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TOXICITY OF SYNTHETIC HIGH DENSITY AND CONVENTIONAL HYDROCARBON JET FUELS TO A SOIL BACTERIUM

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FOR THE COMMANDER

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Jet Fuels	RJ-5	Growth Rate
JP-4	MCH	High Density
JP-8	Bacteria	Synthetic
JP-10	Turbidity	
RJ-4	Viability	

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The effects of selected high density and conventional jet fuels on the growth kinetics of a soil microorganism were determined. A culture of Enterobacter cloacae isolated from soil was exposed to various concentrations of each fuel in a mineral salts medium and bacterial growth was monitored turbidimetrically and by viable count techniques. Effects were indicated by observing changes in maximum bacterial growth, growth rate, lag time, and death rate. The majority of the fuels studied manifested their effects by decreasing the number of viable organisms during the stationary growth period. (CONTINUED ON BACK)

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Stable emulsion formation resulted in erroneous turbidimetric determinations. The applicability of bacterial systems as indicators of toxicity of water insoluble jet propellants was discussed.

PREFACE

This study was conducted in the Toxic Hazards Division, Environmental Quality Branch, Air Force Aerospace Medical Research Laboratory. The research was performed in support of Project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Task 04, Work Unit 19, from September 1978 to September 1979.

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INTRODUCTION

The recognition of the consequences of pollution of aquatic systems by hydrocarbons has elicited extensive research efforts concerning the interaction of microorganisms and various simple and complex hydrocarbons, particularly those comprising petroleum (Bartha and Atlas, 1977; Coffey et al., 1977; Ahearn and Meyers, 1973; Bott and Rogenmuser, 1978). Earlier investigations of these interactions described phenomena such as the bacterial utilization of individual hydrocarbons (Bushnell and Haas, 1941; Johnson et al., 1942; Stone et al., 1942) and involvement in geochemical processes (ZoBell, 1945). The development of hydrocarbon/petroleum microbiology as a distinct research area (Beerstecher, 1954; Sharpley, 1964) included both theoretical aspects, i.e., metabolic pathways (Gibson, 1972; Van der Linden and Thijsse, 1965), and pragmatic concerns, e.g., contamination/corrosion in hydrocarbon fuel systems (London, 1974; London et al., 1964; Finefrock and London, 1966). More recent investigations were directed to the development of specifically tailored hydrocarbonoclastic microorganisms as a potential approach for the control or abatement of hydrocarbon pollution (Fall et al., 1979).

The recent introduction of new cruise missiles by the Air Force and Navy initiated a requirement for and subsequent development of lighter, more efficient propulsion systems. A consequential aspect of this effort was the formulation and synthesis of high density, liquid hydrocarbon fuels. The synthesis procedures and chemical properties of the propellants are described by Burdette et al. (1978). MacEwen and Vernot (1979, 1976, and 1974) presented additional information on the chemistry of these compounds. Their structures are presented in Figure 1; some of the more pertinent physical and chemical characteristics are shown in Table 1, including similar information on the conventional, petroleum-derived turbine engine fuels JP-4, JP-5, and JP-8. (JP-9 is included in Table 1 since it is a blend of RJ-5, JP-10, and methylcyclohexane-MCH).

The environmental impact of inadvertent intrusion of potentially toxic molecules can only be ascertained by exposure of representative species or model ecosystems under controlled conditions. The selection of the most appropriate target plant, animal or other bioform (insect, protozoan, cell culture) is determined by many factors including cost, handling constraints, observation/data collection technics, time, and applicability to anticipated field exposure conditions.

The toxicity of conventional and synthetic, high density fuels to mammalian species has been studied by MacEwen and Vernot (1979, 1978, 1976, 1975, and 1974), Klein et al. (1976), and Klein and Jenkins (1979, 1978, 1977, and 1976).

London (1979) described a simple method using bacterial growth kinetics as a dose-dependent measurement of the toxicity of hydrazine and its derivatives. In essence, this method utilized turbidimetric determinations of the growth of a soil bacterium in a mineral salts medium containing various concentrations of the propellant. Since the jet fuels can exert a toxic effect to diverse bioforms, and in view of the various responses of microorganisms to different hydrocarbons, a study was initiated to determine the effect of these fuels on a soil microbe. This report describes the application of the turbidimetric procedure to evaluate the toxicity of the synthetic, high-density fuels to bacterial systems.

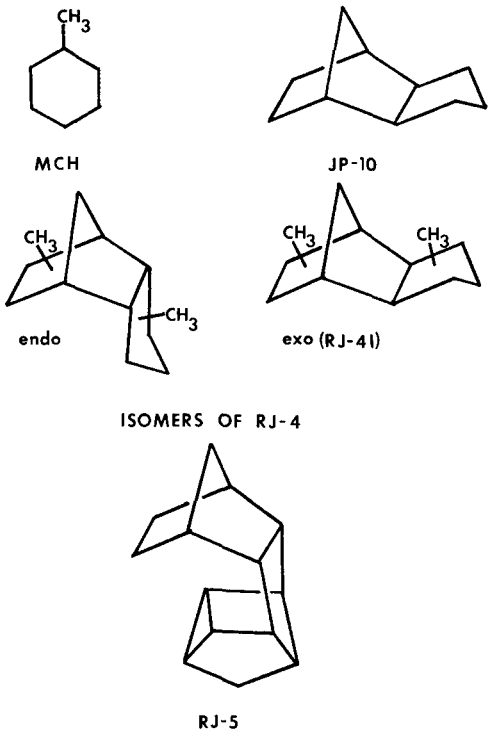


Figure 1. Structures of high-density turbine engine fuels.

