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Intermediate Projects in the Bacterial Decomposition of Hexadecanol and Octadecanol

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INTERMEDIATE PRODUCTS IN THE BACTERIAL DECOMPOSITION OF HEXADECANOL AND OCTADECANOL

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure | Page

CHAPTER I

INTRODUCTION

Water resource planning, development, and utilization encompass the concerns of water quantity and water quality. In a given location a sufficient quantity of water is required to sustain community human activities, provide for industrial use, and allow for agricultural needs. The quality of water must generally be safeguarded in order that community health will not be endangered, that the water will be suitable for recreational purposes, that wildlife will be sustained, that the water will not be rendered unfit for agricultural requirements, or that it will not require extensive treatment for industrial use. Depending on the area of the country, one or the other of these concerns may be dominant. For example, in the Northeast and upper Midwest, an excess of water has been naturally provided which has allowed the development of large population and industrial centers. But the long term and recently accelerated return of water-borne wastes from these centers to water bodies has caused a critical deterioration of water qaulity. In the arid Southwestern region of the United States, the lack of a large natural supply of water and only moderate annual rainfall have retarded the development of the area for both industrial and agricultural purposes. Clearly, the development and maintenance of a sufficient quantity of water has been the dominant concern of this region.

The increased emphasis on conservation and optimum utilization of our water resources has generated a multiplicity of research efforts to

define and suppress the factors which work toward waste or destruction of these resources. In the arid climate of the Southwest, control of water loss by evaporation from impoundments would constitute a major step in suppressing waste, consequently a considerable research effort has been directed toward the investigation of monolayer films on the surface of small reservoirs in attempts to minimize loss by the evaporation process. On the other hand research efforts designed to prevent the destruction of these resources have been concerned with the effects of foreign chemical components on the ecological regimes within the reservoir system. Such research efforts are not contradictory but should rather be complementary if a total understanding is to be gained of the physical, chemical, and biological implications of alteration of a natural process.

When evaporation suppression is achieved by monolayer surface filming, a substance foreign to the water is applied, admittedly in very small quantity relative to the quantity of water in the reservoir. Physical, chemical, or biological forms may then set in to disrupt the film or bring about the disappearance of the added chemical. It is therefore necessary, in order to maintain film integrity, to apply on a continuous or continual basis. Conceivably the eventual application of chemicals might attain a significant level relative to the water, particularly in a small reservoir of low volume to surface area ratio. The chemical added to the water may have several effects with respect to the biological regime in the reservoir, particularly the microorganisms. Obviously, a toxic chemical would be completely unsuitable.

Beyond that the chemical may or may not be biodegradable. Non-biodegradable chemicals, however, are generally considered undesirable as components to be added to a natural environmental system since they would be cumulative with time and might therefore present aesthetic or other unpredictable effects. Biodegradability is desirable, yet it must proceed at a sufficiently slow rate that the effectiveness of the chemical for accomplishing its function is not destroyed.

Complete biodegradability implies the complete assimilation and/or respiration of a given chemical by the aquatic microorganisms. Assimilation may be measured by growth in cell numbers or cell mass while respiration may be measured by some technique for measuring the cumulative oxygen uptake. Neither of these techniques is direct and each is subject to some error or misinterpretation.

In previous work in this laboratory relative to industrial waste treatment processes, gas chromatographic techniques have been developed and employed for the direct measurement of specific substrate removal in aqueous microbial systems. These techniques have been found to be applicable to the low molecular weight hydrocarbon derivatives in aqueous solution. Quantitative gas chromatography possesses a high degree of sensitivity and provides simultaneous analysis for more than one component. It is thus theoretically possible, under the proper conditions, to detect and measure intermediate metabolites during the course of primary substrate utilization. Therefore, if non-biodegradable metabolites appear in the aqueous phase they may be subject to detection and identification by the same analytical technique used for measurement of

substrate disappearance.

It was the objective of this project, in relating the monolayer film forming chemicals hexadecanol and octadecanol to water quality factors:

1. To investigate the behavior of hexadecanol and octadecanol in microbial systems using direct and specific analytical capability, with the behavior studies to include detection, identification, and behavior of any intermediate or end product compounds formed as a result of the biological transformation of the hexadecanol or octadecanol.

2. To correlate the behavior of long chain fatty alcohols with studies of lower molecular weight alcohols in which similar analytical procedures are employed.

CHAPTER II

REVIEW OF LITERATURE

In his review of the literature on water evaporation suppression, Wixon (1) covered the historical development of the use of monolayer film-forming substances for the purpose of decreasing the rate of water evaporation from small reservoirs. The studies cited by Wixon were concerned with:

1. measurement of evaporation retardation by various substances under laboratory or field conditions;
2. physical characterization of the monolayer film imparted on the water surface by various substances and its effect on water characteristics;
3. the effect of molecular structure and natural variables, such as wind, current, temperature on spreading tendency and self-healing characteristics;
4. the effect of the surface film on the ecological system.

Wixon's study was itself concerned with the impact of monolayer films of hexadecanol and octadecanol on the growth characteristics of phytoplankton species under controlled laboratory conditions. He observed that non-filamentous algae in the treated system appeared to have inhibited growth in the first stage of his thirty day experiments, whereas at the conclusion of all experiments growth was significantly greater than in the untreated systems. Filamentous algae appeared to have better growth than the same algae in the untreated system. Although Wixon did

not specifically enumerate bacterial species, he commented that bacterial degradation of the fatty alcohol apparently contributed to a significant increase in cell numbers and commented that such a condition may well lead to a significant demand on the system oxygen requirements.

Parker and Barsom (2) have most recently reviewed the literature of the biological and chemical significance of surface films on aquatic ecosystems. They point out that natural surface films exist on many waters and that these films may serve as a nutrient source for aquatic microorganisms. They further suggest that such natural films may even provide a mechanism for the widespread distribution of persistent chlorinated pesticides. They conclude, "Not only is the knowledge of the natural chemical composition of surface microlayers on lakes, streams, and oceans fragmentary but the influences of substances introduced by man, such as petroleum products, long chain alcohols, synthetic pesticides and surface-active compounds on the biota which inhabit the microlayer cannot be estimated. . . . that the interaction of the microlayer with both the air and the subsurface water may be of sufficient importance as to be a major contributing factor in currently unexplained problems of air and water pollution."

Ludzak and Ettinger (3) investigated the biological degradation of hexadecanol in laboratory test units in which hexadecanol pellets were floated on a water surface. Air which had been scrubbed free of carbon dioxide was continually passed over the surface for periods of up to 40 days and the carbon dioxide resulting from bacterial metabolism was again scrubbed and measured. At the conclusion of the test period the

remaining hexadecanol was recovered by extraction, dried, and weighed. The carbon dioxide equivalent of the hexadecanol lost indicated a high degree of biological degradation through respiration. These tests were conducted on water from five different sources.

Gerhold and Malaney (4) investigated the biological degradation of a large number of aliphatic derivatives by activated sludge from three sewage treatment plants through the use of the Warburg technique. Among the compounds tested were 1-hexadecanol and 1-octadecanol. The oxygen uptake exerted by these substrates over a 24-hour test period was nil with all three sludges, leading them to state that the alcohol of this chain length appeared to be more resistant than the corresponding alkane.

In the present study, three primary analytical techniques were employed in an effort to obtain a direct and specific measure of the rate and extent of degradation of hexadecanol and octadecanol by adapted microorganisms. These techniques, namely gas chromatography, total organic carbon analysis, and recorded oxygen uptake will be discussed in detail in Chapter III.

Gas chromatography has been used extensively in this laboratory for the observation of substrate uptake in single substrate and mixed substrate systems (5, 6, 7, 8). The substrates which have been measured by direct injection techniques have been low molecular weight (eight carbons or less) aliphatic alcohols, ketones, aldehydes, esters, acids, and mixed alkyl-aryl ketones. During the course of these studies it has been observed that in mixed substrate and mixed specie systems adapted to substrate utilization, certain reproducible patterns of substrate response

may be observed. Response to substrate may be independent, competitive, or sequential. In the case of independent response the rate at which one substrate is utilized will be completely independent of the presence of another substrate. This may be the case if they are utilized by a different mechanism or by different species. In the instance of competitive removal, two or more substrates are removed simultaneously at rates proportional to their relative concentrations. This would be the case if they are removed by the same mechanism and there is only statistical competition for the active sites. In the instance of sequential removal, the utilization of one substrate is retarded until another substrate is removed, at which time accelerated removal of the inhibited substrate occurs.

In addition to enabling the observation of the above patterns of mixed substrate removal, gas chromatography may also allow the observation of intermediate metabolite production and determination of subsequent fate. For this reason it was felt that gas chromatography would prove useful in following the course of hexadecanol and octadecanol utilization by adapted microbial cultures.

Gas chromatography has been utilized by others in following the biodehydration of substrates. Patterns similar to those described above were observed by Swisher (9) in relating the biodegration of monoalkylbenzene sulfonates to molecular structure in a study on the effects of phenyl group placement and alkyl chain branching. Mateles and Chian (10) reported gas chromatographic analyses to show acetate production and utilization in natural population consuming glucose. It is possible that products will

appear during the degradation process which will not be detected on chromatographic analysis. To detect the existence of such components and obtain a quantitative measure of their concentration, other analytical techniques are required. For this purpose the American Public Health Association Chemical Oxygen Demand Test (11) has been employed (12). This is a catalyzed potassium dichromate oxidation test responsive to most organic concentrations; a relatively large sample size is required and the time for each analysis is on the order of three hours.

Van Hall, et al (13) described an instrumental technique for measurement of organic components in aqueous solution which, in its various commercial forms, is becomming widely used in water pollution monitoring and research. Busch (14) describes an early modification of this instrument in measuring the removal of biodegradable organics by laboratory activated sludge cultures and suggests that the use of such an instrument might show whether a specific component is truly biodegradable or simply transformed into one or more intermediates. A later modification of this instrument, described in Chapter III, was used in the present study.

CHAPTER III

MATERIALS AND METHODS

This project has been divided into three phases:

I. Analytical Procedures Development
   A. Gas-liquid chromatography
   B. Total organic carbon analysis
   C. Oxygen response measurement

II. Development of Adapted Microbial Culture
   A. Growth of seed organisms of heterogeneous population on mixed alcohol substrate
   B. Demonstration of soluble substrate utilization in mass culture

III. Growth of Seed Microorganisms on Film Forming Alcohol Substrate and Demonstration of Substrate Utilization
   A. Static cultures
   B. Shake flask cultures with incremental substrate addition
   C. Shake flask cultures with total initial substrate addition

Analytical Procedures Development

Gas-liquid chromatography. The gas chromatograph used was a Varian Aerograph Series 1200 with hydrogen flame ionization detector. This instrument contains a single column analytical oven with linear

temperature programming capability. Detector output was recorded on a Sargent Model SRG 10-inch, 1 mV potentiometric recorder equipped with a Disc chart integrator for quantitative measurements. The specific details for analysis of 1-hexadecanol and 1-octadecanol are provided in Chapter IV.

Total organic carbon analysis. A Beckman Model 915 Total Organic Carbon Analyzer was used for non-specific measurement of soluble organics in aqueous solution. This instrument accepts an injected aqueous sample, 10-50 microliters, into a 950°C combustion tube packed with cobalt oxide on asbestos catalyst and through which passes a constant stream of air scrubbed free of carbon dioxide. Under the prevailing conditions all forms of carbon in the sample, whether organic or inorganic, are converted to carbon dioxide gas. The gas stream elutes from the combustion tube, through a water vapor condenser, to the detector cell of a Model 915 non-dispersive infrared analyzer sensitized to carbon dioxide. The absorption signal from the infrared analyzer is read out as a recorder peak whose height may be quantitated by reference to a calibration curve obtained by analysis of a set of standards. A second identical sample may then be injected into another tube maintained at 150°C containing phosphoric acid coated quartz chips and through which passes the purified gas stream. In this tube the dissolved forms of inorganic carbon are converted to carbon dioxide gas, again analyzed by the infrared analyzer producing a second recorder read-out. The difference in total carbon from the first analysis and inorganic carbon from the second is due to organic carbon. This instrument has a detection

limit of 1 mg/l total carbon and is advantageously used in the range 0-100 mg/l organic carbon. Analyses are run in triplicate with a single analysis from time of injection to complete recorder read-out and return to stability requiring less than two minutes.

Oxygen response measurements. For measurement of oxygen response to substrate loading or oxygen uptake during substrate utilization a Gilson Oxygraph was used. This instrument employs an oxygen sensitive membrane electrode in a water jacketed culture reaction cell in recording oxygen uptake versus time. The recorder is an integral part of the instrument. Recorder response may be adjusted by sensitivity and gain controls and calibrated against a water sample whose oxygen content has been determined by Winkler titration.

