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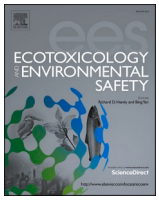

# Effective bioremediation of soil from the Burgan oil field (Kuwait) using compost: A comprehensive hydrocarbon and DNA fingerprinting study

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ABSTRACT

An innovative combination of metagenomic profiling of microbial communities and GC-MS & Pyrolysis-GC-MS fingerprinting methods were used to assess the biodegradation of contaminated soil from the Burgan oil field in Kuwait. The soil was treated with (sludge) compost in microcosms to evaluate the feasibility of this material for bioremediation purposes. The most favourable trial showed a > 80% decrease in TPH, thereby indicating strong potential for full-scale application using a cost-effective technology and thus in line with the principles of the circular economy. The microbial study showed that compost addition enhanced the organic matter and nutrient content of the soil. However, the microorganisms in the compost did not seem to play a relevant role in bioremediation, meaning that compost amendments serve as a biostimulation rather than a bioaugmentation approach. The chemical study of the distinct oil fractions revealed rapidly biodegraded compounds (alkanes, alkyl-aromatics, etc.) and others that were much more refractory (hopanes, benzohopanes, etc.). Of note, although heavy fractions are usually considered recalcitrant to biodegradation, we observed incipient degradation of the asphaltene fraction by means of double-shot thermodesorption and pyrolysis. Finally, chemical fingerprinting also revealed that the treated soil contained some of the compounds found in the compost, such as coprostanol, cholesterol, and plant sterols. This observation would support the use of these compounds as proxies to monitor the effects of compost and to adjust dosages in real-scale bioremediation treatments.

## 1. Introduction

Environmental pollution by petroleum hydrocarbons can originate from various sources, including spillages and use of petroleum products (Cerqueira et al., 2014; Lima et al., 2005; Ortega et al., 2018). In this context, during the Gulf War in 1991, Kuwait experienced what is probably the biggest oil spill on record. This spill caused severe environmental damage, including the formation of multiple oil lakes and partial volatilization of light compounds. In addition, the oil field fires led to huge plumes of black smoke, which eventually settled as soot, tar mats, and tarcrete deposits (Al-Hashem et al., 2007; Al-Dahanii et al., 2015). Covering 838 $km^2$ and located in southeastern Kuwait (Al-Dahanii et al., 2015), the Greater Burgan field is the largest clastic oil field in the world, and it was one of the main areas affected by this environmental disaster.

The choice of remediation technology to tackle soil pollution by hydrocarbons depends on factors such as the type and concentration of oil fractions and the components involved, soil properties, time available to carry out remediation actions, and cost (Haghollahi et al., 2016; Ortega et al., 2018; Yong and Mulligan, 2019). In this regard, bioremediation can be the strategy of choice as it is characterized by low energy consumption, reduced risk of contaminant migration and secondary environmental impacts, and cost-effectiveness (Gallego et al., 2011; Guarino et al., 2017; Miri et al., 2019). Indeed, many microorganisms have developed the capacity to use aliphatic and aromatic hydrocarbons as a source of carbon and energy (Ławniczak et al., 2020). Therefore, biodegradation can be improved by stimulating indigenous microbial populations (biostimulation) or by adding exogenous ones (bioaugmentation). Successful bioremediation treatments have commonly involved aerobic biostimulation processes (Xu et al., 2018;

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Calvo et al., 2019), including the remediation of massive oil spills, both marine (Gallego et al., 2007a) and terrestrial (Brown et al., 2017). Conversely, several diverse issues are often associated with bioaugmentation approaches using exogenous microorganisms (Asquith et al., 2012; Bento et al., 2005).

In this context, biostimulation with compost is an interesting option that can be applied in biopiles/windrows and landfarming (Aguelmous et al., 2019; Lukić et al., 2017). Specific examples of biopiles amended with compost were provided by Kirchmann and Ewnetu (1998), who composted horse manure as a means to degrade two types of oil waste, namely oil sludge from service stations and oil residues from a refinery. In turn, Fountoulakis et al. (2009) composted oil refinery sludge and green waste and obtained an 85% reduction in PAHs in 130 days. Sayara et al. (2010) used compost as an amendment and pyrene as a model contaminant to achieve almost complete degradation in a short period (10 days). Dados et al. (2015) compared biostimulation using compost and bioaugmentation; they found that compost-treated soils showed the highest degradation rates. On the whole, compost appears to simultaneously enrich soil organic matter and nutrient content, stimulate microbial activity, supply additional microorganisms, and foster circular economy approaches (Chen et al., 2015; Castillo et al., 2016). Consequently, comprehensive chemical and microbial studies are required to evaluate these multifaceted effects.

Biological processes do not have the same effect on distinct oil components (Taccaria et al., 2012). In this regard, an early period of far-reaching biodegradation is usually followed by a stage in which the hydrocarbon concentration tends to be residual (Esquinas et al., 2017). Indeed, biodegradation yields are determined by bioavailability constraints associated with soil properties, microbial activity, and weathering processes (Gallego et al., 2011). Given these considerations, the fate of oil components should be addressed using advanced analytics (Gallego et al., 2007b). For instance, reliable diagnostic ratios are required (Yunker et al., 2002) to distinguish between biotic and abiotic (mainly volatilization) processes. Therefore, a complete evaluation requires gas chromatography-mass spectrometry (GC-MS) for pollutant fingerprinting (Stout et al., 2016; Saeed et al., 2021). In this context, the fate of heavy fractions of oil, such as asphaltenes, is not well known, although their degradation has been suggested (Liao et al., 2009; Esquinas et al., 2017). Analytical pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) is a suitable approach to study heavy fractions, as demonstrated in various environmental studies (Miciç et al., 2011; Kruge, 2015). This approach can be also combined with thermodesorption (TD) techniques to provide a fast fingerprint screening method (Kruge et al., 2018, 2020).

Regarding microbial studies, Next-Generation Sequencing (NGS) has recently proved useful for exploring the structures of microbial communities, although it remains expensive and time-consuming (Jami et al., 2014; Patel et al., 2021). As an alternative, molecular fingerprinting techniques are powerful and rapid tools that can be used to estimate diversity and the dynamics of microbial community structures. However, they cannot provide taxonomic information. Automated ribosomal intergenic spacer analysis (ARISA) is a culture-independent molecular fingerprinting method developed by Fisher and Triplett (1999). ARISA involves PCR amplification of the bacterial 16–23 S rRNA hypervariable intergenic spacer region. As the length of this region is heterogeneous, ARISA allows a community-specific banding pattern to be obtained (Dubey et al., 2020). In our context, ARISA provides a rapid and cost-effective method to compare bacterial community structures and infer how they are affected by environmental factors.

Following all these premises, here we used microcosm trials to study the effectiveness of compost as the main additive for the remediation of soil affected by oil spillage in the Burgan field in Kuwait. In addition to addressing the evolution of the concentration of total hydrocarbons, we also used detailed GC-MS fingerprinting to identify the fractions that underwent the greatest biodegradation and thus can be considered truly recalcitrant. Gravimetric measurements and Py-GC-MS analyses were also used to examine the changes in heavy fractions (resins and especially asphaltenes). Furthermore, we performed a microbiological study focused on the DNA fingerprinting of microbial populations using ARISA, which allowed us to infer the roles of bacterial populations in biostimulation involving compost. The combination of these two rapid molecular fingerprinting techniques (chemical and microbiological) allowed us to draw conclusions about the utility of compost in bioremediation and to predict the evolution of hydrocarbon families and microbial populations in the area affected by oil spills in Kuwait. On the basis of our findings, this dual approach emerges as a novel strategy for monitoring bioremediation actions.

## 2. Materials and methods

### 2.1. Samples and initial soil analysis

The samples comprised an oiled sandy soil partially protected for years from weathering effects in very dry/anoxic conditions. They were taken in 2019 in one of the areas (Burgan field) affected by the huge spills that took place in the first Gulf war. The samples were taken from a “dry oil lake”. In this regard, the polluted sand taken from a depth of approx. 5–40 cm was encrusted with a surface layer of weathered heavy oil, such that the soil was sealed by a tar mat (Balba et al., 1998) about 1 cm thick, which was peeled off to reveal the underlying polluted sand to be sampled (Fig. S1a).

Air-dried soil samples were sieved through a 2 mm mesh. They were then disaggregated by a roller and homogenized. Material with grain size $>$ 2 mm was washed and rubbed off to recover fine particles, whereas gravel and pebbles were excluded from the study. Sandy particles impregnated by hydrocarbons were observed using the DINO-LITE AM7915-MZT digital microscope (Fig. S1b). Various representative batches of the final fraction $<$ 2 mm were obtained by quartering in a channel separator. These batches were characterized following standard methods. The pH was measured in a suspension of soil and water (1:2.5) with a glass electrode, while electrical conductivity was determined in the same extract (diluted 1:5). The Kjeldahl method was used to measure nitrogen content and the Olsen method to determine available phosphorus content. Organic matter was determined by the Loss on Ignition method (LOI). The Bouyoucos Densimetry method was used to determine texture. TOC (Total Organic Carbon) and IC (Inorganic Carbon) were measured in a Total TOC-V CSH (Shimadzu) and Total N in a LECO CN-200 analyzer. For multi-element analysis, 0.250-g representative subsamples were leached by means of an ‘Aqua regia’ digestion (HCl + $HNO_3$) in an Anton Paar 3000 microwave, then passed through a 0.45-μm filter and diluted for the quantitative determination of major and trace elements by Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7700, Agilent Technologies, California, USA) using IDA (Isotopic Dilution Analysis), with a spike solution from ISC Science, Spain.

In brief, the samples had (Table S1) an alkaline pH, high salinity, sandy texture (less than 10% of silt and clay), and very low concentrations of nitrogen and phosphorus, typical of a desert environment. Consequently, the C/N ratio was close to 100 (the ideal range for bioremediation is between 10 and 40). The organic matter content was high, which was attributed to the presence of hydrocarbons. Given that none of the inorganic elements quantified showed notable concentrations, we concluded that the samples comprised siliceous sand with minor clayey and carbonate components. Also, these measurements ruled out hypothetical inhibition of degrading microorganisms as no heavy metal(loid)s were present in sufficient concentration to exert this effect.

### 2.2. Experimental design of bioremediation treatments

The oil-polluted soil from the Burgan field was divided into 6 batches, each with 400 g. The soil was then placed in a set of bioreactors (borosilicate glass recipients of 3000 $cm^3$, 40 cm long $\times$ 25 cm wide $\times$ 3

cm high) following the conditions shown in Table 1. Sampling was done at 0, 10, 30, 60 and 90 days after the beginning of the experiments.

