Research Article

Biodegradation of isoprenoids, steranes, terpanes and phenanthrenes during *in situ* bioremediation of petroleum contaminated groundwater[†]

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Abstract

The objective of this study was to stimulate microbial biodegradation of petroleum pollution in groundwater and to analyze changes in the abundance and distribution of organic compounds detectable in petroleum. Bioremediation was conducted in a closed bipolar system, by bioaugmentation with consortia of hydrocarbon degrading microorganisms (HD) and biostimulation with nutrients. Comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS) was used to visualize all fractions simultaneously. During the study, the content of total petroleum hydrocarbon (TPH) in groundwater decreased by 92.7% of the initial level, and the average rate of biodegradation was 0.1 mg/L per day. Increased numbers of HD were observed and the dominant genera were *Pseudomonas*, *Rhodococcus*, *Achromobacter*, *Bacillus*, and *Micromonospora*. In the first 30 days of bioremediation, there was no significant biodegradation of *n*-alkanes and petroleum biomarkers - isoprenoids such as pristane and phytane, and polycyclic saturated hydrocarbons such as terpanes and steranes. However, after 60 days of bioremediation, more than 95% of *n*-alkanes, terpanes and steranes were biodegraded. Phenanthrene and its methyl-, dimethyl-, and trimethyl-isomers were biodegraded and reduced by more than 99% of their initial levels. However, their decomposition had clearly commenced after just 30 days. This is a somewhat surprising result since it follows that the phenanthrenes were more susceptible to biodegradation than the *n*-alkanes and isoprenoids. Depending on the microbial community used for bioaugmentation, biodegradation of phenanthrene can preceded biodegradation of saturated hydrocarbons.

Keywords: Closed bipolar systems, Petroleum biomarkers, Polycyclic aromatic hydrocarbons, Two-dimensional gas chromatography–mass spectrometry, Zymogenous microorganisms

Abbreviations: **CFU**, colony forming unit; **GC×GC-MS**, comprehensive two-dimensional gas chromatography–mass spectrometry; **HD**, hydrocarbon degrading microorganism; **PAH**, polycyclic aromatic hydrocarbon; **TC**, total chemoorganoheterotroph; **TIC**, total ion chromatogram; **TPH**, total petroleum hydrocarbon;

1 Introduction

Organic geochemical studies of the biodegradability of some classes of organic compounds from oil have shown that *n*-alkanes and isoprenoid aliphatic alkanes are the most susceptible to biodegradation [1, 2]. Among the oil compounds, the most resistant to microbial biodegradation are monoaromatic steroids, diasteranes, diahopanes, 25-norhopanes, tricyclic terpanes, C_{21}--C_{22} steranes, oleanane and gammacerane. Very close to them, by resistance to biodegradation, are the C_{27}--C_{35} hopanes and C_{27}--C_{29} regular steranes [3]. Research on microbial biodegradation of oil pollutants has shown that these trends are similar in the environment [4, 5]. Bioremediation is a process which can use either naturally occurring autochthonous microorganisms which are isolated from a particular polluted place to be treated by bioremediation, or allochthonous microorganisms isolated from other polluted habitats. However, it is known that autochthonous microorganisms are much more potent and with a higher survivability rate compared to allochthonous microorganisms [5]. The zymogenous fraction of autochthonous microorganisms exhibits the highest activity toward transformation of the pollutants present [6]. Through their normal life functions, biodegradation or complete mineralization of hazardous organic substances can occur. To date, bioremediation has been proven to be efficient in the removal of oil hydrocarbons [7], polychlorinated biphenyls [8], polycyclic aromatic hydrocarbons (PAH) [9], pesticides [10], some heavy metals [11] and even radionucleotides [12]. The result of bioremediation of oil pollutants in the environment is usually assessed on the basis of differences in the total petroleum hydrocarbons (TPH) content before and after the process.

In studies of simulated biodegradation using a zymogenous microbial consortium (microorganisms isolated from a site exposed to a longer period of oil pollution), on one paraffinic crude oil, on different media and under different conditions, biodegradation of *n*-alkanes and isoprenoid aliphatic alkanes, pristane (C_{19}) and phytane (C_{20}) occurred [13]. This biodegradation was the most intense when the inorganic media were used and experiments were performed in the light [13]. However, polycyclic alkanes of the sterane (C_{27}--C_{29}) and terpane (C_{27}--C_{34}) types, oleanane and gammacerane remained non-degraded [13]. The same conclusion was drawn after simulated biodegradation of a paraffin oil by a consortium of zymogenous bacteria and fungi over a period of 75 days [14]. After decomposition of *n*-alkanes and isoprenoids, the abundance and distribution of steranes and terpanes remained unchanged [14]. Biodegradation of isoprenoids, steranes and terpanes was recently confirmed during *ex situ* bioremediation of mazut on an industrial level using consortia of zymogenous microorganisms [4].

