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Distribution in the environment of degradative capacities for gasoline attenuation

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Abstract

A methodology allowing the detailed assessment of the capacities of microflorae to degrade gasoline in aerobic conditions has been developed. It consisted in the determination of the degradation of a gasoline model mixture in liquid cultures in optimal conditions. The gasoline model mixture contained 23 representative hydrocarbons of gasoline (GM23). The kinetics and extent of biodegradation were evaluated by continuous overall monitoring of CO_2 production and final chromatographic analysis (usually after about 30 days) of the consumption of each hydrocarbon. The methodology was used with soil and water samples from polluted and non polluted sites. The experimentation aimed at assessing the distribution of the degradative capacities in the environment and the prospects for natural attenuation of gasoline. Nine microflorae were tested. The intrinsic biodegradability (existence of mechanisms of biodegradation) appeared total for GM23 as shown by the results obtained with several microflorae. The degradative capacities of microflorae from non polluted samples were high (total degradation rates at least 85%). Incomplete degradation was observed essentially for trimethylalkanes (2,2,4-trimethylpentane and 2,3,4-trimethylpentane) and for cyclohexane. In several cases, samples from polluted sites exhibited more extensive degradative capacities, with total degradation of all hydrocarbons being observed for three out of the six samples.

Introduction

It is presently recognised that integrated knowledge of environmental systems is needed to provide adequate solutions to complex problems such as pollution of soils and aquifers. For a defined pollutant, knowledge of its intrinsic biodegradability (existence and mechanisms of biodegradation processes) and of the distribution in the environment of the capacities to degrade the pollutant, are critical for this goal (Logan & Rittman 1998).

In the case of petroleum products, which constitute the most conspicuous organic pollutants of soils, this question is difficult because of the complexity of the products and requires to devise appropriate methodologies. We devised such methodologies to assess first the extent of intrinsic biodegradability of gasoline in aerobic conditions and then to evaluate the specific capacities of microflorae from various origins. We used a gasoline model-mixture of the 23 most representative hydrocarbons of gasoline (GM23) and assessed in optimal conditions the degradation of individual hydrocarbons of the mixture (Solano-Serena et al. 1998). We report here on the results of a survey of the degradative capacities of microflorae from various environmental sites using the GM23 test.

Materials and methods

Culture media

The nutrient solution was the vitamin-supplemented mineral salt medium described by Bouchez et al.

(1995). The sole carbon and energy source was the hydrocarbon mixture GM23 which contained equal volumes of the 23 most representative hydrocarbons of the C_6–C_{10} gasoline fraction (Solano-Serena et al. 1998). GM23 was added at 400 mg l^{-1} to the nutrient solution to perform the degradation tests.

Microflorae

Several microbial suspensions were used for the gasoline-model degradation test. These microbial suspensions were prepared from various microflorae:

- A microflora from a waste water treatment plant (sample 1). It was obtained by centrifugation and re-suspension in the nutrient solution as described by Solano-Serena et al. (1999b).
- Microflorae from polluted and non polluted soils (samples 2 to 8). Microbial suspensions were directly prepared with 20 g of soil per litre of nutrient solution.
- A microflora from gasoline-polluted ground water (sample 9). It was obtained by centrifugation of the polluted water at 15000 g for 10 min. The microbial suspension was obtained after re-suspension in the same volume of nutrient solution.

Biodegradation of the gasoline model mixture GM23

The biodegradation tests were performed as described by Solano-Serena et al. (1998). 500-ml flasks with sidearms equipped with Mininert valves containing 50 ml of microbial suspension were used to carry out the biodegradation tests. 25 μl of GM23 were weighed and added to the flasks. After incubation at 30 °C, under alternative shaking, the remaining hydrocarbons were extracted by CH_2Cl_2, with dodecane as internal standard. The organic phases from each flask were analyzed by gas chromatography. Abiotic controls and flasks without added substrate (GM23-free flasks) were run simultaneously with test flasks.

Biodegradation kinetics were followed with CO_2 monitoring on additional flasks incubated in the same conditions as the flasks used for hydrocarbon analysis. 250 μl of the gas from the head space were sampled with a gas-tight syringe and CO_2 was determined by gas chromatography.

