Journal of Industrial Microbiology, 4 (1989) 365–374
Elsevier

SIM 00200

Comparison of autochthonous bacteria and commercially available cultures with respect to their effectiveness in fuel oil degradation

Wolfgang Dott*, Doris Feidieker, Peter Kämpfer, Hans Schleibinger and Stefan Strechel

Department of Hygiene, Technical University of Berlin, Berlin, F.R.G.

Received
Accepted 10 January 1989

Key words: biodegradation, fuel oil, bacterial cultures, activated sludge

SUMMARY

This study examined the microbial degradation of fuel oil by nine highly adapted different commercially available mixed bacterial cultures (DBC-plus™, Flow Laboratories, Meckenheim, F.R.G.) and a bacterial community from a domestic sewage sludge sample. All mixed cultures were cultivated under aerobic batch conditions shaking (110 rpm) at 20°C in a mineral base medium containing 1 or 5% (v/v) fuel oil as the sole carbon source. Percent degradation of fuel oil and the n-alkane fraction was recorded for the nine DBC-plus cultures and the mixed population of the activated sludge sample. The increase in colony counts, protein, and optical density was studied during a 31-day incubation period for DBC-plus culture A, DBC-plus culture A2 and the activated sludge sample. The activated sludge mixed culture was most effective in degrading fuel oil, but various isolated bacterial strains from this bacterial community were not able to grow on fuel oil as the sole carbon source. In contrast, the n-alkane degradation rates of the DBC-cultures were lower, but single strains from the commercially available mixed cultures were able to mineralize fuel oil hydrocarbons. Strains of *Pseudomonas aeruginosa* were isolated most frequently and these organisms were able to grow very rapidly on fuel oil as a complex sole carbon source. The results indicate that fuel oil degradation in domestic sewage sludge is performed by mixed populations of naturally occurring bacteria and does not depend on the application of highly adapted commercially available cultures.

Correspondence: Dr. Wolfgang Dott, Department of Hygiene, Technical University of Berlin, D-1000 Berlin 65, Amrumerstr. 32, F.R.G.

INTRODUCTION

Many different microorganisms isolated from soil or water have been found to be able to utilize hydrocarbons as the sole source of energy and carbon. The most important bacterial genera of hydrocarbon utilizers are *Pseudomonas, Alcaligenes, Flavobacterium, Acinetobacter, Vibrio, Micrococcus, Corynebacterium, Arthrobacter, Brevibacterium,* and *Nocardia* [5]. Numerous studies report the metabolic pathways of different individual hydrocarbons [6,8,12,13,15] using mixed or pure cultures. Fuel oil, like petroleum, is an extremely complex mixture of hydrocarbons including *n*-alkanes, branched alkanes, cycloalkanes and aromatic compounds. An environmental perspective of the microbial degradation of petroleum covering all important aspects is given by ATLAS [3].

In recent years a considerable market dealing with 'bioaugmentation' products has developed. Commercial types of bacteria are offered for various applications, e.g. control of odor problems of waste, treatment of domestic and industrial waste water, biogas production, clean up of polluted land and groundwater [1,2,4,10,14]. Bacteria are isolated from sites highly contaminated with organic material. By means of selection and induced mutation an increase in the degradation activities is achieved. Pure strains are preserved by air- or freeze-drying techniques and then brought into mixed cultures.

In our experiments we used nine different formulations of selected mixed bacterial cultures from Flow Laboratories, Meckenhein, F.R.G. These mixed cultures are called DBC-plus™ (dried bacterial cultures) and mainly applied as supplements to domestic and industrial waste water depending on the waste composition.

As we found no yeasts or only few filamentous fungi in the DBC-plus cultures by enrichment procedures, we focused our investigations on the bacterial populations.

The investigations reported in this paper are mainly concerned with the biodegradability of fuel oil as the sole source of carbon and energy using 9 different dried bacterial mixed cultures and a mixed bacterial population from a domestic treatment plant during a 31-day incubation period.

MATERIALS AND METHODS

Mixed cultures

For initial investigations 9 DBC-plus cultures: A, A2, B, F, H1, H2, L, L1, R5. (Flow-Laboratories, Meckenheim, F.R.G.) were used. For comparison we also conducted experiments on samples of activated sludge taken from the domestic sewage treatment plant Berlin Ruhleben (F.R.G.). In the more detailed investigastions, further experiments were carried out for the DBC-plus cultures A and A2 and the activated sludge sample. Table 1 lists the applications for the DBC-plus cultures as described by the manufacturer.

