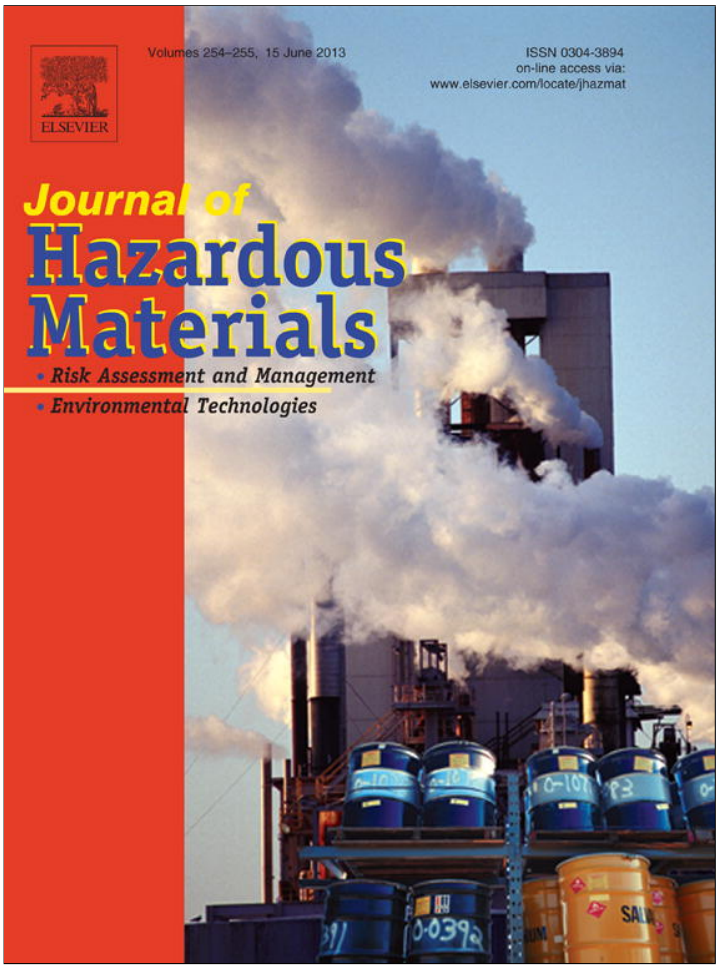

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Chemometric assessment of enhanced bioremediation of oil contaminated soils

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HIGHLIGHTS

- The CHEMSIC method is an accurate tool for assessing the bioremediation efficiency.
- Bacterial enrichment and addition of nutrients enhance removal of TPHs in soil.
- Bacterial enrichment increases the degradation of *n*-alkanes and some PACs.
- Chemometrics is a comprehensive approach for monitoring of the degradation of petroleum hydrocarbons.

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ABSTRACT

Bioremediation is a promising technique for reclamation of oil polluted soils. In this study, six methods for enhancing bioremediation were tested on oil contaminated soils from three refinery areas in Iran (Isfahan, Arak, and Tehran). The methods included bacterial enrichment, planting, and addition of nitrogen and phosphorous, molasses, hydrogen peroxide, and a surfactant (Tween 80). Total petroleum hydrocarbon (TPH) concentrations and CHEMometric analysis of Selected Ion Chromatograms (SIC) termed CHEMSIC method of petroleum biomarkers including terpanes, regular, diaromatic and triaromatic steranes were used for determining the level and type of hydrocarbon contamination. The same methods were used to study oil weathering of 2 to 6 ring polycyclic aromatic compounds (PACs). Results demonstrated that bacterial enrichment and addition of nutrients were most efficient with 50% to 62% removal of TPH. Furthermore, the CHEMSIC results demonstrated that the bacterial enrichment was more efficient in degradation of n-alkanes and low molecular weight PACs as well as alkylated PACs (e.g. C_3–C_4 naphthalenes, C_2 phenanthrenes and C_2–C_3 dibenzothiophenes), while nutrient addition led to a larger relative removal of isoprenoids (e.g. norpristane, pristane and phytane). It is concluded that the CHEMSIC method is a valuable tool for assessing bioremediation efficiency.

1. Introduction

Oil pollution is a major environmental challenge in oil producing countries. Iran is the world's fourth largest producer of crude oil and oil pollution is therefore widespread in this region during production and transport activities. Biological methods such as enhanced microbial degradation and phytoremediation are promising green and cost effective tools for large scale remediation [1–3]. However, the time span for biological methods is often long and the techniques are less efficient on highly polluted sites and for remediation of heavier oil products [4–6]. Therefore, finding new approaches to enhance efficiency of bioremediation is desired.

Biostimulation (i.e. nutrient-enhanced bioremediation) is a promising approach, which has been shown to increase biodegradation rates of petroleum hydrocarbons among other organic pollutants by providing limiting nutrients (e.g. nitrogen and phosphorous) for activity of indigenous degrading microorganisms in soil [2,7,8]. Introduction of a group of natural microbial strains or a genetically engineered variant to treat contaminated soil or water, which is called bioaugmentation has also been shown to be an effective method for elimination of organic pollutants in contaminated media [2,8]. Zhang et al. [9] among others have demonstrated an

Abbreviations: CHEMSIC, CHEMometric analysis of Selected Ion Chromatograms; PACs, Polycyclic Aromatic Compounds; TPH, Total Petroleum Hydrocarbons; SICs, Selected Ion Chromatograms.

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increase in bioaugmentation efficiency of soils contaminant with oil products by addition of wheat straw. Other studies have demonstrated that the addition of surfactants and hydrogen peroxide as well as organic wastes into the oil-contaminated soils increases bioremediation efficiency [10–12].

While considerable studies have been carried out to show enhancement of bioremediation of oil-contaminated soils, they are mostly based on bulk properties such as total petroleum hydrocarbon (TPH) concentrations and gravimetric analysis. Furthermore, only a few selected aliphatics, and polycyclic aromatic compounds (PACs) are typically used as indicators for oil pollution. Gas chromatography with mass spectrometry detection (GC-MS) combined with multivariate statistics for oil hydrocarbon fingerprinting (chemometrics) can provide a more comprehensive and accurate tool for monitoring the changes in the oil hydrocarbon profiles during bioremediation [13–16]. Two-dimensional gas chromatography has been used as a powerful technique for characterization of biological and physical weathering processes of oil complex mixtures at a molecular level in surface waters and sediments as well as marine and land oil spills [17–19].

A detailed characterization and understanding of oil weathering at the molecular level can be considered as an essential part of tiered approaches for forensic oil spill identification, risk assessment of terrestrial and marine oil spills, and the evaluation of bioremediation efficiencies [20]. Chemometrics is the application of statistical and mathematical methods to chemistry that allows for a more advanced treatment of data derived from complex chemical mixtures [20] and can be used to estimate the importance of the contributing weathering processes including physical, biological and chemical weathering [15].

