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USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMIMANTS

FIRST ANNUAL REPORT

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FOR THE COMMANDER

Roger C. Inman

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Chief, Toxic Hazards Division

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Selenastrum capricornutum was used as test algae in bioassays to determine the toxic effects of potential aquatic contamination which might result from the use of conventional JP-4, JP-8 and Shale-Derived JP-8 with and without clay treatment. Chemical analyses were performed to evaluate the fate of water soluble fractions, and preliminary investigations were conducted to determine the relative toxicities of the emulsified versus dissolved fractions of the compounds studied. Techniques and protocols are described and the results are discussed.

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SUMMARY

This report presents detailed results from the work conducted during 1980/81 to evaluate and quantitate the relative toxicities of certain jet propellants that may be accidentally released into water bodies of different nutrient status. The results will be used by the USAF/USN to determine how activities involving such compounds can be conducted in conformance with the National Environmental Policy Act, and also provide a quantitative basis for the selection of alternative fuels.

The specific propellants investigated were RJ-4, RJ-5, shale-derived JP-8 with clay treatment, JP-9 and JP-10. The bioassays were conducted to determine the No Effect Levels (NOEL), and the amount of fuel which would cause a 50 percent growth reduction measured by total algal cell volume (EC_{50}).

During the year, there was a shift in emphasis towards more detailed investigations of the jet fuels' water soluble fractions. Specifically, studies were conducted to provide a better understanding of the relative toxicities of the dissolved versus emulsified fractions of JP-4, JP-8, and shale-derived JP-8 with and without clay treatment.

CONCLUSIONS

Results from these studies supported the following conclusions:

1. A preliminary screening experiment was conducted to determine the range of toxic effects of RJ-4, RJ-5, conventional RJ-8, shale-derived JP-8 (with clay treatment), JP-9, and JP-10. The screening was conducted by exposing bioassay media (simulating typical lake waters) to 5, 4, 3, 2, and 1 percent of each fuel, mixing briefly by hand, separating the fuel and media, and exposing the test organisms to the media now containing dissolved and emulsified compounds from the fuels. The results showed no effect of RJ-4, JP-9, and JP-10 in this range.

2. RJ-4 and JP-8 showed no measurable growth reductions at initial mixtures of greater than 2 percent fuel.

3. Shale-derived JP-8 (clay treated) showed a very strong effect with essentially no growth occurring in the media exposed to 5 percent fuel and significant reductions in growth in the media exposed to 4 and 3 percent fuel, respectively.

4. Particle counting and optical microscopy evaluations of water algal growth media after contact with the fuels revealed the presence of emulsified droplets.

   Results indicated that as time elapsed, fuel composition and mixing procedure influenced the semi-equilibrium status reached.

5. A standard Automated Extraction System (AES) was developed to obtain reproducible extractions/emulsions of JP-4, JP-8, shale-derived JP-8, and clay-treated shale-derived JP-8 over various fuel/deionized water ratios. Total carbon in solution and emulsion combined remained constant over 192 hours compared to substantial variations over time with direct fuel layering or manual extraction.

6. Results of exposure of S. capricornutum to JP-8, monitored through total carbon and GC analysis, indicate that the toxic effects were manifested by the algae test organisms even after the purgeable volatiles left the solution. Apparently, non-purgeable components were responsible for the documented growth limiting properties of the fuels.

7. The effect of regular fuel additives was determined by comparison of JP-4 with and without additives. The presence of additives increased the amount of organic compounds into solution/emulsion by 26.6 mg TC/1 for JP-4, 1.5%, and 19.5 mg TC/1 for JP-4, 5% (measured 192 hours after initial fuel layering).

8. Following exposure of S. capricornutum to the WSF of various fuels, a sudden total carbon increase occurs 132 $\pm$ 12 hours after initial mixing and cell seeding. Since cell growth was obstructed by the toxicity of several of the fuels, it is possible that powerful emulsifiers may be released into the medium by active cell lysing process, and increase the fuels' solubility very significantly.

RECOMMENDATIONS

Based on the conclusions derived in this investigation, the following recommendations are made:

1. Additional investigations should be conducted to improve the GC/purge and trap procedure in order to extend the range of recovery from C_4 to C_{19}.

2. Qualitative and quantitative evaluations should be emphasized to determine the relative toxicity and significance of emulsified versus dissolved fractions of the fuels, and evaluate changes in "effect levels" as a result of propellant aging/decay rates.

3. Current batch algal assays should be completed and continuous culture investigated in order to establish NOEL, EC_{50}, and non-lethal cell structural modifications, if any.

4. Larger-scale experiments should be conducted to verify results obtained with the experimental protocols in simulated natural conditions, and to provide a basis for models to be developed in years 3, 4, and 5.

PREFACE

This is the First Annual Report of work performed under the Air Force contract AF 33615-80-C-0512 and covers the period June 1, 1980, to May 31, 1981. The project is entitled "Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants". Research was conducted by the Water Resources Laboratory, School of Engineering, University of California, Irvine. The investigation was designed to expand the knowledge of toxic and biostimulatory responses of unicellular algae to conventional and shale-derived jet propellants and to aid Air Force personnel in assessing the environmental impact of compounds which may be released into the aquatic environment.

Contract monitor was James M. Livingston, Chief, Environmental Quality Branch of Toxic Hazards Division, AFAMRL, Wright-Patterson Air Force Base, Ohio. Principal investigators were Professor Jan Scherfig, Civil and Environmental Engineering, and Professor Peter S. Dixon, Department of Ecology and Evolutionary Biology, University of California, Irvine. Dr. Marc A. Petty served as project supervisor while Mr. Ching Kuo was responsible for development of the chemical procedures.

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INTRODUCTION

Research investigations have been directed towards the formal evaluation of short- and long-term toxicity of the many jet propellants, additives and/or lubricants being introduced regularly by USAF/USN. Their use presents the possibility of spillage or chronic release into various water bodies, and the direct and recurrent effects of these compounds must therefore be determined.

The most recent review of the effects of oils and oil components on algae (O'Brien and Dixon, 1976) lists almost 200 references, but few of these provide quantitative evaluations of specific fuels or components. This review demonstrates the paucity of detailed information, surprising in view of the significance of algae as the major primary producers of marine and freshwater environments.

Algal bioassays provide a firm basis for assessing the impact of possible aquatic contaminants on algae over a wide range of nutrient and salinity levels. Phytoplankton constitutes one of the most important elements of ecosystems in natural waters and unicellular algae are recommended by US-EPA for use as bioassay test organisms; such organisms may also be used to evaluate both the possibility of toxic compound bioaccumulation and mutagenesis phenomena.

OBJECTIVES

During the past year, major emphasis was allocated to the formal study of the relative toxicities of jet fuels' water soluble fractions. Analytical investigations were carried out to determine the physical and chemical composition of jet fuels' volatile, dissolved and emulsified fractions. Compounds studied included in particular conventional JP-4 and JP-8, shale-derived JP-8 (SDJP-8) and shale-derived JP-8 with clay treatment (CTSDJP-8).

Quantitative bioassays were utilized to determine dose/concentration responses of a unicellular green algae (Selenastrum capricornutum) in various aquatic environments as a function of time, in conformance with Algal Bioassay Procedure (1) and the American Public Health Association standard methods (2). The overall goals have been to provide information about relative safety of these compounds for environmental impact statements, determine operational threshold limits, and submit data which can be used for waste treatment system engineering design and accidental spillage clean-up procedures.

Specific Objectives

1. Determine the no effect level (NOEL) for each of the compounds under the various test conditions. The NOEL is defined as the highest concentration of test compound that can be administered without causing a statistically detectable difference in maximum standing crop. Maximum standing crop is considered to have been reached when the increase in algal growth is less than five percent per day.

2. Determine the median effective concentration (EC_{50}) for the compounds under the various test conditions. The EC_{50} is that concentration of test compound which causes a fifty percent reduction in algal growth when compared with controls with no test compound added.

3. Develop optimal analytical procedures to concentrate and recover volatile aliphatic hydrocarbons.

4. Develop a standard procedure to obtain reproducible jet fuel water soluble fractions (WSF).

5. Assess WSF relative toxicities under bioassay conditions.

WORK PLAN

The work plan was divided into three main parts related to the specific objectives.

1. Completion of NOEL and EC_{50} determinations initiated partly during the 1979/80 research period (WSF without fuel layer).

2. Develop standard methods and analytical procedures to investigate the stability and composition of the jet fuel water soluble fraction/emulsion (WSF/E) under various experimental conditions.

3. Initiate detailed investigations of the jet fuel WSF/E relative toxicities in simulated natural conditions (bioassay flasks containing growth medium and WSF/E with fuel layering).

METHODS AND PROCEDURES

CONCEPTS

Two key concepts were used in this work to form the basis for the conclusions regarding the effects of jet fuel WSF/E in the aquatic environment.

Biological Growth Measures

The main concept used was the measure of biological activity. Several measures can be used including oxygen production rates, specific growth rates, and maximum biomass produced. During the early periods of this investigation, extensive work was done to evaluate the applicability and methods to interpret the results obtained with each of these three parameters. Based on that work it was decided that two measures should be used to evaluate the effects of jet fuel WSF/E.

The first measure is the maximum standing crop. One major difficulty encountered with this measure of the effects of jet fuels is related to the instability of the WSF/E in natural waters versus the normal 10-15 days required to reach the maximum standing crop. In order to determine the absolute and relative toxicity of the WSF/E compounds, it was therefore decided to determine the effects by relative growth compared to a control sample after six, eight, and ten days of growth. The relative growth figures were then compiled to determine the toxic concentrations of the jet fuel WSF/E.

Toxic Concentrations

Two complementary measures were selected to quantify the toxic levels of jet fuel compounds. The first of these is the No Effect Level (NOEL). The NOEL is the maximum concentration of a jet fuel which can be present without causing a statistically detectable difference in maximum standing crop.

The second measure used was the median effective concentration (EC_{50}) which is that concentration resulting in a 50 percent reduction in algal growth on the sixth day of growth when compared with the control. In both cases, the EC_{50} and NOEL were determined on the basis of Analysis of Variance and t-tests combined with interpolation between concentrations of jet fuel.

METHODS

Algal Bioassays

Algal bioassays were conducted in accordance with Algal Assay Procedure (1) and Standard Methods (2) to determine jet fuel WSF/E NOEL and EC_{50} under various experimental conditions.

Modifications of the Algal Assay Procedure included the following:

1. A larger volume of medium was used (250 ml/500 ml flasks) but this was shown to require no change in the auxiliary aeration system used.

2. Temperature control was 25 $\pm$ 1°C.

3. All compounds contained in the growth medium were added in a particular order before filtration in order to prevent iron precipitation. The order of additions was sodium bicarbonate, magnesium sulfate, calcium chloride, potassium orthophosphate (mono-H), magnesium chloride, sodium nitrate and trace metals including a chelating agent.

   Algal bioassays were conducted in two steps: (a) a broad screening series and (b) a fine evaluation analysis. First, a preliminary series of replicate flasks containing the algal growth medium was dosed with a broad range of concentrations (e.g. from 0.001 to 10 ppm) of the test compound. Flasks were seeded with the appropriate test organism and algal growth (both total cell

and total algal volume) was monitored with an electronic particle counter (Coulter model TA II with population accessory) until at least the control flasks reached the maximum standing crop. This facilitated approximation of the NOEL and EC_{50} concentrations. Another series of flasks containing Standard Algal Assay Medium (SAAM) was dosed with this narrow range of concentrations of the test compound. All flasks were seeded with the freshwater bioassay test organism S. capricornutum to an initial concentration of 10^6 cells/1. Algal growth was monitored as described above and the NOEL and EC_{50} concentrations were determined.