Chromatographic analyses

CO_2 and hydrocarbon analyses were performed as described by Solano-Serena et al. (1998). CO_2 was determined with a Girdel Série 30 chromatograph equipped with a Porapak Q column and a thermal conductivity detector. The remaining hydrocarbons were determined after CH_2Cl_2 extraction as described above using a Varian 3400 chromatograph equipped with a CP-Sil PONA CB column and a flame ionization detector.

Chemicals

n-Alkanes, cyclohexane, benzene, toluene and xylenes were purchased from Prolabo (Fontenay-sous-Bois, France). Other hydrocarbons and vitamins were from Fluka-Sigma (Saint-Quentin-Fallavier, France).

Results

Methodology

The gasoline-model degradation test was developed to determine the intrinsic capacities of microflorae from various environments to degrade gasoline in aerobic conditions (Solano-Serena et al. 1998, 1999a). Microbial degradation was performed in optimal conditions concerning in particular substrate toxicity and accessibility, nutrient and O_2 availability. The molar ratio of available dioxygen to the carbon of GM23 in incubation flasks was 3.6. The assessment of the biodegradative capacities consisted in two points:

1. Monitoring of the biodegradation kinetics using CO_2 measurement, in particular to indicate the biodegradation end point,

2. Quantitative determination by gas chromatography of the final degradation of each individual component of the hydrocarbon mixture when CO_2 production had stopped.

The gasoline model mixture GM23 used is shown in Table 1. It was more simple to handle than the corresponding gasoline-cut since all compounds were nearly present at the same concentration (Solano-Serena et al. 1998). It also allowed a sizeable representation of each hydrocarbon family whereas benzene, toluene, ethylbenzene, xylenes (BTEX) represented as much as 67% of a topped-gasoline cut shown in comparison (Figure 1). The biodegradation of each hydrocarbon family could be then properly measured in GM23. In addition, the intrinsic biodegradability of whole gasoline could be adequately evaluated since the 23 major compounds of the gasoline model mixture represented on a quantitative basis nearly 90% of the whole topped-gasoline.

Table 1. Detailed analysis of GM23 degradation by a microflora of kerosene-polluted sand.

Compounds	Retention time (min)	Degradation rate for test flasks[a]	Remaining hydrocarbons in abiotic controls[a]
n-heptane	19.12	100	95
n-octane	32.59	100	112
2-methylhexane	15.66	89	92
3-methylhexane	16.51	100	90
3-methylheptane	28.70	100	104
2,4-dimethylhexane	23.25	97	104
2,5-dimethylhexane	23.01	92	102
2,2,4-trimethylpentane	17.87	16	102
2,3,4-trimethylpentane	25.25	17	104
cyclohexane	14.80	85	88
benzene	14.02	23	96
toluene	25.77	100	107
ethylbenzene	39.70	100	106
m-xylene	41.06	100	101
p-xylene	41.23	100	99
o-xylene	44.54	88	102
n-propylbenzene	54.52	100	99
1,2,3-trimethylbenzene	64.98	33	104
1,2,4-trimethylbenzene	60.80	100	103
1,3,5-trimethylbenzene	56.97	92	99
1-methyl 2-ethylbenzene	58.42	100	100
1-methyl 3-ethylbenzene	55.74	100	99
1-methyl 4-ethylbenzene	56.04	100	97
Total for 23 compounds		85	101

[a] Average value of two flasks; % of initial amount.

