

Microbial Utilization of Crude Oil

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The utilization of two crude oil samples of different quality at 4 and 30 C has been studied by using pure and mixed bacterial cultures obtained by enrichment procedures. Growth, emulsification, and utilization occurred readily at both temperatures. The crude oil residue is increased in specific gravity and readily sediments out of solution. A comparison of the chemical analysis of the oils by liquid and gas-liquid chromatographic procedures before and after growth showed that the *n*-saturate fraction had been preferentially used. Some utilization of the aromatic fraction also occurred. Enrichments obtained with a high-quality crude oil were not as effective in utilizing a lower quality crude oil as sole carbon source as a population enriched on the low-quality crude oil.

In the last 15 years, there has appeared a large number of publications concerning the use of hydrocarbons as substrates for microbial growth (1, 4, 11, 12; J. A. Williams and J. C. Williams. Microbial alteration of crude oil in the reservoir, Amer. Chem. Soc. Annu. Meet. N.Y., 7-12 September 1969). Such studies, however, usually involved the use of purified substrates, such as alkanes of specified chain lengths. Bacteria have been implicated as agents of whole oil catabolism both in oil-bearing formations (in situ) and in artificial fermentation systems by Davis (2) and Williams and Winters (Microbial alteration of crude oil in the reservoir, Amer. Chem. Soc. Annu. Meet. N.Y., 1969). It is also apparent that whole oils spread artificially, or accidentally, on soil are degraded within months or years of application depending on the quality and frequency of the oil spilled and upon the prevailing environmental conditions.

A study of the relationship between crude oil quality and its biodegradability was carried out by comparing the effect of growth of pure and mixed bacterial cultures obtained by soil enrichment on the composition of crude oils of different quality. The effect of temperature on this relationship also was studied, as psychrophilic environmental conditions prevail during the greater part of the year in northern areas which could affect the rate of biodegradation of crude oils.

MATERIALS AND METHODS

Microbiological methods. All cultures used in these studies were obtained by enrichment procedures from soils which had been contaminated with

crude oil over a period of time. Soil samples were obtained from a producing well site in the Judy Creek area of north-central Alberta, from a diesel fuel spill near Salmon Arm, British Columbia, and from a green house soil which had been saturated with crude oil 2 years prior to sampling.

Enrichments were carried out in 2-liter Erlenmeyer flasks containing 1 liter of basal salts medium consisting of K_2HPO_4 , 0.5 g; NH_4Cl , 1.0 g; Na_2SO_4 , 2.0 g; KNO_3 , 2.0 g; $CaCl_2 \cdot 6H_2O$, 0.001 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; and a trace of $FeSO_4$ in 1,000 ml of distilled water. Fifty milliliters of a 10% (w/v) suspension of soil plus 1.0 ml of crude oil was added to each flask which was incubated at 4 or 30 C on a rotary shaker (300 rev/min, 1 inch eccentricity). Transfers were made into the same medium at appropriate intervals with 50 ml of culture as inoculum.

Growth was monitored by using the plate count technique and a medium consisting of the above salts solution (minus oil), to which was added yeast extract (Difco), 1.0 g/liter; sodium lactate (60% solution), 2.5 ml/liter; and 15.0 g of agar (Difco) per liter. This medium also was used for the isolation of pure cultures. An incubation temperature of 4 C was used for the psychrophilic studies and 30 C for the mesophilic experiments.

Different levels of aeration were obtained by varying the amount of medium in the 2-liter Erlenmeyer flasks and keeping the agitation constant, i.e., 300 rev/min and a 1 inch eccentricity. The sulfite oxidation method (3) was used to determine the rate of oxygen transfer into solution.

Analytical methods. Residual crude oils were extracted from cultures by using *n*-pentane as a solvent. Cultures were divided into two equal fractions and extracted three times with a volume of solvent equal to 30% of the original divided culture volume. The aqueous phase was removed each time by using separatory funnels and draining to the interfacial area which contained the salts, asphaltenes, and sol-

vent. This interfacial material was removed after the last solvent extraction, pooled with the *n*-pentane washes, and evaporated to dryness in a fume hood. The residual petroleum was recovered from the beakers by using 10 to 20 ml of benzene. The walls of the beaker were rigorously washed with this benzene prior to its removal and storage for analysis.