Soils were tilled to optimize aeration. Soil moisture was measured regularly with a thermobalance and recovered by adding distilled and sterilized water in order to maintain a range of 60–80% water holding capacity (around 10% moisture).

Regarding the biostimulants, the slow-release fertilizer (SRF) selected was Sierrablen 31:5:7 (N:P:K), manufactured by Scotts and previously used in other bioremediation treatments (Peláez et al., 2013). This product is a granulated fertilizer that requires 2–3 months for complete release. The amount applied was adjusted to achieve the ideal 10:1 C:N ratio for bioremediation (Forján et al., 2020). In turn, the compost used was supplied by COGERSA (Asturias, Spain). It is produced from selected sludge from wastewater treatment plants (WWTPs) and shredded wood, using composting piles with forced aeration. Its main characteristics are summarized in Table S1.

### 2.3. Hydrocarbon analysis

#### 2.3.1. TPH quantification

Representative soil samples were sent to an ISO/IEC 17025 certified laboratory (Eurofins Analytico, The Netherlands) to quantify semi-volatile hydrocarbons and obtain partitioning data in terms of carbon chain length (C12–C16, C16–C22, C22–C30, C30–C40) by means of GC-FID (Gas Chromatography-Flame Ionization Detection).

#### 2.3.2. GC-MS qualitative screening

10-g representative subsamples were extracted with hexane: dichloromethane (1:1, v/v) in a Soxtherm system (Gerhardt). The extract was concentrated by rotary evaporation. Direct extracts were analyzed by GC-MS (Gas Chromatography-Mass Spectrometry). The extracts were injected into a 7890 A GC System coupled to a 5975 C Inert XL MSD with Triple-Axis Detector (Agilent Technologies). A capillary column DB-5 ms (5% phenyl 95% dimethylpolysiloxane) 30 m × 0.25 mm i.d. × 0.25 μm film (Agilent Technologies) was used, with helium as carrier gas at 1 mL/min. The initial oven temperature was 40 °C (held for 5 min), and it was ramped up at 5 °C $min^{-1}$ to 300 °C (held for 20 min). The mass spectrometer was operated in electron ionization mode (EI) at 70 eV and was calibrated daily by auto-tuning with perfluorotributylamine (PFTBA). The chromatograms were acquired in full-scan mode (mass range acquisition from 45 to 500 *m/z*).

#### 2.3.3. Oil extraction, LC fractionation, and comprehensive GC-MS and Py-GC-MS study

10-g representative subsamples were extracted with dichloromethane:methanol (3:1, v/v) in a Soxtherm system (Gerhardt, Germany) at 150 °C for 3 h. The extracts were concentrated by rotary evaporation, and aliquots were fractionated and gravimetrically quantified by deasphaltening and liquid chromatography (LC) into four fractions following SARA (Saturates, Aromatics, Resins and Asphaltenes) procedure (Lara-Gonzalo et al., 2015). In brief, maltenes and asphaltenes were separated by passing the extracts through 0.45-μm PFTE filters, first using *n*-hexane to remove maltenes, followed by dichloromethane to mobilize asphaltenes. Maltenes were then separated into three fractions from lesser to greater polarity in an LC column (Corning 7078–5 N serological disposable glass pipette) filled with activated (dried overnight at 240 °C) silica gel (4/5) and alumina (1/5). Saturates (SAT) were eluted with hexane, aromatics (ARO) with dichloromethane:hexane (7:3, v/v), and resins (polars, POL) with dichloromethane:methanol (1:1, v/v). The desaphaltening and LC methodologies are based upon published sources (Kruge et al., 2018). Blanks, duplicates, and representative standard materials were routinely used for quality control and assurance purposes (QC/QA).

The three maltene fractions were analyzed by GC-MS. Extracts were injected into a GCMS-QP2010 Plus (Shimadzu Europe, Germany). A DB-5MS capillary column (5% phenyl, 95% dimethylpolysiloxane; 60 m × 0.25 mm i.d. x 0.1 μm film) from Agilent Tech. was used, with He as carrier gas at 1 mL/min. The initial oven temperature was 50 °C (held for 2 min), and it was ramped up at 2.5 °C $min^{-1}$ up to 310 °C and then held for 45 min. The mass spectrometer was operated in EI mode at 70 eV and calibrated daily by auto-tuning with PFTBA. The chromatograms were acquired in full-scan mode (mass range acquisition: 45–500 *m/z*). For quality control, solvent blanks were periodically injected. Compounds were identified using the W8N08 (John Wiley & Sons, Inc., New York), NIST27, and NIST47 mass spectral libraries.

Thermodesorption and pyrolysis were performed on a measured amount of asphaltene fraction (0.2 mg) for 20 s at 350 °C using a PY-2020iD double-shot pyrolyzer (Frontier Lab) connected to the same GC-MS device described above. The oven temperature of the gas chromatograph was programmed from 50 °C to 310 °C (at 2.5 °C $min^{-1}$), with an initial hold of 2 min at 50 °C and a final hold of 45 min at 310 °C. The mass spectrometer was operated in full-scan mode (50–600 *m/z*). After the thermodesorption run had ended, the sample (which had remained untouched in the pyroprobe) was heated at 610 °C for 20 s, thereby pyrolyzing the post-thermodesorption residue ("double-shot" or "sequential pyrolysis"). Pyrolysis products were analyzed by GC-MS (Py-GC-MS) using the conditions employed for the TD products, while the compounds were identified using the aforementioned libraries.

Ratios of compounds were compared to define diagnostic ratios. Selection criteria consisted of abundance in the Burgan oil, ease of identification in SIM (Single Ion Monitoring) chromatograms, and representability of the predominant hydrocarbon families in oil. To eliminate the potential varying response of the mass spectrometer, the ratios were obtained with compounds whose peak areas were measured at the same *m/z* values, as suggested by Kienhuis et al. (2016). Chromatograms with poorly resolved peaks or noisy baselines were discarded.

**Table 1**
Experimental setup for microcosms.

| Code | Treatment and amendments | Temperature | Watering | Tilling |
|---|---|---|---|---|
| BG0 | Control (no amendments) | Room temperature (~ 20° C) | To hold approx. 10% moisture | Every 3 days |
| BG1 | 1% (w/w) Slow-release fertilizer (SRF) | Room temperature (~ 20° C) | To hold approx. 10% moisture | Every 3 days |
| BG2 | 20% (w/w) Compost | Room temperature (~ 20° C) | To hold approx. 10% moisture | Every 3 days |
| BG3 | 1% (w/w) SRF 20% (w/w) Compost | Room temperature (~ 20° C) | To hold approx. 10% moisture | Every 3 days |
| BG4 | 20% (w/w) Compost | Room temperature (~ 20° C) | To hold approx. 15% moisture | Daily |
| BG5 | 20% (w/w) Compost | 35° C (closer to average conditions in Kuwait) | To hold approx. 10% moisture | Every 3 days |

### 2.4. Microbiology

#### 2.4.1. Microbial counts

Microbial counting for initial soil and compost was carried out by conventional plate-counting using serial dilutions. To this end, soil and compost were spread on agar plates of Tryptic Soy Broth (Merck, Germany) diluted 1/10, and supplemented with 15% of agar (1/10 TSA). Plates were incubated at 30 °C for 7 days. Cultivable bacteria were then monitored over time in the six microcosms using the same procedure described above.

#### 2.4.2. Bacterial Automated Ribosomal Intergenic Spacer Analysis

Automated ribosomal intergenic spacer analysis (ARISA) employs the natural variability in length of the bacterial 16–23 S rRNA intergenic

spacer region to provide a bacterial community profile. ARISA was used to assess changes in bacterial community structure during the microcosm experiments. DNA was extracted with the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA). The 16–23 S rRNA intergenic spacer region was amplified using specific ARISA Primers ITSReub (5′-GCCAAGGCATCCACC-3′) and ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′), with the latter labeled with a fluorescent dye (either HEX or 6FAM) (Cardinale et al., 2004). The PCR reaction contained 12.5 μL of Speedy Supreme NZYTaq 2x Colorless Master Mix (Nzytech genes & enzymes, Lisbon, Portugal), 1 μL of each primer at 10 μM, 9.5 μL of DNase free water, and 5–10 ng of genomic DNA. Thermal cycling consisted of an initial denaturation of 5 min at 95 °C, followed by 30 cycles of 2 s at 94 °C, 5 s at 55 °C, 10 s at 72 °C, and a final extension at 72 °C for 10 min. Fragments were analyzed by the Scientific and Technical Services of the University of Oviedo, using the internal size standard Map Marker 500ROX on an ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientifics, Waltham, Massachusetts, USA). Electropherogram data were visualized and examined with Peak Scanner™ Software v1.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Background noise thresholds were set at 50 fluorescence units; any peak below this threshold was discarded.

R interactive binning script (Ramette et al., 2009) was used for OTU binning similarly to the procedure described by Castaño et al. (2021). Principal component analysis (PCA) was used to evaluate similarities between communities and was implemented in R package FactoMineR (Lê et al., 2008) using the PCA function. This method allows evaluation of the components that have a greater weight in the variation and their plotting as a two-dimensional graph from the data matrix containing the abundances of each OTU (Operational Taxonomic Unit) identified. A non-parametrical statistical test analysis of similarities, ANOSIM (Clarke, 1993), was performed to evaluate significant differences between groups. To estimate the effect of abiotic soil proprieties, the Mantel test (Mantel, 1967), based on Spearman's correlation, with 9999 permutations was carried out. To characterize alpha-diversity, Hill numbers (Hill, 1973) of orders q= 0 (richness), q= 1 (corresponding to exponent of Shannon index) and q= 2 (equal to multiplicative inverse of the Simpson index) were calculated. E2.0 community evenness was calculated as a ratio of Hill numbers q= 2 and q= 0.

## 3. Results and discussion

### 3.1. Evolution of bioremediation microcosms

A summary of the quantitative results obtained after 90 days of microcosm experiments is shown in Figs. 1 and 2. All treatments revealed a marked decrease in the concentration of TPH, except the control experiment, in which a much lower growth of microbial populations was also consistently observed. The microcosms involving SRF showed a lower yield than those in which compost alone was used (Fig. 1a). This observation thus suggests the greater effectiveness of the latter amendment, as evidenced by both the decrease in the initial hydrocarbon concentration (to values close to 2000 ppm) and the increase in the microbial population by more than four orders of magnitude. The combination of compost with SRF (BG3) did not improve the results of compost alone (BG2). This result could be explained by the relevant role of the microorganisms present in the compost (see Section 3.2) and their possible "over-stimulation" with the SRF nutrients, resulting in augmented bacterial activity on the organic matter in the compost (a highly accessible carbon source) instead of increased hydrocarbon degradation. In addition, the composition of the SRF could have had an adverse effect on relevant autochthonous hydrocarbon degraders.

