The Microbial Degradation of a Diesel Oil in Multistage Continuous Culture Systems

Joseph Michael Suflita B.S.
S.U.N.Y. Center at Albany, 1973

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science in Biological Sciences
at
State University College at Brockport
1976

The State University of New York
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Department of Biological Sciences

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Acknowledgements

The author would like to express his appreciation to:

- My friend and advisor, Dr. Hap Pritchard, for his patience, guidance, enthusiasm, courage and honesty during the past years.

- My committee members, Dr. John Kowalski and Dr. Robert Ellis, for their assistance and scrutiny throughout my graduate career.

- My benefactors, the New York State Sea Grant Institute and the New York State Assembly Scientific Staff, for their financial support through the Sea Grant Institute Traineeships.

- My lover, Miss J. Taylor Bond, for her patience and typing assistance.

- My brother, Mr. George Suflita, for his technical support and advice.

- My friend, Mr. William Mahaffey, for his moral support.

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I. Introduction

Millions of barrels of crude and processed oil are transported across the world's waterways annually. Spillage of oil is an inevitable outcome and represents a serious threat to our aquatic resources. Once spilled, the effects of oil pollution are often chronic because oil degrades very slowly in aquatic environments. Consequently, many studies have been initiated in attempts to determine the fate of oil in a natural ecosystem. Such studies have revealed that bacteria are the ultimate transformers of petroleum hydrocarbons. However, most of these laboratory studies have involved batch culture enrichment techniques. These techniques so simplify the degradative system that extrapolation of the data to the natural environment cannot be justified. The objectives of this study were to: 1. develop a laboratory continuous flow system that more closely resembles a natural aquatic environment; 2. to study the degradative fate of a diesel oil using this system; 3. to illucidate some of the environmental parameters that affect the degradation process.

Classically, studies of hydrocarbon and oil degradation have employed batch culture techniques. Cultures of microorganisms are usually added to flasks containing minimal salts media and a small amount of hydrocarbon substrate. This mixture is then allowed to incubate for a specific time period while constantly being aerated, usually by a shaker apparatus. This technique has been extremely useful in the enrichment and isolation of new hydrocarbon metabolizing organisms and in studies of the specific mechanism by which pure cultures of bacteria oxidize hydrocarbons. Unfortunately, environmental factors which affect the degradation process (i.e. pH, oxygen concentration, nutrient depletions and metabolite buildup), are time dependent variables in batch culture enrichment experiments (34). Thus the growth of microbial populations is a function of the batch culture technique itself, and not a function of those parameters inherent in a natural environment (34). In addition, the batch culture degradation studies in the literature (7, 13, 20, 21, 22, 25, 39), have not reported detecting substantial degradation after the primary attack. Most reports fail to demonstrate the subsequent stages of oil degradation under either laboratory or field conditions (7, 21).

This project has attempted to avoid the experimental pitfalls associated with batch culture and has dealt with the use of a multistage continuous culture system. This system was designed to model the fate of a diesel oil in Lake Ontario. The multistage system consists of three different sized continuous flow vessels connected in series so that the degradation products of one vessel serve as the nutrient source for a larger succeeding vessel. In this way, the dilution capacity of an aquatic body of water is simulated. Furthermore, one can examine what happens to the oil after it is initially attacked by bacteria and subsequently dispersed into the water column. This model system, as has been developed, accomodates a series of three continuous culture vessels designated as Q1, Q2, and Q3 (see Materials and Methods, Figure 1.).

The multistage system has four important advantages regarding a study of the fate of oil in an aquatic environment. First, it closely approximates a natural situation which would be an open system and which would possess a large dilution capacity. The multistage system possesses both of these properites. Second, the multistage system allows one to examine the subsequent stages of degradation. The system is designed to have the effluent from the primary vessel fed into a second continuous culture vessel. In this way, the continued secondary degradation can be monitored and its ultimate fate more closely observed. Simliarly, the sequence can be continued with a third vessel.

Third, the multistage system is not subject to the time dependant variables associated with batch culture experiments. Unlike batch cultures or like the natural environment, the multistage system is buffered, nutrients are constantly supplied, and dilution prevents the buildup of metabolic endproducts.

Fourth, the multistage continuous culture system is highly amenable to studies involving the effects of environmental parameters on the degradation process. Factors such as pH, temperature, organic matter, detergents, seeding, etc. can be readily tested for their effects on the degradation pattern. These inherent advantages of multistage continuous culture make it a system better suited to the study of oil biodegradation.

Through the use of this system, it has been found that the oil undergoes extensive and very characteristic physical changes. Accompanying these physical changes were chemical changes as evidenced by gas chromatographic analysis. The chromatographic profiles suggested a transformation process in which oil hydrocarbons are converted to a variety of high molecular weight endproducts, the exact nature of which is currently under investigation. In addition, the effect of various environmental parameters (i.e. nutrient concentration, increased surface area, and continuous inoculation) were also examined for their affect on the degradation process.

II. Literature Review

A. Composition of Oil

Petroleum is a highly complex organic mixture consisting largely of hydrocarbons of several different molecular types, the three principle classes being: alkanes, cycloalkanes, and aromatics (see Table 1). Alkanes, hydrocarbons which contain only single bonds, account for a large proportion, 36% to 65% of the total weight of most petroleum distillates (38). The most abundant series, the normal or straight chain alkanes, contain from one to seventy-eight carbon atoms. Iso- or branched alkanes, especially the 2, 3, and 4-methyl alkanes occur in relatively high concentrations (10 to 15 weight percent) and individual members up to C40 have been isolated from crude oil (36). Highly branched compounds are not as common in crude oil, although three particular compounds, pristane (2,6,10,14-tetramethylpentadecane), phytane (2, 6,10, 14-tetramethylhexadecane), and farnesane (2,6,10-trimethyldodecane) are found in concentrations ranging from 0.7 to 1.8 weight percent (38).

The cycloalkanes contain at least one saturated ring structure. Although they are not as prevalent as the normal and iso-alkanes (less than 15 weight percent total), they are present throughout the entire boiling range of crude oil (38).

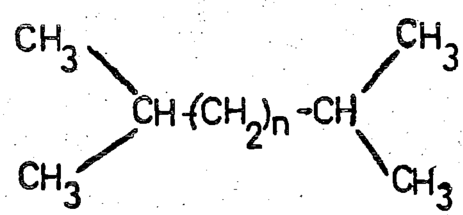
Aromatic compounds, on the other hand, contain at least one unsaturated benzene moiety in their structure. Benzene, phenanthrene, and anthracene as well as many tetra- and penta-

Table 1 - Examples of the Various Petroleum Hydrocarbon Classes Found in Oil

ALKANE

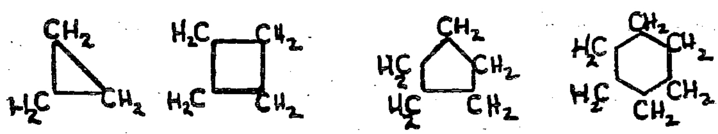
$CH_3-(CH_2)_n-CH_3$ n = 3,4,5,...

ISO-ALKANE

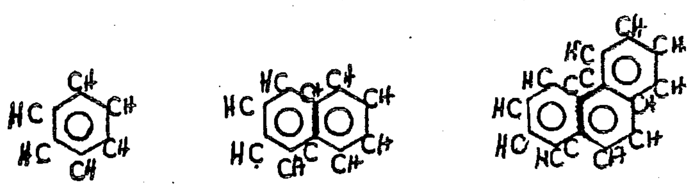


n = 1,2,3....

CYCLOALKANES



AROMATICS



ring compounds can be found in this aromatic series. The predominant aromatics in crude oil, however, are the alkyl-substituted benzenes and napthalenes. Individual compounds which have been isolated are 1-methyl-3-ethylbenzene, 1,2,4-trimethylbenzene, and 1-, 2-, and 3-methylnapthalenes.

There are also many sulfur, nitrogen, and oxygen containing compounds found in varying amounts in most oils (38). Sulfur compounds usually comprise one to two weight percent whereas oxygen and nitrogen compounds are usually found in concentrations of less than 0.9 weight percent.

B. Physical and Chemical Factors Affecting the Disappearance of Oil

Auto-oxidation, a chemical reaction in which liquid hydrocarbons are oxidized in the presence of gaseous oxygen and light, is one of the initial chemical changes that occurs in oil spilled in the environment (26). Organic hydroperoxides appear as the initial product, but are converted to ketones, acids and aldehydes in the presence of the sulfur compounds and metals usually found in oil (30). Although auto-oxidation is a continuous process, it is extremely slow and does not contribute significantly to the ultimate fate of the oil.

Sinking, in contrast, is a very rapid process which removes large quantities of spilled oil from the water surface and deposits it in the sediments (10). It is generally a process whereby the oil becomes impregnated with debris (e.g. clay particles, bacteria, plankton, etc.) giving a brown soft mass which is heavier than water. Once in the sediment, microbial degradation, dissolution, evaporation, and auto-oxidation slow down and may even be completely inhibited. Thus, sinking may actually increase the residence time of oil in the environment.

Dissolution and evaporation are responsible for loss of many volatile components (boiling point less than 300°C) of oil (45), even some of the higher molecular weight water-soluble polar components (27). It has been shown that dissolution and evaporation have been responsible for the loss of up to two-thirds of a Venezuelan crude oil within a few days in the ocean (13).

The use of mechanical and chemical clean up procedures to remove the polluting oil have met with varying degrees of success. Absorbents, such as straw, clay, and polyurethane have been used to collect oil. These are useful in that they facilitate removal of the oil from the shore but they must be used immediately before the oil has a chance to spread over a large area. Oil herders both chemical and mechanical have been used in attempts to contain the oil to a relatively small area, but have met with only limited success (5, 44), due to rough seas and/or various structural problems. Suction pumps have been tried but are only useful when the oil layer is several inches thick (10).

Solvents and emulsifiers have been used to a much greater extent than the above mentioned processes (44). Addition of these chemicals causes the oil to spread over a much larger surface area. This then increases the degradation rate of oil by microorganisms. Unfortunately, many of these chemical dispersants and emulsifiers are toxic or bacteriostatic in even a few parts per million (45) and consequently their value is limited.

C. Role of Bacteria in Physically Removing Oil

The emulsification of oil is probably one of the first processes mediated by oil degrading bacteria. It is simply a process whereby the oil is broken up into a permanent suspension of tiny oil droplets. Emulsification has been commonly observed in many oil degradation studies. In addition, there are indications that the emulsification may be the result of a proteinaceous type compound elaborated by bacteria. Since it appears that bacteria associate themselves with oil droplets as a means of attacking it (16), emulsification, which produces a suspension of tiny oil droplets, is a very important initial step in the microbial degradation of oil.

In addition to emulsifying oil, it has been found that bacteria are capable of sequestering oil and individual petroleum hydrocarbons (12). In the sequestering process, the hydrocarbons or oil is taken up and stored in inclusion bodies within the cells. Finnerty et al (12) have shown that under natural conditions bacteria were able to sequester crude oils, tar residues, and n-alkanes. Presumably these sequestered hydrocarbons served as a suitable carbon source for the metabolic activities of the organisms. Such findings indicate that the sequestering process may play some role in the removal of oil from the surface of a body of water.

D. Microbial Hydrocarbon Degradation

Although it has been well documented that fungi are capable of degrading oil and hydrocarbons (1, 23), it appears that bacteria are the prime degraders in the aquatic environment (45). It is clear from many studies that a large number of bacterial species are capable of degrading hydrocarbons and participating in the degradation of oil (45). Hydrocarbon metabolism is certainly not a unique aspect of microbial populations. In fact, bacteria isolated from different ecosystems often show hydrocarbon degradation capabilities (14).

In terms of substrate specificities, individual bacteria species attack only a narrow range of hydrocarbons. Heterogeneous bacterial populations, however, demonstrate an impressive array of hydrocarbon oxidative mechanisms. As has been established numerous times, n-alkanes are utilized with greater frequency and rapidity than other hydrocarbons in liquid cultures (23). There is apparently a specificity within the n-alkane series as well. Alkanes of 10 carbons or less are not degraded as frequently, or by as many bacterial species, as those hydrocarbons with carbon chain lengths of 10 to 24 (14, 32).

Branched alkanes are generally assumed to be more resistant to microbial degradation than n-alkanes. This may be a reflection of the fact that they have not been studied as extensively. There is, however, no question that the branched alkanes can be broken down by a variety of different bacterial species.

McKenna (28) has shown that microbial utilization of alkanes was affected by the degree of branching and the size and position of the branches. For example, if properly placed, multiple methyl branches did not render an alkane unpalatable to the species tested. However, a quaternary carbon atom occurring at the end of an alkane molecule, resulted in a structure quite resistant to microbial degradation.

The cycloalknaes are very difficult hydrocarbons to breakdown, and no one to date has isolated an organism which will use cyclohexane as a sole source of carbon and energy. Even the addition of short chain n-alkyl-substitution does not render them susceptible to microbial oxidation (31). However, long chain n-alkyl substituted cycloalkanes can be degraded by several strains of bacteria (3). In the same study, it was noted that there was a greater probability of ring cleavage when the side chain contained an odd number of carbon atoms.

The aromatic hydrocarbons represent the most diverse group of compounds and are probably the most recalcitrant fraction of the oil. However, aromatic hydrocarbon oxidation is well established, especially for the benzene and napthlene related hydrocarbons. The primary differences in microbial degradability depends on the type of functional group substitution (15, 24). Methyl group substitution at certain positions has been shown to slow up degradation and even completely inhibit it. On the other hand, the substitution of various alkyl groups on a benzene ring renders the resulting compound more susceptible to degradation (28, 12).

Another aspect of hydrocarbon utilization which will strongly affect the degradation of oil in aquatic ecosystems is the phenomenon of co-metabolism (19,35). Co-metabolism is the process by which a microorganism can oxidize a substrate which cannot or will not be used as an energy source. Many researchers have shown that certain hydrocarbons, which will not suffice as a source of carbon and energy for isolated organisms, are oxidized when mixed with soil, especially if some other readily utilizable substrate is supplied (35). For example, Beam and Perry have suggested that recalcitrant cycloalkanes may be degraded via co-oxidation while other hydrocarbons serve as the primary growth substitutes.

