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Characterisation of biodegradation capacities of environmental microflorae for diesel oil by comprehensive two-dimensional gas chromatography

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Abstract

In contaminated soils, efficiency of natural attenuation or engineered bioremediation largely depends on biodegradation capacities of the local microflorae. In the present study, the biodegradation capacities of various microflorae towards diesel oil were determined in laboratory conditions. Microflorae were collected from 9 contaminated and 10 uncontaminated soil samples and were compared to urban wastewater activated sludge. The recalcitrance of hydrocarbons in tests was characterised using both gas chromatography (GC) and comprehensive two-dimensional gas chromatography (GC×GC). The microflorae from contaminated soils were found to exhibit higher degradation capacities than those from uncontaminated soil and activated sludge. In cultures inoculated by contaminated-soil microflorae, 80% of diesel oil on an average was consumed over 4-week incubation compared to only 64% in uncontaminated soil and 60% in activated sludge cultures. As shown by GC, *n*-alkanes of diesel oil were totally utilised by each microflora but differentiated degradation extents were observed for cyclic and branched hydrocarbons. The enhanced degradation capacities of impacted-soil microflorae resulted probably from an adaptation to the hydrocarbon contaminants but a similar adaptation was noted in uncontaminated soils when conifer trees might have released natural hydrocarbons. GC×GC showed that a contaminated-soil microflora removed all aromatics and all branched alkanes containing less than C_{15}. The most recalcitrant compounds were the branched and cyclic alkanes with 15–23 atoms of carbon.

Introduction

As a result of human activities, large quantities of hydrocarbons are released into the environment. It is estimated that about 0.1% of the world wide production (3650 million tons in 2003 according to www.cpdp.org) is discharged through the processes of recovery, transport, refining and product usage (Ward et al. 2003). Due to their negative impact on health and environment, released hydrocarbons are considered as major contaminants of soil and groundwater. Therefore, detailed information on their biodegradation properties is required to implement efficient removal treatments or monitoring of natural attenuation (Gallego et al. 2001; Salanitro 2001).

The biodegradability of hydrocarbons can be assessed through laboratory tests, which indicate, in particular, if microorganisms from the polluted site have the capability to transform the contaminants (Röling & van Verseveld 2002). Some soil microorganisms may exhibit unusual extended degradation capacities especially when they are able to degrade molecules such as hydrocarbons

with quaternary carbon atoms or consecutively methylated carbon chains (Solano-Serena et al. 1999). In microflorae, intrinsic efficiency of individual microorganisms is often reinforced by the co-operation processes prevailing among the members of microbial communities (Beam & Perry 1974; Penet et al. 2004).

The identification of hydrocarbons recalcitrant to biodegradation is a real challenge for a detailed assessment of degradation capacities of microflorae. Owing to the high-resolution capacities of capillary column (Di Sanzo et al. 1988; Blomberg et al. 2002), which enables separation of more than 500 components (Teng et al. 1994), gas chromatography (GC) has become the tool of choice for analysis of hydrocarbons. However, GC fails to separate all individual hydrocarbons from petroleum cuts such as those of diesel oil.

To overcome the limitations of conventional GC, on-line hyphenated analytical procedures were performed in the recent years (Mondello et al. 2001), leading to comprehensive two-dimensional gas chromatography (GC×GC). The GC×GC technique relies on the coupling of two GC columns with different selectivity properties. A device, called the modulator, samples the effluent from the first column and focuses small portions of solutes that are released into a second column for further separation.

The GC×GC approach is truly comprehensive in that no information gained in the first separation is lost during the second one. Assuming that n_1 and n_2 are the individual peak capacities of the first and second columns respectively, the resulting peak capacity of the comprehensive GC×GC system will be $n_1 \times n_2$. The resolution efficiency is achieved by selecting in the first dimension a non-polar column, which provides a separation based on the volatility of analytes (boiling point separation). All analytes elute from the first column at different temperatures, but volatility properties are very similar at a given retention time. The second separation is usually performed using a polar column and is fast enough to be considered as essentially isothermal at any elution temperature from the first dimension column. The second separation is completely determined by the activity coefficient that can be itself related to polarity, molecular geometry and size (Bertsch 1999; Bertsch 2000). As a result, GC×GC was found to be a powerful analytical technique for the characterisation of hydrocarbons in complex matrices since a breakdown according to the carbon atom number and to the chemical class (alkanes, mono-, di- and tri-aromatics) is easily achieved. Considering crude oil analysis, GC×GC was successfully applied to the characterisation of the so-called ''unresolved complex mixture'' (UCM) of petroleum-contaminated sediments, UCM describing the raised of baseline hump observed in petroleum chromatograms (Frysinger et al. 2003).

