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Aerobic bacterial degradation of selected polyaromatic compounds and *n*-alkanes found in petroleum

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Abstract

Biodegradation in vitro studies were carried out on selected aromatic and aliphatic hydrocarbons in aqueous medium. Biotic and abiotic samples were analysed by GC and/or GC–MS. It was clearly shown that abiotic losses for selected compounds were much higher than might have been expected from the literature. The experiments were undertaken using the higher-relative-molecular-mass compounds; e.g. phenanthrene, chrysene, benzo[*a*]pyrene and a series of *n*-alkanes. Although *n*-C_{17} proved too volatile, *n*-C_{22} and *n*-C_{30} were shown to be extensively biodegraded, and these *n*-alkanes were degraded preferentially to the polyaromatic compounds. Chrysene and benzo[*a*]pyrene were less affected by abiotic losses but did not biodegrade significantly during a four-month incubation. A similar experiment with diesel oil confirmed the above findings.

1. Introduction

Widespread and already frequent contamination by toxic organic chemicals in water, soil and sediment ranges from industrial chemical waste contamination [1–3] to halogenated hydrocarbons and pesticides in ground water [4,5] and oil spills [6,7]. In situ biodegradation processes are focusing attention on the role that microorganisms may play in alleviating environmental pollution [8–13].

Biodegradation is a form of biotransformation which by simplification of an organic compound's structure through the breaking intramolecular bonds, alters its toxicity and transport properties [14]. The simplification may be subtle, involving merely a substituent functional group, or severe, resulting in mineralisation. The process of biotic reactions is accompanied by abiotic reactions, which include all of the reactions not encompassed by biotic reactions, i.e. inorganic, organic, photolytic, surface-catalysed, sorptive and transport processes. Abiotic contribution to the losses of organic compounds, especially those with lower relative molecular mass, have been underestimated in the past.

Several laboratory-based studies have been undertaken to assess the fate of polycyclic aromatic hydrocarbons (PAHs) in the soil [15–18]. These indicate that biodegradation is the key process leading to the loss of most PAHs in the soil system when abiotic losses are controllable. Generally, low-molecular-mass compounds are reported to be affected by volatilisation and/or

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abiotic degradation processes [18]. A broadly inverse relationship has been shown between the rate of biodegradation and the number of benzene rings [15], probably due to changes in solubility, bioavailability and stability of PAHs depending on their molecular structure [17]. Low-molecular-mass PAHs such as naphthalene have higher aqueous solubilities and are less lipophilic than the higher-molecular-mass compounds and can act as a sole carbon/energy source for some soil microbes [16]. The polyringed species cannot act as a sole source of carbon/energy, but may be degraded by co-oxidation processes [19].

It has been shown that PAH mineralisation can be accelerated in soils and sediments that have previously been contaminated; this implies that an adapted microbial population has been developed [16,20]. PAH degradation may also be enhanced by the addition of organic supplements that stimulate the general level of biomass activity. Many other factors are important for the process of bacterial degradation. The presence of the microorganisms in the soil is fundamental. One way to enhance biodegradation of organic compounds is to inoculate the environment with microorganisms, which are known to readily metabolise these chemicals. The rate of microbial decomposition of organic compounds in soils is a function of three variables: (a) the availability of the chemicals to the microorganisms that can degrade them, (b) the quantity of these microorganisms and (c) the activity level of these organisms. Factors such as organic matter and clay content, moisture level, temperature, pH, aeration and nutrient status, are of importance as moderators and driving factors [21].

In the present study, in vitro biodegradation experiments were carried out on both, single compound and mixtures of aromatic and aliphatic hydrocarbons. In addition as a real sample biodegradation of diesel oil (DO) was examined. Single bacterial species *Pseudomonas putida* and *Pseudomonas fluorescens* Texaco were used. Known concentrations of hydrocarbons were added to mineral media paying attention not to exceed the limit of solubility in water.

