

Degradation of long-chain *n*-alkanes in soil microcosms by two actinobacteria

CLAUDIO DE PASQUALE¹, ERISTANNA PALAZZOLO¹, LUCA LO PICCOLO² and PAOLA QUATRINI²

¹Dipartimento dei Sistemi Agro-Ambientali (SAgA), Università degli Studi di Palermo, Palermo, Italy

²Dipartimento di Scienze e tecnologie Molecolari e Biomolecolari, Università degli Studi di Palermo, Palermo, Italy

The ability of two recently isolated actinobacteria, that degrade medium and long chain *n*-alkanes in laboratory water medium, was investigated in soil microcosms using different standard soils that were artificially contaminated with *n*-alkanes of different length (C₁₂- C₂₀- C₂₄- C₃₀). The two strains, identified as *Nocardia* sp. SoB and *Gordonia* sp. SoCp, revealed a similar high HC degradation efficiency with an average of 75% alkane degraded after 28 days incubation. A selectivity of bacteria towards *n*-alkanes of different length was detected as well as a consistent effect of soil texture and other soil physical chemical characteristics on degradation. It was demonstrated the specific aptitude of these selected strains towards specific environmental conditions.

Keywords: Bioremediation, soil microcosms, GC-MS, *n*-alkanes, *Nocardia*, *Gordonia*.

Introduction

Bioremediation, defined as the use of various organisms to detoxify or remove pollutants, is an emerging method for the degradation of many environmental organic pollutants, including those resulting from the petroleum industry.^[1–3] When bioremediation can be carried out *in situ*, it appears as a cost-effective means to remove many of those chemical pollutants that adversely impact human health and environmental quality. Various organisms, especially bacteria, are able to degrade HCs and can be used to detoxify and remove pollutants in various “*in situ*” treatments. The success of biodegradation depends on several factors.

Among them, specific metabolic capabilities of microbes and bioavailability of the contaminating compound as well as its chemical structure are very remarkable.^[4]

Mixtures of HCs, including those resulting from oil-refining processes, are often extremely complex. In fact, they may contain variable amounts of different components such as saturated HCs, cyclic alkanes, mono-, di- and aromatic compounds bearing various degrees of substitution, unsaturated HCs such as *n*-alkenes, iso-alkenes, and cyclic alkenes.^[5]

Although it is well known that unsaturated HCs do not occur in natural oils, they are, however, formed in variable amounts during thermal and catalytic cracking processes. The biodegradation degree of aliphatic HC is typically lower than that of aromatics; nevertheless most studies have focussed on the fate and behaviour of aromatic compounds in soil. Alkanes are non-polar virtually water insoluble HC that interact with the soil mineral and organic fraction reducing their chemical and biological availability. Understanding the microbial ecology and biodegradation constraints of oil contaminated sites in different environments, would help to find the best degrading conditions, resulting in a complete environmental cleanup.

HCs degraders have been isolated from contaminated and pristine soils. Most are *Bacteria* belonging to the α -, β - and γ -Proteobacteria and to the Actinomycetales (high G+C Gram-positive bacteria).^[6] *Pseudomonas putida* GPo1 was the most extensively characterized strain for alkane degradation. Here a membrane-bound monooxygenase (encoded by *alkB*), and soluble rubredoxin and rubredoxin reductase, convert the alkane into the corresponding alcohol that is further oxidized to aldehyde and acid, prior to proceeding into the β -oxidation pathway. Depending on the HC molecules, other degradation pathways have also been elucidated.^[7, 11] Different enzymatic systems may be involved in the degradation of the highest and lowest molecular weight HCs respectively.^[12]

Oil-contaminated sites are dominated by Gram-negative bacteria.^[13, 14] Our previous study on assessment of bioremediation potential of a contaminated site,^[15] revealed that Gram-positive actinomycetes are dominating in harsh and

Address correspondence to Claudio De Pasquale, Università degli Studi di Palermo, Dipartimento dei Sistemi Agro-Ambientali (SAgA) Ed. 4, viale delle Scienze 90128, Palermo, Italy; E-mail: claudio.depasquale@unipa.it
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arid environments suggesting that they might be a good alternative to the most studied Gram-negative strains for HC degradation. Gram-positive alkane degraders have been also detected in uncontaminated soils, they are generally adapted to resource-limited conditions and do not fluctuate in response to the addition of a C source such as HC.^[16–18]

