NEW YORK STATE ASSEMBLY

PUBLIC SERVICE LEGISLATIVE STUDIES PROGRAM

Stanley Steingut
Speaker of the Assembly

Program and Committee Staff
Assembly Scientific Staff
Dr. Seville Chapman

Sequential Continuous Culture Systems as Simulatory Models for The Fate of Oil in Aquatic Ecosystems

Joseph M. Suflita

Prepared under the direction of Dr. Parmely H. Pritchard
Associate Professor of Microbiology in the Department of Biological Sciences, State University of New York, College at Brockport, Brockport, New York 14420

This study was administered by the New York State Assembly Scientific Staff as part of its Public Service Legislative Studies Program which is supported by the Assembly, a grant from the Office of Intergovernmental Science and Research Utilization of the National Science Foundation to the New York State Assembly, and the New York Sea Grant Institute.

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Portions of the research implemented for the preparation of this report will be applied toward the partial fulfillment of requirements for a Master's Degree of Science in the Department of Biological Sciences. Parts of this report will also be directly used in the preparation of a thesis document which will ultimately be presented to this department for consideration and approval.

I heartily endorse and support the findings and research experiments presented in this report. The work Mr. Suflita has carried out under this project has been entirely under my supervision and the results he has reported have been carefully scrutinized and rigorously substantiated. I personally commend him for the contributions he has made.

Parmely H. Pritchard, Ph.D.
Associate Professor

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I. Summary

This project has dealt with the use of sequential continuous culture systems to model the fate of diesel oil in Lake Ontario. We have attempted to determine what happens to the oil after it is initially attacked by bacteria and dispersed into the water column. Our study has successfully generated information which heretofore has not been obtainable in laboratory experiments. We have 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 we have never seen complete degradation in our systems, although it has been substantially modified by the bacterial activities.

We have 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 hydrocarbons 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.

We also present evidence 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. Consequently, we have generated a predictive model for the fate of oil in freshwater ecosystems using this information and we are presently testing this model in field experiments.

II. Brief Synopsis

The primary goal of this project has been to determine the fate of oil once it is dispersed into the aquatic environment. Using the information and the culture techniques presented in a previously reported project (Ventullo and Pritchard, 1975), we have developed and tested a sequential continuous culture system designed to resemble the natural dispersion of oil in a water column and its subsequent attack by bacteria. These culture systems consist of three different sized continuous flow vessels connected in series so that the degradation products of one vessel serves as the nutrient source for the succeeding vessel. In this way the large deletion capacity of an aquatic body of water is simulated.

By observing the physical and chemical changes in a light diesel oil as it is attacked by bacteria, we have been able to characterize to a certain degree, the fate of the oil relative to bacterial transformations. Several important generalizations have been derived from this work:

a) As the oil in the primary vessel was initially attacked by hydrocarbon degrading bacteria, small masses of bacterially impregnated oil droplets flaked off the oil layer (equivalent to a stagnant oil slick) and passed through our sequential continuous culture systems. As a result of its passage through the subsequent two vessels, the oil undergoes an extensive degradation of the normal alkane fraction (the most predominant type of

hydrocarbon in the diesel oil used in these experiments) and the branched alkane/aromatic fraction. The degradation of this latter fraction has not been observed by other types of laboratory experiments despite the fact that it is known to occur in the natural environment. Its observation in our experimental system indicates a functional similarity to the natural aquatic situation. However, in our experiments the complete degradation of all fractions of the oil has not been observed.

b) Our results have shown that while degradation was occurring bacterial activities resulted in the appearance of gas chromatographable components which appeared to be synthetized or derived from the breakdown products of the oil. Two types of synthesis have been observed: one involving the production of high molecular weight and high boiling alkanes which were not originally present in the oil and a second, involving the production of compounds corresponding to high molecular weight aromatics and branched alkanes, that are also not found in the undegraded oil. These synthetic processes may be responsible for the generation of organic materials which are more recalcitrant and perhaps more toxic than the oil itself.

c) Degradation of oil in our sequential continuous culture systems under elevated nitrogen and phosphorous concentrations did not accelerate the degradation process or significantly alter its pattern. However, it did stimulate the synthesis phenomena resulting in more types and greater amounts of the degradation end products.

d) The operation of our sequential continuous culutre systems under a constant input of unsterilized Lake water (i.e. the constant addition of fresh bacterial populations) did not significantly alter the degradation pattern, thus indicating that our system closely resembles the natural bacterial activities occurring in a lake. Likewise, an increase in surface area and/or an increase in the volume of the vessels did not alter the degradation pattern observed.

e) Our analysis of oil adhering to the glass tubing and the walls of the culture vessels has shown that this type of sequestering increases the rate of degradation and may therefore play a major role in determining the fate of oil in aquatic environments.

III. Introduction

Lake Ontario, like many other large bodies of water, is beset with a burgeoning onslaught of organic pollutants from a large variety of sources. For example, petroleum hydrocarbons from motorized vessels, spillages and bilge discharges, pesticides from agricultural run off of fruit and vegetable farms, organic solvents and chemical processing wastes from industrial operations in cities like Buffalo, Hamilton, Toronto, Rochester, and sewage from a large number of municipalities all represent significant organic loadings. Many of the pollutants would be classed as readily biodegradable while others would fall into the "recalcitrant" category meaning very slow or non-existant biodegradation.

The input of these organic materials, is of course regulated by government agencies, local public officials and state and federal legislation. As our experience with environmental pollution will atest, however, the regulatory processes are difficult to maintain at 100% efficiency due to economic considerations, time constraints, and a pavicity of the proper information. As a result, one is faced with the realization that many of these pollutants will still find their way into Lake Ontario and other bodies of water. Thus, these aquatic environments serve, intentionally or unintentionally, as an apparent bottomless repository. Lake Ontario, in this sense, can therefore be considered as a natural resource in that it is a sink which can accept and ultimately do away with organic pollutants up to a certain limit without any obvious adverse effects on the environment itself. The central question. of course, is where does this limit lie. In the case of DDT, which represents a classic recalcitrant organic material, the limit was very low because of the concentration effect that occurred at the upper end of the food chain. Unfortunately, with this particular chlorinated hydrocarbon, the amount that any aquatic environment could accept was determined only after it had reached proportions which, in many situations, irreversibly damaged the aquatic ecosystem. How many more chemical compounds like DDT are we going to belatedly realize have passed the threshold acceptance level of the particular ecosystem involved? Probably quite a few since we in fact know relatively little about their actual fate once they are introduced into the environment.

Many factors, of course, will affect this threshold acceptance level but one of the more crucial factors will be the degradative abilities of bacteria. If the particular pollutants are rapidly turned over or readily transformed into innocuous products, the acceptance limit is going to be much higher, simply because the pollutant will not accumulate. If these transformations do occur, then it is essential to know the mechanisms involved and how they are affected by environmental parameter. Such knowledge can then

be used to evaluate a particular aquatic environment for its resource potential for each particular type of pollutant. The degree to which the environment can cope with its pollutant load will very critically determine the extent and magnitude of the regulatory policies that have to be implemented and legislated.

It is our contention that the processes of bacterial degradation play a preeminant role in determining the fate of many organic pollutants especially oil pollution. We feel very strongly however, that there is a great lack of knowledge in regard to these natural bacterial activities and it is in fact very short sited not to consider their potential when making decisions about the regulation of the input of organic pollutants into an aquatic body of water. One must examine the problem from both sides: if there exists sound and efficient degradation activities for a particular pollutant, there is no reason not to take full advantage of this potential. On the other hand if sufficient degradative activities do not exist, considerable restraint must be exercised.

We have concentrated our efforts over the past two years on attempting to determine the degradative potential for oil and petroleum hydrocarbons in Lake Ontario and we have been focusing our efforts on generating enough information so that we can make some reasonable predictions about the fate of oil in aquatic ecosystems. Because of the particular cultural techniques which have been used in the past to show that oil can be degraded by bacteria isolated from Lake Ontario and other freshwater and marine environments, there is no assurance that this same type of degradation will occur in the natural environment. We have therefore concentrated on developing a laboratory model of the oil degradation process which may occur in Lake Ontario such that we might be able to monitor the fate of oil and subsequently determine the environmental factors which impinge on its fate. As the results presented in this report will atest, we feel that sequential continuous culture systems provide just such a model and we feel that the information we have generated can be reliably used to make many predictions and generalizations about the fate of polluting oil in Lake Ontario. At the very least we have raised questions about the oil degradation process which have not been provoked by other laboratory or field studies and which demand answers before sensible decisions can be made about the handling of oil pollution and oil pollution abatement problems.

IV. Materials and Methods

Most of the materials and methods used in this study have been previously reported (Ventullo and Pritchard, Final Report to New York State Assembly Scientific Staff, 1975).

The type of sequential continuous culture system used in this study is shown in Figure 1. Three vessels were normally employed using a volume ratio of 1:3:9. As can be seen the oil layer serves as the carbon and energy source for the first vessel. The reservoir contained sterile distilled water and phosphate, ammonium nitrogen and magnesium chloride salts as described previously. Its contents were always pumped into the first vessel at a dilution rate = 0.05 hr^{-1}. 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.

A typical sequential continuous culture experiment was commenced by adding a 200 ml sample of fresh Lake Ontario water to the first vessel and then incubating it as a batch culture for 15 hours with a 2mm 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 of the bacteria growing in the second and third vessels came from the original 200 ml inoculum in the first vessel.

The sampling schedule used was the same as the one previously described. For oil analysis, 200 mls of culture fluid was removed from either the second or third vessel and replaced with 200 mls of sterile water.

In the sequential continuous culture systems which were continuously inoculated, 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 in at a dilution rate of 0.025 hr^{-1}. The reservoir containing the inorganic nutrients was also pumped in but at one half the normal dilution rate, i.e. 0.025 hr^{-1}. The concentration of the inorganic nutrients was therefore doubled to maintain similar conditions.

The analysis of oil adhered to the walls of the vessels and the connecting tubing was carried out in the following manner. To remove material from the vessel walls, the contents of each vessel was gently poured into sterile flasks and any material left on the walls was scraped off with a rubber policeman. Sterile water was used to wash the loosened material off the walls and the suspension

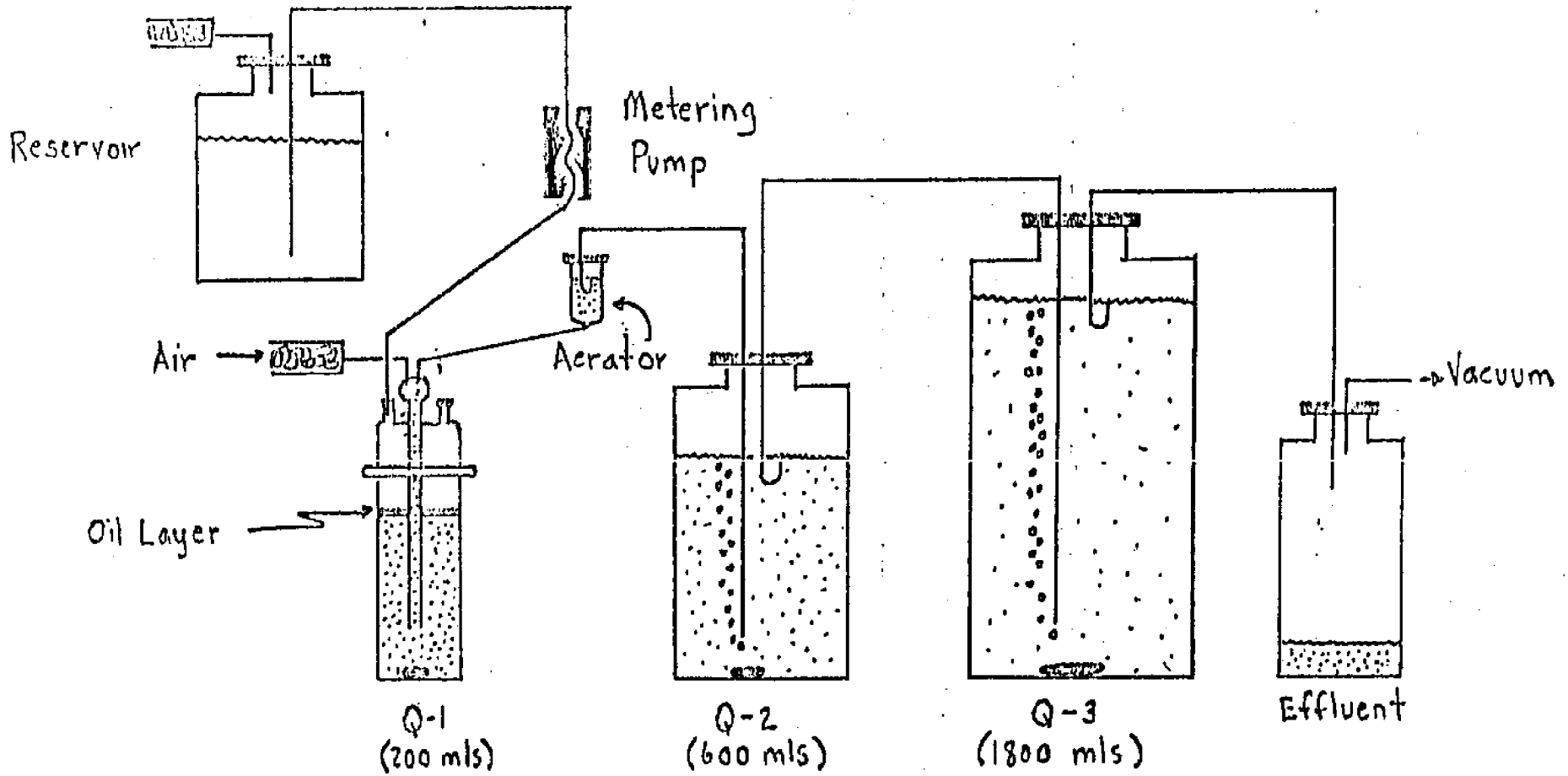


Figure 1 - Sequential Continuous Culture System Used in Oil Degradation Studies

was poured into a sterile tube. This suspension was 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 benzene-pentane solvent through the tubing. This was done by removing the tubing and replacing it with sterile tubing.