The current study attempted to conduct a more efficient decomposition of those compounds from oil pollutants that have proved to be, in earlier attempts of simulated biodegradation in the laboratory, the most resistant to the action of zymogenous microorganisms. To this end, an *in situ* biodegradation process was applied to groundwater contaminated by oil pollutants. In the current study, detailed characterization of the biomarkers in petroleum pollutant extracted from groundwater [15] was conducted and two instrumental techniques were applied: comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC-MS) and GC-MS. GC×GC-MS is suitable for the determination of various organic pollutants in complex environmental matrices [16] and in complex mixtures [17, 18], and it involves the separation of compounds by two orthogonal GC columns [19, 20]. This technique was applied to analyze groundwater extracts before, during and after bioremediation for easy visualization of changes in the abundance of the chosen biomarkers/contaminants.

2 Materials and methods

2.1 Site description and bioremediation methodology

The investigated area was located in the industrial complex Nitex, in the city of Niš, on the terrace sediments of the Nišava River, Serbia. The aquifer at this location was proven to be contaminated by petroleum hydrocarbons from an underground storage tank. A detailed hydrogeological description of this locality was previously published [15].

The typical *in situ* bioremediation process of groundwater requires biostimulation that encourages growth and reproduction of zymogenous microorganisms to metabolize target contaminants and use them as a source of carbon and energy [21]. The groundwater investigated in this study, which contained dissolved hydrocarbons and a floating layer of an oil pollutant, was treated with a filtration--adsorption remediation technique, using columns filled with natural inorganic hydrophobic adsorbents, and *in situ* bioremediation based on the principle of a bipolar model. Enhanced *in situ* bioremediation of groundwater and soil layers in contact with groundwater was performed by a combination of biostimulation and bioaugmentation within the closed bipolar system (one extraction and two injection wells), with adsorption in the external unit. This combination of methods is original and 1158 m^3 of groundwater were treated through the system [15]. In this study, besides biostimulation (by addition of nutrients, nitrate and phosphate, and stimulation of oxidation processes by injection of H_2O_2, into the aquifer), bioaugmentation with consortia of zymogenous microorganisms isolated and selected from contaminated groundwater was applied. The biomass of zymogenous microorganisms was produced on-site in a mobile bioreactor previously described [15].

2.2 Microbiological and chemical analyses

Selected members of the hydrocarbon degrading zymogenous consortia used for bioaugmentation were isolated based on morphological differences and identification of isolated strains was achieved by API tests (Biomeriex, France), and by sequence analysis of 16S rRNA genes. The API 50CH/E, API 20NE and Coryne kits were utilized in accordance with instruction from the manufacturer (Biomerieux, France). The data were interpreted using apiwebTM software. The genomic DNA of each bacterium was extracted [22] and the 16S rRNA genes were amplified by PCR using 27F (50-AGAGTTTGATCMTGGCTCAG-30 [23], and 1492R (50-CGGCTACCTTGTTACGACTT-30 [24] primers. Amplified fragments were sequenced by a commercial service Macrogen Europe (Netherlands). Taxonomic analysis was conducted by the GenBank basic local alignment search tool (BLAST) program.

The amount of TPH was determined according to the standard method, which includes solvent extraction followed by gravimetric determination and GC-MS. TPH from groundwater samples was extracted as per method ISO 9377-2 (2000) [25] and determined gravimetrically in accordance with DIN EN 14345 (2004) [26]. TPH was determined in groundwater on days 0, 30 and 60.

The number of microorganisms in the groundwater was determined by plating appropriate serial dilutions on agar plates incubated at 28 °C. The media used were nutrient agar (15 g/L peptone, 3 g/L meat extract, 5 g/L NaCl, 0.3 g/L K_2HPO_4, 18 g/L agar; Torlak, Serbia) for total chemoorganoheterotrophs (TC) and mineral base medium (1 g/L NH_4NO_3, 0.25 g/L K_2HPO_4, 50 mL soil extract, 16 g/L agar) containing 2 g/L standard D2 diesel fuel for hydrocarbon degraders (HD), according to Bossert et al. [27].

For GC×GC-MS analysis, TPH extracts were dissolved in *n*-hexane and analyzed directly using GC×GC-MS (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan and GC×GC modulator, Zoex) in the form of a total ion chromatograms (TIC). An Rtx®-1 (RESTEK, Crossbond® 100% dimethyl polysiloxane, 30 m × 0.25 mm id, 0.25 µm film thickness) and a BPX50 (SGE Analytical Science, 2.6 m × 0.1 mm id, d_f = 0.1 µm) column were connected through the GC×GC modulator as the first and second capillary columns, respectively. The MS data were collected with GCMSsolution (Ver. 4.11 & 4.2 update) (Shidmazu, Japan) and processed using GC-MS real time analysis program. The GC×GC-MS data were analyzed using GC Image Ver. 2.1 (Zoex), which is capable of directly reading GC×GC data obtained with GC-MSsolution software, converting it to a 2-dimensional image.