Recently, five Gram-positive G+C rich actinobacteria have been isolated from oil-contaminated Sicilian beach. The bacteria were found able to degrade long-chain *n*-alkanes up to hexatriacontane.^[15] The present study reports about the ability of two isolates, *Nocardia* sp. SoB and *Gordonia* sp. SoCp, in degrading medium and long chain *n*-alkanes (*n*C₁₂, *n*C₂₀, *n*C₂₄, *n*C₃₀) in artificially contaminated soil microcosms. Standard soils with different physical-chemical characteristics were used in order to evaluate the effects of environmental parameters on degradation of *n*-alkanes added as pure or mixed solutions. The strains were inoculated in the contaminated microcosms singly and in consortium. The aim of this work is to check the possibility to use these strains as bio-degraders in HC contaminated soil sites characterized by different conditions and to detect the main environmental factors that influence HC degradation in soil. The study evidence different degradation paths for each considered compound considering the microcosm conditions related to the soil's physicochemical characteristics.

Materials and methods

Experimental design

Surface standard soils were purchased from Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUF) Speyer (Germany) and their physico-chemical characteristics are listed in Table 1. Soils were aliquot into glass dishes to give soil layers of <2 cm thickness, and the dishes, covered with aluminium foil, were sterilized in a Fedegari autoclave (Type FVS 9110E, Tecnomara, Italy)

for 20 minutes at 121±1°C. The procedure was repeated twice, with a 24-hour incubation period in a laminar flow unit at room temperature, and afterwards, the samples were considered to be sterile and stored and handled under sterile conditions in a laminar flow unit.

Linear HC *n*C₁₂, *n*C₁₉, *n*C₂₀, *n*C₂₄, and *n*C₃₀ (HCs) all 99% purity grade were obtained by Fluka, Sigma-Aldrich Chemie, Switzerland. Hexane (HPLC fluorescence grade) was purchased from Fisher Scientific. Standard solutions of *n*-alkanes C₁₂, C₂₀, C₂₄, and C₃₀ in hexane (1000 µg mL⁻¹) were used during the study as HC contaminants. Hexane was chosen as solvent after checking that it was neither toxic to the bacterial strains nor used as a growth substrate.

Soil microcosms were set in 15 mL polypropylene conical sterile tube (Falcon, 2 g of air dried soil per tube) and artificially contaminated with 1 mL of the single HC solutions, or with 1 mL of the solution mixture (*n*C₁₂, *n*C₂₀, *n*C₂₄, *n*C₃₀) at a concentration of 1000 µg mL⁻¹ each. In order to get a good and homogeneous distribution of contaminants in the soil microcosms, the HC solutions were added to soil systems in a rotary agitator for 48 hours at 20°C and 30 rev min⁻¹. The hexane residue was then evaporated by a gentle nitrogen stream at 20°C with 3 mL min⁻¹ gas flow.

After the contamination step, soils were inoculated with the bacterial cultures as described in the following section. Abiotic control tubes (without bacterial inoculation) were set in order to evaluate the oxidation processes of contaminants in the absence of bacteria. The soil microcosms were incubated at 30°C for 28 days, during which soil moisture according to weight was maintained at 50% of their WHC by adding sterile distilled water when necessary. All treatments were repeated in triplicates. Combining five different contamination conditions (*n*C₁₂, *n*C₂₀, *n*C₂₄, *n*C₃₀ and Mix solution), four inoculation treatments (*Nocardia*, *Gordonia*, *Nocardia* + *Gordonia*, and not inoculated) and four soils (see Table 1) a total of two hundred forty tubes were set and taken into account for the evaluation of HCs degradation.

Table 1. Soil physicochemical characteristics.