Liquid chromatographic fractionation. Samples containing 0.05 to 0.4 g of residual or crude petroleum were placed in a tared beaker and "topped" by exposing them to forced draft conditions for 19 hr at either 32 or 36.5 C. This treatment removed volatile materials, e.g., light aromatics, naphthenes, and *n*-alkanes up to and including C₁₅ chain length, leaving a weight referred to as the "topped weight of oil." The asphaltenic component of the "topped oil" was precipitated by addition of *n*-pentane. The pentane solubles and precipitated asphaltenes were then applied to a 1 by 15 cm bed of Hyflo Super-Cel (Fisher Scientific Co.) suspended in *n*-pentane. The column was sequentially developed with 50 ml of *n*-pentane and 40 ml of benzene to elute the deasphalted oil and benzene-soluble asphaltenes. The weight of benzene-insoluble asphaltenes remaining on the column was calculated by difference. The deasphalted oil (*n*-pentane-soluble) was fractionated by adsorption chromatography by using a dual-phase column (1 by 40 cm) containing in the bottom half 10 ± 0.2 g of activated 28-200 mesh silica gel (Matheson) and in the upper half 10 ± 0.2 g of activated F-20 alumina gel (Matheson). Both phases were suspended in *n*-pentane. The deasphalted oil was layered on the top of the column and then eluted sequentially with 65 ml of *n*-pentane, 100 ml of benzene, and 100 ml of a 1:1 mixture of benzene-methanol. This procedure (Fig. 1) eluted the saturate, aromatic, and soluble NSO components in that order. (NSO component-fraction recovered by elution with a 1:1 benzene-methanol mixture. This fraction should contain more polar compounds than those eluted with benzene which yields the aromatic fraction of crude oil.) The sum of these three weights subtracted from the deasphalted weight originally applied to the dual-phase column yielded the insoluble NSO component weight. This procedure is similar to that used by Imperial Oil Research Laboratories, Calgary, Alberta.

Gas-liquid chromatographic analysis of saturate hydrocarbons. The separation and determination of *n*-saturate alkanes was achieved by using a Varion Aerograph Chromatograph model 1740-1 equipped with a flame ionization detector and a 10 foot (1/8 inch inner diameter) stainless-steel column containing (100 to 200 mesh) chromosorb P pre-coated with 3% OV-1. Columns were conditioned at 325 C for 72 hr prior to use, and all carrier gases were purified by passage through Hydro-Purge Molecular Sieve 5A filters prior to passage through column or detector. The instrument was programmed as follows: linear temperature program 50 to 325 C; rate of programming, 10 C per minute; injection block temperature, 300 C; nitrogen flow rate, 12.0 ml/min; hydrogen flow rate, 15.0 ml/min; air flow rate, 300 ml/min.

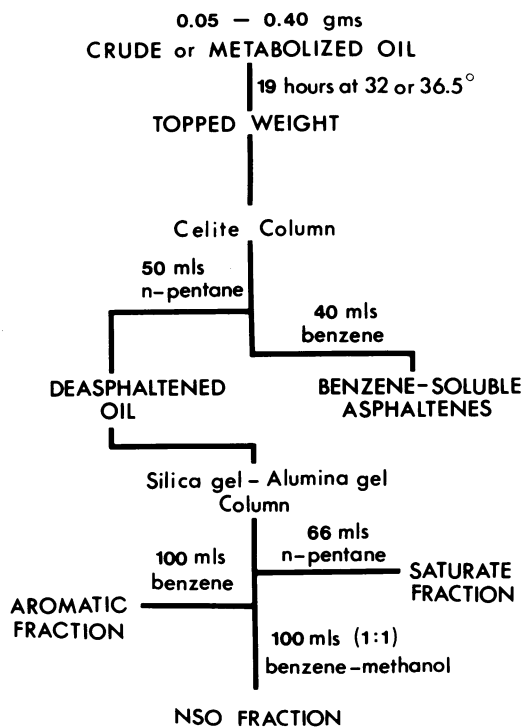


FIG. 1. Flow sheet for liquid chromatographic separation of petroleum fractions.