In the present work, the overall degradation capacities of contaminated- and uncontaminated-soil microflorae were determined by degradation tests performed in closed systems. In order to characterise the recalcitrance of hydrocarbons, the residual compounds were identified and quantified by using GC×GC with flame-ionisation detection (FID).

Materials and methods

Culture media and microflorae

The vitamin-supplemented mineral salt medium described by Bouchez et al. (1995) was used as a nutrient solution. Diesel oil from a city service station was used as only carbon source at 400 mg l^{-1}. The microbial suspensions of the biodegradation tests were prepared by dispersing directly into the nutrient solution either 5 g l^{-1} of soil sample or 100 mg l^{-1} (dry weight) of activated sludge.

Characterisation of soils

Samples were collected from upper layers of contaminated and uncontaminated soils. They were stored at 4 °C in closed plastic boxes. No significant modification in the biodegradation capacities of the microflorae was noted over a period of several months.

Dry mass of soil samples was determined by weight loss after heat treatment according the norm NF ISO 11465 (1993). Hydrocarbon content of soils was obtained by GC equipped with FID after cyclohexane/acetone (85/15 v/v) extraction. Ten grams of soil were extracted twice for 1 h with 100 ml of the solvent mixture. The hydrocarbon content of the pooled organic phase was measured using an external standard method and

a calibration curve drawn with diesel oil standards. The Pollut-Eval^{TM} (Vinci Technologies, Nanterre, France) method was also used (Ducreux et al. 1997). Portions (300 mg) of soil samples were heated at 200 °C in a di-nitrogen stream and hydrocarbons were determined by FID. The pH values of soils were determined according to the norm NF ISO 10390 (2005) and their granulometric characteristics according to the method of Musy & Soutter (1991).

Biodegradation tests

Biodegradation tests were performed in 120-ml flasks closed with Teflon-coated stoppers and sealed with aluminium caps. A 5 μl of commercial diesel oil was added to 10 ml of inoculated culture medium. After an incubation period of 28 days at 30 °C with alternative shaking (60 strokes per min), 10 ml of CH_2Cl_2 were introduced in the flasks, which were stored one night at −20 °C before extraction. The CH_2Cl_2 phase of each flask was analysed by GC-FID after evaporation to 1.5 ml without drying. Dotriacontane (nC_{32}) (50 mg l^{-1} in CH_2Cl_2) was used as an internal standard. Experiments were carried out at least in triplicate and abiotic controls supplemented with $HgCl_2$ were run under similar conditions. The degradation extents were calculated as the ratios of substrate degraded in test flasks to substrate recovered in abiotic controls.

Kinetics of CO_2 production during diesel oil degradation was determined at 30 °C by GC of the headspace. The final amount of CO_2 produced in cultures was determined similarly at the end of the incubation after acidification with 200 μl of 4 N HCl. Mineralisation yield was referred to as the carbon molar ratio of the difference of total CO_2 in test flask and in hydrocarbon-free flask to the consumed hydrocarbons.

Chromatographic analyses

CO_2 was measured with a chromatograph equipped with a thermal conductivity detector and a Porapak Q column (80/100 mesh, 2 m) using an external standard method. The carrier gas was helium and the column temperature was 50 °C. The temperatures of injector and of detector were 100 °C. Samples (100 μl) of the headspace gas in culture flasks were withdrawn with a gas-tight syringe and injected into the chromatograph for CO_2 analysis.

Residual diesel oil was analysed and quantified with a 3400 chromatograph (Varian, USA) equipped with a flame ionisation detector and a 60-m DB-5 column. The carrier gas was helium. The detector temperature was 310 °C. The column temperature was first set at 50 °C for 10 min and increased to 310 °C at 2 °C min^{-1}. The injector temperature was first set at 50 °C for 0.2 min and then increased to 280 °C at 180 °C min^{-1}. Quantification of diesel oil was obtained by global integration of the separated peaks and unresolved complex mixture using BORWIN software (JMBS France).