Standard soil type no.	2.1	2.2	2.3	6S
Physicochemical characteristics				
Particle size (mm) distribution according to USDA (%)				
Clay %	3.7 ± 0.7	7.9 ± 1.1	9.2 ± 0.9	42.0 ± 1.8
Loam %	8.7 ± 2.2	13.5 ± 2.3	29.8 ± 3.2	36.0 ± 2.6
Sand %	87.6 ± 1.9	79.1 ± 3.3	61.0 ± 3.2	21.9 ± 1.6
Soil type	sand	loamy sand	sandy loam	clay
Org C in %	1.5 ± 0.29	2.29 ± 0.12	0.90 ± 0.17	1.79 ± 0.2
pH (0.01 M CaCl ₂)	6.0 ± 0.7	5.6 ± 0.4	6.2 ± 0.3	7.0 ± 0.2
Cation exchange capacity (meq / 100g)	6 ± 3	11 ± 2	9 ± 1	20 ± 3
Water holding capacity (g/100g)	35.1 ± 4.0	48.0 ± 3.0	34.9 ± 3.0	42.3 ± 2.8
Weight per volume (g/1000ml)	1380 ± 38	1170 ± 52	1315 ± 84	1245 ± 78

Bacterial inoculation

After HCs contamination and immediately after hexane evaporation, soil microcosms were inoculated with the two alkane-degrading bacterial strains *Nocardia* sp. SoB and *Gordonia* sp. SoCp, previously isolated in our laboratory.^[15] The two strains were grown for two days in mineral Bushnell–Haas broth (BH, Difco) with 1% *n*-hexadecane as sole C source to a concentration of 10^9 CFU (Colony-Forming Units) mL^{-1} and inoculated alone (500 μL) or co-inoculated (250 μL each strain) in each microcosm tube. Sterile BH broth was added to the non inoculated control tubes (abiotic controls).

Soil HC solvent extraction

After incubation, the soil microcosms were shaken vigorously using a horizontal shaker (Universal Table Shaker 709) at 400 rev min^{-1} for 10 minutes. Then, 10 mL hexane was added and the tubes were shaken in a mechanical rotary agitator for 2 hours at 20°C 80 rev min^{-1} . The tubes were centrifuged using a Mistral 2000 with a rotation radius of 200 mm and a speed of $4027 \text{ rev min}^{-1}$ for 30 min. After soil settling, the aliquot of the supernatant hexane phases were transferred in sample vials. The fraction was dried over 2 g of Na_2SO_4 . The extraction was repeated twice and both fractions were collected in a 20 mL volumetric flask. Then, 100 μL of a hexane solution of nonadecane ($10 \mu\text{g mL}^{-1}$) was added as an internal standard in order to normalize the efficiency of the analytical procedures.

GC-MS analysis

GC-MS analyses were performed on a Hewlett-Packard 5890 GC system interfaced with an HP 5973 quadrupole mass spectrometer. An HP5-MS column was used (5% diphenyl- 95% dimethylpolysiloxane $30 \text{ m} \times 0.2 \text{ mm}$, 0.25

μm film, J&W Scientific, Folsom CA, USA). A 4 mm ID straight glass injector liner packed with glass wool was used. Analyses were carried out in splitless injection (1 μL) mode with helium as carrier gas at 1 mL min^{-1} . Chromatographic conditions were: injector temperature 240°C , oven temperature program 2 minutes of 150°C isotherm followed by a linear temperature increase of 5°C min^{-1} up to 280°C held for 10 minutes. The MS full-scan conditions were: source temperature 230°C , interface temperature 280°C . The electron impact ionization spectra were obtained at 70 eV, recording mass spectra from m/z 42 to 550.

HCs were identified with the NIST 2005 mass spectral database and confirmed by comparing mass spectra and retention times with those of standard compounds. A standard mixture of *n*-alkanes ($n\text{C}_{12}$ – $n\text{C}_{30}$) in hexane was used as an external standard to verify retention times (RT) for each compound and quantify the degradation paths.

The linearity of the analytical method as above describe was assessed and the recovery of solvent extractions was evaluated for each *n*-alkane and two standard soils 6S and 2.1. The analytical recovery was tested at four concentrations, 1000, 100, 25 and 10 $\mu\text{g mL}^{-1}$ and was 70–99% (Fig. 1) and the solvent extraction of HC was constant at increasing concentrations. The limit of quantification (LOQ) was fixed using a standard grade HC solution ($0.1 \mu\text{g mL}^{-1}$ each). The limit of detection (LOD) for each analysis was defined by measuring progressively more diluted concentrations of the studied HCs until a signal-to-noise ratio of 3:1 was reached.^[19]

On the above-mentioned results, the quantitative amounts of each *n*-alkane residue after 28 days of incubation in microcosms were determined by the GC-MS method by using five points calibration curve with concentrations ranging from 0.5 to $50 \mu\text{g mL}^{-1}$. For each calibration curve the regression coefficient (R^2) was determined and linearity considered achieved when R^2 was greater than 0.995. The RSD for each compound was lower than 15%.