TABLE 1

CHEMICAL AND PHYSICAL PROPERTIES OF CONVENTIONAL AND SYNTHESIZED JET FUELS

	JP-4	JP-5	JP-8	JP-9	JP-10	RJ-4	RJ-5
Chemical Formula, Av.	C9.5H18.9	C10H19	C10H19	C10.6H16.2	C10H16	C12H20	C14H18
C:H Ratio	0.50	0.53	0.53	0.65	0.62	0.60	0.78
Molecular Weight, Av.	132.90	139.00	139.00	143.00	136.00	164.00	186.00
Heating Value, KBTU/Gal	118.00	125.00	123.00	142.00	142.00	140.00	161.00
Specific Gravity, $60^{\circ}F$	0.77	0.83	0.83	0.94	0.94	0.94	1.08

MATERIALS AND METHODS

The organism used in this study, designated as D-31, is a soil isolate identified as Enterobacter cloacae. Its characteristics upon initial isolation (London, 1979) resulted in a tentative identification of Paracoccus denitrificans; however, after repeated subculture, changes in biochemical determinants occurred (probably due to selection) permitting a definitive identification of E. cloacae. Since the primary isolation, the growth pattern in minimal medium (SMS) and response to hydrazine and its methylated derivatives has not changed. The culture is maintained in the lyophylized state and on slants prepared from Tryptic Soy Broth (Difco) supplemented with 1.5% Bacto-Agar (Difco), and stored at 10°C. New slants are prepared every six months.

Samples of the high density fuels and several conventional hydrocarbon fuels were obtained in small quantities from H. Lander, Air Force Aero Propulsion Laboratory. The synthesized, high density fuels were portions of pilot production batches from various sources. JP-8 was a representative sample of commercial jet fuels. JP-4NA designates a production sample of Air Force standard jet fuel without the various additive compounds (e.g. corrosion inhibitor and anti-icing agent) included in operational fuel. A less refined sample of fuel, identified as JP-4CD, was also included for study. The fuel designated RJ-4 is a mixture of exo and endo isomers; RJ-4I is the exo isomer only (refer to Figure 1). Each sample was stored in dark bottles at room temperature, and sterilized by membrane filtration (Gelman No. 60170, 0.45 μm) before use.

All growth studies were conducted in Nephelco Culture Flasks (Bellco #2574-19135) containing SMS supplemented with glucose as described by London (1979). In range-finding experiments, the sterile fuel was added aseptically through the rubber serum stopper by microliter syringe in concentrations of 1, 10, 100, and 1000 μl/ 100 ml of medium. Since 1 and 10 μl additions did not result in any observable effect, subsequent investigations were concerned with 100 and 1000 μl additions only. Each experimental condition was established in duplicate; the same inoculum was used for all flasks in each of the three summary experiments which included all the fuels studied. Turbidity determinations were made with the Coleman Junior spectrophotometer at 570 nm at 2-hr intervals until the cultures attained stationary growth and periodically thereafter. Since emulsion formation occurred to a greater or lesser extent in all the flasks, they were placed in a horizonal position in test tube racks supported by the side-arms for 5 minutes prior to reading to allow separation of the emulsion. It was not possible to completely remove the emulsion agglomerates from the surface of the side arm in all flasks; thus, some error was incurred in obtaining culture density measurements.

Viable counts were obtained by removing 0.1 ml aliquots from the flasks via the serum stopper and diluting in 9.9 ml Tryptic Soy Broth (Difco). Dilutions of 1×10^{-6} were prepared in duplicate sets and 0.1 ml drops plated in triplicate on Plate Count Agar (Difco) in three compartment Y Plates (Falcon). Viable counts are reported as colony forming units (cfu)/ml and are the average of 6 determinations. Standard deviations of the viable count data from all experiments did not vary usually more than $x \pm 15\%$ and were in most cases $\pm$ 10% or less.

RESULTS

The growth responses of D-31 to conventional and synthetic jet fuels, as determined turbidimetrically, are presented in Figures 2A and 2B. These curves,