GC×GC analyses were carried out using a HP6890 gas chromatograph (Agilent technologies) equipped with a flame ionisation detector. Cryogenic modulation was performed with a dual-stage CO_2 jet modulator built in-house according to the description given elsewhere (Beens et al. 2001). A 6-sec modulation period was chosen. The column set was an apolar polydimethylsiloxane column (20 m×0.2 mm, I.D.: 0.5 μm, PONA, Agilent Technologies) connected to a narrow-bore semi-polar (50% phenylpolysilphenylene-siloxane) column (1 m×0.1 mm I.D.: 0.1 μm, BPX50, SGE). Both columns were placed in the chromatograph oven, programming of which increased from 50 to 310 °C at 2 °C min^{-1}. Helium was the carrier gas and the column inlet pressure was kept constant at 240 kPa. Diesel oil or extracts obtained after degradation were injected (1 μl) in the splitless mode at 280 °C. Data acquisition was performed at 100 Hz using ChemStation software (Agilent Technologies). A Matlab program written in-house allowed data conversion for visualisation of the two-dimensional chromatograms and integration of the elution peaks displayed as spots on the retention plane.

Results

Characterisation of the soil samples

The environmental microflorae were obtained from samples of 10 uncontaminated soils and nine contaminated soils taken at low depth from the soil surface. Samples were characterised by

their moisture content, structural granulometry, pH and total organic carbon (TOC) (Table 1). Most of the soils were sandy and pH values ranged from 4.63 to 8.39. The TOC content was variable (from 0.79 to 73 mg per g of soil). In order to discriminate between contaminants and humic fraction, Pollut-Eval analyses were carried out. As an example, Figure 1 shows the FID response of soil sample 14 to the increasing temperature profile used. Contaminants were eluted at temperatures lower than 350 °C and humic acid fraction was eluted above. Contaminants were extractable from soil matrix using apolar solvents whereas humic fraction was not (Figure 1). Elution temperature profile of the first chromatogram peak allowed the characterisation of the contaminants involved. Various oil cuts were found to contaminate the soil samples since jet fuel (soil samples 11, 12 and 13), diesel oil (soil samples 14, 15, 16 and 19) or crude oil (soil samples 17 and 18) were detected (Table 1). As calculated by peak integration, they amounted to between 2 and 10 g per kg of dry soil.

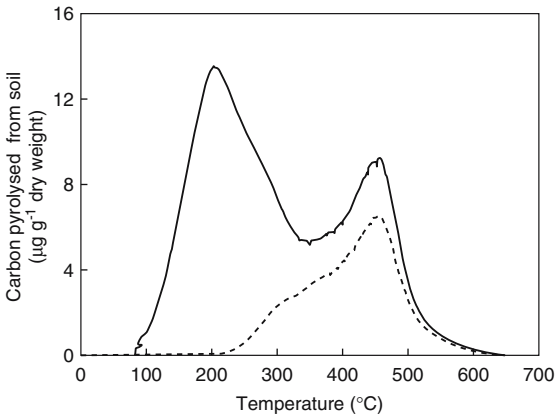


Figure 1. Pyrolysis spectra from contaminated soil sample. Without solvent extraction (continuous line); after removal of extractable contaminants by solvent (dotted line).

Biodegradation capacities of soil microflorae

The biodegradation capacities of soil microflorae were determined with cultures carried out in non-limiting conditions, in particular regarding oxygen, available in excess in flask headspace. The end point of test periods (usually 28 days) was determined by