The aim of this study is to determine the most efficient short-term strategy for bioremediation of heavily oil contaminated soils from three refinery areas in Iran: Tehran, Arak and Isfahan. Six bioremediation strategies (both biostimulation and bioaugmentation) were tested in a two-month laboratory experiment. These included bacterial enrichment; planting; addition of nutrients, hydrogen peroxide, molasses and the surfactant Tween 80. The assessment of bioremediation efficiency was based on TPH concentrations; and more detailed oil hydrocarbon fingerprinting using the CHEMSIC (CHEMometric analysis of Selected Ion Chromatograms) method developed by Christensen et al. [20–22]. The CHEMSIC method consists of principal component analysis (PCA) of pre-processed and combined sections of GC-MS/SIM chromatograms [20]. In this study, we used 4 selected ion chromatograms (SICs) of petroleum biomarkers for source comparison; and 25 SICs of PACs for assessment of oil weathering.

2. Materials and methods

2.1. Soil sampling

Soil samples were collected from three locations: Tehran refinery, Isfahan refinery and Arak refinery located in the central part of Iran. The soils were contaminated with crude oil and heating oil due to transportation accidents, leakage from oil pipes and reservoirs. Tehran and Isfahan are referred to as the oldest refineries operating from 1968 and 1979, respectively), while Arak is one of the newest refineries in Iran beginning operation in 1993. Three composite samples made from five sub samples from each location were collected. Samples were taken down to 20 cm depth, after discarding the upper 3 cm of the soil surface which was heavily weathered due to evaporation and photooxidation processes. Each soil sample was crushed, thoroughly mixed, homogenized and then sieved through a 2 mm pore size sieve to remove large debris. Samples were stored at 4 °C.

Soil characteristics such as nitrogen, phosphors and organic carbon contents, pH, cation exchange capacity (CEC), the amounts of silt, clay and sand were measured [23] at the biotechnology facility at Isfahan Science & Technology Town, Iran.

2.2. Isolation, identification and selection of bacteria for bacterial enrichment

Soil bacteria were isolated according to the method of Saadoun [24]. Briefly, sub samples of 1 g soil were suspended in 100 ml of sterile distilled water, agitated in an incubator-shaker (Innova 4430, GMI, USA) at 100 rpm for 30 min, then serially diluted from 10^{-1} to 10^{-6}. Aliquots of 0.1 ml from each dilution were spread over the surface of nutrient agar plates and incubated in 30 °C for 24 h. Colonies of the bacterial isolates were transferred into 50 ml mineral salts medium, which was a modification of Leadbetter and Foster [25], supplemented with 0.05% (v/v) crude oil sterilized by filtration through 0.45 μm membranes and incubated at 30 °C in an incubator-shaker (Innova 4430, GMI, USA) at 200 rpm for 21 days. Bacterial growth was determined at a 7-day interval by the physical appearance (i.e. turbidity) and by measuring the optical density (OD) at 540 nm using a spectrophotometer (Milton Roy Spectronic 21D, Rochester, USA).

Growth on crude oil was also determined by the 'hole-plate diffusion method' that was reported by Saadoun [24]. The results were recorded daily by the physical appearance of the bacterial growth surrounding the holes during 6 days. Monooxygenase biodegradation pathway was also used to detect the biodegradation of oil by bacteria as described by Saadoun [24].

The morphological characterization including colour, size, and colony form as well as biochemical tests (e.g. Gram stain test, oxidase, catalase, indole formation and glucose fermentation) were used for bacterial identification. Bacterial strains were identified based on Bergey's manual of systematic bacteriology [26,27]. Finally, the bacteria that tolerate, and degrade oil were selected for using in the experiment according to the results of the "hole-plate diffusion method" and monooxygenase biodegradation test.

2.3. Experimental setup

The experimental setup consisted of 63 microcosms including 9 control samples (triplicates of each soil type–Tehran, Arak and Isfahan); and triplicates for each soil type and treatment ($3 \times 3 \times 6 = 54$ samples). Each sample contained 0.5 kg in a plastic pot. The samples were incubated for 2 months under controlled conditions (28 ± 2 °C, 12 h light, 75% water holding capacity ensured by gravimetric method and maintained by adding distilled water). Sub samples were collected after 2 months of incubation. Initial soil samples from Tehran, Arak and Isfahan (day 0) were kept at −20 °C until extraction. Soils in microcosms were homogenized before sub-sampling except for planted soils, which were sub-sampled from the rhizosphere. All types of amendments were added to the soil by spraying them into a thin layer of soil and mixing thoroughly by hand. This ensured proper homogenization of amendments with soil [28].

Treatments were as follows:

(i) Control: No treatment except for homogenization and wetting. The controls included samples from day 0 (CNT_0) and after 2 months (CNT).
(ii) Microbial enrichment (ENT): Nutrient broth (Merck, Germany) was used for preparing the inoculation of oil degrading bacteria. The isolated bacteria were grown in nutrient broth (having 20 μL L^{-1} crude oil as a carbon source, which was sterilized by filtration through 0.45 μm membranes) in an incubator-shaker (Innova 4430, GMI, USA) for

3–5 days (100 rpm, 30 °C). After that the liquid samples were centrifuged at 10,000 rpm for 10 min and clear supernatant were discarded. The inoculants were prepared immediately before experimental setup. Then 5 mL of bacterial inoculants (10^8 bacteria mL^{-1}) were added into 0.5 kg of each soil by spraying on a thin layer of soil and mixing thoroughly.

(iii) Addition of molasses (MS): Four ratios (v/m) including 0, 1, 2 and 5% of molasses were added to 0.5 kg of each soil sample. After 1 week of incubation at 28 °C the microbial numbers were counted using the serial dilution method [24]. The ratio which caused the highest numbering of microbial count in soil (i.e. 5% v/m) was chosen for the microcosms experiments.

(iv) Addition of hydrogen peroxide (H_2O_2): Selection of hydrogen peroxide concentration to use in the microcosms experiment were based on the microbial numbers in a pilot design using four hydrogen peroxide percentages: 0, 0.1, 1, 2 and 5% (v/w) of H_2O_2 (3%). The screening was based on triplicate incubations of one composite and homogenized sample from each sampling site. Five hundred g soil was used for each hydrogen peroxide percentage and soil samples were incubated at 28 °C for 1 week. Microbial numbers were determined before and after the incubation. The setup with the highest microbial count (i.e. 0.1% v/w) was chosen for the microcosms experiments.

(v) Nutrient addition: Nitrogen and phosphorous (NP) were added to the soils to reach the C:N:P= 100:10:1. Ammonium nitrate and ammonium phosphate were used as chemical salts for providing N and P. The required amounts depended on C content of the soils, which measured by method of Walkley and Black [29].

(vi) Addition of surfactant (TWN): Tween 80 was added to the soil in 0.05% (v/w), which was based on pilot experiments (results not published).

(vii) Planting (PLN): Twenty seeds of *Festuca arundinacea* were grown in the pots containing 0.5 kg soil for 2 months. *Festuca arundinacea* has been used by others as a model plant for phytoremediation of oil contaminated soils [30,31] and Soleimani et al. [3] showed that it was effective in enhancing dissipation of petroleum hydrocarbons in contaminated soil from Tehran refinery.

2.4. Extraction and chemical analysis

Integrated extraction and cleanup was performed by pressurized liquid extraction with a Dionex ASE 200 accelerated solvent extractor, and TPH concentrations were measured using an Agilent 6890 GC–FID. The methods were described in detail by Soleimani et al. [3].