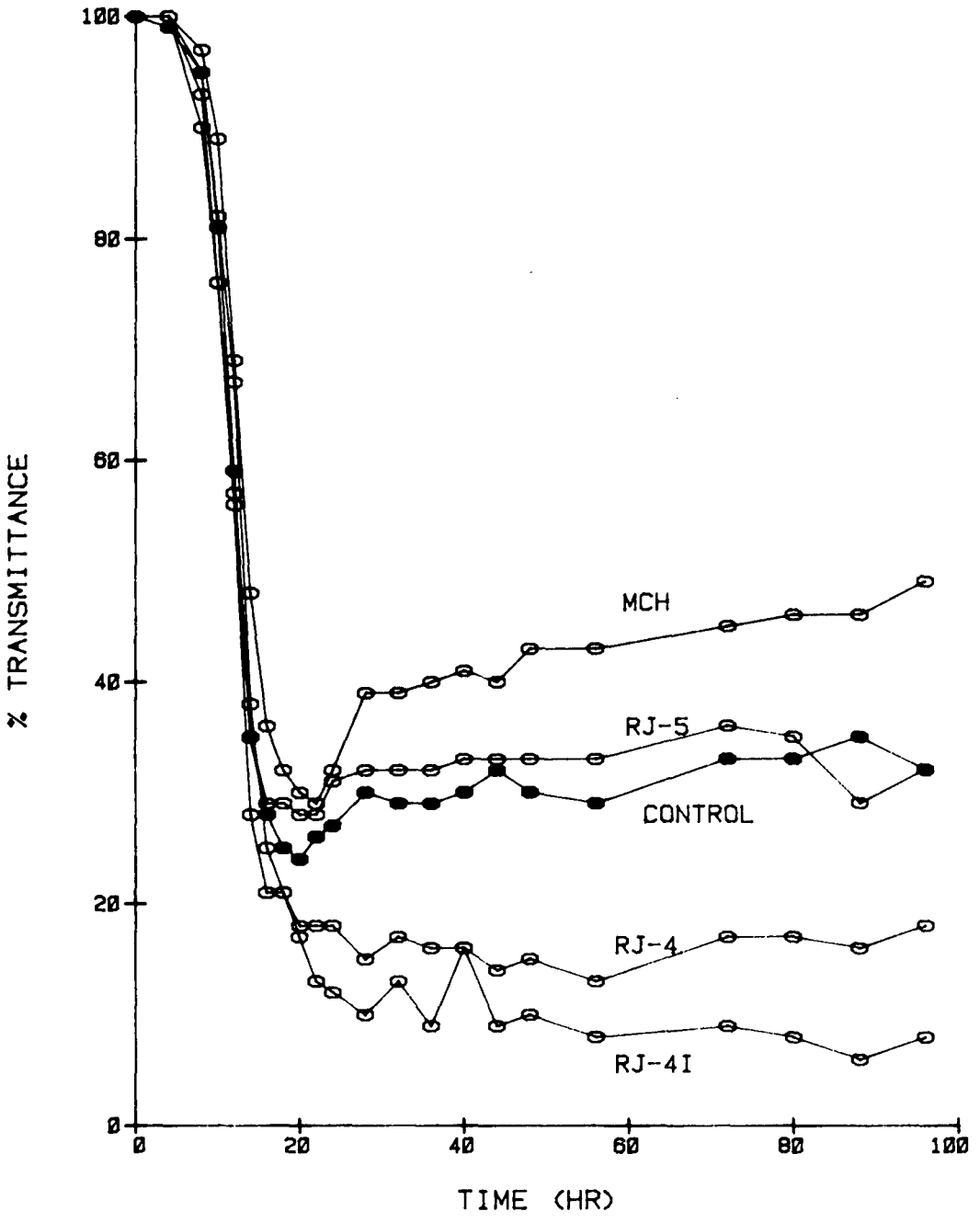


Figure 2A. Growth response of D-31 in the presence of 1% (v/v) of selected jet fuels.

representing the effect of 1.0 ml fuel/100 ml medium, indicate a lack of inhibition in the lag and log phases of growth since all curves exposed to a fuel responded identically to the control culture during these periods (approx. the first 15 hours). After 25 hours of growth, i.e., when the stationary phase was

reached, distinct differences were apparent: two fuels, RJ-5 and JP-10, had no effect on culture turbidity; growth in MCH resulted in a reduced culture density; and RJ-4, RJ-4I, JP-8, and JP-4 appear to have induced an increase in final growth yield.

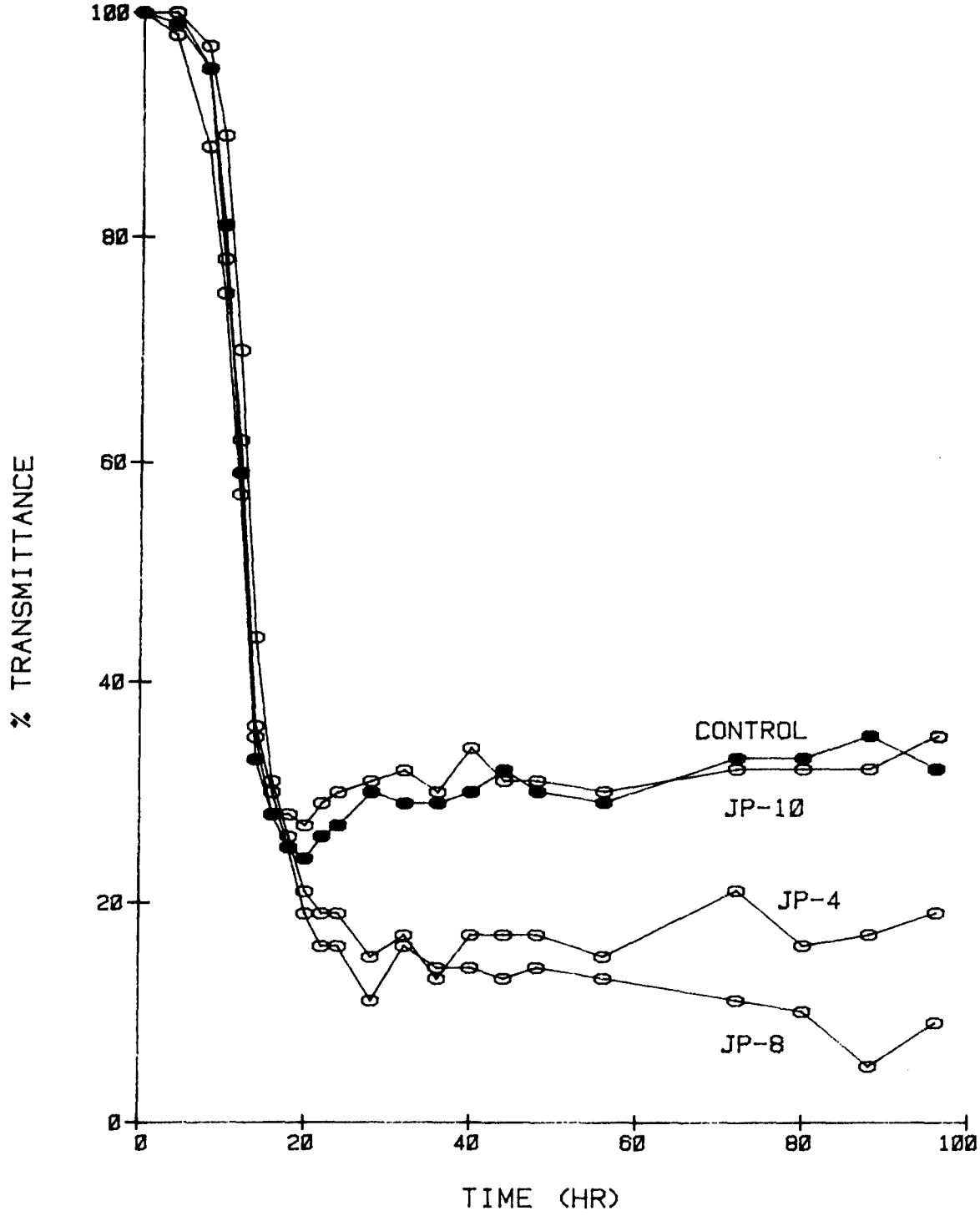
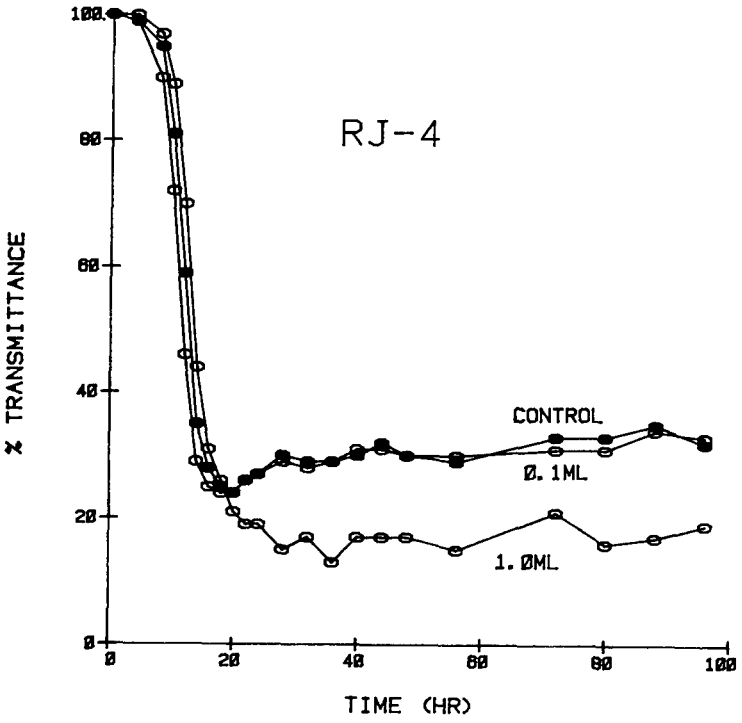
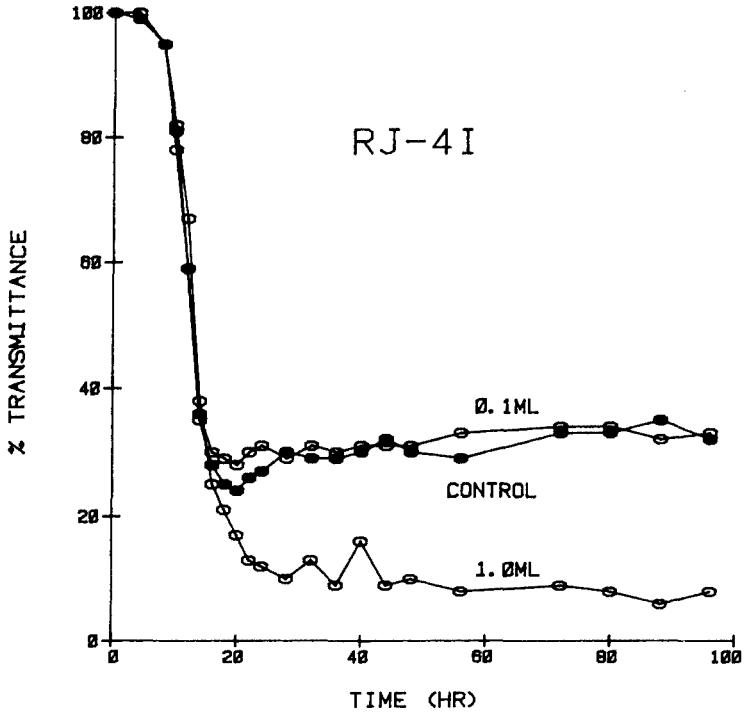


