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# Assessment of three approaches of bioremediation (Natural Attenuation, Landfarming and Bioagumentation – Assistited Landfarming) for a petroleum hydrocarbons contaminated soil

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## HIGHLIGHTS

- Three approaches of bioremediation for a petroleum hydrocarbons contaminated soil through a plot experiment.
- Biodegradation capacity of native microbial community in TPH contaminated soils was evaluated.
- Bioagumentation – assistited Landfarming lab tests allowed to achieve, after 90 days, a contaminant reduction up to 80%.

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## ABSTRACT

Contamination with total petroleum hydrocarbons (TPH) subsequent to refining activities, is currently one of the major environmental problems. Among the biological remediation approaches, landfarming and *in situ* bioremediation strategies are of great interest. Purpose of this study was to verify the feasibility of a remediation process wholly based on biological degradation applied to contaminated soils from a decommissioned refinery.

This study evaluated through a pot experiment three bioremediation strategies: a) Natural Attenuation (**NA**), b) Landfarming (L), c) Bioaugmentation-assisted Landfarming (LB) for the treatment of a contaminated soil with petroleum hydrocarbons (TPHs). After a 90-days trial, Bioagumentation – assistited Landfarming approach produced the best results and the greatest evident effect was shown with the most polluted samples reaching a reduction of about 86% of total petroleum hydrocarbons (TPH), followed by Landfarming (70%), and Natural Attenuation (57%). The results of this study demonstrated that the combined use of bioremediation strategies was the most advantageous option for the treatment of contaminated soil with petroleum hydrocarbons, as compared to natural attenuation, bioaugmentation or landfarming applied alone. Besides, our results indicate that incubation with an autochthonous bacterial consortium may be a promising method for bioremediation of TPH-contaminated soils.



## 1. Introduction

Petroleum-based products still represent a major source of energy for daily life and industrial activity. During exploration, production, refining, transportation and storage of oil and its product derivatives, leakages are frequently. The release of hydrocarbons into the environment causes significant damage to ecosystems and accumulation of these pollutants in animal and plant tissues can cause serious genetic mutations (Alvarez and Vogel, 1991; Taiwo, 2011).

Conventional techniques used for *in situ* remediation of hydrocarbon-contaminated soils comprise chemical and electrochemical oxidation, electrokinetic separation, soil flushing, soil vapor extraction, solidification/stabilization and thermal treatments. These technologies are generally expensive, and also, in some cases, they simply transfer pollutants from one phase to another or, in other cases, they are not able to achieve the complete falling-off of the contaminants becoming even more environmentally unsustainable. Bioremediation processes, defined as the exploitation of the metabolic capabilities of microorganisms, are constantly evolving due to their easiness, higher efficiency and cost-effectiveness (Cappello et al., 2015).

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Biodegradation performed by indigenous microorganisms is one of the major mechanisms by which the oil and other hydrocarbon pollutants are naturally removed from the environment. The success of a bioremediation process basically depends on the intrinsic ability of the system to create and maintain the conditions promoting the pollutants biodegradation with a sufficiently high rate. Several scientific works have dealt with studying different factors affecting the oil biodegradation in the various ecosystems (Das and Chandran, 2011; Sihag et al., 2014).

One of the most important requirements is certainly the presence of microorganisms with the appropriate metabolic skills, but even the chemical physical characteristics of the oil and the interactions between the oil phase and the aqueous phase (containing the microorganisms) are very important for the success of bioremediation which relies on augmenting the natural biodegradation rate of oil (Atlas, 1995; Vogel, 1996; Xu and Obbard, 2004; Cunliffe and Kertesz, 2006; Karamalidis et al., 2010; Jain and Bajpai, 2012; Torlapati and Boufadel, 2014; Ron and Rosenberg, 2014).

Although bioremediation for decontamination of large oil spills is definitely of great social impact, the evaluation of this technology effectiveness cannot be limited to these events but should be addressed to different fields of application. Studying the usefulness of bioremediation of soils and waters contaminated with hydrocarbons is certainly more useful and effective for promoting acceptance. In the environment, hydrocarbons are biodegraded by bacteria, yeasts and fungi and their biodegradation rates varies significantly (Leahy and Colwell, 1990; Bagia et al., 2013).

Several studies report that the presence of mixed populations (consortia) showing various and extensive metabolic capacities is essential for the degradation of complex mixtures of hydrocarbons such as crude oil in soil, in fresh water or in marine environment (Brune and Bayer, 2012).

Strategies to speed up hydrocarbons biodegradation in the soil comprise stimulation of the indigenous microorganisms (biostimulation) by optimizing factors such as nutrients, oxygenation, temperature, pH, possible addition of biosurfactants and inoculation of an enriched mixed microbial consortium into the soil (bioaugmentation) (Mariano et al., 2009).

The aim of this work was to perform a assessment of three bioremediation approaches of a TPH contaminated soil. A series of laboratory scale experiments with different experimental conditions (natural attenuation, biostimulation and bioaugmentation) were carried out. Hydrocarbon degraders native bacteria were selected from soil samples and these bacterial isolates were further characterized considering the opportunity to use them in an assisted bioaugmentation approach.