Culture-conditions

The 9 DBC-plus products in the granulated form were reactivated according to the manufacturer's instructions. The activated sludge samples were collected from the outflow of the aeration tanks in sterile screw-capped bottles and were treated immediately on return to the laboratory. After a 6-day

Table 1

List of dried bacterial cultures (DBC-plus™, Flow Laboratories) used in this investigation and industrial applications according to the instructions of the manufacturer

DBC-plus culture	Applications
A	treatment plants, odor and grease control, improve aerobic sludge digestion
A2	process control in treatment facility, improve waste oil degradation
B	process control under anaerobic conditions
F	odor control
H1	process control in treatment facility, improve degradation of waste from the petrochemical industry
H2	improve degradation of wastes with high content of proteins and hydrocarbons
L	improve degradation of wastes with high content of lipids
L1	odor and grease control, improve waste oil degradation
R5	improve degradation of wastes with high content of aromatic hydrocarbons, improve waste oil degradation

adaptation period during which the reactivated cultures and the activated sludge were continuously transferred and grown on low oil concentration (0.2%, v/v), cultures were inoculated into 250 ml Erlenmeyer flasks containing approximately 100 ml of mineral salt medium so as to obtain an optical density of 0.1 at 600 nm. Summer fuel oil was then added first in a concentration of 1% (v/v) and later we used 5% (v/v) of the fuel oil. The mineral salt medium contained (g $\times$ l^{-1} glass distilled water): $Na_2HPO_4 \times 2H_2O$, 6.99; KH_2PO_4, 0.8; $(NH_4)SO_4$, 1.8; $MgSO_4 \times 7H_2O$, 0.123; $CaSO_4 \times 2H_2O$, 0.017; Yeast extract (OXOID), 0.01; and 1 ml trace element solution SL8 according to Pfennig and Lippert [13]. The pH of the medium was 7.1. The flasks were aerobically incubated at 20°C in a shaker incubator at 110 rpm in the dark.

Chemical measurements

The samples were subjected to ultra sonication to separate the oil-water-bacteria flocs. After adding 10 ml of pentane, each sample was centrifuged for 30 min at about 18 000 $\times$ *g*. The organic layer was removed. The aqueous layer was shaken once again with 20 ml pentane for 5 min. The two organic layers were combined and contained about 99% of the organic material. The samples of the batch cultures containing 1 and 5% oil were evaporated, filled up with hexane to a volume of 10 and 50 ml respectively and further diluted (1:5) with hexane prior to measurement.

Gas chromatography was done using a Shimadzu GC 9A with a flame ionization detector. The 0.32 mm I.D. $\times$ 50 m fused silica column was coated with SE 54. A volume of 1 μl extract was injected. To monitor the decrease of single compounds such as *n*-alkanes a moderate temperature rise (5°C/min starting from 60°C) was chosen. Most compounds were separated to baseline and could be quantified by an external standard. To evaluate the degradation of the total oil (given as total hydrocarbons throughout the manuscript), a high temperature program rate (20°C/min) was chosen to elute the sample without baseline resolution. The total peak areas were calculated by an integrator (Shimadzu C-R3A) according to a GC distillation analysis method. The final temperature of 260°C was held until all compounds were eluted.

Growth parameters

The plating media used for bacterial enumeration was tryptone-soya agar (Oxoid). Bacterial growth was followed up by measuring optical densitgy at 600 nm using a spectrophotometer (Beckmann, DU 7). Due to the development of a flocky layer, samples for the estimation of colony count and optical density were taken from the aqueous phase. Protein concentration was determined by a modified method of Herbert et al. [9].

Identification of bacterial pure cultures

The colony count plates (30–100 CFU) at the beginning and end of the experiments (31 days) were studied for groups showing different colony types. Pure cultures isolated from plates at the end of incubation period (31 days) were identified using the numerical methods described by Dott and Kämpfer [7].

Degradation activities of pure isolates were also studied: Colonies were suspended in 25 ml of the basal medium containing 1% (v/v) oil. The same culture conditions were followed as before and growth was observed over a period of 21 days.