V. Results and Discussion

A. The General Pattern of Degradation Sequential Continuous Culture Systems

From the experiments described in our previous report (Ventullo and Pritchard, Final Report to New York State Assembly Scientific Staff, 1975) it was clear that continuous culture techniques could be used to follow oil degradation. Our results indicated that considerable amounts of environmentally meaningful information could be generated and subsequently employed as a basis for decision making processes and as a basis for environmental impact policies at all levels of government.

However, our results at that time really only represented the primary stages of degradation and did not reflect on the subsequent phases of degradation which would naturally occur throughout the water column. In this primary stage we were undoubtedly seeing a process very similar to the initial attack on an oil slick by bacteria in which the oil is emulsified and dispersed and only partially degraded. What happens to this partially degraded oil once it is dispersed is entirely unknown. In fact, of those oil degradation studies which have been reported in the literature (2, 3, 5, 6, 7, 8, 9), very few have reported detecting substantial degradation after the primary attack and most reports have failed to demonstrate the complete degradation of oil under either laboratory or field conditions (2, 6).

With continuous culture techniques, we have the unique capability of monitoring the secondary and tertiary phases of degradation which normally would be difficult to observe in a natural situation. This can be accomplished thru the use of sequential continuous culture systems in which the effluent of one vessel (carrying partially degraded oil and other degradation products) acts as the substrate or nutrient source for a second larger vessel connected series to the first. This model system as we have developed it, accomodates a series of three continuous culture vessels with the volume of each vessel being three times that of the vessel preceeding it. A schematic diagram of this system is shown in Figure 1.

Our sequential 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. Our sequential continuous culture system possess both of these properties. Second, during our current studies with breakdown of oil in continuous culture, we have observed that much of the oil, in a partially degraded form, flakes-off from the original oil layer and washes out in the effluent. If this effluent is now fed into

a second continuous culture vessel, its continued secondary degradation can be monitored and its ultimate fate more closely observed. Similarly, the sequence can be continued with a third vessel.

Third, the mechanism of degradation designated "co-metabolism" can be readily detected and evaluated as to its contribution to the ultimate fate of the oil. Conditions in the secondary and tertiary phases of degradation should be ideal for the "co-metabolism".

Fourth, the sequential continuous culture system is highly amenable to studies involving the effect of environmental factors on the degradation process. Factors such as pH, temperature, organic matter, dilution rates, inorganic particles, nutrient concentrations, detergents, seeding, etc. can be readily tested for their effects at all levels of degradation.

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

As would be expected, the physical changes in the first vessel were very similar to what we had previously observed. Within 4-7 days after inoculation of the first vessel (see materials and methods) heavy bacterial growth (as indicated by turbidity) occurred in the second and third vessels. Some of this turbidity was due to cells washing out from the first vessel but this only accounted for about one half of the turbidity observed.

Accompanying this 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, but it is an extremely consistent event accompanying the initial events of most of our degradation experiments. Analysis of the bacterial populations did reveal yellow pigmented colonies, but these bacteria did not elaborate the pigment into the medium nor do they represent a significant percent of the mixed bacterial population. The yellow color, although present in the primary vessel, was not nearly as intense.

As incubation continued (6-10 days) the turbidity in the second and third vessel decreased. The decrease occurred in a sequential manner with the third vessel being the last one 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 when considerable flaking of bacterial-oil masses off the oil layer occurred.

When there wasn't substantial flaking occuring 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 which might have been expected due to washover from the first vessel. Bacterially impregnated oil droplets did however 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 vessel were vigorously aerated and stirred, yet this material accumulated 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 amorphorous material.

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

Analysis of the bacterial populations in each of the three sequential vessels using standard plate count techniques revealed two characteristic patterns. First, as we have reported for oil degradation with just a single vessel, predominant colony types appeared within the rather heterogeneous populations in each vessel. These predominant types varied from 40-90% of the total population at various times throughout any experiment and in most cases they did not persist for more than 2-3 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 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 and 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 seen were usually the result of a change over in predominant species which was eventually identically reflected in all three vessels.

As with many of the plate count assays, there is a diverse background of minor colonial types, which for practicality and efficiency 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.

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

Our original contention for the use of sequential continuous culture systems was that it offered a means of following the degradation of oil subsequent to the initial attack by bacteria. We reasoned that once the oil was dispersed in the water column as small partially degraded droplets, continued degradation would occur because of the renewal of nutrients to the adhering bacteria. Our sequential continuous culture system models this process and thus by following chemical changes in the oil with gas chromatography, the actual degradation steps could be pinpointed.

Sequential systems were set up and sampled as described in the materials and methods section. Gas chromatographic analysis of the samples from a typical sequential experiment are shown in Figures 2-7. All sequential experiments were designated by the symbol Q and the vessels are numbered accordingly. Samples from Q-1 represent material from the oil layer and not the culture fluid below the oil layer. All other samples (Q-2, Q-3, Q-effluent) are taken from the culture fluid.

As indicated below, we have attempted to express our results as a function of the incubation time primarily because this seems to be the simplest method at present. However, many of the results we have obtained do not fit into a nice continuous time period and it therefore becomes difficult to relate this information around a progression-type of theme. Much of this problem originates from the nature of our 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 out into the second and third vessels. The inconsistency of this process is obvious and thus 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 thus, of course, superficially upsetting any abstract degradation pattern.

None the less, it is obvious from an overview of data relating to bacterially induced chemical changes that a progression of degradation can be detected whether it be from vessel to vessel or 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, eventually appearing in significantly larger concentrations than undegraded oil. This would result, in part, in the progressive type of degradation that we have observed.

Thus, the chemical changes in oil have been broken down into general time equalivancy stages - early, middle and late - but 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 and the events therein which we mean to emphasize.

1. Early Stages of Degradation

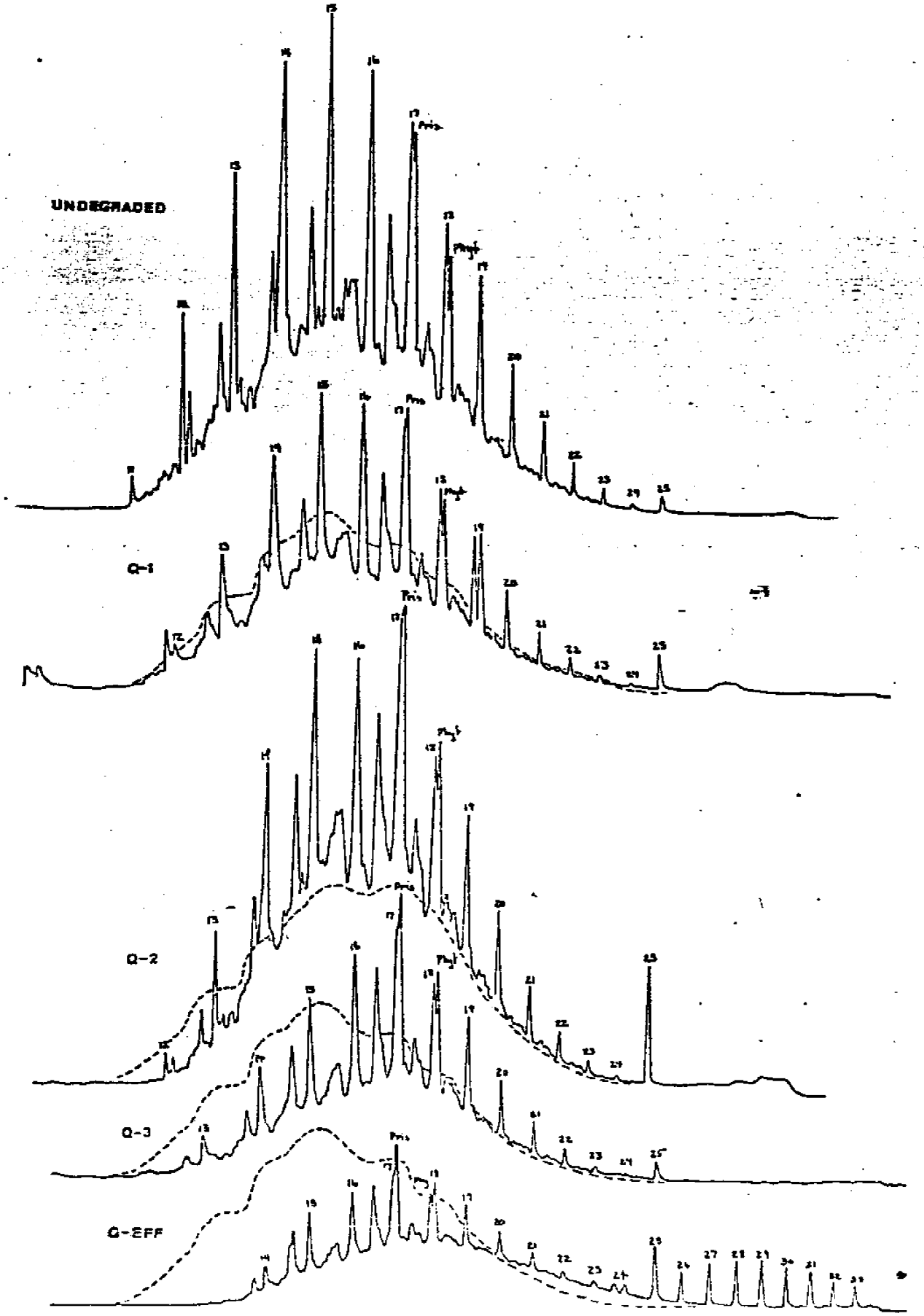
This stage corresponds to a time period of approximately 100 to 600 hours of incubation. It would normally correspond to the events in the physical changes of the oil layer through the formation of a thick film of bacterial growth under the oil and the initial impregnation of the oil layer with bacteria.

The major chemical change of this stage was a significant decrease (presumedly by bacterial degradation) in the major n-alkane peaks. Figure 2 is a composite of gas chromatographs from each of the three vessels (Q-1, Q-2, Q-3) and the effluent from the third vessel (Q-eff) which are representative of this early stage of degradation (about 300 hrs incubation). The ratios of C-17 n-alkane to pristane and C-18 n-alkane to phytane typically decreased by 5-10% in Q-1 (actually the oil layer) and by 10-25% in Q-2, Q-3 and Q-effluent. The C-20 to envelop ratio typically decreased by 15-20% in the first vessel (Q-1) and 20-30% in the other vessels. Also at this stage, the peaks corresponding to C-11 and C-12 n-alkanes, which were detectable in undegraded oil, were almost completely missing. This reflects the general trend of relatively early preferential degradation of the low molecular weight alkanes.

Also apparent during this early state of degradation was the alteration of the envelope 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 envelope as it will be called, encompasses most of the branched alkanes, the cyclic alkanes and the aromatic hydrocarbons found in the diesel oil. These envelope components are apparently not resolvable because of their great variance in molecular structure and their relative concentration. In terms of the degradation pattern observed within the envelope components it was important to study the shape of the envelope profile. The actual size or height of the envelope 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 envelope components except to visually compare the shape of the envelope profile with that from undegraded oil (indicated as dotted line on the figures). This has been done for gas chromatographs in Figure 2 and it was clear that substantial alteration of the envelope profile shape had occurred on the left hand side which corresponds to the lower boiling alkanes and aromatics. This

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



change was particularly noticeable in the latter vessels of the sequential continuous culture system during this early stage of degradation. In figure 2, for example, it can be seen that in Q-eff and to a lesser extent in Q-3, 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 populations present.