Figure 2B. Growth response of D-31 in the presence of 1% (v/v) of selected jet fuels.

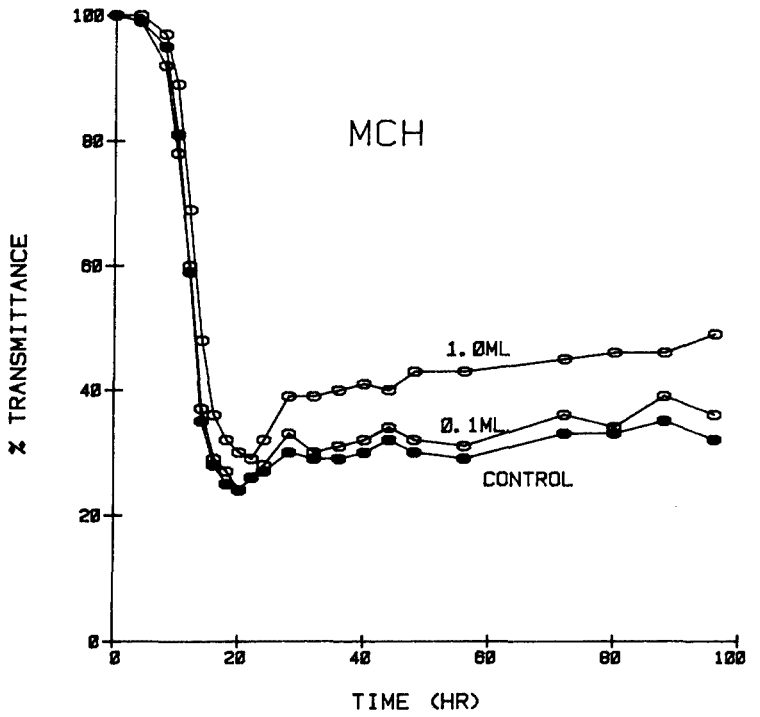
Figures 3A-3G are comparisons of the effects on culture turbidity of 0.1 and 1.0 ml/100 ml exposures of D-31 to each fuel. These curves show a varied concentration response with each fuel, i.e., the effect of 1.0 and 0.1 ml amounts when compared with the control culture turbidity in stationary phase. The 0.1 ml levels of RJ-4, RJ-4I, and MCH (Figures 3A-3C) did not affect the growth yield of D-31; however, 1.0 ml of RJ-4 and RJ-4I produced an apparent increase in final culture density while 1.0 ml of MCH resulted in a reduction in turbidity. The curves for JP-8 (Figure 3D) indicate a concentration dependent increase in stationary growth response. For JP-4 (Figure 3E), the 0.1 ml level provided a final growth response slightly less than or equal to the control while the 1.0 ml exposure resulted in an increase in culture turbidity. RJ-5 exposure (Figure 3F) induced a response inverse of that observed for JP-4: 1.0 ml of fuel resulted in a stationary turbidity slightly less than or equal to the control while the 0.1 ml exposure resulted in a final response somewhat greater than the fuel-free culture. Figure 3G presents the effect of JP-10 on final culture density: D-31 inoculated in 1.0 ml of fuel/100 ml of medium produced a growth response identical to that of the control culture; after approximately 50 hours, 0.1 ml of JP-10 resulted in a decrease in final turbidity. Thus the data presented in Figures 2 and 3 show that all the fuels studied at the 0.1 and 1.0 ml levels did not alter the normal growth pattern of D-31 during the lag and log stages of growth but did exert an effect during the stationary phase.