RESULTS AND DISCUSSION

During aerobic growth, the bacterial cultures showed disruption and break-up of the oil film followed by the development of variable strong emulsions depending on the added mixed culture. Microscopic studies showed a tight adhesion of the bacteria to the oil droplets, whereupon the droplets disappeared and the agglomerations of bacteria remained. A flocky layer of oil-bacteria agglomerations of about 3 mm thickness was formed on the surface of the aqueous phase. In some cultures a light yellow coloration of the aqueous phase occurred. The suspension of the activated sludge showed an intensive yellow coloration after 1-day incubation. The nature of those colorations was not examined. They could have probably developed as

products of various enzymatic oxidation reactions. Zajic et al. [16] observed the coloring effect of a surface active agent isolated from a culture grown on dodecane and so the coloration in our experiments could also be due to production of bioemulsifiers.

Percent degradation of total fuel oil hydrocarbons using different initial oil concentrations is given in Figures 1 and 2 for all cultures examined. DBC-plus F and H1 exhibited no growth and no degradation of any hydrocarbons. The higher oil concentration (5% v/v) had an inhibitory effect on DBC-plus L1 (Fig. 2). In comparison, the other cultures yielded similar degradation patterns of *n*-alkanes with a 1% or 5% oil concentration. However, the degradation of the other hydrocarbons by these cultures using a 5% (v/v) substrate concentration was insignificant due to preferential utilization of *n*-alkanes. On the other hand, the activated sludge mixed culture and DBC-plus B were able to metabolize about 50% of the total substrate (5% v/v fuel oil). Further experiments using winter fuel oil (Oil Company Aral) have shown better degradation rates because of the higher content of small molecular weight alkanes than in summer fuel oil. (unpublished results).

Cultures DBC-plus R5, A2, H2, and L1 are specifically used to enhance petroleum waste removal (see Table 1). However, cultures A and B degraded the oil as well as those cultures recommended by the manufacturer. These findings led to further experiments on the rate of fuel oil degradation by culture DBC-plus A2 (recommended by the manufacturer to enhance petroleum degradation), culture DBC-plus A (not specified for petroleum wastes), and the mixed culture of the activated sludge sample.

Growth parameters such as optical density, protein concentration and colony count were determined over a growth period of 31 days. In addition, *n*-alkane-degradation was determined. The growth curves of DBC-plus A, DBC-plus A2 and the mixed culture from activated sludge are presented in Figures 3–5. After a 5-day period, each for lag and exponential phases, cultures A reached the stationary phase. During the exponential phase a decrease in *n*-alkanes up to 75% and a marked increase in protein concentration was found (Fig. 3). In contrast, culture A2 showed a slight increase in protein concentration during the whole period of 31 days. As a consequence, the *n*-alkane concentration decreased slowly and constantly (Fig. 4). Culture A2 was distinghuished by a linear increase in all parameters studied except for the colony count, and by a slow degradation of the *n*-alkanes (Fig. 4). In comparison, the mixed culture from activated sludge showed a more rapid growth and faster *n*-alkane degradation (Fig. 5). The most rapid degradation of the paraffins was achieved by the activated sludge.

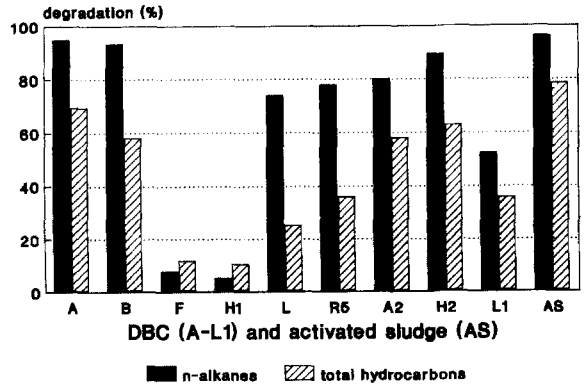

Fig. 1. *n*-Alkane degradation (%) and total hydrocarbon degradation (%) by all DBC-plus cultures and the mixed population of activated sludge in mineral salt medium containing 1% (v/v) fuel oil after 31 days incubation at 20°C.