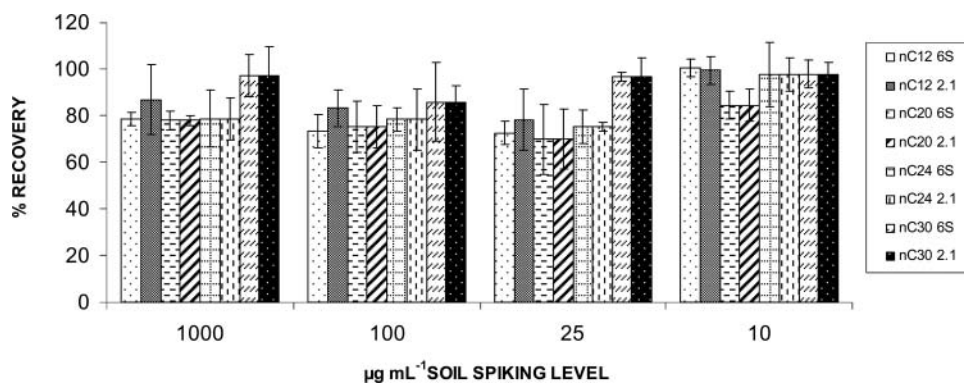


Fig. 1. Recovery of hydrocarbons (HC) from spiked soils (2.1; 6S); error bars represent the Standard deviation ($n = 3$).

Statistical analyses

The amounts of HC residues recovered from each microcosm after the incubation period were expressed in terms of μg and the relative standard deviation (RSD%) were also calculated and considered versus control samples. The amounts of HC recovered from each experimental condition were also analyzed by *t*-tests.

The effect of the microcosm environmental factors: single or consortium strain inoculum, soil types (% of clay), single or mixed HC solution versus the HCs residues were compared by a multifactorial analysis of variance (MANOVA), and the means of residues were separated by the Student–Newman–Keuls pairwise multiple comparison procedure.

Principal component analysis (PCA) was applied to investigate the relationships between the physicochemical characteristics of soils and *n*-alkanes degradation. The HCs residues were considered as the dependent variable of the considered experimental parameters. Five variables were considered and the method of Pearson parametric correlation was used: μg of HCs residues after the incubation period in each microcosm, CEC, % particle size < 0.002 mm, %C and pH of soils. The obtained principal components were considered as significant if their Eigen values were >1. All the statistical analyses were performed by using Statistica 6.0 for Windows (Stat Soft Italia).

Results and discussion

The extent to which a chemical partition into the organic matter is described by K_{oc} , but it may also be described by K_{ow} ; aliphatic hydrocarbons can strongly be partitioned into organic matter and diffuse into the three-dimensional structure of the supramolecular organization of humic and fulvic substances which represent the most abundant fraction of soil organic matter.^[20] Hydrocarbons may be sequestered within the soil through sorption to organic matter and mineral fractions and/or diffuse into the three-dimensional structure of the soil. The degree to which these physical interactions occur increases with time, and has been termed 'ageing'.^[21, 22]

The average amount of the recovered HC in inoculated microcosms under the different experimental conditions after 28 days of incubation was 267.44 μg corresponding to a degradation of 75%.

HC residues found in soil microcosms contaminated with *n*-alkanes in single and mixed solution mode, inoculated with *Nocardia* sp. SoB, *Gordonia* sp. SoCp and the consortium (*Nocardia* + *Gordonia*), in soils 2.1, 2.2, 2.3 and 6S, respectively, are fully elucidated in Figure 2. The two bacterial strains have comparable degradation ability on each single HC showing a preference for the shortest (C_{12}) and the longest one (C_{30}) (Figs. 2 A, B, C; Fig. 4). HC degradation is clearly influenced by the soil type and the

presence of other HCs. An increasing degradation trend is generally detectable in the four soils in the following order: 2.1 < 2.2 < 2.3 < 6S (Fig. 4). Degradation of the long-chain alkane triacontane in microcosms contaminated with the HC mixture (Fig. 2 D, E) is positively influenced by the presence of shorter alkanes suggesting that the latter may function as inducers of the catabolic enzymes. When the soils contaminated with the HC mixture are incubated with the consortium this effect is not detected probably due to competition between the two strains (Fig. 2F).