It should also be emphasized at this early stage of degradation that there was some evidence for successive degrees of degradation as the oil cascaded down the chain of vessels. For example, samples from Q-1 (i.e. the oil layer) showed relatively little degradation (about a 7% change in the pristane and phytane ratios) and an insignificant alteration of the envelop profile, whereas the effluent samples (Q-eff) showed substantial degradation (about a 23% drop in the pristane and phytane ratios) even in the envelop components. In some analyses no signs of successive degrees of degradation were apparent (except relative to the oil layer) and in many cases the Q-eff samples showed only minimal differences.

The most surprising aspect of the sequential degradation process was the sudden appearance of gas chromatographic peaks corresponding to normal alkanes of carbon length C-26 to C-34 (see Figure 2 Q-eff). These peaks never detected in undegraded oil and all appropriate control experiments have indicated that they resulted from bacteria activities occurring 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 in that all eight peaks invariably appeared as a group at about the same relative concentrations. Occasionally there was some variation in the last peaks; in some samples the peak corresponding to a C-34 alkane would be missing, especially in the early stages of degradation.

Our present interpretation is that these extra peaks have been synthesized by bacteria involved in the degradation process. They are most likely some type of metabolic end product, and although we have no evidence for their exact chemical nature, we suspect that they are in fact normal alkanes.

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 end product, and it again corresponded most closely with an n-alkane. In Figure 2 in the Q-effluent there was a decided enrichment in the peak corresponding to C-25 alkane. This enrichment phenomena generally occurred hand in hand with the synthesis of the extra peaks but there were examples where the C-25 peak was enriched independently 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 hours of incubation) but they 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 Q-3 for example the pristane-phytane ratios have changed by 20-25% relative to undegraded oil and the C-20/envelop ratio has changed by 40%. The peak corresponding to a C-13 normal alkane was gone and the peaks for C-14-16 were all greatly reduced. The envelop profile was also substantially modified particularly at the end corresponding to the low molecular weight, low boiling fraction.

There was no indication of any synthesis of the extra peaks except in the effluent samples. However, the enrichment phenomena was considerably magnified. In Q-3 there was a tremendous increase in the peak co-chromatographing with C-25 normal alkane. This extensive enrichment was observed numerous times and generally appeared farther up the chain of vessel as the incubation period increased. Note, also that no other enrichments appeared, thus indicating a very specific metabolic process taking place. In Q-eff the extent of enrichment of the C-25 peak has been reduced but now C-23 and C-24 have also been enriched.

And lastly, 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 since the envelop profile in Q-3 extended 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 which are hydrocarbon in nature and of a higher molecular weight than the original substrate. As will be seen this process was considerably magnified in the latter stages of degradation.

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 consisted of masses of oil droplets and bacteria and it 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 compared with analyses without wall growth (see Figure 3) was obtained. This is shown in Figure 4. It can be seen that the peak synthesis

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

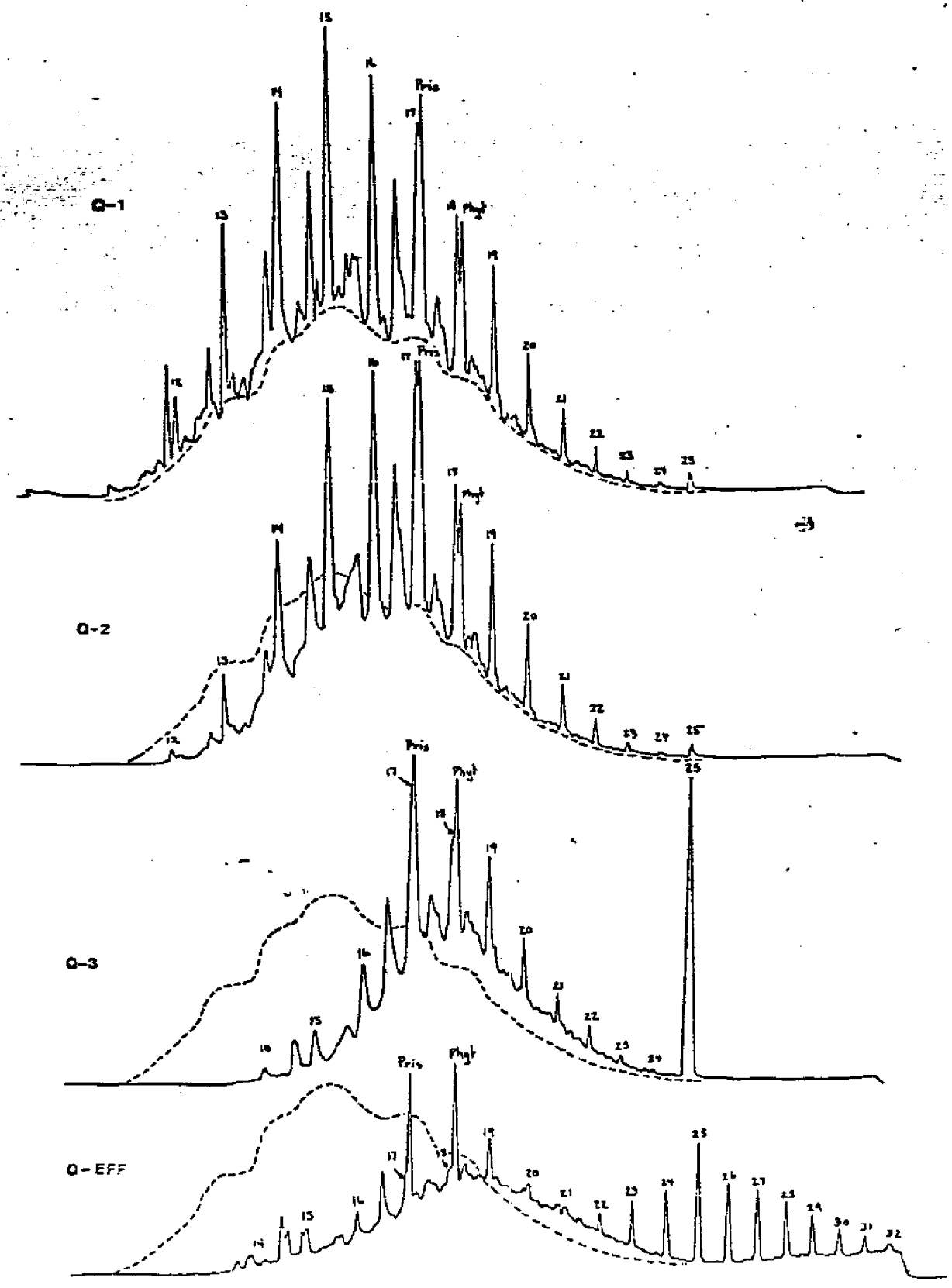
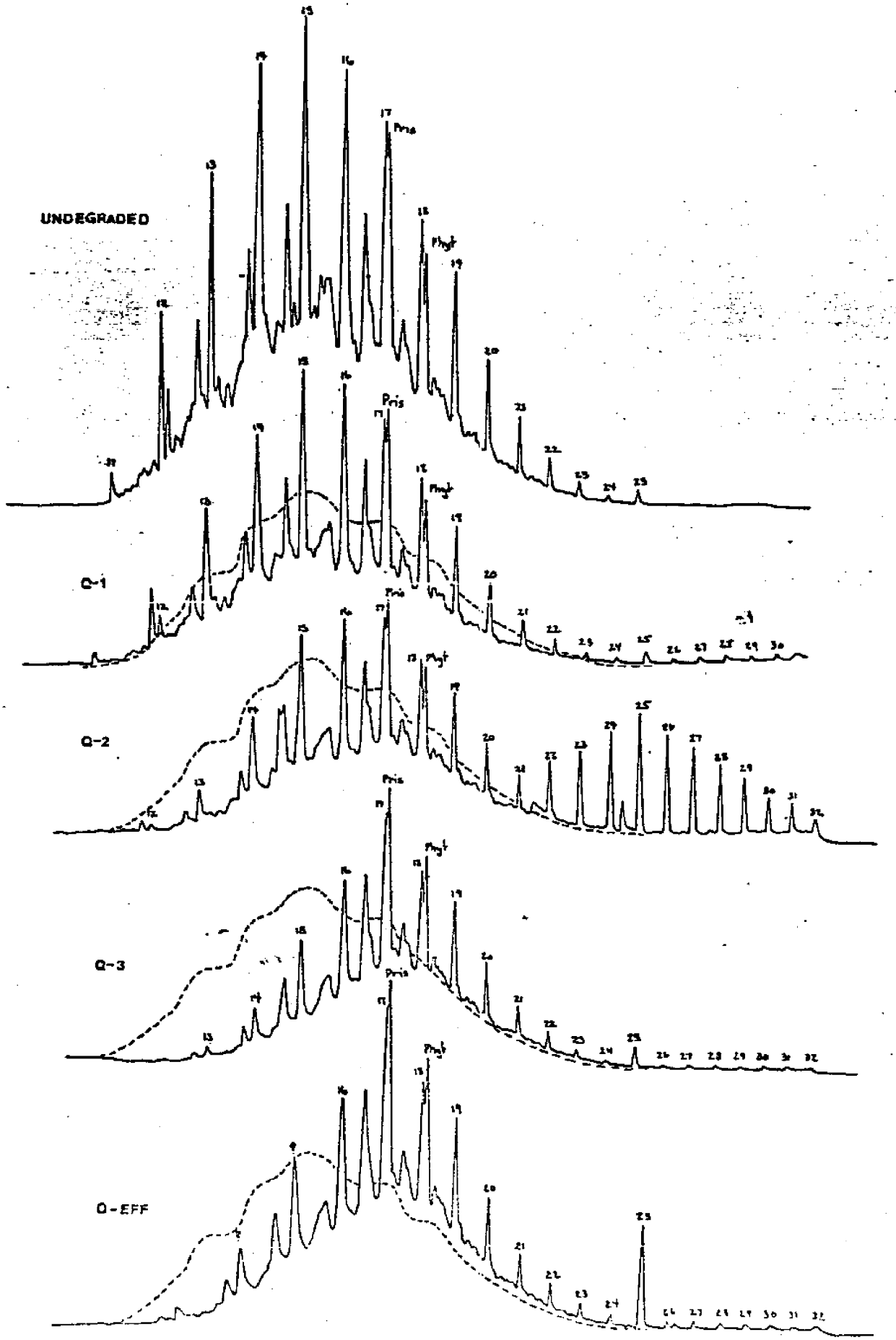


Figure 4. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluid and the Wall Growth of Sequential Continuous Culture Vessels During Early Stages of Degradation (total incubation period equals 700 hours).



and enrichment occurs at all stages of the sequential continuous culture system. In this particular experiment, the amount of enrichment and synthesis was greatest in Q-2 but it was definitely detectable in all the other vessels, including the first vessel. It should be reemphasized that the chemical nature of these peaks is still unknown but that by their behavior during gas chromatography, it would appear that they are high boiling normal alkanes. Thus the phenomena of peak synthesis and enrichment occurs at all stages of the sequential continuous culture system and it can be detected in oil adhered to the sides of the vessels before it it seen in the culture field.

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 arbitary. However, a number of significant degradation events can be used to generally 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 C-12 to C-19 were either completely gone or extensively degraded to the point of being barely detectable. The branched alkanes, pristane and phytane, still stand out in most cases but they too were extensively degraded.

b. Almost 75% of the components comprising the envelop profile have been degraded or have at least disappeared from the chromatographic profile. This removal was again confined primarily to the low boiling fraction but has now 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 C-26 through C-34 were now present in all three vessels (Q-2, Q-3, and Q-eff) but again were not seen in the relatively undegraded oil still present in the oil layer (vessel Q-1). There was also peak enrichment in all three vessels; components corresponding to C-22 thru C-25 were generally increased relative to undegraded oil and C-25 stood out as being 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 but instead appears to be the generation of metabolic endproducts which are unresolvable as a group on the gas chromatograph and which 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 possible surmise that they were aromatics and branched alkanes.