Table 1. Characteristics of the collected soil samples

Soil sample	Origin	Moisture[a]	pH[b]	TOC[c]	Pollution type[d]	Granulometry[e]
1	Garden	10	8.11	22	None	Sand:Clay (79:19)
2	Field	10	8.36	10	None	Sand:Clay (64:21)
3	Garden	7	8.27	5	None	Sand:Clay (56:43)
4	Pine forest	16	4.63	21	None	Sand:Clay (68:27)
5	Garden	18	7.55	29	None	Sand:Clay (67:27)
6	Garden	25	7.79	38	None	Sand:Clay (53:43)
7	Vineyard	36	7.25	30	None	Sand:Clay (19:78)
8	Forest	32	5.65	27	None	Sand:Clay (14:82)
9	Garden	16	8.34	20	None	Clay:Gravel (43:23)
10	Garden	23	7.26	19	None	Sand:Clay (39:55)
11	Polluted site	18	8.21	1.37	Jet fuel (2.0)	Sand:Clay (18:81)
12	Polluted site	21	7.95	2.34	Jet fuel (2.4)	Sand:Clay (65:23)
13	Polluted site	19	8.31	0.79	Jet fuel (2.2)	Sand:Clay (38:61)
14	Polluted site	27	6.05	36	Diesel oil (10)	Gravel:Clay (42:36)
15	Polluted site	14	8.15	3	Diesel oil (2.7)	Sand:Clay (29:71)
16	Polluted site	18	8.39	12	Diesel oil (4.4)	Sand:Clay (53:34)
17	Polluted site	0.40	6.93	9	Crude oil (9)	Sand (100)
18	Polluted site	9	6.41	73	Crude oil (10)	Sand (100)
19	Polluted site	19	8.12	8	Diesel oil (3)	Sand:Clay (48:28)

[a]Moisture content (%) was determined from the weight loss after heat treatment according to the norm ISO11465.
[b]According to the norm ISO10390.
[c]Total organic carbon in mg of carbon per gram of dry soil.
[d]Amounts of contaminating hydrocarbons in g per kg of soil dry weight are indicated between parentheses.
[e]Relative composition is indicated between parentheses.

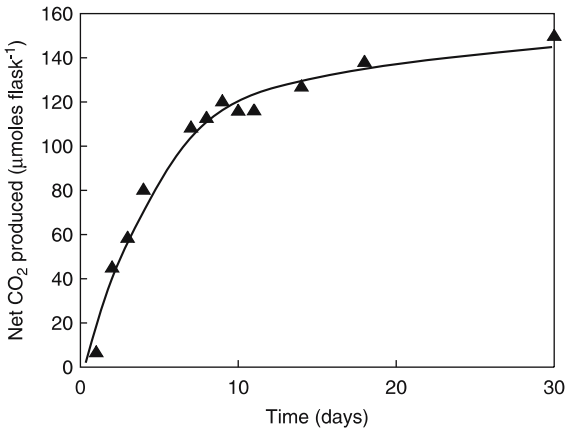


Figure 2. Kinetics of CO_2 production during diesel oil biodegradation by microflora from soil sample 14. CO_2 from endogenous respiration (substrate-free flask) are deducted. Tests were performed in 120-ml closed flasks, with 10 ml of medium, 400 mg l^{-1} of commercial diesel oil and 5 g l^{-1} of soil.

monitoring of CO_2 production in test-flask headspaces. A typical time course of CO_2 production is presented in Figure 2. The reaction was stopped when no additional CO_2 was produced. Degradation capacities of microflorae were assessed by residual hydrocarbons in test and abiotic flasks using GC.

The degradation capacities of microorganisms from different soils and their related mineralisation yields are shown in Table 2. In all abiotic controls, the recovery rate (calculated as final diesel oil recovered and measured by GC to diesel oil initially introduced) was higher than 75%. This indicated that the methodology used for the biodegradation measurements was quite satisfactory. The mean-value of biodegradation capacities of the uncontaminated soils (67%) was close to that of activated sludge (64%). However, significant disparities were observed within results, which varied from $50 \pm 8\%$ to $96 \pm 1\%$. Degradation performances could not be related on soil granulometry but rather to the organic matter content. The mean value of biodegradation capacities of microflorae from contaminated soils (80%) was higher than that of the uncontaminated ones (67%). Large variations in biodegradation capacities were observed as well. Contaminated marsh samples 17 and 18 exhibited biodegradation capacities (69% and 65% respectively) close to those of uncontaminated-soil samples. In contrast, diesel-impacted soils (samples 14, 15, 16, 19) were the most efficient for substrate biodegradation. In particular, soil sample 19 that had been contaminated for about 20 years by domestic-fuel leaks displayed a remarkable high biodegradation capacity (98%).