The PCA reduces the number of total variables to only few retaining the major part of the information on the systems variability. The amount of variables was reduced to only two (PC1 and PC2) which retained 86.1% of the total variance (Fig. 3). The soil with largest amount of clay (6S) retained positive score on PC1. Sandy soils (2.1, 2.2, 2.3) were placed in the directly opposite position respect to soil 6S. Moreover soil 2.2 was separated from 2.1 and 2.3 by the increase of SOC content and CEC on PC2. The HC degradation increases with the increase of pH, SOC, CEC and with the decrease of soil particle size.

The MANOVA results were $F = 89.1$, $df = 3$, $P < 0.001$; $F = 151.15$, $df = 3$, $P < 0.001$; $F = 6.94$, $df = 1$; $P < 0.001$ for soil clay%, linear HC compound residues, and mixed or single solution experimental factors used to contaminate soil microcosms, respectively. The interaction among all factors (soil clay%* HC*solution type) was also significant ($F = 4.81$, $df = 9$, $P < 0.001$) (Fig. 4). The interaction between factors (bacterial strain*HC*solution type) was not significant ($F = 0.17$, $df = 6$, $P = 0.98437$). The environmental complexity of soil systems requires the screening of several parameters, both chemical and physical, each showing a significant overall multivariate effect on the degradation of HCs.

The percentage of soil clay, HC compounds and the solution, mixed or single used to contaminate soil microcosms, showed significant influence on the long-chain HC degradation. Moreover it was evidenced how different parameters related to the environment can relevantly affect the degradation of the organic pollutant.

Soil 6S revealed the highest degradation rate of all HC (Fig. 2, Fig. 4) probably due to neutral pH, higher CEC and higher clay content as compared with the other soils. The higher clay contents are associated with high surface areas which allow a better interaction between supramolecular aggregates and aliphatic hydrocarbons. Natural organic matter is, in turn, a good substrate for bacterial growth. Moreover clay soil systems are also characterized by larger porosities.^[23, 24] These favour better air and water circulation. Good air/water circulation is the factor which enhances bacterial growth. Our results appear to confirm previous data by Ghazali et al.,^[25] showing that larger amount of sand particles in soils disadvantage the microbial biomass growth. The HCs residues confirm how the environmental parameters directly affect the bioremediation result.