Test Compound Concentration

Test compounds were freshly prepared by serial dilution from the stock bottle immediately before being added to the bioassay flasks seeded with algal cells. At least three replicate flasks were prepared for each of the desired initial concentrations of test compound. Total carbon (TC) concentrations were checked in each flask at the beginning of each growth cycle experimental run. In most cases, the desired and actual initial test compound concentrations were in agreement.

Analytical Procedures

Total Carbon Analysis Investigations were conducted to determine the portions of test compound that were actually dissolved and/or emulsified into the SAAM growth medium. To determine this, samples taken from the experimental system were analyzed for actual fuel content. The TC concentration of the samples was used as an indirect measurement of the fuel content. Since the SAAM portion of the medium contained no significant amount of carbon and no other process could substantially introduce carbon into the sample, the use of this indicator was reasonable.

Coulter Counter Determinations Counts were performed with a Coulter Counter to determine algal cell number and cell volume on growth days 6, 8, and 10 in the bioassay flasks initially seeded with S. capricornutum. Results were analyzed statistically to determine NOEL and EC_{50}. The Coulter Counter was also used to investigate the fuels' emulsion droplet size distribution greater than 0.22 μm, which was its limit of resolution.

Optical Microscopy Procedure Optical microscopy evaluations were conducted to determine and quantify the presence and eventual stability of emulsified droplets in the water phase after the addition of fuel to the growth medium. All samples were examined under identical conditions with a phase-contrast Leitz Ortholux microscope (resolution 0.3 μm). Observations were carried out on growth day 8 and "particle" sizes were determined using standard microscopy scales.

Jet Fuels' WSF/E Stability A series of tests was performed to determine the relative stabilities of the WSF/E of fuels under various simulated environmental conditions. The experimental design for these studies of WSF/E stability consisted of monitoring Total Carbon content as a function of fuel type, concentration and time, using a WSF Automated Extraction System developed at UCI

Water Resources Laboratory and the direct fuel layering technique. Details of the specific methods used and the environmental conditions simulated are presented in later sections of this report.

RESULTS AND DISCUSSION

Investigations during the present year concentrated on three major interrelated areas. The first concerned a NOEL for the development of methods and protocols to increase the reliability and reproducibility of experimental results. The second involved test compound exposure alternatives with particular emphasis on JP-4, JP-8, CTJP-8 and CTSDJP-8. The third area of investigation related to a series of experiments conducted to provide a better understanding of the relative toxicity of additives and the significance of the emulsified versus dissolved fraction of the fuels in the aqueous phase.

METHODS DEVELOPMENT

Gas Chromatographic Purge and Trap System Improvement

Gas chromatographic analysis of different types of fuels' WSF and SAAM were made to give a preliminary identification of the hydrocarbon content. Analyses were conducted in accordance with the following parameters and procedures.

The purging device was supplied by the Tekmar Company. A porous disc installed at the base of sample reservoir permitted fine carrier gas (N_2) bubbles to pass through the sample which was kept above the disc. Gas inlet port, sample inlet port and exit port were constructed with removable connection between stainless steel tube fittings and the glass purging device.

The trap was made of two pieces of 1/4" O.D. stainless steel tube connected in series. The longer one (17.5 cm) contained 0.4 g of 60/80 mesh Tenax GC. The shorter one (9 cm) was packed with 0.15 g 100/120 mesh Porapak Q. Two pieces of 3/8" O.D. copper tube wrapped with heating tape were placed outside the two trap columns so that they could be heated independently by two Powerstat variable transformers. Independent heating of the two column materials allowed a greater desorbing temperature (350°C) to be applied to the Tenax GC material without destroying the Porapak Q. This ability to increase desorbing temperature ultimately improved system efficiency.

The trap column was placed horizontally with the Tenax GC end towards the GC sample injection port. Volatile materials were transported by the purging gas from the purging device into the trap column. The gas initially flowed into the column containing Tenax GC, then through the Porapak Q column and finally expelled from the system through a vent.

The purge and trap system was constructed as shown in Figure 1. Two nitrogen tanks were used as the source of carrier gas. A flow of nitrogen at 10 ml/min was fed directly into the back of the Varian model 1200 gas chromatograph (GC) in

order to maintain a constant back pressure in the GC column during temperature programming.

The following is a list of purging, trapping and desorbing procedures.

- Purging and Trapping
  1. Check flow rate:

GC column:	10 ml/min,
purging device:	20 ml/min
trap column:	30 ml/min
run GC:	30 ml/min (20+10)

  2. Open valve V-3 and close valves V-1 and V-2.

  3. Connect purging device exit port stainless steel tubing to the end of trap column (Tenax GC end).

  4. Introduce sample into purging device.

  5. Seal sample introducing port.

  6. After 25 min, close V-1.

- Desorption

  7. Disconnect tubing from purging device at trap column (Tenax GC end). Connect with the tubing leading to the GC sample injection port.

  8. Close V-3.

  9. Turn both Powerstats on:

     a. Set pointer of #2 Powerstat, which controls the temperature of Tenax GC trap column, to 130. The temperature of the column will reach 350°C in 3 min.

     b. Set pointer of #1 Powerstat, which controls the temperature of Porapack Q trap column, to 45. The temperature of the column will reach 210°C in 10 min.

  10. After Powerstats have been on for 2 min, turn GC temperature program mode on.

  11. After three min open valve V-2. While keeping flow level of carrier gas at 20 ml/min, turn pointer of Powerstat #2 to 90.

  12. After 10 min, turn both Powerstats off.

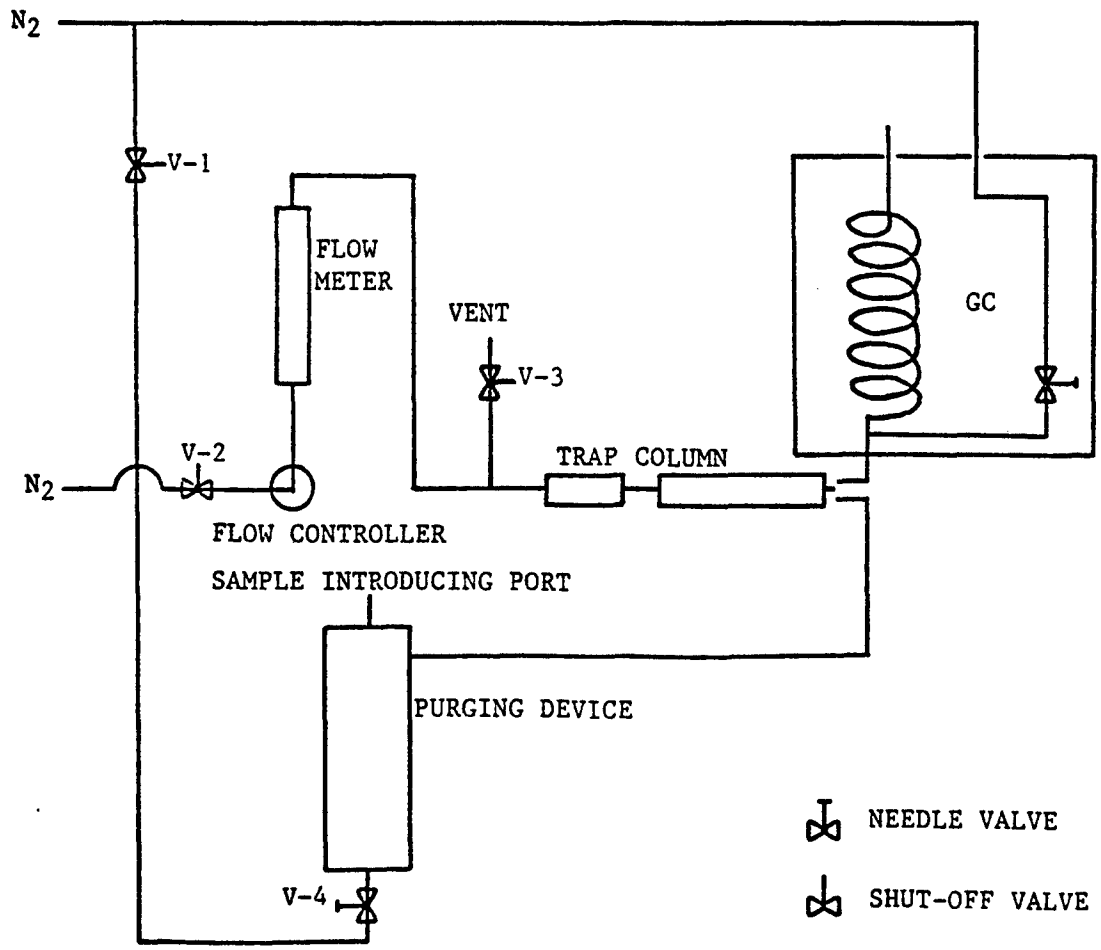


Figure 1 PURGE AND TRAP SYSTEM

Table 1 gives a summary of the GC-purge system parameters after incorporating the new system design, which included the following alterations:

- the gas chromatograph attenuation setting was raised to 128 and the detector temperature increased to 260°C.

- the gas chromatograph temperature program mode was started one min after the Powerstat #2 was turned on.

- the valve V-2 was opened two min after Powerstat #2 was actuated.

Preliminary testing indicated that the modified purge and trap system and operation procedures yielded a marked improvement over the original system design.

Efficiency tests were run comparing differences between chromatograms obtained from saturated hydrocarbon standards injected directly into the GC, and chromatograms obtained by introducing the standard mixture into the purge and trap system. Direct injection replicates allowed the formal evaluation of area and area/weight ratios of each individual hydrocarbon standard, ranging from hexane to pentadecane. The percent recovery was calculated as the ratio of individual peak area obtained by using purge and trap system as opposed to the equivalent peak area obtained by direct injection.

Analyses were run to compare hydrocarbon recovery efficiency obtained from two purging systems with a purging device heated to 65°C. Three separate 25 and three separate 50 min were run for a single test sample each preceding GC analysis, and creating three distinct chromatograms from which a composite chromatogram of the different fractions was derived.

Chromatograms obtained by 25 min purging indicated that components C_6 to C_{13} were completely purged in the first purging segment. Two standard mixture component peaks (C_{14} and C_{15}) were recovered in the second fraction purging segment. In the third fraction, the standard mixture component peaks were barely recognizable. For both 50 min purging segments, components C_6 to C_{14} were completely purged in the first segment and C_{15} was recovered in the second fraction.

In conclusion, results summarized in Table 2 indicate that two fractions of 25 min purging, each in conjunction with a heated purging device at 65°C, yield the most satisfactory results with hydrocarbon percent recoveries up to 100%. Furthermore, this system proves to be comparably efficient with a previously tested non-heated dual fraction 50 min system, thus reducing purging time by 50 min. With the heated dual fraction 25 min purging system, the complete purge/GC system analysis can be completed within 130 min.