GC-MS was used for detailed analysis of *n*-alkanes, isoprenoids, terpanes, steranes, as well as phenanthrene and its methyl-, dimethyl- and trimethyl-isomers. For GC-MS analyses, an Agilent 7890N gas chromatograph fitted with a HP5-MS capillary column (30 × 0.25 mm, 0.25 µm film; oven temperature: 80 °C for 0 min, temperature increase by 2°C/min to 300°C and held for 20 min) with helium as the carrier gas (flow rate 1 mL/min) was used. Detailed analyses of the target compounds were conducted in the selected ion monitoring mode, comprising the following ion chromatograms: *m*/*z* = 71 (*n*-alkanes and isoprenoids), *m*/*z* = 191 (terpanes), *m*/*z* = 217 (steranes), *m*/*z* = 178 (phenanthrene), *m*/*z* = 192 (methyl-phenanthrenes), *m*/*z* = 206 (dimethyl-phenanthrenes) and *m*/*z* = 220 (trimethyl-phenanthrenes).

In order to compare the changes in the distribution and abundances of selected target compounds, prior to the instrumental analyses, all groundwater TPH extracts were dissolved in the same volume of solvent (1 mL), and the same volume of each dissolved sample was injected into the instrument (1 µL).

3 Results and discussion

3.1 Isolation and taxonomic identification of the bacterial strains

Bacterial HD strains (*n* = 8) were isolated from the zymogenous consortium of microorganisms used for bioaugmentation and subjected to detailed analysis. After inspection of colonies grown on nutrient agar and comparison of morphological, physiological and biochemical characteristics, these eight bacterial strains were determined as the predominant ones. Four of them were motile, Gram-negative rods, catalase and oxidase positive, out of which one produced a fluorescent pigment (NI-03) and the others did not (NI-01, NI-04 and NI-07). The other four were Gram-positive, out of which two were nonsporulating, non-motile bacteria, oxidase negative, catalase positive (NI-02, NI-05) and two were spore-forming, catalase positive, one beta-hemolytic (NI-06) and one with branched mycelium (NI-08).

According to physiological-biochemical properties (API tests), the HD bacteria were preliminarily identified as *Pseudomonas sp.* (NI-01, NI-03, NI-07), *Rhodococcus* sp. (NI-02, NI-05), *Achromobacter* sp. (NI-04), *Bacillus* sp. (NI-06) and *Micromonospora* sp. (NI-08).

The final identification was performed by sequence analysis of the 16S ribosomal RNA gene. Isolated strains were identified as: NI-01: *Pseudomonas aeruginosa* (98%); NI-02 *Rhodococcus rhodochrous* (97%); NI-03 *P. fluorescens* (97%); NI-04 *Achromobacter xylosoxidans* (97%); NI-05: *R. erythropolis* (98%); NI-06: *Bacillus cereus* (98%); NI-07: *P. aeruginosa* (97%); NI-08 *Micromonospora carbonacea* (98%); sequence alignment identity values are given in the brackets.

3.2 Change in TPH content and number of microorganisms

During two months of *in situ* bioremediation, the concentration of TPH was reduced by 92.7 %, from 6.8 mg/L to <0.5 mg/L, as previously published [15], which means that the average rate of biodegradation was 0.1 mg/L per day.

Due to biostimulation and bioaugmentation using zymogenous consortium of microorganisms, the number of TC and HD microorganisms started to increase. The number of TC microorganisms increased from 10 000 colony forming units (CFU)/mL at day 0 day, to 25 000 and 80 000 CFU/mL after 30 and 60 days, respectively. HD microorganisms reached their maximum population density after 30 days of activity (5000 and 15 000 CFU/mL on days 0 and 30, respectively), and by the end of the process their number had decreased (3000 CFU/mL). This decrease in concentration of HD microorganisms was very likely a result of depletion of oil hydrocarbons (the source of carbon for these microorganisms).

3.3 Fate of individual hydrocarbons in the polluted groundwater - GC×GC-MS analysis

The changes in the distribution and abundances of *n*-alkanes, isoprenoids, steranes, terpanes, phenanthrene, methyl-phenanthrenes, dimethyl-phenanthrenes and trimethyl-phenanthrenes were monitored by analyzing the extracts isolated from the samples by GC×GC-MS and GC-MS.

TPH extracts before and after *in situ* bioremediation were analyzed using comprehensive GC×GC-MS and results as TIC are shown in Fig. 1. All fractions including *n*-alkanes, steranes, terpanes and phenanthrenes are marked on the chromatogram based on MS analysis and they were eluted following the elution protocol [17]. Important biomarkers in petroleum, terpanes co-elute with C_{28}--C_{30} *n*-alkanes steranes with C_{26}--C_{29} *n*-alkanes, and phenanthrenes with C_{17}--C_{20} *n*-alkanes [17]. However, they can be found at different second-dimension retention times (Fig. 1).

After 60 days of intensive biodegradation by the applied zymogenous microbial consortium, TPH was reduced to almost the background noise level of GC×GC, which can be seen from the 3D chromatograms (Fig. 1). The concentrations of all fractions of *n*-alkanes, steranes, terpanes, and phenanthrenes were reduced to the background level of GC×GC.