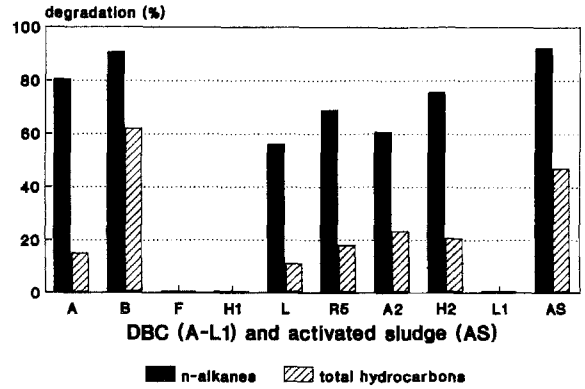

Fig. 2. *n*-Alkane degradation (%) and total hydrocarbon degradation (%) by all DBC-plus cultures and the mixed population of activated sludge in mineral salt medium containing 5% (v/v) fuel oil after 31 days incubation at 20°C.

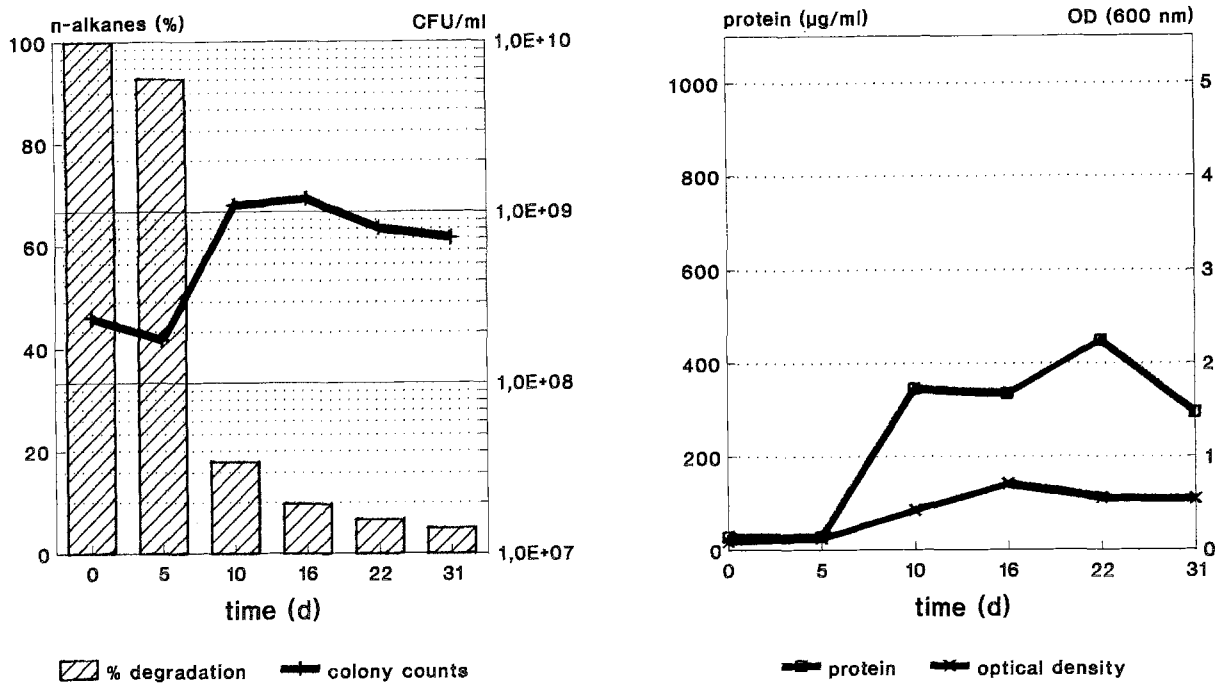

Fig. 3. *n*-Alkane concentration (%), optical density at 600 nm, protein concentration, and colony forming units (CFU) on TS-agar of culture DBC-plus A in a mineral salt medium containing 1% (v/v) fuel oil.