Forty micrograms of saturates could be readily resolved by using these conditions. Benzene was used as solvent as it passed through the OV-1 column well in advance of all other components and is less volatile than *n*-pentane.

Chemicals. The solvents used in the fractionation of the crude oil were of spectral quality and obtained from Fisher Scientific Co. The two crude oil samples used in these studies were obtained through Imperial Oil Research Laboratories, Calgary, and originated in the Weyburn area of south-east Saskatchewan. The sample from the North Cantal field represents a high-grade crude oil having more saturates and less sulfur than the sample from the Lost Horse Hill field which represents an inferior grade of crude oil. Both samples are classified as having the same origin and their analyses are presented in Table 1.

RESULTS

Microbial utilization of a high-grade crude oil. The utilization of crude oil, i.e., North Cantal sample, at mesophilic temperatures, i.e., 30 C, is shown by the increase in number of viable cells as a function of time (Fig. 2). The most striking effect of bacterial action on the crude oil was a change in its specific gravity. Repurified, residual crude North Cantal oil showed a specific gravity of 1.046 after 21 days of incubation as compared to its

TABLE 1. Liquid chromatographic analysis of North Cantal and Lost Horse Hill crude oil samples

Oil fraction	Weight (%) of "topped" crude oil	
	North Cantal	Lost Horse Hill
Sulfur	0.97	2.56
Asphaltenes	10.4	17.7
Saturates	50.8	36.8
Aromatics	31.9	37.9
NSO ^a	8.1	13.3

^a NSO component: fraction recovered by elution with a 1:1 benzene-methanol mixture. This fraction should contain more polar compounds than those eluted with benzene which yields the aromatic fraction of crude oil.

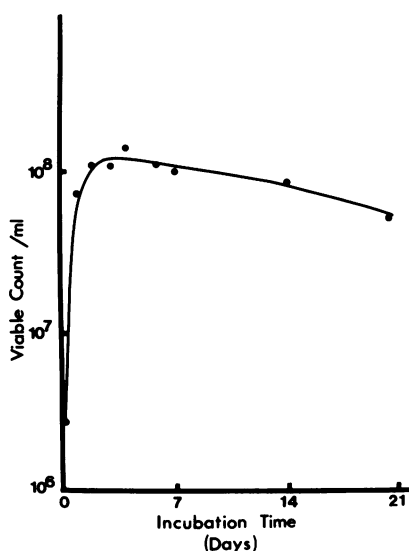


FIG. 2. Viable counts of soil mixture organisms incubated with 0.1% North Cantal oil at 30 C.

original specific gravity of 0.827 (as determined by pycnometric measurement). This resulted in a residual material which gradually settled to the bottom of the flasks. However, even before this specific gravity change had occurred, emulsification of the petroleum was observed.

Similar growth patterns were obtained through seven successive transfers at which time the population consisted of at least three distinct morphological types. These were identified as being a nonpigmented *Pseudomonas* species, a *Flavobacterium* species, and an *Achromobacter* species. A fourth colonial type appeared, which was classified as a *Bacillus* species, but its incidence suggested that it

could have arisen as a contaminant rather than being part of the original enrichment mixture. Since their initial enrichment, this mixture has been maintained for over a year by semi-weekly transferring on salts medium plus 0.1% crude oil without any detectable changes in the proportion of colonial types comprising the mixed population.

Liquid chromatographic analysis of the crude oil residue after the growth of the mesophilic mixture is presented in Table 2. The saturate fraction is the primary source of carbon and energy for growth as there has been about a 30% reduction in weight of this fraction during the 21-day incubation period. However, a calculation of residual weights which would be expected if only the saturates were used shows a decrease in weight of the aromatic fraction. This suggests that the aromatic fraction was also subjected to microbial attack.

The utilization of the components of the saturate fraction was followed by gas-liquid chromatography and the results are presented in Fig. 3. The short-chain saturates present, i.e., C₁₆ to C₂₅, are used before the longer chain ones although they all disappear within 14 days of incubation at 30 C. The isoprenoids pristane and phytane are more resistant to microbial attack, requiring at least 2 weeks of incubation before being utilized.