On the other hand, in the compost-only trials (Fig. 1b), a more frequent aeration and watering regime (BG4) did not give rise to significant additional effects, while increasing the incubation temperature (BG5) moderately improved the final results (clearly below 2000 ppm), although the microbial counts were similar. On the whole, these results

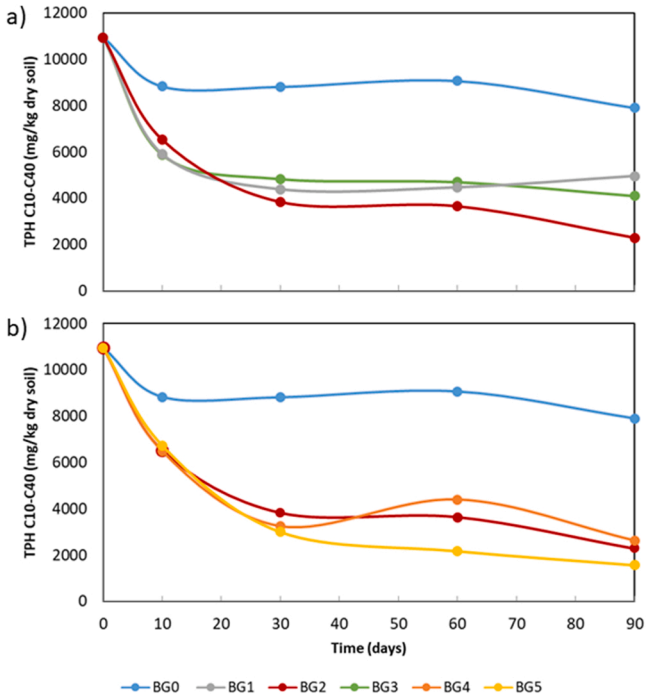


**Fig. 1.** Evolution of the TPH content (mg/kg) of polluted soil (BG0) and soil treated with SRF (BG1), compost (BG2), SRF + compost (BG3), compost under higher moisture and aeration (BG4), and compost under higher temperature (BG5). (a) Compost vs. SRF, and (b) compost-only trials. The relative standard deviations (RSD) were below 5% in all cases.

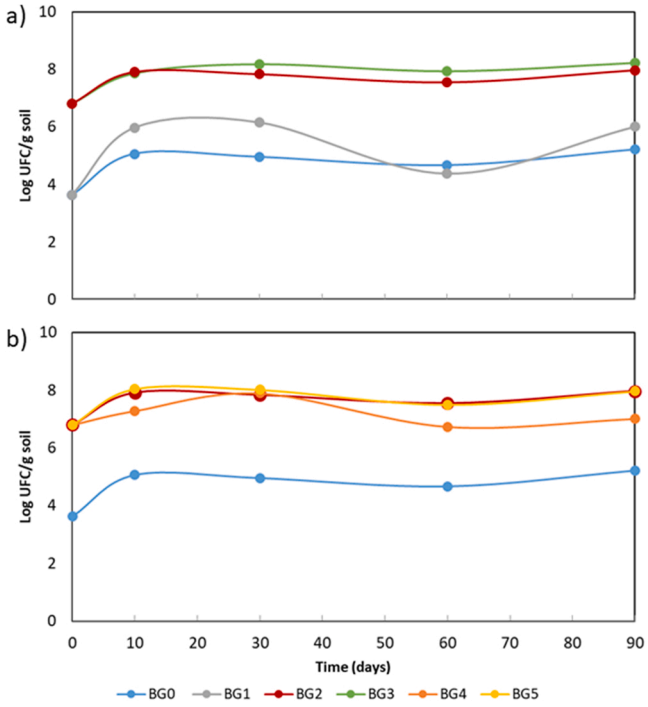


**Fig. 2.** Changes in bacterial counts during 90 days of microcosm experiments under the different conditions tested: (a) Compost vs. SFR, and (b) compost under different conditions. Microcosms: polluted soil (BG0), soil treated with SRF (BG1), compost (BG2), SRF + compost (BG3), compost under higher moisture and aeration (BG4), and compost under higher temperature (BG5).

are concordant with other bioremediation studies on hydrocarbon-polluted soils from Kuwait (Al-Awadhi et al., 1996), particularly those involving the use of compost as an amendment (Al-Daher et al., 1998; Al-Daher et al., 2001).

Originally, compost showed a much higher bacterial load ($5.2 \times 10^8$ CFU/g) than the initial soil ($2.9 \times 10^3$ CFU/g). Thus, the compost contributed a significant amount of bacteria to the soil (Fig. 2a)—an effect that was maintained until the end of the experiments. The modifications of the compost treatment, in terms of aeration & moisture (BG4), and temperature (BG5), did not substantially alter the values obtained in BG2 (Fig. 2b), and even caused a slight decrease in the number of bacteria in BG4.

Concerning the evolution of hydrocarbon fractions with distinct molecular weights, in all cases the proportion of lighter fractions decreased compared to the heavier ones (Fig. S2; Table S2), as found in other studies (Bento et al., 2005; Curiel-Alegre et al., 2022; Gallego et al., 2011). These differences were more pronounced in the experiments that registered the greatest overall reduction in TPH (BG4, and especially BG5), although in all cases the intermediate fraction (C21-C30) remained the most abundant. Although GC-MS data (see below) are useful to achieve a comprehensive view of the degradation process, the GC-FID results for the different fractions are also relevant as they are usually employed in risk assessment procedures and in the drawing up of environmental regulations.

Furthermore, a qualitative screening by GC-MS (Fig. S3) showed how the high abundance of alkanes observed in the control test gave way in all cases to the preponderance of hopanes (recalcitrant hydrocarbons) wherever amendments were applied. Of note, the size of the UCM

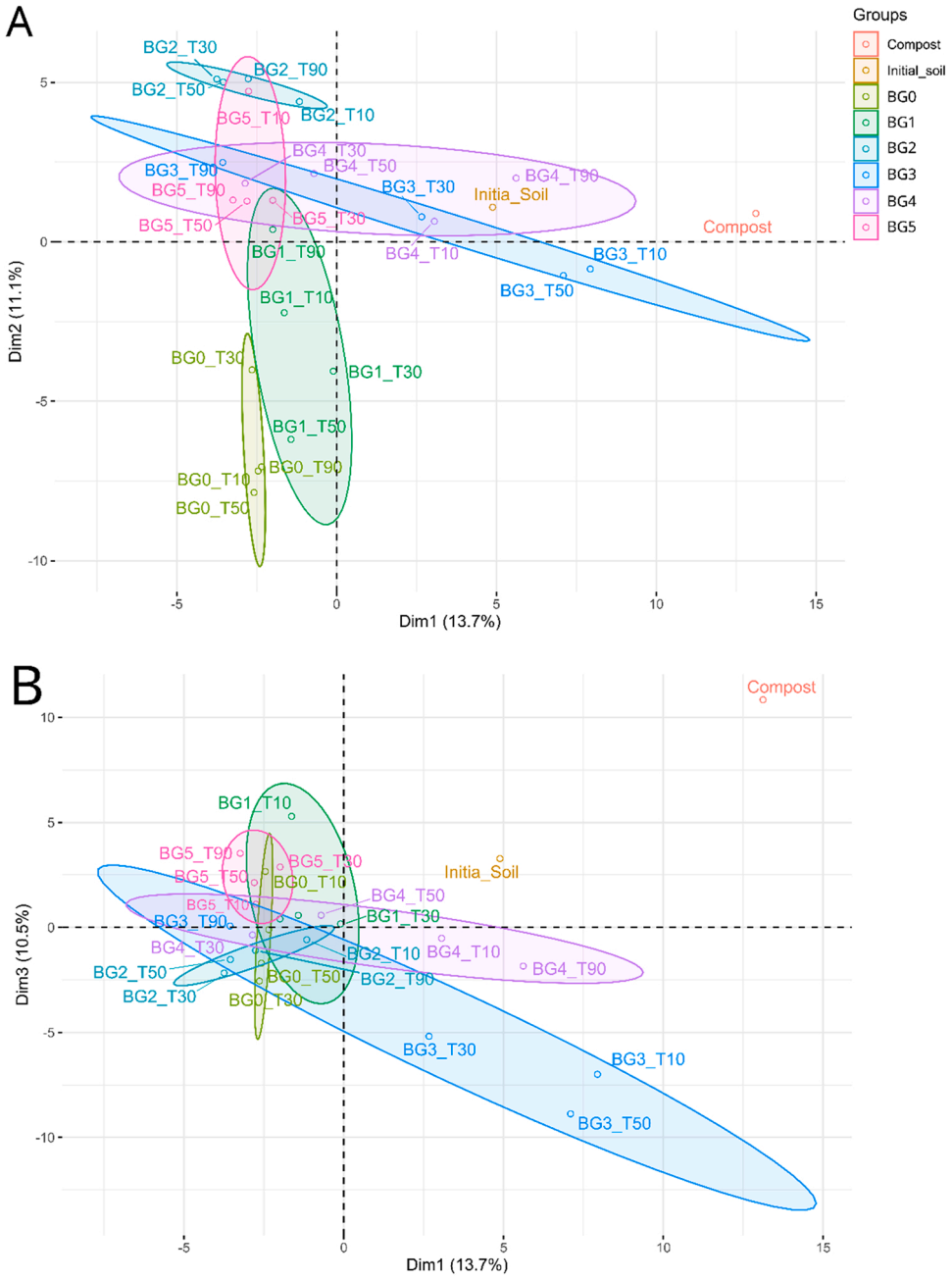


**Fig. 3.** PCA ordination of bacterial community structures of microcosms based on ARISA profiles. A: differences between bacterial communities along the first and second principal components; and B: differences between bacterial communities along the third and second principal components. Statistical ellipses represent 75% confidence for treatments (see Table 1 for experimental setup).

("hump") was smaller in the microcosms in which a higher reduction of TPH was achieved (BG4, BG5). Also, note the presence of a peak corresponding to elemental sulfur in the treatments involving SRF.