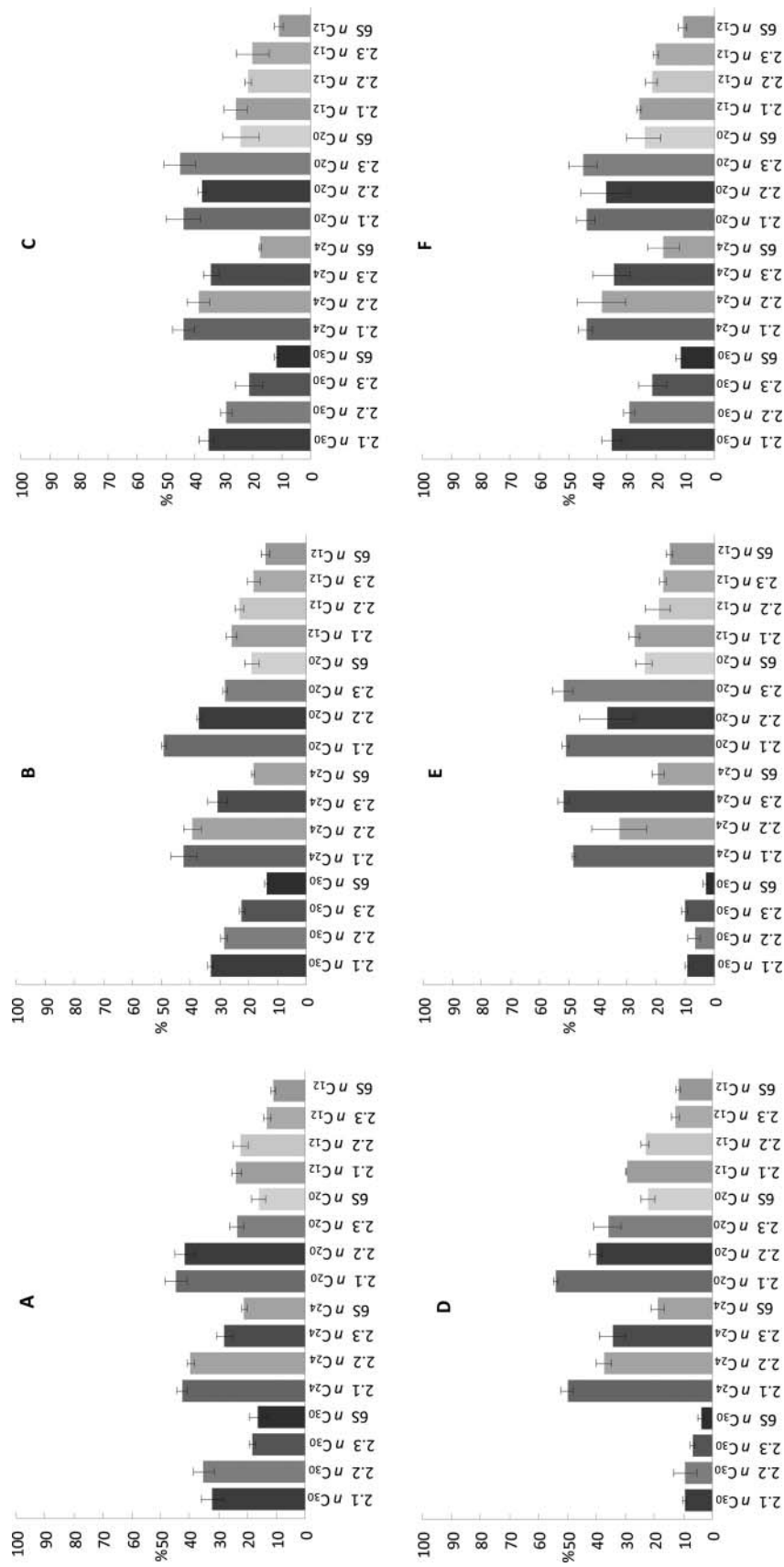


Fig. 2. Descriptive statistics of the % hydrocarbon residues (HCs) in different soil systems contaminated with single HC (A, B, C) or mixtures of HC (D, E, F) after 28 days of incubation with *Nocardia* SoB (A, D), *Gordonia* SoCp (B, E), the bacterial consortium (C, F). Error bars represent the Standard deviation (n = 3).

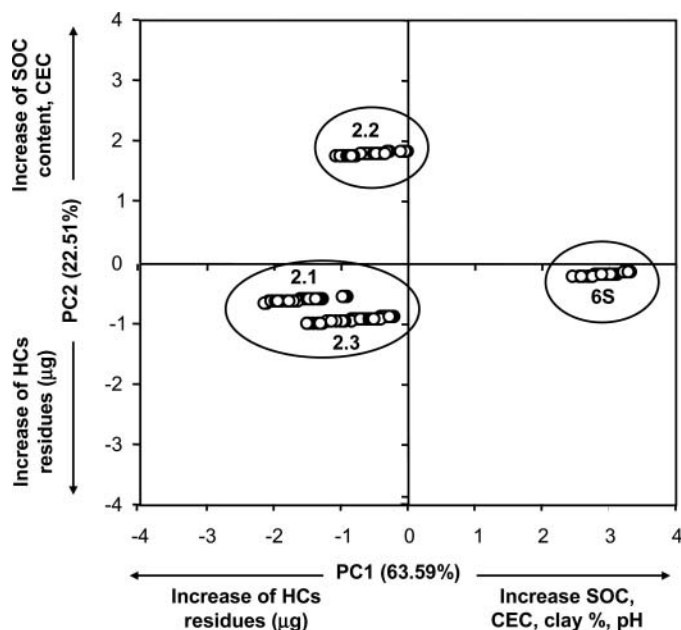


Fig. 3. Principal component analysis (PCA) graph representation of the soils physicochemical characteristics and HCs residues after the incubation period ($n = 3$).