## 2. Materials and methods

### 2.1. Collection of soil samples for experimental treatments

Soils samples were collected from a decommissioned refinery of 400.000 $m^2$ located in northern Italy with a history of contamination by petroleum hydrocarbons, mostly diesel. Based on the available geological and morphological soil features and considering neighboring plots of land already remediated, the area was divided into 9 sub-areas of about 20,000 $m^2$ each. Among these nine areas, four geomorphologically homogeneous lots were chosen for the trial: lots 2, 4, 5 and 8. The lots 2 and 4 characterized by high concentrations of petroleum hydrocarbons C > 12 and lots 5 and 8 characterized by low concentrations of petroleum hydrocarbons C > 12.

For each lot, two soil samples were collected, a shallow one (0–1.5 m) and a deeper one (1.5–3 m). To maximize the representativeness of these eight soil samples (B1-B8), each of them was made from five subsamples taken within the same homogeneous area. These subsamples were homogenized and subdivided to obtain the required amount for the experimentation. All soil samples (B1-B8), collected with a small digger, were air dried and ground to pass through a 2 mm sieve before soil analysis.

### 2.2. Selection of the native hydrocarbons degrading bacteria for bioagumentation treatment

For the isolation, 1 g of sieved (2 × 2 cm) and homogenized soil from B1-B8 samples was incubated in duplicate in 50 ml of a mineral medium (MM: $KH_2PO_4$ 1.5 g $l^{-1}$, $NaHPO_4$ 0.5 g $l^{-1}$, $NH_4Cl$ 1 g $l^{-1}$, NaCl 0.1 g $l^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.2 g $l^{-1}$, $CaCl_2 \cdot 2H_2O$ 0.0264 g $l^{-1}$, $FeCl_3$ 0.01 g $l^{-1}$ $MnCl_2 \cdot H_2O$ 5 mg $l^{-1}$, $ZnCl_2$ 3 mg $l^{-1}$, $CuCl_2 \cdot 2H_2O$ 0.9 mg $l^{-1}$, $CoCl_2 \cdot 6H_2O$ 1 mg $l^{-1}$, $NaMoO_4 \cdot 2H_2O$ 1 mg $l^{-1}$, $NiCl_2 \cdot 6H_2O$ 0.3 mg $l^{-1}$, $H_3BO_3$ 3 mg $l^{-1}$, $Na_2O_3Se \cdot 5H_2O$ 0.2 mg $l^{-1}$) at pH 6.8 with the addition of 5% of diesel oil as unique C source. Enrichment cultures were incubated in 250 ml conical flasks, at 28 °C in an orbital shaker (200 rpm) for five days. Cultures were then diluted at 1% and reincubated for five days repeating this step once more. After this selection, in order to isolate the larger number of strains, 100 μl of serial tenfold dilutions of bacterial cultures were propagated on two different solid media: LB and R2A (Sigma Aldrich) with the purpose to not overlook slower growing colonies. First colonies were visualized after 4 days of incubation at 28 °C and after additional 4 days a total count was performed. Forty colonies per medium and per samples were randomly selected and maintained as pure cultures. A collection of twenty-two isolates was obtained.

#### 2.2.1. Characterization of the bacterial isolates

Genomic DNA from the isolates was extracted by Wizard® Genomic DNA Purification Kit (Promega) and used as template for 16S rRNA gene amplification with universal eubacterial primers (F27a: AGAGTTTGATCCTGGCTCAG; R1492a: GGTTACCTTGTTACGACTT). Polymerase chain reaction (PCR) were performed with GoTaq® Polymerase (Promega) according to the supplier's instructions. PCR-amplified DNA was sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc. USA) using an automated DNA sequencer (ABI model 3500 Genetic Analyzer). Nucleotide sequences were edited and assembled with Lasergene version 11.2.1 (DNASTAR®) and subjected to homology comparison (BLAST analysis) at the National Center for Biotechnology Information (NCBI) server (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Partial 16S rRNA gene sequences (1200–1400 bp) from the isolates were deposited in the GeneBank database and the accession numbers assigned are from KT204469 to KT204491.

The identified isolates were then evaluated *in vitro* for the assessment of PGP potential. Indolacetic acid (IAA) production was estimated following the protocol described by Brick et al. (1991) by colorimetric reaction. Briefly, medium supplemented with 5 mM L-tryptophan is inoculated with isolates of interest, overlaid with a nitrocellulose membrane, and then incubated until bacterial colonies reach 1 to 2 mm in diameter. The membrane is removed to a filter paper saturated with Salkowski reagent and incubated until distinct red haloes form around the colonies. The colorimetric reaction to IAA is limited to a region immediately surrounding each colony, is specific to isolates producing IAA, occurs within 1 h after the membrane is placed in the reagent, and is sensitive to as little as 50 pmol of IAA in a 2-$mm^2$ spot. We have used this assay for quantifying epiphytic populations of IAA-producing isolates of bacteria. The assay provides a rapid and convenient method to screen large numbers of bacteria.

Siderophore release was determined as described by Milagres