### 3.2. DNA fingerprinting analysis of the bacterial communities

Given that the levels of cultivable bacteria in the compost were much higher than in the study soil, it can be assumed that the levels determine the intensity of the degradation. However, the composition of the bacterial populations in the compost, and not only the count, may also be a determining factor. In this regard, the compost may have hosted communities capable of eliminating TPH more effectively than those present in the soil. This notion can be verified only by characterization of the total bacterial populations using molecular techniques (Steen et al., 2019). Therefore, genomic-molecular techniques to analyze such diversity are required. To address this point, DNA was extracted from the initial soil and compost, and then from the soil samples taken from each microcosm at different time points. A total of 32 samples were thus processed. The structure of the bacterial community was characterized using ARISA fingerprinting.

A total of 134 OTUs were obtained after binning. The PCA of ARISA data showed that 35% of total variance was explained by the first three principal components (Fig. 3 and Fig. S4). The overlapping of statistical ellipses (with 75% confidence) indicated that, for most treatments, there was some degree of overlap in the composition of their bacterial communities. In this regard, the treatments that showed higher overlap were BG3, BG4 and BG5, whereas BG1 and BG2 had a very small area of intersection, thus indicating higher divergence of community structures. BG0 (control) emerged as a clearly differentiated group along the first two principal components. Variations along the first and third principal components indicated that the communities from all treatments had a significant degree of overlap. At the same time, in both cases the bacterial community of the compost itself appeared separated from the rest of the communities (Fig. 3), indicating its dissimilarity to the rest of the samples.

Microbial communities added with the compost contributed the most (20%) to the variance explained by the first two principal components (Fig. S5). These observations strongly suggest that the microbial community structure differed in the microcosms amended with compost. In turn, while PCA analysis showed some degree of overlap in the composition of bacterial communities between different treatments, the differences between them were still sufficiently large. This was corroborated by the ANOSIM test ($R = 0.5257$; $p = 0.0001$), which indicated statistically significant (even if not very large) differences between treatment groups but not between samples over time. These differences could be clearly attributed to the compost added to the treatments. Moreover, the Mantel test showed that these differences were not correlated with environmental factors such as temperature ($R = -0.0727$; $p = 0.6996$), humidity ($R = 0.1453$; $p = 0.1326$), or TPH concentration ($R = 0.0702$; $p = 0.2296$). In addition, when samples were grouped by sampling time ($R = 0.0460$; $p = 0.2333$), an even distribution within and between groups, without significant differences, was observed.

Soil bacterial diversity is not necessarily related to the level of hydrocarbon degradation observed but it influences the initial selection of the most effective bacteria during bioremediation treatment (Bell et al., 2013). That is to say, the presence of specific bacterial genera is pivotal for the effectiveness of bioremediation (Romantschuk et al., 2000). Although compost is a source of both bacteria and organic matter, the changes observed in the bacterial populations are probably attributable to the latter, i.e., the input of organic matter (Saison et. al, 2006; Abis et. al, 2021), because allochthonous bacteria cannot compete with indigenous soil bacteria (Fodelianakis et al., 2015; Garbisu et al., 2017; Radwan et al., 2019). Furthermore, according to the Mantel test, the distribution of the populations was not correlated with the environmental parameters measured in the microcosms. Therefore, the differences may be due to the factors already mentioned, that is to say, the source and composition of the organic matter. To verify this hypothesis, we selected the OTUs that contributed the most to the variance in the first two principal components and analyzed their relative abundance (Fig. 4).

Some OTUs that were present in the initial soil disappeared in almost all the treatments, whereas several others, poorly represented in the initial samples, were boosted relatively intensely during the treatments and irrespective of the addition of SRF or compost (for instance OTU_2, OTU_80, and others). Some OTUs that were abundant in compost (e.g., OTU_13, OTU_21, or OTU_50) were present in much lower abundance or even disappeared in microcosms containing compost during the 90 days of the experiment. This observation therefore further supports the hypothesis that compost serves mainly as a reserve of organic matter and nutrients for indigenous bacteria.

Finally, Hill numbers were calculated in order to characterize alpha-diversity, thereby helping to determine whether any treatment had an impact on richness and evenness of bacterial communities (Fig. 5).

The addition of compost may lead to an increase in alpha-diversity (Wu et. al, 2020). However, in our case, overall diversity, as represented by Hill numbers of orders $q = 1$ (equivalent to an exponent of Shannon index) and $q = 2$ (a multiplicative inverse of the Simpson index), decreased slightly compared to the initial soil but remained broadly similar to the control group in all treatments. Community evenness was not negatively affected. Thus, it can be summarized that, despite the changes in community structure caused by proliferation of hydrocarbon-degrading bacteria, the overall impact on diversity was very limited.

Our approach is partially limited as it was not possible to identify the indigenous microorganisms that should be promoted to optimize bioremediation yields, nor the conditions that favor the most effective bacterial genera (Bell et al., 2013). These limitations, coupled with the fact that microbial communities vary between different treatments, contribute to the difficulty in selecting the most effective treatments. The prediction could be improved with the development of more incisive methods of functional analysis, both of individual bacteria strains and populations as a whole.

### 3.3. Comprehensive GC-MS fingerprint of remaining hydrocarbons

Analysis of the results of the microcosm experiments and the evolution of the microbial populations revealed that the best bioremediation yields were achieved in BG5. Therefore, we performed a specific fingerprinting study of hydrocarbon evolution for the initial oil, BG0 (control), and BG5 microcosms. Prior to the GC-MS analyses, the separation of fractions on the basis of polarity by SARA was helpful when examining the fate of heavy fractions of the oil (Table 2).

The results were initially consistent with those observed in similar cases (Gallego et al., 2007b; Esquinas et al., 2017), in that the saturate and aromatic fractions were notably depleted. In contrast, an increase in the relative proportions of polars (resins) and principally asphaltenes was observed. A detailed study of each fraction was then performed.

#### 3.3.1. Saturate fraction

The fingerprint of saturates observed in Fig. 6 is reminiscent of that previously shown in Fig. S3. The SIM (Single Ion Monitoring) trace of the oil's alkanes ($m/z = 57$) displayed the following: prominent normal alkanes from n-$C_{15}$ to n-$C_{37}$, with their peaks crowning in n-$C_{21}$; isoprenoids such as phytane and pristane; and $C_{29}$-$C_{35}$ hopanes (Fig. 6a), with a predominance of the C29 17α(H),21β(H)− 30-norhopane over the C30 17α(H),21β(H)-hopane (peaks H29 and H30 respectively in Fig. 6). Indeed, notable degradation of linear alkanes and other saturated compounds (including isoprenoids, see Gallego et al. (2010)) was evident in microcosm BG5 after 90 days, whereas the process was smoother in the control (BG0). At the same time, the hopane family ($m/z = 191$) did not show any evolution in any case, thereby emerging

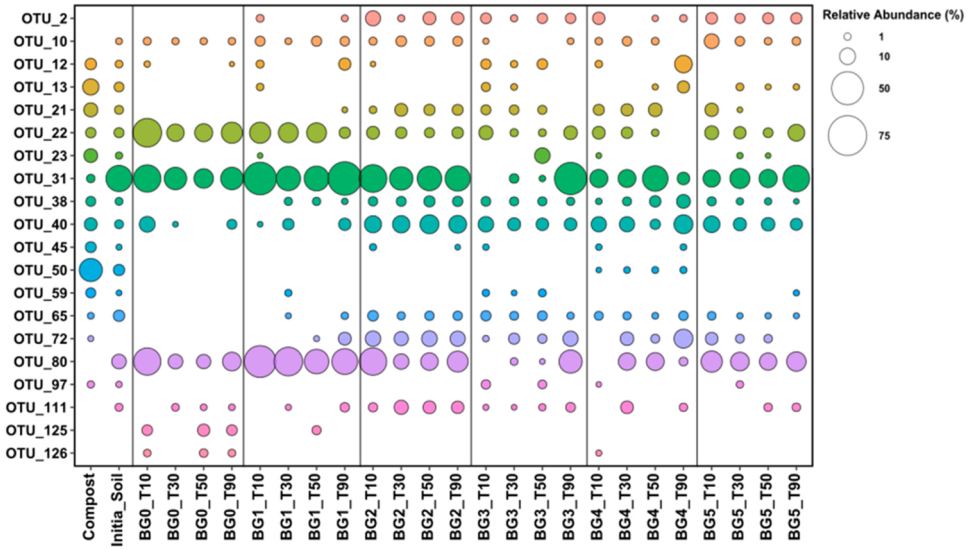


**Fig. 4.** Relative abundance of OTUs with a higher percentage of contribution to variance in the first two principal components (see Table 1 for experimental setup).

as the most abundant and recalcitrant part, as commonly described (Stout and Wang, 2007; Kruge et al., 2018).

For the alkanes, several ratios were also calculated to assess both the extent of biodegradation and volatilization processes. Table 3 shows how the C18/phytane ratio (Peters et al., 2005) decreased markedly in the case of BG5 (to a lesser extent also in BG0), thereby revealing the usual differential degradation of linear alkanes vs. isoprenoids; this also applies to high molecular weight linear alkanes (indices with C23 and C29 shown in Table 3). However, particularly in the case of the latter (C29), the results were very similar for BG0 and BG5, which may point to a reduced bioavailability of these heavy linear compounds, as previously described (Gallego et al., 2006; Kruge et al., 2018). The other index calculated between n-Pr (nor-pristane) and Ph (phytane) also showed an ostensible decrease in the case of BG5. This reduction thus points to the (bio)degradation of branched alkanes (isoprenoids). Moreover, given the moderate differential volatility between n-Pr and Ph (Esquinas et al., 2017), it might also suggest a certain role of volatilization. Nonetheless, it must also be considered that a substantial part of lighter hydrocarbon fractions evaporated or were partially recovered in liquid form in the first weeks after the spills in Kuwait (Ud Din et al., 2008). In this regard, another indicator of evaporation is the GC fingerprint itself, as the GC retention time is a consistent indicator of the liquid-vapor pressure of compounds. In our case, these evaporation trends were not evident since the lightest compounds found were in the range of 13–14 C atoms (Fig. 6). At any case, note that the low sample mass compared to overall surface area of the test vessels used (i.e., a high surface area: volume ratio) might have favored greater volatilization in this study than in large-scale field studies.

