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Intrinsic capacities of soil microfloræ for gasoline degradation

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Abstract

A methodology to determine the intrinsic capacities of a microflora to degrade gasoline was developed, in particular for assessing the potential of autochtonous populations of polluted and non polluted soils for natural attenuation and engineered bioremediation. A model mixture (GM23) constituted of the 23 most representative hydrocarbons of a commercial gasoline was used. The capacities of the microfloræ (kinetics and extent of biodegradation) were assessed by chromatographic analysis of hydrocarbon consumption and of CO_2 production. The degradation of the components of GM23 was assayed in separate incubations of each component and in the complete mixture. For the microflora of an unpolluted spruce forest soil, all hydrocarbons of GM23 except cyclohexane, 2,2,4- and 2,3,4-trimethylpentane isomers were degraded to below detection limit in 28 days. This microflora was reinforced with two mixed microbial communities selected from gasoline-polluted sites and shown to degrade cyclohexane and 2,2,4-trimethylpentane. With the reinforced microflora, complete degradation of GM23 was observed. The degradation patterns of individual components of GM23 were similar when the compounds were present individually or in the GM23 mixture, as long as the concentrations of 2-ethyltoluene and trimethylbenzene isomers were kept sufficiently low (≤ 35 mg.l^{-1}) to remain below their inhibitory level.

Introduction

The aerobic degradation of hydrocarbons has been studied for four decades, but the degradation of the complex mixtures constituting the commonly used petroleum products such as gasoline, kerosene, diesel oil, which frequently are released as pollutants in the environment, is only partially understood. Concerning gasoline biodegradation, most of the present knowledge still rests on the utilization of individual hydrocarbons as carbon sources by isolated strains or, when strain isolation could not be achieved, by microbial consortia (Atlas 1984). Judging from degradation velocities and from frequency of selection of active microfloræ, in particular from gasoline-contaminated soils, it appears that common aromatic hydrocarbons – such as toluene, *m*-xylene and *p*-xylene, which are major gasoline constituents — and *n*-alkanes are clearly more easily degraded than *iso*-alkanes and cycloalkanes (Jamison et al. 1975; Jamison et al. 1976; Perry 1979; Ridgway et al. 1990).

In the present work, we aimed at obtaining information on the potential of the microfloræ from polluted and non polluted soils to degrade gasoline in non limiting conditions, i.e. to evaluate intrinsic degradative capacities of the microfloræ. Such information and the methodologies to obtain it are of prime importance to define an appropriate strategy concerning polluted soils in terms of possibility of bioremediation and prospects for natural attenuation. The performances (kinetics and extent of the biodegradation) of the microfloræ on a complex mixture constituting a model gasoline were investigated by chromatographic analysis of hydrocarbon consumption and of CO_2 production. The capacities of the microfloræ to degrade individual components of the mixture were also characterized.

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Materials and methods

Culture media

A nutrient solution consisting of mineral salts and vitamins described by Bouchez et al. (1995) was used. The carbon source of the culture media was either individual hydrocarbon compounds or a hydrocarbon mixture. The latter was prepared by mixing equal volumes of the main compounds making up a topped gasoline-cut obtained by distillation at 76 °C of a French commercial unleaded gasoline. The compositions of the hydrocarbon mixture (GM23) and of the topped gasoline were determined by gas chromatography.

Preparation of soil suspensions

The soil sample was taken from a spruce forest (Foulain, France). It contained mineral and superficial organic matter. The native soil suspension (NSS) was prepared with 20 g of homogenized soil per litre of nutrient solution.

A cyclohexane-degrading microflora and a 2,2,4-trimethylpentane-degrading microflora were added to the soil suspension (5% (v/v) each) to prepare the microbially-reinforced soil suspension (MRSS). The cyclohexane-degrading microflora was obtained from a few grams of a sandy soil sample taken from a gasoline storage site. Four successive enrichment cultures on the nutrient solution containing 350 mg.l^{-1} of cyclohexane as the sole carbon source were carried out. The 2,2,4-trimethylpentane-degrading microflora was obtained by the same enrichment procedure from a ground water which had been polluted by unleaded gasoline, using 2,2,4-trimethylpentane as the sole carbon source.