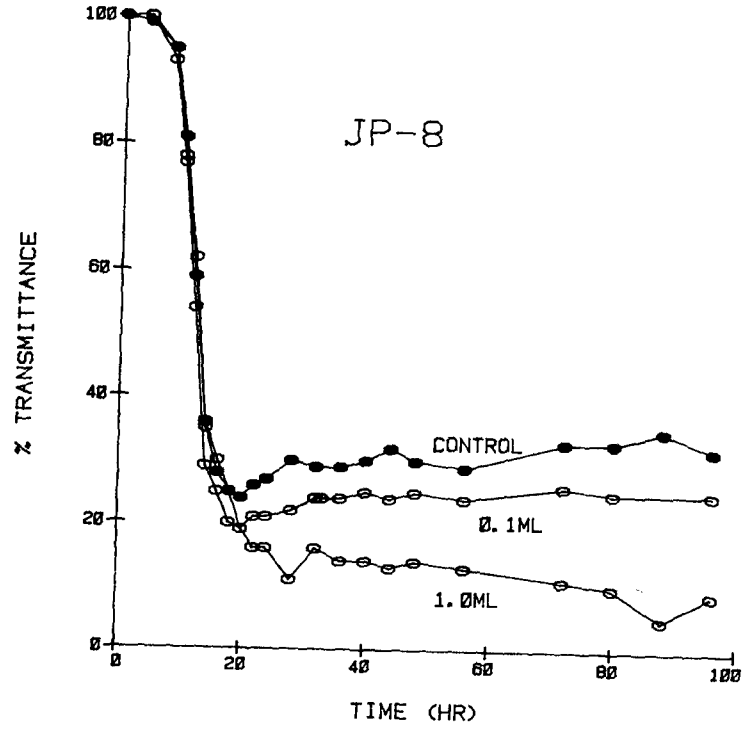
3A. Growth curves of D-31 in 0.1 and 1.0 ml of selected jet fuels per 100 ml of SMS medium.