### 3.3.2. *Aromatic fraction*

The aromatic fraction of the initial crude oil (Fig. 7a) presented a large variety of compounds with a wide range of molecular weights. In terms of biodegradability and molecular weight, these compounds can initially be divided into the following two main groups (Peters et al., 2005; Kruge et al., 2018): the first includes series of alkyl-benzenes, alkyl-PAHs, and heterocyclic compounds (mainly alkyl-dibenzothiophenes); and the second group, which can be labeled "aromatic biomarkers", includes triaromatic steroids, secohopanoids, and benzohopanes. This pattern was modified after 90 days in the BG0 experiment, in which compounds of the first group appeared in lower proportions, an aspect that was fostered in the BG5 profile. In the latter, most of the compounds were highly degraded and even the UCM (Unresolved Complex Mixture or "hump") was reduced in complexity. This behavior was also supported by the fingerprint of one of the major compound families (alkylbenzenes) (Fig. 7b) (left), as well as by the evolution observed in other families of interest (Fig. S6), namely alkyl-phenanthrenes and alkyl-dibenzothiophenes. This last figure reveals not only that the families of compounds vary from one to the other but also that some isomers are degraded more rapidly than others within each family (Esquinas et al., 2017). On the other hand, recalcitrant biomarkers remained stable irrespective of the overall degree of biodegradation achieved (Fig. S7), except triaromatic steroids (Aeppli et al., 2014), which showed slight but appreciable differential modifications when biodegradation advanced significantly (sample BG5).

### 3.3.3. *Polar fraction*

The non-derivatized samples revealed little beyond phthalates (which were possibly introduced during sample handling) and a Unresolved Complex Mixture, together with some traces of alkanones and oxy-PAHs (Figs. 8a and 8b). Therefore, this fraction did not appear to be relevant in terms of the variety of compounds present. However, the BG5 sample showed several predominant peaks that were coincident with some of those most marked when the compost was analyzed (see Figs. 8c and 8d). This observation indicates that most of the constituents of the compost remained after 90 days of notable biodegradation (BG5) and thus emerge as potential markers to monitor the evolution of this amendment. Recalcitrant compounds, such as markers of human physiology (cholesterol, coprostanol, and squalene), indicate the use of sewage sludge during compost production, whereas other plant markers (stigmastanol, β-sitosterol) are associated with the use of woody material. Finally, other readily biodegradable compounds found in the original compost (Fig. 8d, e.g., palmitic and stearic acids) were not detected in the amended soil. On the whole, these findings strengthen the notion that the organic matter included in the compost is a major factor favoring biodegradation.

### 3.3.4. *Asphaltene fraction*

As previously explained, the asphaltene fraction was studied by sequential pyrolysis. The thermodesorption step (Figs. 9a, 9b and 9c) revealed the presence of alkanes and other compounds occluded within the asphaltene structure (Snowdon et al., 2016). These compounds showed different degrees of biodegradation with a strong predominance of linear alkanes (up to more than 42 C atoms) and, secondarily, hopanes. The fingerprint of BG0 showed few variations when compared with the original product. In contrast, in BG5 (Fig. 9c), the linear compounds were fully degraded. These findings indicate that the asphaltene

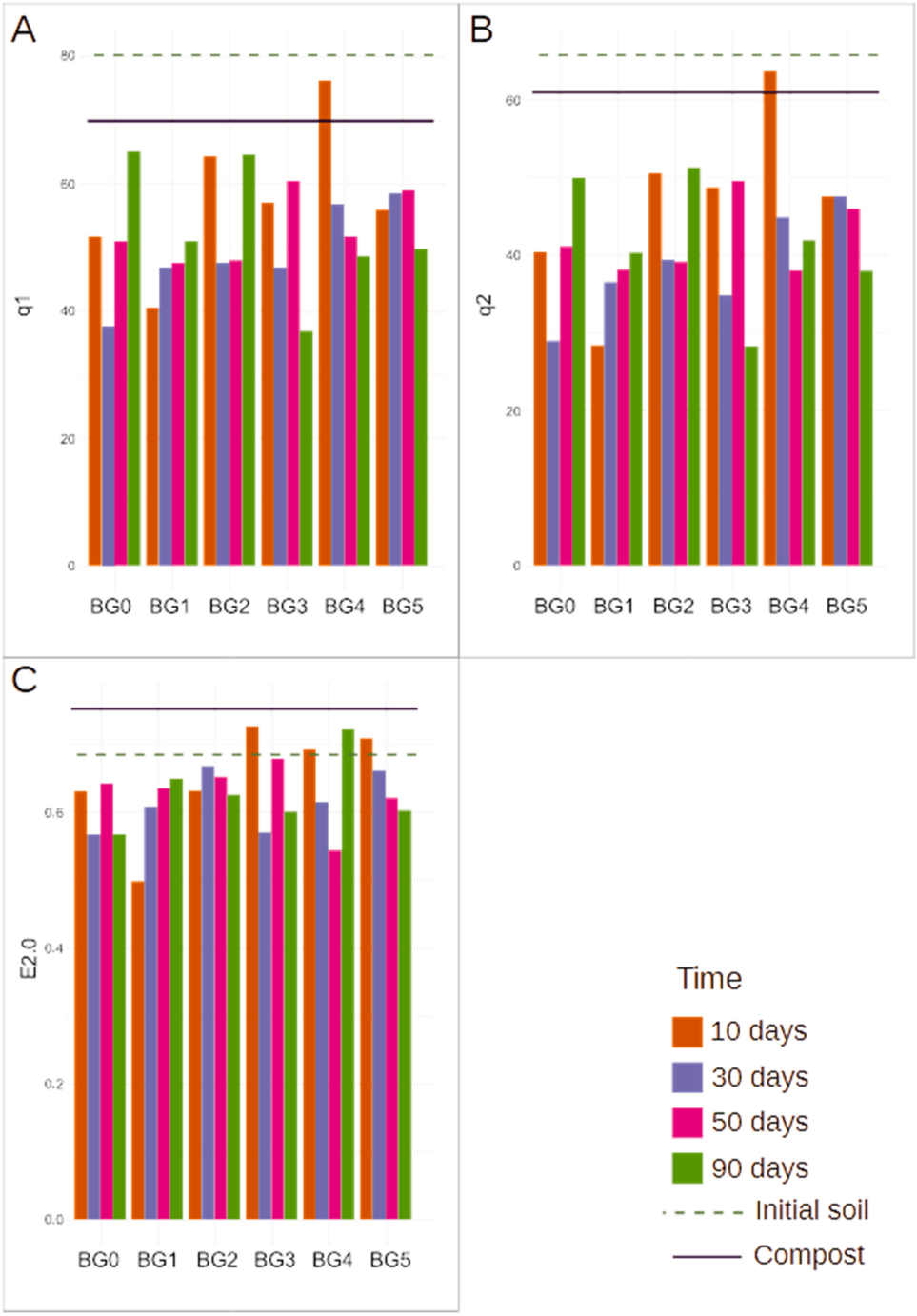


**Fig. 5.** Alpha-diversity indices (Hill numbers) for microbial communities of the initial soil, compost, and microcosms on the basis of the ARISA profiles (see Table 1 for experimental setup). A: Hill number of the order q= 1, equivalent to an exponent of Shannon index; B: Hill number q= 2, equivalent to multiplicative inverse of the Simpson index; and C: E2.0 community evenness measure, calculated as a ratio of q= 2 to community richness.

**Table 2**
Percentage composition of the four fractions studied by SARA. Errors within 5%.

| Sample | SARA fractions | | | |
|---|---|---|---|---|
| | SAT | ARO | POL | ASP |
| *Initial* | 22.5 | 17.7 | 20.1 | 39.7 |
| *BG0* | 16.0 | 15.5 | 23.9 | 44.6 |
| *BG5* | 6.9 | 12.2 | 24.9 | 55.9 |

fraction was also modified in the biodegradation process.

As regards pyrolysis products, upon first examination, the pyrolyzates of the extract's asphaltene fraction (Figs. 9d, 9e and 9f) were very similar in all cases. They showed prominent n-alk-1-ene/n-alkane pairs, caused by the "cracking" of aliphatic long-chain compounds, and also some monoaromatic compounds derived from the pyrolysis of aromatic components of the complex asphaltene structures. These observations point to recalcitrance, a notion supported by SARA fractioning data (relative decrease in saturates and aromatics and a significant increase in resins and asphaltenes as biodegradation progresses, see Table 2). Nevertheless, some qualitative differences were noticeable in sample

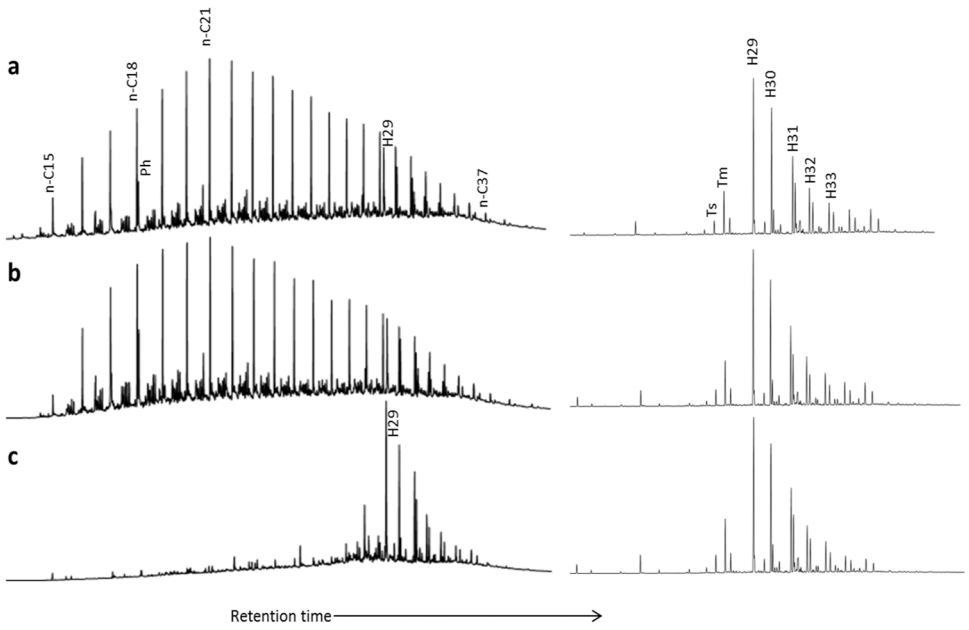


**Fig. 6.** Representative SIM chromatograms of different groups of compounds; alkanes (left, $m/z = 57$) and hopanes (right, $m/z = 191$). (a) Initial soil, (b) BG0 microcosm after 90 days, and (c) BG5 microcosm after 90 days. (n-$C_n$: Linear alkanes; Ph: Phytane; H29: C29 17α(H),21β(H)− 30-norhopane; H30: C30 17α(H),21β(H)-hopane; H31, H32, H33: Homohopane series, Ts: (18α(H),21β(H)− 22,29,30 Trisnorneohopane, Tm: (17α(H),21β(H)− 22,29,30 Trisnorneohopane).