Experimentally, this instrument may be used in two ways. First, for oxygen uptake during substrate utilization a culture sample is transferred to the culture reaction cell and a recording is obtained of oxygen depletion over a certain time interval. This information may be obtained periodically during the period of time that a culture is consuming an applied substrate. A plot of oxygen uptake rate versus reaction time during the course of substrate consumption may be integrated to obtain the total oxygen required for complete substrate removal. Secondly, for immediate oxygen response to substrate loading, a starved culture sample is transferred to the reaction cell and an endogenous rate of oxygen utilization is recorded. A specific quantity of substrate is then applied to the reactor with a microsyringe and a recording is obtained of the rate of oxygen utilization due to the

consumption of exogenous substrate. This type of response measurement is valuable in rapidly screening cell activity to a number of different substrates.

Development of Adapted Microbial Culture

Growth of seed organisms of heterogeneous population on mixed alcohol substrate. To develop and maintain a seed culture of mixed population for the duration of these studies, an inorganic salts medium contained in a four liter capacity reactor as described by Langley (15) was inoculated with 250 ml of a settled sewage supernatant. Growth of a diverse population selected to the utilization of primary alcohols was accomplished by the inclusion of 100 mg/l each of ethanol, 1-propanol, 1-butanol, and isobutanol in the medium as a source of organic carbon for reproduction and energy requirements. The resulting microbial culture was maintained on a daily schedule which consisted of withdrawal and discard of 1 liter of mixed culture fluids from the total mixture of three liters maintained in the reactor, addition of primary alcohol mixture to re-establish the initial concentration of 100 mg/l of each of the four components, replenishment of inorganic salts, and addition of distilled water to restore the three liter total volume. Through the general practice of one-third daily wasting of cell culture, the mean cell age in the reactor was maintained at three days.

The inorganic salts medium used in the culture reactor is described below. Four aqueous solutions of the following composition were

prepared:

Solution 1: Ammonium Sulfate, $(NH_4)_2SO_4$; 117 g/l

Solution 2: Potassium Dihydrogen Phosphate, KH_2PO_4; 10.9 g/l

Potassium Monohydrogen Phosphate, K_2HPO_4; 14.0 g/l

Solution 3: Calcium Chloride, $CaCl_2$; 69.3 g/l

Magnesium Chloride, $MgCl_2 \cdot 6H_2O$; 83.5 g/l

Sodium Chloride, NaCl; 43.6 g/l

Solution 4: Ferric Chloride, $FeCl_3$; 2.9 g/l

Three ml of each solution in three liters of fluid volume provided the following minimum ionic or elemental concentrations:

Sodium, Na^+	17 mg/l
Potassium, K^+	9 mg/l
Calcium, Ca^{++}	25 mg/l
Magnesium, Mg^{++}	10 mg/l
Iron, Fe^{+++}	1 mg/l
Chloride, Cl^-	100 mg/l
Nitrogen, N	25 mg/l
Phosphorus, P	5 mg/l
Sulfur, S	28 mg/l

The phosphates as applied to the medium were for nutrient purposes only and were insufficient to provide buffer capacity to prevent the medium from becoming acid due to metabolic activity. To neutralize the acids formed during intermediary metabolism and maintain a slightly basic pH, one gram of sodium bicarbonate was added to the three liters of reactor fluids each time the medium was changed and substrate charge added.

Microbial growth and substrate utilization. Modifications of four types of bacterial growth and substrate utilization tests were run during the course of these studies:

1. Soluble substrate uptake: tests with low molecular weight primary alcohols were conducted directly in the mass culture reactors using techniques developed by Davis, Langley, and Whealy (5). These tests were primarily for the demonstration of microbial adaptation to primary alcohols and to obtain a relative basis for comparison of the microbial utilization of the high molecular weight primary alcohols, 1-hexadecanol and 1-octadecanol. The four alcohols used as soluble substrate in these tests were 1-butanol, isobutanol (2-methyl-1-propanol), 1-pentanol, and isopentanol (3-methyl-1-butanol). The culture reactor was operated in a batch mode wherein a specific concentration of the desired components was applied at the beginning of the test. Periodic samples were withdrawn for measurement

of component concentration by gas chromatography, total organic carbon analysis, oxygen uptake rate, biomass concentration by a gravimetric membrane filter procedure, and pH with a Beckman expanded scale pH meter. These tests were conducted at a controlled room temperature of 22°C ± 2°C. Tests were terminated on complete disappearance of substrate from aqueous solution.

2. Static culture for higher alcohols: the higher alcohols, 1-hexadecanol and 1-octadecanol, presented several problems with regard to measurement of substrate uptake. They are, of course, insoluble in water but will form a film on the water surface. They could not be tested directly in the batch culture reactor as they would tend to be transported by agitation to the upper walls of the vessels where they would adhere and thus be removed from the microbial environment. Furthermore, periodic sampling for analysis of substrate remaining in a single reactor is possible only for a soluble substrate. Microbial tests for higher alcohol utilization thus required the use of a series of flasks containing medium, each treated identically. For periodic analyses, flasks could then be sacrificed at certain intervals with contents subjected to a prescribed set of analyses. Several procedures were attempted. The first of these was a static culture technique. To each of a series of twenty 250 ml flasks, 99 ml of inorganic salts

medium was added. This medium was seeded with 1 ml of adapted culture to provide approximately 5 mg/l biomass. To each of 10 flasks selected for controls, 2 ml hexane were added. To each of the remaining 10 test flasks, 2 ml hexane containing 5 mg/ml 1-hexadecanol was added. The flasks were loosely plugged with cotton gauze and incubated at 23°C for up to ten days with one control and one test flask sacrificed each day. Under the above conditions, the hexane evaporated rapidly leaving nc residue in the controls and leaving a homogeneous 1-hexadecanol film on the water surface in the test flask. Flasks were analyzed by the following procedure. Each day the contents of one control and one test flask were transferred to a 500 ml separatory funnel. The contents were made acid with 1 ml concentrated HCl. The flasks were thoroughly rinsed with successive 10 ml washings of hexane which were in turn transferred to the respective separatory funnels. The contents of the closed funnels were vigorously agitated for two minutes and the water-hexane phases allowed to separate. The hexane phase was then analyzed by gas chromatography for 1-hexadecanol remaining. The extraction represented a five-fold concentration of hexadecanol from the equivalent 100 mg/l added to water to a possible 500 mg/l in hexane. As this was a preliminary procedure for

measuring 1-hexadecanol uptake by seed organisms no other parameters were measured.

3. Shake flask culture with incremental substrate addition: following more preliminary experimentation to determine satisfactory conditions for organism growth, it was determined that shake flask cultures would be desirable to provide the agitation for keeping organisms in contact with substrate and provide an unlimited oxygen supply. To each of four two liter flasks 495 ml of medium and 5 ml seed organisms were added. To the control flask 10 ml hexane was added. To the first test flask 10 ml hexane containing 5 mg/ml 1-hexadecanol was added. To the second was added 10 ml hexane containing 5 mg/ml 1-octadecanol and to the third test flask was added 5 ml hexane with 5 mg/ml 1-hexadecanol and 5 ml hexane with 5 mg/ml 1-octadecanol. These flasks were placed on an environmental shaker maintained at 25°C. Flasks were observed periodically for evidence of growth which was noticeable after 24 hours. At 24 hours, 48 hours, and 120 hours, samples were removed for total organic carbon analysis, gravimetric biomass measurement, and aqueous phase chromatographic analysis for volatile acids. Also at these times additional substrate was added in identical amounts to the initial substrate charge.

At 168 hours tests were terminated, total organic carbon analyzed, gravimetric biomass measured, aqueous phase analyzed, and final pH measured. In addition the flask contents were extracted with 50 ml hexane and the hexane extract analyzed for residual alcohol.

4. Shake flask culture with total initial substrate addition, granular form: to each of ten 2 liter flasks, 495 ml of medium was added together with 5 ml seed organism to provide 5 mg/l biomass. To each of these flasks 50 mg 1-hexadecanol in granular form was added and the flasks were incubated on the environmental shakers at 25°C for up to thirty days with flasks being sacrificed periodically for total organic carbon analysis, volatile acid analysis in the aqueous phase, pH measurement and hexane extract analysis. Sacrificing was necessary due to the hexane extraction to recover remaining alcohols. The quantity of hexane utilized in extraction was 50 ml with extraction performed in 500 ml separatory funnels after sacrificing a portion of the flask contents to other analyses. Biomass was not measured due to the possibility of unavoidably trapping some of the granular hexadecanol on the filters.

CHAPTER IV

RESULTS

Analytical Response

Gas chromatographic analyses for 1-hexadecanol and 1-octadecanol. After considerable experimentation an effective analytical procedure was developed for 1-hexadecanol and 1-octadecanol. Inasmuch as these compounds have virtually no solubility in water, a direct aqueous injection technique, as is used for soluble alcohols, could not be employed. The higher alcohols are solid at room temperature, thus to prepare them for gas chromatographic analysis it was necessary to dissolve them in solvent immiscible with water and compatible with the gas chromatographic analytical technique. Hexane was found to be a good solvent for this purpose. The gas chromatographic analysis posed the problem of column selection, sample volume, and oven temperature operation. It was determined by experimentation that a 5' x 1/8" five percent SE-30 on 60/80 Chromosorb W column accepting a three microliter sample injected on column at 90°C allowed rapid elution of hexane but no elution whatsoever of 1-hexadecanol and 1-octadecanol. At temperatures high enough to allow elution of 1-hexadecanol and 1-octadecanol (150°C) good separation of the latter components was not obtained and solvent interference with the analysis was experienced. The temperature programmer was thus employed and the following procedure evolved.

1. A three microliter aliquot of hexane solution of 1-hexadecanol and 1-octadecanol was injected on-column at 90°C.
2. The solvent was allowed to elute isothermally for six minutes. This allowed virtually complete solvent elution and return of the recorder to the baseline.
3. Temperature programming was initiated at 15°C/minute and terminated at 200°C.
4. Under the above conditions, 1-hexadecanol eluted at 10.6 minutes with the column oven reading 156°C; 1-octadecanol eluted at 12.6 minutes with the column oven reading 186°C. Baseline separation was attained between the two component peaks.

The type of trace obtained during a gas chromatographic analysis of 1-hexadecanol and 1-octadecanol is illustrated in Figure 1.

Calibration of the recorder chart integrator was obtained by performing analyses (three injections each) on a set of hexane standards containing 500 mg/l, 400 mg/l, 300 mg/l, 200 mg/l, and 100 mg/l 1-hexadecanol and 1-octadecanol. For each concentration level, the integrator counts were averaged for each component and the average taken as the integrator volume for that concentration. Figures 2 and 3 illustrate a calibration curve for 1-hexadecanol and 1-octadecanol, respectively, showing the linearity of the director/recorder response with component concentration. The detector-recorder response is somewhat sensitive to various parameters

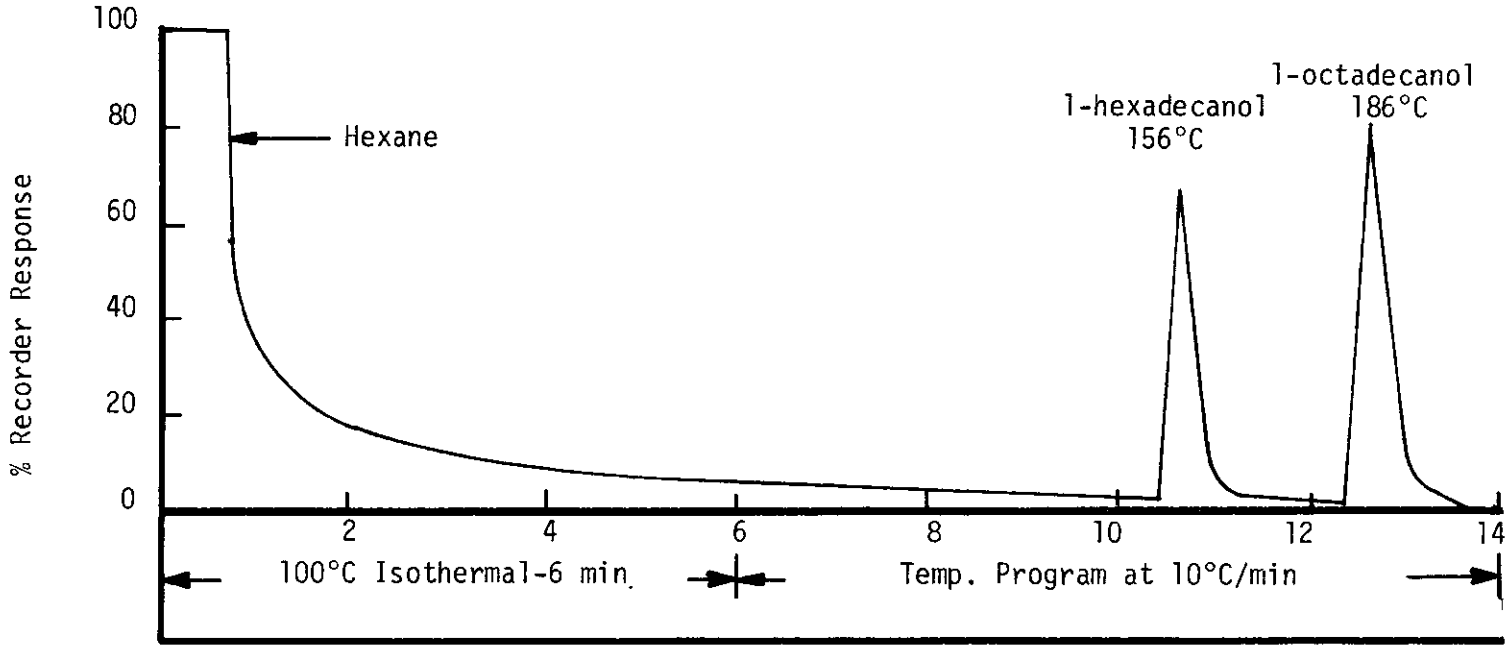

Figure 1: Typical Gas Chromatograph of 1-hexadecanol and 1-octadecanol in Hexane Extract.