The chromatograms showing hydrocarbons of the petroleum pollutant extracted from groundwater at the beginning of the study are given in Fig. 2, and the identification of the most important peaks is given in Table 1. In the alkane fraction, *n*-alkanes were characterized by bimodal distribution with peak maximums at C_{18} and C_{23}. The bimodal distribution of *n*-alkanes suggests contamination by different oil pollutants. The isoprenoid aliphatic alkanes, pristane and phytane, were similar in abundance to *n*-C_{17} and *n*-C_{18} (Fig. 2; *m*/*z* = 71). This ratio of isoprenoids and *n*-alkanes shows that significant biodegradation of pollutants in natural conditions did not occur before the start of the study [3, 28].

Terpanes (*m*/*z* = 191) and steranes (*m*/*z* = 217) in the alkane fractions (Fig. 2) had distributions that are typical for crude oils [29]. The same can be said for phenanthrene and its methyl-isomers (Fig. 2; *m*/*z* = 178, 192, 206, 220) [30].

3.4 Changes in the distribution and abundance of hydrocarbons

Changes in the distribution of *n*-alkanes and the isoprenoid aliphatic alkanes pristane and phytane during the 60-day *in situ* bioremediation are given in Fig. 3.

At the beginning of the process, the alkanes *n*-C_{17} and *n*-C_{18} and the closely eluting isoprenoids, pristane and phytane, were present in similar concentrations. Comparing GC-MS chromatograms of *n*-alkanes in the hydrocarbon fraction after 30 days with the initial peaks at day 0, it is clear that *n*-alkanes lower than C_{21} were the most affected by the microorganisms and that the loss of the *n*-alkanes was mainly due to a decrease of homologues in the C_{15}–C_{21} range. During the first 30 days, peaks for *n*-alkanes and isoprenoids did not reduce significantly (Fig. 3; days 0 and 30). However, in the second month of bioremediation, biodegradation of these compounds had occurred, and they remained only in trace amounts (Fig. 3; 60 days). Along with the very small, barely noticeable peaks belonging to pristane and phytane, peaks for *n*-C_{17} and *n*-C_{18} were observed to have the same intensity (Fig. 3). This is evidence that the microbial biodegradation of *n*-alkanes and isoprenoids occurred at the same speed.

Changes in the distribution and abundance of tricyclic diterpanes and pentacyclic terpanes in alkane fractions of the petroleum pollutant extracted from groundwater during the 60-day *in situ* bioremediation are given in Fig. 4. In the first 30 days, there was no significant change in abundance or distribution of tricyclic diterpanes or pentacyclic terpanes, based on the almost identical fragmentograms of *m*/z = 191 shown in Fig. 4 (days 0 and 30). It is well known that polycyclic alkanes of the sterane and terpane types in crude oils in the subsurface are decomposed by biodegradation after the degradation of acyclic hydrocarbons [28]. Furthermore, it has also been shown that crude oil biodegradation under aerobic conditions can follow completely different sequences than oil in reservoir rocks [31]. In addition, it was reported that under laboratory conditions, sterane and terpane biomarkers were not affected by biodegradation and that the biodegradation of saturated hydrocarbons was restricted to the acyclic aliphatic compounds (*n*-alkanes and isoprenoids) [32]. However, in the present study, zymogenous microbial consortia in a non-sterile, open system on an industrial scale was applied, leading to complete biodegradation of these polycyclic hydrocarbons as well as *n*-alkanes and isoprenoids, as a result of microbial activity in the second month (Fig. 4; 60 days). Only traces of C_{27} – 18α (H)-22,29,30-trisnorhopane (Ts) and C_{27} – 17α(H)-22,29,30-trisnorhopane (Tm) remained, as these had been the most abundant at the beginning of the bioremediation process.

An almost identical trend was confirmed for biodegradation of steranes (Fig. 5). In the first 30 days, the sterane abundance did not significantly change (Fig. 5; days 0 and 30), while at the end of the study, peaks originating from steranes were barely distinguishable from background peaks in the GC-MS analysis (Fig. 5; 60 days).

Recently, it was reported that successful *in situ* biostimulation of petroleum hydrocarbon biodegradation without bioaugmentation but by nitrate and phosphate injection was conducted, in which dissolved hydrocarbon concentrations including benzene decreased to non-detectable levels in less than three months [33]. However, according to present knowledge it is clear that biostimulation and bioaugmentation can have a synergistic effect on the bioremediation process.

The biodegradation of crude oil is often viewed as a stepwise process in which various compounds are removed/reduced in an organized and recognized sequence [32]. However, several compound classes are destroyed simultaneously but at different rates. This reflects differences in the rate of their catabolism under varying conditions [34]. In reservoir oils, studies have shown that polycyclic aromatic compounds such as phenanthrenes cannot be degraded prior to or concomitantly with sterane and terpane biomarkers [35]. It is also important to emphasize that many compounds can be degraded only by co-metabolism [36, 37]. However, based on the literature, after 75 days of simulated microbial biodegradation by zymogenous microorganisms,

phenanthrene and methyl phenanthrenes were degraded in polluted soil, while steranes and triterpanes retained their original abundance and distribution [32]. This suggests that polycyclic alkanes, steranes and triterpenes showed higher resistance toward biodegradation by zymogenous microorganisms compared with phenanthrene and its methyl-isomers. In natural bioremediation conditions, biodegradation of dimethyl-, and especially trimethyl-phenanthrene is slower in comparison with phenanthrene. In contrast, if bioremediation is carried out by bioaugmentation and biostimulation, with the addition of sawdust and biosurfactants, trimethyl- and dimethyl-phenanthrenes are degraded much faster than phenanthrene [30]. It was also confirmed that during natural ex situ bioremediation, phenanthrene and its methyl-isomers were degraded before complete decomposition of n-alkanes [38] and this is in line with results presented in this paper.