**Table 3**
Evolution of diagnostic ratios selected for estimating volatilization and biodegradation effects within the saturate fraction (calculated in SIM mode, $m/z = 57$).

| Samples | n-$C_{18}$/Ph | | n-$C_{23}$/Ph | | n-$C_{29}$/Ph | | n-Pr/Ph | |
|---|---|---|---|---|---|---|---|---|
| | Ratio | % Change | Ratio | % Change | Ratio | % Change | Ratio | % Change |
| Initial | 1.92 | – | 2.03 | – | 1.01 | – | 0.79 | – |
| BG0 90 days | 1.41 | 26.6 | 1.06 | 47.8 | 0.64 | 36.6 | 0.70 | 11.4 |
| BG5 90 days | 0.27 | 85.9 | 0.77 | 62.1 | 0.61 | 39.6 | 0.19 | 75.9 |

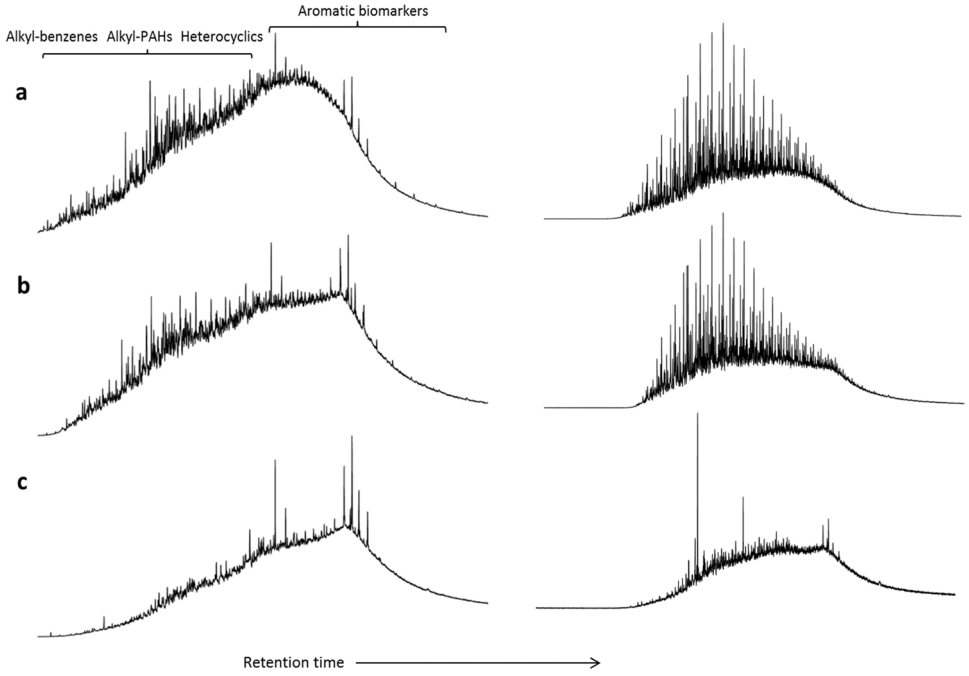


**Fig. 7.** Representative TIC (aromatics, left) and merged SIM (alkylbenzenes, $m/z = 105 + 119$) chromatograms of the aromatic fraction: (a) Initial soil, (b) BG0 microcosm after 90 days, and (c) BG5 microcosm after 90 days.

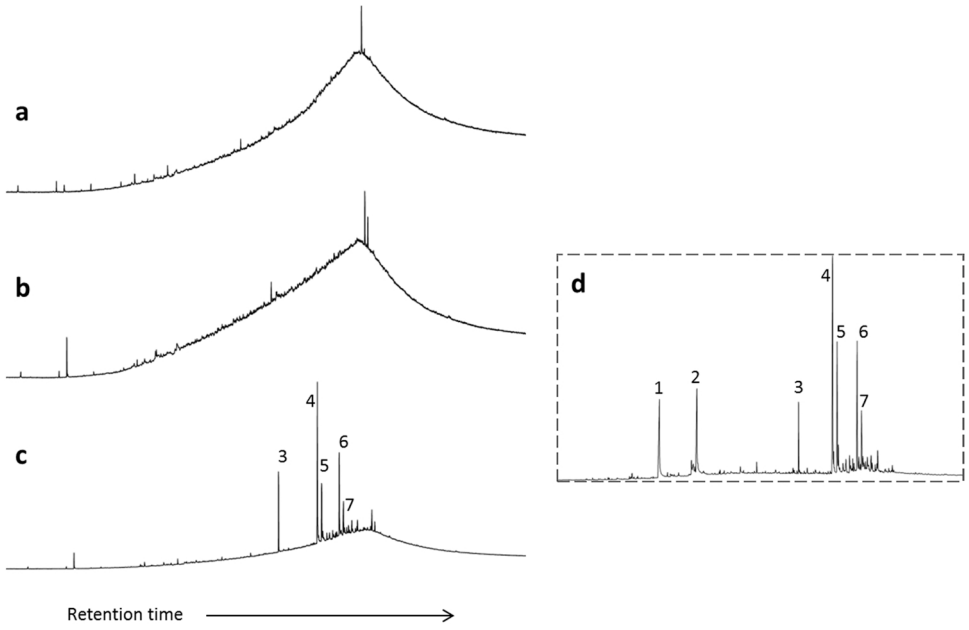


**Fig. 8.** Total ion current traces of the polar fractions: (a) Initial soil, (b) BG0 microcosm after 90 days, and (c) BG5 microcosm after 90 days. On the left, (d) the compost TIC is shown and the main peaks are labeled (1: Palmitic acid; 2: Stearic acid; 3: Squalene; 4: Coprostanol; 5: Cholesterol; 6: Stigmastanol; 7: β-Sitosterol).

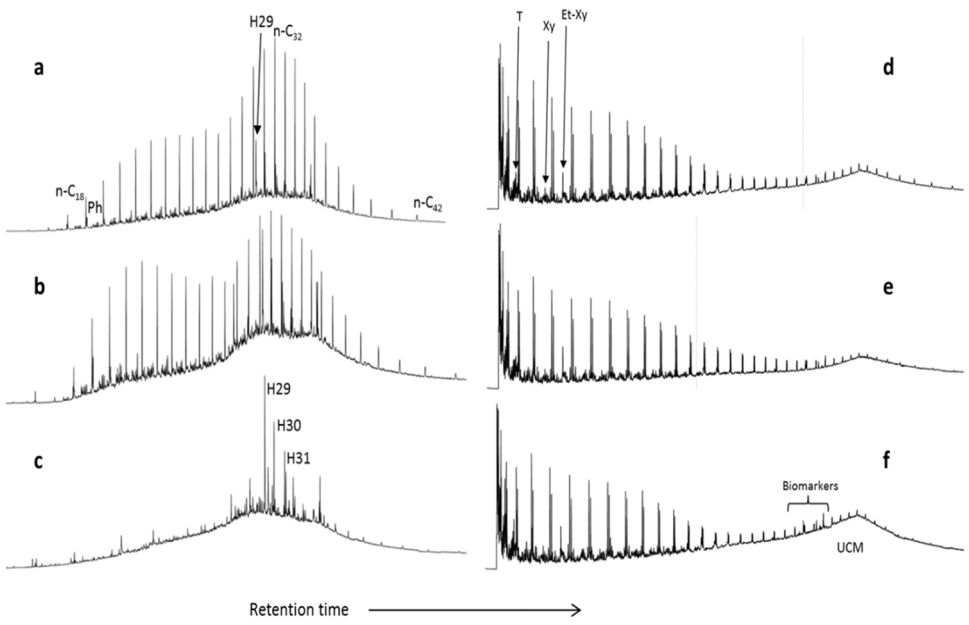


**Fig. 9.** Total ion current traces of the asphaltene thermodesorption of initial soil, BG0, and BG5 after 90 days (a, b, c), and pyrochromatograms (e, f, g) of the thermodesorption residues of the same samples. (n-$C_n$: Linear alkanes; Ph: Phytane; H29: C29 17α(H),21β(H)− 30-norhopane; H30: C30 17α(H),21β(H)-hopane; H31: Homohopane series, T: toluene; Xy: Xylene; Et-Xy: Ethyl-xylene; UCM: Unresolved Complex Mixture).

BG5 (Fig. 9f), as an emerging UCM was detected and a relative increase in the amount of some biomarkers (mainly hopanes) was also evident. This finding again points to the abovementioned evidence of incipient asphaltene degradation, which could be linked to biodegradation processes but also to photooxidation and other singularities (Gallego et al., 2006; Esquinas et al., 2017; White et al., 2016).

## 4. Conclusions

Here we demonstrated that fast yields of hydrocarbon degradation (more than 80% of TPH reduction in 90 days) can be achieved in soil bioremediation using compost as the sole amendment. This strategy could be a high-efficiency and low-cost nature-based solution for large areas of contaminated soil, such as those of the Burgan oil field in Kuwait. Compost is a source of nutrients and microorganisms, and especially of organic matter, which is very useful for bioremediation. The composting process, from sludge of wastewater plants of local refineries or other oil installations, could support the cost-effective and sustainable remediation of large spills in soils, and, in turn, contribute to the circular economy.

An innovative combination of microbial and chemical fingerprint screening methodologies was used to analyze the bioremediation processes from different but complementary perspectives. In this regard, the PCA of bacterial community DNA fingerprints (ARISA) showed that the

bacterial community in the compost differed greatly from that of the initial soil. However, after compost amendment and several weeks of treatment, no great differences were found. This observation is attributed to the following: i) allochthonous bacteria in the compost were not able to compete with indigenous soil bacteria; and ii) changes in bacterial community structures were induced mainly by the input of organic matter supplied by the compost. Consequently, we can consider soil amendment with compost to be a biostimulation rather than a bioaugmentation approach. Regarding chemical monitoring, GC-MS & Py-GC-MS fingerprinting methods identified biodegraded compounds (alkanes, alkyl-aromatics, etc.) and others that were much more recalcitrant (hopanes, benzohopanes, etc.). Notably, evidence of incipient degradation of the heaviest oil fractions was found using double-shot thermodesorption and pyrolysis of the asphaltene fraction. At the same time, the fingerprinting study of the treated soil revealed traces of the most abundant compounds in the compost. On the one hand, this observation supports the hypothesis that the organic matter in the compost fosters oil biodegradation and, on the other hand, it validates the use of these analytics to adjust and monitor compost performance and dosages in real-scale treatments.

## CRediT authorship contribution statement

**José Luis R. Gallego:** Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition. **Verónica Peña-Álvarez:** Investigation, Writing – original draft. **Luis M. Lara:** Investigation, Visualization. **Diego Baragaño**: Investigation, Writing – review & editing. **Rubén Forján**: Investigation, Writing – review & editing. **Arturo Colina:** Writing – review & editing, Visualization. **Alexander Prosenkov**: Investigation, Writing – review & editing. **Ana Isabel Peláez**: Investigation, Methodology, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114267.