Sample Volume: 3 microliters
N_2: 42 psi, 30 cc/min; H_2: 34 psi, 30 cc/min; Air: 6 psi, 300 cc/min
5' x 1/8" 5% SE-30 on 60/80 chromosorb W
Range 1, Att x 128 Varian Aerograph Series 1200

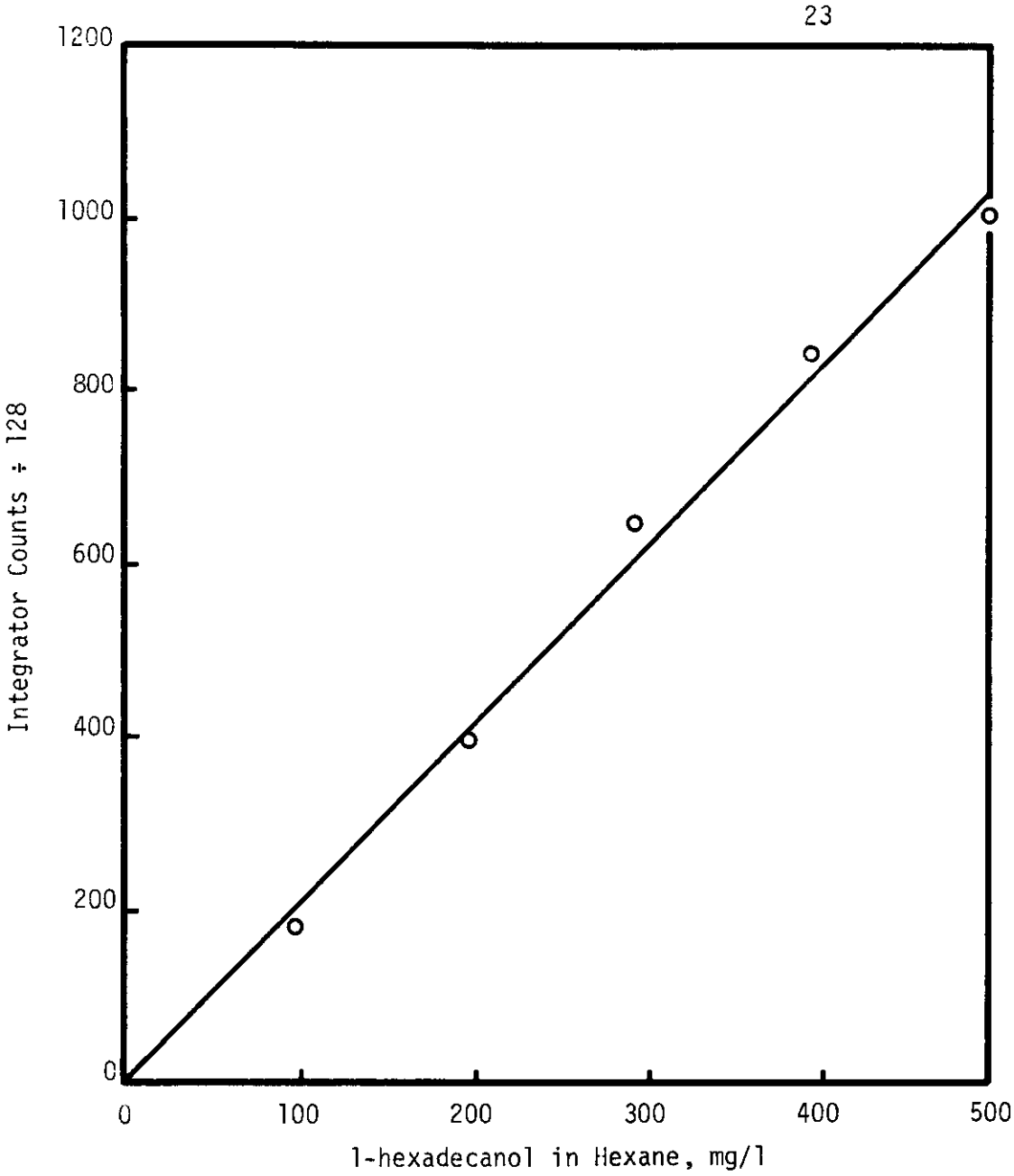

Figure 2: Linearity of 1-hexadecanol Detector-Recorder Response.

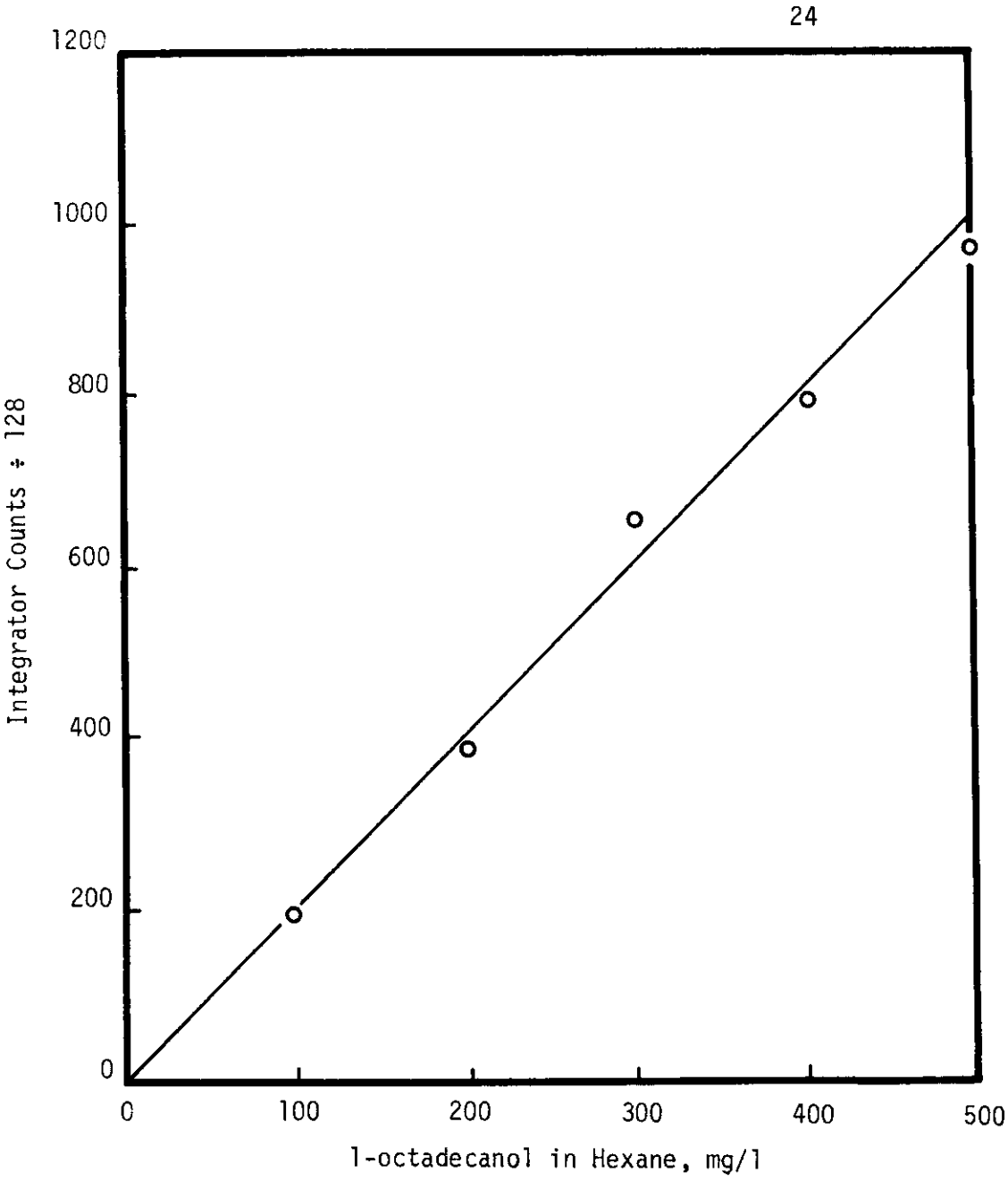

Figure 3: Linearity of 1-octadecanol Detector-Recorder Response.

which may vary daily, for example, the ratio of nitrogen carrier gas to hydrogen detector gas. For this reason a calibration is required with each set of analyses run. However, once linearity has been demonstrated three injections at a single concentration level are sufficient to provide a calibration base.

The precision of gas chromatographic analyses is shown in Table 1 and Table 2. The relative standard deviation for three injections varied from a low value of 1.4 percent to a high of 9.2 percent. During the course of experimental tests values for 1-hexadecanol were measured at concentrations as low as 2.5 mg/l in hexane extract in which the 1-hexadecanol was concentrated five fold from the water from which it had been extracted. Therefore, values as low as 0.5 mg 1-hexadecanol per liter of water were measurable.

Total organic carbon analyses. The Total Organic Carbon Analyzer has a sensitivity adjustment (gain control on the 215A infrared analyzer) which allows a wide variation in response settings. A convenient setting is to adjust for 100 percent recorder response for a 100 mg/l total carbon standard. Total carbon calibration standards for this instrument were prepared from a stock solution of potassium biphthalate diluted to contain 100 mg/l, 75 mg/l, 50 mg/l, and 25 mg/l total carbon as well as a sample of dilution water containing 0 mg/l carbon. Boiled, deionized water was used as dilution water. The peak heights for three injections at each concentration were averaged to obtain values for the calibration curve. Inorganic carbon standards must also be obtained in the same manner using a set

TABLE 1

PRECISION OF ANALYTICAL RESPONSE

1-HEXADECANOL IN HEXANE

Concentration 1-Hexadecanol mg/l	Integrator Counts ÷ 128	Standard Deviation	Relative Standard Deviation
500	1050		
	960		
	1002		
mean of 3	1004	±44	4.4%
400	842		
	830		
	800		
mean of 3	824	±22	2.7%
300	625		
	655		
	670		
mean of 3	650	±23	3.5%
200	400		
	400		
	410		
mean of 3	403	±5.8	1.4%
100	200		
	170		
	200		
mean of 3	190	±17.3	9.2%

TABLE 2

PRECISION OF ANALYTICAL RESPONSES

1-OCTADECANOL IN HEXANE

Concentration 1-Octadecanol mg/l	Integrator Counts ÷ 128	Standard Deviation	Relative Standard Deviation
500	990 970 960		
mean	973	±15.3	1.6%
400	825 745 820		
mean	797	±45	5.7%
300	645 665 640		
mean	650	±12.9	2.0%
200	400 400 390		
mean	397	± 8.0	2.0%
100	210 176 196		
mean	194	±17.2	8.8%

of standards prepared by dilution from a stock sodium carbonate--sodium bicarbonate solution. In performing a set of analyses, a standard calibration curve for both total carbon and inorganic carbon must always be obtained. The curves will generally be linear but will not necessarily pass through the origin, therefore a single calibration concentration is not acceptable. Also, although instrument sensitivity settings are unchanged, total carbon response will generally be slightly greater than inorganic carbon response for the same equivalent carbon concentration. On an actual sample analysis, total carbon is obtained by projecting the percentage response to the total carbon calibration curve and reading concentration in mg/l. Inorganic carbon is obtained in the same manner from the inorganic carbon calibration curve. Total organic carbon is then obtained by difference in the two values.

