were captured using a Gel Doc 2000 apparatus (Bio-Rad, Hercules, CA, USA).

### Amplified ribosomal DNA restriction analysis (ARDRA-PCR)

ARDRA-PCR was applied on total DNA of the microbial community as described by Weisburg et al. [17]. The reaction mixture consisted of 2 μl of diluted sample (1:5), 250 μM of each dNTPs, 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.2 μM of each primer, and 2.5 U of TaqGold, in a final volume of 50 μl. 16S regions amplified were digested by restriction enzymes Sma I, or Taq I, or Hind III, according to the supplier's instructions (Amersham Pharmacia Biotech). Restriction patterns were resolved by electrophoresis in 2% (w/v) agarose TBE (0.9 M TRIS - borate, 0.004 M EDTA, pH=8) gels. Images were captured using a Gel Doc 2000 apparatus. Chemicals, where not differently indicated, were purchased from Sigma – Aldrich (St. Luis, MO, USA).

**TABLE 1 - Physical and chemical characteristics of soil sampled in the summer 2001.**

| Soil properties | Unit | Uncontaminated soil | Contaminated soil |
|---|---|---|---|
| Sand [18] | % | 54.4 | 56.5 |
| Silt [18] | % | 25.9 | 24.6 |
| Clay [18] | % | 19.7 | 18.9 |
| pH ($H_2O$) [19] | | 7.2 | 7.7 |
| Electrical Conductivity [20] | mS $cm^{-1}$ | 0.18 | 0.32 |
| Organic Carbon [21] | % | 1.41 | 1.7 |
| Total N [22] | % | 0.16 | 0.14 |
| C/N | | 8.75 | 12.14 |
| Total P [23] | mg $kg^{-1}$ | 0.60 | 1.2 |

## RESULTS AND DISCUSSION

### Site and soil characteristics

The mean values of physico-chemical properties of the representative soil sampled in the summer 2001, analysed in triplicate, are reported in Table 1.

The studied soil is an uniform sandy-loam soil, well drained and aerated. The equilibrated dotation of clay and the good value of the C/N ratio let us expect an excellent response of microbial biomass to stress effects from oil contamination. In fact, soil organic matter has been implicated as the primary factor governing the interactions between organic contaminants and soil particles, and hydrocarbons have been indicated to have a strong affinity for organic matter [24, 25]. The hydrological parameters of non-contaminated soil are reported in Table 2. The importance of water availability for the potential degradation of crude oil by microbiological processes is correlated with the oil solubility, and efficient microbial growth and proliferation in the soil. Climatic conditions, temperature, physical and chemical soil characteristics, and resistance and degradative capability of microbes in the presence of organic pollutants influence the degradation of hydrocarbons [10].

**TABLE 2 - Hydrological parameters of non-contaminated soil collected in the summer 2001.**

| Hydrological parameter | | |
|---|---|---|
| Field capacity[26] | $cm^3\ cm^{-3}$ | 0.22 |
| Wilting point[26] | $cm^3\ cm^{-3}$ | 0.13 |
| Bulk density [27] | g $cm^{-3}$ | 1.40 |

A medium annual rainfall (838 mm) and a range of seasonal temperatures compatible with a good microbial activity, characterize the studied area, contributing to create the expectation of a good reaction of soil biomass to hydrocarbon pollution.

### Gas-chromatographic analyses

Quantification of TPH in soil, measured 1 month after the accidental event (summer 2001) by GC/MS, was 327 mg $kg^{-1}$ for the contaminated sample and 0 mg $kg^{-1}$ for the control. GC/MS results showed the presence of more than 550 compounds in the contaminated soil. These substances included the volatile fraction as well as the semi-volatile one. One year after (summer 2002), the amount of TPH in contaminated soil was reduced to 215 mg $kg^{-1}$. Figure 1 shows the GC/MS chromatogram of TPH extracted from the representative samples collected from the contaminated soil during (A) summer 2001 and (B) summer 2002.

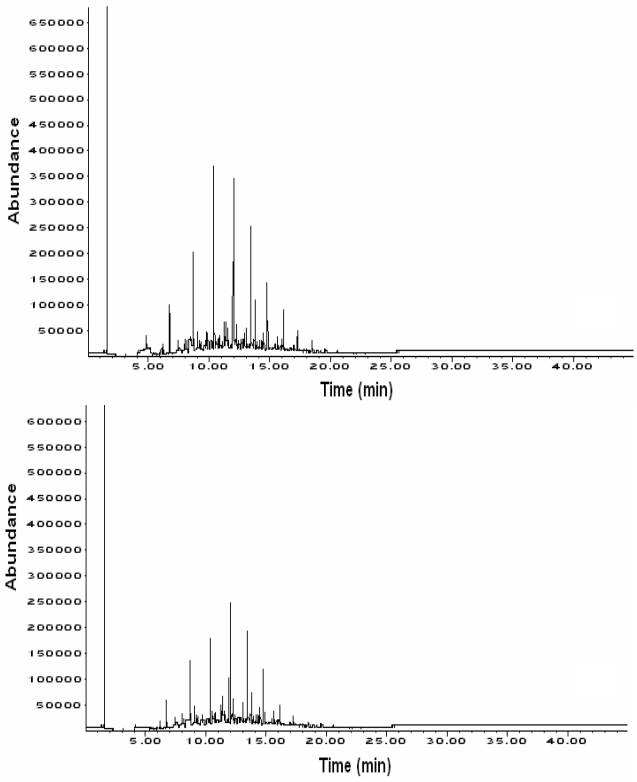


**FIGURE 1 - GC/MS chromatogram of TPH extracted from the representative sample collected from the contaminated soil during (A) summer 2001 and (B) summer 2002.**