Similar results were obtained at 30 C with a pure culture (tentatively identified as a *Micrococcus* species) except that there was no preferential earlier digestion of the shorter chain saturates. Phytane and pristane were also metabolized at a faster rate by this *Micrococcus* than by the mixed culture (Fig. 4).

Enrichment at psychrophilic temperatures,

TABLE 2. Liquid chromatographic analysis of crude oil residue after growth^a of mixed population at 30 C

Oil fraction	Weight (%) of "topped" weight of oil	
	Control	Residue
Benzene-soluble asphaltenes	2.5	5.1 (3.2) ^b
Benzene-insoluble asphaltenes	6.8	8.1 (8.6)
Saturates	51.0	35.6 (-)
Aromatics	31.2	36.8 (40.3)
Soluble NSO ^c	8.6	10.9 (11.2)
Insoluble NSO	0.3	3.3 (0.4)

^a Grown for 21 days at 30 C.

^b Values in parentheses are weight percentages calculated on the basis of the saturate fraction only being utilized.

^c See Table 1, footnote a.

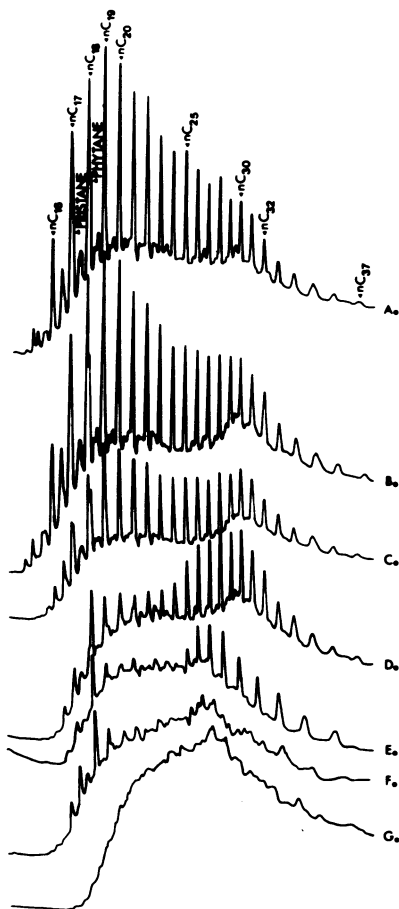


FIG. 3. Gas-liquid chromatographic analyses of residual saturate fractions from sequential utilization of oil by mixed population at 30 C. (A) Saturate fraction of control North Cantal oil. (B) Residual saturates after 2 days of incubation. (C) Residual saturates after 3 days of incubation. (D) Residual saturates after 4 days of incubation. (E) Residual saturates after 5 days of incubation. (F) Residual saturates after 14 days of incubation. (G) Residual saturates after 21 days of incubation.

i.e., 4 C, resulted, after several subcultures, in the establishment of a population consisting of a small gram-negative pseudomonad, a small, thick, unidentified gram-negative rod, and a gram-positive rod tentatively identified as a *Bacillus* species. Similar mixtures were observed in enrichments from both the Judy Creek and Salmon Arm soil samples. These were considered to be nonobligate psychrophiles as they grew as well at 30 C as at 4 C. The growth of this mixed population on whole crude oil is shown in Fig. 5.

Liquid chromatographic analyses of the crude oil residue after growth of the Judy