The precision of analytical response for potassium biphthalate in water is shown in Table 3. The relative standard deviation over the range of the standards varied from 0.5 percent to 1.4 percent. The instrument should be capable of this precision when operating properly and when samples are being injected by an experienced technician. Accuracy of the instrument, however, may be greatly dependent on the type of compounds in solution, therefore may show some variation between compounds.

Soluble substrate removal by adapted microorganisms. The microbial culture adapted to the lower molecular weight, water soluble primary alcohols was maintained as a source of seed organisms. In addition a

TABLE 3

PRECISION OF ANALYTICAL RESPONSE

TOTAL CARBON ANALYSIS

POTASSIUM BIPHTHALATE IN WATER

Concentration Potassium Biphthalate mg/l	Recorder Response %	Standard Deviation	Relative Standard Deviation
100	99.2 98.8 100.2		
mean	99.4	± .72	0.7%
75	78.4 80.1 78.0		
mean	78.8	±1.10	1.4%
50	53.7 53.8 54.2		
mean	53.9	± .26	0.5%
25	28.8 28.8 29.3		
mean	29.0	± .28	1.0%

number of substrate removal experiments were conducted with this mass culture in various stages of development and at various biomass and soluble substrate concentrations employing gas chromatographic analytical procedures previously developed in this laboratory. The total organic carbon analyzer was employed for total substrate measurements and the oxygraph was used for periodic oxygen uptake determinations.

Through a plate streaking isolation procedure, the predominant microorganisms in this culture were identified as three strains of Pseudomonas Group IV species and a Coryneform species (see Appendix I).

Response of microbial culture to 1-butanol. In Figure 4 the response of the culture at an average biomass concentration of 112 mg/l is illustrated. A thirty minute lag was noted before detectable utilization or disappearance of 1-butanol began. Beyond this time a linear rate of 1-butanol removal was noted with complete disappearance occurring approximately four and one-half hours into the test. The total organic carbon values also reflected the substrate uptake lag then decreased linearly to the minimum value approaching the background carbon value of 18 mg/l at four and one-half hours.

The oxygen uptake curve did not reflect a substrate removal lag. It increased immediately from an endogenous rate of 4 mg/l per hour prior to substrate addition to 25 mg/l per hour after substrate addition. After thirty minutes oxygen utilization increased gradually for two and one-half hours then increased rapidly to 80 mg/l per hour at four hours. This type of oxygen utilization curve has previously

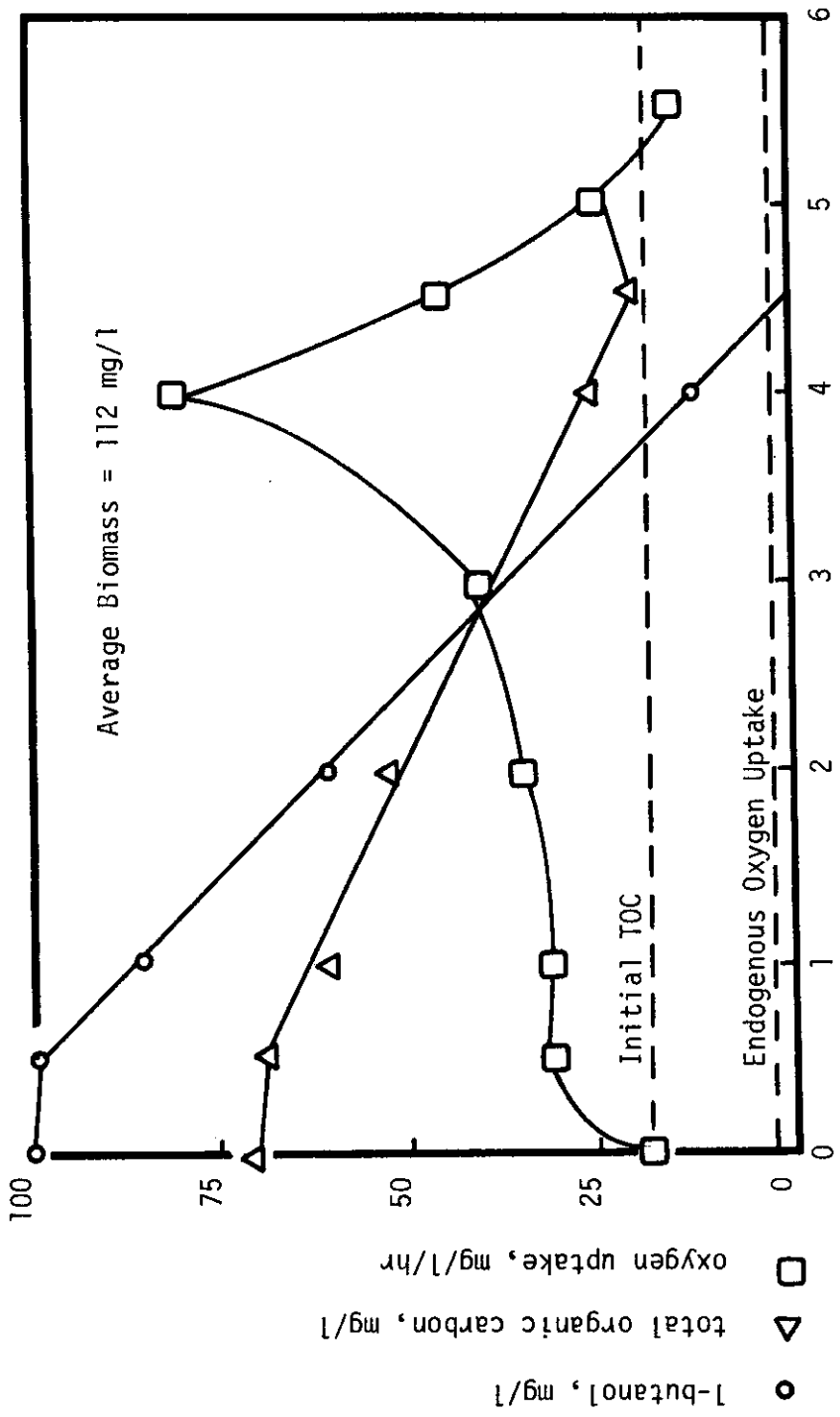

Figure 4. Microbial Response to 1-butanol.

been noted with organic acids by Tyer (16) and may reflect the presence of some butyric acid accumulation, although none was detected in the extracellular medium. After soluble substrate had disappeared from the medium the oxygen utilization rate rapidly returned to a low value.

Graphical integration of the area under the oxygen uptake curve provided a cumulative value of 216 mg O_2 consumed over a period of five and one-half hours. If butanol were completely converted to CO_2 and H_2O by the hypothetical reaction:

$$C_4H_9OH + 6\ O_2 \rightarrow 4\ CO_2 + 5\ H_2O$$

the theoretical oxygen requirement for 100 mg 1-butanol would be 260 mg O_2. The actual value of oxygen consumed, 216 mg, is thus 83 percent of the theoretical oxygen requirement, indicating a respiratory predominance in the culture metabolism, with very little assimilation. This was borne out by very little change in cell mass about the average value of 112 mg/l.

Microbial response to 1-butanol, isopentanol, and 1-pentanol. Figure 5 illustrates the component removal pattern as measured by gas chromatography during the course of culture response to a substrate mixture of 50 mg/l each of 1-butanol, isopentanol, and 1-pentanol. Isopentanol is a primary five carbon alcohol with methyl branching on the number 3 carbon of a four carbon chain. Its IUPAC name is 3-methyl-1-butanol. There appeared to be a selectivity of response among these three alcohols which favored 1-butanol and 1-pentanol.

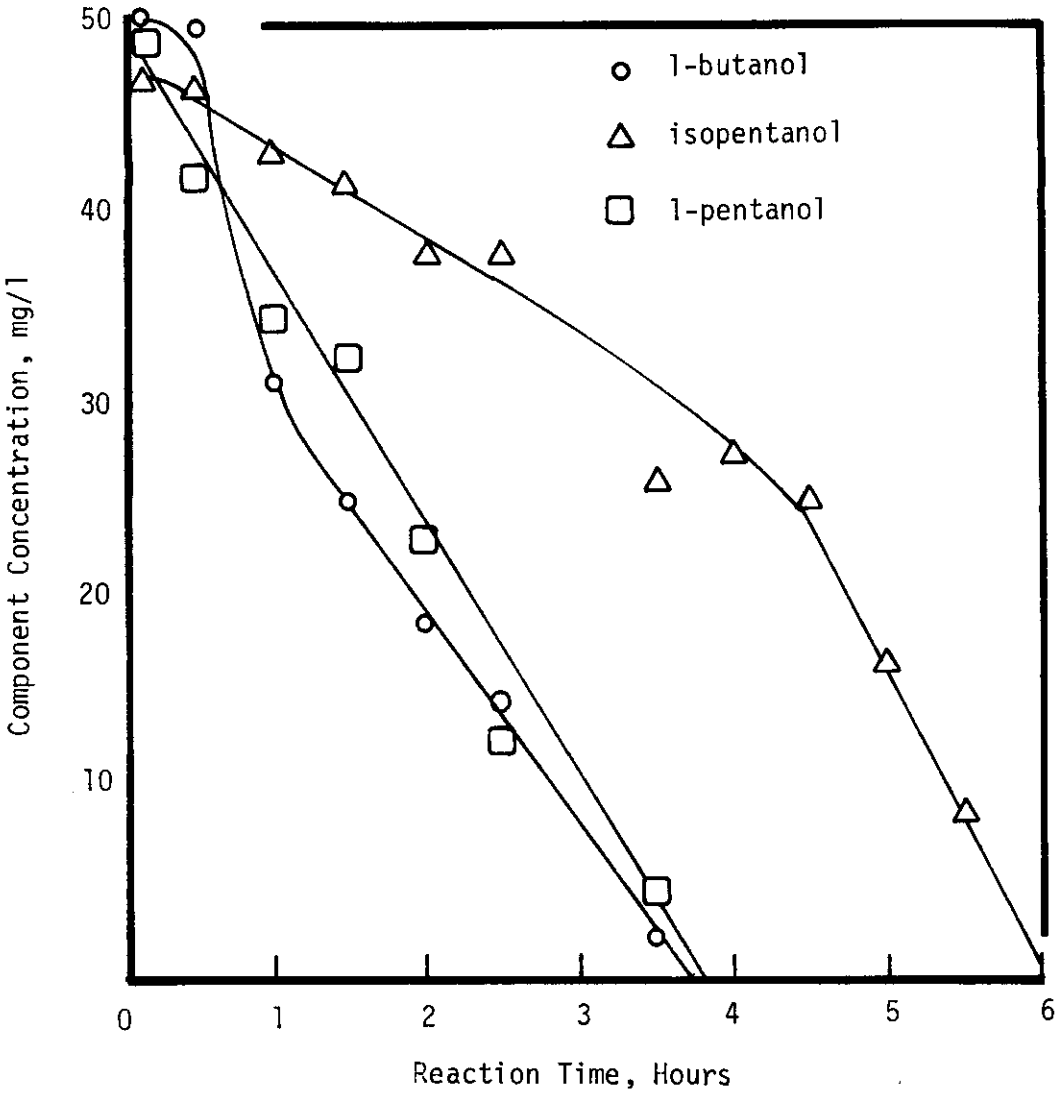

Figure 5: Component Removal During Microbial Response to 1-butanol, isopentanol, and 1-pentanol.

The removal or conversion rate of isopentanol was approximately half as great in the presence of 1-butanol and 1-pentanol (first four hours) as it was in their absence (final two hours). This type of removal retardation has previously been noted among a group of aliphatic ketones (7).

Referring to Figure 6, the total organic carbon removal during the course of microbial response approaches a first order relation with an approximate half-life of two hours in the medium. The oxygen uptake showed an immediate rise from the endogenous value of 7 mg O_2/l per hour to 25 mg/l per hour. It then leveled off at 18 mg/l per hour for about two hours then increased rapidly to 31 mg/l per hour, decreasing gradually as final substrate was removed. Integration of the oxygen uptake curve provided a cumulative quantity of 162.5 mg O_2 consumed over a six and one-half hour period. The theoretical oxygen requirement for the combined substrates, assuming complete respiration was 391 mg. The actual value is 41.5 percent of the theoretical requirement indicating a balance between respiration and substrate assimilation. The cell biomass increased from approximately 360 mg/l to 410 mg/l during this test.