RJ-4I
% TRANSMITTANCE
TIME (HR)
Ø.1ML
CONTROL
1.ØML
100
80
60
40
20
0
0
20
40
60
80
100

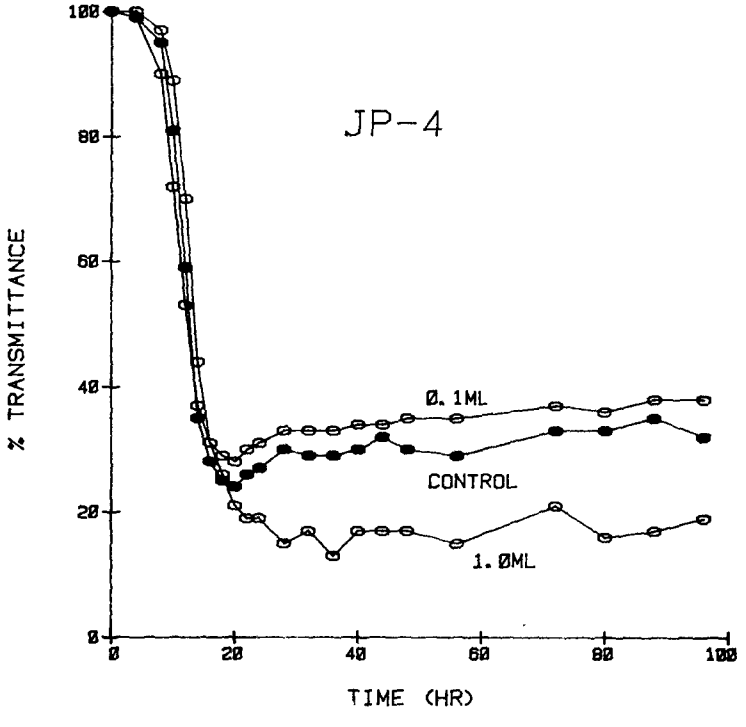
3B


MCH
% TRANSMITTANCE
TIME (HR)
1.ØML
Ø.1ML
CONTROL
100
80
60
40
20
0
0
20
40
60
80
100

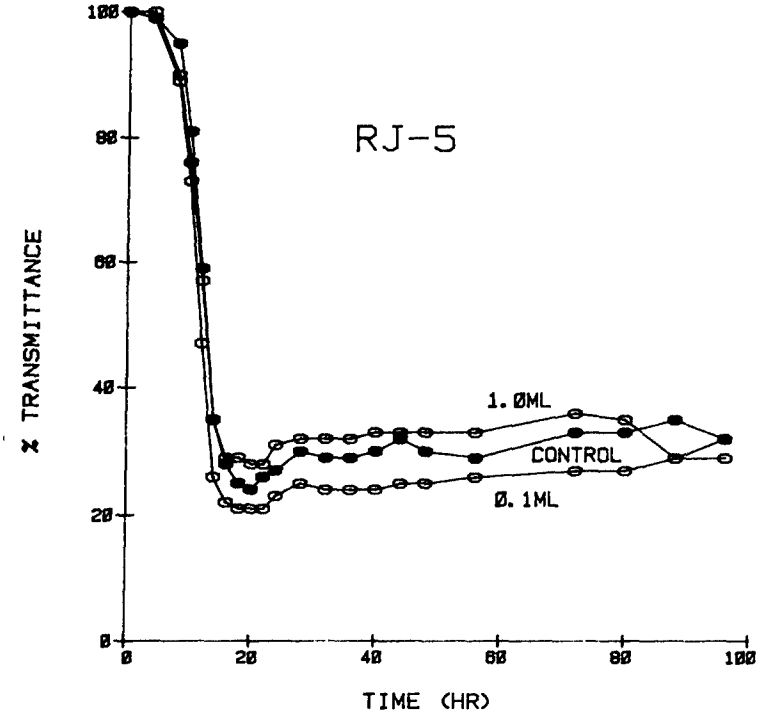
3C


JP-8
% TRANSMITTANCE
TIME (HR)
CONTROL
Ø.1ML
1.ØML
100
80
60
40
20
Ø
Ø
20
40
60
80
100

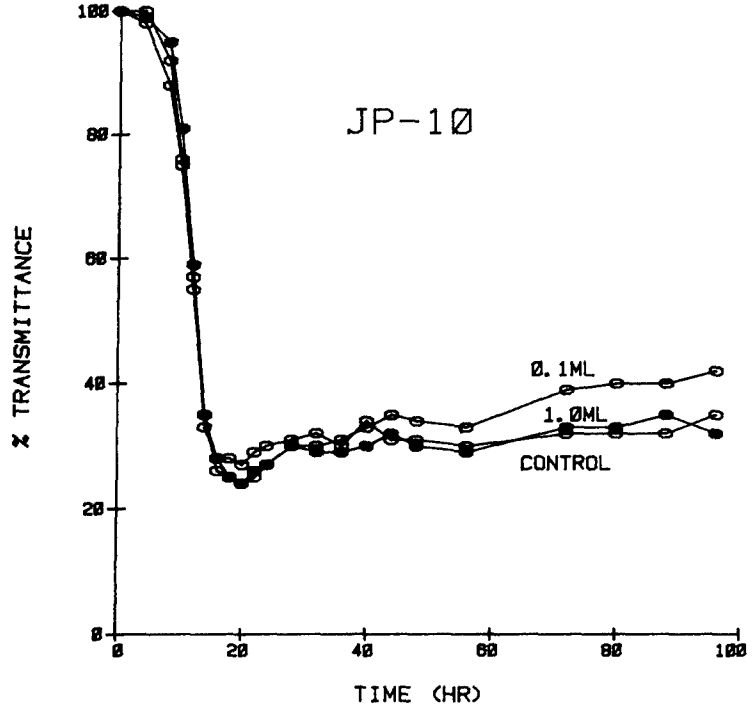
3D


JP-4
% TRANSMITTANCE
TIME (HR)
Ø.1ML
CONTROL
1.ØML
100
80
60
40
20
Ø
Ø
20
40
60
80
100

3E


RJ-5
% TRANSMITTANCE
100
80
60
40
20
0
0
20
40
60
80
100
TIME (HR)
1.0ML
CONTROL
0.1ML

3F


JP-10
% TRANSMITTANCE
100
80
60
40
20
0
0
20
40
60
80
100
TIME (HR)
0.1ML
1.0ML
CONTROL

3G

The viable count data obtained during the stationary growth phase are presented in Figures 4A-4D. With the exception of RJ-5 exposure and the 96-hr count for RJ-4, which resulted in viable cell counts essentially the same as the control culture, all fuels caused a reduction in cell number. The difference between the 1.0 and 0.1 ml levels of each fuel is not as pronounced as would be expected if the effect in cell reduction were concentration dependent (in terms of volume of fuel added to the growth flasks). The 1.0 ml exposures (Figures 4A and 4B) resulted in a range of reduction in cell viability with JP-8 initiating the smallest effect and MCH the greatest decrease. Note that the control culture remained stable during the stationary period while the fuel-exposed cultures exhibited changes in cell viability. The cultures grown in the presence of 0.1 ml amounts of the fuels (Figures 4C and 4D) showed a reduction in viable count to a lesser extent than the 1.0 ml exposures and with some differences in the relative degree of reduction from each fuel. The 0.1 ml concentrations showed similar results with some changes in order of inhibitory effect.

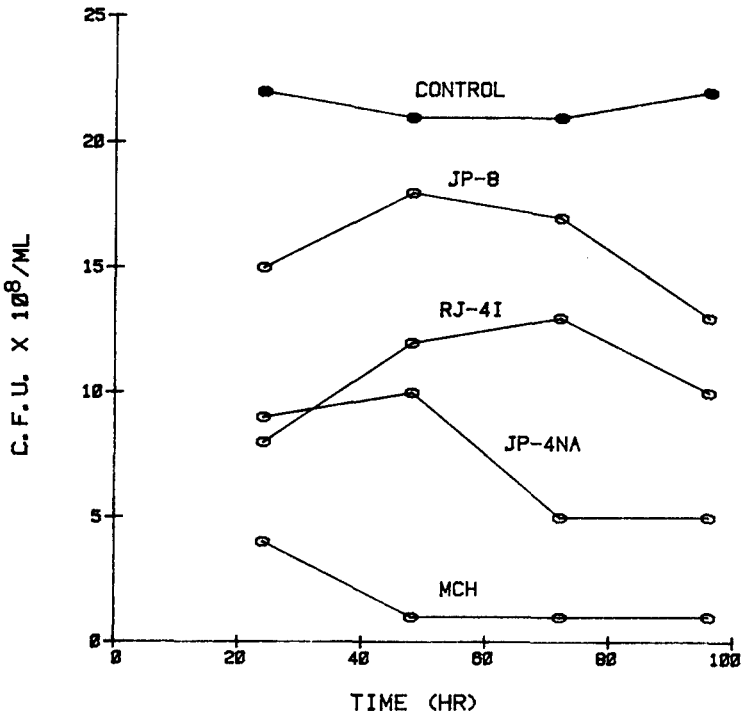


Figure 4A. Viability of D-31 grown with 1.0 ml/100 ml (A and B) and 0.1 ml/100 ml (C and D) of selected jet fuels during stationary phase.