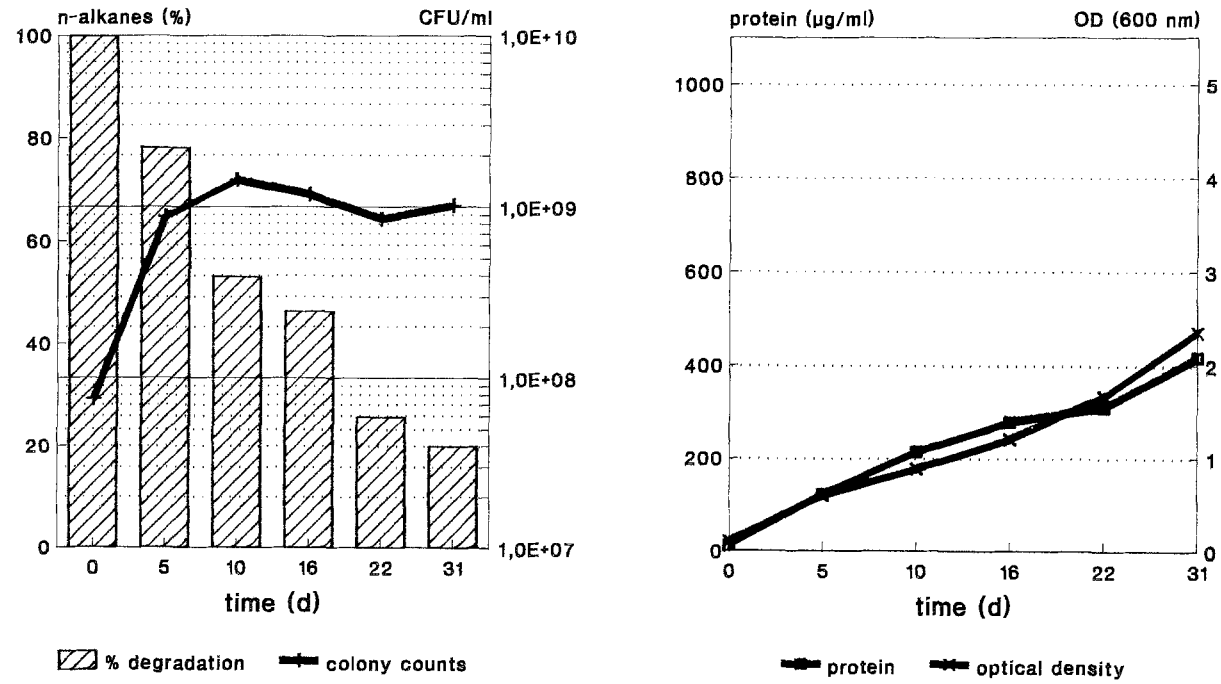

Fig. 4. *n*-Alkane concentration (%), optical density at 600 nm, protein concentration, and colony forming units (CFU) on TS-agar of culture DBC-plus A2 in a mineral salt medium containing 1% (v/v) fuel oil.