Biodegradation of gasoline model mixture (GM23)

The biodegradation tests were performed with NSS and MRSS in 500 ml flasks with sidearms equipped with Mininert valves (Pierce, Oud-Beijerland, The Netherlands). 25 μl of GM23 were added to 50 ml of soil suspension through the Mininert valve with a Hamilton syringe. The precise quantities of GM23 introduced in each flask were determined by the weight difference between the full and the emptied syringe. After an incubation period of 14 or 28 days at 30 °C, 5 ml of CH_2Cl_2, containing dodecane at 600 mg.ml^{-1} as internal standard, were introduced in the flasks through the Mininert valve, and the remaining hydrocarbons were extracted for 1 h with shaking. The flasks were refrigerated over night at 4 °C before opening, and the suspensions was centrifuged at 4 °C and 35000 g for 30 min. The CH_2Cl_2 phase of each flask was then analyzed by gas chromatography.

Abiotic experiments were performed and analyzed in similar conditions for NSS and MRSS, 1 g.l^{-1} $HgCl_2$ being added to the flasks before incubation.

Kinetics of CO_2 production

The kinetics of CO_2 production during the degradation of GM23 were also studied at 30 °C over 28 days. 18 ml of NSS or 18 ml of MRSS were introduced into 240-ml flasks that were closed by butyl rubber stoppers covered with teflon film. 8 μl of GM23 were dispensed in the sealed flasks with a 10 μl syringe and weighed as described above. A 250 μl gas-tight syringe was used to sample gas from the head space, and CO_2 was determined by gas chromatography.

The kinetics of endogenous-like respiration of NSS and of MRSS were determined in the same conditions with flasks incubated without hydrocarbon.

Mineralization yields

At the end of biodegradation kinetics experiments, final mineralization yields of GM23 were determined by measuring CO_2 produced. Total CO_2 was determined by gas chromatography after addition of 0.5 ml HNO_3 (68%). Mineralization yields (Y) were calculated as molar ratios of the difference between the carbon quantity of the total CO_2 produced in the test flask ($C_{CO_2}^{TF}$) and the carbon quantity of the total CO_2 produced in the hydrocarbon-free flask ($C_{CO_2}^{HFF}$) to the carbon quantity of the supplied hydrocarbon (C_{HC}^{TF}):

$$Y = \frac{C_{CO_2}^{TF} - C_{CO_2}^{HFF}}{C_{HC}^{TF}}$$

The mineralization yields of the 23 individual hydrocarbons were determined as for GM23. Cultures were carried out for 34 days in 125-ml flasks with 20 ml of the soil suspension and 5 μl of each hydrocarbon.

Chromatographic analyses

CO_2 was measured by an external standard method, with a Girdel Series 30 chromatograph equipped with a thermal conductivity detector and a Porapak Q column (80/100 mesh, 2 m). Helium was the carrier gas,