Microbial response to isobutanol, 1-butanol, and 1-pentanol. Isobutanol is a four carbon primary alcohol with methyl branching on the middle carbon of a three carbon chain. Its IUPAC name is 2-methyl-1-propanol. In Figure 7 is illustrated the culture response to a combined substrate of approximately 100 mg/l each of isobutanol, 1-butanol, and 1-pentanol as measured by gas chromatographic methods.

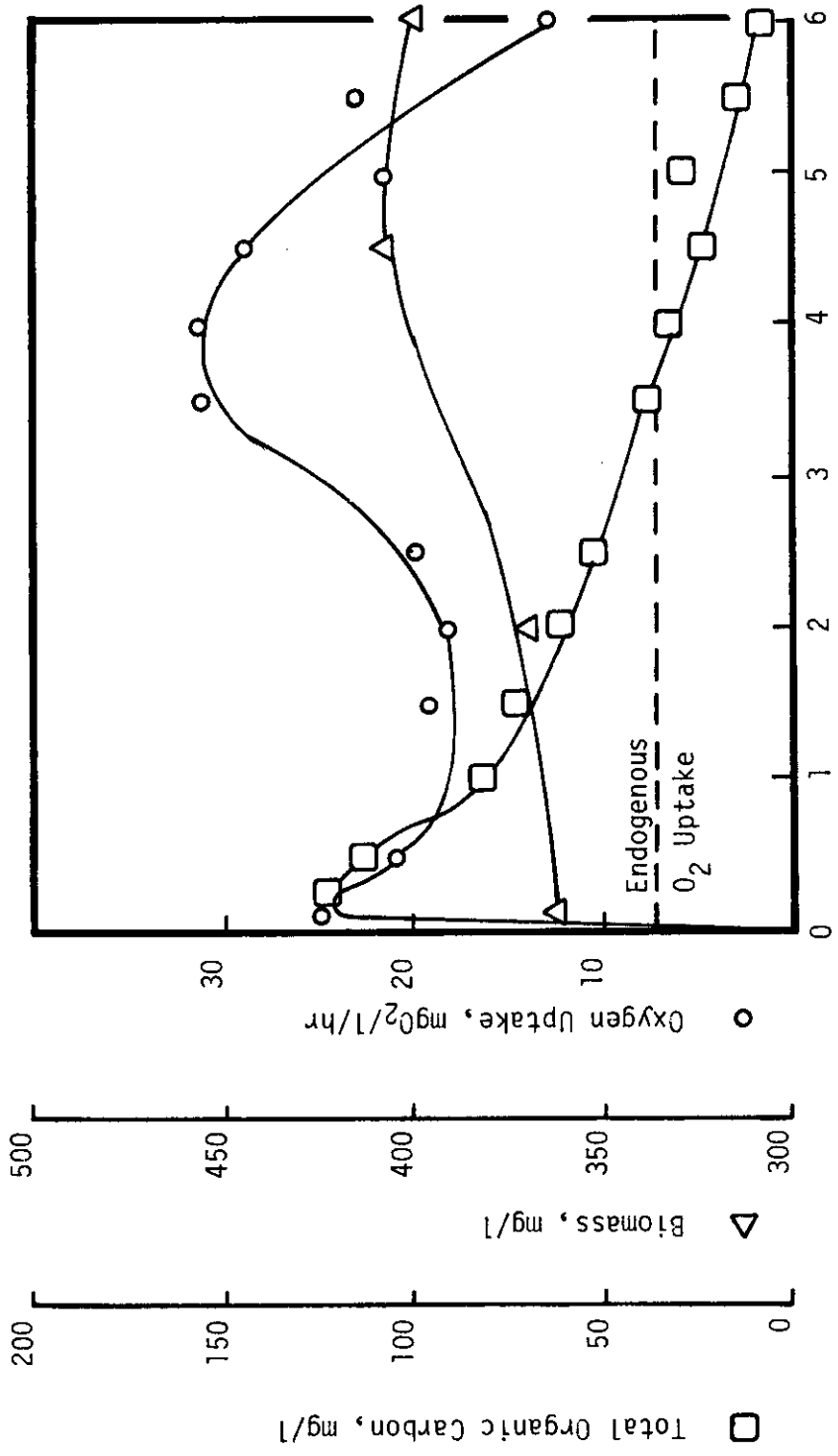

Figure 6: Total Organic Carbon, Oxygen Uptake, and Biomass During Microbial Response to 1-butanol, isopentanol, and 1-pentanol.

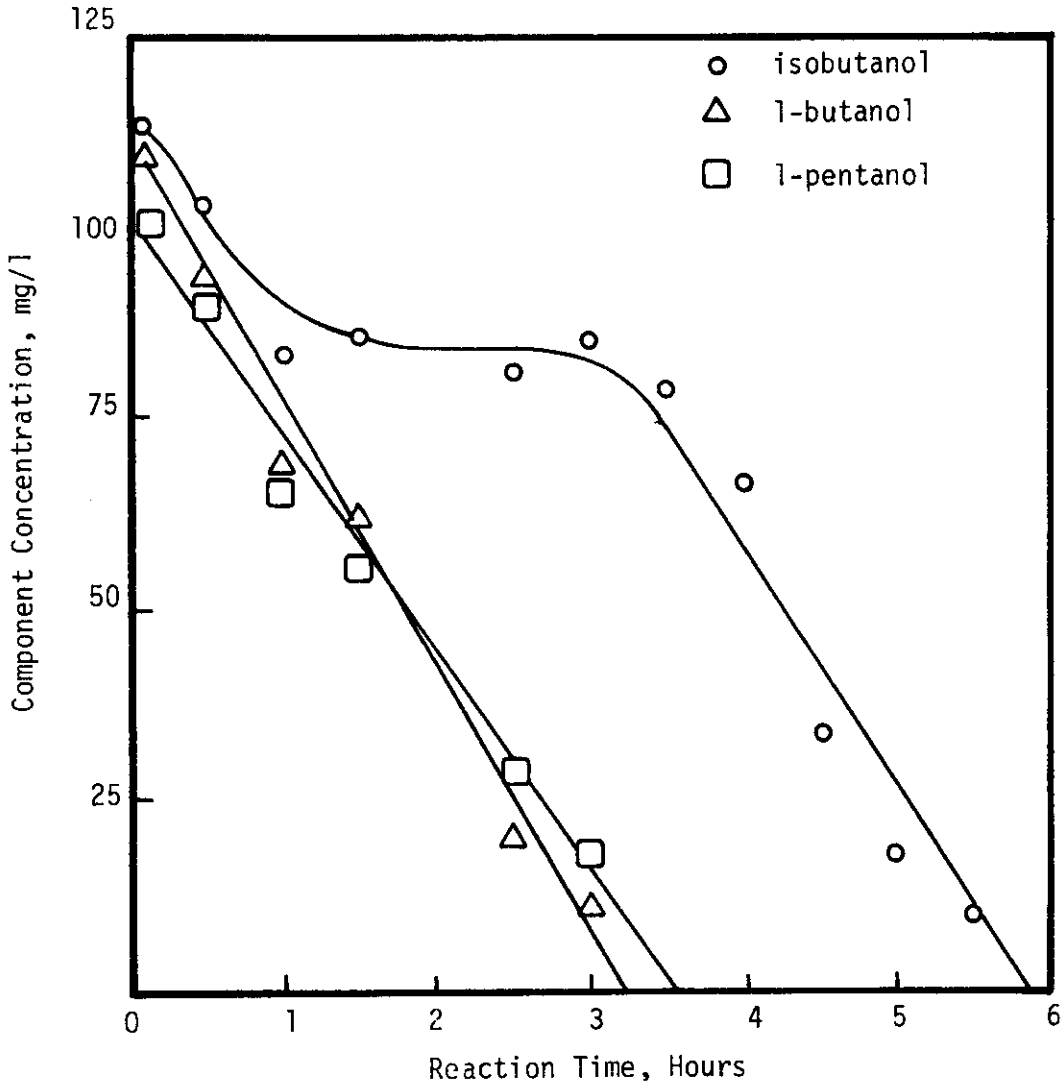

Figure 7: Component Removal of Isobutanol, 1-butanol and 1-pentanol.

Again response to the straight chain alcohols was virtually non-selective but following an initial response to isobutanol, its removal virtually stopped until all 1-butanol and 1-pentanol were removed or converted. After their complete disappearance from the media, removal of the remaining branched chain isobutanol proceeded normally.

Referring to Figure 8, the pattern of total organic carbon removal during this test reflected the possibility of an accumulation of soluble intermediates, however delayed chromatographic analyses detected only the presence of isobutyric acid in a late phase of the test, after isobutanol removal had begun. Although a quantitative value was not obtained this most likely accounts for the failure of organic carbon to return to the initial background level within six hours. Oxygen uptake data were scattered in a cyclic pattern about a line rising from 50 mg/l per hour to 63 mg/l per hour. Integration of the oxygen rate curve over a six hour period provided a cumulative oxygen consumed value of 345 mg O_2. The calculated theoretical value for the combined substrate was 864 mg based on complete oxidation. Therefore, approximately 40 percent of the theoretical oxygen requirement was consumed, again representing a balance between respiration and assimilation. The net increase in biomass was about 270 mg/l during the course of the experiment.

Microbial response to 1-butanol, isobutanol, isopentanol and 1-pentanol. As illustrated in Figure 9, the combined four substrates were added to the microbial culture at a concentration of approximately

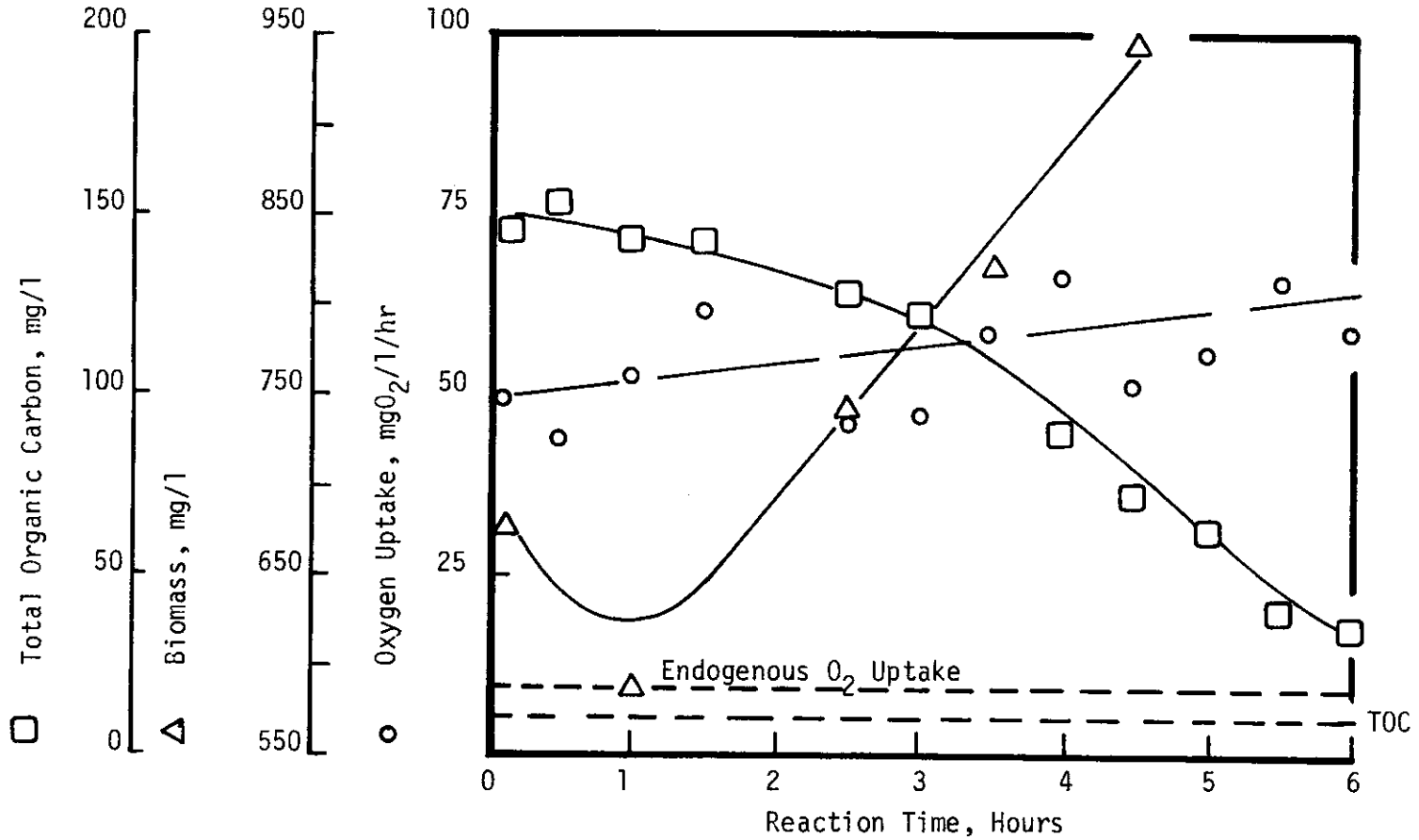

Figure 8: Total Organic Carbon Removal, Oxygen Uptake and Biomass During Microbial Response to Isobutanol, 1-butanol and 1-pentanol.