Table 2. Degradation capacities of the microflorae used

Microflora	Abiotic recovery[a](%)	Degradation extent[b](%)	Mineralisation yield[c](%)
Soil 1	88 ± 5	58 ± 5	66 ± 12
Soil 2	83 ± 3	77 ± 1	73 ± 2
Soil 3	79 ± 4	58 ± 8	54 ± 7
Soil 4[d]	80	87	55
Soil 5	82 ± 2	31 ± 5	71 ± 5
Soil 6	90 ± 5	59 ± 5	64 ± 8
Soil 7	90	87 ± 1	54 ± 6
Soil 8	83	69 ± 3	44 ± 5
Soil 9	84	96 ± 1	69 ± 7
Soil 10	76	50 ± 8	66 ± 8
Soil 11	91 ± 6	82 ± 1	55 ± 1
Soil 12	92 ± 2	81 ± 2	70 ± 1
Soil 13	94 ± 7	70 ± 5	57 ± 5
Soil 14	88 ± 0	93 ± 2	54 ± 10
Soil 15	84 ± 2	81 ± 3	70 ± 4
Soil 16	84 ± 1	83 ± 4	67 ± 1
Soil 17	97 ± 0	69 ± 2	40 ± 2
Soil 18	78 ± 0	65 ± 7	39 ± 9
Soil 19	76 ± 5	98 ± 0	64 ± 6
Activated sludge 1 [d]	90	61	54
Activated sludge 2 [d]	95	73	63
Activated sludge 3	84 ± 0	59 ± 5	57 ± 5

[a]Hydrocarbons recovered in the abiotic flasks with respect to initial hydrocarbons supplied.
[b]Calculated as the ratio of the amount of substrate degraded in test flasks to the amounts of recovered substrate in the abiotic controls. Test was performed in triplicate and standard deviation is indicated.
[c]Carbon molar ratio of produced CO_2 to consumed hydrocarbons.
[d]Only one test was performed.

The yield of CO_2 production calculated with respect to consumed hydrocarbons did not depend on the presence of contaminants in soils. The mineralisation yields were higher than 50% in all assays except for soil samples 17 and 18 where they were only 40% and 39%, respectively. In the latter cases, metabolic intermediates derived from hydrocarbons might accumulate in the culture broth in addition to the biomass increase (Penet et al. 2004).

Determination of residual hydrocarbons

The residual hydrocarbons after biodegradation by the microflorae of an uncontaminated soil, a contaminated soil and an activated sludge were determined and compared to those initially present in the diesel oil (Figure 3). Figure 3a and a′ show the patterns of initial diesel oil obtained by GC and GC×GC, respectively. The GC chromatogram (Figure 3a) displayed a quite satisfactory resolution for all *n*-alkanes and some particular branched alkanes such as farnesane, pristane or phytane. However, a large part of branched and cyclic alkanes and of aromatics remained unresolved and was eluted in the hump of UMC. In comparison to GC, the GC×GC chromatogram of initial diesel oil (Figure 3a′) was more detailed thanks to the discriminative effect of the second-dimension column towards the chemical structures. As previously indicated (Vendeuvre et al. 2005), all chemical classes of compounds in diesel oil could be unambiguously localised in the retention plane and integrated since the group-type quantification was relevant to diesel oil analysis. The chemical classes were delimited consequently using retention data of standard compounds and a specific program function that adjusted spot outlines thanks to density increase of the peak valleys at boundaries of adjacent groups. Table 3 indicates the composition into saturated (linear, branched and cyclic) alkanes and mono-, di-, and tri-aromatics found for diesel oil.

Parts b, c and d in Figure 3 show the GC profiles of residual hydrocarbons after biodegradation by activated sludge, uncontaminated-soil sample 1 and contaminated-soil sample 14, respectively. Parts b′, c′ and d′ in Figure 3 show the homologous profiles obtained by GC×GC. *N*-alkanes were completely degraded by each microflora, as shown both by GC and GC×GC analyses. The residual chemical classes were detailed by GC×GC using the elution zones drawn from initial diesel oil as references and assuming stable retention times in both dimensions. In the diesel oils biodegraded by microflora from activated sludge or uncontaminated soil, the relative amounts of alkanes, mono-, di-, and tri-aromatics were similar those of initial diesel oil. However, a distinct pattern including lower amounts of di- and tri-aromatics was noted for contaminated-soil microflora (Table 3). The detailed analysis of GC×GC patterns of biodegraded diesel oils indicated that aromatics with less than 12 carbon atoms and di-aromatics with less than 13 carbon atoms were extensively biodegraded. The recalcitrant hydrocarbons were mainly the branched and cyclic alkanes ranging from 15 to 23 carbon atoms.