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

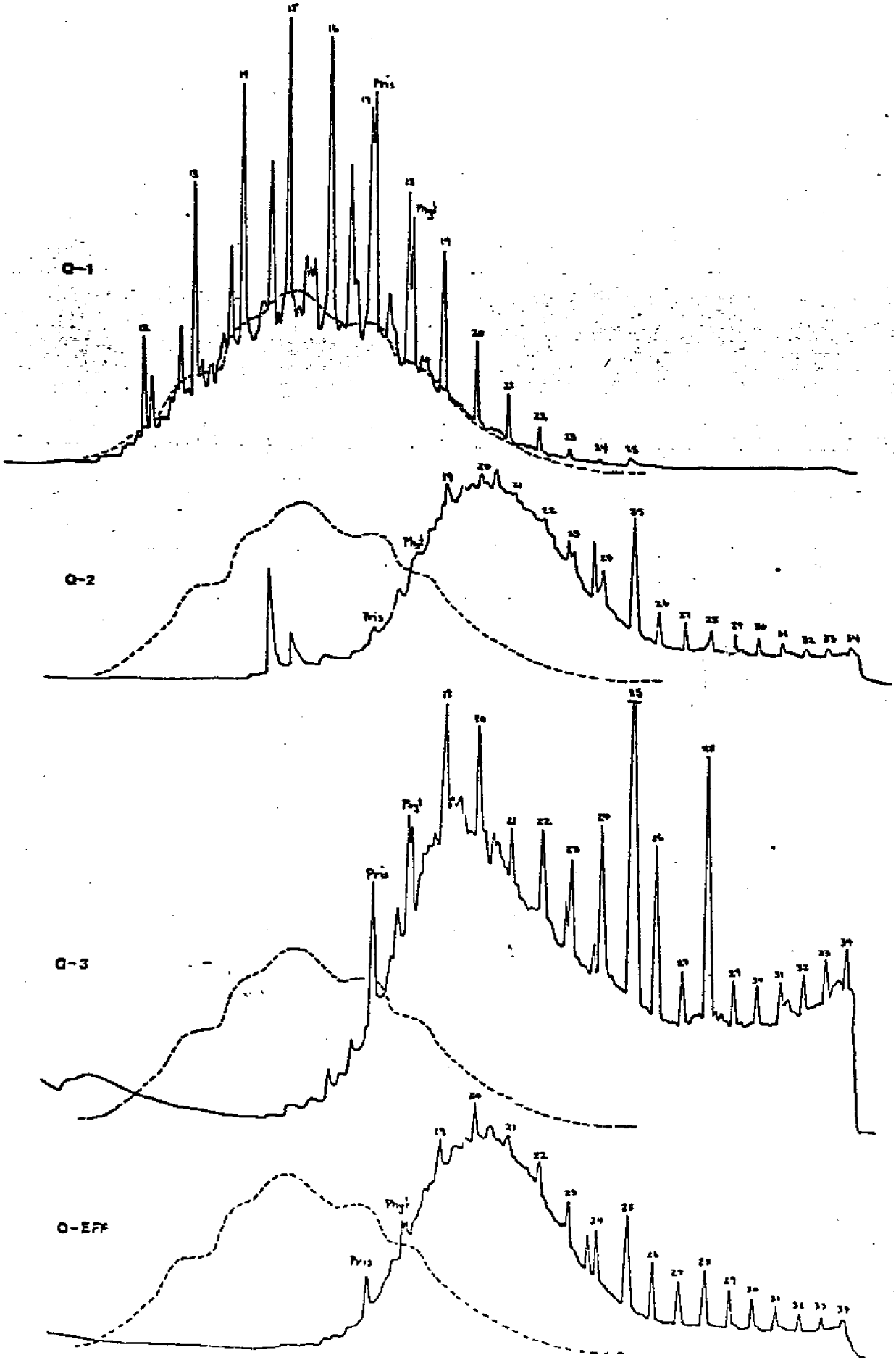
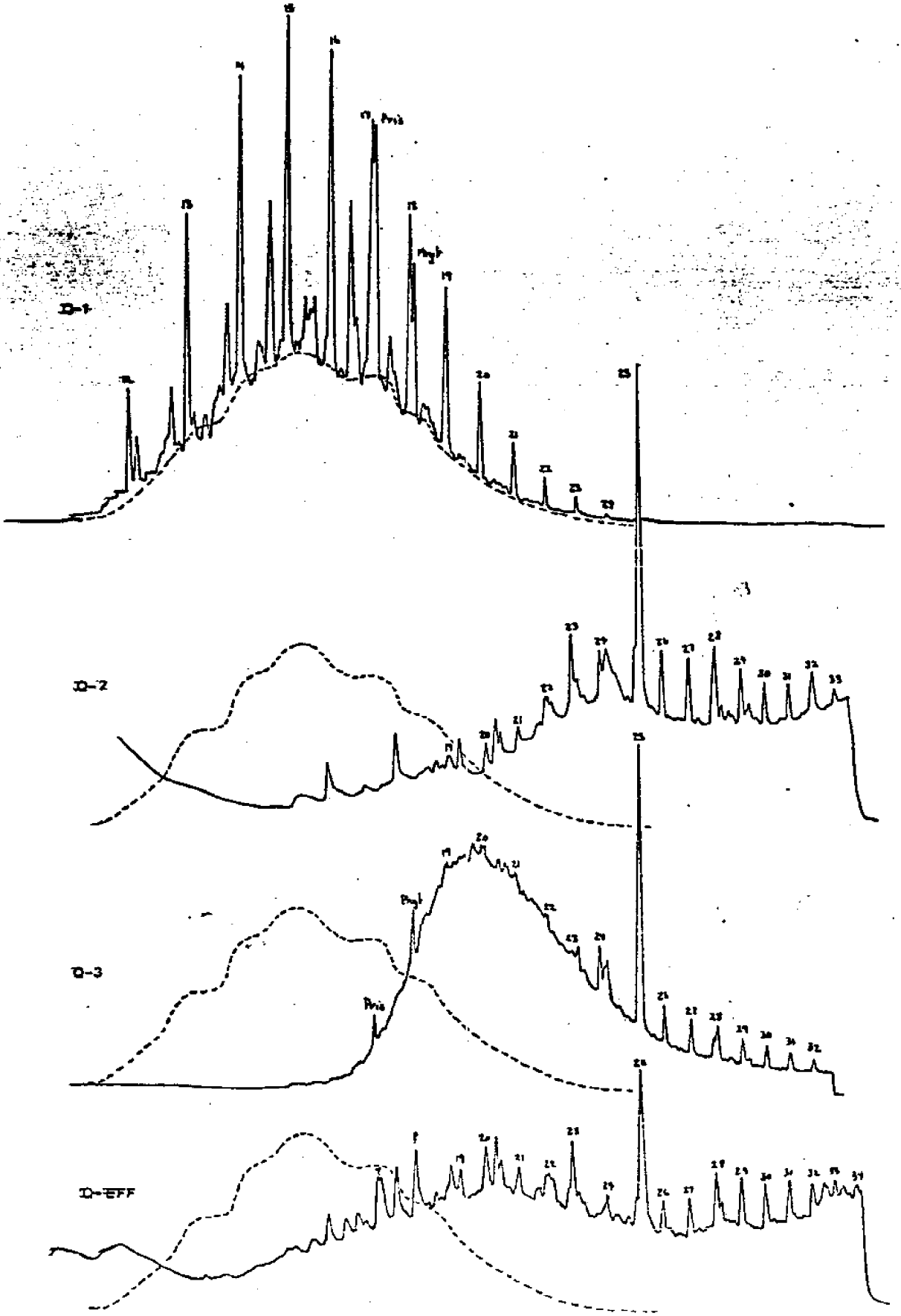


Figure 6. Gas Chromatographic Profiles of Diesel Oil Extracted From the Culture Fluids of Sequential Continuous Culture Vessels During Early Stages of Degradation (total incubation period equals 1500 hours).



e. There was relatively little difference in the extent of degradation from one vessel to the next. All of the gas chromatographic profile data indicate only minor differences in Q-2, Q-3 and Q-eff. Many of the differences can be attributed to sample size while a few others appear to be legitimate occurrences of new peaks. On the whole, there actually was little difference in gas-liquid chromatographic profiles from time period to time period during this middle phase of degradation. On the other hand, in some analyses there was also gas chromatographic profiles which differed drastically from typical results normally seen. An example is shown in Figure 6. In this particular analysis it appeared that most of the synthesis of new hydrocarbon components, both unresolved in the envelop and projecting out of the new envelop, was in the extreme high boiling range. This was not pronounced in Q-2 and Q-eff in Figure 7, and it would further indicate that even though many extra metabolic endproducts are being produced, these too eventually undergo degradation in our sequential systems. However, close examination does show that many of the peaks projecting out of the envelop do not correspond to a known hydrocarbon peak and thus they were possible synthesized by bacterial activities or simply enriched because many of the other components were being removed.

iii. Late Stages of Degradation

This corresponds approximately to the point where the oil in the first vessel has been significantly decreased in volume. The oil is now a brownish, 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, significant modification of the oil in the oil layer (Q-1) is apparent presumedly because the amount of undegraded oil was sufficient to dilute out any degraded oil. This degraded oil reflected the same general degradation observed previously, i.e. removal of n-alkanes and envelop components, synthesis and enrichment of alkane-like peaks and a shifting of the envelop profile into the high boiling range. The branched alkanes, pristane and phytane are still quite prominant and have not undergone the extent of degradation seen in the next vessels in the chain.

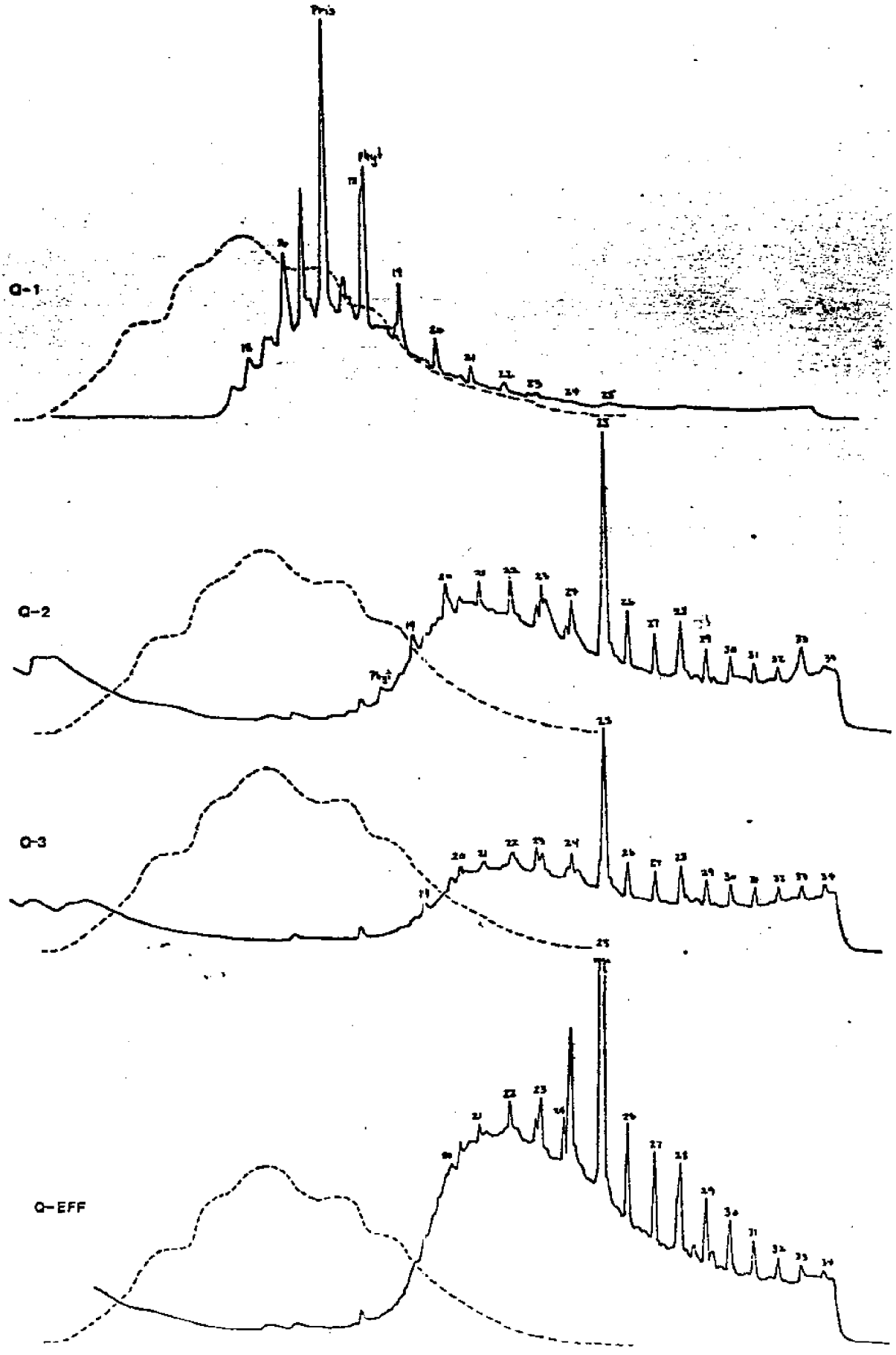
The other vessels all showed a general decrease in the remaining components. The peak corresponding to C-25 alkane, however, continues to be produced in large quantities. In most cases hydrocarbons up to C-20 in carbon length are gone, even the pristane and phytane.