## References

Abis, L., Sadet-Bourgeteau, S., Lebrun, B., Ciuraru, R., Lafouge, F., Nowak, V., Loubet, B., 2021. Short-term effect of green waste and sludge amendment on soil microbial diversity and volatile organic compound emissions. Appl. Microbiol. 1, 123–141. https://doi.org/10.3390/applmicrobiol1010010.

Aeppli, C., Nelson, R.K., Radović, J.R., Carmichael, C.A., Valentine, D.L., Reddy, C.M., 2014. Recalcitrance and degradation of petroleum biomarkers upon abiotic and biotic natural weathering of deepwater horizon oil. Environ. Sci. Technol. 48, 6726–6734. https://doi.org/10.1021/es500825q.

Aguelmous, A., El Fels, L., Souabi, S., Zamama, M., Hafidi, M., 2019. The fate of total petroleum hydrocarbons during oily sludge composting: a critical review. Rev. Environ. Sci. Biotechnol. 18, 473–493. https://doi.org/10.1007/s11157-019-09509-w.

Al-Awadhi, N., Al-Daher, R., ElNawawy, A., Salba, M.T., 1996. Bioremediation of oil-contaminated soil in Kuwait. I. landfarming to remediate oil-contaminated soil. Soil Sediment Contam. 5, 243–260.

Al-Dahanii, H., Watson, P., Giles, D., 2015. A geotechnical and geochemical characterisation of oil fire contaminated soils in Kuwait. In: Lollino, G., Giordan, D., Thuro, K., Carranza-Torres, C., Wu, F., Marinos, P., Delgado, C. (Eds.), , Engineering Geology for Society and Territory - Volume 6: Applied Geology for Major Engineering Projects. Springer, Cham, pp. 249–253. https://doi.org/10.1007/978-3-319-09060-3_40.

Al-Daher, R., Al-Awadhi, N., 1998. Bioremediation of damaged desert environment using the windrow soil pile system in Kuwait. Environ. Int. 24, 175–180.

Al-Daher, R., Al-Awadhi, N., Yateem, A., Balba, M.T., ElNawawy, A., 2001. Compost Soil Piles for Treatment of Oil-Contaminated Soil. Soil Sediment Contam.: Int. J. 10, 197–209.

Al-Hashem, M.A., Brain, P.F., Omar, S.A., 2007. Effects of oil pollution at Kuwait's greater Al-Burgan oil field on polycyclic aromatic hydrocarbon concentrations in the tissues of the desert lizard *Acanthodactylus scutellatus* and their ant prey. Ecotoxicology 16, 551–555. https://doi.org/10.1007/s10646-007-0161-1.

Asquith, E.A., Geary, P.M., Nolan, A.L., Evans, C.A., 2012. Comparative bioremediation of petroleum hydrocarbon-contaminated soil by biostimulation, bioaugmentation and surfactant addition. J. Environ. Sci. Eng. 1, 637–650. https://doi.org/10.1007/s10646-007-0161-1.

Balba, M.T., Al-Daher, R., Al-Awadhi, N., 1998. Bioremediation of oil-contaminated desert soil: the Kuwaiti experience. Environ. Int. 24, 163–173. https://doi.org/10.1016/S0160-4120(97)00132-3.

Bell, T.H., Yergeau, E., Maynard, C., Juck, D., Whyte, L.G., Greer, C.W., 2013. Predictable bacterial composition and hydrocarbon degradation in Arctic soils following diesel and nutrient disturbance. ISME J. 7, 1200–1210. https://doi.org/10.1038/ismej.2013.1.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049–1055. https://doi.org/10.1016/J.BIORTECH.2004.09.008.

Brown, D.M., Okoro, S., van Gils, J., van Spanning, R., Bonte, M., Hutchings, T., Linden, O., Egbuche, U., Bruun, K.B., Smith, J.W.N., 2017. Comparison of landfarming amendments to improve bioremediation of petroleum hydrocarbons in Niger Delta soils. Sci. Total Environ. 596–597, 284–292. https://doi.org/10.1016/j.scitotenv.2017.04.072.

Calvo, C., Rodríguez-Calvo, A., Robledo-Mahón, T., Manzanera, M., González-López, J., Aranda, E., Silva-Castro, G.A., 2019. Biostimulation of crude oil-polluted soils: influence of initial physicochemical and biological characteristics of soil. Int. J. Environ. Sci. Technol. 16, 4925–4934. https://doi.org/10.1007/s13762-019-02269-8.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C., Daffonchio, D., 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Environ. Microbiol. 70, 6147–6156. https://doi.org/10.1128/AEM.70.10.6147-6156.2004.

Castaño, A., Prosenkov, A., Baragaño, D., Otaegui, N., Sastre, H., Rodríguez-Valdés, E., Gallego, J.L.R., Peláez, A.I., 2021. Effects of in situ remediation with nanoscale zero valence iron on the physicochemical conditions and bacterial communities of groundwater contaminated with arsenic. Front. Microbiol. 12, 580. https://doi.org/10.3389/fmicb.2021.643589.

Castillo, J.M., Beguet, J., Martin-Laurent, F., Romero, E., 2016. Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes. J. Hazard. Mater. 304, 379–387. https://doi.org/10.1016/j.jhazmat.2015.10.056.

Cerqueira, V.S., Peralba, M., do, C.R., Camargo, F.A.O., Bento, F.M., 2014. Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge. Int. Biodeterior. Biodegrad. 95, 338–345. https://doi.org/10.1016/j.ibiod.2014.08.015.

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: applications, microbes and future research needs. Biotechnol. Adv. 33, 745–755. https://doi.org/10.1016/j.biotechadv.2015.05.003.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x.

Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A.H.A., Tamayo-Ramos, J.A., Rad, C., Gallego, J.R., Barros, R., 2022. Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil (https://doi:). Chemosphere 307, 135638. https://doi.org/10.1016/j.chemosphere.2022.135638.

Dados, A., Omirou, M., Demetriou, K., Papastephanou, C.H., Ioannides, I.M., 2015. Rapid remediation of soil heavily contaminated with hydrocarbons: a comparison of different approaches. Ann. Microbiol 65, 241–251. https://doi.org/10.1007/s13213-014-0856-5.

Dubey, R.K., Tripathi, V., Prabha, R., Chaurasia, R., Singh, D.P., Rao, C., El-Keblawy, A., Abhilash, P.C., 2020. Methods for exploring soil microbial diversity. Unravelling the soil microbiome. SpringerBriefs in Environmental Science. Springer, Cham, pp. 23–32. https://doi.org/10.1007/978-3-030-15516-2_3.

Esquinas, N., Rodríguez-Valdés, E., Márquez, G., Gallego, J.R., 2017. Diagnostic ratios for the rapid evaluation of natural attenuation of heavy fuel oil pollution along shores. Chemosphere 184, 1089–1098. https://doi.org/10.1016/j.chemosphere.2017.06.087.

Fisher, M.M., Triplett, E.W., 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. https://doi.org/4630-4636. Appl. Environ. Microbiol. 65 https://doi.org/10.1128/aem.65.10.4630-4636.1999.

Fodelianakis, S., Antoniou, E., Mapelli, F., Magagnini, M., Nikolopoulou, M., Marasco, R., Barbato, M., Tsiola, A., Tsikopoulou, I., Giaccaglia, L., Mahjoubi, M., Jaouani, A., Amer, R., Hussein, E., Al-Horani, F.A., Benzha, F., Blaghen, M., Malkawi, H.I., Abdel-Fattah, Y., Cherif, A., Daffonchio, D., Kalogerakis, N., 2015. Allochthonous bioaugmentation in ex situ treatment of crude oil-polluted sediments in the presence of an effective degrading indigenous microbiome. J. Hazard. Mater. 287, 78–86. https://doi.org/10.1016/j.jhazmat.2015.01.038.

Forján, R., Lores, I., Sierra, C., Baragaño, D., Gallego, J.L.R., Peláez, A.I., 2020. Bioaugmentation Treatment of a PAH-Polluted Soil in a Slurry Bioreactor. Appl. Sci. 10, 2837. https://doi.org/10.3390/app10082837.

Fountoulakis, M.S., Terzakis, S., Georgaki, E., Drakopoulou, S., Sabathianakis, I., Kouzoulakis, M., Manios, T., 2009. Oil refinery sludge and green waste simulated windrow composting. Biodegradation 20, 177–189. https://doi.org/10.1007/s10532-008-9211-5.

Gallego, J.R., González-Rojas, E., Peláez, A.I., Sánchez, J., García-Martínez, M.J., Ortiz, J.E., Torres, J.F., Llamas, J.F., 2006. Natural attenuation and bioremediation of prestige fuel oil along the Atlantic coast of Galicia (Spain). Org. Geochem. 37, 1869–1884. https://doi.org/10.1016/j.orggeochem.2006.07.022.

Gallego, J.R., García-Martínez, M.J., Llamas, J.F., Belloch, C., Peláez, A.I., Sánchez, J., 2007a. Biodegradation of oil tank bottom sludge using microbial consortia. Biodegradation 18, 269–281. https://doi.org/10.1007/s10532-006-9061-y.

Gallego, J.R., Fernández, J.R., Díez-Sanz, F., Ordoñez, S., Sastre, H., González-Rojas, E., Peláez, A.I., Sánchez, J., 2007b. Bioremediation for shoreline cleanup: In situ vs. on-site treatments. Environ. Eng. Sci. 24, 493–504. https://doi.org/10.1089/ees.2006.0091.

Gallego, J.R., Sierra, C., Villa, R., Peláez, A.I., Sánchez, J., 2010. Weathering processes only partially limit the potential for bioremediation of hydrocarbon-contaminated soils. Org. Geochem. 41, 896–900. https://doi.org/10.1016/j.orggeochem.2010.04.021.

Gallego, J.R., Sierra, C., Permanyer, A., Peláez, A.I., Menéndez-Vega, D., Sánchez, J., 2011. Full-scale remediation of a jet fuel-contaminated soil: Assessment of biodegradation, volatilization, and bioavailability. Water, Air, Soil Pollut. 217, 197–211. https://doi.org/10.1007/s11270-010-0579-6.

Garbisu, C., Garaiyurrebaso, O., Epelde, L., Grohmann, E., Alkorta, I., 2017. Plasmid-mediated bioaugmentation for the bioremediation of contaminated soils. Front. Microbiol. 8, 1966. https://doi.org/10.3389/fmicb.2017.01966.