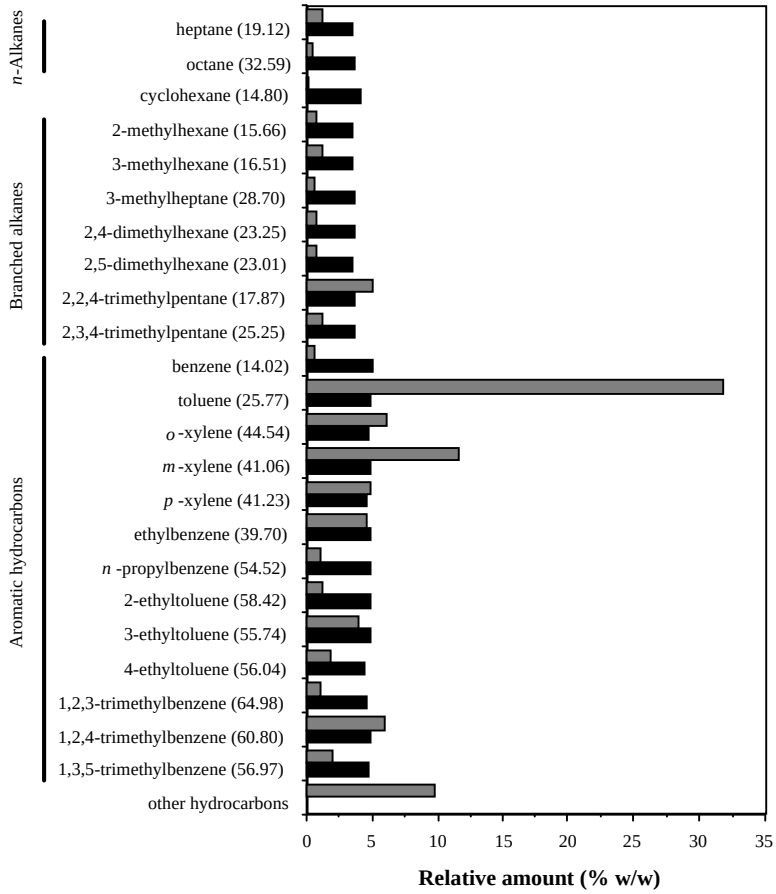

Figure 1. Comparative composition of topped gasoline and gasoline-model mixture GM23. Retention times (in min) are mentioned for each compound. Topped gasoline (■) – Gasoline-model mixture GM23 (■).

and the column temperature was 50 °C. A 250-μl gas-tight syringe was used to inject gas from the flask head space in the chromatograph.

Analyses of hydrocarbons and topped gasoline were performed with a Varian 3400 chromatograph equipped with a flame ionization detector and a CP-Sil Pona CB column (0.25 mm × 100 m) (Chrompack). Helium was the carrier gas. The operating temperature of the detector was 300 °C and that of the injector was 250 °C. The column temperature was set at 35 °C for 10 min, increased to 115 °C at 1.1 °C.min^{-1}, and then to 280 °C at 1.7 °C.min^{-1}. The detection limit of each individual hydrocarbon was 0.5 mg per litre of medium.

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

Composition of the gasoline-model mixture

The gasoline sample used as a reference was a topped gasoline-cut free of compounds lighter than C6 and containing no oxygenated compounds. It contained 203 measurable components, 23 of which were present at a relative concentration higher than 0.5% (w/w). These represented together nearly 90% of the whole topped gasoline-cut. Important differences in the relative amounts of each component were noted: For example, cyclohexane and toluene amounted to 0.5 and 32% respectively. The necessity to perform biodegradation experiments in closed flasks set constraints on the amount of gasoline that could be used in order

Table 1. Mineralization of individual hydrocarbons by the native soil microflora

Substrates	Mineralization yields[a]
heptane	0.74
octane	0.49
cyclohexane	0
2-methylhexane	0.47
3-methylhexane	0.71
3-methylheptane	0.69
2,4-dimethylhexane	0.56
2,5-dimethylhexane	0.44
2,2,4-trimethylpentane	0.02
2,3,4-trimethylpentane	0
benzene	0.56
toluene	0.63
o-xylene	0.49
m-xylene	0.61
p-xylene	0.48
ethylbenzene	0.48
n-propylbenzene	0.34
2-ethyltoluene	0[b]
3-ethyltoluene	0.38
4-ethyltoluene	0.34
1,2,3-trimethylbenzene	0[b]
1,2,4-trimethylbenzene	0.18
1,3,5-trimethylbenzene	0[b]

The mineralization yields were determined after 34 days of incubation at 30 °C as described in Materials and methods. Two flasks for each substrate and six control flasks were used.
(a) Mineralization yields within ± 8% according to CO_2 measurements and hydrocarbon supplies.
(b) CO_2 production lower than for hydrocarbon-free flasks.

to operate in conditions of oxygen excess on the one hand and to remain above detection limits of gas chromatography for all components on the other hand. For this reason, instead of gasoline, a gasoline model mixture (GM23) was used. It was prepared by mixing equal volumes of the 23 main components of topped gasoline (Figure 1). In the conditions used, the molar ratio of available di-oxygen to the carbon of GM23 in incubation flasks was 3.6.