The extracts were also analyzed using an Agilent 6890N/5975 GC–MS operating in electron ionization mode. The GC was equipped with a 60 m ZB-5 (0.25 mm I.D., 0.25 μm film thickness) capillary column. Helium was used as carrier gas with a flow rate of 1.1 mL s^{-1}. Aliquots of 1 μL were injected in pulsed splitless mode with injection temperature of 315 °C. The column temperature programme was as follows: Initial temperature 40 °C held for 2 min, 25 °C min^{-1} to 100 °C then followed by an increase of 5 °C min^{-1} to 315 °C (held for 13.4 min). The transfer line, ion source and quadrupole temperatures were 315 °C, 230 °C and 150 °C, respectively. A total of 55 groups of oil compounds (mass-to-charge ratios, *m*/*z*'s) and deuterated standards were analyzed in selected ion monitoring (SIM) mode. The *m*/*z*'s were divided into 12 SIM groups (Table 1). The dwell time for each *m*/*z* was 25 ms with 2.81 scans s^{-1}. The number of monitored ions in each group (13 *m*/*z*'s) was consistent between groups to avoid differences in the scanning frequency.

2.5. Quality control

The extracts were analyzed in 9 batches. In the analytical sequence, dichloromethane, an oil reference sample (1:1 mixture of heavy fuel oil from the Baltic Carrier and North Sea crude oil from the Brent oil field) [32], a mixture sample (1:1:1 mixture of soil extracts from Tehran, Arak and Isfahan) and a test solution containing DFTPP, 4,4′-DDT, pentachlorophenol, and benzidine (50 μg mL^{-1} of each standard) were analyzed between batches. These test solutions were used for quality control by daily monitoring for cross-contamination; changes in peak shapes, chromatographic resolution and sensitivity; and to verify tuning, injection port inertness and GC column performance, respectively. Furthermore, the mixture sample was used as a validation sample during the chemometric data analysis mainly for ensuring a sufficient quality of the data pre-processing [20].

2.6. Data

The data set consisted of retention time windows of 55 SICs per sample (Table 1), including the deuterated standards. 121 samples were analyzed and split into a 'training' set of 86 sample extracts (25 samples from the Arak, 26 samples from the Isfahan and 35 samples from Tehran) and two 'validation sets': one comprised of 13 analytical replicates of the reference oil sample 'Ref' in PCA plots); one consisting of nine mixture samples ('Mix' in PCA plots), and 8 extracts of a blank sample prepared using Ottawa sand (20 to 30 mm mesh, from AppliChem, Darmstads, Germany) as an inert matrix instead of oil contaminated soil.

2.7. Data preprocessing

Data consisting of 55 GC–MS/SIM chromatograms for each sample were exported to the AIA file format using the commercial software ChemStation (Agilent technologies). NetCDF was used to retrieve relevant data in MATLAB. All m-files were written in MATLAB using tools from the PLS_Toolbox (Eigenvector Inc, WA, USA), and the warping toolbox (www.models.life.ku.dk).

The chromatograms comprising between 344 and 8710 data points were reduced before data processing, by visual inspection, eliminating parts with low signal-to-noise ratio and retention time sections where target compounds or compound groups were not detected.

The CHEMSIC method described by Christensen et al. [14,21] and Christensen and Tomasi [20] and recently named by Gallotta and Christensen [33] was utilized in this work. The method aims at reducing variation that is unrelated to the chemical composition (viz. baseline removal, retention time alignment, and data normalization) followed by PCA of the pre-processed and combined SICs.

Briefly, the baseline was removed by calculating the first derivatives of the SICs (point-by-point subtraction). The retention time alignment was performed in two steps: (i) applying rigid shifts (i.e., without compression or expansion) on the chromatograms, and (ii) employing correlation optimized warping (COW). COW aligns a sample chromatogram towards a target chromatogram by stretching or compressing sample segments along the retention time axis using linear interpolation [34]. The optimal warping parameters (i.e., the length of the segments, in which the signals are divided, and 'slack parameter', how much it is allowed to change) were determined by the use of a grid search in the parameter space followed by a discrete simplex-search on maximum values for warping effect function [35]. The target for the alignment was selected from the training set samples, using the one with the highest sum of correlation coefficients with the others.

Table 1
List of compound group names, *m*/*z*'s and SIM group number.

Compounds	*m*/*z*	Group(s)	Compounds	*m*/*z*	Group(s)
n-alkyl cyclo hexanes	83	I to XII	C4-decalins C2-fluorenes	194	I + II + III + IV + VI + VII
Alkanes	85	I to XII	C2-dibenzofurans	196	V + VI + VII
alkyl toluenes	105	I to XII	C1-dibenzothiophenes	198	VI + VII
sesquiterpanes	123	I to VI	C0-fluoranthene C0-pyrene	202	VII + VIII + IX
naphthalene	128	I	C2-phenanthrenes/anthracenes	206	VII + VIII + IX
benzo(b)thiophene	134	I	C3-fluorenes	208	VI + VII
d8-naphthalene	136	I	C2-dibenzothiophenes d10-fluoranthene[a] d10-pyrene[a]	212	VII + VIII
C0-decalin	138	I	C1-fluoranthenes/pyrenes	216	VIII + IX
C1-naphthalenes	142	II	Steranes	217	VIII + IX + X + XI + XII
C1-benzo(b)thiophenes	148	I + II	Steranes	218	X + XI + XII
C1-decalins acenaphthylene	152	I + II + III	C3-phenanthrenes/anthracenes	220	VII + VIII + IX
acenaphthene	154	II + III + IV	C3-dibenzothiophenes	226	VII + VIII + IX
C2-naphthalenes	156	III	C0-benzo(a)anthracene C0-chrysene	228	X
d8-acenaphthylene[a]	160	III	C2-fluoranthenes/pyrenes	230	IX + X
C2-benzo(b)thiophenes	162	II + III	triaromatic steranes	231	X + XI + XII
d10-acenaphthene	164	III + IV	C4-phenanthrenes/anthracenes retene C0-benzonaphtothiophene	234	VIII + IX + X
C2-decalins C0-fluorene	166	I + II + V	C4-dibenzothiophenes d12-benzo(a)anthracene[a] d12-chrysene	240	VIII + IX + X
C0-dibenzofuran	168	II + III + IV	C1-chrysenes	242	X + XI
C3-naphthalenes	170	IV + V	d14-p-terphenyl	244	VIII
C3-benzo(b)thiophenes d10-fluorene[a]	176	IV + V	C1-benzonaphtothiophenes	248	X + XI
C0-phenanthrene C0-anthracene	178	VI	5 Rings PAHs	252	XI + XII
C3-decalins C1-fluorenes	180	I + II + III + V	C2-chrysenes	256	XI
C1-dibenzofurans	182	IV + V + VI	d12-benzo(k)fluoranthene[a] d12-benzo(a)pyrene[a] d12-perylene	264	XI + XII
C4-naphthalenes C0-dibenzothiophene	184	IV + V + VI	C3-chrysenes	270	XI + XII
d10-phenanthrene d10-anthracene[a]	188	VI	6 Rings PAHs	276	XII
C4-benzo(b)thiophenes	190	IV + V	6 Rings PAHs	278	XII
tricyclic terpanes hopanes	191	IX + X + XI + XII	d12-indeno(1,2,3-cd)pyrene[a] d12-benzo(g,h,i)perylene[a]	288	XII
C1-phenanthrenes/anthracenes d8-dibenzothiophene[a]	192	V + VI + VII			

[a] Deuterated standards *m*/*z*'s analyzed but not added to the samples in this particular case study.