TABLE 1

GAS CHROMATOGRAPH AND PURGE AND TRAP SYSTEM PARAMETERS

GC column:	10% OV101 on CW/HP 80/100 mesh support, 1/8" x 20'	
Trap column:	Tenax GC (0.4 g) 60/80 mesh in 0.635 cm x 17.5 cm stainless steel tubing;	
	Porapak Q (0.15 g) 100/120 mesh, in 0.635 cm x 9 cm stainless steel tubing	
Detector:	Flame ionization	
Range:	10	
Attenuation:	128	
Hydrogen:	30 ml/min	
Air:	300 ml/min	
Desorption and GC analysis settings:		
	Injector temperature:	280°C
	Detector temperature:	260°C
	Temperature program initial:	50°C
	Temperature program final:	200°C
	Temperature program rate:	4°C/min
	Carrier gas flow rate:	30° ml/min (20+10)
Direct Injection:		
	Injector temperature:	260°C
	Detector temperature:	260°C
	Temperature program initial:	50°C
	Temperature program final:	200°C
	Temperature program rate:	4°C/min
	Carrier gas flow rate:	30 ml/min
Purging device:		
	Housing temperature:	65°C

TABLE 2

PERCENT RECOVERY OF A STANDARD MIXTURE, USING PURGE-AND-TRAP SYSTEM (25 MINUTES, TWO-STAGE PURGING: HEATED PURGING DEVICE AT 65°C)

n-alkane	Area 1st Purging	Area 2nd Purging	Total	Percent Recovery
C_6	0.85	-	0.85	102
C_7	0.57	-	0.57	100
C_8	0.78	-	0.78	103
C_9	0.92	-	0.92	99
C_{10}	0.99	-	0.99	97
C_{11}	1.00	-	1.00	101
C_{12}	1.05	-	1.05	102
C_{13}	0.96	-	0.96	108
C_{14}	0.73	0.15	0.88	101
C_{15}	0.42	0.46	0.88	98

A series of preliminary investigations was conducted to compare the chromatogram of the standard mixture and the chromatograms of JP-8, SDJP-8 and CTSDJP-8 WSF with various fuels to SAAM ratios using the purge and trap system. Early results indicated a lack of low boiling components (C_6H_{12} to C_8H_{16}) in the SDJP-8 chromatogram; such low boilers were present in chromatograms of conventional JP-8 and CTSDJP-8.

Possible theories explaining the lack of low boilers in SDJP-8 are:

- a difference in component make-up of SDJP-8 compared with either JP-8 or CTSDJP-8
- the loss of low boiling components due to partial evaporation of SDJP-8
- the delay of SDJP-8 component(s) GC detection creating an artificial "chromatogram shift".

Initial results provided some evidence of effective operation of the purge and trap system. However, some of the results raise unanswered questions and thus dictate further improvements of the operating procedures during the 1981/82 research work plan. Such improvements include in particular, the extension of the hydrocarbon recovery from C_4 to C_{19}.

Additional testing of the fuels' WSF composition indicated significant variations of the WSF's TC content as a function of mixing procedures and time elasped. Consequently, there was a shift in emphasis towards more detailed

investigations of the jet fuels' WSF and chromatogram evaluations were postponed until experimental results would provide a better understanding of the mechanisms regulating the dissolved WSF versus emulsified fractions of JP-4, JP-8, SDJP-8 and CTSDJP-8.

Automated Water Soluble Fraction Extraction System (AES)

An automated fuel extraction system was designed to supply the WSF of the test fuels on a continuous basis. The new system improved the extraction efficiency and the reproducibility of the WSF of the test fuels previously obtained by manual shaking methods.

The system's principal components include a Technicon proportioning pump, mixing coils and a mechanical mixer. The proportioning pump is one of the modules used in the Technicon Autoanalyser II system. The pumping rate is fixed although the flow of the liquid is controlled and proportioned by the inside diameter of the pump tubes.

As the two liquid streams meet in the mixing coils, the denser deionized water portion falls through the fuel portion by gravity. In order to enhance mixing of the two phases, a mechanical vibrator was used to shake the mixing coils which were attached to the platform mixer head. A schematic diagram of the AES is shown in Figure 2. Fuels can be pumped directly to the system instead of using displacement methods as shown in Figure 2 if organic-resistant pump tubes are available.

The behavior and efficiency of this new extraction system are based on the actual portion of fuel injected into the system that is dissolved and/or emulsified into the medium (experiments were conducted with both deionized water and SAAM). To determine this, samples produced from the system were analysed for actual fuel content. The total carbon content of the samples was used as an indirect measurement of the fuel concentration. Variables introduced in the testing of the AES included the length of time between creation of the mixture and its carbon analysis, the level of vibration inflicted on the mixing coils, the number of coils and the fuel-to-deionized-water or SAAM ratios. Intensive testing indicated that the best results were achieved with 60 loop mixing coils vibrated at 1460 rpm.

Preliminary tests were conducted to obtain the WSF/E of each fuel investigated for various fuel/deionized water mixing ratios. In order to prevent the loss of any or part of the volatile components, the fuels were initally pumped out of closed containers prior to the mixing process. TC values were measured in the separatory funnels after 30 min gravity separation of the fuels following the actual AES mixing procedure. Figure 3 demonstrates that with the exception of JP-4 for a 9.2% fuel/deionized water mixing ratio indicating a slight TC increase after 72 hours, the AES allows for reproducible experimental conditions during at least 192 hours (stability measured as TC) for all fuels and mixing ratios. Additional tests were conducted to determine whether the WSF/E obtained through

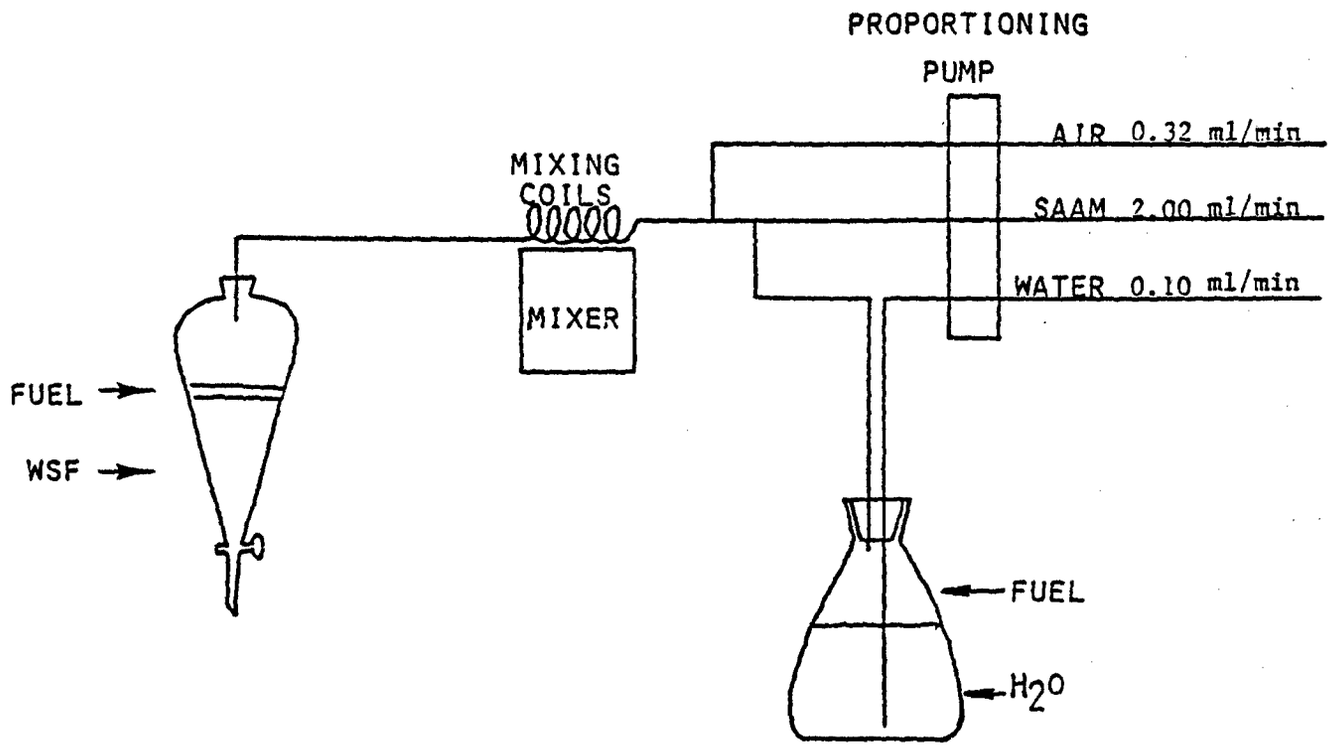


Figure 2 DIAGRAM OF AUTOMATED CONTINUOUS EXTRACTION SYSTEM

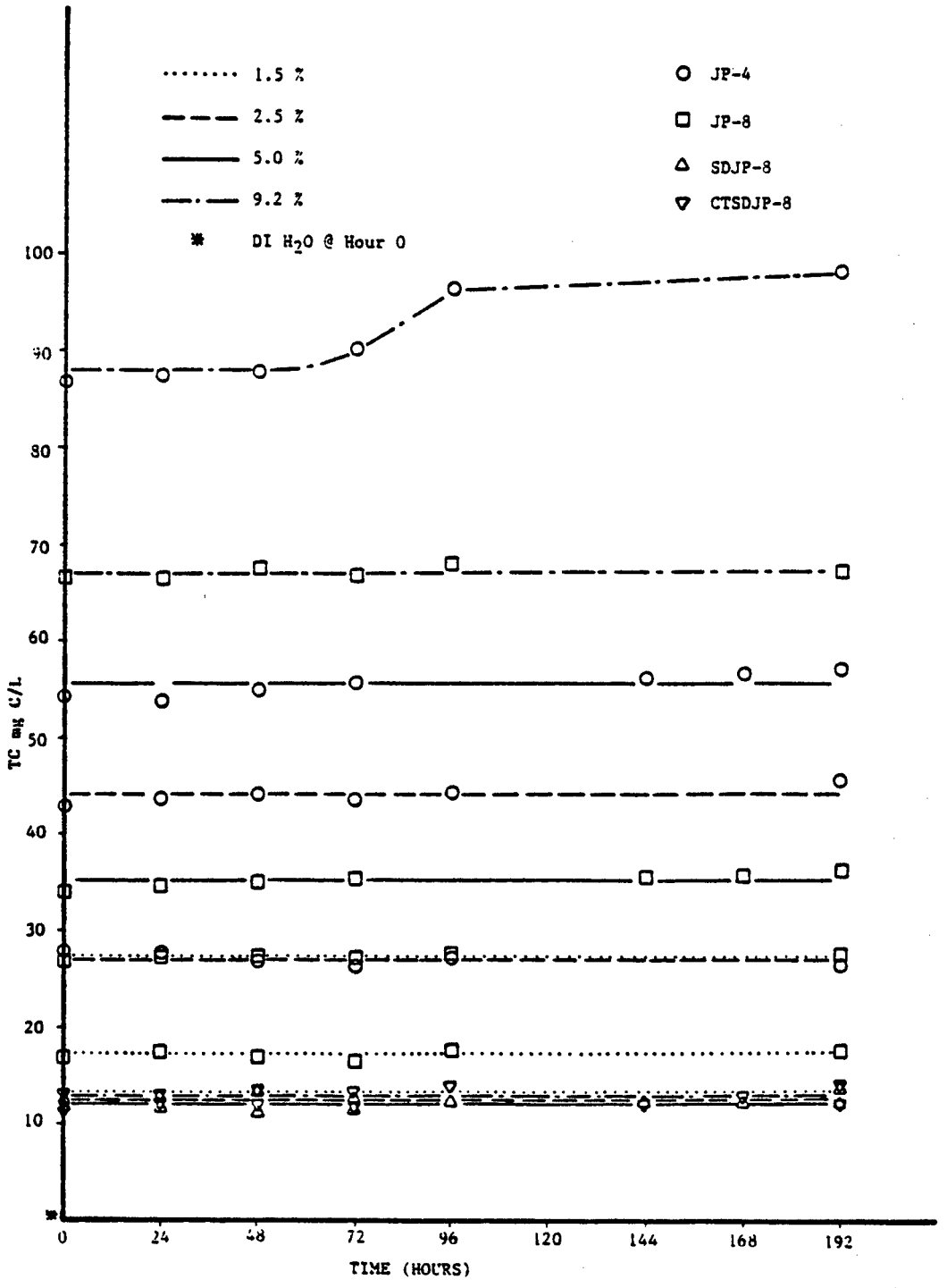


Figure 3 STABILITY OF WSF/E AS FUNCTION OF FUEL/DEIONIZED WATER MIXING RATIO, AND TIME (PREPARED WITH AES)

AES would remain as stable over 192 hours if 33% SAAM were used instead of deionized water. The results shown in Figure 4 indicate that the TC values of each fuel WSF/E (5% in 33% SAAM) increased moderately as a function of elapsed time.