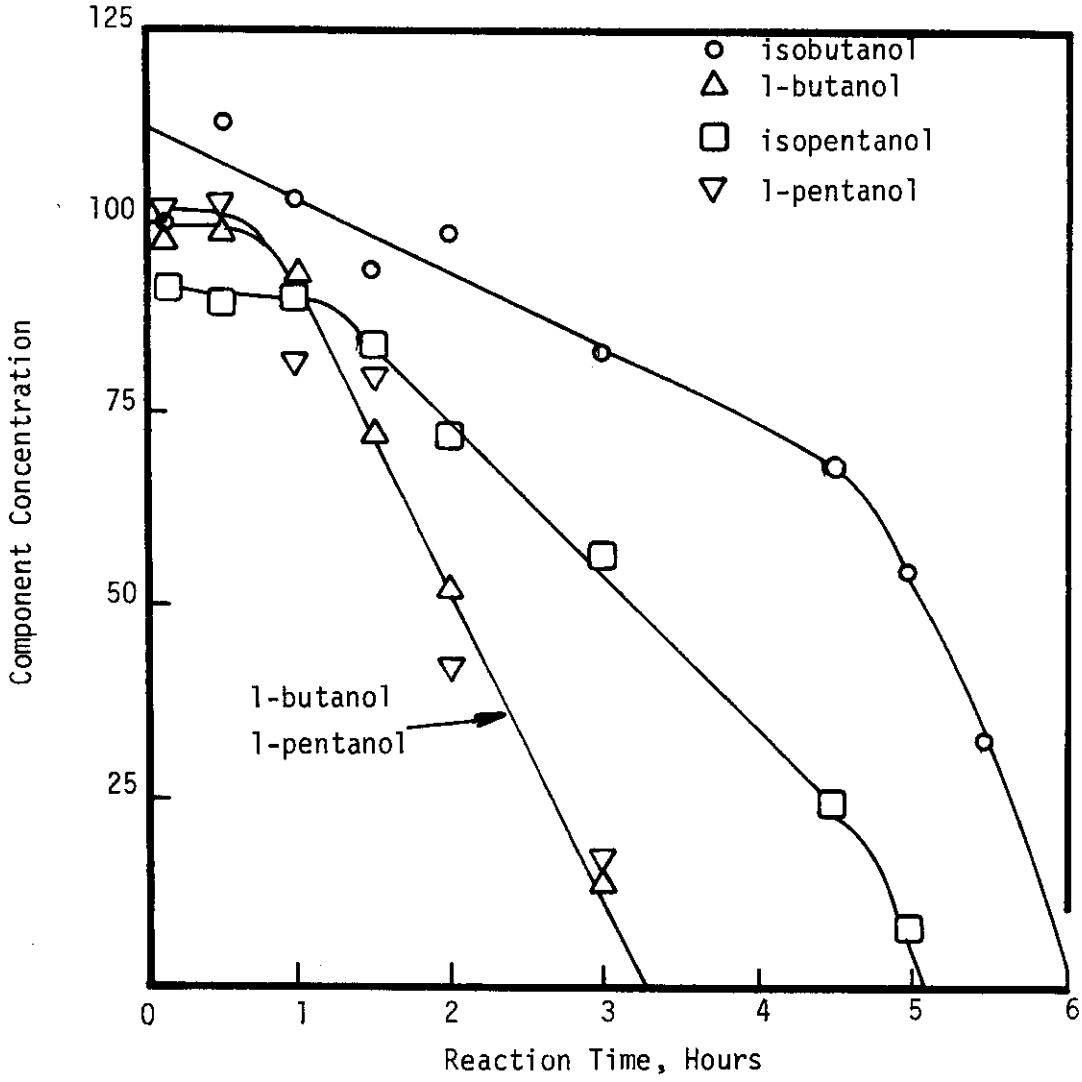

Figure 9: Component Removal of 1-butanol, isobutanol, isopentanol, and 1-pentanol.

100 mg/l each. Again there was virtually no selectivity between the straight chain alcohols while both isopentanol and isobutanol, the branched chain alcohols, were selectively retarded in their removal, isobutanol removal being retarded much more than isopentanol. As in the other tests an increased rate of removal of the branched chain alcohols occurred following the disappearance of the straight chain alcohols.

Referring to Figure 10, total organic carbon removal was only slight for the first two hours, then approached a first order decline during the remainder of the experiment. Oxygen utilization increased rapidly during the initial phase to 244 mg/l per hour then declined slightly to 180 mg/l per hour. After three hours oxygen utilization rate cycled about a line of 140 mg/l per hour ±40. Integration of the oxygen uptake curve provided a cumulative value of 1175 mg O_2 consumed. The theoretical value based on total respiration of substrate added was 1064. Based on these figures the value for oxygen consumed was 110 percent of the value required for theoretical substrate respiration. This would represent a net loss of biomass due to endogenous respiration during the course of the experiment. The average biomass of 1483 mg/l was in fact less than the initial measured value of 164 mg/l; however, biomass data were erratic.

Utilization of 1-hexadecanol and 1-octadecanol. Many problems arise in attempting to apply the techniques described above to an insoluble substrate. The higher alcohols could not be maintained in intimate contact with the media and biomass in aerated reactors due

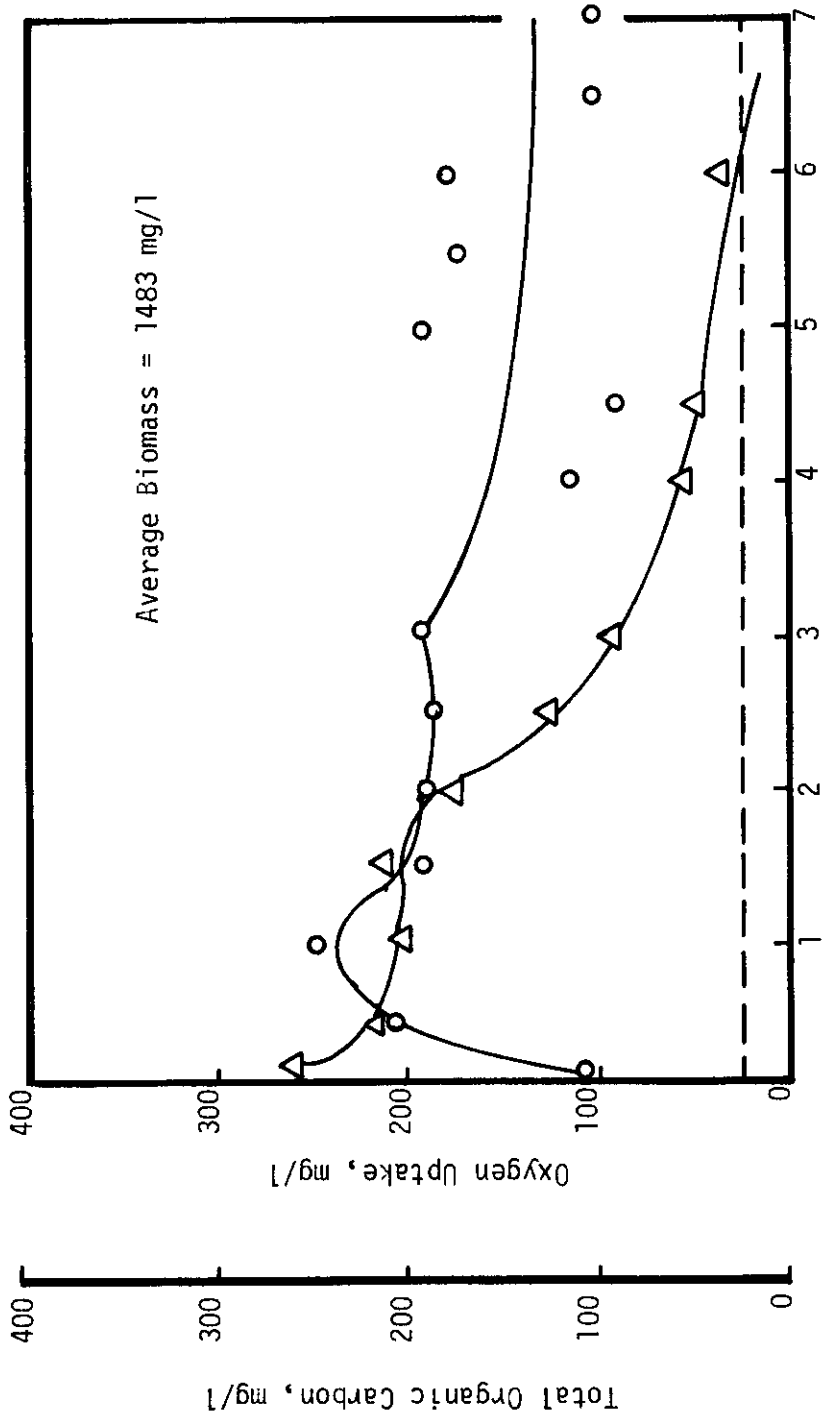

Figure 10: Total Carbon Removal and Oxygen Uptake During Microbial Response to 1-butanol, isobutanol, isopentanol, and 1-pentanol.

to deposition on the reactor walls above the fluid surface. Also the gas chromatographic analytical procedures require solvent extraction to retrieve the unreacted insoluble alcohols. A large quentity of biomass interferes with this extraction procedure by emulsifying the water and solvent phases. Also extraction procedures necessitate complete sample sacrifice for a single analysis and inject an unknown factor into total organic carbon measurements of the water phase.

The systems which were best observed with 1-hexadecanol and 1-octadecanol were then growth systems started from seed organisms in individual shake flasks; such flasks could then be sacrificed for analysis as required.

Uptake of 1-hexadecanol in static culture. Figure 11 represents the initial attempts to measure rate of 1-hexadecanol removal under static conditions in media inoculated with organisms from the reactor maintained in soluble alcohols. The primary purpose of the experiment was to investigate the extraction and gas chromatographic procedure and to investigate the feasibility of static culture as opposed to shake flasks. The hexadecanol added was allowed to film uniformly over the media surface by evaporation of hexane carrier solvent. Over a ten day period a significant removal of 1-hexadecanol did occur. Its concentration relative to water was decreased from 100 mg/l to 75 mg/l. Since only 100 ml samples of media were contained in the flasks this represents a loss of 2.5 mg of the applied hexadecanol or 0.25 mg/day. By the end of the ten day period, the unseeded control samples had begun to lose some hexadecanol, possibly through atmospheric