Two types of normalizations were applied to remove variations unrelated to the chemical information such as time related changes in sensitivity; and to focus the subsequent chemometric data analyses on different aspects (viz. differences between groups of compounds (SICs) or differences in relative concentrations within SICs). In scheme I, SICs are combined and then normalizing to constant Euclidean norm (i.e., corresponds to normalization to the sum if data were consisting of only positive values). This normalization scheme focuses the analysis on variations between SICs and to some extent variations within SICs [20]. In scheme II, data are normalized to constant Euclidean norm within each SIC before SICs are combined. This normalization scheme will focus the PCA on chemical variations within each SIC such as differences in isomer PAC patterns and biomarker fingerprints [20].

2.8. Chemometric data analysis

The preprocessed data are collected in a two-way data matrix (X) of size I (samples) $\times$ J (preprocessed chromatographic abundances). Subsequently, X is bilinearly decomposed by PCA into products of scores, t ($I \times 1$), and loading vectors, p^T ($1 \times J$) (i.e. T in superscript means the transposed matrix of p), plus residuals, E ($I \times J$). The bilinear decomposition with K principal components is defined in Eq. (1).

$$X = \left(\sum_{k=1}^{K} t_k \times p_k^T \right) + E \quad (1)$$

In addition, PCA was fitted according to a weighted-least squares criterion (WLS-PCA). We applied the weighted PCA (PCAW) algorithm [36] for this purpose using the inverse of the relative standard deviations of the analytical replicates (RSD_A) of the mixture sample as weights for each signal. All chemometric data analyses were performed in MATLAB 7.10. The PCAW algorithm was downloaded from http://www.bdagroup.nl/.

The RSD_A were calculated from the combined SICs after data preprocessing and normalization to the Euclidean norm for the nine replicate analyses of the mixture sample. The RSD_A's were used for selection of the most certain variables for normalization and as weights in WLS-PCA (RSD_A's = Weights^{-1}).

Table 2
Selected properties of the soil samples from Arak, Tehran and Isfahan.

	Calcareous content (% T.N.V.)	TPH ($\pm$SD) $\times 10^4$ mg kg^{-1}	Organic carbon (%)	N (%)	P (mg kg^{-1})	CEC[a] Cmol(+) kg^{-1}	pH	Sand[b] (%)	Silt (%)	Clay (%)
Arak	39	1.32 ($\pm$0.06)	7.1	0.6	7	13.1	6.4	62	19	19
Tehran	17	1.19 ($\pm$0.18)	3.4	0.4	4	12.6	6.8	59	26	15
Isfahan	33	0.25 ($\pm$0.02)	6.9	0.3	4	7.3	4.5	53	35	12

[a] Cation Exchange Capacity.
[b] Note: % sand, silt and clay do not include organic carbon.

2.9. Statistical tests

Analysis of variance (ANOVA) for all treatments was conducted using the SAS program (Release 9.1) and the difference between specific pairs of means was identified using a Tukey test ($p < 0.05$).

3. Results and discussion

3.1. Soil characteristics

Soil properties and TPH concentrations are listed in Table 2. The TPH concentrations were highest in soil from Arak and Tehran refineries ($1.32 \pm 0.06 \times 10^4$ and $1.19 \pm 0.18 \times 10^4$ mg TPH kg^{-1} soil, respectively) while the TPH concentrations in soil from Isfahan were lower ($0.25 \pm 0.02 \times 10^4$ mg TPH kg^{-1} soil). Soils from Arak and Tehran refineries had a higher sand and clay content than soil from Isfahan, which had a higher amount of silt. The CEC's were about 1.8 times higher in soils from Arak and Tehran than CEC in soil from Isfahan.

3.2. Isolated bacteria

A total of 27 bacterial strains mostly belonging to the *Bacillus* genus were isolated from the soils. The bacterial genera in Tehran samples were *Bacillus*, *Pseudomonas*, *Listeria*, *Rothia*, *Corynebacterium* and *Rhodococcus*. Soils from Arak refinery contained *Bacillus*, *Listeria*, *Nocardiform*, and *Rothia*. All bacterial genera in Isfahan samples were *Bacillus*. The bacterial consortium used in the experiment includes 5 strains (belong to *Bacillus*, *Pseudomonas*, *Rothia* and *Corynebacterium* genera): 2 isolated from the Tehran soil, 2 from Arak and 1 from Isfahan. The results of "hole-plate diffusion method" and monooxygenase biodegradation test confirmed a high potential of these strains to tolerate and degrade oil.

3.3. Classification and source comparison

Mineral oil contains a large number of petroleum biomarkers, of which terpanes (e.g., *m*/*z* 191), steranes (*m*/*z* 217, 218) and triaromatic steroids (*m*/*z* 231) are among the most abundant in crude oils. The relative concentrations of biomarkers in mineral oils depend on source, maturation, and in-reservoir weathering and biodegradation processes [37]. Petroleum biomarkers are recalcitrant when released to the environment and can therefore be used to classify samples, and to compare the type of hydrocarbon contamination. The biomarker fingerprints in soil samples from Tehran, Arak and Isfahan are shown in Fig. 1.

The biomarker fingerprints in 86 samples from Tehran, Arak and Isfahan were compared using the CHEMSIC method. The baselines of the four biomarker SICs (*m*/*z* 191, 217, 218 and 231) were removed by calculating the first derivative. The retention time shifts in the data set were between 7 and 12 scan points, depending on the SIC. The four *m*/*z*'s were aligned separately to the SICs of the training set sample with highest sum of correlation coefficients with the others. The rigid shift procedure took care of the main part of the shift within each SIC. The optimal segment lengths in COW were between 49 and 300 scan points and the optima for the slack parameter were between 1 and 3 points.

The biomarker SICs contain a large amount of instrumental and chemical noise. Data noise affects the ability of the PC model to extract reliable components. To reduce the effects of noise in the modelling, the four pre-processed SICs (after baseline removal and alignment) were combined and data were normalized to the Euclidean norm of the data with lowest RSD_A (20% fractile). The pre-processed data were then analyzed by weighted PCA using $RSD_A{}^{-1}$ as weights. The combination of a modification of normalization scheme I and weighted PCA led to a strongly improved separation of samples from Tehran, Arak and Isfahan into three distinct clusters in the score plots (Fig. 2) compared to the results obtained when using standard normalization scheme I and PCA (Fig. S1). In the latter analysis, samples from the three locations were not fully separated.