E. Microbial Degradation Of Oil

The degradation of oil by bacteria has been extensively studied through the use of batch culture enrichment experiments (7, 20,22, 25). Analysis of the data obtained from these types of experiments yield certain trends which are routinely encountered. Besides the initial emulsification step described above, bacteria invariably attack the normal alkanes first. In a gas chromatographic profile, the n-alkanes usually represent the most predominant peaks. These peaks are always the first to disappear during the degradation process. In fact, oils which are low in saturates show a much slower rate of degradation (43).

In most oils the alkanes, pristane and phytane, are also present in high concentrations. These iso-alkanes invariably take much longer to be degraded when compared to the n-alkane fraction. However, it is only a short lived occurance since, after sufficient incubation, the pristane/phytane peaks also disappear (20,33). The cycloalkane and aromatic fractions are the most recalcitrant fraction of the oil. In many batch culture experiments these fractions do not disappear, even after extensive incubation. This is undoubtedly a function of the particular cultural conditions used to study oil biodegradation. In batch culture enrichment experiments the microorganisms with the maximum specific growth rate or maximum final cell concentration are favored under the imposed selection pressures. Thus alkane oxidizers, which grow rapidly and reach high cell concentration on oil, have been selected for and examined in greatest detail. Bacteria that grow more slowly or oxidize minor components of the oil never come to fore in batch culture ecperiments. The activity of these other microorganisms may be of special significance in natural environments. In natural situations, the more recalcitrant fractions disappear. However, there is no convincing evidence that they are actually being degraded. In the laboratory, Horowitz et al (18) have been able to demonstrate some utilization of this more recalcitrant fraction. In addition there was also the concommitant production of new alkane-like materials as revealed by gas chromatography.

The time course involved in these degradations is highly variable. Generally, in the laboratory the n-alkanes are entirely degraded in 20-40 days. In the field, it may take 1-3 months to degrade the saturates and as long as 12-15 months to completely degrade the entire amount of oil present (7).

F. Environmental Factors Affecting Oil Degradation

There are various interrelated parameters which influence the microbial degradation of oil in the aquatic environment. Generally, most hydrocarbon utilizers require free dissolved oxygen. For example, Zobell (46) has determined that the biological oxygen demand of Barataria Bay bottom samples, inoculated into an oil-mineral salts media, was three to four milligrams per liter. Thus, the complete oxidation of 1 mg of hydrocarbon to carbon dioxide and water required approximately 4 mg of oxygen. Partial oxidation of the hydrocarbon to form alcohols, acids, aldehydes, or esters requires much less oxygen than does the complete oxidation to carbon dioxide and water. In situations where the organisms are in contact with the normal atmosphere, as at an air-water-oil interface, the supply of oxygen is quite adequate. However, oxygen can become a limiting factor especially in areas below the water's surface, particularly in bottom sediments.

There is still a great deal of controversy over whether bacteria can oxidize and degrade hydrocarbons under anaerobic conditions. Several investigators have reported the slow disappearance of oil in the complete absence of oxygen, using sediment samples (46, 9), but the actual mechanism of degradation has never really been fully clarified. Since sulfate and nitrate can serve as alternate electron acceptors, there would appear to be no problem in degrading a hydrocarbon once it was initially oxidized. However, this initial oxidation step is the most troublesome point since it is a mechanism involving molecular oxygen. Despite the fact that Kallio (29) has argued that primary oxidation under anaerobic conditions is thermodynamically impossible, several lines of evidence now indicate that water can be added over a double carbon moiety to produce an alcohol derivative of an alkane without the use of molecular oxygen (29). Whether this is a common reaction still remains to be established. In any case, it has been well established that the rate of degradation is drastically reduced if the oil is deposited in an anaerobic environment. (29).

Temperature also affects oil degradation rates and there is now considerable evidence to indicate that temperatures below 10°C slow degradation substantially (1). Westlake and his colleagues (43) have demonstrated the existence of different degrading bacteria at 4°C. Furthermore, there was an apparent decreased rate of aromatic degradation at these temperatures, but not a substantial effect on the n-alkane utilization.

Nitrogen and phosphorous are, of course, required for oil degradation and are probably the first factors to become rate limiting in the natural environment (45). For example, Atlas and Bartha (2,4) found that the addition of either nitrogen or phosphorous to sea water did not stimulate biodegradation or mineralization. However, the addition of both increased degradation by 79% and minerlaization by 42%. Jobson et al (21) have shown that the addition of ureaphosphate to oil-impregnated soil significantly increases the utilization of both the saturate and the aromatic fractions of oil. The requirements of an oil-utilizing bacterial population, then, can be supplied by a few parts per million nitrogen and phosphorous.

Dibble and Bartha (11) have also documented the effect of iron on a Southern Louisiana crude oil. They demonstrated that the addition of nitrogen and phosphorous allowed very rapid biodegradation (72% in 3 days) in polluted seawater in which the concentration of iron was very high (5.2 uM). The further addition of iron did not affect the biodegradation rate. However, in a less polluted seawater sample (1.2 uM iron) biodegradation of the oil was considerably slower (21% in 3 days) and the additon of chelated iron and ferric octoate had a marked stimulating effect.

The presence of low concentrations of organic material also may promote the growth of hydrocarbon utilizing organisms by providing needed co-factors such as vitamins or amino acids. However, Atlas and Bartha (3) found that the utilization of petroleum by two marine isolates was inhibited by the presence of fatty acids, particularly short chain fatty acids. It is feasible that catabolite repression by glucose or other organic compounds may have an effect on oil degradation, but this has not been determined.

G. Methods of Oil Degradation Analysis

One of the big problems in oil degradation studies has been the lack of good analytical methods for monitoring the degradation process. Since so many different methods have been used, it is often difficult to compare results. Many are also limited in value, either because they are not quantitative or because they do not show to what degree the oil has been degraded.

Column chromatography has been somewhat successful in analyzing chemical changes occurring in oil (8). Silica gel and silica gel-alumina dual phase column chromatography have been used to separate alkane and aromatic fractions of crude and diesel oils (20,22). Once separated, bacterially mediated changes in the two fractions can then be determined by gravimetric means (20) or by gas chromatography (20,8).

When used in conjunction with liquid chromatography, gravimetric analysis allows an absolute determination of the extent of degradation by comparing weight loss of the alkane and aromatic fractions relative to a stock oil. Both Kator (22) and Jobson et al (20) have used this method and have obtained substantial information on the oil degradative process. However, gravimetric measurements are not qualitative and cannot be used to determine the utilization of individual components of a mixture.

Gas chromatography or gas chromatography used in conjunction with liquid chromatography can provide both qualitative and quantitative measurements of changes brought about by microbial action on oil. The separation of components in a very small sample can be obtained rapidly and with a high degree of resolution. Qualitative analysis is based on comparison of the retention time of the unknown compound with that from a known hydrocarbon under identical conditions (17). Various workers have used gas chromatography to study oil degradation and it is now considered a standard technique. Soli and Bens (37) for example, have determined the extent of utilization of an artificial oil by analytical gas chromatography. Walker and Colwell (41) have recently used motor oil and an artificial oil in laboratory experiments. Gas chromatography has also been found to be extremely useful in field studies also, as exemplified by the work of Blumer (7) and Jobson et al (20).

There is however one problem with gas chromatography as an analytical tool. Researchers have been unable to resolve the gas chromatographic profile which is left in the residue of degraded oil. Mass spectral data have indicated that the undegraded envelope profile is comprised of one to six ring cycloalkanes and various polyaromatic compounds which cover a large boiling range (41).

Simple visual observation of a gas chromatographic profile is generally not enough to verify actual chemical change brought on by bacterial degradation. Instead, quantitative relationships between certain components in the oil must be evaluated. The peak height ratios of n-C17/pristane and n-C18/phytane have been established as a valid indication of compositional changes occurring in the oil. Furthermore, this method appears to be a sensitive indicator of the bacterial degradation of oil (8). The method is based on the recalcitrant nature of the iso-alkanes, pristane and phytane. Since they are much less susceptible to microbial attack, the n-alkane peak height changes can be related. If there is a decrease in either ratio, a preferential decrease in the concentration of the respective n-alkane is indicated, whereas an increase in the ratios would indicate a preferential utilization of the isoalkanes. One drawback with this technique is that if both pristane and phytane are degraded along with their respective n-alkanes, the ratios would indicate no degradation. Indeed, there are reported cases of branched alkane oxidizing bacteria (33).

Thus, in order to further substantiate the peak height method, peak envelope ratios were also calculated (8). The ratio of the peak height to the envelope components depends on the recalcitrant nature of the envelope materials (20,41). The peak height above the envelope and the envelope height itself would then give another ratio to determine the extent of degradation of the oil by bacteria.

III. Materials and Methods

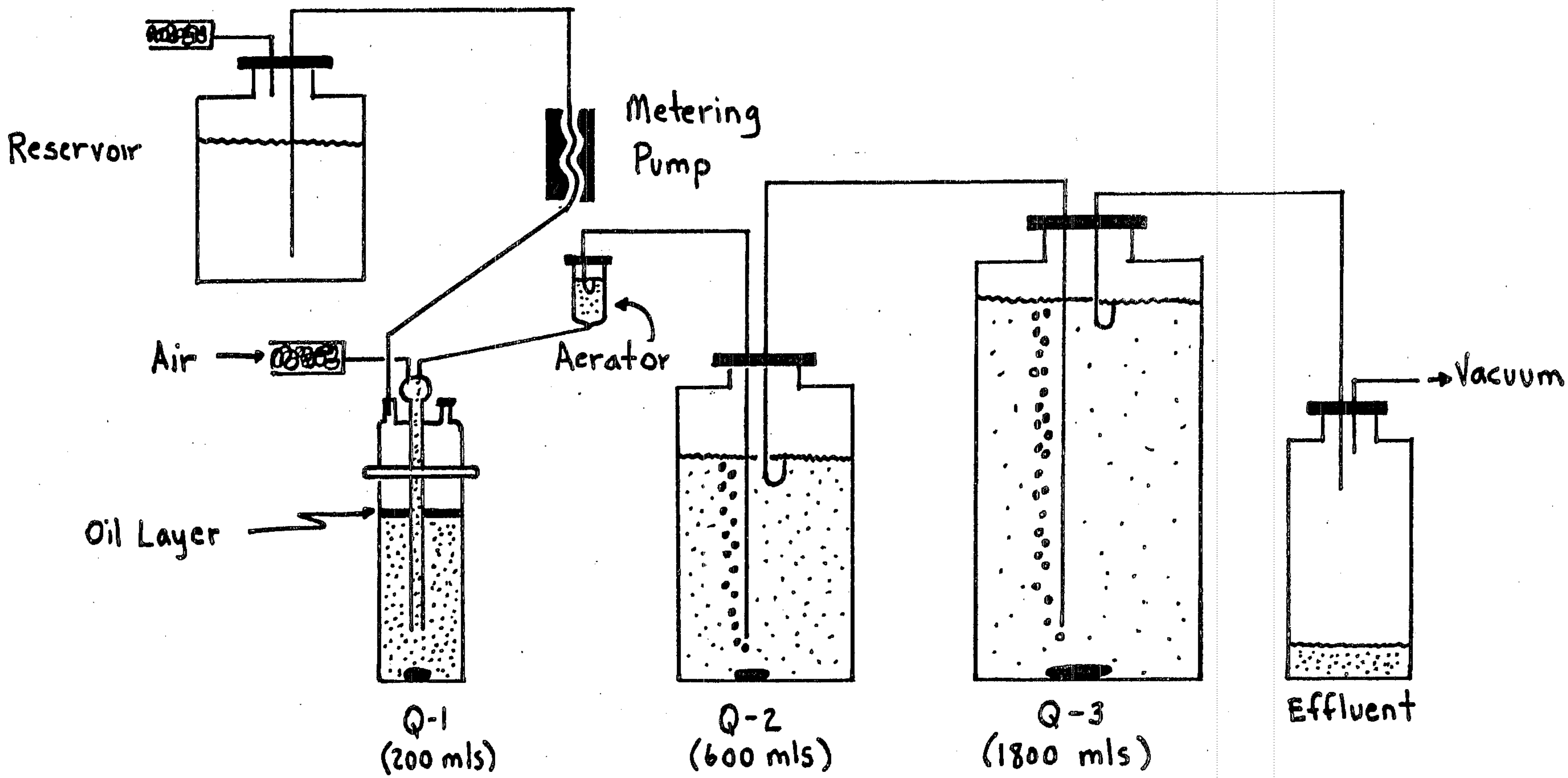
The type of sequential continuous culture system used in this study is shown in Figure 1. Three vessels were employed using a volume ratio of 1:3:9. The oil layer served as the carbon and energy source for the first vessel. The carbon and energy source for the second and third vessels consisted of the oil degradation products which were in the effluent of the preceeding vessel. The second and third vessels were not supplemented with inorganic nutrients except for what entered from the first vessel.

The basic design of the continuous culture apparatus allowed me to study oil degradation in an undisturbed two phase system (34). Oil was floated on the surface of a water column while a continual flow of media was passed beneath the oil layer. Flow rates from the reservoir were controlled by a peristaltic pump (Harvard Apparatus, Model 1203).

The medium used in the reservoir of the continuous culture systems contained 0.3 mM K_2HPO_4-KH_2PO_4, pH 7.2 and 0.2 mM NH_4CL and 0.08 mM $MgSo_4$. For all experiments, salts were added as stock solutions to 14 liters of distilled water which had been steam sterilized for 4-6 h. Its contents were always pumped into the first vessel at a dilution rate of 0.05 h^{-1}.

In the multistage continuous culture experiments, designed to continuously inoculate themselves, a second reservoir was employed. This consisted of unsupplemented, unsterilized Lake Ontario water that was continuously stirred and maintained at 10°C by a refrigerated water bath. The lake water was pumped

Figure 1. A Schematic Representation of the Multistage Continuous Culture System used in Oil Degradation Studies.