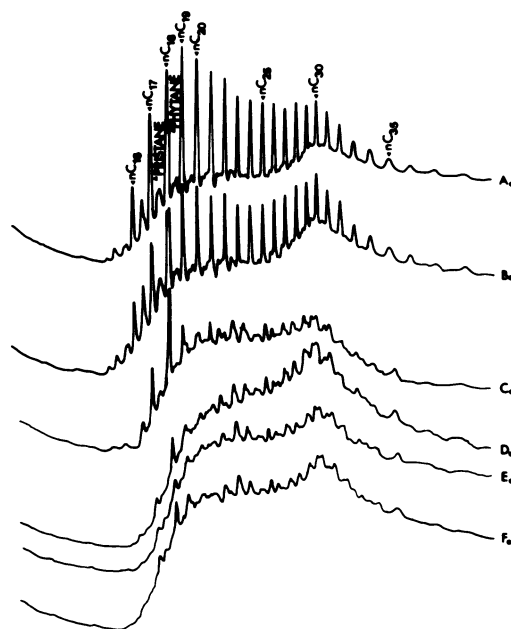


FIG. 4. Gas-liquid chromatographic analyses of residual saturate fractions from sequential utilization of crude oil by *Micrococcus* sp. Grown at 30 C. (A) Saturate fraction of control North Cantal oil. (B) Residual saturates after 2 days of incubation. (C) Residual saturates after 5 days of incubation. (D) Residual saturates after 7 days of incubation. (E) Residual saturates after 14 days of incubation. (F) Residual saturates after 21 days of incubation.

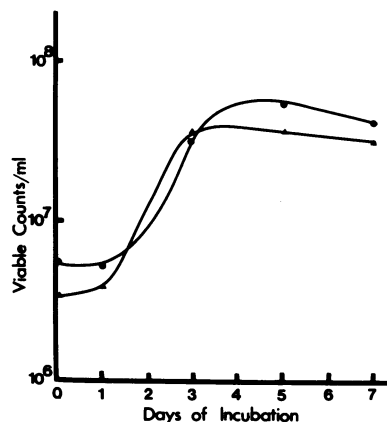


FIG. 5. Growth of two nonobligate psychrophilic soil mixtures growing on North Cantal oil. Symbols: Christmas tree psychrophilic mixture (●); Salmon Arm psychrophilic mixture (▲).

Creek mixture shows that the saturate fraction is preferentially used (Table 3). However, calculation of expected weights of various fractions if only the saturates were degraded indicates that, in contrast to mesophilic growth,

TABLE 3. Liquid chromatographic analysis of crude oil residue after growth^a of mixed population from the Judy Creek enrichment at 4 C

Oil fraction	Weight (%) of "topped" weight of oil	
	Control	Residue
Benzene-soluble asphaltenes	2.5	3.7 (3.1) ^b
Benzene-insoluble asphaltenes	6.8	4.8 (8.3)
Saturates	51.0	39.5 (-)
Aromatics	31.2	37.2 (38.4)
Soluble NSO ^c	8.6	12.6 (10.6)
Insoluble NSO	0.3	2.2 (0.4)

^a Grown for 21 days at 4 C.

^b Values in parentheses are weight percentages calculated on the basis of the saturate fraction only being utilized.

^c See Table 1, footnote a.

under psychrophilic conditions very little of the aromatic fraction is used.

Gas chromatographic analysis of the saturate fraction as a function of psychrophilic growth is presented in Fig. 6. The utilization pattern is more similar to that of the mixed mesophilic population than is the *Micrococcus* species pattern. The isoprenoids phytane and pristane again show a greater resistance to microbiological degradation than the *n*-alkanes of the saturate fraction.

Effect of aeration on utilization of crude oil. The effect of aeration on increase in viable counts is presented in Fig. 7. Sulfite oxidation values (millimoles of O₂ per liter per hr) of 27.5, 19.0, and 12.5 millimoles of O₂ per liter per hr were obtained with liquid volumes of 250, 500, and 750 ml/2-liter flask, respectively. Increasing the liquid volume to 1,000 ml did not further reduce the sulfite oxidation value. It would appear that the rate of growth and total yield of cells are not affected by aeration levels under these conditions. However, analyses of the crude oil by liquid chromatography (Table 4) and by gas-liquid chromatography (Fig. 8) indicate a differential utilization of crude oil components. The results of both analytical techniques show a more rapid utilization of the saturate fraction under conditions of maximum aeration.