2. Experimental

2.1. Experimental design

Mineral media and standard compounds of interest were added to a series of Erlenmeyer flasks. Bacterial broth was added only to biotic samples. Hg_2Cl_2 (Aldrich, Dorset, UK) was added to the remaining samples to ensure that only abiotic conditions prevailed. The concentrations of each hydrocarbon in aqueous solution (0.8 mg/l) was kept below the limit of solubility of one of the common PAHs in water samples —phenanthrene. Such high concentration levels are expected in case of fuel spills where we wish to apply our results. For that reason we did not use lower concentration levels, although biodegradation has been mainly studied at these lower levels [22,23]. All the compounds, that is phenanthrene (Ph), chrysene (Chr), benzo[*a*]pyrene (BaP), *n*-C_{17}, *n*-C_{22} and *n*-C_{30} from Aldrich and BaP from Sigma, Dorset, UK, except the internal standard stock solution [2-methylphenanthrene (2MPh) for aromatics and *n*-C_{25} for aliphatics, Aldrich] were added to the mineral media solution on day 0. Internal standards were injected to the sample on the day of analysis prior to extraction. The selected hydrocarbons were chosen because they can be found abundantly in petroleum.

Flasks, stoppered with non-adsorbent cotton wool, were then incubated aerobically in the absence of the light on a shaker, at room temperature and at a lower temperature (4–5°C) for up to four months. Control abiotic flasks containing mineral salts, hydrocarbons, but no bacterial inoculum, were incubated under the same conditions to monitor abiological losses (e.g. evaporation). The experiments were carried out in duplicates. During incubation on each day of analysis, two biotic and two abiotic samples were extracted three times with 10 ml of dichloromethane (all the solvents were purchased at Rathburn, Peebleshire, UK) from the salt solution, dried with anhydrous Na_2SO_4 (Aldrich) for 60 min and filtered. After transferring the samples into separation funnels, the flasks were

rinsed three times with dichloromethane (DCM) to avoid any compound left in the flask due to the adsorption to glass walls. The volume was reduced (nitrogen) to 1 ml prior to analysis by capillary gas chromatography.

Initial experiments were set up with compounds (benzene, naphthalene) that were proved as too volatile for such a kind of experiment. Therefore following studies were performed using single compounds phenanthrene and n-C_{17} and mixtures of three aromatic (Ph, 2MPh and BaP) and three aliphatic (n-C_{17}, n-C_{22} and n-C_{30}) hydrocarbons. As a real sample DO (Plinsko olje D2; Petrol, Koper, Slovenia) was tested. DO was separated into aromatic and aliphatic fraction by wet column chromatography. The column was filled with dried (450°C, 24 h) and activated silica. For 1 g of sample 25 g of silica were required. Aliphatic fraction was eluted first with hexane. Aromatics were eluted with hexane–diethyl ether (10:1).

P. putida and *P. fluorescens* Texaco [24] were chosen for this study because of their known ability to degrade hydrocarbons even at low temperatures [25]. They are also common in the soil contaminated with petroleum products. Cultures were prepared in an OXOID nutrient broth (1.3%) for 24 h at 20°C before use.

All glassware and mineral media were sterilised by autoclaving (120°C, 20 min). Bacterial broth (1 ml, approx. 10^7 bacteria) was added to a conical flask (250 ml) containing 100 ml of sterilised salt solution where 5‰ NH_4Cl, 1‰ NH_4NO_3, 2‰ Na_2SO_4; 3‰ K_2HPO_4; 1‰ KH_2PO_4 and 0.1‰ $MgSO_4 \cdot 7H_2O$ (Aldrich) were dissolved in 1000 ml of deionised water [22].

A set of experiments to prove evaporation losses was undertaken (evaporation test). Three samples containing examined hydrocarbons and mineral media were incubated under different conditions. One sample was refrigerated and the other two were observed at room temperature; one on a bench top and the other in the fume cupboard. It was suspected that high abiotic losses resulted from a higher air flow in the fume cupboard where experiments were set up at the beginning. All the samples were extracted after 5 days of incubation. Despite the higher air flow in the fume cupboard, no significant difference was revealed between with cotton wool stoppered samples carried out at room temperature. The comparison between the samples incubated at room temperature and the refrigerated one showed clearly that abiotic losses were diminished greatly at lower temperature.