Reservoir
Metering Pump
Air
Aerator
Oil Layer
Vacuum
Q-1 (200 mls)
Q-2 (600 mls)
Q-3 (1800 mls)
Effluent

in at a dilution rate of 0.025 h^{-1}. The resevoir containing the inorganic nutrients was also pumped in but at one half the normal dilution rate, i.e. 0.025 h^{-1}. The concentration of the inorganic nutrients was therefore doubled to maintain similar conditions.

A typical multistage continuous culture experiment was initiated by adding a 200 ml sample of fresh Lake Ontario water to the first vessel. This inoculum of bacteria was then incubated as a batch culture (i.e. no flow of media) for 15 h. with a 2mm-thick oil layer on the surface. When the flow rate was started, the second and third vessels were empty and were eventually filled by the effluent from the first vessel. All bacteria growing in the second and third vessels usually originated in the 200 ml inoculum in the first vessel.

All samples of water used to inoculate the continuous culture systems were obtained from Lake Ontaria at Hamlin Beach State Park, Hamlin, New York. Samples were taken in 15 liter Nalgene carboys and were either used immediately upon return to the lab or stored for up to three weeks at 4°C.

The aeration system employed a constant vacuum source to suck filter-sterilized air and culture fluids throughout the system. The volumes of fluid in the respective culture vessels were maintained at appropriate levels by using a U-tube in the aeration system. The first vessel was equipped with a special aeration chamber to prevent the mechanical disturbance of the oil layer. The design of this first vessel has been previously described by Pritchard and Starr (34).

The culture fluids were also monitored for bacterial growth. Cell numbers were determined by standard quantitative spread plate techniques (Difco Standard Methods Agar). Plates were incubated at room temperature for up to 15 days before examining. Isolates were distinguished entirely by colony morphology and were stored on Standard Methods agar slants at 10°C. The colony type which make up the largest percentage of the total bacterial population was considered predominant.

Diesel oil used in this study was obtained from the commissary at S.U.C. Brockport, Brockport, New York. The oil was artificially weathered by placing 1 liter of the oil in a tarred beaker and exposing it to forced draft conditions at 37°C. This process was continued until the rate of weight loss was negligible, i.e. about two weeks. This process removed volatile materials up to and including normal dodecane, leaving approximately 65% of the weight of the original oil. The topped oil was then filter sterilized through 0.45 u Millipore filters and stored in sterile ground glass bottles. This oil was used for all experiments.

The analysis of compositional changes in the oil during the degradation process was accomplished either by sampling oil directly from the oil layer itself, or by extracting resiual oil from culture fluids and effluent samples. Approximately 0.2 ml of the oil layer was asceptically removed during the sampling procedure with a capillary pipet. The sampled oil was then placed in a 2 x 10 mm conical tipped centrifuge tube containing 10 ml of dry pentane-benzene solvent (1:1 by volume).

To sample the culture fluids and effluents, 200 ml of culture fluid or effluent were collected and twice extracted with 25 ml of the benzene-pentane solvent in a 250 ml separatory funnel. The 200 ml of culture fluid that were removed from either the second or third vessel, were then replaced with 200 ml of sterile water, The organic phases in the extractions were saturated with tiny air bubbles interdispersed in a white emulsion. In order not to contaminate the extracted oil with this emulsion, the solvent layer was carefully decanted into a 3 x 15 mm round bottom test tube, leaving the emulsion in the funnel.

All extracts were concentrated to approximately 0.5 ml under forced air conditions in test tubes suspended in a 32°C water bath. These concentrates were placed in 1.1 ml conical tipped storage vials (Bellco glass) along with a 0.5 ml solvent rinse of each test tube. These solutions were further concentrated by a forced air draft. If visual examination of the concentrate demonstrated little further loss of volatile solvent, the samples were sealed with teflon, silicone, or aluminum foil lined caps.

The analysis of oil that adhered to the walls of the vessels and the connecting tubing was carried out in the following manner: first, to remove material from the vessel walls, the contents of each vessel were gently poured into sterile flasks. Secondly, any material remaining on the vessel walls was scraped off with a rubber policeman. Sterile water was then used to wash the loosened material off the walls and the resultant suspension was poured into a sterile tube. This suspension was then thoroughly mixed and then analyzed for bacteria and oil as previously described.

Material on the walls of the tubing was not scraped off but was extracted for oil directly by passing the benzene-pentane solvent through the tubing. This was done by removing the tubing and replacing it with sterile tubing. After concentrating, all the samples were analyzed by gas chromatography.

Gas chromatographic analyses were carried out using standard high temperature non-polar columns, 3 m in length, with a 0.3 cm inner diameter and packed with OV-1 methyl silicone as prepared and described by Ventullo and Pritchard (40).

A Hewlett-Packard Model 5750 gas chromatograph equipped with a dual flame ionization detector was used in all analyses. The instrument was programmed as followa: linear temperature program 60°-670°C at a programming rate of 4°C per minute; injection port temperature, 300°C; flame detector, 300°C; helium carrier flow rate, 20 ml/min; hydrogen flow rate, 12.5 ml/min; air flow rate, 350 ml/min. A benzene-pentane solvent was used for injctions since they passed through the column well in advance of other compounds.

Characterization of most of the projecting peaks in the oil chromatographic profile was possible by comparing peak retention times with a known standard hydrocarbon mixture (40). Thus, it was possible to identify all of the n-alkane series, the two isoalkanes, pristane and phytane, and several aromatic peaks within the oil chromatogram.

To monitor chemical changes in the oil during the degradation process, samples were removed from the oil layer at various times, chromatographed, and analyzed by the peak height ratio method of Blumer et al (8). Peak heights for the n-C17/pristane, and n-C18/phytane ratios were measured from the baseline to the top of the peaks. The envelope is defined as that area enclosed by the baseline and a line drawn along the bottom of all peaks in the profile. Profiles of degraded oil were also visually compared with profiles of undegraded oil in order to check for any obvious signs of degradation of particular fractions of the oil. The term profile refers to the entire chromatographic pattern.

IV. Results

A. The General Pattern of Degradation in Multistage Continuous Culture Systems

1. Physical Changes Occurring During Oil Degradation in Multistage Continuous Culture Systems

As expected, the physical changes in the first vessel were very similar to those observed by Ventullo and Pritchard (40). A sumary of these changes is presented in Table 2. Within 4-7 days after inoculation of the first vessel, heavy bacterial growth (indicated solely by turbidity) occurred in the second and third vessels. Some of this turbidity was undoubtedly due to cells washing out from the first vessel.

Accompanying the large increase in turbidity in the second and third vessel was the appearance of a bright yellow coloration of the culture fluids. It's relationship to the bacteria present or to the degradation process underway is unknown. However, it is an extremely consistent event accompanying the initial events of the degradation experiments. Analysis of the bacterial populations did reveal yellow pigmented colonies, but these bacteria did not elaborate the pigment into the medium. Furthermore, these bacteria represented a large proportion of the mixed bacterial population. The yellow color, although present in the primary vessel, was much less intense.

As incubation continued (6-10 days), the turbidity in the second and third vessel decreased. The decrease occurred

Table 2 - Description of the Physical Changes Associated with the Oil Layer in the Multistage Continuous Culture System

Stage of Degradation	Occurrance After Inoculation (days)	Duration (days)	Description
Adjustment Phase	1-2	2-10	Rapid change over in predominant bacterial species Initial fluguation in cell density Eventual clearing of culture fluid under oil layer General lag period - no gross physical change
Surface Growth Phase	4-6	4-25	Accumulation of bacterial film on bottom surface of oil layer Oil layer becomes stagnant Bacterial film thickens Wall growth
Impregnation Phase	10-30	10-until termination	Oil layer becomes turbid, milky white in color Oil layer highly impregnated with bacteria Oil layer breaks up into mass of floating droplets Culture fluid relatively clear
Flaking-Off Phase	25-35	25-until termination	Masses of cells and oil flake off oil layer Oil layer disappearing Oil layer could be easily dispersed by increased turbulance at any time
Washout-Out Phase	40-50	40-until termination	Most of oil layer gone Most of the bacteria washed out Some wall growth remains

in a sequential manner with the third vessel being the last to lose its turbidity. At about this time a bacterial film was well formed under the oil layer and the bacteria had begun to impregnate the oil. From this point on, the turbidity remained relatively low in all vessels throughout the experiment. Bacterial population densities remained relatively constant ranging from 10^6-10^7 cells/ml and did not differ appreciably from one vessel to the next. The only time that turbidity increased was toward the latter parts of the experiments. This occurred only when considerable flaking of bacterial-oil masses from the oil layer was evident.

When there wasn't substantial flaking occurring in the first vessel, the culture fluids in the second and third vessels remained relatively free of particulate material and droplets of oil. There was also no sign of floating bacterial-oil masses in either vessel. This might have been expected due to washover from the first vessel. However, bacterially impregnated oil droplets did accumulate to a small degree on the walls of each vessel. This material was darkish brown in color and of a floccy, sometimes stringy consistency. It was readily sluffed off with a solid object or with shaking. Both the second and the third culture vessels were vigorously aerated and stirred, yet this material continued to accumulate on the side walls. When the floccy material was scraped off, it readily reappeared in several days. Microscopic analysis of the floc indicated large numbers of bacteria and oil droplets amassed in an unknown amorphous material.

2. Morphological Composition of Mixed Bacterial Populations in the Multistage Continuous Culture Systems

Analysis of the bacterial populations in each of the three multistage vessels using standard plate count techniques revealed two characteristic patterns. First, as has been reported for oil degradation with just a single vessel (40), predominant colony types appeared within the rather heterogeneous populations in each vessel. These preodominant types varied from 40-90% of the total population at various times throughout any experiment. In most cases, these populations did not persist for more than two or three weeks. Most of the predominant types were eventually replaced by some other colony type never to appear again.

A second pattern was the virtual absence of any differences in population composition from one vessel to the next. It was expected that as oil was degraded in the first vessel, various types of degradation products would be washed out into the second vessel. As a result, different types of bacteria would be enriched as a function of the type of degradation product present. It would therefore be expected that the bacterial populations would be different in each vessel. This, however, was not the case; a predominant species in one vessel was also predominant in the other two vessels. Any differences that were observed were usually the result of a change over in predominant species. This new predominant species was eventually reflected in all vessels.

As with many of the plate count assays, there is a diverse background of minor colonial types. For practicality and efficiency these minor types were generally overlooked in favor of the more predominant types. The members of these minor populations, if they could be feasibly analyzed, may in fact reflect the expected enrichment brought on by the different degradation endproducts.

In general, however, there was little variation in the morphological composition of the bacterial populations once predominance was established. This was true not only as the experiments progressed with time, but also with samples of Lake Ontario water taken at different times of the year.

3. Chemical Changes in Oil as a Result of Degradation in the Multistage Continuous Culture Systems

Gas Chromatographic analysis of the samples from a typical experiment are shown in Figures 2-7. All multistage experiments were designated by the symbol Q and the vessels are numbered accordingly. Samples from Q1 represent material from the oil layer and not the culture fluid below the oil layer. All other samples (Q2, Q3, Q-effluent) are taken from the culture fluid.

As indicated below, the results are expressed as a function of the incubation time primarily because this seems to be the simplest method at present. However, many of the results obtained do not fit into a continuous time period, this making it difficult to relate this information around a progression type of theme. Much of this problem originates from the nature of the system. As the oil undergoes the initial attack in the first vessel, droplets of various sizes containing oil and bacteria sluff off the oil layer and washout into the second and third vessels. The inconsistency of this process is obvious and it should not be expected that any type of uniform product is going to be supplied to the second and third vessels. The degree of degradation which has occurred in a droplet of oil released from the oil layer in the first vessel will also vary considerably. If, for example, a quantity of relatively undegraded oil was washed over into the second and third vessels, it could in fact obscure a sample of extensively degraded oil. Consequently, it would superficially upset any abstract degradation pattern.

None the less, it is obvious from an overview of the data that bacterially induced chemical changes do occur. A progression of degradation can be detected from vessel to vessel and from time period to time period. Certainly as the oil layer is more and more extensively attacked by the bacteria, the degraded oil becomes a greater and greater proportion of the oil layer. Degraded oil eventually appears in substantially larger concentrations than undegraded oil. This then results in the progressive type of degradation that has been observed.

The chemical changes in oil have been broken down into general time equivalency stages - early, middle, and late stages. However, it should be stipulated that the actual time period involved may be quite variable and there may be significant degrees of overlap between stages. However, it is the general pattern of degradation which is meant to be stressed.