To evaluate the volatilization and leaching loss of petroleum hydrocarbons, further experiments were performed. For volatilization assessment, an aliquot of the sample, collected in summer 2001 in the layer 0-40 cm of the soil, was sieved (2 mm) and sterilized by adding 0.05% of sodium azide. The sterilized soil sample was kept for 1 year in an incubator (FTC 90E Refrigerated Incubator, Velp Scientifica, Milan, Italy), equipped with a xenon lamp and an electric fan to simulate the solar irradiation and natural

airflow. The simulated daily irradiation, measured with a radiometer (Eppley Lab., Newport, RI, USA) in the wavelength range 285-800 nm, was 438 W $m^{-2}$ with a mean sunshine of 8 h, a mean temperature of 18.4 °C, and a total air replacement of 10 s. For leaching evaluation, other soil samples were collected from the layer 40-60 cm of the soil in summers 2001 and 2002. The GC/MS analyses of TPH evidenced a volatilization loss of 6% and a non-significant leaching effect.

#### Molecular analyses

Soil microbial diversity has been defined as the range of significantly different types of microorganisms, and also as the amount and distribution of information in a microbial community [28]. In the last years, microbial diversity in terrestrial ecosystems has obtained a significant consideration both for the decline of various ecosystems in the world and the lack of knowledge of soil microbial communities. Soil microflora transforms reserve substances from organic matter through its own metabolisms and contributes to soil changes and modifications of its composition. Since life of every community member is subjected to chemical-physical conditions of habitat and presence of other microorganisms, microbial community composition is not always the same, but it is subjected to changes. In this context, the restoration of hydrocarbon-contaminated sites relies on the collective ability of microorganisms to degrade the contaminants under prevailing environmental conditions.

Microbial degradation of organic contaminants, such as petroleum-derived hydrocarbons, has been monitored by analyzing changes in chemical parameters. Until recently, methods of analyzing soil microbial ecosystems were mainly focused on measuring biomass as well as respiration, or total or culturable bacteria. These methods are coming to be combined with molecular ones, which are less laborious and more reliable for studying microbial diversity at the molecular level in soil ecosystems [29-31].

In this way, naturally occurring microbial populations in soil (not grown in laboratory nor soil-inoculated) were analyzed. Total DNA was extracted and purified from soil samples and subjected to amplification by PCR. Total DNA from complex microbial communities is a complex mixture of DNA from different bacterial types that contain too much information to be analysed directly [14]. Composition and complexity measure of total community DNA permits to assess the gross genetical structure and diversity in bacterial communities [32]. The 16S ribosomal DNA genes are useful for such studies, since these genes are present in all bacteria. Extraction of total DNA from soil followed by PCR amplification of the 16S rDNA gene should ideally yield a mixture of DNA fragments representing all species present in that soil sample [13].

The analysis of ribosomal DNA genes allowed to discriminate microbial structures genetically differentiated in the petroleum-contaminated soil. Polymerase chain reaction (PCR) fingerprinting of microbial community DNA extracted from the contaminated soil has provided qualitative estimates, and comprehensive information on global pattern of microbial diversities.

Amplification of spacer 16S-23S, as an important supplement to 16S rRNA sequencing, was able to show polymorphism of this region and to discriminate microbial population. Primers used in our experiment are able to amplify highly conserved nucleotide sequences in many microorganisms, and this behaviour allows to use only one reaction run for realizing amplification of a great number of bacterial groups. Many papers [13, 30] reported that ARDRA-PCR is a useful tool to discriminate microorganism at level of species. We speculate that the complexity of microflora community in sampled soils can be qualitatively evaluated through the whole amplification pattern obtained from each sample.

In order to determine relationships between the community fingerprints, similarities were evaluated on the basis of the absence or presence of bands. Figure 2 shows the polymorphism of 16S ribosomal DNA genes on the uncontaminated soil (collected in summer 2001) obtained