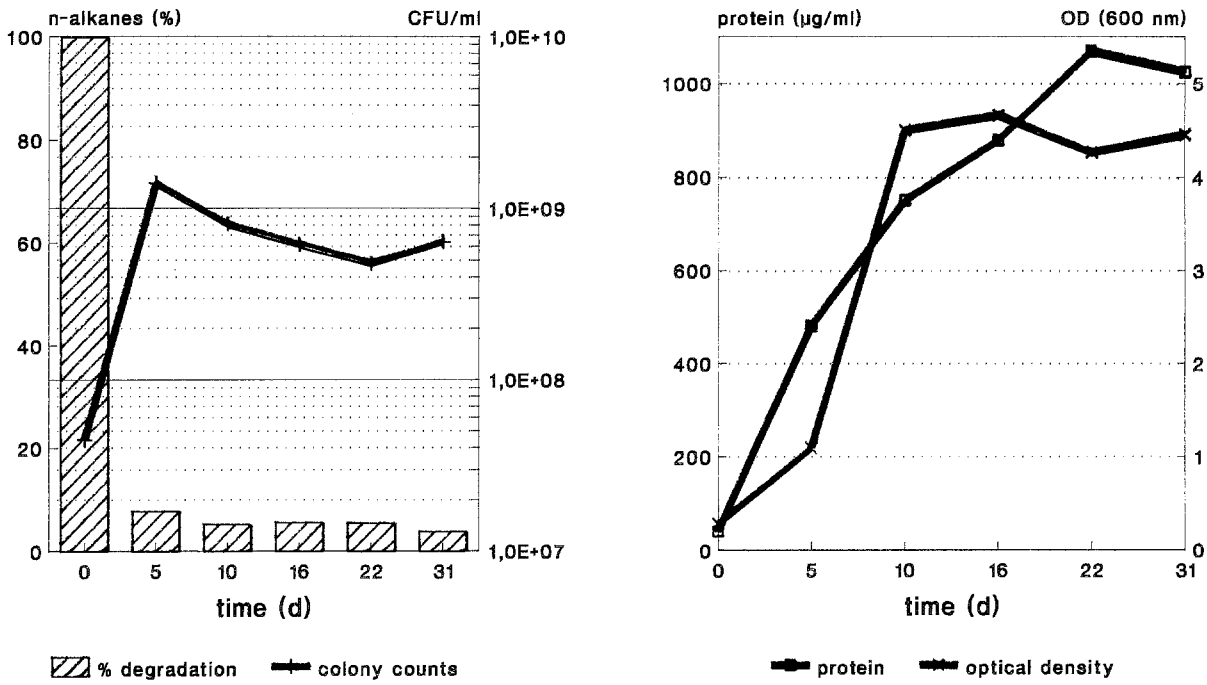

Fig. 5. *n*-Alkane concentration (%), optical density at 600 nm, protein concentration, and colony forming units (CFU) on TS-agar of a mixed culture from activated sludge in a mineral salt medium containing 1% (v/v) fuel oil.

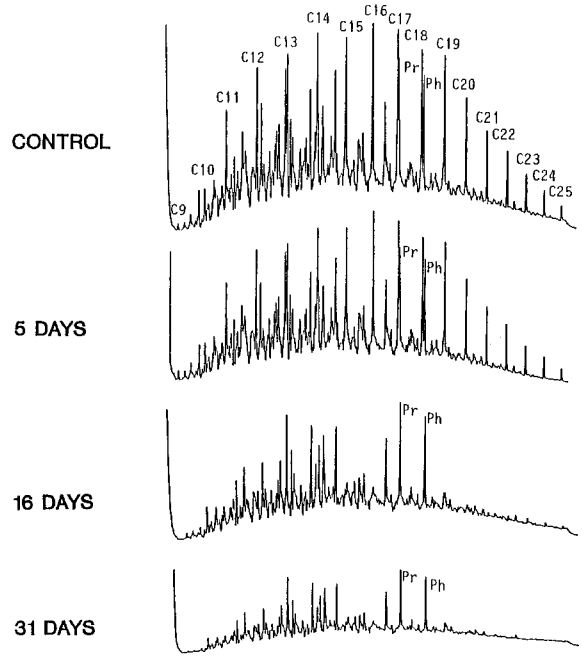

Fig. 6. Total gas chromatograms of the mineral basal medium containing 1% fuel oil as complex carbon source after 0, 5, 16 and 31-day growth of DBC-plus A mixed bacterial culture in this medium. See text for analytical conditions. Ph = phytane; Pr = pristane.

After 5 days, 92% of the *n*-alkane were utilized. The biomass examined by protein concentration continued to increase remarkably and reached a 3 fold higher protein concentration than cultures A and A2.

The maximum absorbance values of the optical density are too high (Fig. 5). They were probably caused by dispersed oil droplets and coloured metabolites. Colony counts of cultures A, A2 and activated sludge showed only slight variation during the 31-day incubation period.

The chromatographic profiles presented in Fig. 6–8 show differing degradation activities by the three cultures. The profile presented in Fig. 6 shows a slow degradation of *n*-alkanes by culture A, and can be still identified after 31 days. Successive degradation of *n*-alkanes according to length of carbon-chain was found for culture A2. A degradation of more than 50% was recorded for the C9–C15 alkanes after 5 and 16 days incubation while a lower degradation ($< 50\%$) was found for the C16–C21 alkanes during the same period (Fig. 7). The mixed culture from activated sludge showed a complete