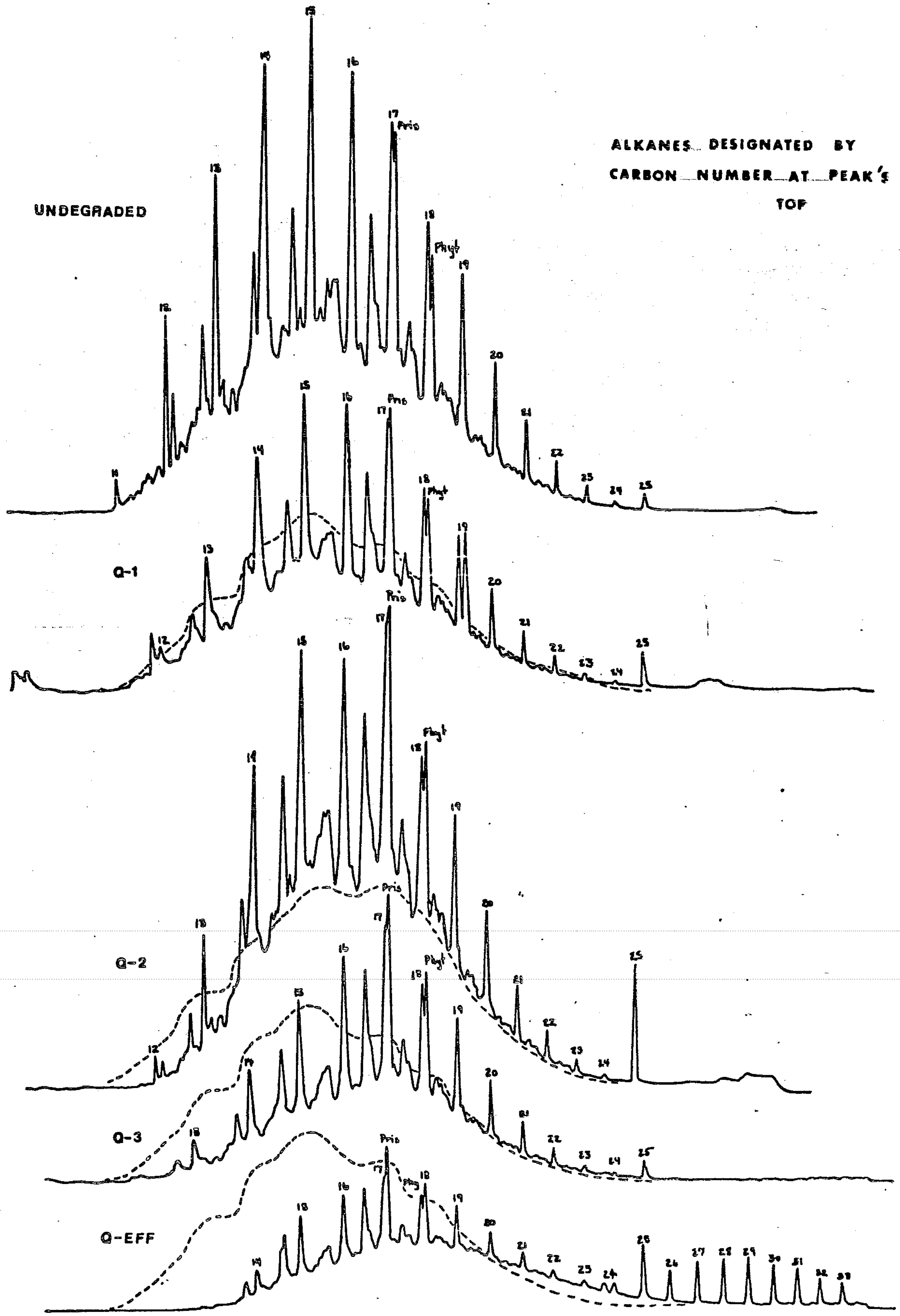
i. Early Stages of Degradation

This stage corresponds to a time period of approximately 100 to 600 h of incubation. It would normally correspond to the events in the physical changes of the oil layer through the formation of a thick film of bacterial growth under the oil and the initial impregnation of the oil layer with bacteria.

The major chemical change of this stage was a significant decrease in the major n-alkane peaks. Figure 2 is a composite of gas chromatographs from each of the three vessels (Q1, Q2, Q3) and the effluent from the third vessel (Q-eff) which are representative of this early stage of degradation (about 300 h of incubation). The ratios of C17 n-alkane to pristane and C18 n-alkane to phytane typically decreased by 5-10% in Q1 and by 10-25% in Q2, Q3, and Q-effluent. Also at this stage, the peaks corresponding to C11 and C12 n-alkanes, which were detectable in undegraded oil, were almost completely missing. This reflects the general trend of early preferential degradation of the low molecular weight alkanes.

Also apparent during this early stage of degradation was the alteration of the envelop profile. This profile constitutes the unresolved hump which is outlined by drawing a continuous line at the base of all the peaks which project out of the hump. This hump or envelop encompasses most of the branched alkanes, the cyclic alkanes and the aromatic hydrocarbons found in the diesel oil. These envelop components are apparently not resolvable because of their great variance in molecular structure and their relative concentration. In

Figure 2. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Multistage Continuous Culture Vessels During Early Stages of Degradation (total incubation period equals 300 hours).


ALKANES DESIGNATED BY CARBON NUMBER AT PEAK'S TOP
UNDEGRADED
Q-1
Q-2
Q-3
Q-EFF
Pris
Phyt

terms of the degradation pattern observed within the envelop profile it was important to study the shape of the profile itself. The actual size or height of the envelop profile is not important because it is directly related to the sample size used for injection into the gas chromatograph.

At present, there is no way to quantitate the degradation of the envelop components except to visually compare the shape of the envelop profile with that from undegraded oil (indicated as a dotted line superimposed on the figures). This has been done for the gas chromatographs in Figure 2. It is clear that substantial alteration of the envelop profile shape had occurred on the left hand side, corresponding to the lower boiling branched alkanes and aromatics. This change was particularly noticeable in the latter vessels of the multistage continuous culture system during this early stage of degradation. For example, in Figure 2 it can be seen that in Q-eff and to a lesser extent in Q3, there was obvious deviation in shape from the envelop profile of undegraded oil in the low boiling range. This would presumedly correspond to a preferential degradation of these components by the bacterial population present.

It should also be emphasized at this early stage of degradation that there was some evidence for successive degrees of degradation. For example, samples from Q1 showed relatively little degradation (about a 7% change in the pristane and phytane ratios) and an insignificant alteration of the envelop profile. However, the effluent sample showed substantial degradation (23% drop in the pristane and phytane ratios) particularly in the envelop components. In some analyses no signs of successive degrees of degradation were apparent and in many cases the Q-eff samples showed only minimal differences.

The most surprising aspect of the multistage degradation process was the sudden appearance of gas chromatographic peaks. These peaks corresponded to normal alkanes of carbon lengths C26 to C34 (see Figure 2, Q-eff). These peaks were never detected in undegraded oil. All appropriate control experiments have indicated that they resulted from the bacterial activities that occurred during the degradation process. In the early stages of degradation these extra peaks appeared only in the effluent and were most prominent in oil samples taken from the walls of the effluent bottle. The make-up of these extra peaks was quite specific. All eight peaks invariably appeared as a group at about the same relative concentrations. Occassionally there was some variation in the last peaks. In some samples the peak corresponding to a C34 alkane would be missing, especially in the early stages of degradation.

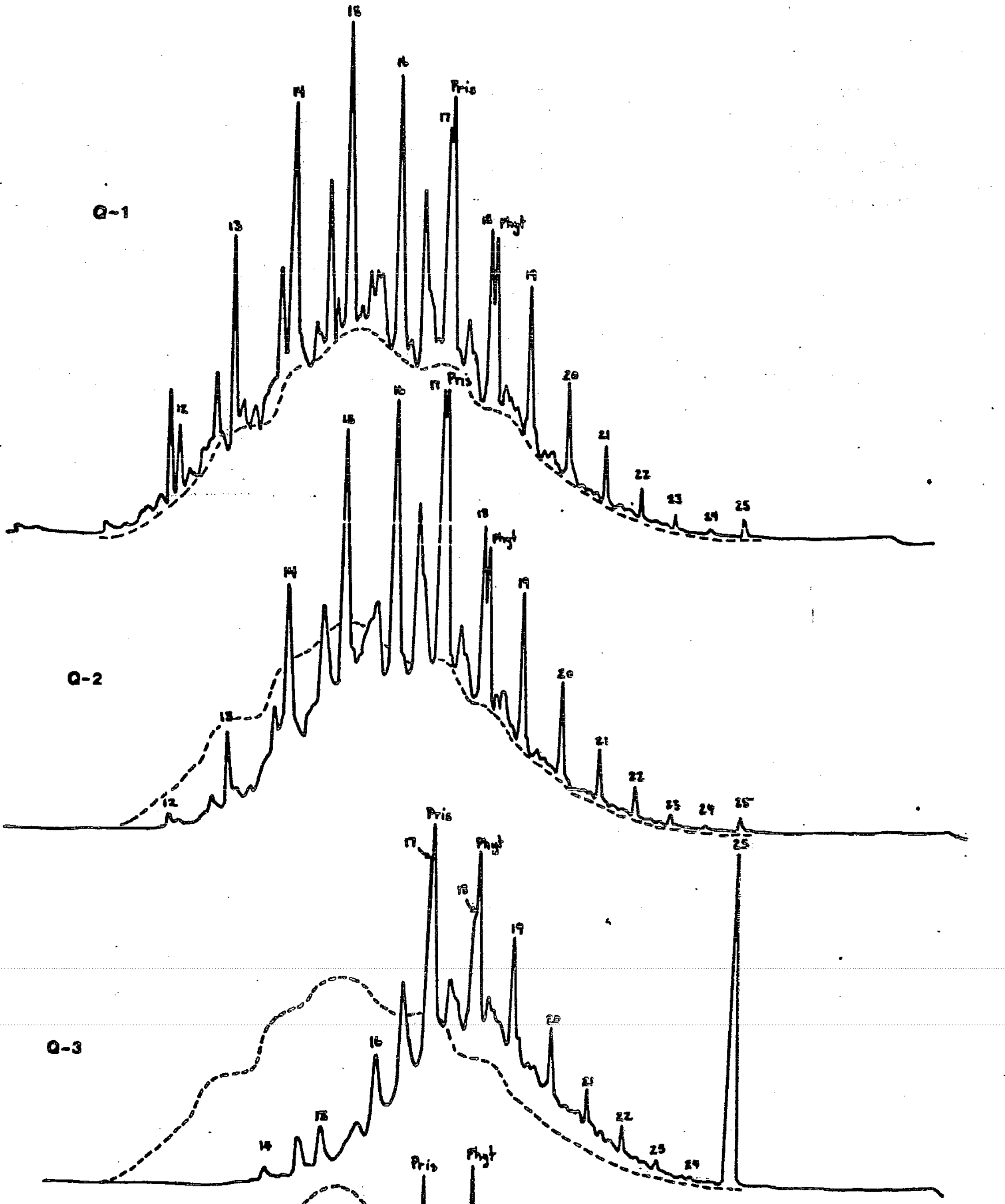
Along with the apparent synthesis of these eight extra peaks, there was also an enrichment of certain peaks originally present in undegraded oil. This enrichment also involved synthesis of a particular compound, probably as a metabolic endproduct. This peak again corresponded most closely with an n-alkane. In Figure 2, Q-eff, there was a decided enrichment in the peak corresponding to C25 n-alkane. This enrichment phenomena generally occurred concommitant with the synthesis of the extra peaks but there were examples where the C25 peak was enriched independantly of the other peak appearances.

Figure 3 shows another series of gas chromatographs which are again representative of the early stages of degradation (about 700 h of incubation). However, they do correspond to the latter part of this stage. It should be noted that the degradation pattern is about the same as that seen in Figure 2, but the extent of degradation was considerably greater. In Q3 for example, the pristane-phytane ratios have changed by 20-25% relative to undegraded oil. The peak corresponding to a C13 normal alkane was gone and the peaks for C14 through C16 were all greatly reduced. The envelop profile was also substantially modified. This was particularly true at the low molecular weight, low boiling end of the envelop profile.

There was no indication of any synthesis of the extra peaks except in the effluent samples. However, the enrichment phenomena was considerably magnified. In Q3 there was a tremendous increase in the peak co-chromatographing with the C25 normal alkane. This extensive enrichment was observed numerous times and generally appeared farther up the chain of vessels as the incubation period increased. Note also, that no other enrichments appeared. This apparently indicates a very specific metabolic process taking place. In Q-eff the extent of enrichment of the C25 peak has been reduced but now C23 and C24 have also been enriched.

Furthermore, another type of extra synthesis was also apparent. In this case, components making up the envelop profile appear to have been synthesized in the higher boiling range. This is particularly manifested in Q3. This profile

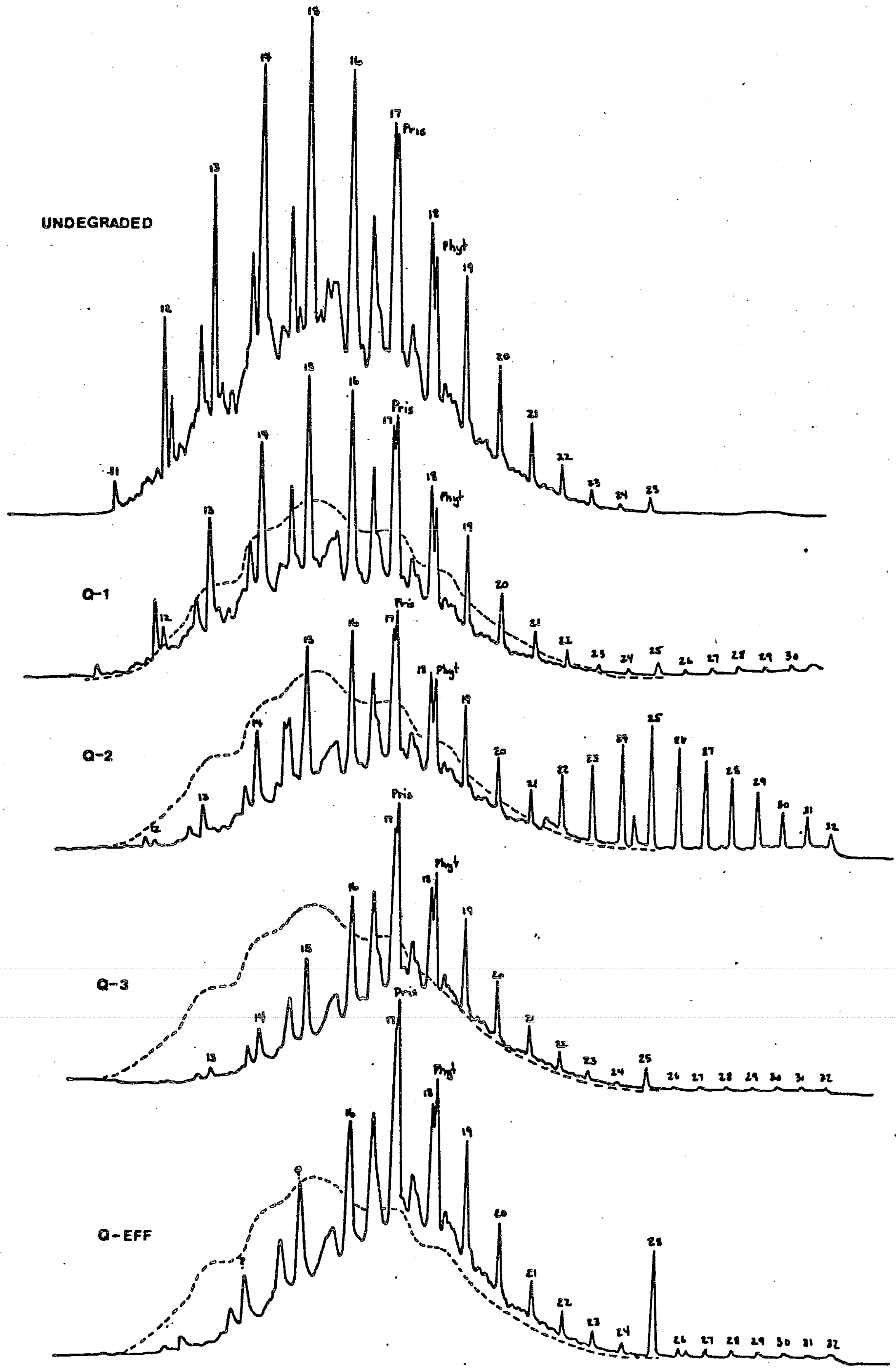
Figure 3. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Multistage Continuous Culture Vessels During Early Stages of Degradation (total incubation period equals 700 hours).