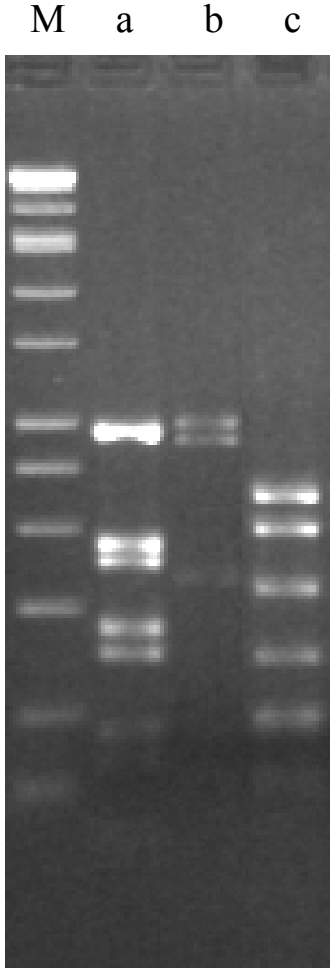


**FIGURE 2 - Polymorphism of 16S ribosomal DNA genes obtained on total DNA of soil microbial community: M = marker; a = *Hind* III; b = *Sma* I; c = *Taq* I.**

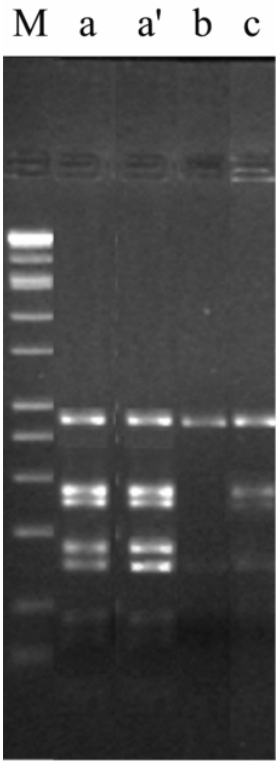


**FIGURE 3 - Polymorphism of 16S ribosomal DNA genes obtained on total DNA of soil microbial community: M = marker; a = uncontaminated soil (2001); a' = uncontaminated soil (2002); b = contaminated soil; c = contaminated soil after 1 year.**

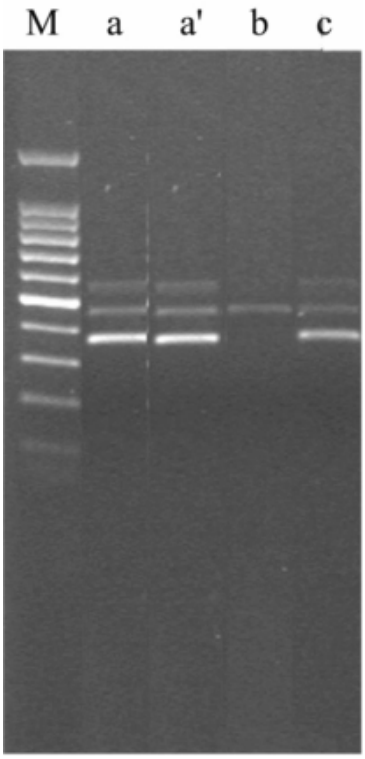


**FIGURE 4 - Polymorphism of 16S-23S spacer regions on total DNA of soil microbial community: M = marker; a = uncontaminated soil (2001); a' = uncontaminated soil (2002); b = contaminated soil; c = contaminated soil after 1 year**

using *Hind* III, *Sma* I and *Taq* I. The pattern obtained with *Hind* III shows a greater number of bands and a better separation of them, so this enzyme has been used in successive soil microbial tests.

The results of the polymorphism for *Hind* III-digested 16S ribosomal DNA genes on the contaminated and the uncontaminated soils are shown in Figure 3.

It is important to observe the decrease of the bands' number in the contaminated-soil sample with respect to the uncontaminated soil, which is demonstrating a comprehensive genetic reduction of the microbial population. Figure 3 shows the results obtained on (c) the contaminated, and (a') the uncontaminated soil, sampled after one year. In this case, an important improvement of some bands took place, showing a recovery of the initially present microbial community.

From the same DNA directly extracted from soil, it has been estimated also the polymorphism of the region spacer 16S-23S. The results are shown in Figure 4 and confirm those obtained with ARDRA-PCR.

## CONCLUSIONS

Petroleum hydrocarbons exert a detrimental effect on soil biological communities. Sizes of microbial biomass are strongly reduced by the presence of such toxic compounds in soil environment. Accumulation of hydrocarbons in a soil produces harmful effects to microbiological level, such as induction of genetic mutations and damaging of local flora and fauna.

Selected strains of soil microflora can show, however, resistance to petroleum by-products due to various systems of adaptation to toxic concentrations of chemicals. Biological crude oil degradation represents the major pathways through which hydrocarbons are removed from contaminated environments.

Measuring the rate of adaptation or evolutionary change of microbial populations, as consequence of altered ecological factors, can give information about sensitivity or resistance showed by the whole soil microflora, when exposed to toxic pollutants.

In this study, the analysis of the rDNA polymorphism obtained by direct soil extraction of genetic material has been demonstrated as a useful tool to quickly verify the effects of the oil contamination on the microbial community.

An evolution of the used method could be the employment of the species-specific PCR for various microbial groups in order to estimate the species more sensible to the pollutants, and those that are effectively utilizable for recovery purposes.

## ACKNOWLEDGMENTS

We acknowledge Prof. M. D'Auria and Dr. G. Mauriello for their analytical assistance. This work was supported by the Italian Ministry of Education and Research.

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**Received:** March 13, 2006
**Accepted:** March 16, 2006

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