Preliminary investigations were conducted to determine whether the WSF/E obtained through AES would yield an equivalent TC increase after 192 hours if either the micronutrient or macronutrient fractions of SAAM were omitted. The results shown in Figure 5 indicate that WSF/E in 33% SAAM devoid of macronutrients appears to be more stable.

The modified standard algal assay medium (33% SAAM) was prepared using procedures set forth in the Algae Assay Procedure Bottle Test. Concentrations of essential nutrients added to deionized water are shown in Table 3.

TABLE 3

CONCENTRATION OF ESSENTIAL NUTRIENTS IN SAAM

Macronutrients		Micronutrients	
Element	Concentration (mg/1)	Element	Concentration (µg/1)
N	1.400	B	32.460
P	0.062	Mn	115.374
Mg	2.904	Zn	15.691
S	1.911	Co	9.354
C	2.143	Cu	0.004
Ca	1.202	Mo	2.878
Na	11.001	Fe	33.051
K	0.469		

The 33% SAAM devoid of micronutrients was prepared according to standard procedures, except that micronutrients were omitted from the medium and a second test medium was prepared without macronutrients.

There is a similar rate of TC increase between standard 33% SAAM and 33% SAAM devoid of micronutrients. This suggests that the TC increase observed in Figure 5 is due solely to macronutrients and that micronutrients present in a 5% fuel mixing and 33% SAAM have no influence upon the TC increase.

Exposure Alternatives

In natural circumstances, toxic materials may be released into the aquatic environment in either of two ways. Addition may represent a single accidental release of toxic compounds (i.e., spill), or release may be occurring repetitively from a storage facility. The biological effects of these two different situations may be very different, particularly in the case of compounds unstable in aqueous

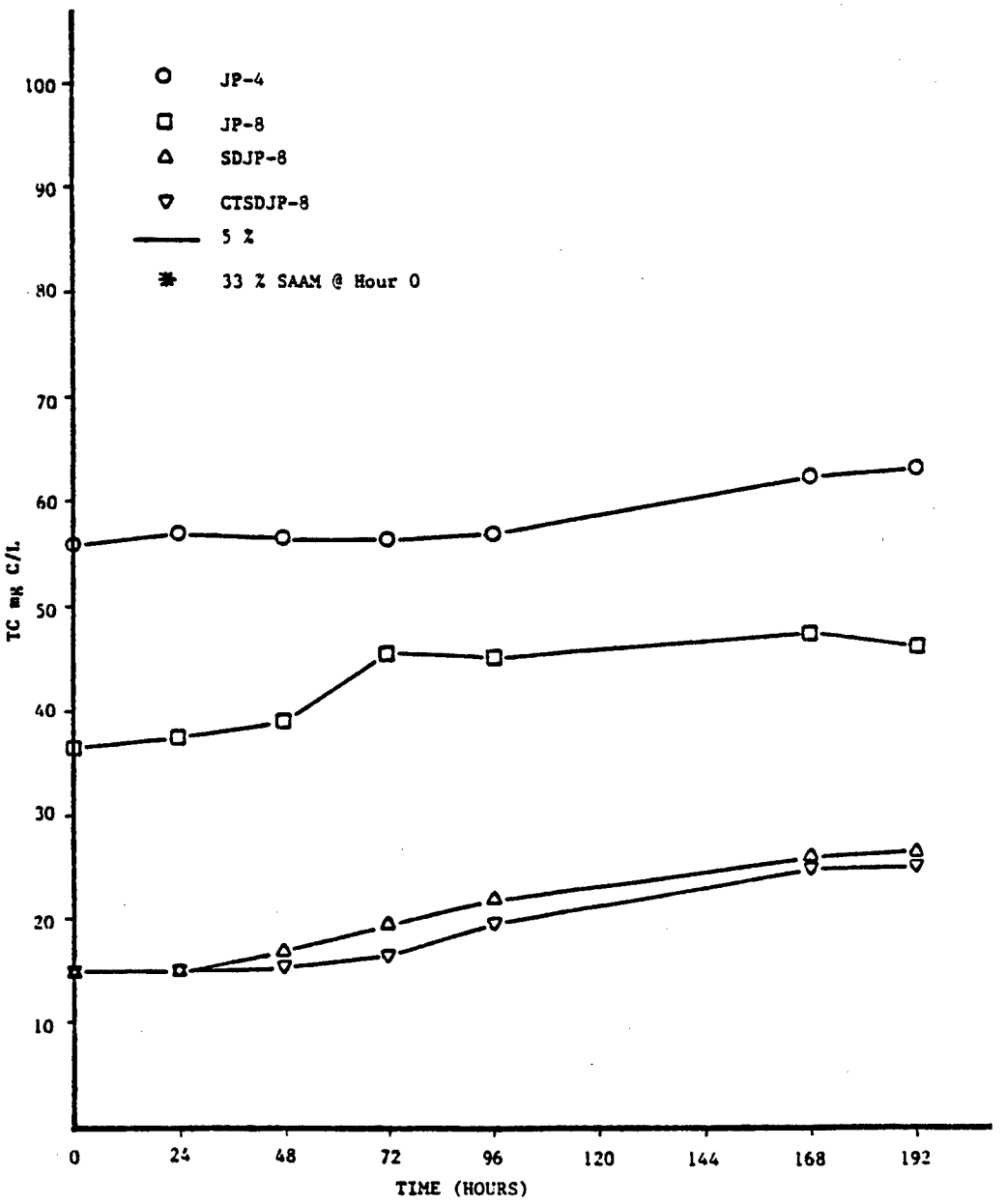


Figure 4 STABILITY OF WSF/E AS FUNCTION OF FUEL/33% SAAM MIXING RATIO, AND TIME (PREPARED WITH AES)

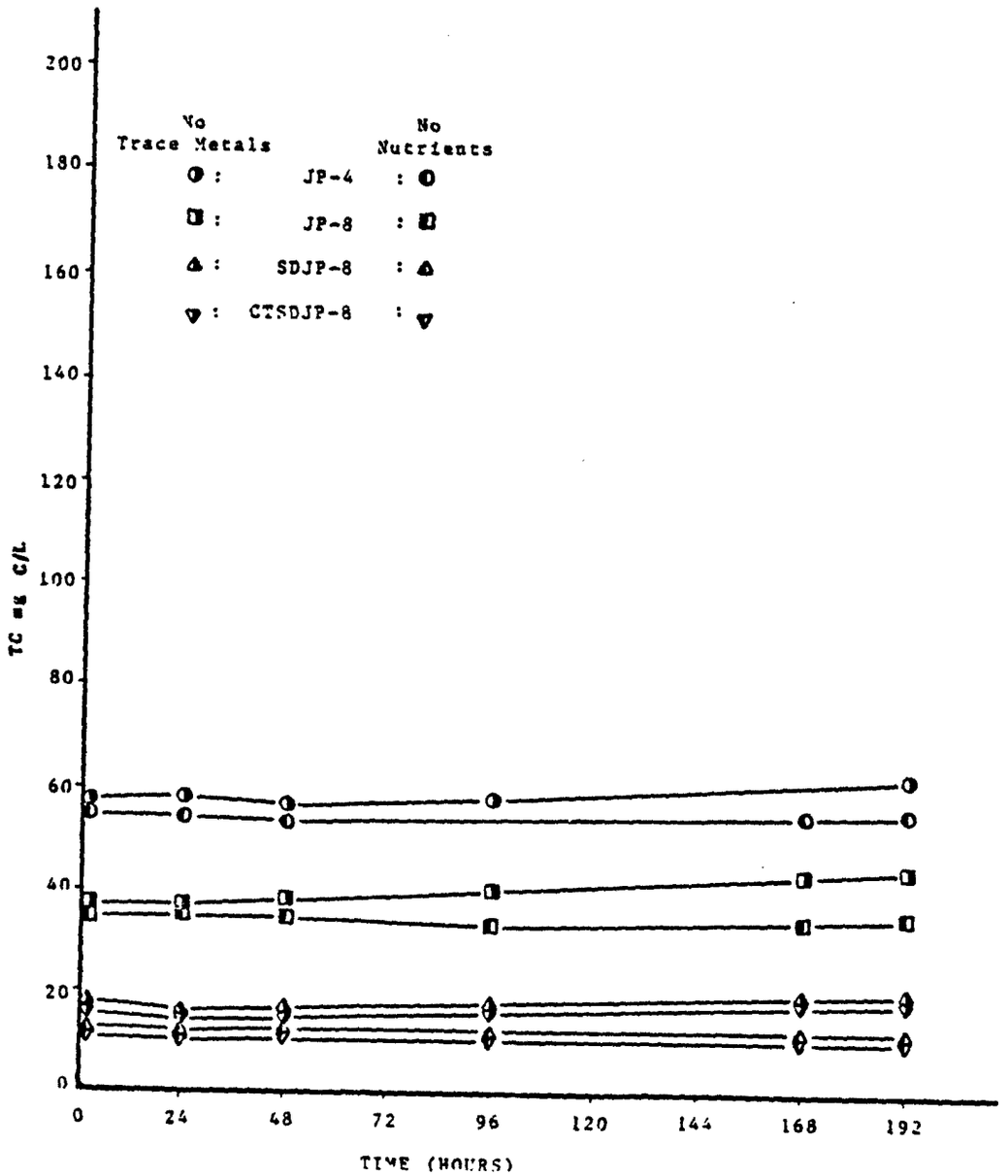


Figure 5 STABILITY OF WSF/E AS FUNCTION OF FUEL/33% SAAM (DEVOID OF TRACE METALS OR NUTRIENTS), AND TIME (PREPRED WITH AES)

media. Major emphasis was allocated to the development of comparable and reproducible experimental exposure alternatives, in order to provide reliable data simulating natural conditions.

Spill conditions were investigated and the experimental exposures of S. capricornutum to the test fuels were modified as shown in Figure 6. Bioassays were still run according to standard methods (1,2) except that a fuel layer corresponding to the jet fuel concentrations tested appeared in the experimental flasks. Previously, in earlier determinations of NOEL and EC_{50}, the fuel/SAAM mixtures were hand shaken and separation of two phases occurred by gravity. The WSF/E was then diluted with SAAM (i.e.: 5%, 4%, 3%, 2%, 1%) and seeded with S. capricornutum without any fuel layer.

In simulated spill responses, a fuel layer would undoubtedly create an obstructive film interfacing with the air phase, possibly limiting O_2 and CO_2 exchanges and volatile fraction escape from the water phase. Consequently, subsequent exposures of unicellular green algae to test fuels included fuel layer interactions. The WSF/E was obtained either by the AES or by direct fuel layering of an aliquot of the test fuel.