Mineralization of individual compounds

The ability of the native soil microflora to mineralize separately the 23 individual compounds composing the gasoline model mixture was investigated. The mineralization yields were measured after 34 days of incubation (Table 1). 16 compounds exhibited mineralization yields higher or equal to 0.34. *n*-Alkanes and methyl and dimethyl alkanes were readily mineralized, whereas cyclohexane and trimethyl alkanes were not. Mineralization of benzene and mono-substituted aromatics (toluene, ethylbenzene, propylbenzene) and xylenes was also observed. 3-ethyltoluene and 4-ethyltoluene were mineralized, but not 2-ethyltoluene. Considering trimethyl benzenes, only the 1,2,4-trimethyl isomer was slightly mineralized.

The reason for the absence of mineralization observed with several substituted aromatics was investigated. In fact, CO_2 production resulting from the degradation of organic components of the soil suspension was also noted in substrate-free flasks, accounting for an endogenous-like respiration (data not presented). 1,3,5-trimethylbenzene, 2-ethyltoluene and 1,2,3-trimethylbenzene appeared to lower CO_2 production resulting from endogenous-like respiration. Inhibitory properties of 1,2,3-trimethylbenzene on mineralization were also observed in a cometabolism experiment. When used as putative co-substrate (220 $mg.l^{-1}$) with each of the mono-aromatics composing GM23 (220 $mg.l^{-1}$) as carbon source, 1,2,3-trimethylbenzene was not cometabolized (data not shown). The degradation of the three inhibitory substrates was then examined when added individually to NSS at a lower concentration (35 $mg.l^{-1}$). Chromatographic analysis showed that 1,2,3-trimethylbenzene, 1,2,4-trimethylbenzene, and 2-ethyltoluene were totally degraded (below detection limit of chromatographic analysis, see Materiels and methods) by NSS in 28 days in these conditions.

No inhibition phenomena were apparent for the experiments in which mineralization of trimethylpentanes and cyclohexane did not occur. The persistence of these compounds can therefore, be attributed to a deficiency of the native soil microflora. Overcoming this deficiency was attempted by preparing a microbially reinforced soil suspension with a cyclohexane-degrading microflora and a 2,2,4-trimethylpentane-degrading microflora (see Materials and Methods). The respective capacities of these microfloræ to mineralize pure cyclohexane and pure 2,2,4-trimethylpentane were determined when incubated in the nutrient solution. The mineralization yields after 20 days of incubation were found to be, respectively, 0.54 and 0.45.