In comparison with the *n*-alkanes, isoprenoids, terpanes and steranes, biodegradation of phenanthrene and its methyl-, dimethyl- and trimethyl-isomers (Fig. 6), followed a different pattern. In fact, only these hydrocarbons underwent changes after the first 30 days of bioremediation. The intensity of these peaks after 30 days was significantly lower than the intensity of the corresponding peaks on day 0 (Fig. 6). At the end of the study, all these hydrocarbons (phenanthrene and its methyl-, dimethyl- and trimethyl-isomers) were degraded by more than 99% of their initial levels (Fig. 6; 60 days). This result is consistent with current knowledge, according to which phenanthrene compounds are more susceptible to biodegradation than polycyclic alkanes such as terpanes and steranes in environmental conditions during oil spills [39]. However, it is still somewhat surprising, since it follows that these aromatic phenanthrene compounds were more susceptible to biodegradation than the *n*-alkanes and isoprenoid aliphatic alkanes (Fig. 3), which is contrary to the generally accepted knowledge about biodegradability of petroleum compounds [3].

4 Concluding remarks

This study describes enhanced *in situ* bioremediation of groundwater contaminated by petroleum hydrocarbons. The efficiency of bioremediation was evaluated on the basis of changes in the content of TPH, TC and HD microorganisms, as well as on the basis of changes in the abundance and distribution of *n*-alkanes, isoprenoids, terpanes, steranes and phenanthrene and its methyl-, dimethyl- and trimethyl-isomers.

During bioremediation, the TPH content in groundwater was reduced dramatically. At the same time, the numbers of TC and HD microorganisms increased substantially. In the first 30 days of bioremediation, there was no significant biodegradation of *n*-alkanes, pristane, phytane, terpanes or steranes. However, after 60 days of bioremediation, saturated hydrocarbons, including alkanes and polycyclic-type steranes and terpanes, were biodegraded and reduced for more than 95% of their initial levels, which was not observed in a previous study of *ex situ* bioremediation. Interestingly, phenanthrene and its methyl-, dimethyl-, and trimethyl isomers were biodegraded and reduced for >99% of their initial levels after 60 days. However, their decomposition had clearly commenced after just 30 days. This is a somewhat surprising result, since it follows that the PAH, such as phenanthrene, were more susceptible to biodegradation than the *n*-alkanes and isoprenoid aliphatic alkanes. This is in contrast with generally accepted knowledge about the biodegradability of petroleum compounds. This suggests that utilization of a well-selected microbial community for bioaugmentation may stimulate biodegradation of some PAH such that it could precede biodegradation of saturated hydrocarbons. All these factors and results together provide evidence of the high efficiency of the applied method for removing oil pollutants from contaminated water by an *in situ* bioremediation treatment in a closed bipolar system.

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Fig. 1 GC×GC-MS 3D chromatograms of groundwater extracts before (day 0) and after (60 days) *in situ* bioremediation

Fig. 2 Distribution and abundances of *n*-alkanes and the isoprenoid aliphatic alkanes (pristane and phytane) (*m*/*z* = 71); terpanes (*m*/*z* = 191); steranes (*m*/*z* = 217); phenanthrene (and its methyl-, dimethyl- and trimethyl-isomers) (*m*/*z* = 178 + 192 + 206 + 220); at the beginning of the study (day 0)

Fig. 3 Changes in the distribution and abundance of *n*-alkanes and isoprenoid aliphatic alkanes pristane (Pr) and phytane (Phyt) during the 60-day *in situ* bioremediation

Fig. 4 Changes in the distribution and abundance of tricyclic diterpanes and pentacyclic terpanes in alkane fractions of the petroleum pollutant extracted from groundwater during the 60-day *in situ* bioremediation

Fig. 5 Changes in the distribution and abundance of diasteranes and C_{27}--C_{29} steranes in alkane fractions of the petroleum pollutant extracted from groundwater during the 60-day *in situ* bioremediation

Fig. 6 Changes in the distribution and abundance of phenanthrene and its methyl-, dimethyl- and trimethyl-isomers in the aromatic fractions of the petroleum pollutant extracted from groundwater during the 60-day *in situ* bioremediation