Q-1
Q-2
Q-3
Q-EFF
Pris
Phyt

extends out beyond the envelop profile of undegraded oil. This extension was very difficult to account for by any other mechanism except the generation of metabolic endproducts. Presumedly, these materials are hydrocarbon in nature and of a higher molecular weight than the original substrate.

During this early stage of degradation it was discovered that peak enrichment and peak synthesis was not confined entirely to the effluent. The prevalance of these phenomena was further indicated by examining the wall growth on each of the continuous culture vessels. This wall growth material was sticky enough to remain adhered to the walls and thus would not be analyzed during a normal sampling procedure. If however, this material was gently scraped off the walls with a wire loop and then sampled along with the culture fluid, a considerably different gas chromatographic picture was obtained (see Figure 4). It can be seen that the peak synthesis and enrichment occurs at all stages of the multistage continuous culture system. In this particular experiment, the amount of synthesis and enrichment was greatest in Q2. However, it was definitely detectable in all the other vessels, including the first vessel. It should be re-emphasized that the chemical nature of these peaks is still unknown, but it would appear that they are high boiling normal alkanes. Thus the phenomena of peak synthesis and enrichment occurs at all stages of the multistage continuous culture system. Furthermore, it can be detected in oil adhered to the sides of the vessels before it is seen in the culture fluids.

Figure 4. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluid and the Wall Growth of Multistage Continuous Culture Vessels During Early Stages of Degradation (total incubation period equals 700 hours).


UNDEGRADED
Pris
Phyt
Q-1
Q-2
Q-3
Q-EFF

ii. Middle Stages of Degradation

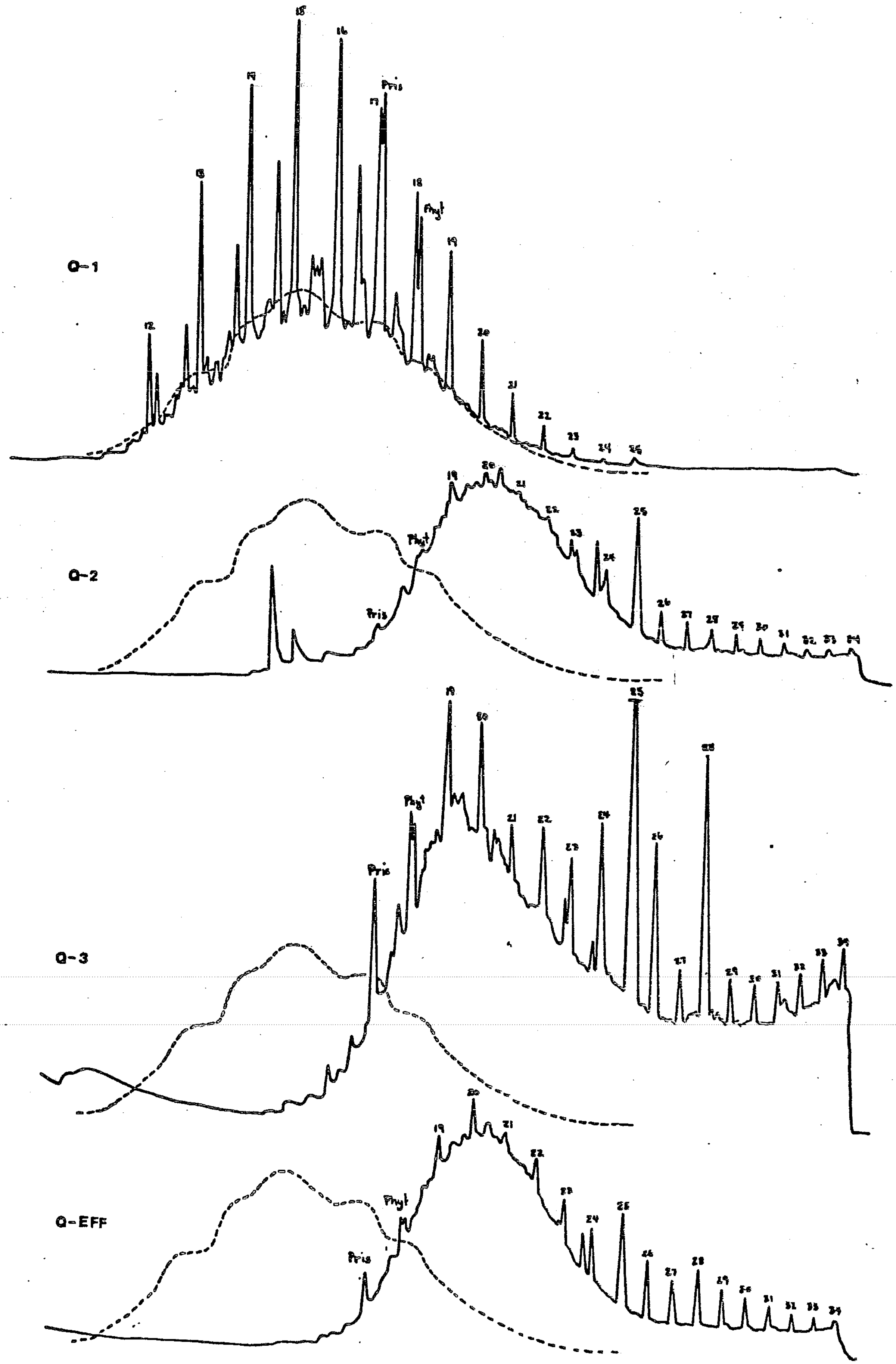
This stage corresponds to the point in the physical changes where the oil was highly impregnated with bacteria and considerable flaking off of bacteria-oil masses was occurring. The duration of this phase was variable and its limits are more or less arbitrary. However, a number of important degradation events can be used to characterize this stage. Typical gas chromatographic profiles during this stage (for incubation periods of 1200 and 1500 hours) are shown in Figures 5 and 6. The important events were as follows:

a. Most of the peaks which co-chromatograph with n-alkanes of C12 to C19 were either completely gone or extensively degraded. The branched alkanes, pristane and phytane, still stand out. However, they too were extensively degraded.

b. Almost 75% of the components comprising the envelope profile have been degraded or have at least disappeared from the chromatographic profile. This removal was again primarily in the low boiling fraction. However, it progressed to a point where more and more of the high boiling components are being degraded.

c. Peaks which co-chromatographed with n-alkanes of C26 through C34 were now present in all three vessels (Q2, Q3, and Q-eff.). They were not seen in the relatively undegraded oil still present in the oil layer (Q1). There was also peak enrichment in all three vessels; components corresponding to C22 through C25 were generally increased relative to undegraded oil. Once again, the C25 stood out as being

Figure 5. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Multistage Continuous Culture Vessels During Middle Stages of Degradation (total incubation period equals 1200 hours).

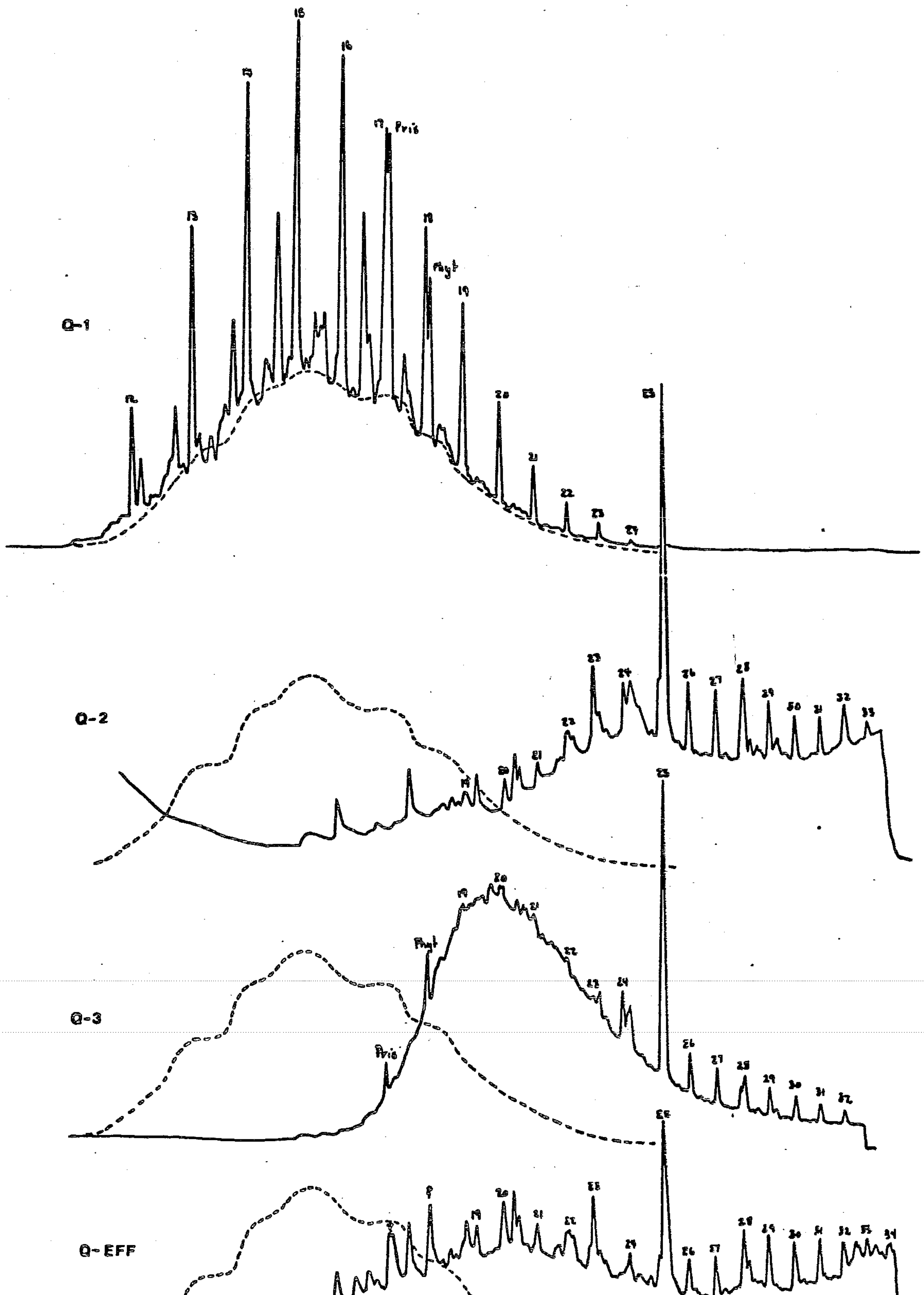

Q-1
Pris
Phyt
Q-2
Phyt
Pris
Q-3
Phyt
Pris
Q-EFF
Phyt
Pris

the most massively increased.

d. A whole envelop profile has apparently been generated which now lies in the higher boiling range. This does not seem to be a shifting of the original envelop profile. Instead, it appears to be the generation of metabolic endproducts which are unresolvable as a group in the gas chromatograph. These apparently are of a more complicated molecular structure than the substrates from which they were derived. The chemical nature of the components in this new envelop profile is not known but one would possibly surmise that they were aromatic and branched alkane-like material.

e. There was relatively little difference in the extent of degradaiton from one vessel to the next. All of the gas chromatographic profile data indicate only minor differences in Q2, Q3, and Q-eff. Many of the differences can be attributed to sample size while a few others appear to be legitimate occurrances of new peaks. On the whole, there was little difference in the gas-liquid chromatographic profiles from time period to time period during this middle phase of degradation. On the other hand, in some analyses there were also gas chromatographic profiles which differed drastically from the typical results normally seen (see Figure 6). In this particular analysis it appeared that most of the synthesis of new hydrocarbon components was in the extreme high boiling range. This was not as pronounced in Q2 and Q-eff in Figure 6. This would seem to further indicate that even though many extra metabolic endproducts are being produced, these too eventually undergoe degradation in

Figure 6. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Multi-stage Continuous Culture Vessels During Middle Stages of Degradation (total incubation period equals 1500 hours).