The problem of evaporation was confirmed with Soxhlet extraction of non-absorbent cotton wool used for stoppering conical flasks. Discernible amount (about 10%) of observed compound was found in the cotton wool after extraction. Reference cotton wool did not contain this compound. The head space analysis of the same sample also confirmed the above findings.

The bacteria in the samples were tested to ascertain if they were alive prior to analysis by spreading an agar plate with a loopful of sample. The count of the bacteria was performed on the day of analysis by the Miles and Misra drop counts method with serial of dilutions of the organisms [26]. It was shown that experiments with higher biodegradation losses gave higher counts in general. The bacteria incubated in the cold room had visibly lower counts, but the chosen bacteria did grow and multiply.

A quick agar plate test was performed in order to determine the biodegradation potential of selected compounds with chosen bacterial species. Agar plates were prepared especially for this experiment with nutrient agar with no usable carbon substrate for bacterial growth. These plates were spread with the culture. A thin layer of the compound was sprayed over the surface of the inoculated plate using an aerosol spray [27]. The plates were left to dry before being incubated at 30°C. Blanks included an inoculated plate without compounds, a plate with compounds and no bacteria and an empty plate. No growth of bacteria was observed on any of the blanks, even after 10 days of incubation. Only plates sprayed with the n-docosane, n-eicosane and DO gave visible growth of bacteria within the incubation time. This proved that only simple short chain aliphatic hydrocarbons are readi-

ly degraded in a single compound and single culture degradation experiment without any other organic carbon present.

2.2. Gas chromatography

Gas chromatography was performed using a Carlo Erba (Milan, Italy) 4160 gas chromatograph with on-column injector and flame ionisation detector. Separation was achieved using a 30 m × 0.32 mm I.D. phenyl silica column (1 μm film thickness) DB 5 (J & W Scientific, Loughborough, UK). Oven temperature was programmed from 65 to 300°C at 5°C/min and held for 10 min. Hydrogen was used as the carrier gas at a flow-rate of 1.5 ml/min. The flame ionisation detector's temperature was 330°C. Quantitation of individual hydrocarbons was made by measurement of GC peak areas with a Shimadzu (Kratos Analytical, Manchester, UK) CR3-A integrator and a comparison of these with the responses of known concentrations of internal standards, 2MPh for aromatic hydrocarbons and n-C_{25} for aliphatic ones. In case of DO a mixture of internal standards (squalane and BaP) was used. Compounds were identified by co-chromatography with authentic compounds. For confirmation some samples were run on GC–MS (HP 5890 series II gas chromatograph coupled to 5970 series mass-selective detector) (Hewlett-Packard, Waldbronn, Germany) using HP1 column (12 m × 0.32 mm I.D.), splitless injection and head pressure of 36 kPa. Biological degradation was calculated from differences between biotic and abiotic losses.

3. Results and discussion

Biodegradation studies of selected aromatic and aliphatic compounds showed that the abiotic losses amounted to 78% of the starting concentration within 17 days of incubation when working at room temperature with a single compound (Ph and n-C_{17} separately) under the limit of solubility. This discernible diminished the purely biotic degradation to about 4% only. One possible explanation was evaporation, and this was clearly proven by the evaporation test. An experiment which was set up in the dark (to avoid possible photodegradation) in a cold room (4–5°C) showed lower abiotic losses at lower temperature. The results of the biodegradation were in this case visibly higher —up to 29% within 20 days of incubation. These experiments clearly showed that evaporation plays a decisive role when working with low concentrations and a single compound. It should be born in mind that even if the limit of solubility is exceeded the quantity of the compound available to the microorganism does not increase.