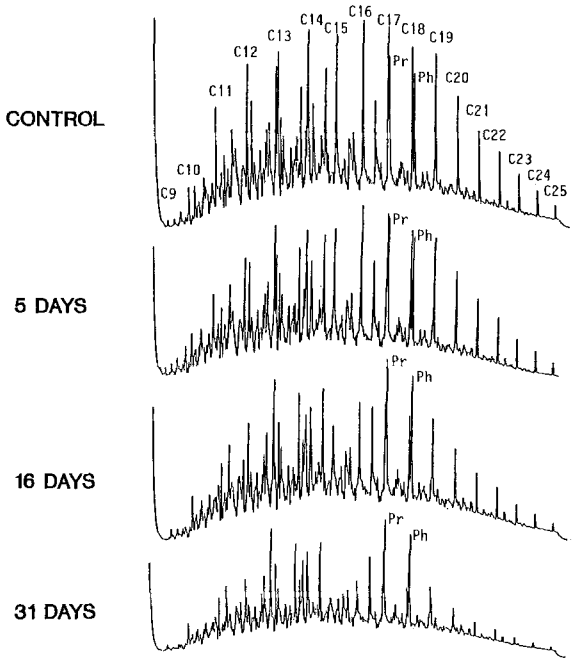

Fig. 7. Total gas chromatograms of the mineral basal medium containing 1% fuel oil as complex carbon source after 0, 5, 16 and 31-day growth of DBC-plus A2 mixed bacterial culture in this medium. See text for analytical conditions. Ph = phytane; Pr = pristane.

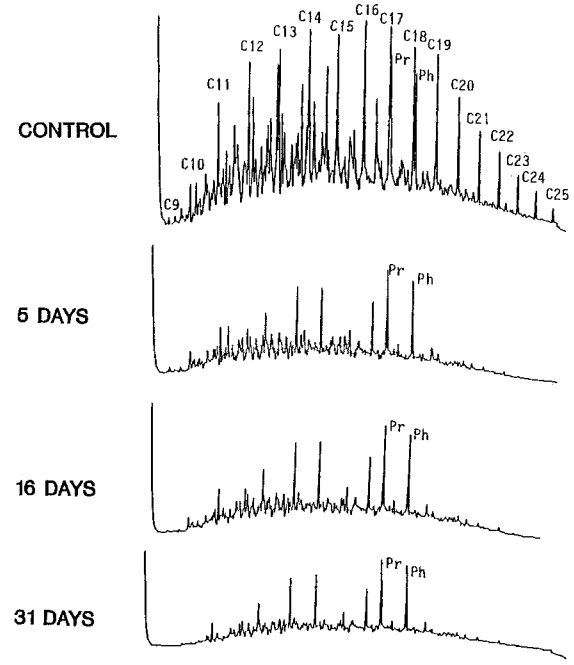

Fig. 8. Total gas chromatograms of the mineral basal medium containing 1% fuel oil as complex carbon source after 0, 5, 16 and 31-day growth of mixed bacterial culture from activated sludge in this medium. See text for analytical conditions. Ph = phytane; Pr = pristane.

elimination of *n*-alkanes after 5 days with 1% (v/v) oil and also an extensive reduction of the remaining hydrocarbons (Figs. 1 and 2). After 31-day incubation, a pattern of resistant substances remained, e.g. the isoprenoid compounds: pristane and phytane. These compounds were reduced to 50% of the initial concentration by the activated sludge mixed culture and DBC-plus A and 20% for DBC-plus A2. New peaks indicating metabolic products could not be found during growth experiments.

Comparing the plate counts at the beginning and end of the growth experiments, a reduction in the variety of different colony types was observed indicating a selection for a few bacterial species. This resulted in an elimination of the different colony-forming types grown on 5% (v/v) oil, with a lower selection (0–30% elimination) in the types grown on 1% (v/v) substrate.

The remaining strains from the DBC-plus cultures A and A2 as well as from the activated sludge sample were identified. (Results are shown in Figs. 9 and 10). Most strains belonged to the genus *Pseudomonas* with the exception of culture A2 which consisted mainly of *Acinetobacter calcoaceticus* after a 31 day incubation with 1% oil (Fig. 9). In contrast to the DBC-plus cultures, the activated sludge was composed of a variety of different strains but the isolates could not grow on fuel oil in pure cultures. Only strains of *Pseudomonas maltophilia, Pseudomonas aeruginosa* and *Flavobacterium species* showed a slight growth on 1% oil after 3 weeks incubation.