Q-1
Q-2
Q-3
Q-EFF
Pris
Phyt

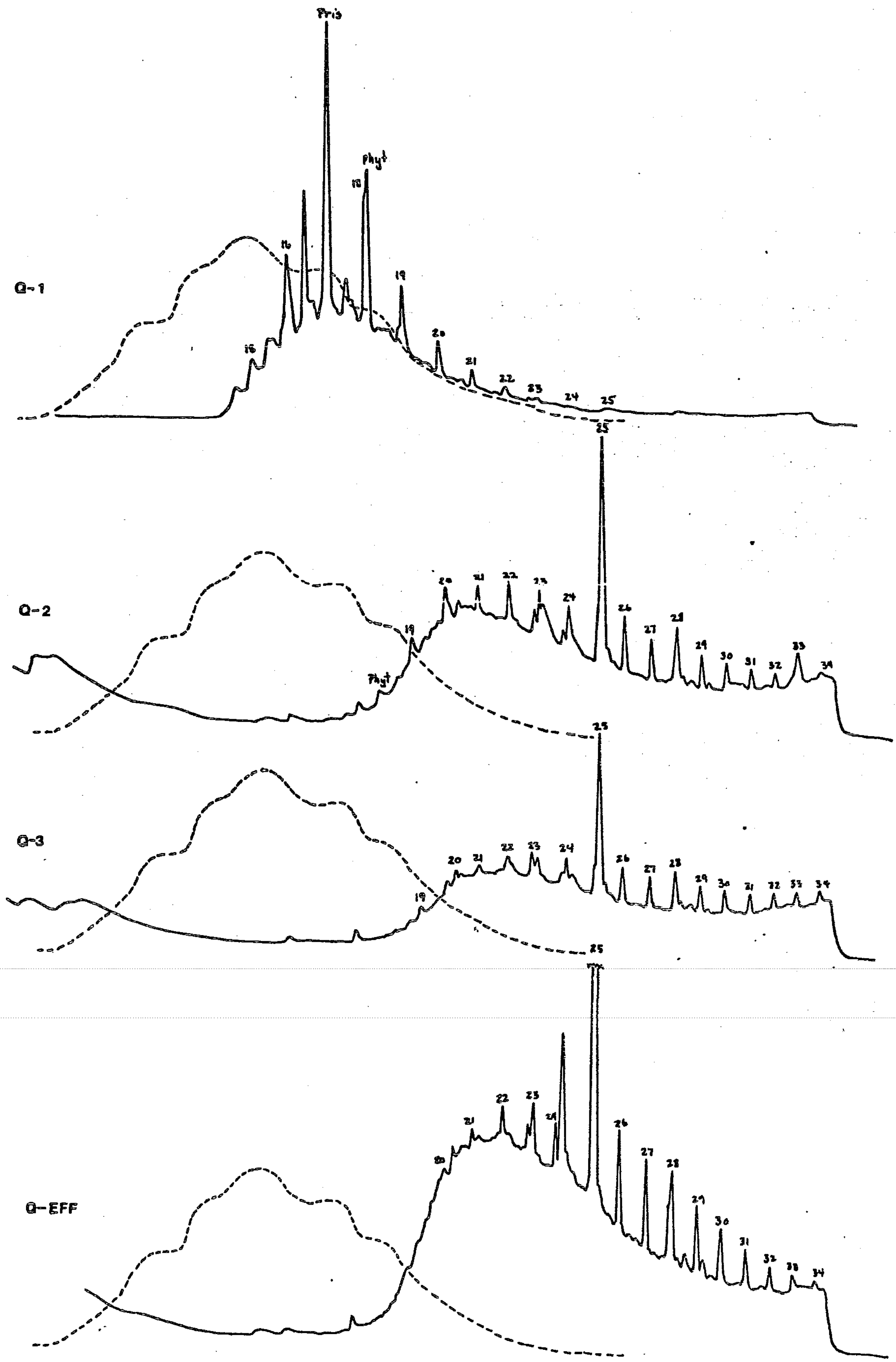
the multistage system. However, close examination does show that many of the peaks projecting out of the envelope do not correspond to any known hydrocarbon peak. Consequently, these peaks were possibly synthesized by bacterial activities or simply enriched because many of the other components were being removed.

iii. Late Stages of Degradation

This stage corresponds approximately to the point where the oil in the first vessel has been substantially decreased in volume. The oil is now a brownish, or whitish, slimy clump of cells and oil floating on the surface. Gas chromatographic analysis of the oil in all vessels is shown in Figure 7. For the first time, substantial chemical modification of the oil layer (Q1) was apparent. Presumedly, this was because the amount of undegraded oil was insufficient to dilute out any undegraded oil. This degraded oil reflected the same general degradation observed previously, i.e. removal of n-alkanes and envelope components, synthesis and enrichment of alkane-like peaks and a shifting of the envelope profile into the high boiling range. The branched alkanes, pristane and phytane are still quite prominant but have not undergone the extent of degradation seen in the next vessels in the chain.

The subsequent vessels all showed a general decrease in the remaining components. The peak corresponding to C25 alkane, however, continues to be produced in large quantities.

Figure 7. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Multi-stage Continuous Culture Vessels During Late Stages of Degradation (total incubation period equals 1700 hours).


Pris
Phyt
Q-1
Q-2
Phyt
Q-3
Q-EFF

In most cases hydrocarbons up to C20 are gone, including the isoalkanes, pristane and phytane.

No further degrees of degradation were observed at this point. This was primarily because the oil concentration becomes so low that any remaining components are not detectable with gas chromatography.

B. The Effect of Increased Nitrogen and Phosphorous Concentrations on Oil Degradation in Multistage Continuous Culture Systems.

The degradation of oil by bacteria that has been demonstrated in the work illustrated previously is actually an accelerated process relative to natural conditions. This is because the amount of nitrogen and phosphorous added to the systems is about 10-100 times greater than that found naturally in Lake Ontario. However, as partially degraded oil passes through the multistage continuous culture system, the bacterial activities may in fact use up the available nitrogen and phosphorous and thereby slow down the degradation rate. To test this possible source of limitation, another multistage system (designated QB) was set up in which the nitrogen and phosphorous concentrations were doubled (200 mg/l potassium phosphate buffer, 50 mg/l ammonium chloride).

Visual changes during the degradation process in the QB system were generally about the same as those seen in the normal Q system. The oil layer was attacked and impregnated by bacteria in the same manner. Any major changes in turbidity, color, or flaking occurred at about the same time in each vessel. The bacterial populations in all vessels showed the same predominant species and about the same degree of heterogeneity.

The only major visual difference between the QB system and the normal Q system was observed during the latter stages of degradations. It had then become obvious that the consistency of the bacterially impregnated oil layer differed. In the QB system, the oil layer was considerably more mucousy and slimy and not as particulate-looking as the oil layer in the normal Q system. In addition, the oil layer in the Q system was dark brown to grey in color. The QB system oil layer was more milky-white in color. It should also be noted that the QB system had slightly more wall growth than the normal Q system.

An examination of the gas chromatographic profiles of oil extracted from the QB system (Fig.8) again indicated relatively little overall difference from the normal Q system. Degradation of the alkanes and the envelop components proceeded to about the same degree. The appearance of extra peaks and the extra envelop profile also occurred at the same time and to about the same extent.

If anything, the QB system showed slightly more synthesis of the extra n-alkane peaks and the extra envelop components. This can be seen in Figure 9. This figure represents the analysis of a sample taken during the middle stages of degradation (1200-1500 h, 50-60 days incubation). Most pronounced in this particular analysis is the increased size of the extra envelop profile. This increase presumedly had

Figure 8. Gas Chromatographic Profiles of Oil Extracted From Culture Fluids of Multistage Continuous Culture Systems Under Conditions of 1 times(Q) and 2 times (QB) Concentrations of Nitrogen and Phosphorous.

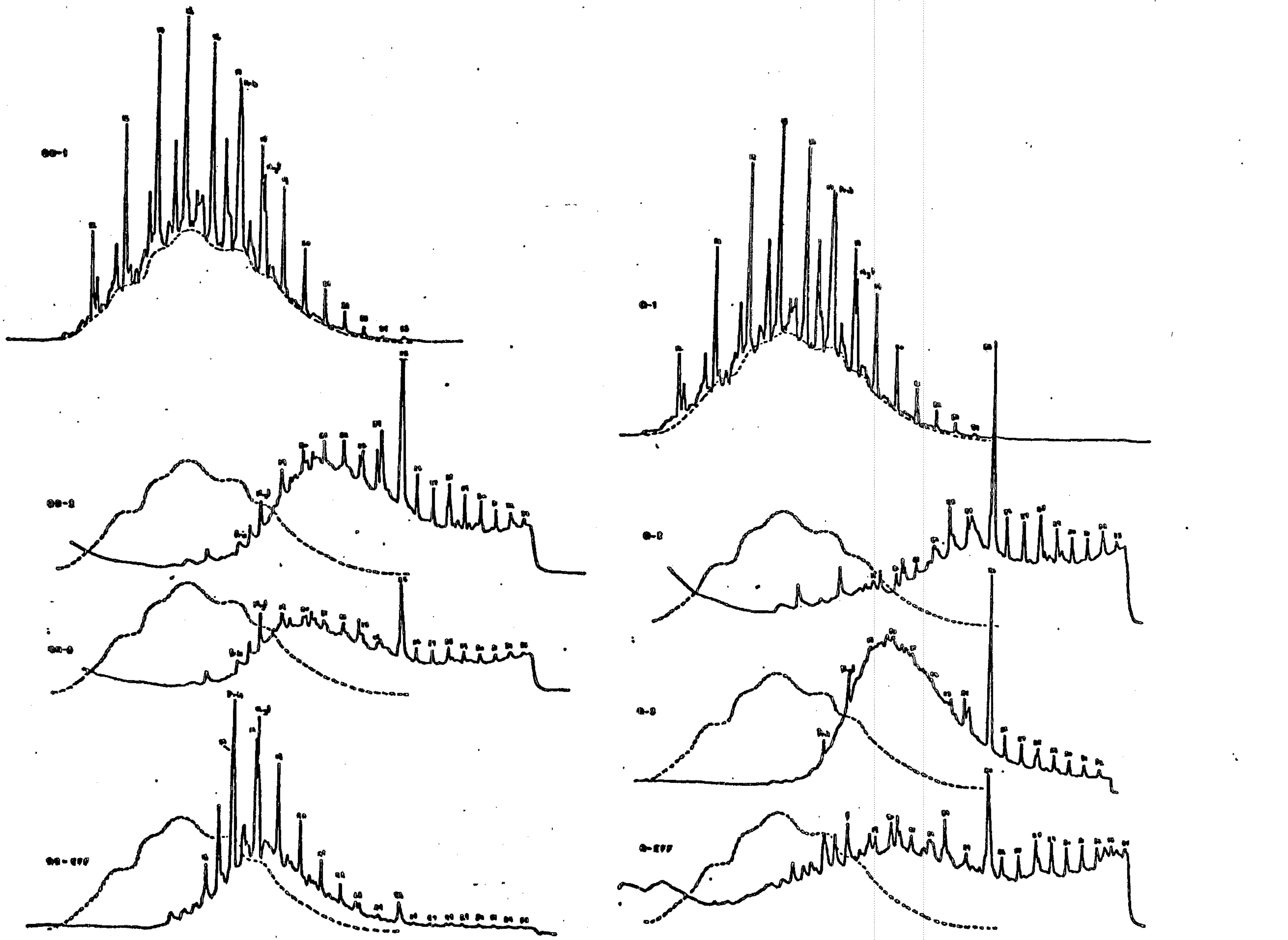
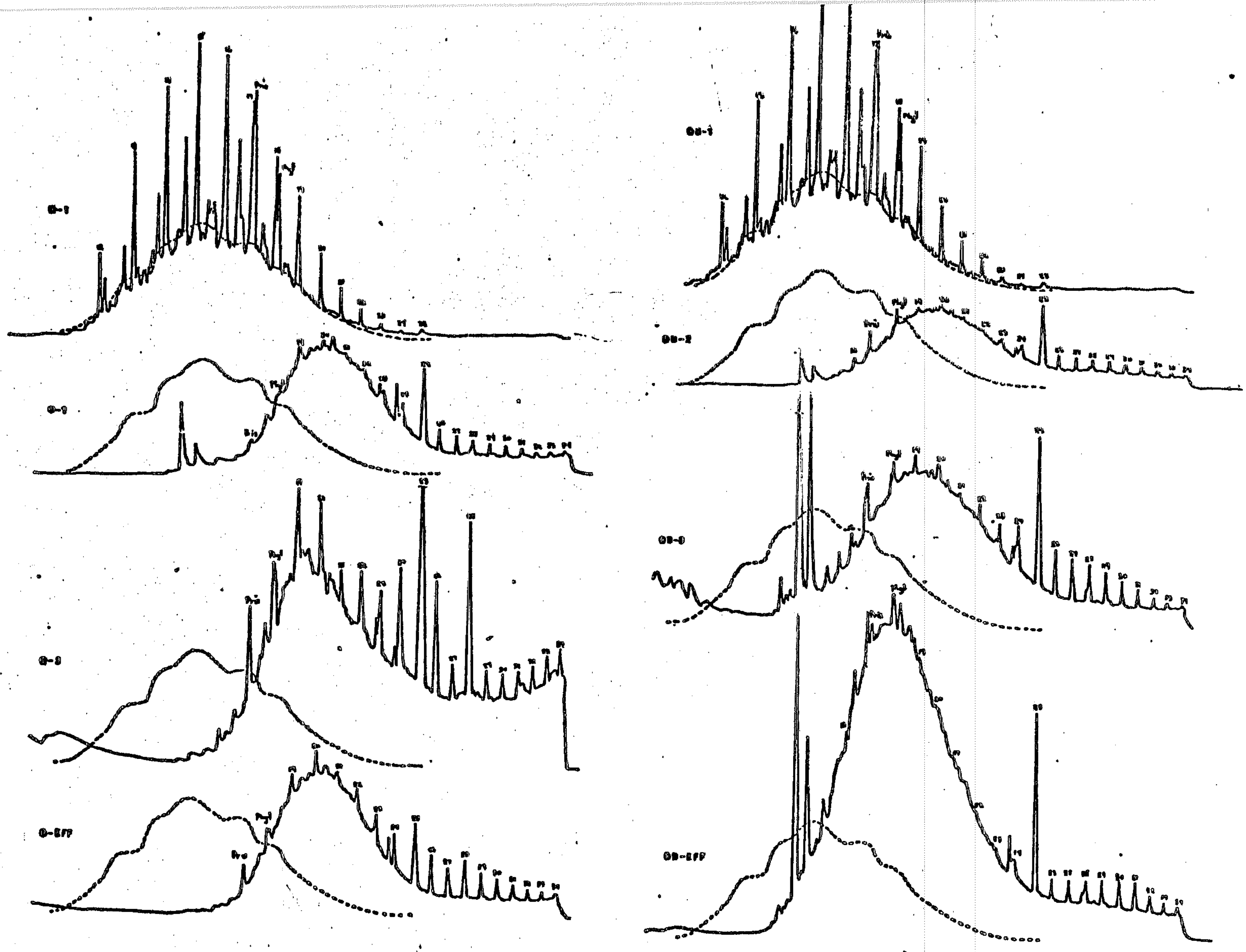


Figure 9. Gas Chromatographic Profiles of Oil Extracted From Culture Fluids of Multistage Continuous Culture Systems Under Conditions of 1 times (Q) and 2 times (QB) Concentrations of Nitrogen and Phosphorous.



resulted from a greater synthesis of extra unresolvable organic substances. Also quite apparent in the QB system is the appearance of two extra peaks in the low boiling range which are not nearly as magnified in the normal Q system.