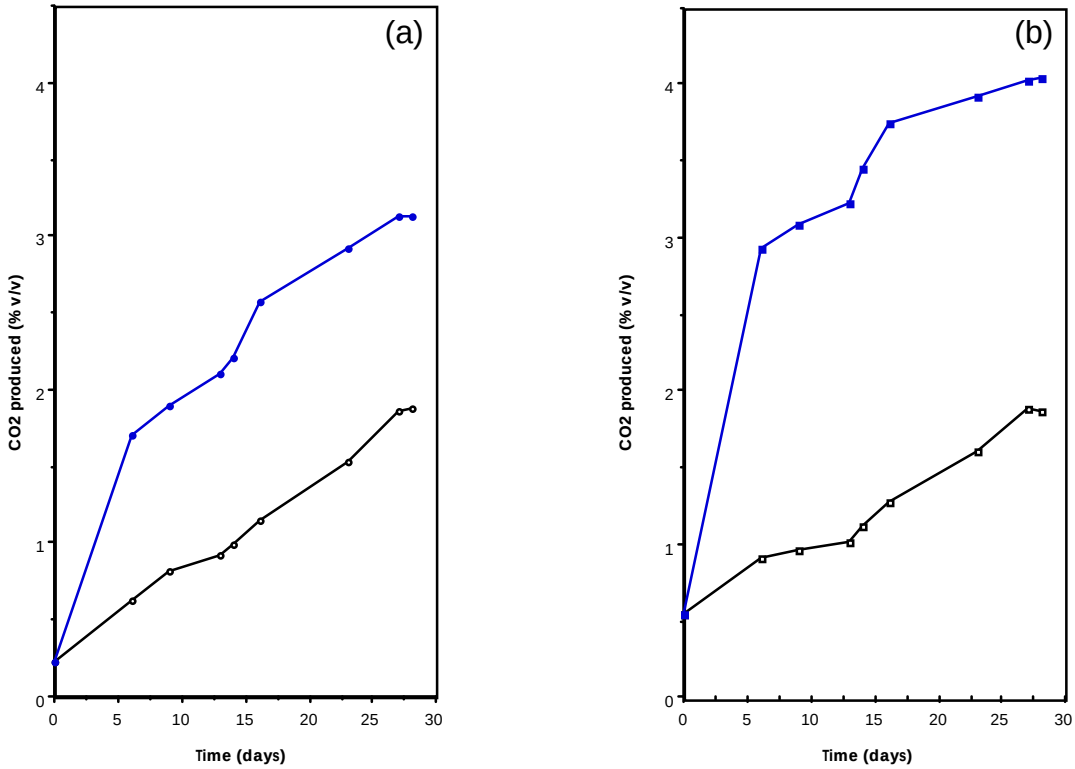

Figure 2. Kinetics of carbon dioxide production during degradation of the gasoline-model mixture. (a) native soil sample without hydrocarbon (○) and with GM23 (●). (b) microbially reinforced soil sample without hydrocarbon (□) and with GM23 (■).

Biodegradation of the gasoline-model mixture (GM23)

The kinetics of CO_2 production were recorded over 28 day periods for NSS and MRSS, with the gasoline-model mixture as substrate (Figure 2). In substrate-free flasks, CO_2 productions were significant for NSS and for MRSS because of the presence of organic matter in the soil suspensions. In flasks containing the gasoline-model mixture, CO_2 production occurred rapidly in the first six days and then diminished. After 16 days of incubation, CO_2 production rates in test flasks were close to those in the substrate-free flasks.

Mineralization yields were estimated at the end of the biodegradation runs by total recovery of CO_2 after acidification of the flask suspensions. The mineralization yield was significantly higher for MRSS (0.58) than for NSS (0.45).

In order to correlate the mineralization yields to substrate consumption, the residual components of the gasoline-model mixture were analyzed. The chromatographic patterns obtained for flasks inoculated with NSS and MRSS and for abiotic flasks are presented in Figure 3. Three compounds (cyclohexane, 2,2,4-trimethylpentane and 2,3,4-trimethylpentane) were only slightly biodegraded by NSS, and three others (3-methylhexane, 2,4-dimethylhexane and 1,3,5-trimethylbenzene) were incompletely biodegraded by NSS. All other compounds, including 1,2,3-trimethylbenzene, 1,2,4-trimethylbenzene and 2-ethyltoluene, were totally consumed by NSS. It is important to notice that in the presence of MRSS, all the components of the gasoline-model mixture were totally consumed.