Guarino, C., Spada, V., Sciarrillo, R., 2017. Assessment of three approaches of bioremediation (Natural Attenuation, Landfarming and Bioagumentation–Assistited Landfarming) for a petroleum hydrocarbons contaminated soil. Chemosphere 170, 10–16. https://doi.org/10.1016/j.chemosphere.2016.11.165.

Haghollahi, A., Fazaelipoor, M.H., Schaffie, M., 2016. The effect of soil type on the bioremediation of petroleum contaminated soils. J. Environ. Manag. 180, 197–201. https://doi.org/10.1016/j.jenvman.2016.05.038.

Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54 (2), 427–432.

Jami, E., Shterzer, N., Mizrahi, I., 2014. Evaluation of automated ribosomal intergenic spacer analysis for bacterial fingerprinting of rumen microbiome compared to pyrosequencing technology. Pathogens 3, 109–120. https://doi.org/10.3390/pathogens3010109.

Kienhuis, P.A., Hansen, A.B., Fakness, L.G., Stout, S.A., Dahlmann, G., 2016. CEN methodology for oil spill identification. In: Stout, S.A., Wang, Z. (Eds.), Standard Handbook Oil Spill Environmental Forensics. Elsevier, London, U.K., pp. 685–728. https://doi.org/10.1016/B978-0-12-803832-1.00014-3

Kirchmann, H., Ewnetu, W., 1998. Biodegradation of petroleum-based oil wastes through composting. Biodegradation 9, 151–156. https://doi.org/10.1023/A:1008355825404.

Kruge, M.A., 2015. Analytical pyrolysis principles and applications to environmental science. In: Barbooti, M. (Ed.), Environmental Applications of Instrumental Chemical Analysis. Apple Academic Press, Oakville, Canada, pp. 534–569.

Kruge, M.A., Gallego, J.R., Lara-Gonzalo, A., Esquinas, N., 2018. Environmental forensics study of crude oil and petroleum product spills in coastal and oilfield settings: combined insights from conventional GC-MS, thermodesorption-GC-MS, and pyrolysis-GC-MS. Oil spill Environ. Forensics case Stud. 131–155. https://doi.org/10.1016/B978-0-12-804434-6.00007-0.

Kruge, M.A., Lara-Gonzalo, A., Gallego, J.L.R., 2020. Environmental forensics of complexly contaminated sites: a complimentary fingerprinting approach. Environ. Pollut. 263, 114645 https://doi.org/10.1016/j.envpol.2020.114645.

Lara-Gonzalo, A., Kruge, M.A., Lores, I., Gutiérrez, B., Gallego, J.R., 2015. Pyrolysis GC-MS for the rapid environmental forensic screening of contaminated brownfield soil. Org. Geochem. 87, 9–20. https://doi.org/10.1016/j.orggeochem.2015.06.012.

Ławniczak, Ł., Woźniak-Karczewska, M., Loibner, A.P., Heipieper, H.J., Chrzanowski, Ł., 2020. Microbial degradation of hydrocarbons-basic principles for bioremediation: a review. Molecules 25 (4), 856. https://doi.org/10.3390/molecules25040856.

Lê, S., Josse, J., Husson, F., 2008. FactoMineR: an R package for multivariate analysis. J. Stat. Softw. 25, 1–18. https://doi.org/10.18637/jss.v025.i01.

Liao, Y., Geng, A., Huang, H., 2009. The influence of biodegradation on resins and asphaltenes in the Liaohe basin. Org. Geochem. 40, 312–320. https://doi.org/10.1016/j.orggeochem.2008.12.006.

Lima, A.L.C., Farrington, J.W., Reddy, C.M., 2005. Combustion-derived polycyclic aromatic hydrocarbons in the environment—a review. Environ. Forensics 6 (2), 109–131. https://doi.org/10.1080/15275920590952739.

Lukić, B., Panico, A., Huguenot, D., Fabbricino, M., van Hullebusch, E.D., Esposito, G., 2017. A review on the efficiency of landfarming integrated with composting as a soil remediation treatment. Environ. Technol. Rev. 6 (1), 94–116. https://doi.org/10.1080/21622515.2017.1310310.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.

Miciç, V., Kruge, M.A., Koster, J., Hofmann, T., 2011. Natural, anthropogenic and fossil organic matter in river sediments and suspended particulate matter: a multi-molecular marker approach. Sci. Total Environ. 409, 905–919. https://doi.org/10.1016/j.scitotenv.2010.11.009.

Miri, S., Naghdi, M., Rouissi, T., Brar, S.K., Martel, R., 2019. Recent biotechnological advances in petroleum hydrocarbons degradation under cold climate conditions: a review. Crit. Rev. Environ. Sci. Technol. 49, 553–586. https://doi.org/10.1080/10643389.2018.1552070.

Ortega, M.F., Guerrero, D.E., García-Martínez, M.J., Bolonio, D., Llamas, J.F., Canoira, L., Gallego, J.L.R., 2018. Optimization of landfarming amendments based on soil texture and crude oil concentration. Water, Air, Soil Pollut. 229, 234. https://doi.org/10.1007/s11270-018-3891-1.

Patel, A.B., Manvar, T., Jain, K.R., Desai, C., Madamwar, D., 2021. Metagenomic insights into bacterial communities' structures in polycyclic aromatic hydrocarbons degrading consortia. J. Environ. Chem. Eng. 9, 106578 https://doi.org/10.1016/j.jece.2021.106578.

Peláez, A.I., Lores, I., Sotres, A., Méndez-García, C., Fernández-Velarde, C., Santos, J.A., Gallego, J.R., Sánchez, J., 2013. Design and field-scale implementation of an "on site" bioremediation treatment in PAH-polluted soil. Environ. Pollut. 18, 190–199. https://doi.org/10.1016/j.envpol.2013.06.004.

Peters, K.E., Walters, C.C., Moldowan, J.M., 2005. The Biomarker Guide. Vol. 2. Biomarkers and Isotopes. In: Peters, K.E., Walters, C.C., Moldowan, J.M. (Eds.), Petroleum Exploration and Earth History. Cambridge University Press, Cambridge.

Radwan, S.S., Al-Mailem, D.M., Kansour, M.K., 2019. Bioaugmentation failed to enhance oil bioremediation in three soil samples from three different continents. Sci. Rep. 9, 1–11. https://doi.org/10.1038/s41598-019-56099-2.

Ramette, A., 2009. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. Appl. Environ. Microbiol. 75, 2495–2505. https://doi.org/10.1128/AEM.02409-08.

Romantschuk, M., Sarand, I., Petänen, T., Peltola, R., Jonsson-Vihanne, M., Koivula, T., Yrjälä, K., Haahtela, K., 2000. Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. Environ. Pollut. 107, 179–185. https://doi.org/10.1016/S0269-7491(99)00136-0.

Saeed, M., Ilyas, N., Arshad, M., Sheeraz, M., Ahmed, I., Bhattacharya, A., 2021. Development of a plant microbiome bioremediation system for crude oil contamination. J. Environ. Chem. Eng. 9, 105401 https://doi.org/10.1016/j.jece.2021.105401.

Saison, C., Degrange, V., Oliver, R., Millard, P., Commeaux, C., Montange, D., Le Roux, X., 2006. Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. Environ. Microbiol. 8, 247–257. https://doi.org/10.1111/j.1462-2920.2005.00892.x.

Sayara, T., Sarrà, M., Sánchez, A., 2010. Optimization and enhancement of soil bioremediation by composting using the experimental design technique. Biodegradation 21, 345–356. https://doi.org/10.1007/s10532-009-9305-8.

Snowdon, L.R., Volkman, J.K., Guoliang, Z.Z., Peng, L., Guoliang, T., 2016. The organic geochemistry of asphaltenes and occluded biomarkers. Org. Geochem. 91, 3–15. https://doi.org/10.1016/j.orggeochem.2015.11.005.

Steen, A.D., Crits-Christoph, A., Carini, P., DeAngelis, K.M., Fierer, N., Lloyd, K.G., Cameron Thrash, J., 2019. High proportions of bacteria and archaea across most biomes remain uncultured. ISME J. 13, 3126–3130. https://doi.org/10.1038/s41396-019-0484-y.

Stout, S.A., 2016. Oil spill fingerprinting method for oily matrices used in the Deepwater Horizon NRDA. Environ. Forensics 17, 218–243. https://doi.org/10.1080/15275922.2016.1177759.

Stout, S.A., Wang, Z., 2007. Chemical fingerprinting of spilled or discharged petroleum-methods and factors affecting petroleum fingerprints in the environment. In: Wang, Z., Stout, S.A. (Eds.), Oil Spill Environmental Forensics. Academic Press, Burlington (MA), pp. 1–53. https://doi.org/10.1016/B978-012369523-9.50005-7.

Taccaria, M., Milanovic, V., Comitini, F., Casucci, C., Ciani, M., 2012. Effects of biostimulation and bioaugmentation on diesel removal and bacterial community. Int. Biodeterior. Biodegrad. 66, 39–46. https://doi.org/10.1016/j.ibiod.2011.09.012.

Ud Din, S., Al Dousari, A., Literathy, P., 2008. Evidence of hydrocarbon contamination from the Burgan oil field, Kuwait-Interpretations from thermal remote sensing data. J. Environ. Manag. 86, 605–615. https://doi.org/10.1016/j.jenvman.2006.12.028.

White, H.K., Wang, C.H., Williams, P.L., Findley, D.M., Thurston, A.M., Simister, R.L., Aeppli, C., Nelson, R.K., Reddy, C.M., 2016. Long-term weathering and continued oxidation of oil residues from the Deepwater Horizon spill. Mar. Poll. Bull. 113, 380–386. https://doi.org/10.1016/j.marpolbul.2016.10.029.

Wu, M., Guo, X., Wu, J., Chen, K., 2020. Effect of compost amendment and bioaugmentation on PAH degradation and microbial community shifting in

petroleum-contaminated soil. Chemosphere 256, 126998. https://doi.org/10.1016/j.chemosphere.2020.126998.

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., Gao, X., Li, F., Li, H., Yu, H., 2018. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. Front. Microbiol. 9, 2885. https://doi.org/10.3389/fmicb.2018.02885.

Yong, R.N., Mulligan, C.N., 2019. Natural and Enhanced Attenuation of Contaminants in Soils, 2nd ed. CRC Press, Boca Raton, FL. https://doi.org/10.1201/9781315159195.

Yunker, M.B., Macdonald, R.W., Vingarzan, R., Mitchell, R.H., Goyette, D., Sylvestre, S., 2002. PAHs in the fraser river basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515. https://doi.org/10.1016/S0146-6380(02)00002-5.