C. The Effects of Adherence to Surfaces on the Oil Degradation Process in Multistage Continuous Culture Systems

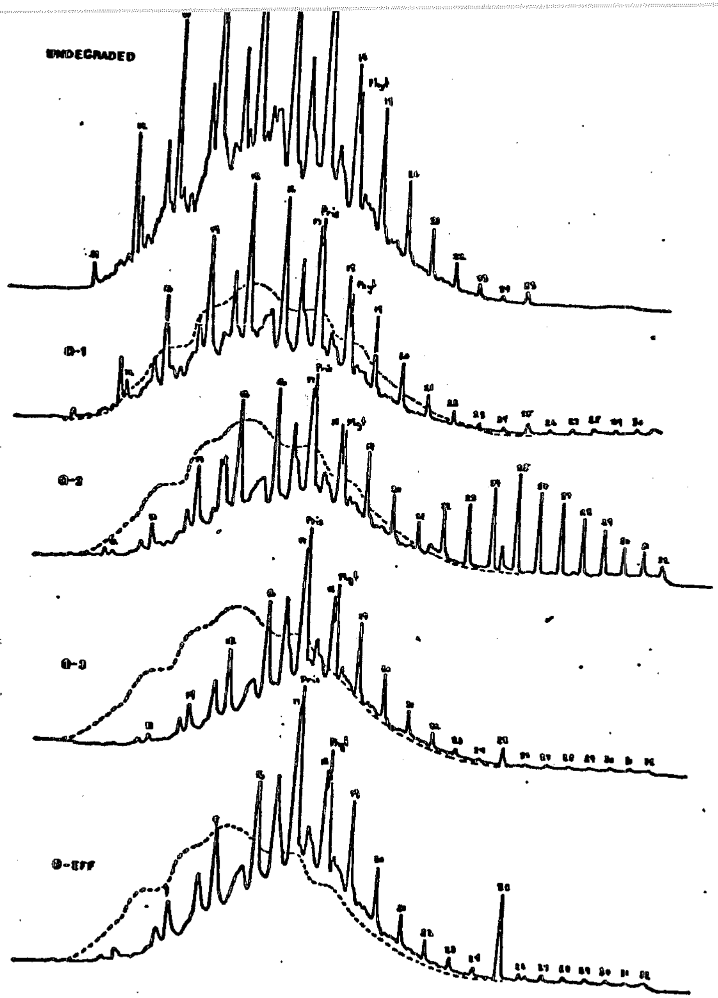
As noted previously, during many phases of the degradation process, significant amounts of material accumulated on the walls of the culture vessels. This material was usually light brown in color, rather gelatinous in consistency, and not very tightly held to the walls. In other kinds of continuous culture experiments this accumulation of material on the walls would be a troublesome problem. Wall growth normally complicates interpretation of continuous culture results. However, in examining the wall material in the multistage continuous culture systems, it was discovered that considerable amounts of small oil droplets and bacteria were part of this material. This meant in fact that bacteria may have been growing on the walls and utilizing the oil under conditions which were still open and in fact continuously maintained. Consequently, it was decided to examine the chemical nature of this oil to see if any different types of degradation pattern had occurred.

As already alluded to, some differences in the degradation process as a function of wall growth was apparent. At that point, it was noted that the synthesis of extra peaks corresponding to n-alkanes of C23 to C34 carbon number, were readily detected in the oil adhered to the vessel walls before it was detected in the oil extracted from the culture fluid. To further verify this difference, wall material had been removed and extracted from a multistage system. In the samples analyzed a decidedly different degradation pattern had been detected.

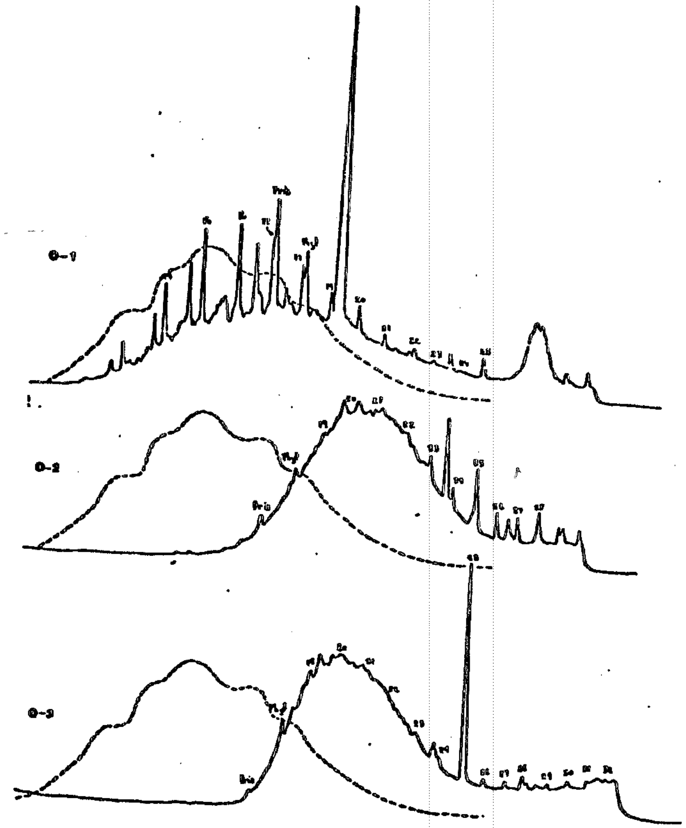
Figure 10 shows the gas chromatographic profiles of oil extracted from the culture fluid and from the material adhering to the walls. It is evident that there was an increased degree of degradation of oil taken from the wall material. In Q1 the oil from the vessel walls showed a considerably greater degree of degradation. The n-alkanes and envelop components were more degraded than the oil taken directly from the oil layer. Some synthesis of components in the boiling range above the C25 normal alkane was also present. The large peak appearing next to the C19 peak is unknown chemically, but appears to be a result of the wall associated degradation.

The oil extracted from the material on the walls of the second vessel (Q2) again showed a substantially different degradation pattern (Figure 10). Unfortunately, the sample obtained from the culture fluid was not sufficient to give the normal gas chromatographic picture. However, if one compares the height of the pristane and phytane peaks to the height of the envelop profile, it can be seen that much more degradation had taken place on the walls of the growth vessel. The amount of envelop components synthesized was also considerably greater in the wall material. Overall, it would appear

Figure 10. Gas Chromatographic Profiles of Oil Extracted From Vessel Walls and Culture Fluid of the Same Multistage Continuous Culture System.



CULTURE FLUID



VESSEL WALLS

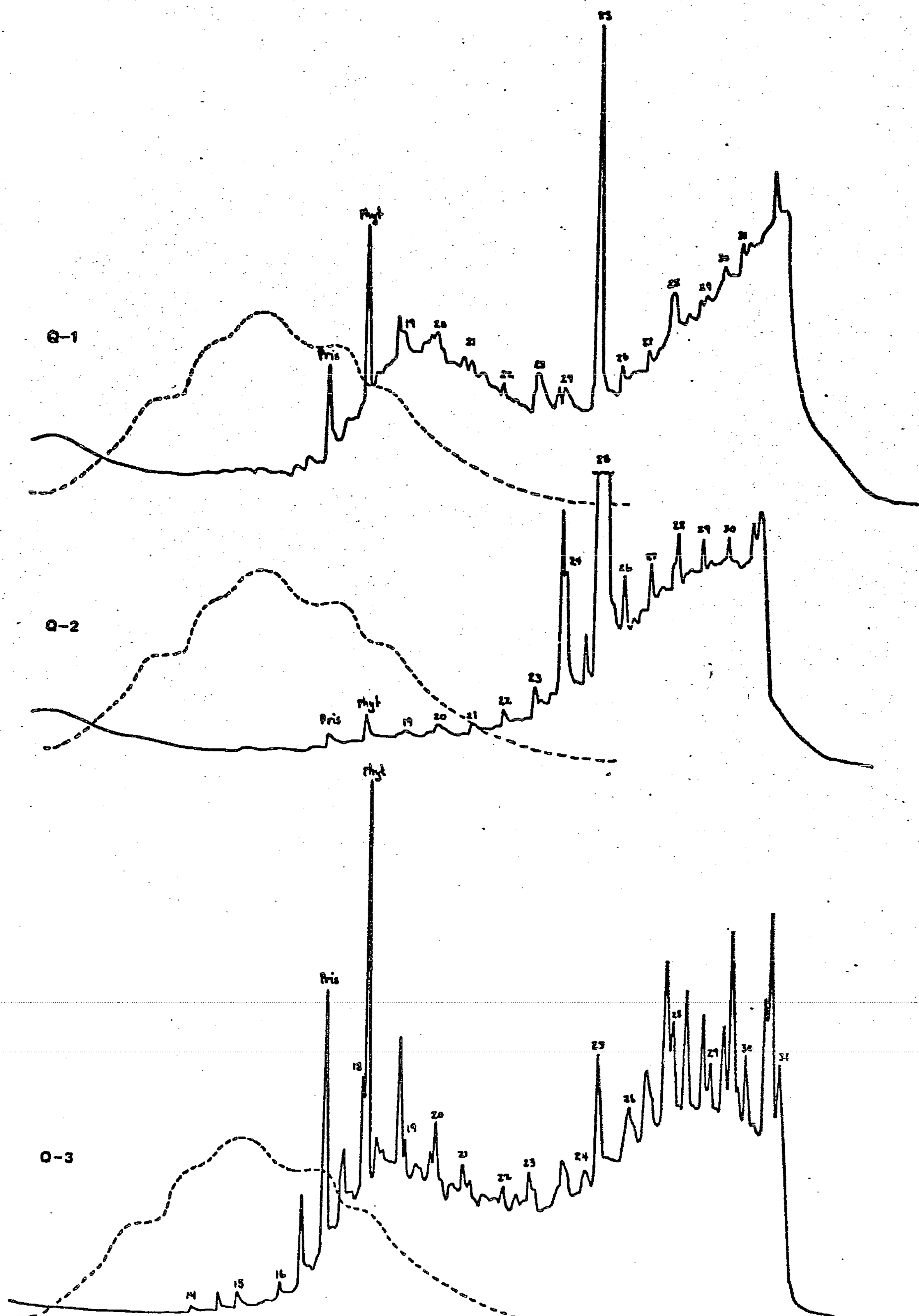
that the whole degradation process has been speeded up as a result of the oils' attachment to the vessel walls. Examination of the chromatographs from Q3 also substantiates this generalization but not to quite as great an extent.

Not only were small droplets of oil found on the walls of the vessels, but significant quantities were found associated with the walls of the glass and silicone tubing which connected each of the multistage continuous culture vessels. The physical appearance of this tubing material was much the same as it was on the vessel walls. However, chemical analysis of the oil extracted from the tubing has indicated the greatest degree of degradation yet obtained in the systems. This is shown in Figure 11. Three results are immediately obvious in examining oil extracted from the tubing connecting the first and second vessel.

First, the envelope profile harboring the branched alkanes and the aromatics were completely degraded away leaving only traces of the pristane and phytane peaks above. Second, the synthesis of extra envelope components appeared to reach an extreme. The relative quantity of material in this higher boiling range was considerably greater than that seen anywhere else. Third, the peak co-chromatographing with the C25 n-alkane was enormous, again being considerably more than that seen previously. This degree of degradation was not seen in samples extracted from the culture fluids.

For the samples taken from the tubing between vessels Q2 and Q3 a similar, but not as extensive, degradation has occurred. Very noticeable on this chromatograph was the large number of peaks which have appeared after the C25 peak.

Figure 11. Gas Chromatographic Profiles of Oil Extracted From Tubing Walls and Culture of the Same Multistage Continuous Culture System


Q-1
Phyt
Pris
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Q-2
Pris
Phyt
19
20
21
22
23
24
25
26
27
28
29
30
Q-3
Phyt
Pris
14
15
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30
31

These peaks do not correspond to any other type of n-alkane.

In the tubing between Q3 and the effluent bottle, no oil was detectable. This could mean either that there was not enough oil to be extracted or that a virtually complete degradation had occurred. It is probably the former case which is true.

D. The Effects of Continuous Inoculation on the Oil Degradation Process in the Multistage Continuous Culture Systems

The results obtained from the multistage continuous culture experiments are unique and not reported in the literature. Thus, it was necessary to show that such a laboratory model was as much like a natural situation as possible. In all previous experiments, the source of bacterial populations for the degradation process had come from the original sample of Lake Ontario water. In other words, the addition of one 200 ml sample of lake water supplied all of the bacteria needed to carry out the degradation pattern observed over a two month period. However, to date it has not been possible to get the complete degradation of oil as it passes through the multistage continuous culture system. Furthermore, there has not been a great difference in degradation as the oil proceeds from one vessel to the next. This could be due to the fact that the right bacterial populations were not present simply because they were washed out of the growth vessels before they had a chance to multiply.

Of course, in a natural aquatic situation, this would not be the case. Continual reinoculation of the oil would always take place. Thus, in an attempt to simulate this reinoculation process, multistage continuous culture experiments were set up in which fresh lake water was continually added. The degradation process was then monitored in the same way.

Results indicate no substantial difference in the multistage oil degradation process when a continuous inoculum was used. Physical changes and changes in the chemical make-up of the oil occurred to about the same degree as observed previously and within about the same time span. Complete degradation of the oil was not observed as it passed through the multistage continuous culture vessels. There was again no overwhelming difference between the degree of degradation in one vessel relative to the one preceeding it. Likewise an analysis of the bacterial populations showed no enrichment of any colony type which was unique to any vessel.