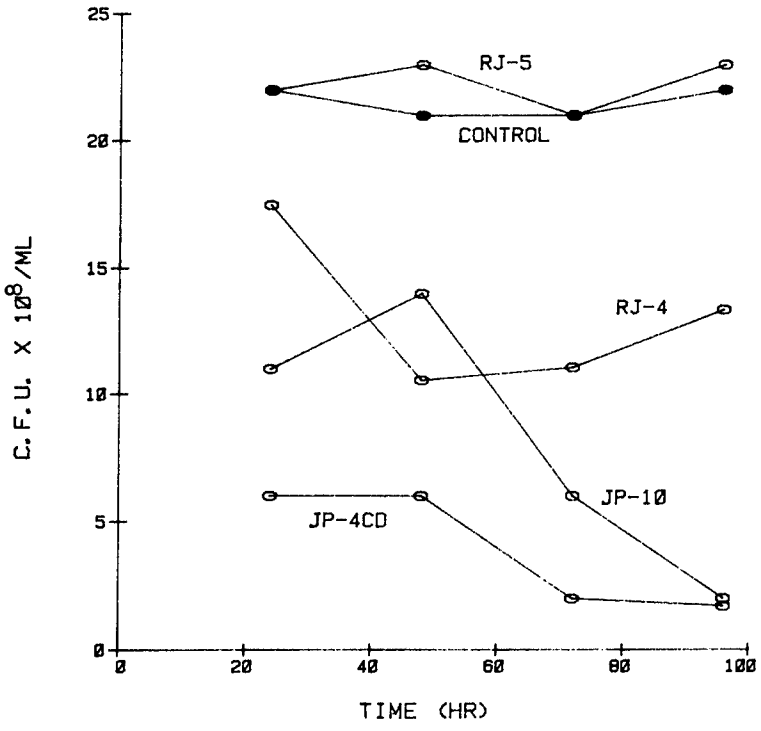


Figure 4B.

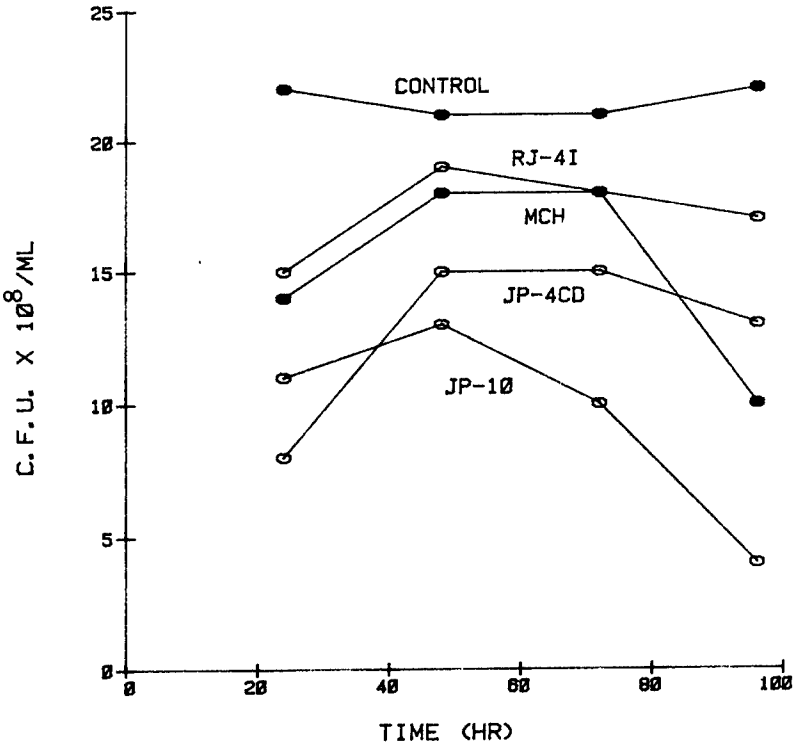


Figure 4C.

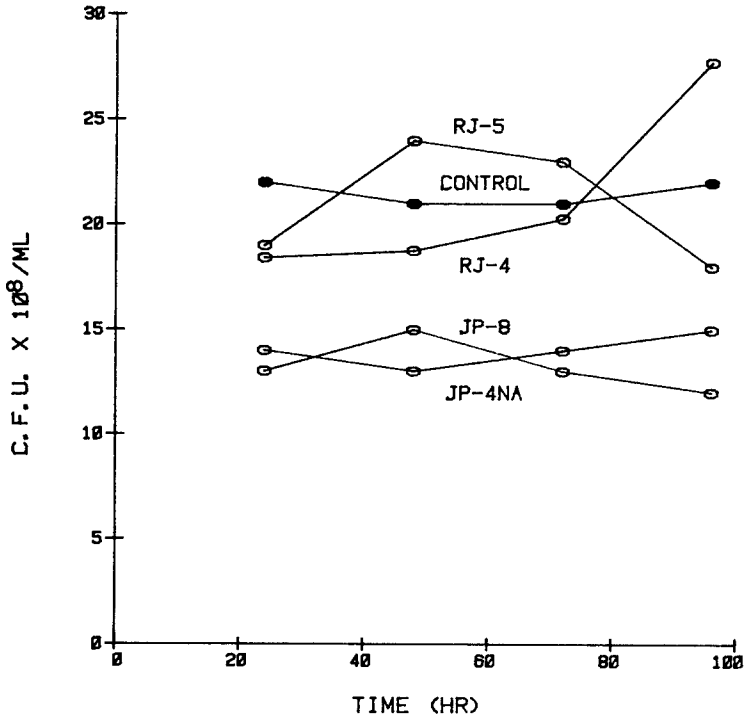


Figure 4D.

DISCUSSION

The measurement of bacterial population density by turbidimetric technics, though simple in execution, is in fact a complex procedure. With the use of a transmittance spectrophotometer, an increase in the number of suspended bacterial cells is indicated by a reduction in the amount of light, usually monochromatic, that impinges upon the light sensitive device such as a photomultiplier tube. The wavelength selected for use is that which is least absorbed by the suspending menstruum and thus permits maximal response range and sensitivity as defined by Beer's Law. A more sensitive approach permitting a greater dynamic range entails the use of a light scattering photometer or nephelometer in which the incident light beam, which may be either mono- or polychromatic, is at a right angle to the light sensing device. With this method, an increase in culture (suspended particle) density results in an increase in light energy transmitted to the sensing element with a concomitant increase in electrical output. In either of these methods, the amount of light energy that ultimately reaches the sensor is dependent not only on the number of suspended particles but on the complex interaction of transmitted and absorbed light as influenced by cell number, cell size, cell shape, and cell constituents. (For a more complete discussion of turbidity measurements, refer to Toennies and Gallant, 1949). Implicit in the application of turbidimetry is the assumption of uniformity or, better, constancy of these bacteriological attributes during the measurement period. However, all of these cell characteristics are changing during the normal growth cycle of any bacterial culture. During log phase, this distri-

bution of cell types is most constant, and it is during the phase of growth that cell number is most accurately indicated by turbidimetric measurements. If a further assumption is made -- that various cultures of the same bacterial strain do not differ in the individual distribution of cell size and shape -- then comparative turbidity determinations made during the log phase are reasonable representations of comparative cell numbers of the different cultures. In this study, light microscopic observation of D-31 grown in the presence of various jet fuels indicated this latter assumption is valid.