Soil samples from Tehran, Arak and Isfahan were classified into three distinct groups in the score plot of PC1 vs. PC2 (encircled in Fig. 2). The TPH concentrations were comparable in soil from Arak and Tehran: 1.32 ($\pm$0.06) $\times 10^4$ and 1.19 ($\pm$0.18) $\times 10^4$ mg kg^{-1}, respectively (Fig. 3) and the biomarker SICs were similar (Fig. 1). Soils from Arak and Tehran contained higher relative concentrations of triaromatic steroids (*m*/*z* 231 SIC) and higher relative concentrations of C_{23}-C_{29} tri-pentacyclic terpanes compared to C_{30}-C_{33} pentacyclic terpanes (*m*/*z* 191 SIC) from the soil of Isfahan, We could conclude this as these biomarkers have negative PC1 loading values (Fig. S2a) and the samples from Arak and Tehran have negative PC1 scores (Fig. 2). Likewise, soil samples from Arak refinery (positive PC2 scores, Fig. 2) contained higher relative concentrations of tricyclic terpanes, C_{20}–C_{21} triaromatic steroids and C_{27}–C_{29} steranes and diasteranes (positive PC2 loadings, Fig. S2b)

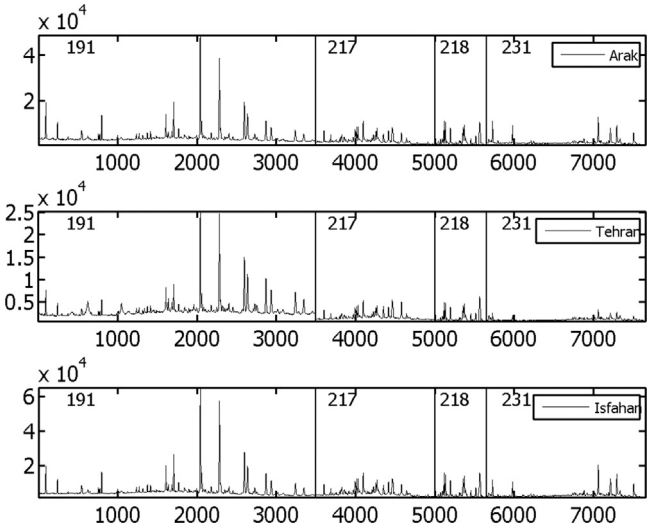

Fig. 1. Biomarker fingerprints consisting of combined SICs of terpanes (*m*/*z* 191), steranes (*m*/*z* 217, 218) and triaromatic steroids (*m*/*z* 231) for an untreated soil sample from Arak refinery (upper), Tehran refinery (middle), and Isfahan refinery (lower).

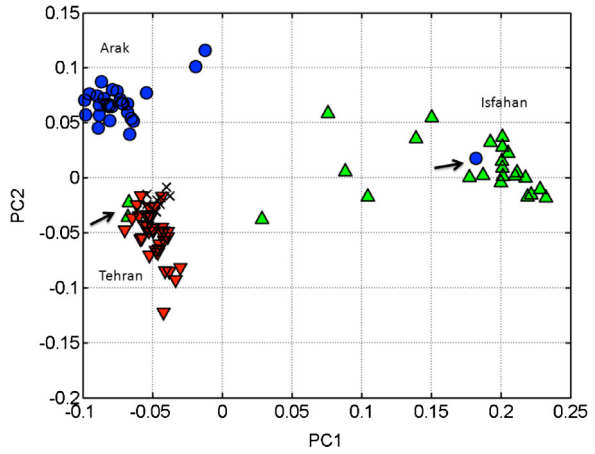

Fig. 2. Score plot of PC1 vs. PC2 based on the sample set (88 × 7654 data points), using weighted PCA and normalization to data points with the 20% lowest fractile of RSDA. Samples from Arak are marked by blue circles; samples from Tehran by red triangles; and samples from Isfahan by green triangles. Black crosses are analytical replicates of the mixture sample. The black arrows indicate mislabelled samples.

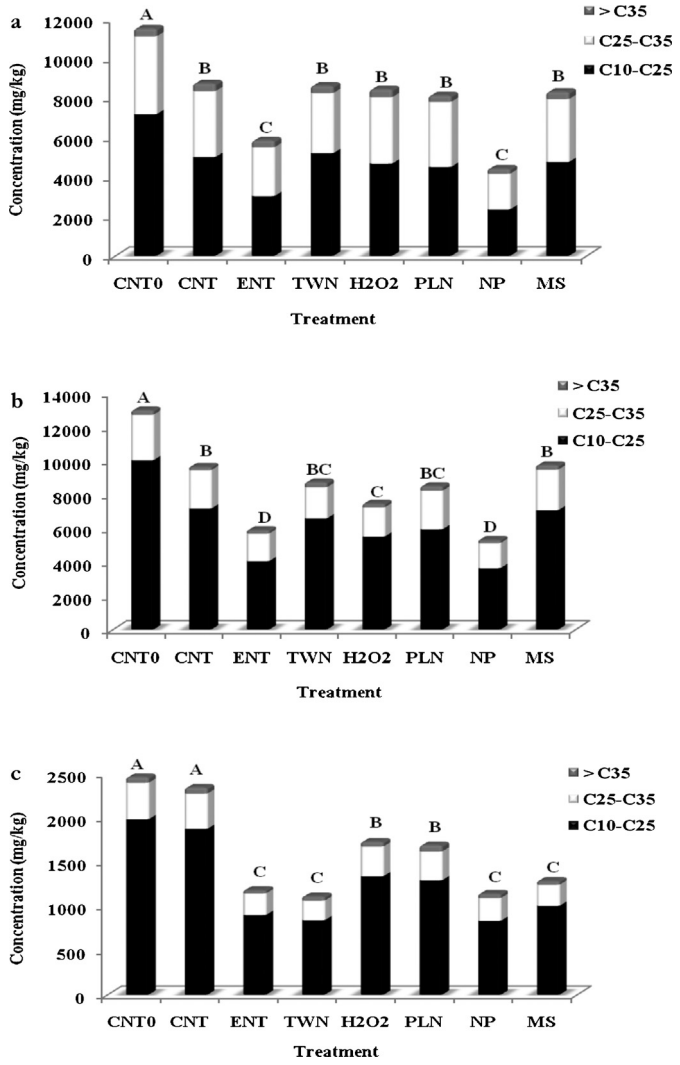

Fig. 3. (a, upper) TPH degradation in soil from Tehran Refinery, (b, middle) TPH degradation in soil from Arak Refinery, and (c, lower) TPH degradation in soil from Isfahan Refinery. Different letters represent statistical difference in means by using Tuckey test ($p < 0.05$). Treatments are shown with CNT0 (control samples in day 0), CNT (control samples after 2 months), ENT (microbial enrichment), TWN (addition of surfactant), H2O2 (addition of hydrogen peroxide), PLN (planting), NP (addition of nutrients including nitrogen and phosphorous), and MS (addition of molasses).

than soil from Tehran refinery (negative PC2 scores, Fig. 2). In summary, these results demonstrate that soils from Arak and Tehran contained a heavier oil fraction with a higher content of aromatics compared to the soil from Isfahan refinery; and soil from Arak was contaminated with a slightly lighter oil fraction than the soil from Tehran refinery.

3.4. Effects of bio-enhancement treatments on soil TPH concentrations

The TPH concentration decreased from 26 to 27% in Tehran and Arak soils during two-months of incubation in the controls (addition of water to the soil and mixing) (see Fig. 3). The removal of petroleum hydrocarbons in the control samples can be explained by an increased activity of the microbial community due to the addition of water and aeration. Another explanation is that the mixing process enhances evaporation of lighter oil components. This interpretation is supported by the analysis of oil fractions, which demonstrates the removal was almost exclusively from the lighter petroleum fraction – C_{10}–C_{25} (Fig. 3).