The abiotic losses were examined at room temperature with the mixture of three aromatic and three aliphatic compounds. The effect of evaporation on the compounds with different vapour pressures was clearly shown. The abiotic losses were very high for the compounds with lower molecular mass (n-C_{17} and Ph). These reached 92 ± 5% within 61 days of incubation at room temperature and affected the others according to their molecular mass (Fig. 1). Of all the compounds tested, n-C_{22} was proven to be the best for biodegradation studies. As an aliphatic compound it was readily degradable —89 ± 4% of the compound was biodegraded— and it was less affected by abiotic losses than n-C_{17} and Ph; abiotic factors resulted in only 9 ± 2% losses overall. n-C_{30} was slightly less degraded (81 ± 5%) than n-C_{22} under the same conditions, but its abiotic losses were diminished to 2 ± 1%. Aromatic compounds experienced lower biotic degradation in general. Ph, the lowest-molecular-mass aromatic compound tested, was found to have only 3 ± 1% losses due to biological degradation and extended abiotic losses to 92 ± 6%. PAHs with higher molecular masses were only slightly affected from both abiotic and biotic losses. This proved that bacteria of the genus *Pseudomonas* utilised alkanes more intensively than PAHs [28] and this was manifested by higher plate counts, higher metabolic activity and hydrocarbon degradation rate. In general all kinds of bacteria would oxidise compounds with a lower molecular mass more easily. Between aliphatic and aromatic compounds they would degrade aliphatic hydrocar-