The turbidimetric growth responses of D-31 in the presence of the conventional and synthetic jet fuels (Figures 2 and 3) indicate: 1) none of the fuels interfered with the initiation of cell replication, i.e., the lag phase of all fuel exposed cultures was essentially the same as the control culture; 2) none of the fuels affected logarithmic growth rate, i.e., the slopes of the curves during log phase were identical and terminated at the same incubation time; and 3) cells in stationary phase were influenced by the presence of a fuel, i.e., the final growth yields as indicated by turbidity differed from the control culture. These differences appear to be specific fuel and concentration related. However, the appearance of a stable emulsion in all but the control cultures during the later growth periods (mid to late log phase) suggested a possible interference with the turbidimetric determinations. In addition, a final growth level greater than the control was indicative of utilization of the fuel as both an energy and carbon source since the glucose, which serves both of these functions in SMS, was exhausted when stationary phase was attained. (Glucose is added at a rate limiting concentration which results in termination of growth after approximately 20 hr and a culture density equivalent to about 30% transmittance). Since JP-4 is a complex mixture of numerous hydrocarbons of varied chain length, saturation, and configuration, and therefore most likely to contain both metabolizable and inhibitory components (the synthetic fuels are essentially single component compounds), it was selected for several experiments to determine if either nutrient enhancement or growth inhibition contributed to culture growth and turbidimetric density. Batch cultures of D-31 were prepared in the usual manner with the exception that glucose was omitted from the medium. To 100 ml of this medium were added 0.1, 1.0, and 10.0 ml of JP-4NA. After 46 hr incubation on the reciprocating shaker, visible growth had not occurred nor had a stable emulsion been formed. Glucose was then added aseptically and after an additional 8 hr, growth was observed in all flasks. Maximal growth was attained 22 hr after glucose addition, indicating: 1) JP-4NA could not serve as carbon and energy source for D-31; and 2) the inoculum was essentially not affected by exposure of the fuel since the lag period was only slightly longer than usually observed.

A second experiment confirmed the absence of inhibition of cell growth by JP-4. Equal volumes of JP-4NA and complete SMS were shaken under sterile conditions for 5 days and then separated. An inoculum of D-31 was added to the aqueous (SMS) phase containing the water extractable substances presented in the jet fuel. Growth of the organism proceeded normally indicating that the fuel sample did not contain water soluble components inimical to bacterial growth. Since some of the fuels appeared to cause a turbidimetric decrease in final growth yield (MCH and RJ-5) and since enhancement of final turbidity by other fuels appeared not to result from fuel utilization, cell concentrations were determined by obtaining viable counts. These counts were performed only during the stationary period since the observed differences occurred at this time.

Comparative viable counts are deemed valid if the assumption is made that the plating efficiency of all cultures is the same, i.e., that exposure to the

compounds of interest does not alter the degree of which individual cells of a given bacterial population can initiate cell reproduction and colony formation. The viable count data obtained in this study do not agree with the turbidimetric data in that exposure of D-31 to all fuels, with the exception of RJ-5, resulted in a reduction in the number of viable cells (cfu) at stationary phase. The erroneous turbidimetric indication of increased final yield was therefore due to microemulsion formation that was not distinguishable from suspended bacterial cells. Such emulsion formation is a well recognized attribute of some hydrocarbon-utilizing microorganisms (Rosenberg et al., 1979) that produce extracellular emulsifying agents. The chemical composition of the hydrocarbon substrate(s) has been shown to affect the extent of emulsification (Rosenberg et al., 1979).

The expression of interference with cell viability only in stationary phase indicates that the mechanism entails some facet of senescent cell structure or activity. The changes in cell wall and capsular (if present) composition that occur in older cells could account for increased susceptibility to cell killing by virtue of increased permeability. Such alterations might also result in a decrease in plating efficiency by inhibition of cell wall synthesis. The data obtained in this study cannot elucidate the mechanism involved; however, the absence of any observable effect in lag and log phases suggests either an attribute of aging cells or perhaps the formation of very low concentrations of toxic substances in older cultures of D-31 growing in the presence of the fuels studied.

The results of this study show that turbidity determinations as a measure of bacterial response may not provide accurate or representative indications of the biological effect of water immiscible compounds. Considerations of the possibility of emulstion formation, degree of solubilization, and stability could negate the applicability of this approach. Klein and Jenkins (1979, 1978, 1977, 1976) demonstrated that with extensive aqueous extractions, components of JP-4 and JP-8 exerted a toxic effect on various aquatic organisms at levels of 1-10 mg/L. Thus, substances that can be solubilized might exhibit a toxic effect in bacterial systems should they by sensitive to the concentrations used. Such was not the case in this investigation. Another difficulty with insoluble materials is the lack of uniform exposure of the bacterial inoculum. Should the material exert an effect by direct action on the cell surface, dose-dependent and reproducible results would be difficult if not impossible to obtain. However, the viability data derived in this study are informative and in reasonable agreement with those studies (Klein and Jenkins (1979, 1978, 1977, 1976) of aquatic toxicity in which two phases were present, i.e., JP-4, JP-8, RJ-4, and RJ-5 were added in various volumes to the test systems directly. In these investigations, concentrations on the order of 0.1 ml/100 ml of water exerted a measurable toxic effect on the fish species exposed and indicated JP-4 was more toxic than RJ-4 and RJ-5. The present study confirms this observation. Thus, bacterial systems have utility as a means of identifying materials potentially hazardous to higher species and quantifying the degree of toxicity if consideration is given to appropriate methods of exposure and observation.

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