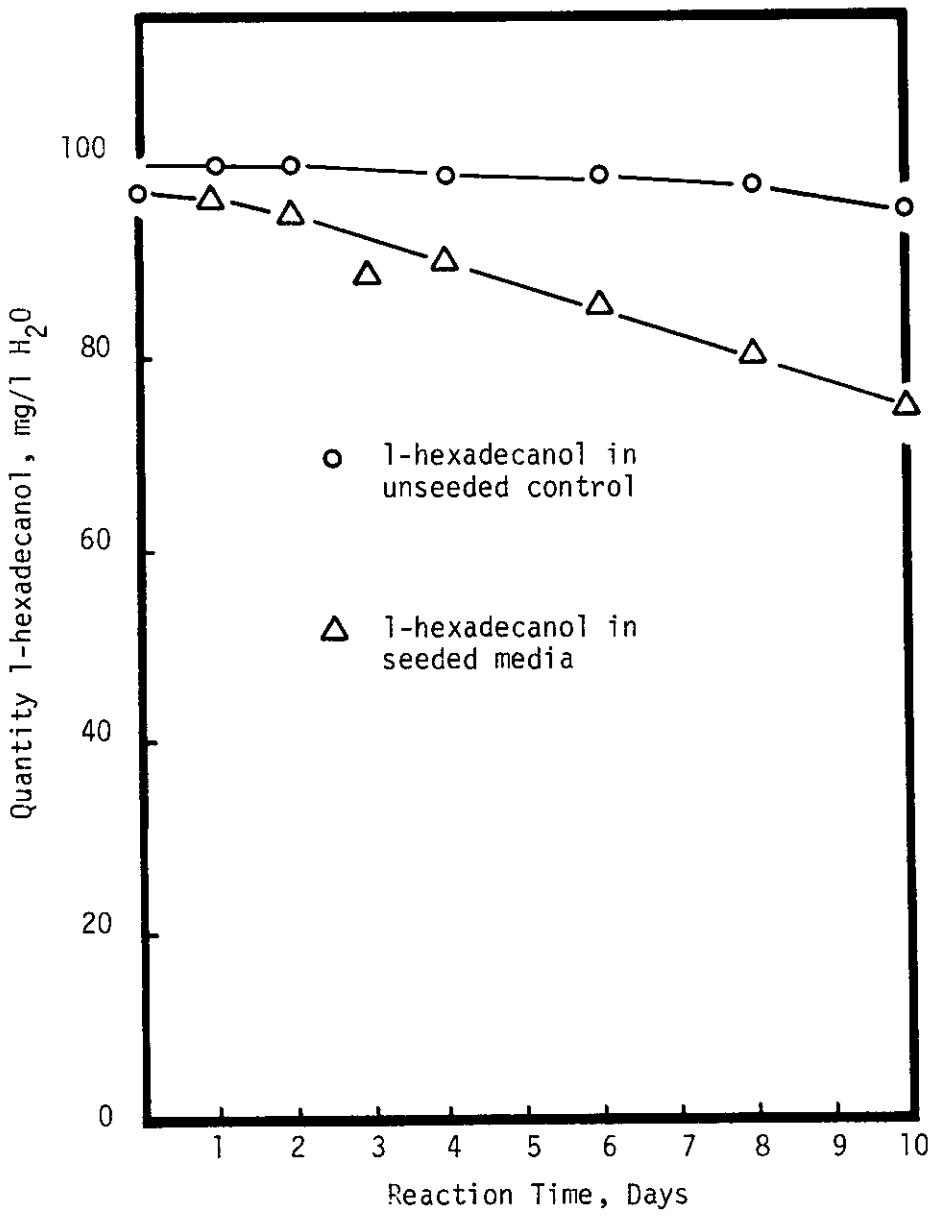

Figure 11: Disappearance of 1-hexadecanol in Static Culture.

contamination. Prior to the initiation of the static culture experiments, considerable operational difficulty had been experienced with attempts to conduct growth and utilization studies in low capacity shake flasks with direct granular substrate addition. The addition through hexane solvent appeared to offer a means of adding 1-hexadecanol to the system in small amounts; therefore, a return was made to the shake flasks.

Growth of microorganisms on 1-hexadecanol and 1-octadecanol in shake flask cultures: incremental substrate addition. Figures 12A, B, C, and D represent a series of experiments in which 1-hexadecanol and 1-octadecanol were added to inoculated media with the substrate by way of a hexane solution. The flasks were placed in a controlled temperature (25°C) shake flask chamber and incubated for seven days. Additional substrate was added after the first, second, and fifth days. Microbial growth lagged through the first day and then increased very significantly through the seventh day. Total organic measurements during the period of growth did not show significant accumulation of intermediates or end products up through the fifth day. At the seventh day, however, the cultures which had been grown on alcohol substrate had from 30 to 45 mg/l soluble organic carbon. The total substrate feed had been 400 mg/l alcohol. The carbon content of 1-hexadecanol and 1-octadecanol is on the order of 80 percent. Therefore, about 320 mg/l total carbon had been applied in the form of alcohols. Thus, about ten to fifteen percent of this returned to the aqueous solution as extracellular carbon by the seventh day. Biomass yield based on the amount of alcohol fed was 66.8 percent for the

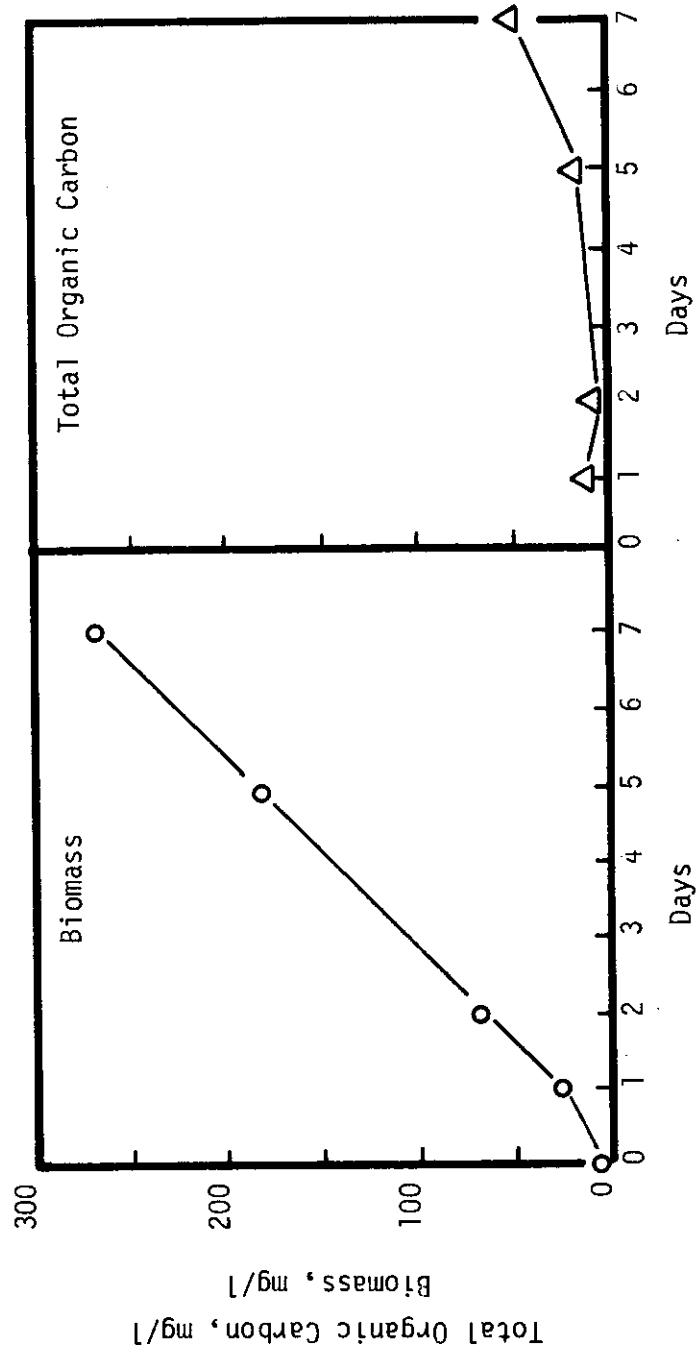

Figure 12A: Microbial Growth and Total Organic Carbon Accumulation on 1-hexadecanol Incremental Substrate Addition.

100 mg/l Hexadecanol added dissolved with 10 ml Hexane on Day 0, Day 1, Day 2, and Day 5

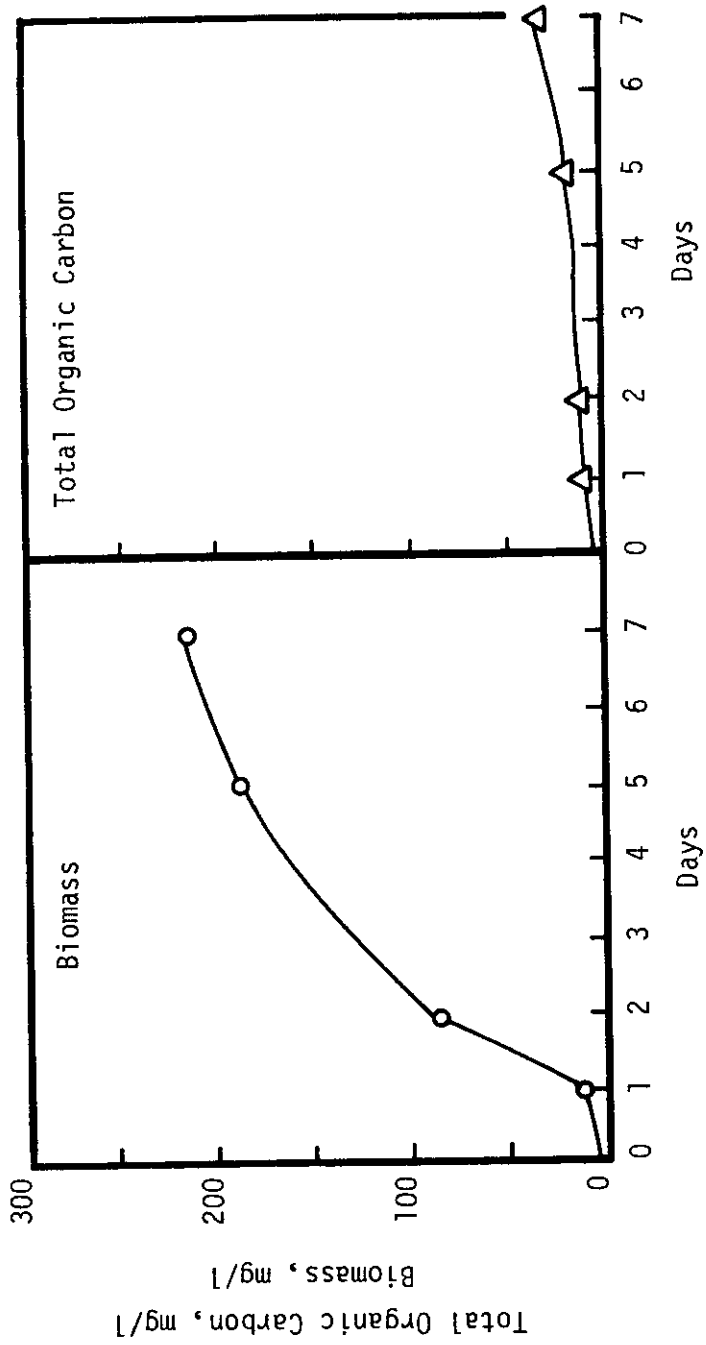

Figure 12B: Microbial Growth and Total Organic Carbon Accumulation on 1-octadecanol.

Incremental substrate addition
100 mg/l 1-octadecanol added dissolved in 10 ml hexane
on Day 0, Day 1, Day 2 and Day 5.

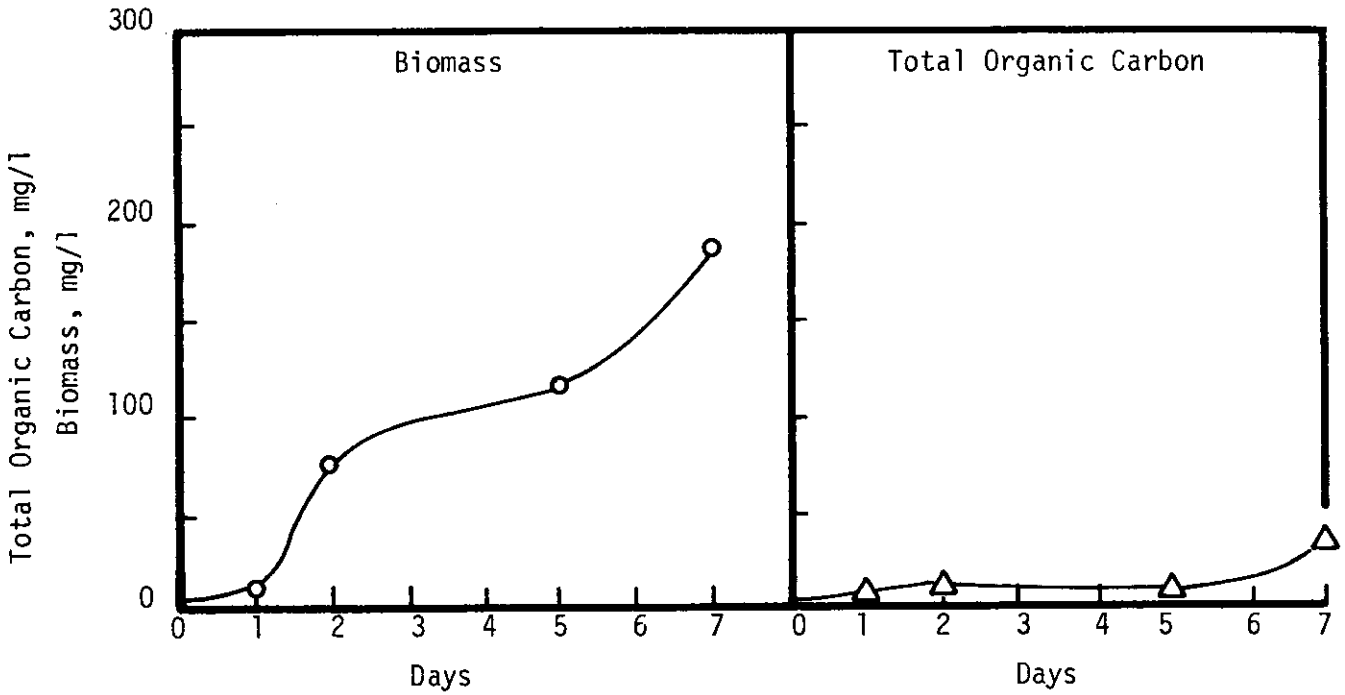

Figure 12C: Microbial Growth and Total Organic Carbon Accumulation on 1-hexadecanol and 1-octadecanol Incremental Substrate Addition.