In contrast, only a slight decrease in the lighter petroleum fraction was observed for the control samples from Isfahan refinery unaffected after two-months of incubation (Fig. 3). The TPH concentration was 5–6 times lower in soil from Isfahan than in soil from Arak and Tehran refineries (Table 2). The soil characteristics CEC, pH and clay content were different (Table 2) as were the composition of the microbial community and the type of contamination. Soil characteristics (e.g. soil fractions, organic matter content and nutrients etc.) and type of contamination are important characteristics for the total removal rate of oil hydrocarbons [38–42].

Bacterial enrichment and supplement of nutrients were the most efficient treatments leading to removal of 50 to 62% of the TPH. The other four treatments were less efficient with removals between 26 and 54% of the TPH. For Arak and Tehran soils, TPH concentrations decreased significantly ($p < 0.05$) in bacterial enrichment and with the addition of nutrients (ENT and NP treatments, respectively). The remaining four treatments did not significantly ($p < 0.05$) differ from the controls (Fig. 3).

In contrast, in the soil from Isfahan the TPH removal was >50% in four of the six treatments including bacterial enrichment and the addition of nutrients, surfactant and molasses compared to a 2% TPH removal in the control. This indicates the limited removal in the control soil from Isfahan refinery after two-months of incubation could be due to low numbers of oil-degrading bacteria.

A recent study by the authors on long-term (7 months) phytoremediation of oil contaminated soils from Iran using plant seedlings showed a positive effect on PAC and TPH removal in the presence of plants (80 to 84% and 64 to 72%, respectively) compared to unplanted controls (56 and 31%, respectively) [3]. In this study, we found no statistical significant effects ($p < 0.05$) of planting, however, the incubation time was shorter (2 months) and seeds were used instead of seedlings. Hence, the short time for plant stabilization and growth is most likely the main explanation for the lack of effect. But, differences in levels and types of oil hydrocarbons could also have affected the results.

As for the controls, the removal was more pronounced for the lighter petroleum hydrocarbons (C_{10}–C_{25}) than for the heavier fractions (C_{25}–C_{35} and >C_{35}). This is in line with previous results [3,4,38,42].

3.5. Effects of bio-enhancement treatments on oil composition

The CHEMSIC method was applied to SICs of 23 groups of PACs and one retention time region of n-alkanes and isoprenoids (nC_{15}–nC_{20}) relevant for source identification and the study of oil

Table 3
List of compound groups, *m*/*z*'s (i.e., SICs) used for the detailed study of oil weathering.

Compounds	*m*/*z*	Compounds	*m*/*z*
Alkanes	85	C3-fluorenes	208
C3-naphthalenes	170	C0-fluoranthenes/pyrenes	202
C4-naphthalenes	184	C1-fluoranthenes/pyrenes	216
C0-dibenzothiophene			
C1-phenanthrenes/anthracenes	192	C2-fluoranthenes/pyrenes	230
C2-phenanthrenes/anthracenes	206	C0-chrysene	228
C3-phenanthrenes/anthracenes	220	C1-chrysenes	242
C4-phenanthrenes/anthracenes	234	C2-chrysenes	256
C1-dibenzothiophenes	198	C3-chrysenes	270
C2-dibenzothiophenes	212	C1-benzonaphtothiophenes	248
C3-dibenzothiophenes	226		
C4-dibenzothiophenes	240	5 Rings PAHs	252
C2-fluorenes	194	6 Rings PAHs	276
		6 Rings PAHs	278

weathering [20]. The compound groups and *m*/*z*'s used in the analyses are listed in Table 3.

The baselines of the 24 SICs were removed by calculating the first derivative, and the SICs were then aligned separately. The maximum rigid shift allowed was ±10 scan points and the search space for COW was 25–175 with 25 point increment for segment length and 1–5 with 1 point increment for slack parameter. The maximum correction allowed for COW was 10 scan points. The initial PC model was calculated on the combined SICs normalized to the Euclidean norm after the SICs were combined (normalization scheme I). PC model was calculated on the training set (86 samples × 12,693 data points) and the two validation sets were projected on the PC model. Cross validation with 10 random subsets were used to estimate the optimal number of PCs. A bend in explained cross validation variance was observed past the third and another past the fifth PC. The Root Mean Square Error of Cross Validation (RMSECV) showed a clear minimum for five PCs and since the loadings above PC5 contain shift patterns [20] in addition to chemical variation, it was concluded that the optimal number of PCs was five. The five-component PC model described 95.2% of the variance in the training set.

The score plots for PC1 vs. PC2 and PC3 vs. PC4 using mean centering are shown in Fig. 4. The PC1 and PC2 loadings are shown in Fig. 5 and PC3 and PC4 loadings in Fig. S3. Each of the five PCs contain information on both oil source and degree and type of weathering.

The PC model confirmed the TPH results (Fig. 3) that the most efficient techniques for enhancing bioremediation were bacterial enrichment (E) and supplement of nutrients (NP) compared to control treatment (Ct) (Fig. 4).

PC1 and PC2 describe the main weathering effects (evaporation, dissolution and biodegradation). PC1 loading coefficients are negative for n-alkanes (marked on Fig. 5a), C_0-C_3-naphthalenes, C_0-C_3-benzothiophenes, C_0-C_1-phenanthrenes and dibenzothiophenes (Fig. 5a, upper) and positive for isoprenoids (e.g., norpristane, pristane and phytane, marked on Fig. 5a) and the more alkylated 2–3 PACs, and 4–5 ring PACs. In contrast, PC2 loading coefficients were negative for both isoprenoids and n-alkanes and positive for all PACs (Fig. 5b, lower). Isoprenoids are less susceptible to microbial degradation than n-alkanes of similar molecular weight; and the rate of both evaporation and microbial degradation decreases with degree of alkylation and number of aromatic rings [43].

Hence, the main effects of weathering within a sample series (Arak, Tehran or Isfahan) can be described by their PC1 and PC2 scores. Samples with negative PC1 scores and PC2 scores close to zero (see Fig. 4a, upper) are the least weathered (Control samples from the start of the experiment); and the degree of weathering increases along a curvature to high positive PC1 and positive PC2