A configuration of these two methods in a bioassay experimental segment allowed for simulations of spill exposures occurring in a still pond (direct fuel layering with shaker tables set at 0 rpm), a slow flowing stream (direct fuel layering with shaker tables set at 100 rpm), and in a high turbulence stream (AES).

COMPOUND TESTING

Fuel Layering

Preliminary tests were conducted to determine the fate of jet fuel WSF in simulated low intensity mixing conditions with direct fuel layering using JP-8. Four different fuel/deionized water ratios of 1.5%, 2.5%, 5% and 9.2% were chosen to simulate ranges of fuel to water under various spill conditions. Each 500 ml Erlenmeyer flask at first contained 300 ml of deionized water to which the required amount of fuel was slowly added at the surface to avoid mixing. Prior to the fuel addition, a sampling tube was introduced in each flask to a depth of 7.5 cm below the surface to allow for successive sampling of water phase from below the fuel layer. Each flask was covered to prevent contamination. The experimental conditions and what they simulate are summarized in Table 4.

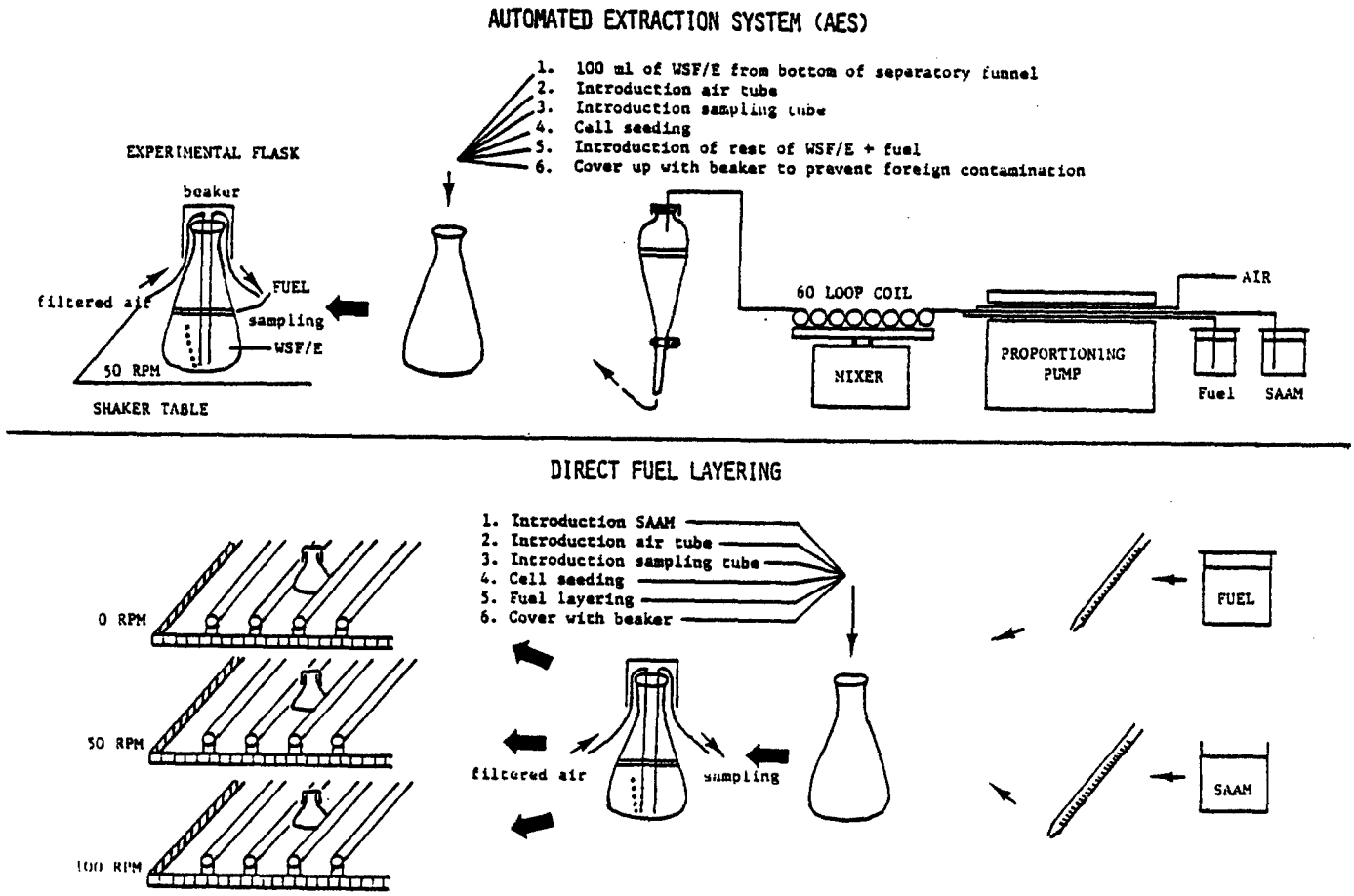


Figure 6 EXPOSURE ALTERNATIVES

TABLE 4

DIRECT FUEL LAYERING: EXPERIMENTAL CONDITIONS

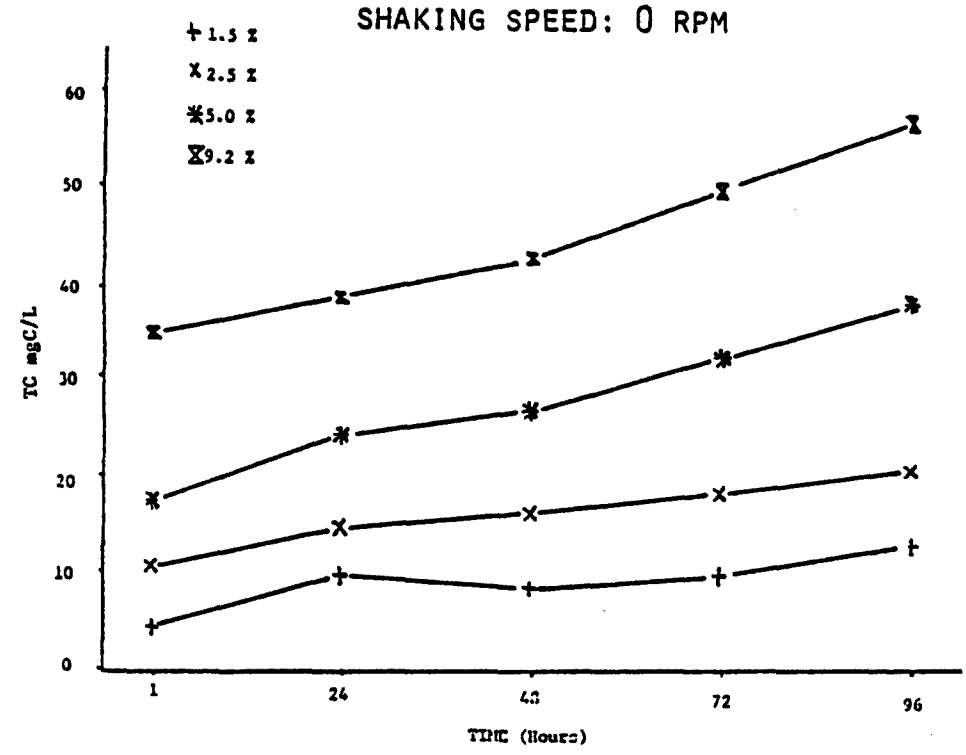
	Shaker Table I Simulated Still Pond Shaking Speed: 0 rpm				Shake Table 2 Moderate Flowing Stream Shaking Speed: 100 rpm			
Fuel Ratio %	1.5	2.5	5	9.2	1.5	2.5	5	9.2
JP-8 (ml)	4.5	7.5	15.0	27.6	4.5	7.5	15.0	27.6
De-ionized water (ml)	300	300	300	300	300	300	300	300
Sampling rate (hours)	1, 24 48, 72, 96				1, 24, 48, 72, 96			

In all cases, the TC increase is proportional to time elapsed and initial fuel concentration (Figure 7). There is very little difference in TC readings between 0 and 100 rpm shaking speed. This observation is of considerable biological importance since 0 rpm conditions simulate the effects of minor to heavy accidental spills over a period of 96 hours upon a steady lake/reservoir non-affected by wind. Comparison of the results shown in Figure 2 with those shown in Figure 7 also indicate that the JP-8 WSF/E obtained from the reproducible AES, and from low intensity shaking conditions have identical TC values in the waterphase at 0 rpm after 96 hours and 100 rpm after 48 hours.

Optical microscopy evaluation was conducted in parallel with WSF/Emulsion TC investigations to determine the presence and stability of emulsified droplets in the water after the mixing of fuel and deionized water. The WSF/E sampled from both the shaker tables and the AES were observed microscopically under identical conditions. Observations of the JP-8 WSF (5% mixing ratio) were carried out following periods of 30 min, 24 hours, 48 hours and 72 hours after the initial mixing.

The initial results summarized in Table 5 indicate the following probable fate of the WSF/E as a function of elapsed time, fuel composition and mixing procedure/intensity factors:

- in all the cases investigated the water phase contained numerous fuel droplets
- fuel droplet size decreased proportionally with elapsed time
- fuel droplet number decreased proportionally with elapsed time
- no difference in droplet size was observed from the low intensity mixing (0 or 100 rpm shaking speeds). The equilibrium reached after 72 hours appears to be similar in all cases, for all fuels (many droplets, range $\leq$ 0.3 µm)



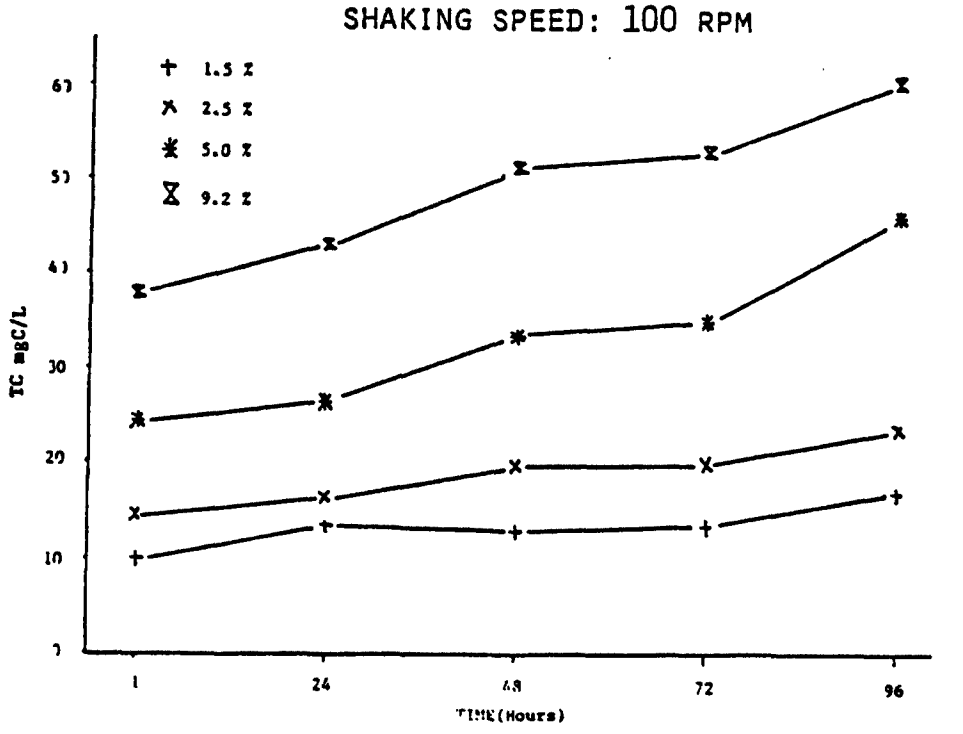


Figure 7 DIRECT FUEL LAYERING - JP-8 WSF TC LEVEL INCREASE AS A FUNCTION OF TIME (FOUR FUEL TO DEIONIZED WATER MIXING RATIOS)

- the high power magnification, limited by the 0.3 μm microscope resolution showed a "coarse granular background" which suggests the presence of many droplets with sizes less than 0.3 μm.