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

iv. Discussion

The use of sequential continuous culture systems does appear to be an important way to study the degradation of oil in aquatic ecosystems. We have shown in these results particular aspects of the oil degradation process which have heretofor never been reported in

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



the literature (1) and it is doubtful that they could have been seen by any other method except in a continuous culture system. It would appear that this present system could be extremely useful in determining the fate of any organic pollutant in aquatic ecosystems especially in accessing the role of environmental factors.

In most oil degradation studies to date, the degradation pattern most often observed is a relatively rapid removal of the easily degraded normal alkane fraction leaving behind a fraction of unresolvable hydrocarbons consisting of a large variety of branched and cyclic alkanes and aromatics hydrocarbons (2, 3, 6, 8, 9). These studies, which in most cases involved batch culture experiments, seldom show any attack on the fractions other than the n-alkanes. Our results on the other hand, show a considerably different pattern of degradation. We have not only shown the typical n-alkane attack but we have also shown a concominant attack on the branched alkanes and aromatic compounds starting with the lower boiling components. This has led us to the general conclusion that if the proper conditions are present, the branched alkanes and aromatics are not nearly as recalcitrant as once thought. In a natural degradation process there is not as severe a preferential attack on the n-alkanes as so many laboratory experiments had once indicated.

The type of oil degradation pattern in our continuous culture systems has proven to be very convenient. In our first vessel the oil layer was attacked by numerous types of hydrocarbon degrading bacteria. This lead 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 and presumedly as these droplets move throughout the water column, the adhering bacteria would slowly degrade the oil from the outside in as inorganic nutrient sources were replenished. This appears to be the exact process we are observing in our continuous culture systems except that we are preventing a rapid mechanical dispersal of the oil. Instead the small droplets of oil with their adhering bacteria were partially retained in the oil layer in the first vessel 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. We thus have the capability of observing the fate of emulsified, bacterially impregnated oil droplets as they are dispersed throughout our sequential continuous culture system, much in the same manner as they would be in a natural aquatic environment. It is also important that we again emphasize the importance of the initial attachment and impregnation of the oil by bacteria in order to initiate any type of speedy degradation. We strongly feel that if oil is mechanically or chemically dispersed before bacteria have had a chance to attack, the whole degradation process is greatly slowed down. This is simply because the individual bacteria must

first collide with the oil droplet and then must begin to grow and divide in order to spread throughout the droplet and commence significant degradation.

The type of oil degradation we have observed in our sequential continuous culture systems is unique. Not only was the original oil degraded but new types of gas chromatographable materials were generated as a result of 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. Our basic conclusions at the present time is that these extra materials are degradation products of the oil itself which may be produced directly from the oil or produced and subsequently transformed by bacterial action 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, we seriously suspect that they are actually hydrocarbons of a chemical structure not represented 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 resulted in the production of essentially more recalcitrant materials. Since we also see the appearance of extra materials corresponding to unresolvable branched and cyclic alkanes and aromatic hydrocarbons (i.e. the envelope profile) it is entirely possible that more toxic or even carcinogenic compounds could be among the products. This insinuation can only be substantiated with further chemical analysis.

As to why these extra materials are produced, it is not at all clear. It could represent a unique type of enzymatic attack which resulted in more of a transformation than an actual degradation. This type of mechanism is not commonly observed in most degradation studies (2, 5, 6, 7, 8). 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 as reported by Stewart and Kallio (9). Whether we are observing ester formation in our 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 thus leading to the formation of normal alkanes of considerably higher molecular weight than the original materials.

Another question which arises, is why hasn't the production of these extra materials ever been detected before in oil degradation studies. The only explanation that we can give is the following. In laboratory experiments, we feel that batch culture experiments lead to such a severe selection of a relatively small number of hydrocarbon oxidizing bacteria of rather low metabolic diversity that 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.

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

The degradation of oil by bacteria that we have demonstrated in the work sighted above is actually an accelerated process relative to natural conditions. This is because the amount of nitrogen and phosphorous added to our systems is about 10-100 times greater than that found naturally in Lake Ontario. However, as partially degraded oil passes thru our sequential 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 sequential system (designated as QB) was set up in which the nitrogen and phosphorous concentrations were doubled (200 mg/l potassium phosphate, 50 mg/l ammonium chloride).

Visual changes during the degradation 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 and any major changes in turbidity, color, or flaking occurred at about the same time in each vessel. The bacterial populations showed the same predominant species and about the same degree of heterogenity.

The only major visual difference between the QB system and the normal Q system was observed during the latter stages of degradation when it had 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. However, the oil layer in the QB system was dark brown to grey in color whereas the Q 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.

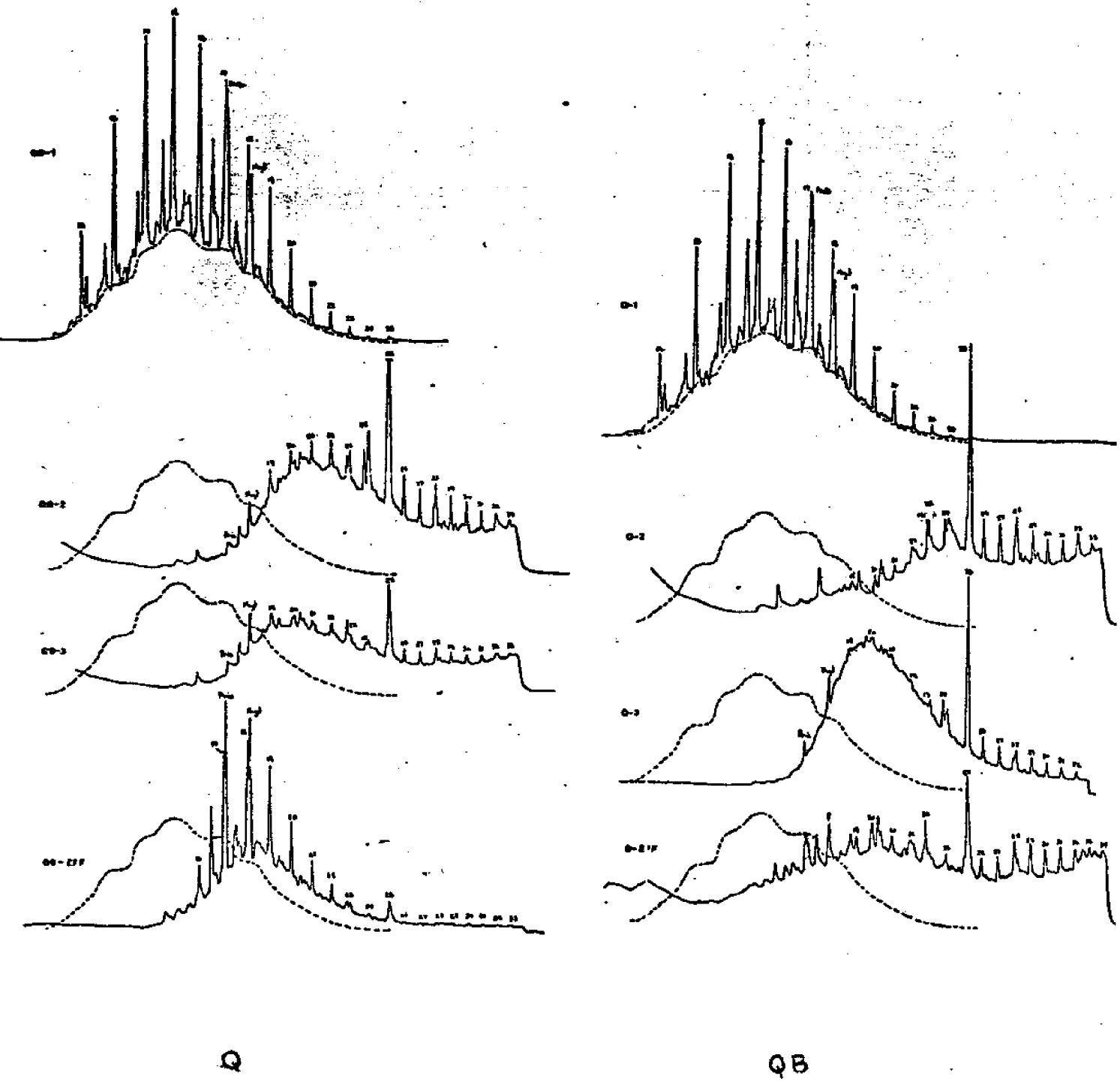
At present, it is difficult to account for these differences in the physical appearances of these systems since analysis of other parameters do not necessarily reflect these differences. For example, the pattern of colony morphologies resulting from analysis of the bacterial populations was strikingly similar. Since it is known from other work that changes in the nitrogen and phosphorous concentrations bring about changes in the composition of bacteria populations our 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 just reflect the stimulation of some bacterial activity which does not greatly effect the overall degradation process.

An examination of the gas chromatographic profiles of oil extracted from the QB system (Figure 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 and 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 which represents the analysis of a sample taken during the middle stages of degradation (1200-1500 hours, 50-60 days incubation). Most pronounced in this particular analysis is the increased size of the extra envelop profile which presumedly had 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. These new peaks were again presumedly 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 in our column. They are certainly not peaks detectable in undegraded oil.

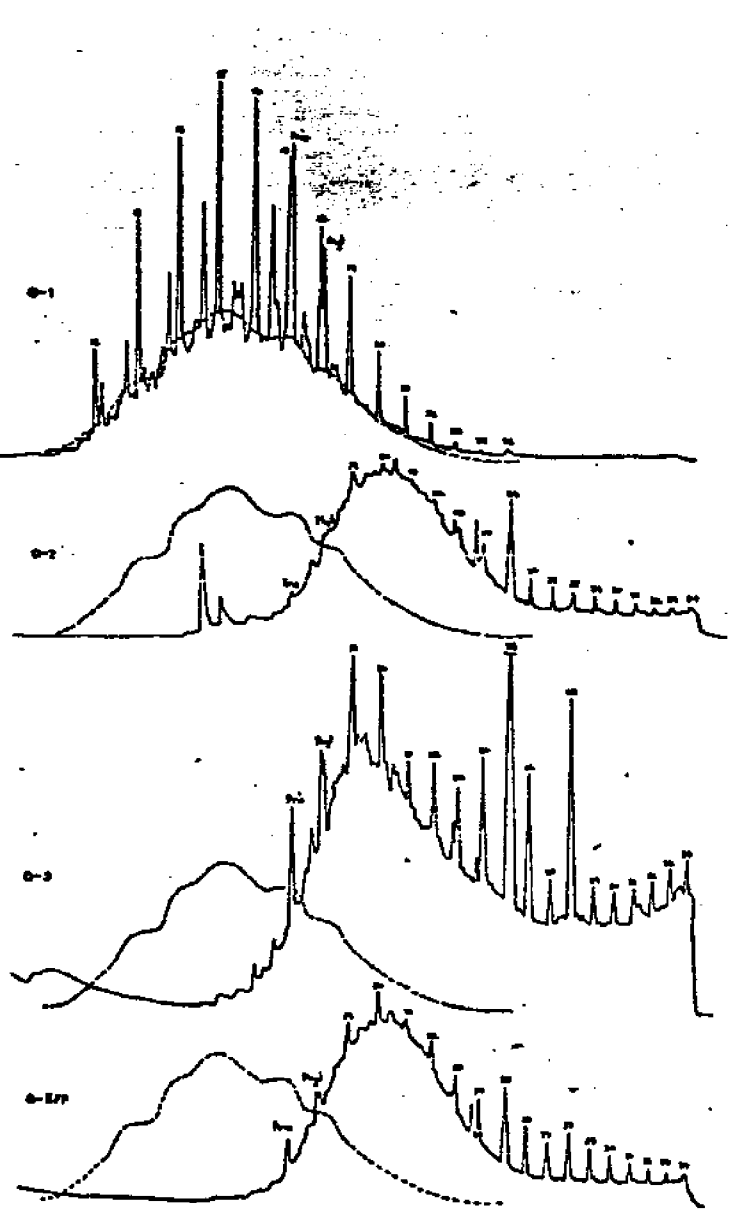
The absence of any significant differences in the degradation patterns of Q and QB (high nitrogen and phosphorous) seemed to indicate that nitrogen and/or phosphorous concentrations were not the limiting factors in the sequential degradation process. Some other particular environmental factor was presumedly needed in order to get any further degree of degradation as the oil cascaded down the sequential vessels. It is possible for example that the bacterial populations of the second and third vessels were not sufficient to promote any further degradation and thus the nitrogen and phosphorous concentrations would impart little effect on 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 undoubtedly 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. Experiments are presently underway to check the effects of even higher nitrogen and phosphorous concentrations and to chemically determine how much of these nutrients are actually consumed by the bacteria.