Consumption of each component by NSS did not occur at the same rate. Table 2 shows the amount of compounds remaining in the culture medium after 2 and 4 weeks of incubation. *n*-Alkanes and most of the mono-aromatics were completely degraded within the 14 first days, whereas cyclohexane and di- and trimethyl alkanes were not. Methyl alkanes and dimethyl hexanes were only slightly consumed over this period of time but they were nearly totally biodegraded after 28 days. Cyclohexane and trimethyl pentanes were little consumed after 4 weeks. An important point is that the recovery of the gasoline-model mixture after 28 days of incubation under abiotic con-

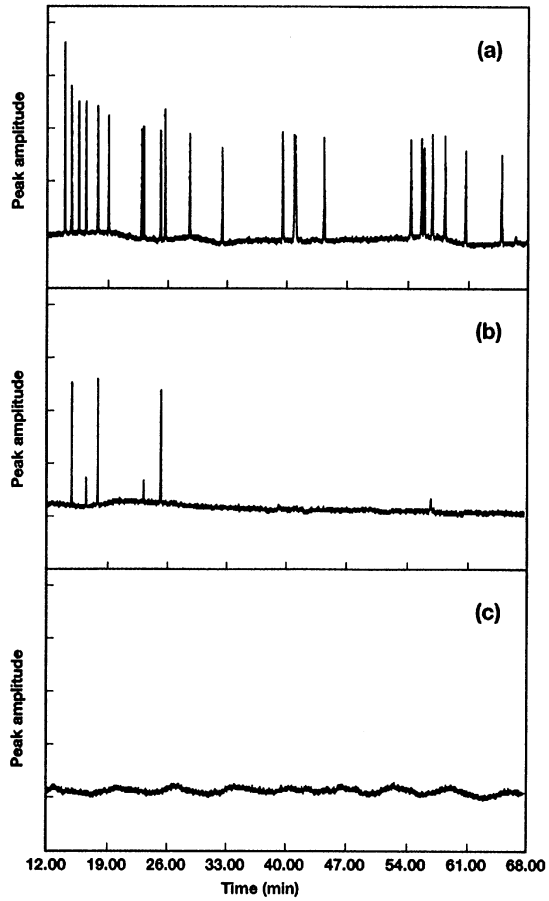

Figure 3. Chromatographic patterns of residual hydrocarbons of GM23 after 28 days of incubation. (a) abiotic control. (b) native soil sample. (c) microbially reinforced soil sample.

ditions was nearly 100%, which means that losses in the inoculated experiments were due solely to biodegradation.

Discussion

Gasoline is a complex mixture composed of several families of hydrocarbons: mono-aromatics and linear, branched, or cyclic alkanes. Most of the studies on gasoline biodegradation have been mainly focused on degradation of a particular class of mono-aromatics, including benzene, toluene, ethylbenzene and xylene isomers (BTEX), and have been performed using polluted soil or ground water as microbial inoculate (Jamison et al. 1976; Zhou and Crawford 1995; Jutras et al. 1997). In contrast, we investigated in this study the capacities of a unpolluted soil sample to degrade a defined gasoline-model mixture composed of the most representative components of each class of hydrocarbon of gasoline. Therefore, methodology was developed to determine the intrinsic capacities for gasoline degradation, in particular for assessing the potential of autochtonous microfloræ of polluted soils for natural attenuation and bioremediation.

The gasoline-model mixture GM23 was degradable about 89% by a native soil microflora. Lack of degradation capacity was observed for 2,2,4- and 2,3,4-trimethylpentane isomers and for cyclohexane. In fact, from 17 polluted or unpolluted soil and ground water samples, we obtained only three cyclohexane-degrading microfloræ and one 2,2,4-trimethylpentane-degrading microflora. These relatively low occurrences for isolation of cyclohexane and 2,2,4-trimethylpentane-degrading populations was already observed by Ridgway et al. (1990) in a study on a gasoline-contaminated coastal aquifer. This corroborates a more general observation that highly branched alkanes are less susceptible to microbial degradation (Thijsse and Zwilling-De Vries 1959; McKenna 1972; Pirnik 1977) and that degradation of cyclohexane requires particular strain associations, involving in some case cometabolism (Beam and Perry 1973, 1974). Supplementation of NSS with a cyclohexane-degrading microflora plus a 2,2,4-trimethylpentane-degrading microflora led to complete degradation of GM23. Since methyl alkanes and cycloalkanes have been identified as the most recalcitrant components in biofilter treatment of gasoline (Wright et al. 1997), a suitable population reinforcement by cyclohexane and 2,2,4-trimethylpentane-degrading microfloræ could have a beneficial effect on the degradation performances of this type of process.