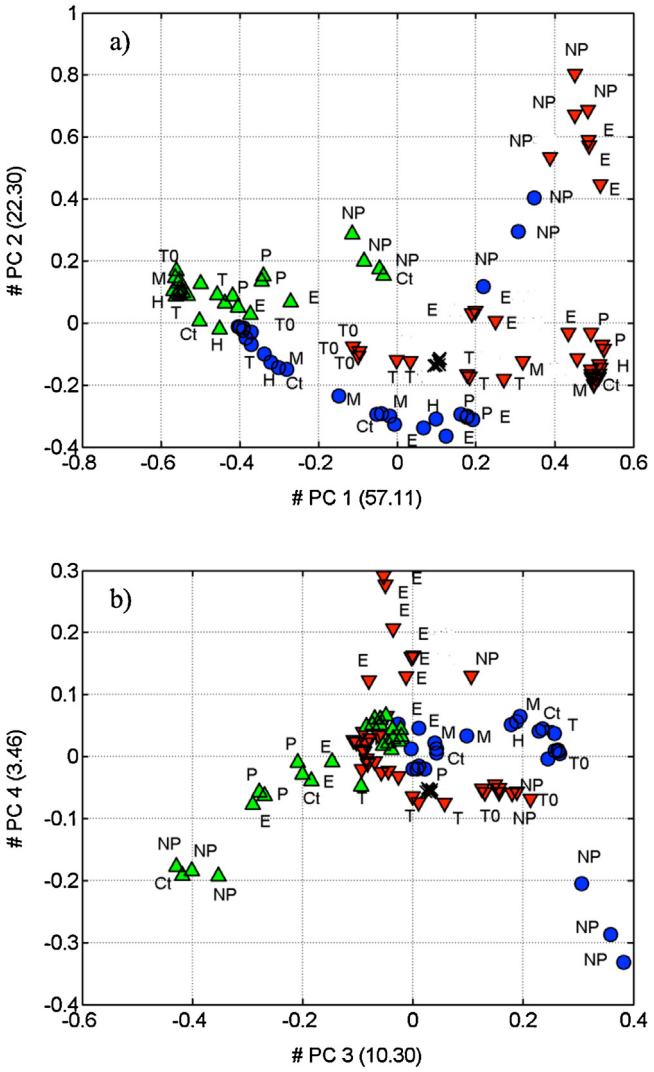

Fig. 4. (a, upper) Score plot PC1 vs. PC2 and (b, lower) PC3 vs. PC4 based on the sample set (86 × 7760 data points), using PCA and normalization to Euclidean norm after combining SICs (normalization scheme I). Samples from Tehran are marked by red triangles; samples from Isfahan by green triangles; and samples from Arak by blue circles. Treatments are shown with T0 (control samples in day 0), Ct (control samples after 2 months), E (microbial enrichment), T (addition of surfactant), H (addition of hydrogen peroxide), P (planting), NP (addition of nutrients including nitrogen and phosphorous), and M (addition of molasses).

score values. This fitted well with the sample labels as supplement of nutrients (NP) and bacterial enrichment (E) were the most heavily weathered, whereas control samples at time zero (with T_0 in their label) and the other treatments (adding molasses, H_2O_2, and planting) were less and moderately weathered, respectively (Fig. 4a, upper).

In summary, while the main effects of bacterial enrichment were an increased degradation of n-alkanes and low-molecular-weight PACs; the nutrients supplement had a more pronounced effect on the degradation of isoprenoids.

The first two PCs of a PC model calculated from the 35 Tehran samples confirmed these conclusions (results not shown). Furthermore, a third minor component (not shown) showed that the relative effects of 'supplement of nutrients' and 'bacterial enrichment' on oil composition were slightly different. The bacterial enrichment seemed to be more efficient in degrading more heavily alkylated PACs: C_3–C_4–naphthalenes, C_2–phenanthrenes and C_2–C_3–dibenzothiophenes, while nutrient addition led to a relative increase in removal of isoprenoids.

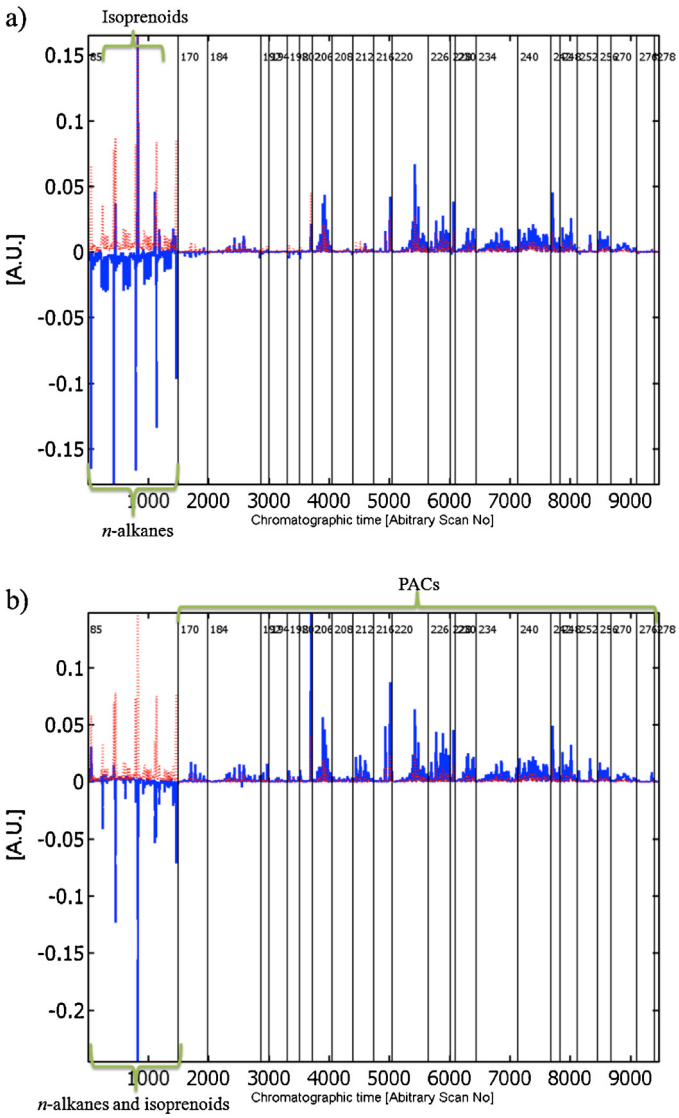

Fig. 5. Loading plots: PC1 (a, upper) and PC2 (b, lower) for the PC model of mean-centred data normalization to Euclidean norm after combining SICs (normalization scheme I). The dotted red lines are the mean chromatogram of the entire training set, while the solid blue lines are the loadings. The *m/z* groups are specified in loading plot.

PC3 and PC4 seem to describe a combination of oil source and minor weathering effects. Chemical interpretation of the scores (Fig. 4) and loadings (Fig. 5 and Fig. S3) confirmed that the contamination in the soil from Isfahan was the most pyrogenic (or heavily refined) followed by that in the soils from Tehran and Arak. This was elucidated from higher relative concentrations of C_0–C_1–pyrene and 5–6 ring PACs (e.g., more positive PC3 loading coefficients for Arak samples).