TABLE 5

OPTICAL MICROSCOPY EVALUATION
JP-8 WSF 5% CONCENTRATION

SHT: Shaker Table (0 = stationary; 100 = 100 RPM)
AES: Automated Extraction System (60 mixing coils)
LP: Low Power
HP: High Power
\- Data not available
C: Many droplets
D: Numerous droplets
E: Light granular background
F: Coarse granular background

1: Range ≃ ≤ 0.5 μm
2: Range ≃ ≤ 0.3 μm
3: 1+ medium droplets, range ≃ ≤ 20 μm
4: 1+ medium droplets, range ≃ ≤ 1.5 μm
5: 2+ medium droplets, range ≃ ≤ 1 μm
6: 2+ medium droplets, range ≃ ≤ 0.5 μm

Ortholux Leitz; LP = 10x10; HP = 10x100;
Resolution: 0.3 μm

	0 RPM		100 RPM		AES	
	LP	HP	LP	HP	LP	HP
30 min	-	-	-	-	D2	F2
24 hours	D1	F4	D2	F1	D2	F2
48 hours	C1	E2	D2	F1	D2	F2
72 hours	C2	E2	C2	F2	C2	F2

The observation that no shaking and 60 coil AES seem to create the same stabilized emulsion is not yet understood. It is most likely to be a result of the presence of specific emulsifiers or surfactants rather than a broad range of compounds. Evidence supporting this hypothesis will be documented on page 35, when comparing the results of the exposure of S. capricornutum to JP-4 and JP-4 labelled "non-additive".

A comprehensive set of experiments was conducted on conventional JP-4 and JP-8, SDJP-8 and CTSDJP-8 to determine simultaneously the amount of hydrocarbon going into solution/emulsion under various conditions of mixing and fuel/water ratios, and the effect of the dissolved/emulsified hydrocarbon from the fuels on S. capricornutum. Two mixing conditions were used, each with two ratios of fuel to 33% SAAM (1.5% and 5% respectively).

Effects on algal growth were monitored at growth day three, six, and eight, with three investigation techniques: TC quantification, particle counting, and optical microscopy. No cells remained eight days after the original seeding in many of the experiments. Significant TC concentration increases in the water phase were observed at the same time for both fuels and all mixing conditions.

Initial contamination of cells by direct contact with the test fuels was avoided by seeding the experimental flasks before the fuel component was added to the assay medium. Each assay was maintained on a lighted shaker table set at 100 rpm (direct fuel layering) or 50 rpm (direct fuel layering and AES initial mixing). Observations from previous bioassay experimental runs indicated that, at a speed of 0 rpm, unicellular algae would cling to the bottom of the bioassay flask. Consequently, in order to collect comparable and representative samples, shaker table speeds were set at a minimum of 50 rpm.

Prior to the addition of fuel, a sampling tube was introduced into each flask, allowing for sampling of the water phase on days 6, 8, and 10. Each flask was covered to prevent foreign material contamination(s), and was aerated continuously throughout the experiment. Particle number and volume for each flask were counted on presumed algal growth days 6, 8 and 10 using the Coulter TA II Particle Counter.

The experimental conditions and what they simulate are summarized in Table 6. All experiments were run with three replicates.

TABLE 6

EXPOSURE OF S. CAPRICORNUTUM TO JP-4, JP-8, SDJP-8, CTSDJP-8
(EXPERIMENTAL CONDITIONS)

	JP-4	JP-8	SDJP-8	CTSDJP-8
	1.5% Fuel Ratio		5% Fuel Ratio	
	50 rpm	100 rpm	50 rpm	100 rpm
	Pond	Moderate Flowing Stream	Pond	Moderate Flowing Stream
Day 6	A	A	A	A
Day 8	B	B	B	B
Day 10	A	A	A	A

A = Total Carbon Analysis + Coulter Counts
B = Total Carbon Analysis + Coulter Counts + Microscope Evaluation

Visual observations revealed a total lack of green pigment associated with algal growth in all test flasks (except controls) throughout the duration of the experiment. Therefore, particle counts and mean volume measurements were not of

algal cells but readings of emulsion globules or other constituents originating from the fuels. Particle analysis of number and volume, together with total carbon analysis of the exposure of S. capricornutum to 1.5 and 5 percent concentrations of the four fuels, are presented in Tables 7 and 8.

Examination of the results of particle analyses shown in Tables 7 and 8 reveals a large variation between replicate values, producing inordinate standard deviations for both particle number and mean particle volume. The source of this lack of uniform replicate data may likely be due to the observed presence, in the majority of test flasks, of a white precipitate that forms in the medium and clings to the assay flask during the bioassay. A small fraction of the total number of assay flasks remained clear (without precipitate) throughout the test. Particle analysis from these precipitate-free flasks showed a substantial decrease in particle number, and a smaller variation between replicate values than in flasks containing the precipitate. Chemical and observational analysis of the white precipitate that forms during the bioassay will be conducted to determine its composition, origin, and mechanism of formation.

In support of the visual evidence of no algal growth, mean particle volumes were significantly lower than the expected algal cell volume which is detected in the experimental controls.

Therefore, particle counts and mean volume measurements did not represent algal cells but were readings of emulsion globules and/or other constituents originating from the fuels. Comparison of Tables 7 and 8 indicates the influence of experimental shaking speed. For all fuels and at all concentrations, the number of particles was greater at 100 rpm mixing than at 50 rpm mixing. Also, particles had larger Mean Particle Volumes at 100 rpm mixing than at 50 rpm, suggesting that increasing mixing speed from 50 to 100 rpm induced particle/emulsion coalescence. Examination of means and standard deviations indicated great variability among replicates, due probably to the limited resolution (0.2 μm) of the Coulter Counter. It is presumed that the Coulter Counter allows for effective measurement only at the end of the Gaussian distribution of emulsion droplet size. This opinion is consistent with evaluations using phase-contrast optical microscopy which suggested that mean droplet size was likely to be below the detection limit of the Coulter Counter.

Total carbon analyses of test samples are presented in Figures 8 and 9 showing a general trend for all samples to increase in level of total carbon as a function of fuel concentration and time elapsed. Regardless of mixing conditions, results indicate higher TC values for the shale-derived fuels. Optical microscopy evaluations indicated the following trends:

- In all flasks except control, no remaining cells could be identified suggesting that fuel toxicity resulting from 5% and 1.5% mixing ratios inhibits all growth and, furthermore, destroys all cells.

- All flasks contained numerous fuel droplets.

TABLE 7

EXPOSURE OF S. CAPRICORNUTUM TO JP-4, JP-8, SDJP-8, CTSDJP-8
PARTICLE COUNT AND TOTAL CARBON OF BIOASSAY (50 RPM MIXING)

Fuel Type	Fuel Percentage	Rep. No.	Growth Day 6			Growth Day 8			Growth Day 10		
			(1) 10^6 part/ℓ	(2) MPV-mm^3	(3) TC-mgC/ℓ	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ
JP-4	1.5	1	1762	30.5	38.5	567.5	37.7	51.5	1635	23.2	61
		2	3691	28.9	57.5	2181	20.6	71.5	1627	20	74.5
		3	1597	23.7	46.5	229.1	27.5	76.5	671.1	30.8	68.5
		X	2350	27.7	47.5	992.5	28.6	66.5	1311	24.7	68
		S	1164.3	3.6	9.5	1043.1	8.6	13.2	554.2	5.5	6.8
JP-4	5	1	5600	182	61	51.7	121.7	75.5	86.7	47.7	91.5
		2	5467.4	33.9	90	187.5	26.8	79	167.3	33.0	88.5
		3	2097	13.4	63.5	107.3	39.1	71	77.4	8.9	83.5
		X	4388.2	76.4	71.5	115.5	62.5	75.2	110.5	29.9	87.8
		S	1985.3	92	16.1	68.3	51.6	4.0	49.4	19.6	4.0
JP-8	1.5	1	4177	17.9	NS	2756	9.0	48.5	10237	7.7	73.5
		2	909	26.9	52.5	8178	11.3	81.5	9269	9.1	NS
		3	6128	10.1	59	5929	5.7	55.5	9147	6.3	67.5
		X	3738	18.3	55.8	5621	8.7	61.8	9551	7.7	70.5
		S	2637.1	8.4	4.6	2724	2.8	17.4	597.2	1.4	4.2
JP-8	5	1	718.4	19.5	42.5	901.1	21.4	54	1122	20.9	NS
		2	516.8	23.0	41.5	651.3	21.1	51.5	673.5	14.3	57
		3	741.6	23.6	40.5	748.4	18.4	56.5	1439	42.6	NS
		X	658.9	22.0	41.5	767.0	20.3	54	1078.2	25.9	57
		S	123.6	22	1.0	125.9	1.7	2.5	384.6	14.8	-
CONTROL		1	2368	60.8	21	702.9	65.8	27	3297	49.6	23
		2	1841	58.7	32.5	650.4	66.8	27	2249	56.6	27
		X	2104.5	59.8	26.8	676.7	66.3	27	2773	53.1	25
		S	372.6	1.5	8.1	37.1	0.7	0	741.0	4.9	2.8
SDJP-8	1.5	1	5638	27.1	41.5	513.8	24.2	59	499.2	22.1	83
		2	510.9	22.9	29	1732	13.5	42	540.1	17.9	61
		3	636.0	11.1	NS	14853	6.55	NS	11810	4.79	NS
		X	2261	25.2	35.3	5699	14.70	50.5	4283	14.9	72
		S	2924	24.3	8.8	7950	8.89	12.0	6518	9.02	15.6
SDJP-8	5.0	1	512.3	16.1	64	2280	9.98	98.5	298.2	18.5	195
		2	761.8	19.9	67.5	892.5	10.9	88	658.5	25.1	190
		3	410.6	16.8	56.5	472.3	14.6	89	319.9	10.7	175
		X	561.6	17.3	62.7	1215	11.8	91.8	425.5	18.1	186.7
		S	180.7	2.13	5.6	946.0	2.44	5.8	202.0	7.20	10.4
CTSDJP-8	1.5	1	2106	20.3	38.5	1555	18.2	62.5	962	8.60	135
		2	7758	16.2	NS	6703	7.41	79.5	5536	7.85	160
		3	8452	10.9	67.5	3741	10.3	NS	13943	3.96	175
		X	6105	14.6	53	3999	11.9	71	6813	6.80	156.7
		S	3481	5.18	20.5	2583	5.59	12.0	6584	2.49	20.2
CTSDJP-8	5.0	1	1355	10.7	21	1189	16.2	42.5	614.4	16.8	115
		2	971.1	11.4	17.5	1993	12.8	33.5	450.3	13.8	75
		3	1101	11.3	18	944.3	20.5	35	1010	10.9	90
		X	1142	11.2	18.8	1375	16.5	37	691.5	13.8	93.3
		S	195.2	3.88	1.9	548.6	3.86	4.8	287.7	2.95	20.2
CONTROL		1	452.5	38.1	11	840.8	36.1	11	851.7	42.9	10.5
		2	2082	58.3	20.5	2330	48.8	15.5	2400	53.7	37
		3	787.6	62.2	16.5	2410	34.3	11	1887	39.1	16.5
		X	1107	52.8	16	1860	39.7	12.5	1713	45.2	21.3
		S	860.5	5.75	4.8	883.8	7.90	2.6	788.7	7.57	13.9