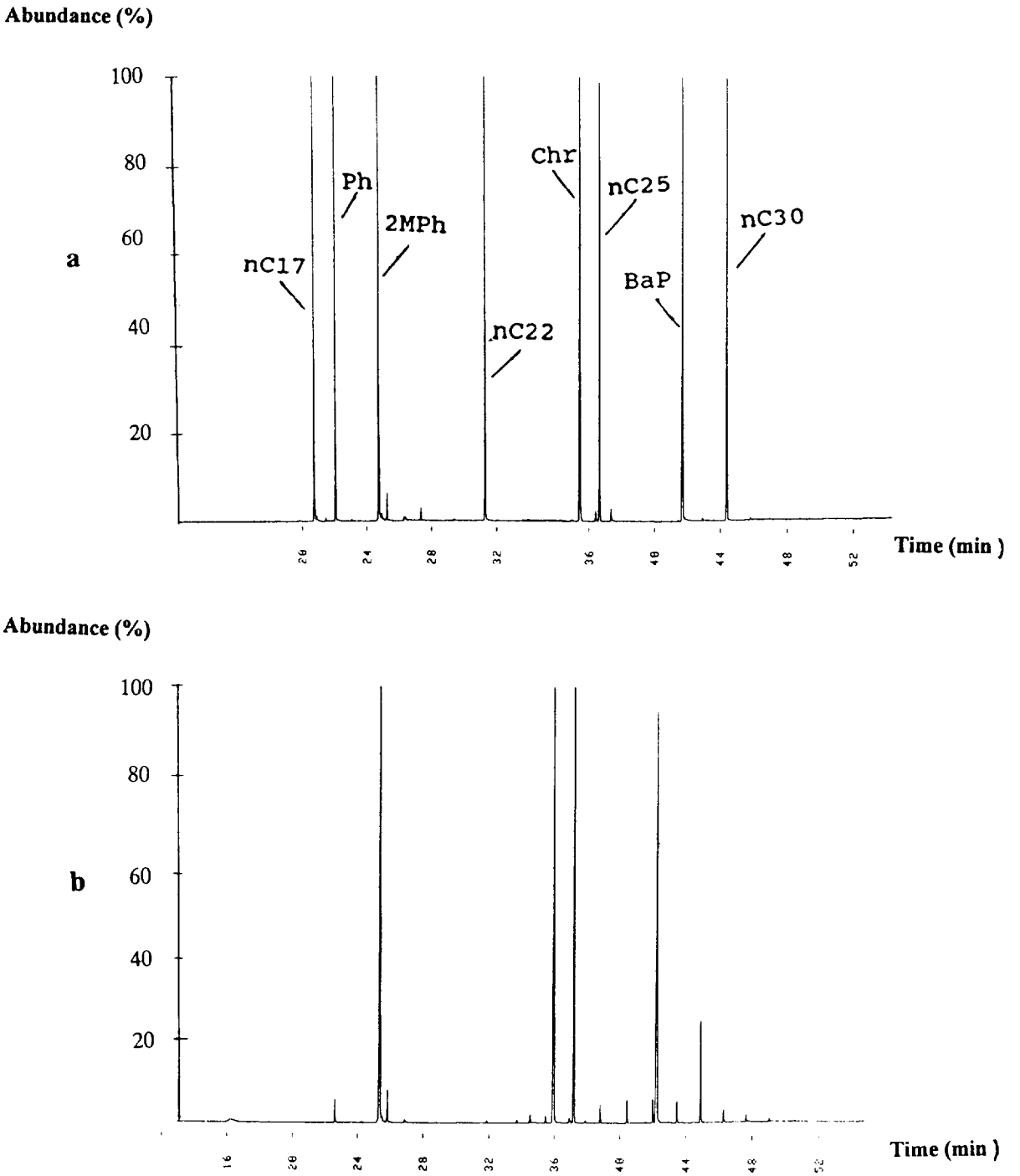

Fig. 1 (*continued on p. 520*).

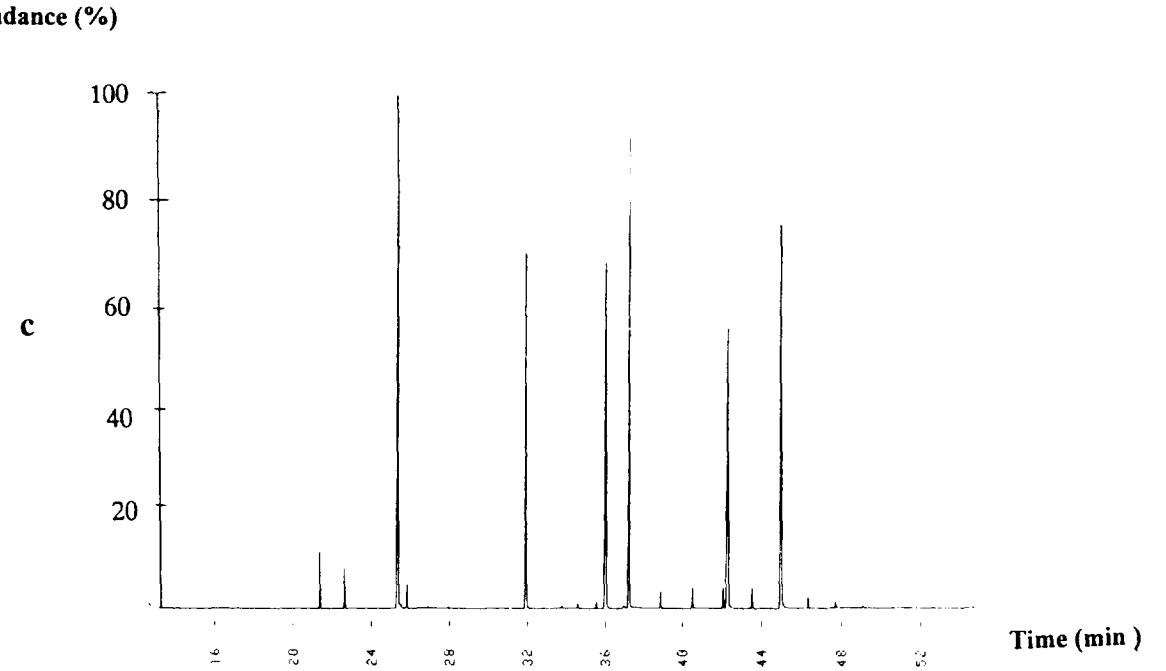

Fig. 1. Biodegradation of selected aromatic and aliphatic hydrocarbons. (a) Gas chromatogram of the mixture of hydrocarbons on day 0; (b) biotic and (c) abiotic samples after 61 days of incubation.

bons first. Bacteria will use compounds that are more difficult to degrade only if they are incubated for long enough and an adopted microbial population is developed.

It has been shown that hydrocarbons, which can not be used for growth by many soil organisms, can be oxidised if present as co-substrates in a system in which another substrate is available for growth [29]. It seems likely that bacteria become modified after attacking lower-molecular-mass compounds in a way so that they are able to oxidise even higher, less degradable compounds. When bacteria are exposed straight away to the heavy less degradable compounds, they quite possibly will not be able to oxidise them. A very interesting study has been reported very recently about a conditional-suicide containment system for bacteria which mineralise aromatic hydrocarbons [30].