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

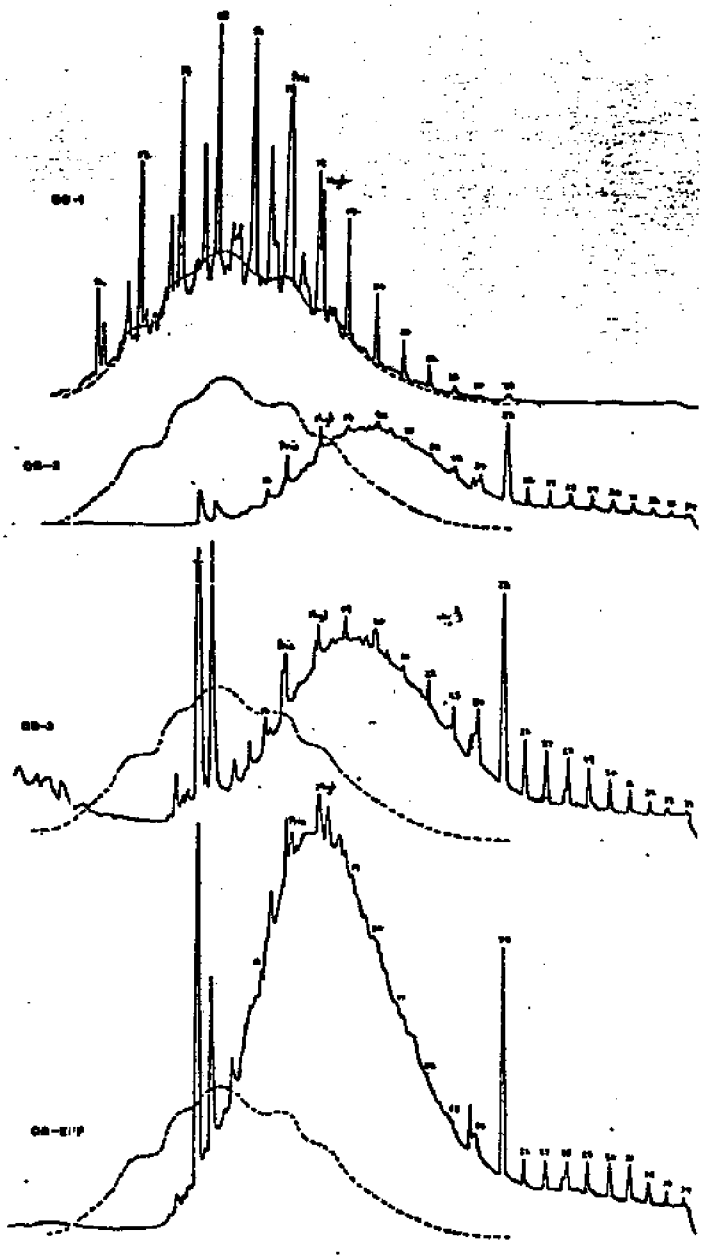


Q QB

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



Q



QB

In field experiments, where you might expect to see the type of degradation we have observed in continuous culture studies it has not been detected because investigators have not looked in the right place. In these field experiments (2,6) the only way in which the degradation of the oil can be monitored is to sample the oil directly from its point of input and 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.

Another aspect of these sequential 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 thru the other vessels in the chain. The reason for this lack of a more complete degradation was not due to a depletion of nitrogen and phosphorous sources since there were ample amounts detected in the effluents of the third vessel. It is quite possible, however, that the right bacterial population were not present in the second and third vessels. As we have mentioned above, the pattern of colony morphology was the same for all three vessels and we would have suspected that different degradation products would have induced enrichments for different types of bacteria (as detected by different colony morphologies). This enrichment obviously did not occur and it is not exactly known at the present time as to what prevented it. In experiments using a continuous inoculum of unsterilized Lake Ontario water into the first vessel, the enrichments in the second and third vessels also did not occur. Further experimentation is needed to determine if this lack of sequential degrees of degradation is a natural phenomena or an artifact of our continuous culture systems.

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

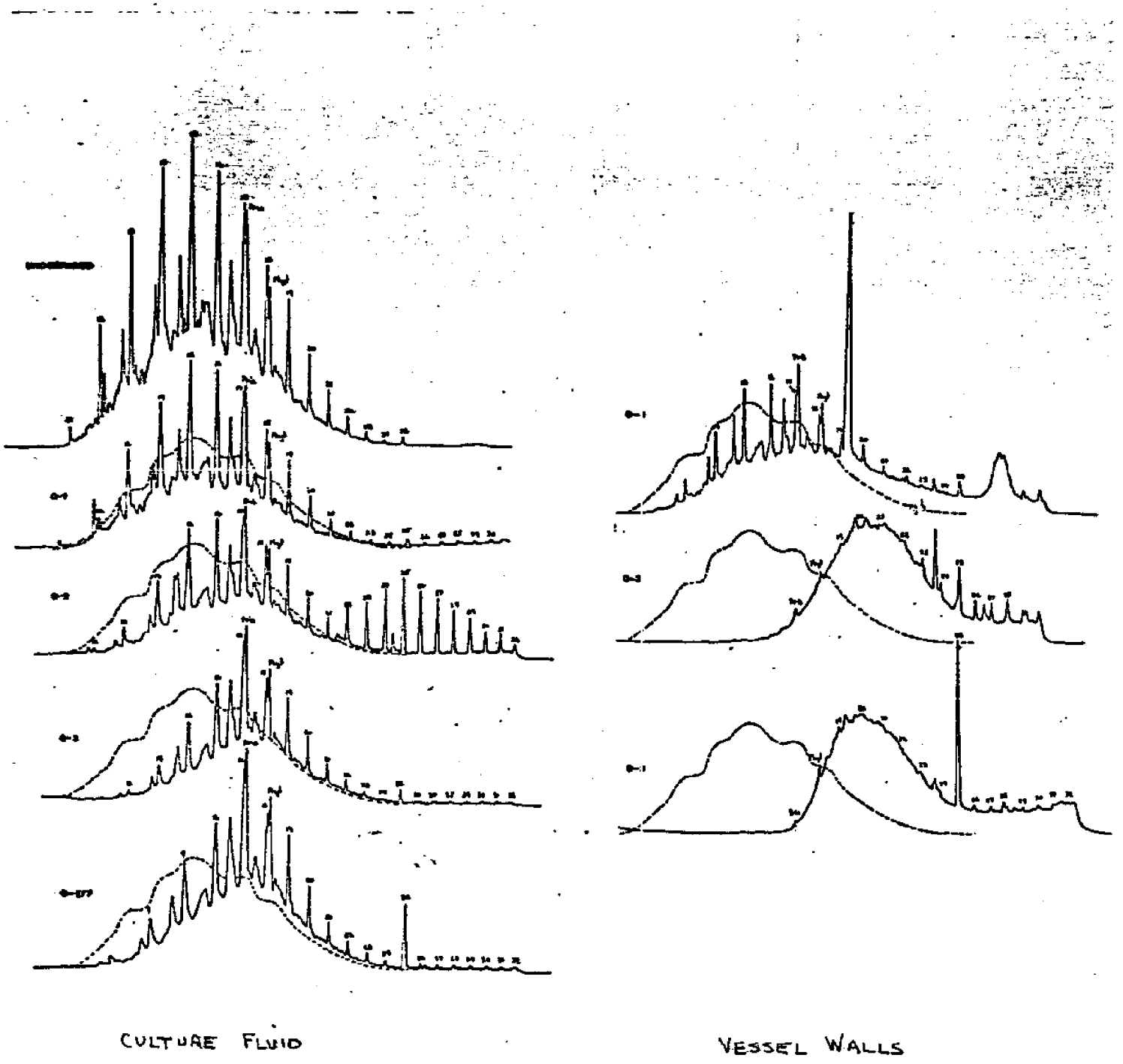
As we have noted above, during many phases of the degradation process significant amounts of material accumulated on the walls of the culture vessels. This material was usually light brown in color, rather gelatinous in consistency and not very tightly held to the walls. In most other kinds of continuous culture experiments this accumulation of material on the walls would be a troublesome problem since it normally complicates interpretation of the results. However, in examining the wall material in our 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. Thus it was decided to examine the chemical nature of the oil that was part of this wall growth to see if any different types of degradation had occurred.

We have already previously alluded to some differences in the degradation process as a function of wall growth (see pages 16-17) At that point it was pointed out that the synthesis of extra peaks corresponding to n-alkanes of C-23 to C-34 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 has been removed and extracted from several different systems. In all samples analyzed a decidedly different degradation pattern has been detected.

Figure 10 shows the gas chromatographic profiles of oil extracted from the culture fluid and from the material adhering to the walls. As can be seen, there was a general increased degree of degradation of oil taken from the wall material. In Q-1 (the first vessel containing the oil layer) the oil from the vessel walls showed considerably more degradation of the n-alkanes and envelop components than oil taken directly from the oil layer. Some synthesis of components in the boiling range above that for the C-25 normal alkane was also present. The large peak appearing next to the C-19 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 (Q-2) again showed a substantially different degradation pattern (Figure 10). Unfortnately the sample obtained from the culture fluid was not sufficient to give the normal gas chromatographi picture but if one compares the height of the pristane and phytane peaks to the height of the envelop profile it can be readily seen that much more degradation had taken place on the walls of the growth vessel. The amount of envelop components synthesized was also con-

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



siderably greater in the wall material. Peaks corresponding to C-26, 27, 28 and 29 normal alkanes are also present in the wall material sample and not in the culture fluid sample. Overall it would appear that the whole degradation process has been speeded up as a result of the oils' attachment to the vessel walls. Examination of the analyses from Q-3 also substantiates this generalization but not to quite as great an extent, i.e., the degree of degradation from each source was more similar.

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 sequential continuous culture vessel. The physical appearance of this wall material in the tubing 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 our 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, which are typically seen in undegraded oil, were completely degraded away leaving only traces of pristane and phytane sticking above.

Second the synthesis of extra envelope components appeared to reach an extreme in that the quantity of material in this higher boiling range was considerably greater than that seen anywhere else.

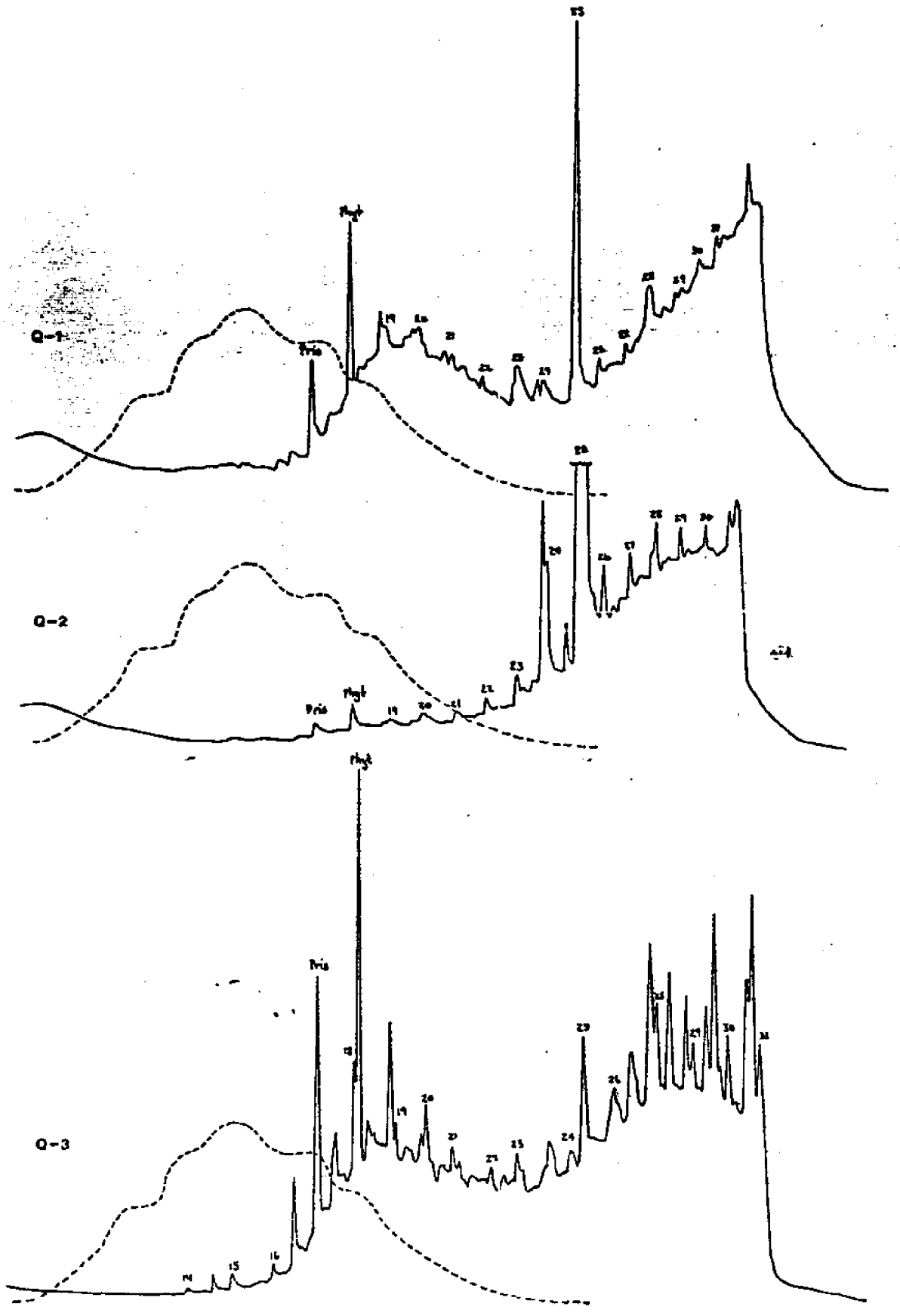
And third, the peak co-chromatographing with the C-25 n-alkane was enormous, again being considerably more than that seen previously. None of this degree of degradation was seen in samples extracted from the culture fluid.