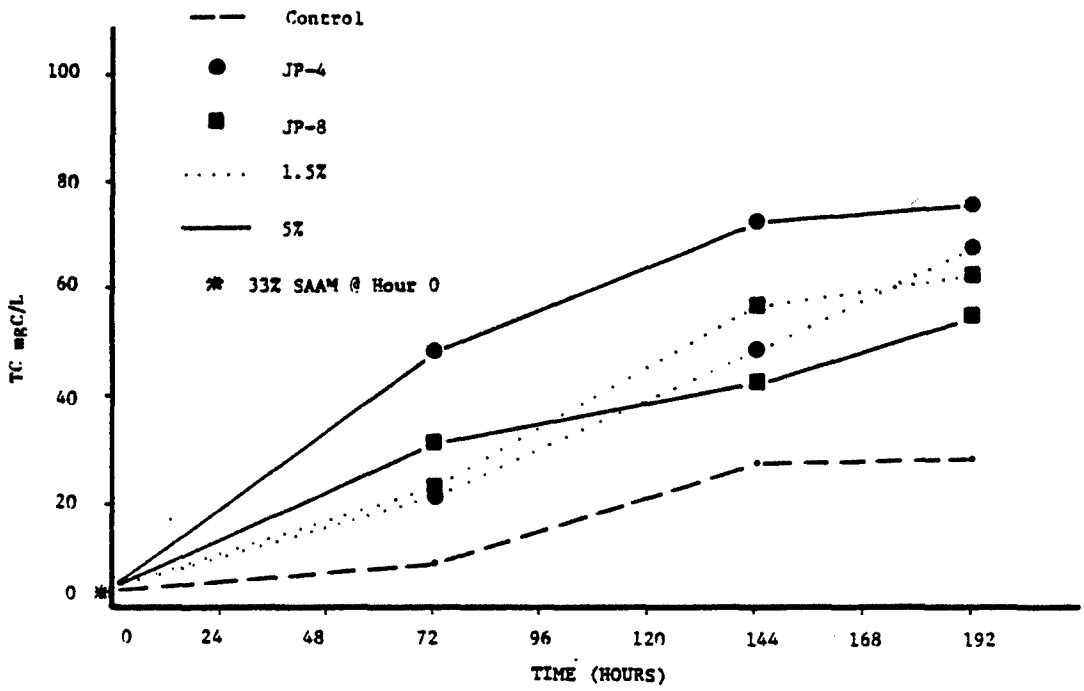
(1) Total Particle Numbers
(2) Mean Particle Volume (MPV)
(3) Total Carbon (TC)
X Mean
S Standard Deviation
NS No Sample

TABLE 8

EXPOSURE OF S. CAPRICORNUTUM TO JP-4, JP-8, SDJP-8, CTSDJP-8
PARTICLE COUNT AND TOTAL CARBON OF BIOASSAY (100 RPM MIXING)

Fuel Type	Fuel Percentage	Rep. No.	Growth Day 6			Growth Day 8			Growth Day 10		
			(1) 10^6 part/ℓ	(2) MPV-mm^3	(3) TC-mgC/ℓ	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ
		1	1596	29.8	42	549.6	65.7	65	751.4	30.3	82.5
		2	406.6	23.7	32.5	51.1	32.8	39.5	69.3	69.7	46.5
JP-4	1.5	3	771.6	37.6	43	392.7	78.1	49.5	1909	32.1	78
		X	924.7	30.4	39.2	331.1	58.9	51.3	909.9	44.0	69
		S	609.3	7.0	5.8	254.9	23.4	12.8	930.0	22.2	19.6
		1	435.7	58.6	46	155.9	26.9	74.5	189.8	65.4	84.5
		2	1364	24.3	57.5	57.8	58.1	69.5	146.5	65.9	78
JP-4	5	3	1235	11.7	61	111.5	37.6	76.5	99.8	89.8	87
		X	1011.6	31.5	54.8	108.4	40.9	73.5	145.4	73.7	83.2
		S	502.9	24.3	7.8	49.1	15.9	3.6	45.0	13.9	4.6
		1	789.7	18.6	32.5	1283	19.8	NS	1541	13.7	68.5
		2	481.2	24.7	27.5	1049	17.7	62	9913	6.8	61.5
JP-8	1.5	3	1110	15.7	31.5	2201	14.4	61	16930	18.1	58
		X	793.6	19.7	30.5	1511	17.3	61.5	9461.3	12.9	62.7
		S	314.4	4.6	2.6	608.9	2.7	0.7	7704.4	5.7	5.3
		1	611.1	29.8	38	864.9	9.6	46.5	1119	13.5	59
		2	761.3	21.1	45.5	820.4	19.3	57	1525	20.3	NS
JP-8	5	3	920.6	18.3	44.5	696.9	13.8	54	879	10.1	61
		X	764.3	23.1	42.7	794.1	14.2	52.5	1174.3	14.6	60
		S	154.8	6.0	4.1	87.0	4.9	5.4	326.5	5.2	1.4
CONTROL		1	1543	63.5	17.5	798.0	60.5	25.5	2683	57.2	33.5
		2	1432	65.6	21.5	829.8	61.5	23.5	2188	67.4	31.5
		X	1487.5	64.6	19.5	813.9	61	24.5	2435.5	62.3	32.5
		3	78.5	1.5	2.8	22.5	0.7	1.4	350.0	7.2	1.4
		1	2615	12.9	47	971.5	11.3	70	1487	12.9	120
		2	1473	16.8	55.5	1919	15.4	82	1262	14.8	130
SDJP-8	1.5	3	1419	24.3	48.5	1362	21.3	67	967.3	11.4	105
		X	1836	15.6	50.3	1417	16.0	73	1239	13.0	118.3
		S	675.4	6.35	4.5	476.2	5.03	7.9	260.6	1.70	12.6
		1	516.7	30.7	59	1197	16.1	95.5	428.6	9.65	190
		2	399.7	12.1	54.5	840.1	22.1	87	234.2	44.2	175
SDJP-8	5.0	3	190.8	36.1	50.5	1048	17.7	86	261.2	34.3	175
		X	369.1	22.4	54.7	1028	18.6	89.5	308.0	29.4	180
		S	165.1	7.29	4.3	179.2	3.11	5.2	105.4	17.8	8.7
		1	1299	11.1	38.5	15431	6.26	63	681.8	11.1	115
		2	1459	7.57	40.5	1180	11.6	68.5	438.4	15.7	115
CTSDJP-8	1.5	3	7376	15.3	58	720.6	21.1	97.5	2517	7.40	170
		X	3378	12.7	45.7	5777	13.0	76.3	1212	11.4	133.3
		S	3463	3.0	10.7	8363	7.52	18.5	1136	4.16	31.8
		1	1041	17.9	29.5	861.8	18.4	73.5	247.0	16.7	140
		2	8829	18.5	38	933.3	11.8	70.5	386.2	14.3	165
CTSDJP-8	5.0	3	10544	24.1	21.5	994.7	14.5	51.5	228.2	15.1	135
		X	6804	20.1	29.7	929.9	14.9	65.2	287.1	15.3	146.6
		S	5064	4.31	8.3	66.51	3.31	11.9	86.31	1.22	16.1
		1	1899	58.8	25.5	2872	62.9	33	3395	48.8	38
		2	3414	48.5	27	3508	50.0	32.5	3445	51.7	39.5
CONTROL		3	2500	44.7	27	3601	45.6	30.5	3389	49.1	37
		X	2665	50.6	26.5	3327	52.8	32	3409	49.9	28.2
		S	681.2	4.27	0.9	396.8	8.99	1.3	30.75	1.59	1.3

(1) Total Particle Numbers
(2) Mean Particle Volume (MPV)
(3) Total Carbon (TC)
X Mean
S Standard Deviation
NS No Sample



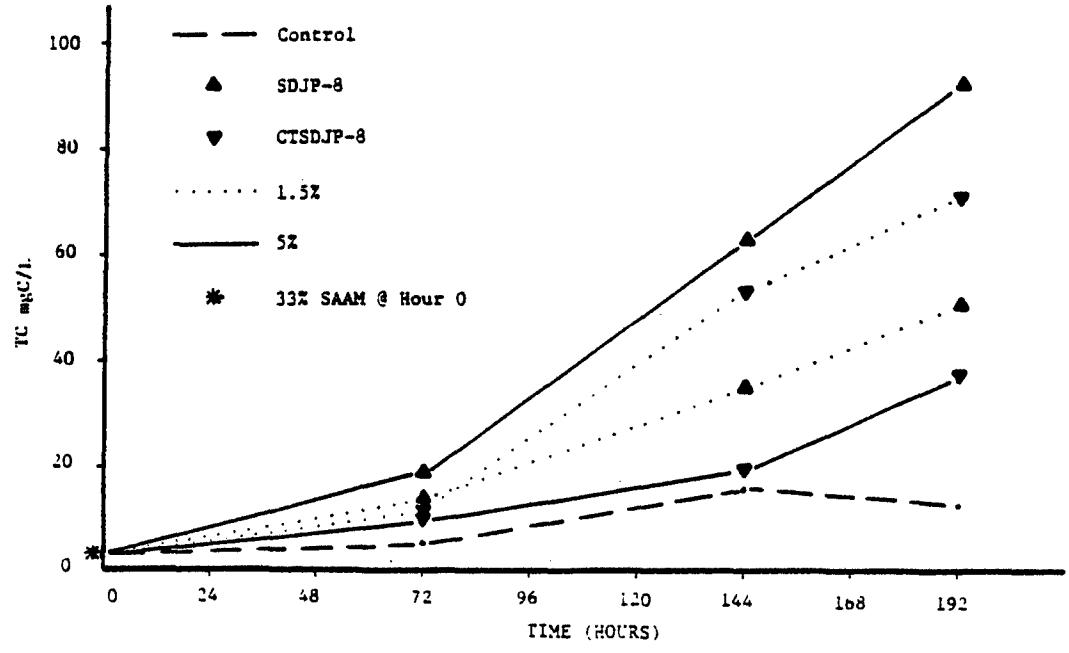
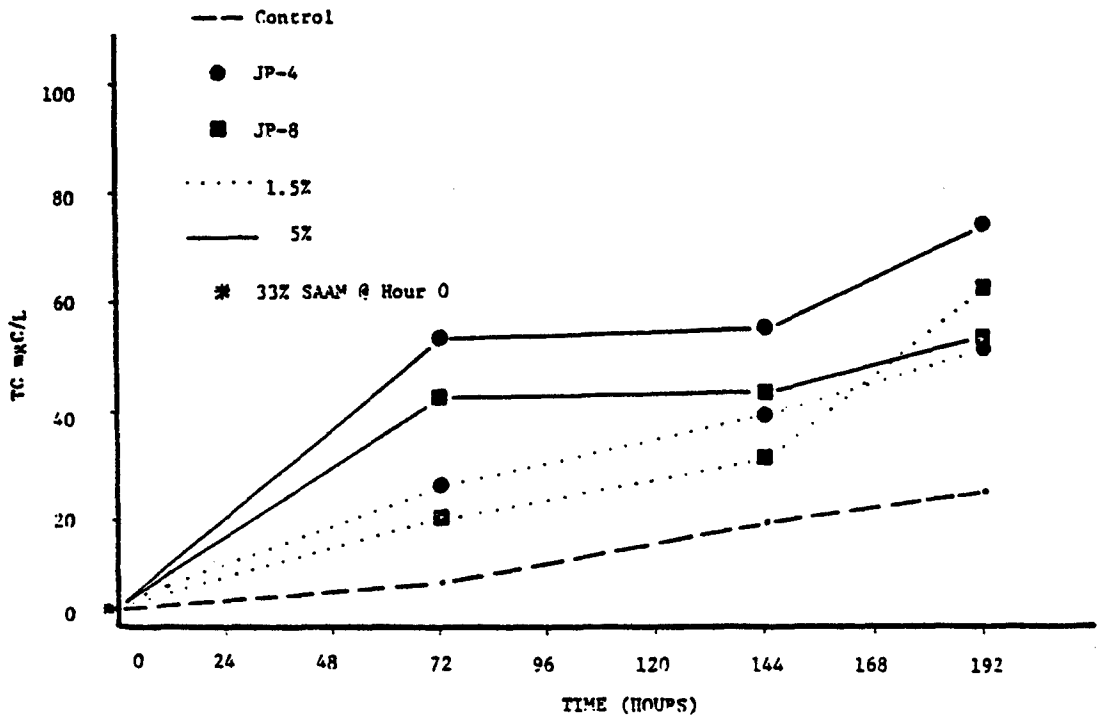