Utilization of purified oil components by mixed cultures at mesophilic temperatures. The utilization of the individual components of the North Cantal crude oil sample as growth substrates is shown in Fig. 9. In the purified state, only the saturate fraction sustained the growth of the mixed population which had been derived by enrichment on whole crude oil

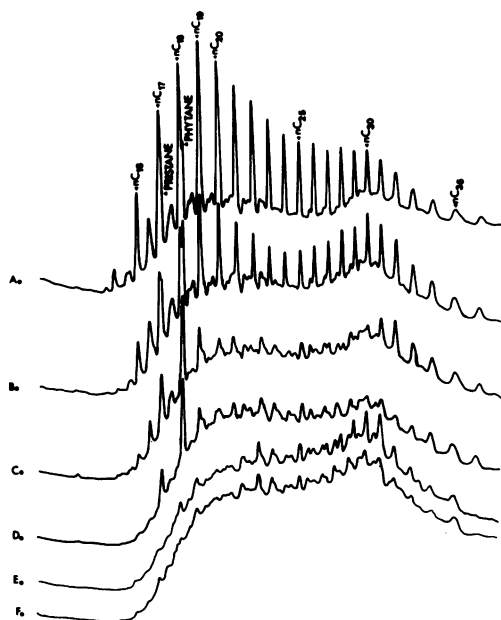


FIG. 6. Gas-liquid chromatographic analyses of residual saturate fractions from sequential utilization of crude oil by the Judy Creek enrichment mixture at 4 C. (A) Saturate fraction of control North Cantal oil. (B) Residual saturates after 3 days of incubation. (C) Residual saturates after 5 days of incubation. (D) Residual saturates after 7 days of incubation. (E) Residual saturates after 14 days of incubation. (F) Residual saturates after 21 days of incubation.

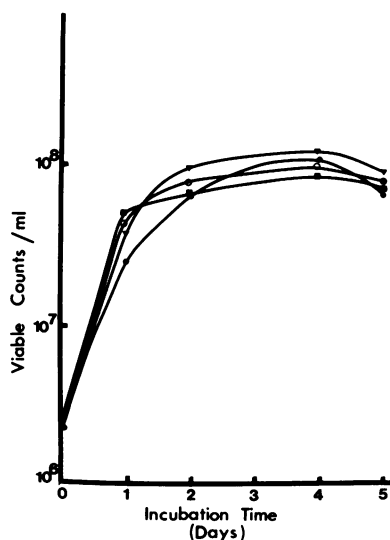


FIG. 7. Effect of aeration level on growth at 30 C. Symbols: 250-ml incubation volume (●), 500-ml incubation volume (▼), 750-ml incubation volume (■), 1,000-ml incubation volume (○).

TABLE 4. Liquid chromatographic analyses of residual oils from the aeration experiment after 5 days of growth at 30 C

Oil fraction	Weight (%) of "topped" oil volume				
	Control	250 ml ^a	500 ml ^a	750 ml ^a	1,000 ml ^a
Benzene-soluble asphaltenes.....	2.5	7.6	4.8	3.3	3.3
Benzene-insoluble asphaltenes.....	6.8	6.1	6.8	5.0	5.5
Saturates.....	51.0	35.9	46.8	45.6	47.2
Soluble NSO ^b	8.6	14.4	10.7	10.2	10.1
Insoluble NSO.....	0.3	1.0	1.0	3.2	1.0

^a Per 2-liter flask.

^b See Table 1, footnote a.

at mesophilic temperatures. In contrast to the suggested concomitant utilization of the aromatic fraction when the whole crude oil was subjected to microbial digestion, very little growth was produced with the aromatic fraction as the sole carbon source.

Utilization of an inferior grade crude oil.

Liquid chromatographic analyses of the residues of Lost Horse Hill crude oil samples after exposure to the mesophilic soil mixture enriched on a high-quality North Cantal oil sample and to the fresh enrichment obtained on the Lost Horse Hill sample are shown in Table 5. The population obtained from enrichment on the high-quality crude oil had very little effect on the composition of the lower quality Lost Horse Hill crude oil sample. However, an enrichment using the same soil as for the North Cantal oil sample brought up a population which readily brought about changes in this lower quality crude oil similar to those obtained with the high-quality North Cantal crude oil sample. Gas-liquid chromatographic analyses of these samples (Fig. 10) confirm the above observations.

DISCUSSION

The first evidence of bacterial activity on a high- or low-quality crude oil is a quick and extensive emulsification of the oil followed by an increase in its specific gravity to a level greater than that of the medium used in these experiments.