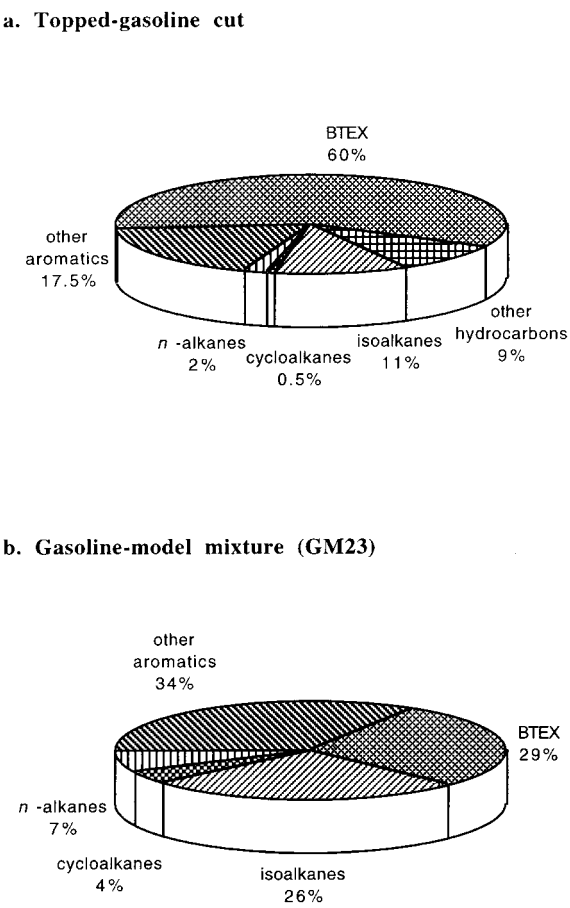

Figure 1. Comparative composition of GM23 and of a topped-gasoline cut. GM23 contained the 23 major compounds listed in Table 1. The gasoline cut was obtained by distillation at 76 °C of a commercial unleaded gasoline to remove more volatile ($\leq C_6$) hydrocarbons. (a) Hydrocarbons of GM23 and others in topped-gasoline cut. (b) Gasoline-model mixture (GM23).

Application to various environmental microflorae

The methodology was applied to different samples from polluted or non-polluted environments. The degradation and the recovery rates for the 23 hydrocarbons of GM23 determined in test flasks and abiotic flasks respectively are detailed for a sandy soil polluted with kerosene (Table 1). The recovery rates determined in abiotic control for each hydrocarbon were quite satisfactory. The microflora of this soil exhibited a broad potential of biodegradation since 19 of the 23 compounds were degraded at final ratio higher than 88%. However, a microbial limitation concerning in particular trimethylpentanes, benzene and 1,2,3-trimethylbenzene biodegradation was observed.

A synthesis of the results obtained with the microflorae of nine samples from various origins is shown in Tables 2 and 3. The characteristics of the samples are presented in Table 2. As also shown in Table 2, final CO_2 productions were determined at the end of the incubation periods for some samples. In GM23-free flasks, CO_2 productions were significant. It was particularly important for sample 6, probably because of the high level of the endogenous pollution which was biodegraded under the optimal conditions of the test. Actually, the amount of endogenous pollutant of sample 6 was nearly equivalent to the amount of GM23 added in the test, resulting in a high CO_2 production in GM23-free flasks. For samples 2 and 9 the levels of CO_2 production in GM23-free flasks could be explained by the organic matter contained in the microbial suspensions. For the other samples, the production of CO_2 in GM23-free flasks was lower (between 1 or 2 mmole l^{-1}).

Table 2. Characteristics and CO_2 production by the microflorae.

Sample	characteristics[a]	Incubation time (days)	CO_2 produced (mmole l^{-1}[b])	
			GM23-free flasks	Test flasks
1	Aerobic sludge from an urban waste water treatment plant	31	1.9	20.1
2	Superficial spruce forest soil	30	9.0	18.9
3	Garden soil	32	nd	nd
4	Gasoline oil-polluted sand	32	nd	nd
5	Diesel oil-polluted soil (2 mg/g)	35	2.1	18.9
6	Diesel oil-polluted soil (31 mg/g)	35	21.5	42.6
7	BTEX-polluted clay (5.8 mg/g)	28	nd	nd
8	Kerosene-polluted sand (4 mg/g)	31	1.3	14.9
9	Gasoline-polluted ground water	68	4.6	29.8

[a] The amounts of endogenous pollutant in mg per g of soil, as indicated in parenthesis, were determined either by gas chromatography after solvent extraction or by Pollut-Eval™ analysis.
[b] Average value (two flasks) of CO_2 produced measured at the end of the incubation period, in mmole per litre of culture media.
nd: not determined.

The values obtained for the degradation of individual hydrocarbons were distributed into sub-classes such as *n*-alkanes, methyl-alkanes, dimethyl-alkanes, trimethyl-alkanes etc... (Table 3) to allow easier comparison of the degradation capacities of the samples. The degradation capacities of the microflorae were high since total degradation was observed for 3 of the samples. Moreover, the degradation rates of whole GM23 were at least 85% for 8 of the 9 samples tested. However, one of them (sample 7) was found to be microbiologically inactive, possibly because of a deleterious effect of the high level of the endogenous pollution by BTEX on the native microflora of the soil.