The mineralization yields of the individual components of GM23 were high for the aromatics and alkanes having no or few methyl groups. Considering a biomass formation yield between 0.2 and 0.5 Cmol.Cmol^{-1} (Wodzinski and Johnson 1968; Geerdink et al. 1996), the data suggest extensive mineralization of these compounds. A few compounds, such as 3- and 4-ethyltoluene and *n*-propylbenzene, which were mineralized at a lower extent, might have been partially transformed into metabolites. Attempts of detecting metabolic acidic derivatives in culture media of GM23 remained unsuccessful (data not shown). Finally, the overall mineralization yield of GM23 determined either with NSS or with MRSS is in agreement with the mineralization data determined for individual compounds.

Table 2. Biodegradation of the components of the gasoline-model mixture GM23 by NSS after different incubation times

Components	Amounts of residual components[a] (%) for:		
	NSS after 14 days	NSS after 14 days[b]	Abiotic control after 28 days[c]
heptane	0	0	96
octane	0	0	102
cyclohexane	98	70	92
2-methylhexane	80	0	93
3-methylhexane	94	18	94
3-methylheptane	63	0	101
2,4-dimethylhexane	104	19	100
2,5-dimethylhexane	100	0	98
2,2,4-trimethylpentane	103	82	97
2,3,4-trimethylpentane	105	91	105
benzene	0	0	92
toluene	0	0	98
o-xylene	0	0	98
m-xylene	0	0	94
p-xylene	0	0	100
ethylbenzene	0	0	95
n-propylbenzene	0	0	96
2-ethyltoluene	0	0	99
3-ethyltoluene	0	0	96
4-ethyltoluene	0	0	94
1,2,3-trimethylbenzene	0	0	97
1,2,4-trimethylbenzene	0	0	94
1,3,5-trimethylbenzene	47	12	100
GM23	29	11	97

The initial amount of GM23 in flasks was 25 μl. The precise initial amount of each compound was calculated with the weight of GM23 dispensed and its relative composition.
(a) determined by the ratio between the residual amount to the initial amount of each component in GM23.
(b) Mean value of two test flasks.
(c) Mean value of four abiotic flasks.

In the degradation runs of GM23 by NSS, *n*-alkanes and aromatics were biodegraded before *iso*-alkanes, confirming the high rate of degradation previously determined for these compounds (Nielsen et al. 1996; Zhou and Crawford 1995). Our study does not provide further information whether the rate of *iso*-alkanes degradation is affected by the high affinity of the linear alkanes for the enzyme systems involved (Geerdink et al. 1996) or whether a diauxic phenomenom takes place as suggested by Pirnik et al. (1974) for pristane (2,6,10,14-tetramethylpentadecane) and hexadecane. Another point of interest is the role of cometabolism in gasoline degradation reported by Jamison et al. (1976). Our results suggest that a cometabolism phenomenom was not involved, for example for cyclohexane, since all the components degraded when supplied as GM23, were also mineralized when supplied individually. Nevertheless, 1,2,3- and 1,2,4-trimethylbenzene isomers and 2-ethyltoluene were biodegraded individually only when supplied at a low concentration. At a high concentration (220 mg.l^{-1}), they were not degraded, and they even inhibited microbial activity.

On the whole, the pattern of gasoline degradation appears as to be a sum of the degradation of individual compounds. Reciprocal kinetic effects on the degradation of individual compounds cannot be excluded but no marked occurence of cometabolism was observed in the degradation of the gasoline model mixture.

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