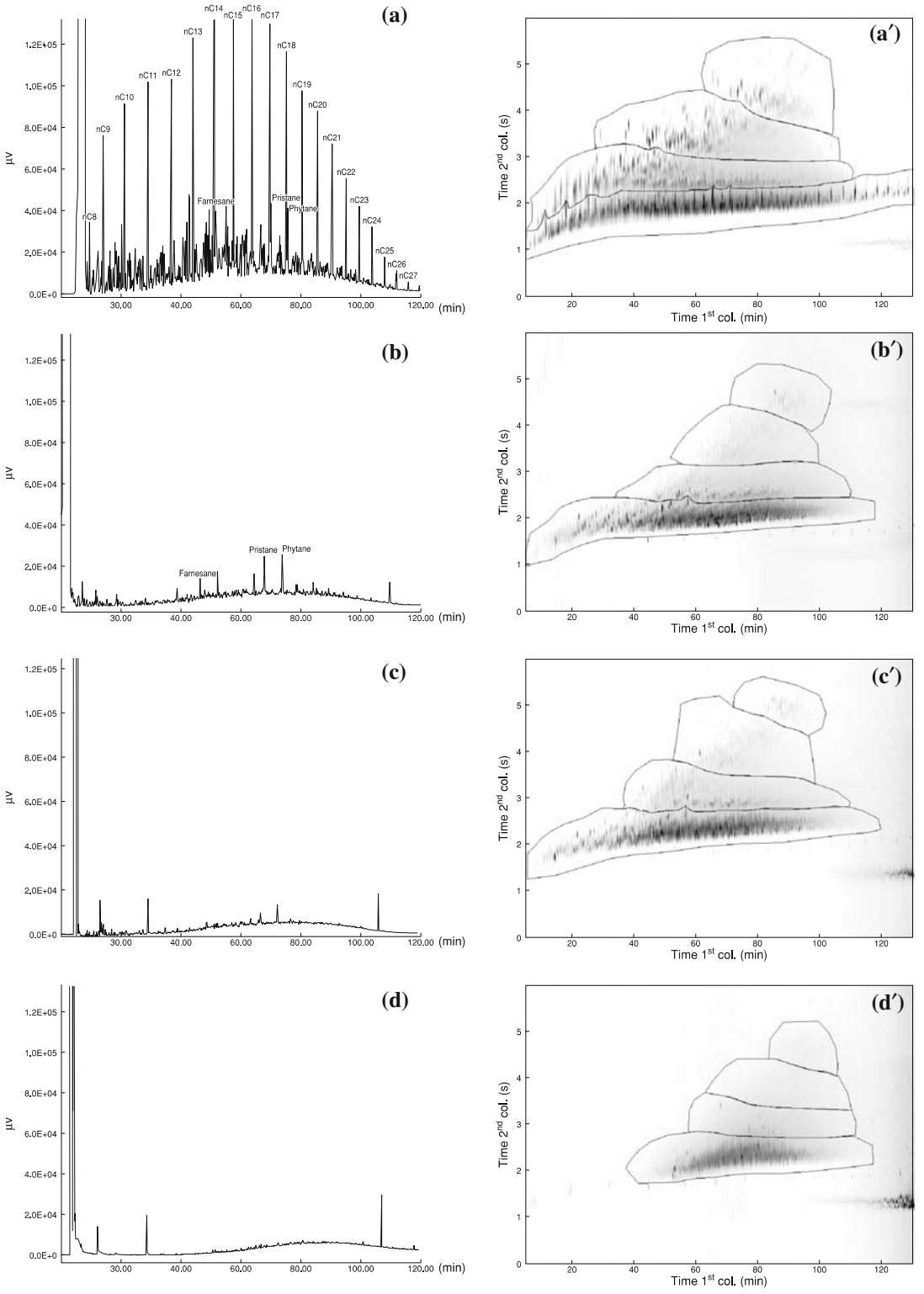
Discussion

GC×GC has been rarely applied to resolve environmental problems (Frysinger et al. 2003). In this study, it was used to discriminate between alkanes and aromatics in the UMC of diesel oil.