3.6. *Effects of bio-enhancement treatments: Isomer-specific degradation*

Several authors have observed that microbial degradation is isomer specific [13,43–45]. Changes in nC_{17}/Pristane and nC_{18}/Phytane have long been used as indicators for biodegradation [46], and their usefulness is also clear from this study. Likewise, preferential degradation of specific isomers within homologue PAC series has been described since the 1980s [44,47]. Recently, Christensen et al. [14] observed differential susceptibility to degradation within series of alkylated PAC homologues of methylfluorenes

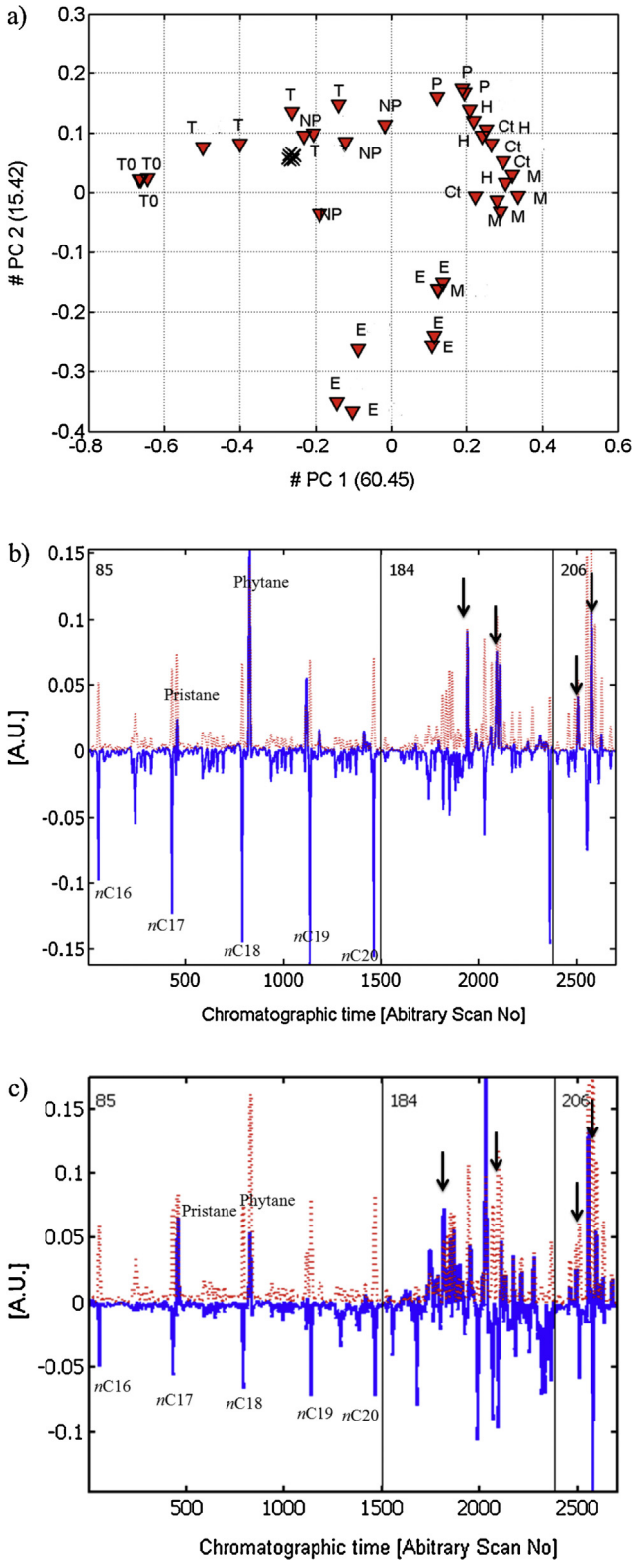

Fig. 6. (a, upper) Score plot PC1 vs. PC2 based on the sample set (35 × 2703 data points), using PCA and normalization to Euclidean norm before combining the SICs (normalization scheme II); (b, middle) PC1 loadings; and (c, lower) PC2 loadings. The red dotted lines are the mean chromatogram of the entire training set, while the blue solid lines are the loadings. The black arrows indicate compounds of special interest (showing preferential degradation). Treatments in part a are shown with T0 (control samples in day 0), Ct (control samples after 2 months), E (microbial enrichment), T (addition of surfactant), H (addition of hydrogen peroxide), P (planting), NP (addition of nutrients including nitrogen and phosphorous), and M (addition of molasses).

(MF), methylphenanthrenes (MP) and methyldibenzothiophenes (MD) by the use of the CHEMSIC method. To assess isomer-specific degradation we normalized to constant Euclidean norm for each SIC and then combined the SICs (normalization scheme II). In the initial PC model 38 SICs were included. Subsequently, SICs that contained only little information (showing shift patterns in the first PC) were successively removed and the PC model recalculated. The final model contained 3 SICs: n-alkanes/isoprenoids, C_4-naphthalenes, and C_2-phenanthrenes (35×2703). The PC model demonstrates preferential degradation of nC_{17} and nC_{18} over pristane and phytane, respectively. This is revealed as both the PC1 and PC2 loadings are negative for the *n*-alkanes (nC16-nC19) and positive for pristane and phytane (Fig. 6b and c). The score and loading plots for the PC model with scheme II normalization should be interpreted with care as the sample moves from negative PC1 to intermediate PC1 and to negative PC2 with increasing degree of degradation. Hence, the reason that the E samples have large negative PC2 is that the pristane and phytane and degraded again decreasing the ratio between the n-alkanes and isoprenoids. In addition to these well-known changes in ratios, isomer-specific degradation of C_4-naphthalenes and C_2-phenanthrenes (Fig. 6a–c) was also observed. Both for PC1 and PC2 clear isomer-specific degradation is seen as some isomers have negative loadings while others have positive loadings. For E treated soil isomers with positive PC2 loadings are preferentially degraded (marked by an arrow in Fig. 6c) while those with negative PC2 loadings are degradation-resistant.

The study also confirms previous observations, that the supplement of nutrients is inefficient in removing PACs as the NP samples have the most similar score values with the control samples. In this study both the biomarker compounds (>100 individual peaks) as well as 38 SICs of PACs (corresponding to >500 individual compounds) were investigated in 86 sample extracts. Hence, in a total of >50.000 peaks were included in this analysis, which would have been impossible to comprehend using PCA on the basis of peak tables obtained using standard integration procedures. Furthermore, as was also described in [20,22] another advantage by the CHEMSIC method is that it is possible to include partially separated peaks in the assessment.

On the other hand, CHEMSIC procedure requires expertise in advanced data preprocessing strategies (e.g., correlation optimized warping) that is not yet widespread in the scientific community. The method is sensitive to inadequate baseline reduction, retention time alignment, and normalization and in cases of inadequate preprocessing the PC model will not describe all the relevant information in the data set and earlier PCs will partly describe noise and therefore be difficult to interpret.

4. Conclusion

Overall, bacterial enrichment and supplement of nutrients were the most efficient treatments among the other treatments (addition of surfactant, hydrogen peroxide, molasses and planting) in removal of soil TPHs (50–62%) compared to 2–27% TPH removal in controls. These conclusions were confirmed by use of a chemometric approach (the CHEMSIC method). TPH removal was more pronounced for lighter petroleum hydrocarbons (C_{10}–C_{25}) than the heavier fractions (C_{25}–C_{35} and $>C_{35}$).

The CHEMSIC results revealed that the bacterial enrichment was more efficient in enhancing the degradation of PACs, whereas nutrient addition had a more pronounced effect on degradation of isoprenoids. In fact, nutrient addition has limited effects on the removal of PACs and clustered with the controls in score plots. Hence, based on reductions in TPH concentrations nutrient supplements seemed an equally efficient method for enhancing bioremediation as bacterial enrichment, but the more detailed assessment by CHEMSIC reveals otherwise. As PACs are considered a toxic part of mineral oil and the isoprenoids are of less concern, the bacterial enrichment is likely the most efficient of the six remediation strategies in reducing the toxicity of the oil contaminated soil from the three refineries in Iran.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2013.03.004.

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