Sequential chemical analyses of the oil residues as microbial growth proceeds indicate that the *n*-alkane components of the *n*-saturate fraction were preferentially utilized as carbon and energy sources. The residue which forms the envelope in the gas-liquid chromatogram

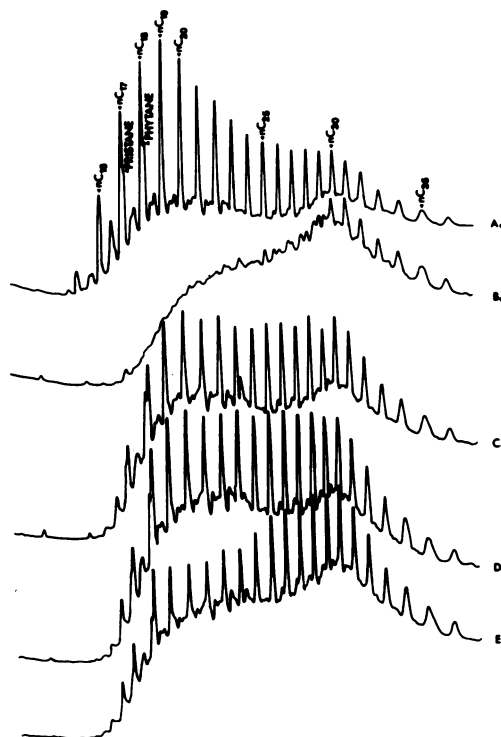


FIG. 8. Saturate gas-liquid chromatogram profiles of residual petroleum recovered from aeration experiment after 5 days growth at 30 C. (A) North Cantal control saturates. (B) Residual saturates from 250 ml of medium per 2-liter flask. (C) Residual saturates from 500 ml of medium per 2-liter flask. (D) Residual saturates from 750 ml of medium per 2-liter flask. (E) Residual saturates from 1,000 ml of medium per 2-liter flask.

profile was comprised of iso- and cycloparaffins. The pattern of *n*-alkane utilization does vary depending on the population present, the longer chain components tending to be more resistant to microbial attack. For example, the psychrophilic mixture preferentially utilized *n*-alkanes of chain length up to C₃₁, whereas the mesophilic mixture used up to C₂₅ prior to metabolism of the rest of this fraction. This suggests that a factor or factors other than solubility determines the utilization of *n*-alkanes by microorganisms. This preference had previously been noted (12).

The isoprenoid compounds, phytane and pristane, are more resistant to microbiological degradation. However, the fact that they were degraded indicates that their use as biological markers has to be interpreted with care. The mechanism by which they were utilized was not investigated but may be similar to that outlined by McKenna and Kallio (8).

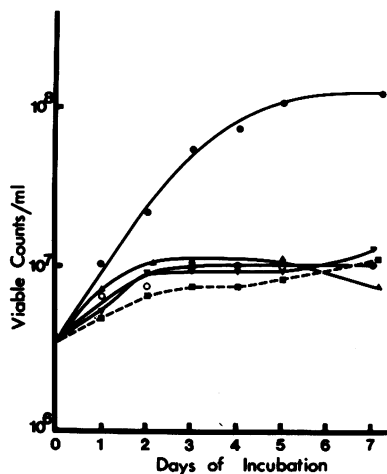


FIG. 9. Growth of soil mixture on purified components of North Cantal oil at 30 C. Symbols: benzene-soluble asphaltenes (\blacktriangledown), saturate fraction (\bullet), aromatic fraction (\circ), NSO fraction (\blacktriangle), control growth (\blacksquare).

TABLE 5. Liquid chromatographic analysis of Lost Horse Hill crude oil sample after growth^a of bacterial soil mixtures derived from different quality crude oils

Oil fraction	Weight (%) of "topped" oil		
	Control	North Cantal enrichment	Lost Horse Hill enrichment
Benzene-soluble asphaltenes	7.8	7.7	9.2
Benzene-insoluble asphaltenes	9.9	5.1	12.2
Saturates	36.8	29.7	17.8
Aromatics	37.9	38.7	40.3
Soluble NSO ^b	13.1	16.8	15.4
Insoluble NSO	0.2	1.9	5.6

^a Growth for 7 days at 30 C.

^b See Table 1, footnote a.