For all active samples (excepted for sample 8), *n*-alkanes, BTEX, *n*-propylbenzene and ethyltoluenes were found to be completely degraded. Microfloræ 2 and 8 were the only samples for which incomplete (although substantial) degradation of methylalkanes, dimethylalkanes and trimethylbenzenes was observed. The most important observation concerns trimethylpentanes and cyclohexane, for which a more or less important limitation of the degradative capacities was observed. In fact, cyclohexane degradation was not complete with microflorae from unpolluted environments (2 and 3) and only in one case with microflorae from polluted sites (sample 8). The microbial deficiency for trimethylpentanes degradation was more common. It was observed for the two microflorae from unpolluted sites and for half (3 out of 6) of the microflorae from polluted environments.

Discussion

Gasoline is one of the most common pollutants of soils and aquifers. However, information on its biodegradation suffered from limitations on several important aspects. Some recent studies have reported extensive overall biodegradation of gasoline considering mainly total hydrocarbons (Zhou & Crawford 1995; Yerushalmi & Guiot 1998). Concerning individual hydrocarbons, as discussed below, except for the study of Jamison et al. (1975), detailed information was available only for a limited number of the numerous (over 200) constituents. Another insufficiently documented aspect was the distribution of the degradative capacities in various environments. In order to obtain information on these aspects, we devised first a methodology

Table 3. Biodegradation of the gasoline-model mixture GM23 by various microflorae.

Hydrocarbons	Hydrocarbon degradation for sample:[a]								
	1	2	3	4	5	6	7	8	9
n-alkanes	100	100	99	99	100	100	0[b]	100	100
Methyl-alkanes	100	94	100	100	100	100	0[b]	97	100
Dimethyl-alkanes	100	90	100	100	100	100	0[b]	95	100
Trimethyl-alkanes	27	14	28	76	100	100	0[b]	17	100
Cyclohexane	100	29	75	99	100	100	0[b]	85	100
BTEX, *n*-propylbenzene, ethyltoluenes	100	100	100	100	100	100	0 [b]	93	100
Trimethylbenzenes	100	96	100	100	100	100	0[b]	76	100
Whole GM23	95	89	94	98	100	100	0[b]	85	100

Samples number refer to the microflorae of Table 2.
[a] Average value of two flasks; % of initial amount.
[b] The recovered amounts were higher than the supplied substrates owing to endogenous pollution.

using a gasoline cut as the substrate and determining the extent of biodegradation of each individual hydrocarbon. The degradative capacities of activated sludge from waste water treatment were characterised in such a way (Solano-Serena et al. 1999b). The use of an hydrocarbon mixture in the test is important as cometabolism is involved in the degradation of several hydrocarbons such as *o*-xylene and cyclohexane (Gibson & Subramanian 1984; Trudgill 1984). The direct inoculation of the test flasks with the microbiological sample to avoid any detrimental strain selection is another important point. Although excellent results were obtained using gasoline, hydrocarbon analysis was complex and time-consuming. For systematic tests of microflorae of the environment, the GM23 mixture (Table 1) representing the main constituents of gasoline and all the types of chemical structures was more suitable. Prior to its use in this study, the GM23 test was validated by showing that it yielded results similar to those obtained with a real gasoline cut. For a given microflora the same limitations in degradative capacities were observed with both substrates (Solano-Serena et al. 1999a).

The results obtained here with the GM23 test show that the intrinsic biodegradability of gasoline can be considered as quasi-total since all tested hydrocarbons including trialkylbenzenes and highly branched isoalkanes (2,2,4- and 2,3,4-trimethylpentanes) were completely degraded by several microflorae. It must be noted however, that the most volatile fraction of gasoline (C4 to C6 hydrocarbons), which is quantitatively significant (about 40% of gasoline), was not included in the present study. For methodological reasons, its biodegradability is currently assessed independently.