50 mg/l 1-hexadecanol and 50 mg/l 1-octadecanol dissolved in 10 ml hexane added on Day 0, Day 1, Day 2, and Day 5

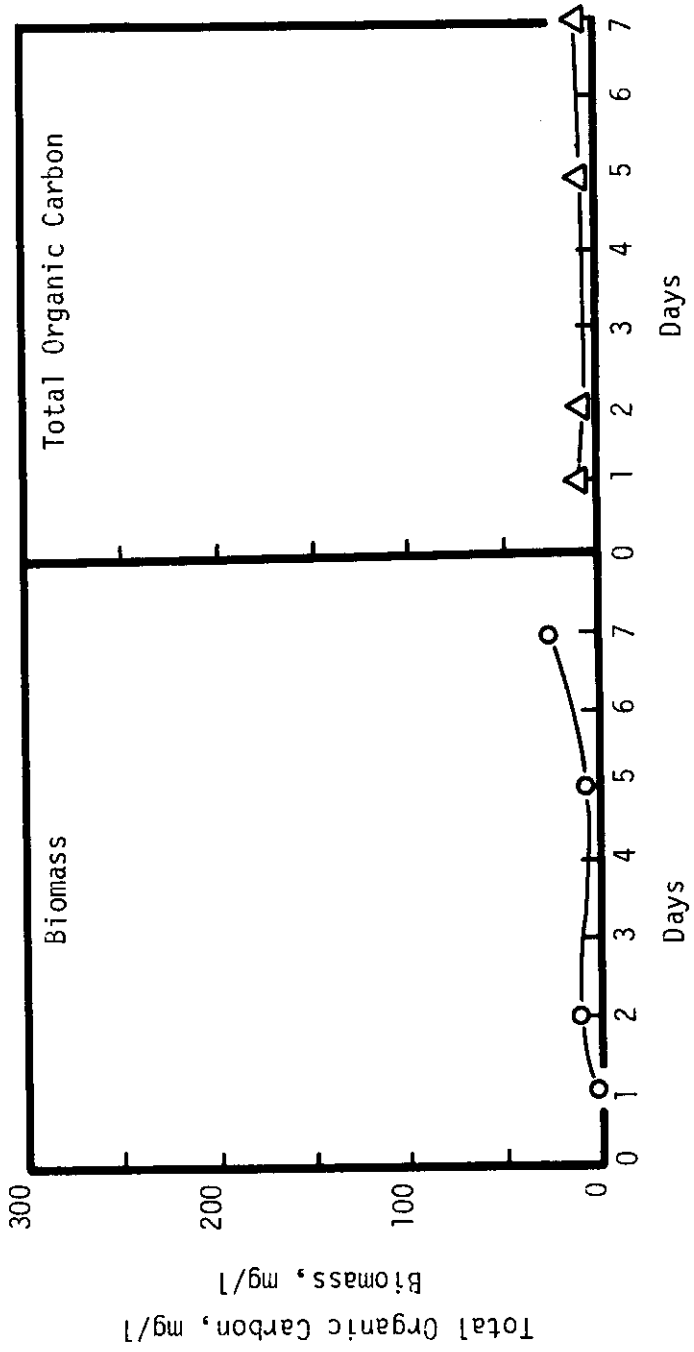

Figure 12D: Microbial Growth and Total Organic Carbon Accumulation on Hexane Control Incremental Substrate Addition.

10 ml Hexane added on Day 0, Day 1, Day 2, and Day 5

culture given 1-hexadecanol, 54.4 percent for the culture given, 1-octadecanol, and 46.5 percent for the culture given a combined substrate.

Gas chromatographic analyses of the aqueous media, neutral and acidified, were not successful in identifying or detecting the source of the organic carbon. There was no residual alcohol in a hexane extract on the seventh day.

Although there was no significant biomass increase of total carbon accumulation in the control medium subjected only to hexane, there was no absolute way to judge the possible adaptation to hexane in the cells growing on 1-hexadecanol and 1-octadecanol.

Uptake of 1-hexadecanol in shake flask cultures: total initial substrate addition in granular form. A fine granular form of 1-hexadecanol was subjected to further testing in two-liter Erlenmeyer flasks in the environmental shaker. In a series of flasks, seeded media was given an amount of 1-hexadecanol equivalent to 100 mg/l. At periodic intervals a flask was sacrificed, subjected to total organic carbon analysis and hexane extraction. Growth of biomass was not measured due to the difficulty of removing a sufficient sample for biomass measurement without disturbing and removing a significant quantity of the granular hexadecanol on the surface. Figure 13 illustrates the removal of 1-hexadecanol over a thirty day period. The final concentration measured, 0.50 mg/l in a 500 ml aqueous sample represents only 0.25 mg hexadecanol of the original 50 mg added available for extraction. The total organic carbon in

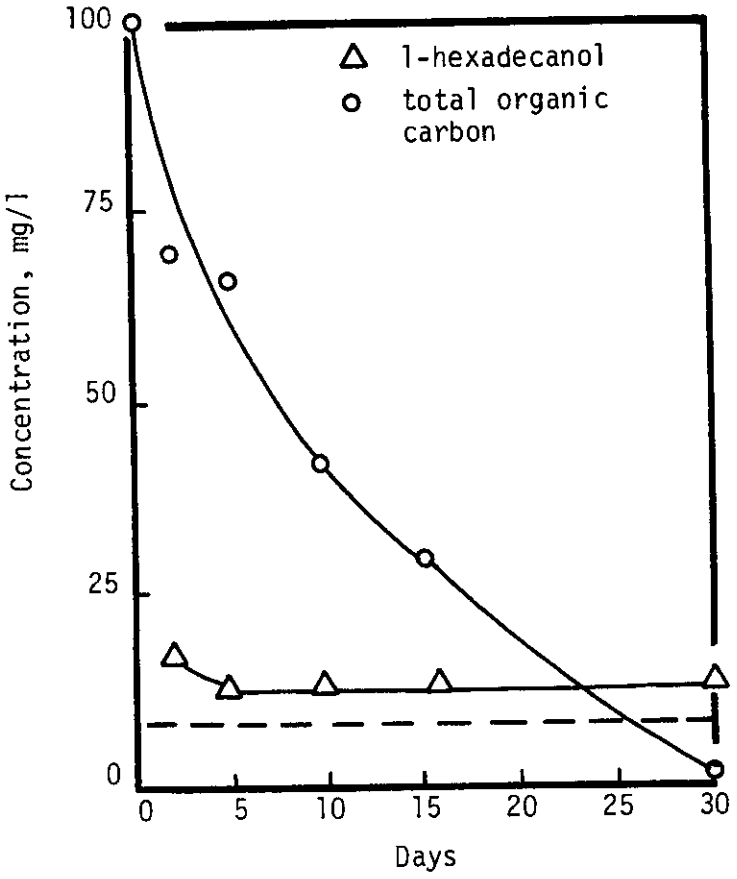

Figure 13: 1-hexadecanol Decline in Seeded Media. 100 mg Granular Hexadecanol Per Liter Mineral Salts Media.

the aqueous phase at the end of thirty days was only 4 mg/l above the total organic carbon in the control flask which had been given no substrate during this time. The highest organic carbon measured occurred at the second day when 17 mg/l, 8 mg/l above the control, was measured.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

The following conclusions are suggested from these studies:

1. Given sufficient time in contact with adapted microbial species under conditions otherwise non-limiting, the complete disappearance of 1-hexadecanol and 1-octadecanol as identifiable molecular species will occur.
2. Gas chromatography possesses sufficient sensitivity and resolving capability as to have potential as a control analytical procedure for measuring hexadecanol and octadecanol in surface films providing sufficiently precise sampling and extraction procedures are employed.
3. Straight chain primary alcohols appear to represent a competitive substrate system with respect to microbial selectivity. In the presence of branched chain primary alcohols, the straight chain forms appear to constitute a preferred substrate system. The branched chain primary alcohols are nonetheless biodegradable. Their rate of degradation is simply retarded.
4. The rate of microbial growth and substrate disappearance is very dependent on substrate availability. They are much more rapid when the insoluble higher alcohol is added in the form of a hexane solution where a homogeneous film is left by evaporation of hexane than when the

higher alcohol is added in granular form so that the film is maintained only by natural replacement.

5. Where the substrate was added in granular form such that disappearance was slow, no significant soluble organic accumulation occurred.
6. Where the substrate was added in dissolved form (hexane solution) and microbial growth was rapid, there was evidence of soluble organic accumulation above the level of controls. Efforts to extract and identify the organics were unsuccessful and final results must generally be held inconclusive due to the presence of the volatile hexane as a complicating factor (for example: some adaptation to hexane could have occurred with the organisms growing on higher alcohols although no such adaptation was observed in the control media; or hexane may have caused some microbial cell dissolution thus releasing cellular products.)

With regard to recommendations for future work, this should be concentrated primarily in field operations. It would be interesting to develop the sampling and extraction procedures for following the fate of applied alcohols in actual reservoirs, conducting, if at all possible or feasible, a complete carbon balance on a small test reservoir. A complicating factor in such a test would certainly be inorganic carbon assimilation by phototrophic organisms. A further field test which should be enlightening would be the investigations of the effect of

application techniques on the rate of biological degradation of the applied monolayers. It should be noted that in Wixon's studies with algae species in laboratory aquaria, he applied hexadecanol and octadecanol in a water soluble isopropanol carrier. The isopropanol itself may have contributed to the increased bacterial cell numbers, a fact which he recognized.

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APPENDIX

IDENTIFICATION PROCEDURES FOR MICROORGANISMS ADAPTED TO PRIMARY ALCOHOLS

Agar plates were prepared with media as in Table A-1. A culture sample was homogenized for 30 seconds in a blender. One ml was serially diluted in a buffered medium and 0.1 ml of each dilution was aseptically inoculated on the plates to yield dilution factors of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}. Uninoculated controls were used as well as a repeat of the above dilution/inoculation procedure with tryptone glucose medium.

After several days incubation at room temperature colonies were counted and streaked for isolation. After three streak platings the organisms were isolated on tryptone glucose agar in screw cap test tubes. This was done with isolates from both the alcohol and tryptone glucose media. The predominant organisms were the same on both media.

Seven organisms were initially isolated. Two of the less predominant species did not survive the isolation procedures. These organisms probably required the metabolic products of the predominant species for survival. Of the five remaining organisms three were decidedly predominant. One of the three remained unidentified.

The organisms and their plate counts were as follows:

Pseudomonas sp. Group IV	600×10^5
Pseudomas sp. Group IV	480×10^5

Unidentified species	320×10^5
Pseudomonas sp. Group IV	100×10^5
Coryneform sp. (not microbacterium)	50×10^5

The organisms were identified by the following characteristics:

Pseudomonas sp. - Aerobic, gram negative, oxidase positive, polar flagellated, no reaction on Hugh Leifson OF medium. E. coli control was positive on OF medium.

Coryneform sp. - Aerobic, gram positive, non-sporing, non acid-fast, unbranched rods, palisades grouping, and does not resist 72°C for 15 minutes.

TABLE A-1

AGAR PLATE MEDIA

Component	Amount Added	Field/Liter
$(NH_4)_2SO_4$ (117 mg/ml)	1 ml	31.2 mg N
$FeCl_3$ (2.9 mg/ml)	1 ml	1 mg Fe^{+++}
$CaCl_2$ (69.3 mg/ml)		25 mg Ca^{++}
$MgCl_2 \cdot 6 H_2O$ (83.5 mg/ml)	1 ml	9.8 mg Mg^{++}
NaCl (43.6 mg/ml)		17.1 mg Na^{+}
		100 mg Cl^{-}
Buffer:	60 ml	786 mg P
KH_2PO_4 (8.5 mg/ml)		27 mg N
K_2HPO_4 (21.75 mg/ml)		66 mg Cl^{-}
$Na_2HPO_4 \cdot 7H_2O$ (33.4 mg/ml)		
NH_4Cl (1.7 mg/ml)		
Alcohols:	32 ml	160 mg of each
ethanol (5 mg/l)		
n-propanol (5 ml/l)		
n-butanol (5 ml/l)		
isobutanol (5 ml/l)		