Table 1. Ion chromatogram characteristic for *n*-alkanes and isoprenoid aliphatic alkanes (*m*/*z* = 71), terpanes (*m*/*z* = 191), steranes (*m*/*z* = 217), phenanthrenes (*m*/*z* = 178 + 192 + 206 + 220)

m/z	**Identification**
71	**1** *n*-C_{15}; **2** *n*-C_{16}; **3** *n*-C_{17}; **4** pristane; **5** *n*-C_{18}; **6** phytane; **7** *n*-C_{19}; **8** *n*-C_{20}; **9** *n*-C_{21}; **10** *n*-C_{22}; **11** *n*-C_{23}; **12** *n*-C_{24}; **13** *n*-C_{25}; **14** *n*-C_{26}; **15** *n*-C_{27}; **16** *n*-C_{28}; **17** *n*-C_{29}; **18** *n*-C_{30}; **19** *n*-C_{31}; **20** *n*-C_{32}
191	**1** C_{19} – tricyclic terpane; **2** C_{20} – tricyclic terpane; **3** C_{21} – tricyclic terpane; **4** C_{23} – tricyclic terpane; **5** C_{24} – tricyclic terpane; **6** C_{25} – tricyclic terpane; **7** C_{24} – tetracyclic terpane; **8** C_{26} – tricyclic terpane; **9** C_{27} – 18α (H)-22,29,30-trisnorhopane (Ts); **10** C_{27} – 17α(H)-22,29,30-trisnorhopane (Tm); **11** C_{29} – 17α(H),21β(H)-hopane; **12** C_{30} – 17α(H),21β(H)-hopane
217	**1** C_{27} – 13β(H),17α(H) diasterane (20S); **2** C_{27} – 13β(H),17α(H) diasterane (20R); **3** C_{27} – 13α(H),17β(H) diasterane (20S); **4** C_{27} – 13α(H),17β(H) diasterane (20R); **5** C_{28} – 13β(H),17α(H) diasterane (20S); **6** C_{28} – 13β(H),17α(H) diasterane (20R); **7** C_{28} – 13α(H),17β(H) diasterane (20S) + C_{27} – 14α(H),17α(H) sterane (20S); **8** C_{29} – 13β(H),17α(H) diasterane (20S) + C_{27} – 14β(H),17β(H) sterane (20R); **9** C_{27} – 14β(H),17β(H) sterane (20S) + C_{28} – 13α(H),17β(H) diasterane (20R); **10** C_{27} – 14α(H),17α(H) sterane (20R); **11** C_{29} – 13β(H),17α(H) diasterane (20R); **12** C_{29} – 13α(H),17β(H) diasterane (20S); **13** C_{28} – 14α(H),17α(H) sterane (20S); **14** C_{29} – 13α(H),17β(H) diasterane (20R) + C_{28} – 14β(H),17β(H) sterane (20R); **15** C_{28} – 14β(H),17β(H) sterane (20S); **16** C_{28} – 14α(H),17α(H) sterane (20R); **17** C_{29} – 14α(H),17α(H) sterane (20S); **18** C_{29} – 14β(H),17β(H) sterane (20R); **19** C_{29} – 14β(H),17β(H) sterane (20S); **20** C_{29} – 14α(H),17α(H) sterane (20R)
178	**1** Phenanthrene
192	**2** Methyl-phenanthrenes
206	**3** Dimethyl-phenanthrenes
220	**4** Trimethyl-phenanthrenes