V. Discussion

A. The General Pattern of Degradation in Multistage Continuous Culture Systems.

The use of multistage continuous culture systems does appear to be an important way to study the degradation of oil in aquatic ecosystems. These results successfully show particular aspects of the oil degradation process which have never before been reported in the literature. It is doubtful that these results could have been duplicated by any other method except in a continuous culture system.

In batch culture oil degradation studies, the degradation pattern most often observed is a relatively simple one. Typically, one sees the rapid removal of the easily degraded normal alkane fraction leaving behind a hydrocarbon fraction consisting of a large variety of branched and cyclic alkanes and aromatic hydrocarbons (7,13,21,25.29). However, the results from multistage continuous culture studies have illustrated a different degradation pattern. Study in this system has shown the typical n-alkane attack as well as the concommitant attack on the branched alkanes and aromatic compounds starting with the lower boiling components. This has led to the general conclusion that if the proper conditions are present, the branched alkanes and aromatics are not nearly as recalcitrant as once thought. Presumedly in a natural process, there is not as severe a preferential attack on the n-alkanes as so many laboratory experiments had indicated.

The type of oil degradation pattern in the continuous culture systems has proven to be very interesting. In the first vessel the oil layer was attacked by numerous types of hydrocarbon degrading bacteria. This led to a process of emulsification and oil droplet formation which under natural conditions would have allowed the oil to be rapidly dispersed into the water column. This dispersed oil exists as small droplets covered with bacteria. Presumedly as these droplets move throughout the water column, the adhering bacteria would slowly degrade the oil from the outside in as inorganic nutrients were replenished. This appears to be the process that is observed in the continuous culture system except that mechanical dispersal of the oil is prevented. Instead, the small droplets of oil with their adhering bacteria were partially retained in the oil layer. This was accomplished in such a way that only small amounts of the oil droplets were dispersed. This controlled degree of dispersal thus occurred continuously, and as such, the extended stages of oil degradation could be observed. Consequently, one has the capability of observing the fate of emulsified, bacterially impregnated oil droplets as they pass through the multistage system.

The type of oil degradation observed in the multistage system is unique. Not only was the original oil degraded, but new types of gas chromatographable materials were generated. Presumedly this results from the bacterial activities taking place. These materials, which represent extra peaks on the gas chromatographs, were not present in undegraded oil and they did not appear to be artifacts.

The present interpretation is that these extra peaks have been synthesized by the bacteria involved in the degradation process. These peaks are most likely some type of metabolic endproduct. Although there is no chemical evidence for their exact nature, they could possibly be normal alkanes. A similar type of synthesis phenomena has been noted by Horowitz et al (18) when various bacterial isolates were sequentially grown on a crude oil. However, their peaks (also interpreted as n-alkanes) appeared in a lower boiling range and corresponded ot C14 to C16. These peaks could possibly be produced directly from the oil or produced and subsequently transformed into some other type of products. The chemical nature of these extra materials is totally unknown at present. However, because of their behavior in a gas chromatograph, it is suspected that they are actually hydrocarbons of a chemical structure not represesnted in the original oil. For example, many of the extra peaks which appeared on the chromatographs corresponded to n-alkanes with higher molecular weights and therefore higher boiling points. If this is actually true, then the oil degradation process may result in the production of essentially more recalcitrant materials. There is also the appearance of extra materials corresponding to unresolvable branched and cyclic alkanes and aromatic hydrocarbons (i.e. the new envelope profile). It is therefore possible that more toxic or even carcinogenic compounds could be among the endproducts.

As to why these extra materials are produced, it is not at all clear. It could represent a unique type of enzymatic attack which results in more of a transformation than an actual degradation. This type of mechanism is not commonly observed in most degradation studies (7,20,21,22,25). The closest reported case of such a transformation is the formation of high molecular weight waxy esters from the growth of a Micrococcus species on heptadecane (39). In addition, as previously noted, the production of new, gas chromatographable alkane-like materials was noted by Horowitz et al (18) when bacteria were grown sequentially on a crude oil. Whether esters are being produced in these experiments is difficult to say. From simple physical observations it would not seem to be the case. It is possible however, that any esters formed could subsequently be reduced. This would lead to the formation of normal alkanes of considerably higher molecular weight than the original material.

Interestingly enough, Walker et al (42) reports on another type of synthesis as a result of oil biodegradation. When growing the hydrocarbonoclastic, achlorophyllous alga Prototheca zopfii on motor oil; resins and resin-like compounds were produced. In addition, asphaltic compounds were also produced. This is indeed surprising because the same alga, when grown under similar conditions on a crude oil, readily degrades the resin and asphaltic components.

Another question which arises, is why hasn't the production of these particular extra materials ever been detected before in oil degradation studies. A possible explanation is the following: In laboratory experiments, it is felt that batch culture experiments lead to such a severe selection of a relatively small number of hydrocarbon oxidizing bacteria which have a rather low metabolic diversity. The synthesis of these extra materials never occurs because the required bacteria are invariably selected against. Thus the experimental design is such that this transformation process is never allowed to occur.

In field experiments, where one might expect to see the type of degradation observed in continuous culture studies, it has also not been detected. However, it is felt that investigators have not looked in the right place. In field experiments (7, 25), the only way in which the degradation of the oil can be monitored is to sample the oil directly from its point of input. This procedure does not give a true indication of the degradation progress. Instead the surrounding water column needs to be extracted and analyzed for the oil degradation products. The magnitude of this task however, makes it prohibitive and thus the synthesis of extra materials during oil degradation essentially goes on unnoticed.

In any event, the results presented herein, successfully establish a new and distinct degradation pattern. Furthermore, because these results were obtained in a system which in all probability is similar to the natural environment, extrapolation of the data is possible. The total effect of oil on the environment can now be more intelligently evaluated. However, the exact social, political, and scientific ramification of this work has yet to be uncovered.

B. The Effect of Increased Nitrogen and Phosphorous Concentrations on Oil Degradation in Multistage Continuous Culture Systems

One aspect of the multistage continuous culture studies is the ~~absence of significant~~ sequential degradation of the oil. Much of the material leaving the first vessel remained relatively undegraded as it passed through the other vessels in the chain. Increasing the concentrations of nutrients to the system did not substantially alter the degradation pattern. This result would seem to indicate that the concentrations of nutrients were not limiting the degradation process. However, differences were seen in the physical appearance of the QB oil layer as compared with a normal Q oil layer.

At present, it is difficult to account for these differences in the physical appearances of these systems. Analysis of other parameters do not necessarily reflect these differences. For example, the pattern of colony morphologies resulting from analysis of the bacterial populations was strikingly similar. It is known from other work that changes in the nitrogen and phosphorous concentrations bring about changes in the composition of bacterial populations. Since this did not occur, the results are difficult to interpret. It would appear therefore that the presence of the oil has more to do with dictating the composition of the bacterial population than the concentration of nitrogen and phosphorous. The differences in the physical appearance of the oil layers may then reflect the stimulation of some bacterial activity which does not greatly affect the overall degradation process.

Another difference in the QB system was the appearance of two extra peaks in the low boiling range (see Figure 9). These peaks were not as intensely magnified in the normal Q system. These new peaks were again presumably produced as a result of the bacterial activities. They are interesting in that their position on the gas chromatograph indicates a low molecular weight product which was not generated by some synthesis process of linking two partially oxidized hydrocarbons together. Instead it appears to be a breakdown product or possibly a partially oxidized hydrocarbon which has a shorter retention time in a gas chromatographic column. They are certainly not peaks detectable in undegraded oil. They do however, agree in retention time to the peaks found by Horowitz et al (18).

The absence of any substantial differences in the degradation patterns of Q and QB seemed to indicate that nitrogen and/or phosphorous concentrations were not the limiting factors in the sequential degradation process. Some other particular environmental factor was presumably needed in order to get any further degree of degradation as the oil cascaded down the sequence of vessels. It is possible that the bacterial populations of the second and third vessels were not sufficient to promote any further degradation. Consequently, the nitrogen and phosphorous concentrations would impart little effect on the transformation process. There is also the further possibility that the increased inorganic nutrient concentration enhanced the degradation of metabolic endproducts such as fatty acids. These acids are undoubtably produced during the degradation and their further metabolism would drain the available nitrogen and phosphorous. Since the detection of these products was not part of our routine chemical analysis it is difficult to access their overall effects on the oil degradation process.

C. The Effects of Adherence to Surfaces on the Oil Degradation Process in Multistage Continuous Culture systems

As noted previously, surface adhesion of bacteria to some solid surface seems to affect the degradation process. This is readily seen by examination of Figures 10 and 11.

The more extensive type of degradation seen in both the connecting tubing and the vessels walls is both interesting and enlightening. It initially indicates that the adherence of oil to solid surfaces may greatly stimulate the degradation process. In addition it indicates that bacteria find this situation much more conducive to greater degradation efficiency. Since analysis of bacterial population on the culture vessel walls showed about the same colony composition as the population in the culture fluid, the extra degree of degradation does not appear to be due to the selection of a special type of bacterial population. Rather, the trapping of the oil on the walls or in the tubing places the oil in a position which makes it more susceptible to microbial attack. This situation is apparently not present when oil is suspended as small droplets in the water column.

The role of solid substrata in any microbial transformation process has been debated for some years. Many investigators believe that the attachment of organic material to particulate matter greatly increases its rate of degradation. For oil, because of its hydrophobic nature, degradation generally takes place on the outside of an oil droplet and works its way inward. However, if the oil droplet was to attach to particulate matter or to some inert surface, it may allow it to flatten out. This process would greatly increase the surface area and therefore increase the degradation rates. This would be a possible explanation for the results obtained in these experiments.

In a natural degradation situation an important question then arises; would it not in fact be better to promote the adherence of oil to particulate surfaces as a means of speeding degradation. Perhaps one of the best places to get maximum degradation would be in the sediments or on beach sand where the availability of particulate surfaces is maximized. Certainly this will depend on the availability of nitrogen and phosphorous and oxygen. The amount of work which has actually gone into investigating the effects of surfaces on oil degradation is relatively sparse and it is clear that more information need be obtained.

D. The Effects of Continuous Inoculation on the Oil Degradation Process in the Multistage Continuous Culture Systems

As noted earlier, there was very little sequential degradation in the multistage continuous culture system. It was theorized that a loss in metabolic potential was the limiting factor. However, when a multistage continuous culture system was constantly innoculated with fresh bacterial types, no substantial difference in the degradation pattern was observed.

Thus it would appear that the bacterial populations present in the continuous culture vessels were sufficient for at least the degradation obtained so far. Some other factor must then be limiting the degradation process.

VI. Conclusions

It can be concluded from the work presented herein that multistage continuous culture techniques represent an extremely useful method for studying the degradation of oil under conditions which are very similar to those found in nature. It has generated information which could not have been obtained through other methods or techniques. All experiments carried out so far tend to support the contention that the laboratory model is a good facsimile of the natural oil degradation process.

Further conclusions are as follows:

a) The rapid visual disappearance of oil from a water surface as a result of initial bacterial attack is very misleading in terms of the ultimate fate of the oil. Despite the fact that this primary bacterial attack is crucial for the initiation of the oil degradation process, the subsequent rate of degradation of the dispersed oil is very slow. It is seriously questioned whether the oil is ever completely broken down within a reasonable span of time (months to years). In these experiments partially degraded oil or oil degradation products were still readily detectable after passage through three continuous culture vessels. Furthermore, the experiments were run under conditions not found year round in a lake (such as high temperature, high nitrogen and phosphorous and high oxygen concentration). Thus, it would appear that oil can persist in natural environments for considerably longer periods of time than once thought.

It is still quite clear that nitrogen and phosphorous must be supplied in order to get any substantial degradation. The availability of these inorganic materials, either naturally or through fertilization, will in fact partially determine the capacity of the lake to handle certain degrees of oil pollution. This would mean that oil pollution in areas high in organic nutrients would probably not need to be as closely scrutinized or regulated. It also may be that certain areas of the aquatic habitat (such as the sediments) may be the ultimate place to obtain the required nitrogen and phosphorous and thereby give the fastest and most complete oil degradation.

b) The oil degradation process is not a simple breakdown mechanism with the eventual release of carbon as carbon dioxide or bacterial biomass. Degradation is instead a more complicated process. This work has shown that petroleum hydrocarbons are transformed into specific metabolic endproducts. Furthermore, it is concluded that these endproducts are actually synthesized from hydrocarbons in the oil. They are apparently of a higher molecular weight and fall within a higher boiling range than do components in the original oil. If this is true, it means that these synthesized products are possibly much more resistent to degradation than their starting material and they could be more toxic than the oil.

c) The degradation of oil may in fact be faster and more complete when oil droplets adhere to solid surfaces such as sediments, rocks or biologically derived substrata. From the continuous culture studies it appeared that oil, which has been initially attacked and dispersed by bacteria and which eventually sticks to the walls of the growth vessels, undergoes a more rapid and cpmplete degradation relative to that oil which remains suspended in the culture fluid. If this can be shown to be a consistent phenomena of the bacterial degradation processes, then it may be possible in the future to recommend that partially degraded oil actually be absorbed on to clay particles and sunk into the sediments. This type of procedure may quite satisfactorily supply the needed surface for degradation as well as a greater supply of nitrogen and phosphorous.

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Abstract

This project has dealt with the use of multistage continuous culture systems to model the fate of diesel oil in Lake Ontario. It has attempted to determine what happens to the oil after it is initially attacked by bacteria and subsequently dispersed into the water column. This study has successfully generated information which heretofore has not been obtainable in laboratory experiments. It has been shown that even under conditions which are more ideal than those in Lake Ontario (i.e. higher amounts of nitrogen and phosphorous) the oil is degraded very slowly. To date there has never been complete degradation in the systems, although it has been substantially modified by the bacterial activities.

It was discovered that the degradation of oil by bacteria does not lead to its complete destruction but instead results in a transformation process in which the oil hydrocargons are converted into various end products. The chemical nature of these endproducts is as yet unknown but they appear to be more resistant to degradation and possibly more toxic than the original oil.

Evidence is also presented indicating that oil droplets adhering to surfaces will undergo a more rapid and complete degradation than oil droplets which are freely suspended in the water column.