The utilization of the remaining components, with the exception of the aromatic fraction, appears to take place slowly, if at all, under these experimental conditions. The apparent increased utilization of the aromatics in the presence of whole crude oil which contains the readily utilized saturates as opposed to the lack of growth when aromatics were the sole carbon source suggest that cooxidation is involved in their metabolism. They also would appear to be more readily utilized at mesophilic than at psychrophilic temperatures.

Similar studies in this laboratory (*unpublished observations*) concerning the metabolism of this aromatic fraction of crude oil confirm this hypothesis. The works of other investigators (7, 9) also confirm the requirement for the presence of an assimilable substrate in order to obtain utilization of certain model aromatic compounds.

The insoluble NSO components in the metabolized oils have been increased beyond that value predicted from the theoretical enrichment calculations. This suggests that some modifications of the crude oil, presumably brought about by microbial action, have taken place yielding an increase in the amount of polar N-, S-, and O-containing materials.

Aeration, although not having a marked effect on either the rate of growth or the total amount of cells produced, did affect the utilization of the *n*-saturate fraction. The highest level of aeration used resulted in a 15 weight % reduction in the saturates present in the residual oil, whereas lower levels of aeration resulted in a reduction of only approximately 5 weight %. The constant yield of cells, however, suggests a more efficient conversion of

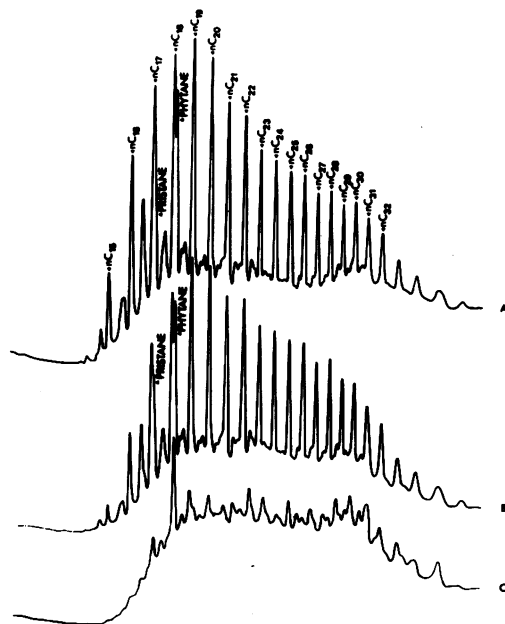


FIG. 10. Gas-liquid chromatographic analyses of residual Lost Horse Hill saturates after metabolism by bacterial soil mixtures derived from different quality crude oils. (A) Lost Horse Hill control saturates. (B) Residual Lost Horse Hill saturates after growth by established population at 30 C. (C) Residual Lost Horse Hill saturates after growth resulting from Judy Creek soil reinoculation.

n-saturates to cell material under conditions of reduced aeration.

The quality of the crude oil used in enrichment studies has a marked effect on the capabilities of the populations to utilize crude oils of lower quality. A mixed population derived from enrichment procedures by using the high-quality crude, i.e., North Cantal, had only a limited capability of utilizing the lower quality crude, i.e., Lost Horse Hill. However, populations derived by using a low-quality crude oil during enrichment procedures can readily metabolize a high-quality crude oil (*unpublished observations*). It is possible that the increased asphaltenic content of the low-quality crude oil or the altered aromatic character inhibits its utilization by a population derived when these components are present in lower concentrations. This result suggests that the successful use of "bacterial cocktails" to deal with accidental oil spills will be dependent, in part, on the mixture being composed of microorganisms capable of utilizing oils of the lowest quality comprising the spill.

So far it has been impossible to degrade a crude oil completely under laboratory conditions. Populations are readily derived which can utilize a significant proportion of the saturate fractions and possibly some of the aromatic fraction. These chemical changes are accompanied by a change in the specific gravity of the oil from lighter to heavier than water and results in a product which forms an emulsion with the medium. This phenomenon has been well documented in the literature (6) and is attributed to a microbial product of hydrocarbon metabolism. There is, however, a substantial portion of the original crude oil material still found within the emulsified material. We suggest that this residual material is analogous to the humic matter remaining after the readily digestible material of organic matter has been metabolized by microorganisms and thus could be regarded as humic

material.

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