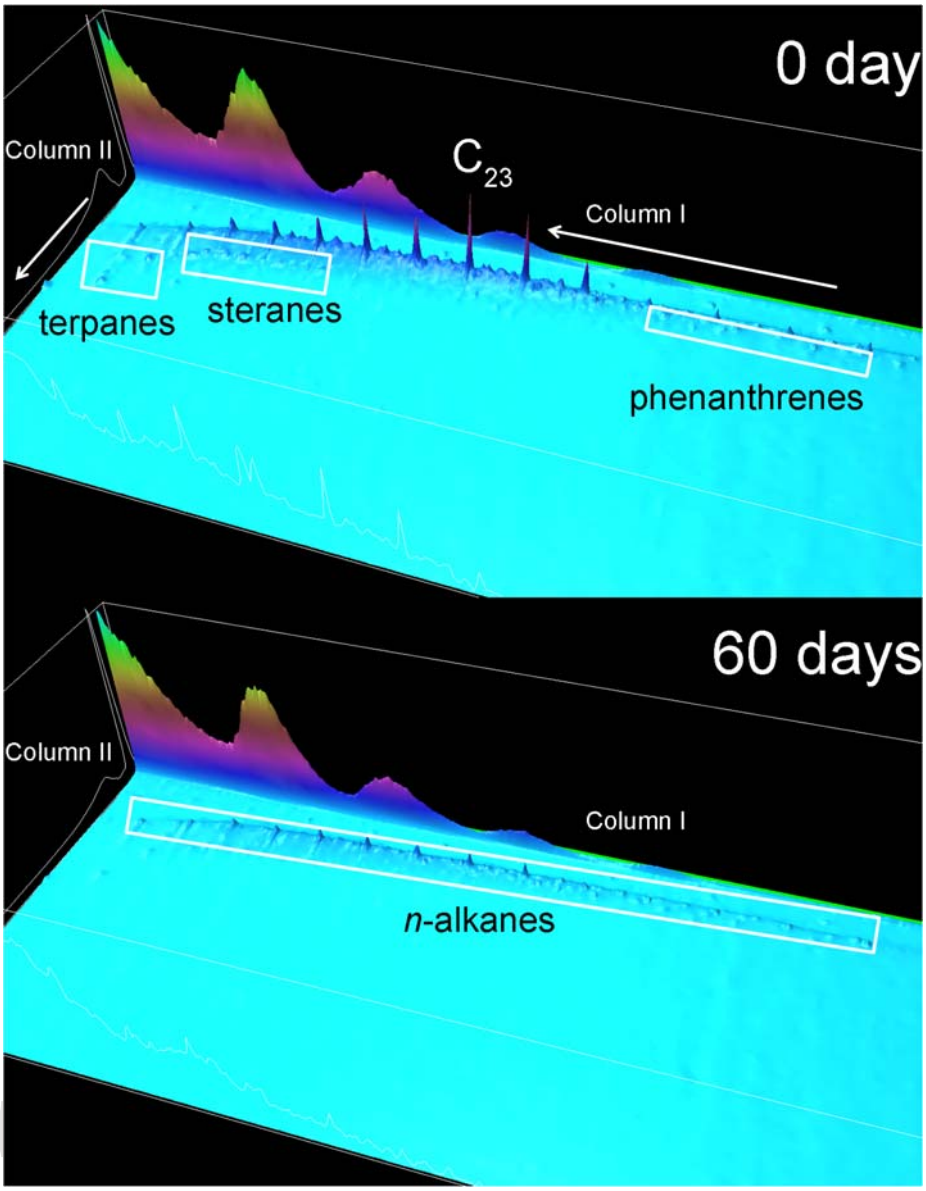


Figure 1

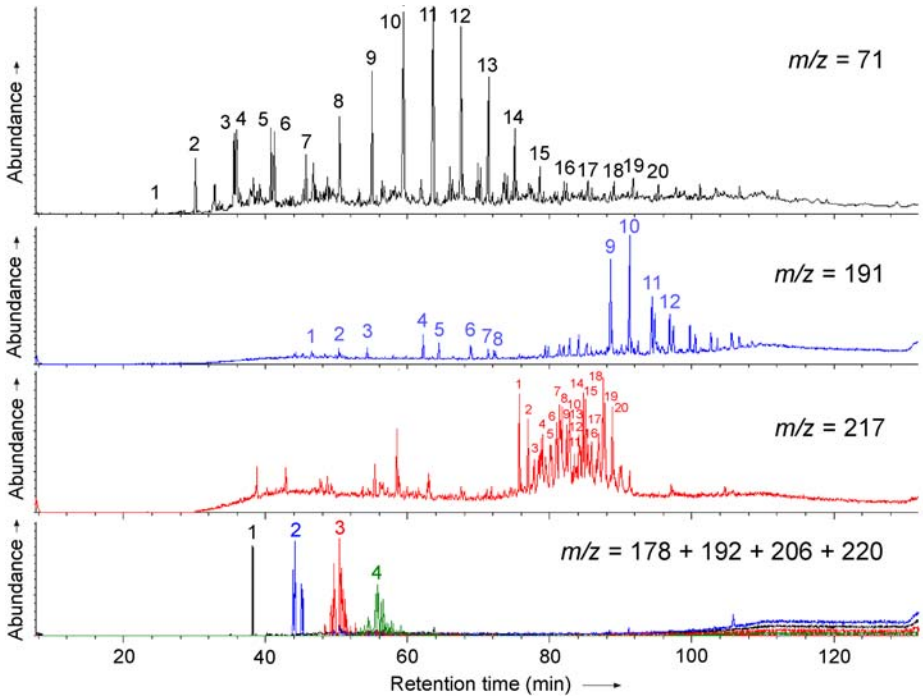


Figure 2

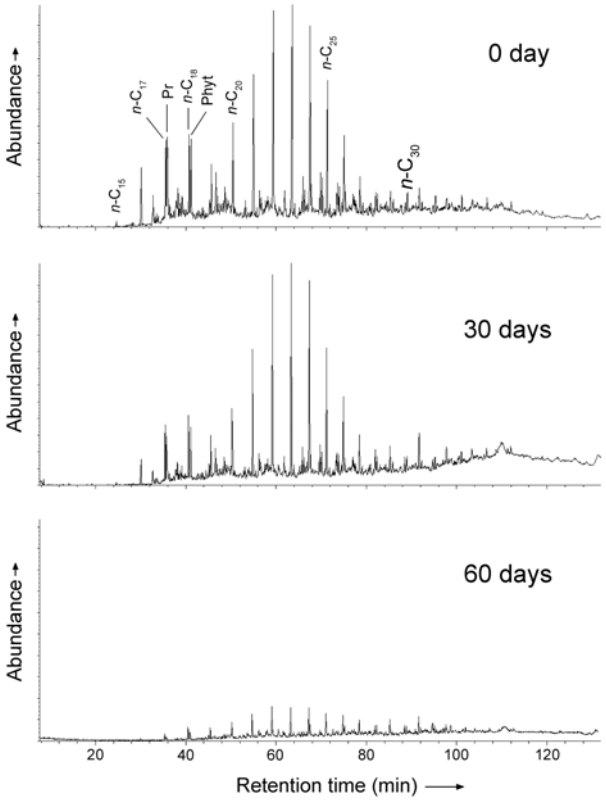
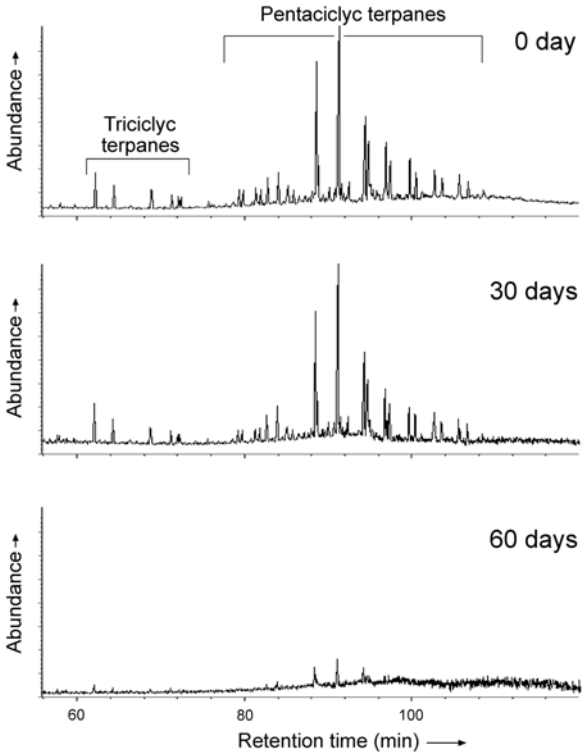