For the samples taken from the tubing between vessels Q-2 and Q-3 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 C-25 peak which do not correspond to another type of n-alkane.

In the tubing between Q-3 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 occcurred It is probably the former case which is true.

This 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 and that for some reason the 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

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



selection of a special type of bacterial population. Rather, the trapping of the oil on the walls or in the tubing places it in a position which makes it more susceptible to microbial attack, a situation which 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 with many investigators believing 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 or spread out greatly increasing the surface area and thereby increasing the degradation rates. This would at least be one explanation for the results we have seen 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 should be obtained. For example, it may be that once oil is dispersed as freely suspended droplets, degradation only proceeds at a slow rate. On the other hand when these droplets become impregnated with particulate matter, such as clay minerals or silicone particles, degradation is greatly enhanced. This would then mean that the controlled addition of particulates to oil spills may infact represent a new approach to oil pollution abatement programs. Unquestionably one must first obtain more information about the role of surfaces in oil degradation and determine the type of degradation which will occur in sediments in beach sand and on particulate matter suspended in the water column.

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

Because the results we have obtained from our laboratory model of Lake Ontario are unique and not extensively reported any where else, it was necessary to show that our laboratory model was as much like a natural situation as possible. In all of our experiments so far, the source of bacterial populations for the degradation process studied has come from the original sample of Lake Ontario water used to inoculate the experiment. In other words the addition of one 200 ml sample of Lake water supplied all of the bacteria which were needed to carry out the type of degradation observed over about a two month period. However, to date we have not been able to get the complete degradation of oil as it passes through our sequential continuous culture system and we have not seen a highly significant 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 grow.

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

To date, no significant difference in the sequential oil degradation process was detected 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 sequential continuous culture vessels and 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.

Thus it would appear that the bacterial populations present in our continuous culture vessels were sufficient for at least the degradation we have obtained so far. Some other factor must then be limiting the degradation and we are currently doing experiments to determine what this factor might be. Once it is elucidated it could be a very important factor to be considered in obtaining the complete degradation of oil in a natural aquatic environment.

E. Oil Degradation Field Experiments

Ever since our conception of this project in oil degradation, we have expounded on the virtues of continuous culture techniques over other experimental designs. We have consistently emphasized the point that because our experiment system has many characteristics in common with a natural aquatic environment, our results reflect a degradation process which is, in fact, very similar to the one observed naturally. Many studies which have tried to relate field and laboratory results have been unsuccessful primarily because one is forced to look at only the initial attack of oil by bacteria and not at the subsequent stages of degradation which occur once the oil is dispersed through the water column. In the field, if you study the degradation of an oil slick, once the slick is gone the remaining oil is so greatly diluted that it becomes virtually impossible to monitor its further degradation.

The results from our sequential continuous culture systems have indicated that this secondary degradation of oil as it is dispersed through the water column represents some rather unique degradation mechanisms. The central problem, however, is to verify that these mechanisms do exist in nature. If this can be done, then it will add tremendous credibility to our sequential continuous systems as models for this natural degradation process.

Toward this objective, a field study program has been developed. Central to this program is the development of a piece of apparatus which will allow us to monitor both the degradation of an oil slick and the degradation of oil which is dispersed into the water column. The designed field apparatus is depicted in figure 12 and plate 1. Basically it consists of three concentric cylinders with perforations in each to allow for the adequate flow of nutrients bacteria and partially degraded oil from one cylinder to the other. This design is in fact an attempt to mimic the sequential continuous culture systems we have tested in the laboratory. The innermost plastic cylinder is designed to maintain a water column under conditions of minimum turbulence. It would thus be very analogous to the first vessel (Q-1) in our sequential continuous culture systems. The two surrounding cylinders are designed to slow down the dilution of dispersed oil so that it can hopefully be chemically analyzed. The amount of exchange between each cylinder is a function of the number of perforations present. The outer two cylinders are thus designed to be analogous to Q-2 and Q-3 in our sequential continuous culture system and there is a three-fold difference in their volumes.

The entire apparatus is fitted with a floatation collar and anchored to a cement slab. It is covered with a clear plastic umbrella to keep splashing at a minimum.

To date we have completely built and tested the apparatus and it very nicely maintains a quiescent oil layer inside the inner cylinder, even in rough water. We are now in the process

Figure 12. Schematic Diagram of Apparatus Used in Field Experiment to Study the Microbial Degradation of Oil in Lake Ontario.

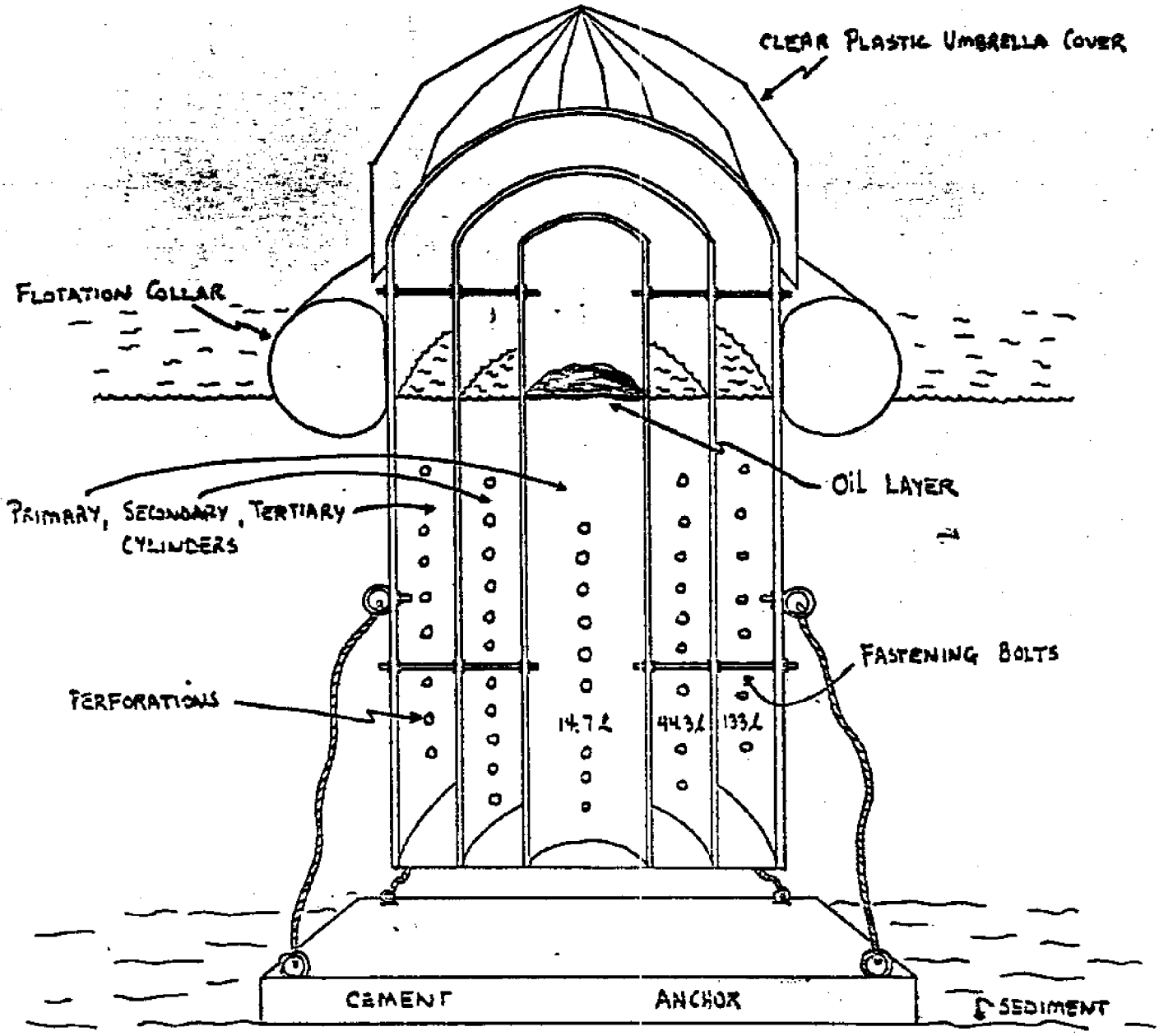
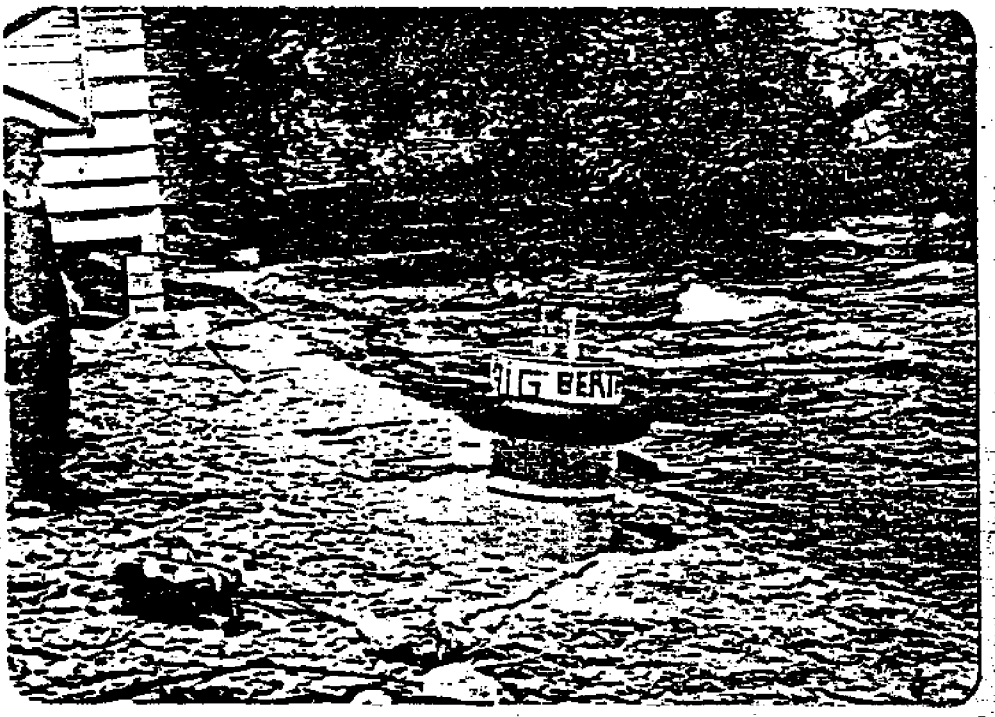
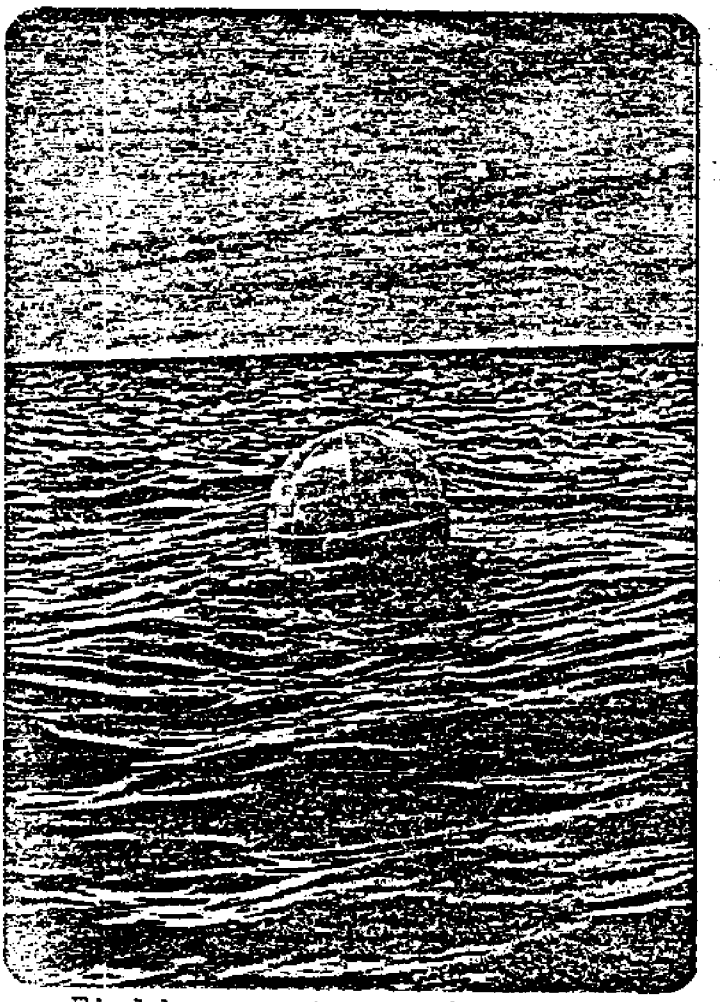


Plate 1. Three views of field apparatus.