Another conclusion, which confirms an earlier report (Solano-Serena et al. 1998), is the high biodegradative capacities of microflorae from non polluted soils (spruce forest soil, garden soil). These results are indicative of the ubiquitous presence of diversified hydrocarbon structures, originating in particular from plants, in the environment. They are also in accordance with the presence in gasoline of a large portion of hydrocarbons known to be well biodegraded.

In relation to the latter point, different levels of information were obtained concerning the various classes of hydrocarbons involved. The excellent degradation of BTEX, *n*-alkanes and 2-methylalkanes could be largely expected in view of the numerous reports showing the efficiency of microflorae from various origins to degrade these hydrocarbons (Watkinson & Morgan 1990; Smith 1990; Nielsen et al. 1996; Jutras et al. 1997; Wright et al. 1997; Yerushalmi & Guiot 1998). The extensive degradation of trimethylbenzenes by almost all microflorae deserves to be noted. Less information is available concerning these compounds. The degradation of some polyalkylated benzenes was reported (Lang 1996; Rozkov et al. 1998) and Solano-Serena et al. (1999b) described extensive, although rather slow, degradation of all tri- and tetraalkylbenzenes of gasoline by activated sludge. Other hydrocarbons were found more recalcitrant in this survey. Cyclohexane was degraded in all cases, although often incompletely. Ridgway et al. (1990) reported a low occurrence of selection of cyclohexane-degrading populations from an aquifer polluted by gasoline. In

fact, their results are compatible with ours considering that cyclohexane can be degraded by mutualism in populations (which are then able to use it as only carbon source), but also by cometabolism in hydrocarbon mixtures (Beam & Perry 1973, 1974; Lloyd-Jones & Trudgill 1989). Accordingly, we selected several microflorae using cyclohexane as only carbon source from our samples, but could not isolate any pure strain (data not shown). Another point is that cyclohexane was completely degraded in three samples from polluted soils, indicating adaptation of the microflorae. The higher capacities of polluted samples for cyclohexane degradation may result from the presence of a larger hydrocarbon-degrading population with broader capacities of degradation by cometabolism, probably because *n*-alkanes, isoalkanes and aromatics were suitable substrates for the degradation of cyclohexane by cometabolism.

The results concerning branched alkanes are of particular interest. Limited information is available on highly branched alkanes which are considered resistant to microbial degradation (Thijsse & Zwilling-De Vries 1959; McKenna 1972; Pirnik 1977). Two features in particular have been shown to make degradation difficult. The first one is the presence of β-methyl branched (*anteiso*) structures leading to 3-methylacylCoA intermediates blocking β-oxidation, an obstacle overcome only by specialised microorganisms (Schaeffer et al. 1979; Fall et al. 1979). The second one is the existence of quaternary-carbon structures or of methyl groups on successive carbon atoms (Kniemeyer et al. 1999; Solano-Serena et al. 1999b). Actually, both types of structures were attacked, in particular in microflorae from polluted samples. Furthermore, the results suggested that trimethylalkanes were degraded by cometabolism in microflorae from non-polluted samples as no microflora growing on isooctane could be selected from these samples (data not shown). On the contrary, one strain growing on isooctane was isolated from gasoline-polluted sample 9. The presence in an adapted microflora of a specialised strain with unusual degradative capacities is an aspect deserving further investigation.

The comparison between polluted and non-polluted samples for the degradation of recalcitrant compounds thus indicated the occurrence of an adaptation of the native microflorae after pollution had occurred. Quantitative adaptation has been documented, as reported by Horowitz & Atlas (1977) who observed a rapid population shift to high numbers of gasoline-degrading microorganisms in soil after pollution by gasoline. Here, a qualitative adaptation was also observed concerning the degradation of trimethylalkanes.

Finally, the GM23 test appears quite useful to assess the potential for natural attenuation of polluted sites. Concerning the limitations in degradative capacities observed, the test allowed a fine discrimination of the specific capacities for the degradation of isoalkanes (these hydrocarbons amounted to 26% of GM23 versus 11% of the gasoline cut). In particular, differences between the various microflorae in the degradation of the trimethylpentane isomers were very clear-cut with the GM23 methodology. The results of the present study illustrate the interest of specific methodologies yielding a comprehensive view of the degradative capacities of a microflora, as pointed out by Logan & Rittmann (1998) and by Chapelle (1999).

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