The two strains inoculated did not behave differently in their degradation efficiency. The interaction between factors (bacterial strain*HC*solution type) was not statically significant. These data are consistent considering similar degradation rate of each strain. The initial step in the aerobic degradation of saturated, aliphatic (n -alkanes) compounds involves the oxygenase enzyme ‘attacking’ the terminal methyl group where a primary alcohol is formed. [8, 26, 27]

Both strains were isolated from a HC contaminated sandy soil in Sicily and confirmed in all kinds of soils the degradation ability on medium and long chain HC that was already detected in mineral medium. [15] This ability depends on the presence of one or more AlkB-like alkane-hydroxylase systems that were detected in both strains [15] and likely, on other undetected systems. In *Nocardia* SoB three quite divergent *alkB* sequences were detected by PCR using degenerated primers. It can be hypothesized that each system is involved in degradation of alkanes of different length. *Gordonia* SoCp and its ‘companion’ isolate SoCg carry a single *alkB* gene in their chromosome that encodes an AlkB-type alkane hydroxylase involved in degradation of hexadecane and triacontane. [28] The two strains inoculated in the microcosms may use different strategies to cope with HC of different length both reaching a similar

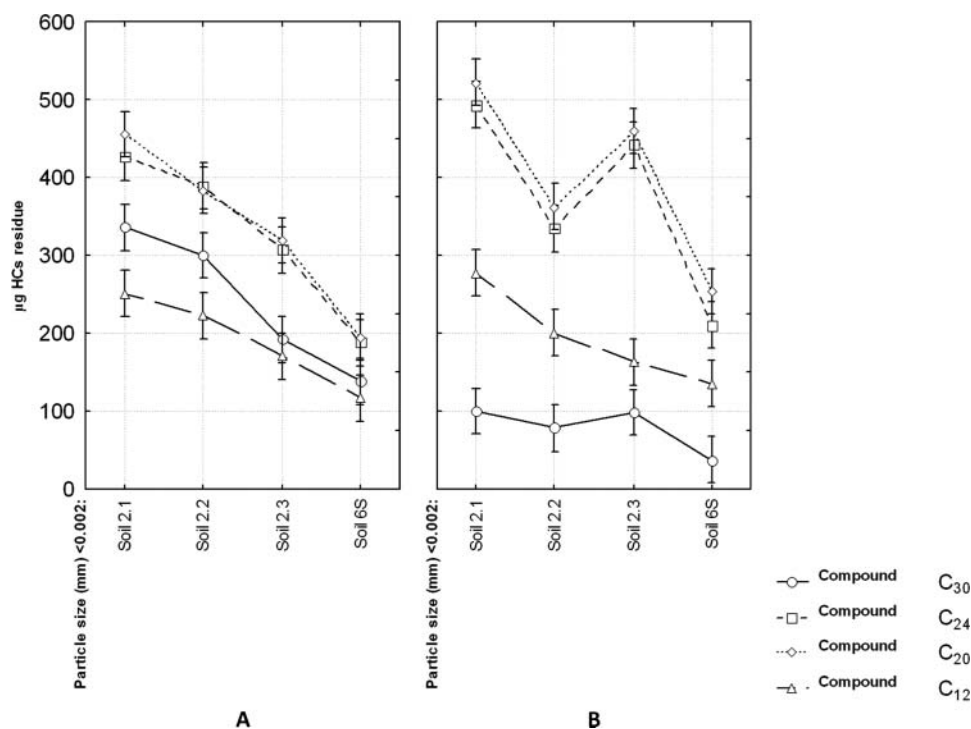


Fig. 4. Amount of hydrocarbons (μg) recovered from soils systems spiked with (A) single or (B) a mixture of HC after incubation with *Gordonia*, *Nocardia* and the bacterial consortium. Soil types were considered by their clay % content (particle size <0.002 mm). Error bars represent confidence values at the 95% confidence level. The P -value is the result of Multifactorial Analysis of Variance (MANOVA).

degradation rate depending on the supramolecular organic aggregate.

Conclusions

In the present study two HC degrading bacterial strains were used on different soils to show how soil–contaminant interactions can affect microbial degradation. Our study has demonstrated as hydrocarbons biodegradation in soils is controlled by hydrocarbon physico-chemistry, environmental conditions, bioavailability and the presence of catabolically active microbes. *Gordonia* and *Nocardia* genera appear to be particularly promising strains for aliphatic hydrocarbon bioremediation programs in all kinds of soils and in particular in those with large clay content. They are suitable for HC degradation because of their drought resistance ability.^[29, 30] Moreover considering the promising results obtained by the selected degraders in different environmental conditions, they may be capable tools in polluted environments for bioaugmentation practices in clay and arid soils.

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