CONCLUSION

DBC-plus cultures showed differences in growth and in the quantity and rate of *n*-alkane degradation. High and rapid degradation rates could be seen by cultures DBC-plus A, DBC-plus B and DBC-plus H2. A high specifity for degrading petrochemical sewage could not be confirmed for the cul-

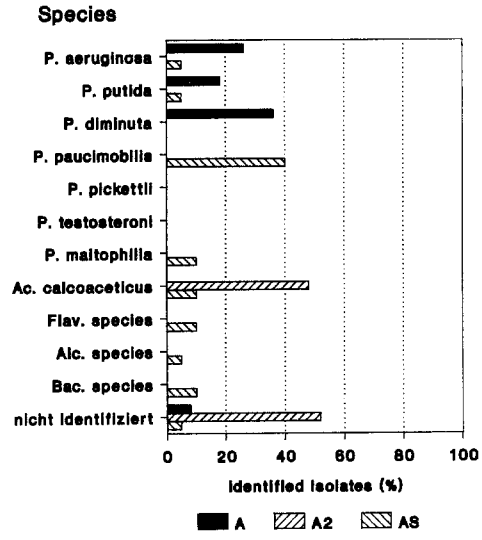

Fig. 9. Number of pure cultures shown as % of total strains, isolated from DBC-plus A, (A); DBC-plus A2, (A2); and the activated sludge sample, (AS) able to grow on the complex carbon source of 1% (v/v) fuel oil after 21 day incubation at 20°C. (P = Pseudomonas; Ac = Acinetobacter; Flav = Flavobacterium; Alc = Alcaligenes; Bac = Bacillus).

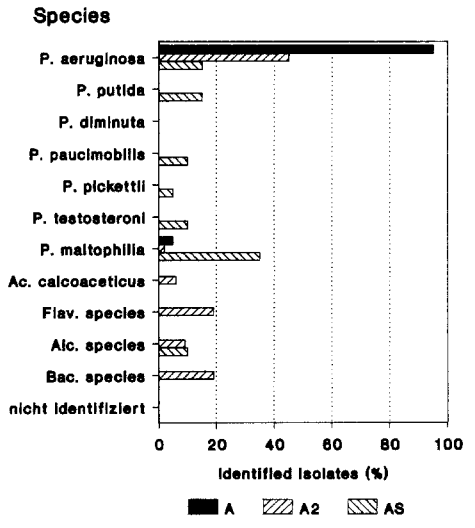

Fig. 10. Number of pure cultures shown as % of total strains, isolated from DBC-plus A, (A); DBC-plus A2, (A2); and the activated sludge sample, (AS) able to grow on the complex carbon source of 5% (v/v) fuel oil after 21 day incubation at 20°C. (P = Pseudomonas; Ac = Acinetobacter; Flav = Flavobacterium; Alc = Alcaligenes; Bac = Bacillus).

tures A2, R5, L1. These cultures showed a slow degradation of the alkanes. Apparently, the high capacity of the activated sludge for n-alkane degradation could be due to synergistic effects among the species as more bacterial groups were recorded in the sludge samples. A selective effect of high oil concentration (5%) was seen on the bacterial composition of the different cultures. (Figs. 9 and 10).

A striking feature of the activated sludge was the further increase in the biomass which confirmed the elimination of the other compounds then *n*-alkanes (Fig. 5). This could not be observed with DBC-plus cultures although the chromatographic profiles showed a reduction of hydrocarbons other than *n*-alkanes. However, there was no way of knowing whether disappearance of a peak actually corresponded to the degradation of this individual compound. By control experiments we could exclude cell-adsorptive effects of hydrocarbons and loss by evaporation. But the method of non-polar extraction poses some difficulties, i.e. polar compounds such as oxidation products and further metabolic intermediates are not detected. In addition, it is also possible that the method for protein determination of biomass was not sufficient as the accumulated intracellular lipids could not be measured by this method.

Although oil contamination at high concentrations is rarely encountered in domestic sewage treatment plants, exposures to hydrocarbons at low concentrations may be chronic and could support a continously acclimated population of hydrocarbon degraders. The more rapid adaptation and higher degree of oil degradation found for the sludge samples in our experiments could be due to the more diverse bacterial population of the activated sludge. As a consequence, the application of commercially available products to enhance biodegradation activities in sewage treatment plants should not always be the recommended method of choice. In many cases the autochthonous bacteria possess sufficient potential for degradation of hazardous chemicals and these capacities should be activated and enhanced.

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