Figure 3



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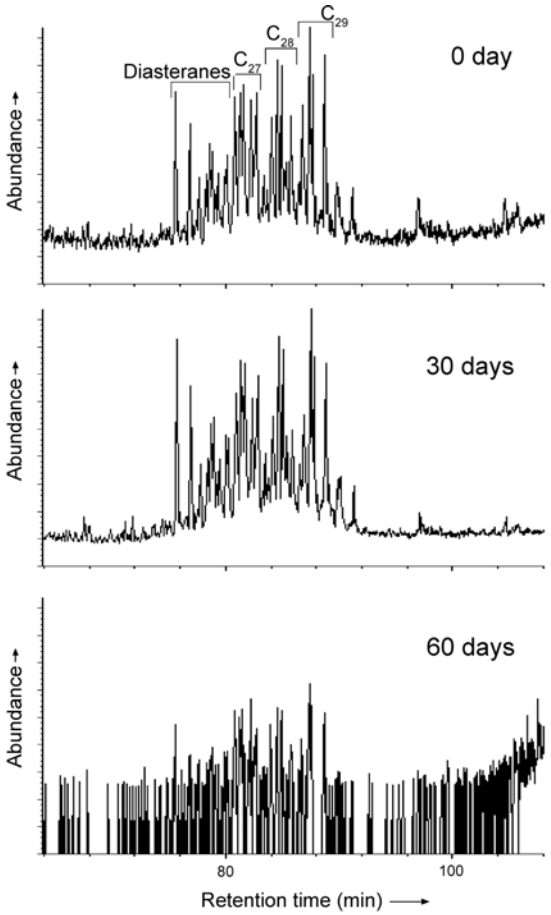

Figure 5

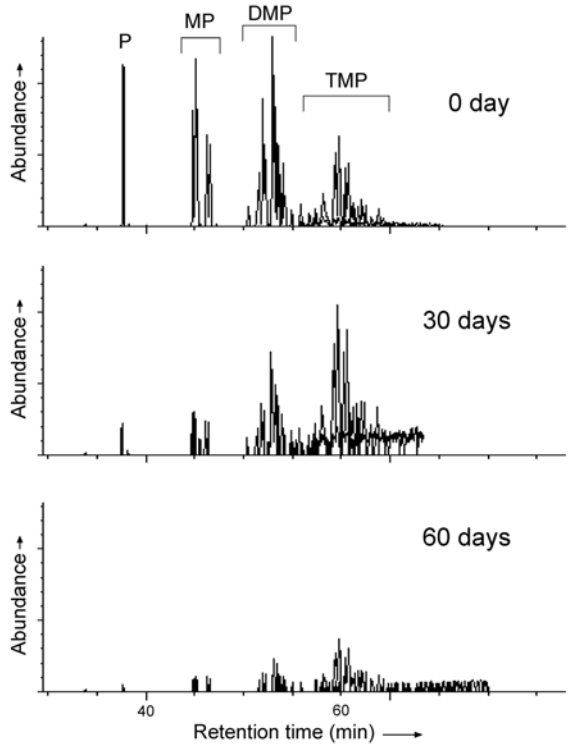

Figure 6