The capacity of GC×GC to resolve hundred or more constituting hydrocarbons of diesel oil was demonstrated. The hydrocarbons from C_8 to C_{32} were distinctly resolved in the first dimension whereas alkanes were separated from mono-, di-, and tri-aromatic hydrocarbons in the second one. The quantitative results obtained were in good agreement with other classical less-discriminative methods since relative amounts determined by HPLC were 64%, 19%, 15% and 2%, respectively (Penet et al. 2004). Subtle variations in hydrocarbon composition could be detected without performing the heavy large-scale separation into structural classes required by the silica-gel liquid chromatography (Olson et al. 1999).

GC×GC was found to be a remarkable tool to detail the biodegradability of diesel oil. In all tests, *n*-alkanes were found thoroughly biodegraded and the residual compounds were principally branched alkanes as shown by the main individual spots identified in alkane-elution zone. Thus, the main characteristics of hydrocarbon biodegradability were globally in accordance with published results (McKenna & Kallio 1971; Cerniglia & Perry 1973; Pirnik et al. 1974; Van Hamme et al. 2003;

Figure 3. GC- (a to d) and GC×GC (a′ to d′) chromatograms of residual hydrocarbons from commercial diesel oil after degradation: abiotic flask (a, a′); flasks inoculated respectively, with activated sludge (b, b′), with uncontaminated soil sample (c, c′), and contaminated soil sample 14 (d, d′). Incubation time was 28 days. The *x*-axis of GC×GC chromatograms is the retention in the first dimension (apolar column) and the *y*-axis is the relative retention in the second dimension (semi-polar column). Elution zones represents the different chemical classes: Alkanes (A), mono- (M), di- (D), tri- (T) aromatics.


(a)
(a′)
(b)
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(c)
(c′)
(d)
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nC17
nC18
nC19
nC20
nC21
nC22
nC23
nC24
nC25
nC26
nC27
Farnesane
Pristane
Phytane
Time 2nd col. (s)
Time 1st col. (min)

Table 3. Chemical classes determined by GC×GC of diesel oil after degradation by various microflorae

Microflora origin	Alkanes (%)	Mono-aromatics (%)	Di-aromatics (%)	Tri-aromatics (%)
No microflora	65.1	22.3	10.0	2.6
Uncontaminated soil	59.8	26.2	11.5	2.5
Activated sludge	64.5	24.2	8.5	2.8
Contaminated soil	66.2	26.7	5.9	1.2

The incubation time was 28 days.

Chaillan et al. 2004). However, particular variations could be noticed. Cyclic or branched alkanes with less than 15 carbon atoms might be completely degraded by some contaminated-soil microflorae whereas they were not degraded by microflorae from uncontaminated soils (Figure 3). Noticeable differences were also observed in the degradation of the di- and tri-aromatics.

The analysis of biodegradation capacities highlights the adaptation capacity of microflorae in impacted soils. The adaptation of microflorae to contaminant has been already mentioned in the case of gasoline degradation (Horowitz & Atlas 1977; Solano-Serena et al. 2000). In this work, comparison between contaminated and uncontaminated soil samples demonstrated such an adaptation to pollution exposure since the degradation capacities of microflorae from contaminated soils were higher than those of uncontaminated soils or activated sludge. Considering uncontaminated soil samples 4 and 9, the conifer trees that grew at the sampling site might have released terpenic compounds acting as selective substrates on microflora compositions.

Field experiments have shown that the rate of natural attenuation of hydrocarbon in impacted-soils depended on the abundance and diversity of microorganisms present in the environment and on physico-chemical parameters such as nutrients, oxygen and temperature (Morgan & Watkinson 1989; Leahy & Colwell 1990). In the engineered bioremediation domain, the selection of a strategy largely depends on the natural degradation capacities of local microflorae (Delille et al. 2004). In this respect, GC×GC is a promising technique to assess in detail the biodegradation capacities of the local microflorae. Hyphenation with mass spectrometry that has already been demonstrated as a powerful tool for investigating chemical structures in complex hydrocarbon matrices (Van Deursen et al. 2000) could be very useful for this purpose. We hope to implement this technique in the future to learn more about what compounds are recalcitrant in spilled oil.

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