View of field apparatus illustrating perforations in outer cylinder.

Top view of field apparatus illustrating concentric cylinders



Field apparatus anchored in place and covered.

of using it in an experiment; water samples from each cylinder are going to be taken and analyzed for bacteria and oil. A sequential continuous culture system is also being simultaneously studied in the laboratory.

The results of our experimentation attest to the technical feasibility of using such an apparatus. During several weeks of water conditions ranging from calm to moderately turbulent, the oil layer was indeed successfully contained. During this calm period the apparatus proved to be ideal in that it allowed the researcher to monitor the oil layer as well as the degradation products released into the aqueous phase as a result of the initial bacterial attack. Furthermore, the oil layer was observed to undergo some of the initial physical changes which were reminicent of processes that occurred in our laboratory studies.

Unfortunately, our experimental system would not tolerate heavily turbulent waters. The subsequent wave action suceeded in disturbing the oil layer, eventually washing the oil out of the system.

Thus it became obvious that our experimental success was dependent on local weather conditions. To overcome the "open-water" failure of the system, the decision was made to move the apparatus to an aqueous system with a considerably more protected physiography.

The new site chosen is McCargo Lake, located in Orleans County, New York, on the Fancher Campus of the State University of New York, College at Brockport. The lake is part of the Ontario-Salmon River system and is characterized by large accumulation of humic and clay materials. The lake is approximately eight acres in surface area, 1,250 ft long and has a maximum width of 350 ft. and a depth of 19 ft. It is generally classified as mesotrophic. The shoreline is almost completely dominated by woodlands, the remainder being swamp area. This particular feature substantially reduces wind action, and therefore wave action, to a minimum level.

McCargo Lake bears little semblance to Lake Ontario and no attempt is being made to infer a similarity. McCargo Lake merely represents an aqueous system which readily lends itself to the study of natural microbial degradative processes "in situ". However, it is believed that oil degradation potential for both environments is similar and the same or similar biodegradation processes will occur in each. Furthermore, McCargo Lake represents an area that can be policed, thus keeping sabotage and theft of materials under control. This was indeed a considerable problem in the Lake Ontario experiments and ultimately led to our decision to move the experiments.

The Lake Ontario efforts, did however, yield a few interesting initial observations. The first was the appearance of substantial amounts of wall growth which covered the surfaces of the three cylinders. This wall growth bore a gross morphological similarity to the wall growth which was established in our laboratory culture vessels and was established "in situ" in approximately the same time span (i.e. 14 days). In the laboratory, when the wall growth was analyzed, it was found that a more complete oil degradation occurred when the microorganism adhered to a solid surface. (See Results, Section C). Whether or not this phenomena occurred in the field, is difficult to determine at this point but further study is being implemented.

During the course of a number of field experiments, it was also noted that microbial association with the oil layer was very slow although similar to our laboratory studies. The "in situ" oil layer became laden with particulate matter and a white microbial film appeared at the oil/water interface. These initial processes were however very slow especially when compared with laboratory experiments. It is felt that this increase in time for microbial attachment is a reflection of the various nutrient limitations of the particular natural environment.

To overcome some of these nutrient limitations, various design modifications are being introduced into the system. These modifications will allow for the controlled input of ammonia nitrogen and phosphate phosphorous directly onto the oil layer. Hopefully, in this way, some of the factors restraining the oil degradation process will be eliminated.

The problems associated with this study of "in situ" microbiology are indeed substantial. However, we have learned a good deal from our previous experiments in Lake Ontario and are optimistic about our experimental success in McCargo Lake. We believe that we will ultimately be able to provide the required degradative information and assess aquatic environments for their oil pollution tolerance limits.

F. Tentative Predictive Model for the Fate of Oil in Aquatic Ecosystems

Many of the problems which have arisen during oil pollution abatement programs have resulted from a poor knowledge of the various natural mechanisms which exist for the self cleansing of an aquatic environment. By far, one of the most important self cleansing mechanisms is the activities of oil degrading bacteria. Because of the complicated process which is involved a great deal more general information is still critically needed before any reasonable assumptions can be made about the role of these bacterial activities in the fate of polluting oil. However, one must be cautious about how the information is generated because if it is difficult to interrelate, no meaningful generalizations or concepts will result. Thus we have felt that some working model of the oil degradation process in aquatic environments must be formulated so that the importance of any information generated can be evaluated relative to an overall picture of oil degradation.

The advantages of such a working model are three fold; first it can be designed such that general predictions can be made about the fate of the oil based on the particular environmental conditions at hand and based on the particular pollution abatement program being implemented.

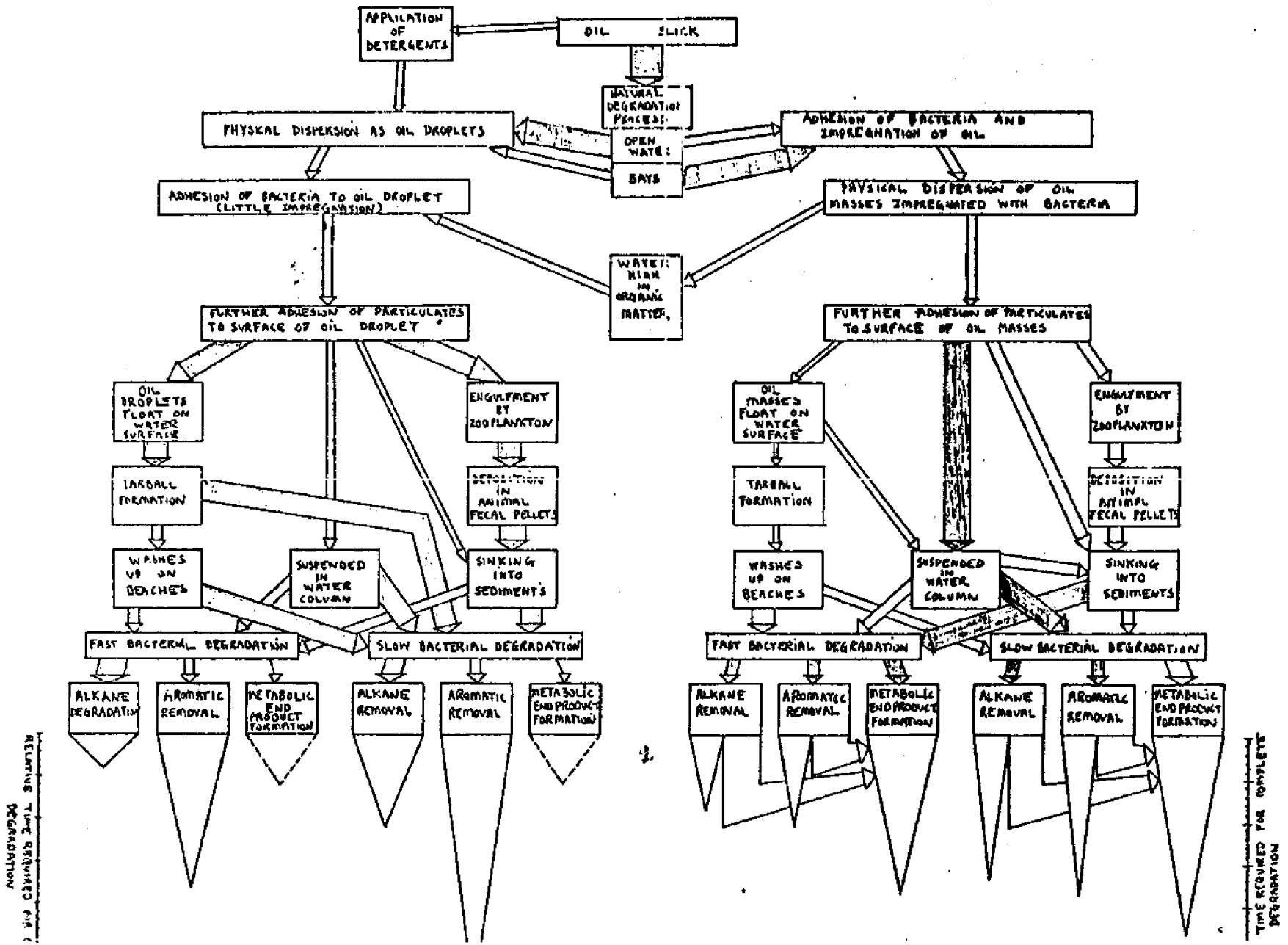
Second, a model of the fate of oil in aquatic environments can act as a guide for public officials at all levels in making policy decisions relative to oil pollution of aquatic ecosystems. Their ability to create meaningful and effective legislation depends on being able to access the problem easily and quickly and a predictive model will give them this flexibility.

And third, the development of any kind of model is going to be tremendously useful to research laboratories and water quality laboratories because it will act as a stimulus for increased and more extensive thought regarding the fate of oil in aquatic environments. And it will hopefully generate new types of experiments which will lead to an even greater understanding of the oil degradation process.

Subsequently, we have attempted to generate an initial predictive model which may ultimately lead to a more comprehensive picture of oil degradation. Our model, as we have developed it, is depicted in Figure 13. It should be emphasized that this is only a very tentative model designed more for generating discussion than for actual practical application. We have taken the liberty to speculate on many aspects, hoping that our generalizations may be challenged and ultimately evaluated experimentally.

The central theme of this model is that the initial bacterial attack on the oil is critical in dictating the eventual degradation

Figure 13. Tentative Predictive Model for the Fate of Oil in Aquatic Environments.



process. We visualize two basic types of attack. In one type the oil is first dispersed as small oil droplets and then these droplets are attacked by bacteria. In the second type, the oil is attacked by the bacteria first and then through their activities the oil is broken up into small masses and ultimately dispersed. The major difference in these two types of attacks is that in the former case bacteria never really extensively impregnated the oil but instead associated only with the outer surface of the oil droplet. Thus, despite the fact that the oil droplet may be completely covered with bacteria, the inside is still highly nonpolar and degradation must take place from the outside inward. This we are speculating results as a slow degradation process.

In the latter type of attack described above, the bacteria actually have the opportunity to impregnate the oil and it is through this process that the oil eventually breaks up into small droplets. These droplets, however, are more like masses of bacteria and oil and thus the oil is dispersed in a form which is thoroughly mixed with bacteria. The degradation process is therefore not restricted to just the outer surface.

As a result of the information generated by this laboratory, we feel that this latter process encompasses a different kind of degradation process.

VI. Conclusions and Recommendations

It can be concluded from the work presented herein that sequential 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. We have 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 our laboratory model is a good facsimile of the natural oil degradation process.

Our 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 and we seriously question whether the oil is ever completly broken down within a reasonable time span (of months to years). Since in our experiments partially degraded oil or oil degradation products were still readily detectable after passage through three continuous culture vessels and since our experiments were run under conditions not found year round in a lake (such as high temperature, high nitrogen and phosphorous and high oxygen concentration) it would appear that oil can persist in natural environments for considerably longer periods of time than once thought.

As we recommended in our previous report, it is still quite clear that nitrogen and phosphorous must be supplied in order to get any significant degradation. The availability of these inorganic materials, either naturally or through fertilization and there accessibility to the site of oil degradation 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 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 but is instead a more complicated process. Our work has shown that petroleum hydrocarbons are transformed into specific metabolic endproducts. From our work we conclude that these products are actually synthesized from hydrocarbons in the oil and that they are 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 considerably more resistant to degradation than their starting material and they could be more toxic than the oil.

We recommend, therefore, that the mechanisms behind these transformations be investigated and that the actual chemical nature of these synthesized products be elucidated to whatever extent possible. The conditions which control the production of these products and a knowledge of their chemical structure will play a very important role in determining how much, if any, oil can be tolerated by an aquatic environment.

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 our 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 complete degradation relative to that oil which remains suspended in the culture fluid. If this can be shown to be a consistent phenomena of bacterial degradation processes, then it may be possible in the future to recommend that partially degraded oil (that is oil which has undergone an initial attack by bacteria) 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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