The study of a real sample DO showed more effective biodegradation than its separated aromatic fraction. This was expected and proved the above conclusions. Abiotic losses were much lower in the real sample and the biological degradation more efficient. Fig. 2 represents the total ion chromatograms of the complex DO on day 0 of incubation (a) and its biotic (b) and abiotic (c) sample after 48 days of incubation. It is noticeable that as a result of evaporation all the light compounds disappeared in both biotic and abiotic samples. Disappearance of several heavier compounds was a result of biological degradation.

4. Conclusions

Results from this study proved that abiotic losses can play more important role than has previously been reported in the literature. Very recently similar findings have been obtained with PAHs only [31,32]. Compounds which are particularly affected are those with lower molecular masses, e.g. PAHs with less than four benzene rings and straight-chain alkanes with less than 20 C atoms. It was shown that higher concentra-

tions of hydrocarbons reduce the abiotic losses, but the availability of those compounds to the biota is then questionable. Abiotic losses in real samples such as DO are much lower. The abiotic losses are expected to be significantly diminished when working in a soil–water system because of

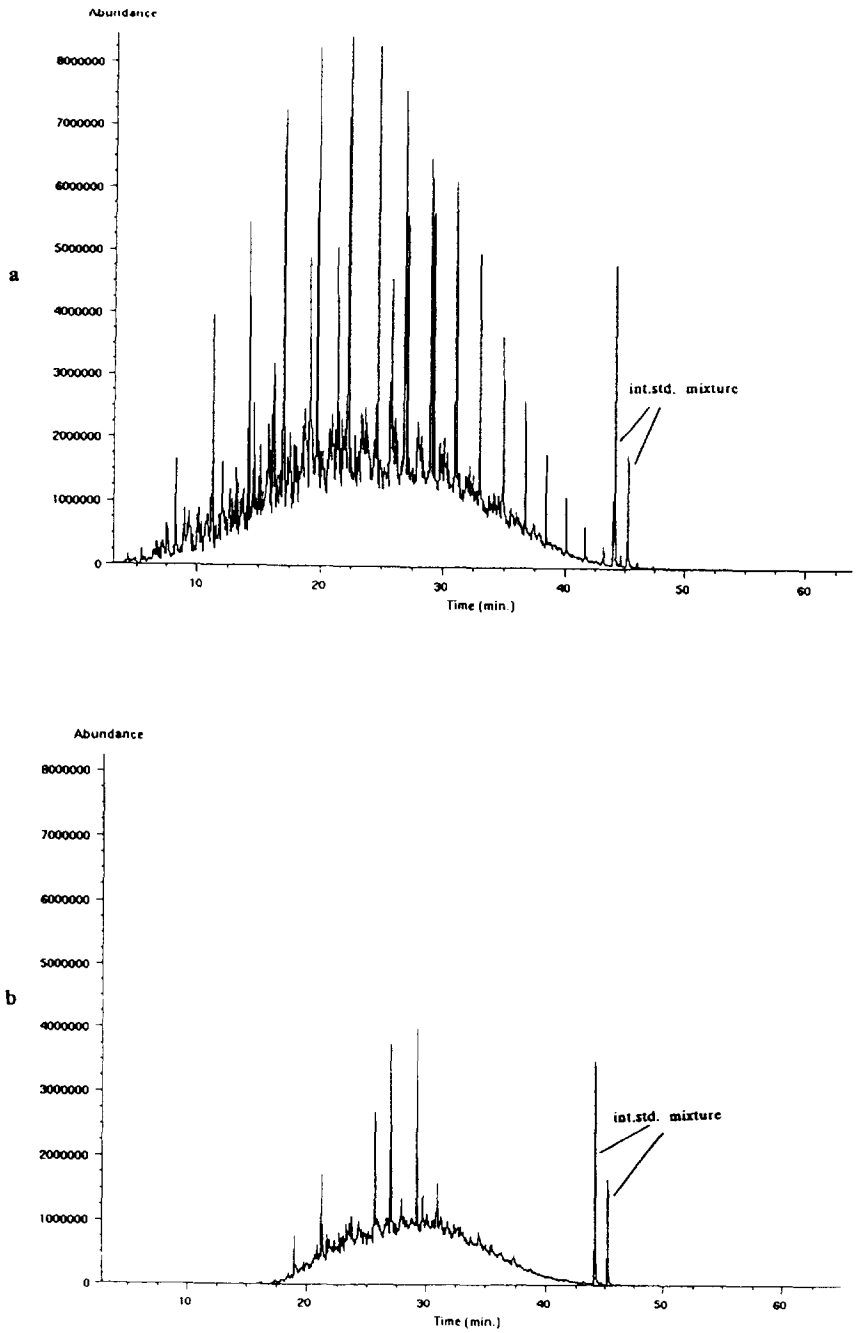

Fig. 2 (*continued on p. 522*).

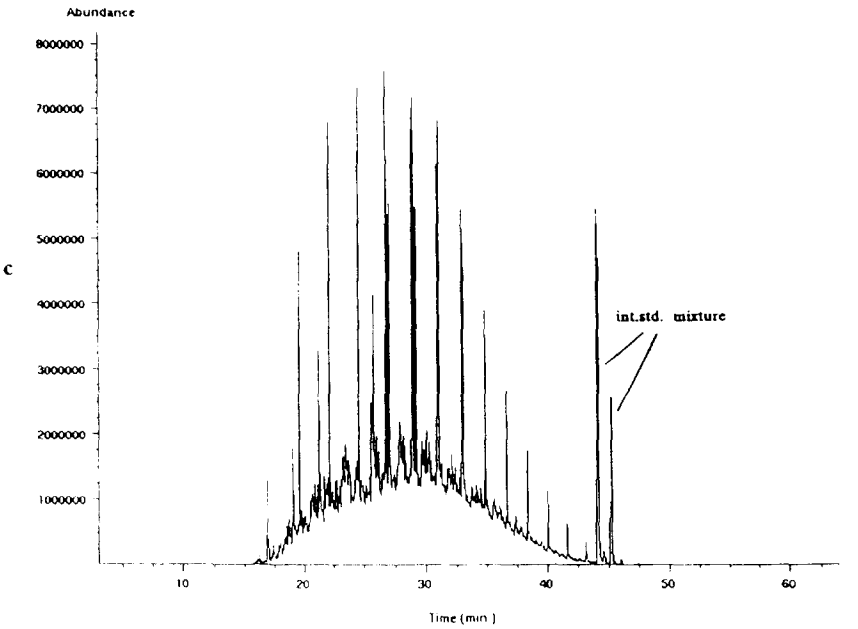

Fig. 2. Biodegradation of diesel oil. (a) Total ion chromatogram of diesel oil on day 0 of incubation; (b) biotic and (c) abiotic samples after 48 days of incubation.

the adsorption processes present. Care should be taken when relating laboratory-based observations concerning hydrocarbon losses to the losses occurring in the field.

In addition to in vitro biodegradation studies it is usually suggested to use chemical and microbiological measurements on samples from a contaminated site. The majority of biodegradation investigations that utilise environmental samples (water, soil and sediments from contaminated and uncontaminated sites) in laboratory flask assays demonstrate biodegradation potential, not in situ biodegradation. A laboratory approach allows the testing of a large number of controlled environment parameters within a relatively short time. But it is necessary to be aware of the limitations of such studies in reproducing field situations. For example, important parameters such as tillage and soil texture cannot be meaningfully tested in a laboratory system. It also remains to be determined whether temperature fluctuations around a daily mean during a 24-h cycle can be equated with a constant incubation temperature. Consequently, laboratory findings need validation and possible adjustment when compared to field studies. Nevertheless, laboratory studies can greatly reduce and simplify any subsequent field tests. The reason for performing the laboratory studies is clear —the control over mass balances and ability to distinguish biotic from abiotic reactions allow definite conclusions to be drawn.

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