Figure 8 EXPOSURE OF S.CAPRICORNUTUM TO JP-4, JP-8, SDJP-8, CTSDJP-8, TOTAL CARBON WSF/E CONTENT AT SHAKING SPEED 50 RPM



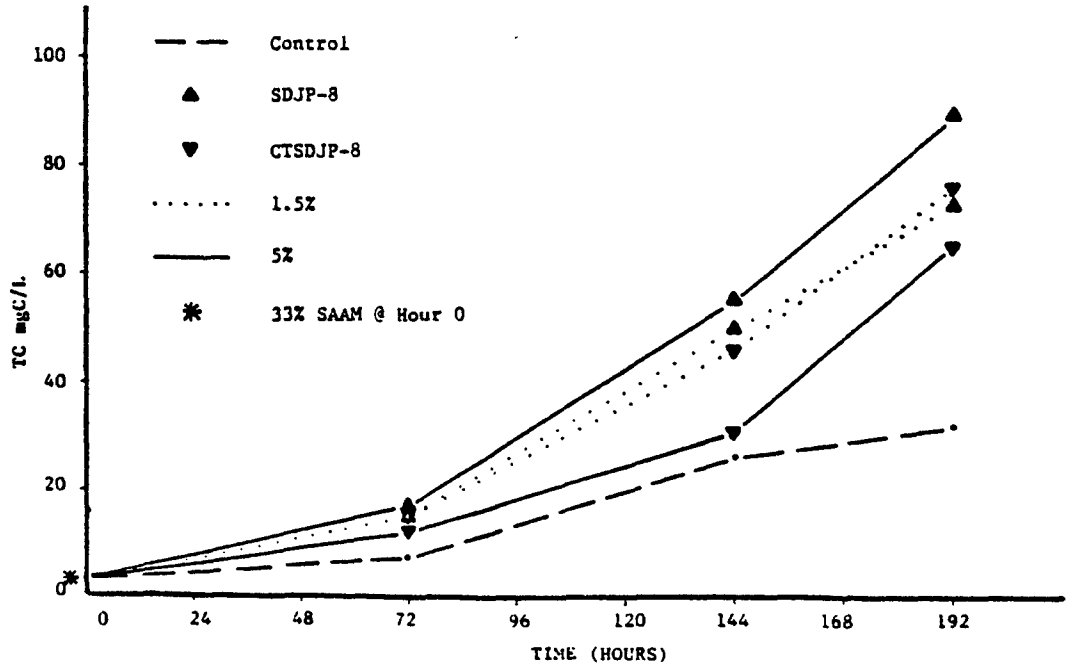


Figure 9 EXPOSURE OF S. CAPRICORNUTUM TO JP-4, JP-8, SDJP-8, CTSDJP-8, TOTAL CARBON WSF/E CONTENT AT SHAKING SPEED 100 RPM

- Shale-derived fuels' WSF contained more medium size droplets than JP-4 and JP-8.

- The cell debris content was light and similar for both SDJP-8 and CTSDJP-8; under the microscope, all samples showed liposoluble chlorophyll pigments, mostly in small globules ranging from 0.3 μm to 0.5 μm even though there was no gross coloration in the flasks. Shale-derived 1.5% fuel ratio WSF/E indicated a denser green coloration for both 50 rpm and 100 rpm mixing systems.

Preliminary investigation was conducted to determine whether TC contents of WSF/E obtained from JP-4 and JP-4 labelled "non-additive" would be significantly different. Assay procedures were similar to those previously described, except only direct fuel layering at 100 rpm was tested. The experimental conditions/results are summarized in Table 9 and indicate clearly that WSF/E obtained from JP-4 "non-additive" contained a lesser amount of total carbon than regular JP-4. If it can be assumed that "regular" JP-4 contains additives, such components probably induce a greater fuel solubility and/or emulsification. This hypothesis is consistent with previous results suggesting emulsifier interaction, yet to be formally investigated.

TABLE 9

EXPOSURE OF S. CAPRICORNUTUM TO JP-4 WITH AND WITHOUT ADDITIVES

	Total Carbon (mg/l)		
	72 Hours	144 Hours	192 Hours
JP-4 non-additive 1.5% fuel ratio	15.2	24.5	24.7
JP-4 non-additive 5% fuel ratio	18.5	37.7	54.0
JP-4 1.5% fuel ratio	25.7	29.2	51.3
JP-4 5% fuel ratio	53.0	54.8	73.5

Bioassays were conducted on a trial basis to determine the EC_{50} and NOEL for JP-4. Exposures of S. capricornutum were limited to 0.5, 0.1, 0.05 and 0.01% concentrations of JP-4 (100 rpm shaking conditions).

Particle analysis of number and mean volume indicated a significant alteration in the distribution curve of both mean volume and population over particle diameter at the 0.05 level. Above 0.05% concentration, population and mean volume increased inversely with size peaking towards the detection limit of the particle counter.

At fuel concentrations of 0.05 and 0.01% both population and mean particle volume distribution peaked at diameters representative of typical algal diameters (3-4 μm). These results indicated that cell growth was effectively obstructed at levels down to approximately 0.1% of JP-4. Substantial growth is observed at lower concentrations.

More detailed investigation of the toxic limits of JP-4 and other test fuels will be carried out during the 1981/82 research period.

Automated Extraction System

The AES components and operational procedure were described previously and experimental reproducibility proved of prime importance during investigations carried out to determine the mechanisms regulating true solution/emulsion phenomena.

Algal batch assays were conducted for studies of relative toxicities through evaluation of growth rates to test the effects of the WSF/E obtained with 33% SAAM, using AES and a 5% mixing concentrations of the four fuels investigated. Effects were monitored through particle counting, TC quantification and optical microscopy observation techniques.

Results of the exposure of S. capricornutum to JP-4, JP-8, SDJP-8 and CTSDJP-8 using AES are presented in Table 10. The mean particle volume measured was consistently lower than the mean particle volume (between 40-60 μm^3) for a normal S. capricornutum cell. Furthermore, visual observations of the total lack of green coloration associated with algal growth in all test flasks (except controls) throughout the duration of the experiment together with the total absence of living algal cells in optical microscope observations led to the conclusion that algal growth was obstructed by the fuels' toxic components. Therefore, particle counts and mean volume measurements were not of unicellular algae but of emulsion globules originating from the fuels.

A comparison of number and size of emulsion globules from Tables 7, 8 and 10 indicates clearly that test solutions mixed by AES had emulsion globules significantly greater in number and smaller in volume than compared with direct fuel layering under low mixing conditions.

The experimental conditions relating to the algal seeding of the fuels' WSF/E were described previously. Figure 10 shows a direct comparison of the WSF/E stability for various fuels as a function of time elapsed. A surprising disruption of the semi-equilibrium state was observed when the WSF/E was seeded with algae. Since particle counting and optical microscopy observations revealed that no cells survived the 5% fuel ration WSF/E, the noticeable TC increase in the WSF/E of the fuel/33% SAAM + algae remains to be explained.

The increased rate cannot be due solely to the limited algal carbon content. It is supposed that an active algal cell lysing process takes place between

TABLE 10

PARTICLE COUNT AND TOTAL CARBON OF BIOASSAY
USING S. CAPRICORNUTUM WITH AUTOMATED EXTRACTION SYSTEM

		Growth Day 6			Growth Day 8			Growth Day 10		
Fuel Type	Rep. No.	10^6 part/ℓ (1)	MPV-mm^3 (2)	TC-mgC/ℓ (3)	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ	10^6 part/ℓ	MPV-mm^3	TC-mgC/ℓ
	1	224.9	16.8	68	511.6	16.4	94	349.4	10.8	100
	2	474.5	21.2	61	905.8	10.2	105	467.8	4.49	85
JP-4	3	1092	17.3	64.5	754.9	8.92	160	539.8	6.22	115
	X	597.1	18.4	64.5	724.1	11.8	119.7	452.3	7.17	100
	S	446.4	2.41	3.49	198.9	4.00	35.4	96.14	3.26	15.0
	1	483.7	12.1	56.5	725.7	8.68	125	740.5	9.07	250
	2	747.8	8.98	60	998.3	18.9	115	779.7	9.98	125
JP-8	3	687.9	17.7	49	961.8	13.9	125	660.9	7.62	85
	X	639.8	12.9	55.2	895.3	13.8	121.7	733.7	8.89	153.3
	S	138.5	4.41	1.73	148.0	5.11	5.77	69.65	1.19	86.1
	1	160.8	28.7	75.5	768.5	NS	135	708.7	7.11	200
	2	1133	8.52	88.5	796.8	7.32	80	1069	11.3	300
SDJP-8	3	1741	9.65	NS	1204	18.8	195	1496	10.4	290
	X	1011	15.6	82	923.1	13.1	170	1091	9.60	263.3
	S	797.1	11.3	9.19	243.7	8.11	31.2	394.1	2.21	55.1
	1	992.0	10.6	NS	3721	11.3	NS	867.2	7.75	390
	2	936.0	13.9	28.5	679.2	14.8	65	722.5	8.14	205
CTSDJP-8	3	1735	7.26	40.5	584.8	8.62	95	534.9	24.3	260
	X	1221	10.6	34.5	1662	11.6	80	708.2	13.4	285
	S	446.0	3.32	8.48	1784	3.10	21.2	166.6	9.44	95
	1	1032	26.8	13.5	94.75	35.5	13.5	810.5	39.9	13.5
	2	881.6	29.6	12.0	80.62	52.1	14.5	273.2	29.2	13.0
CONTROL	3	757.8	14.4	11.0	85.94	34.2	13.0	195.0	8.61	12.5
	X	890.4	23.6	12.2	87.10	40.6	13.7	426.2	25.9	13
	S	137.3	8.09	1.25	7.13	9.98	.707	335.1	15.9	.500

(1) Total Particle Numbers
(2) Mean Particle Volume (MPV)
(3) Total Carbon (TC)
X Mean
S Standard Deviation
NS No Sample

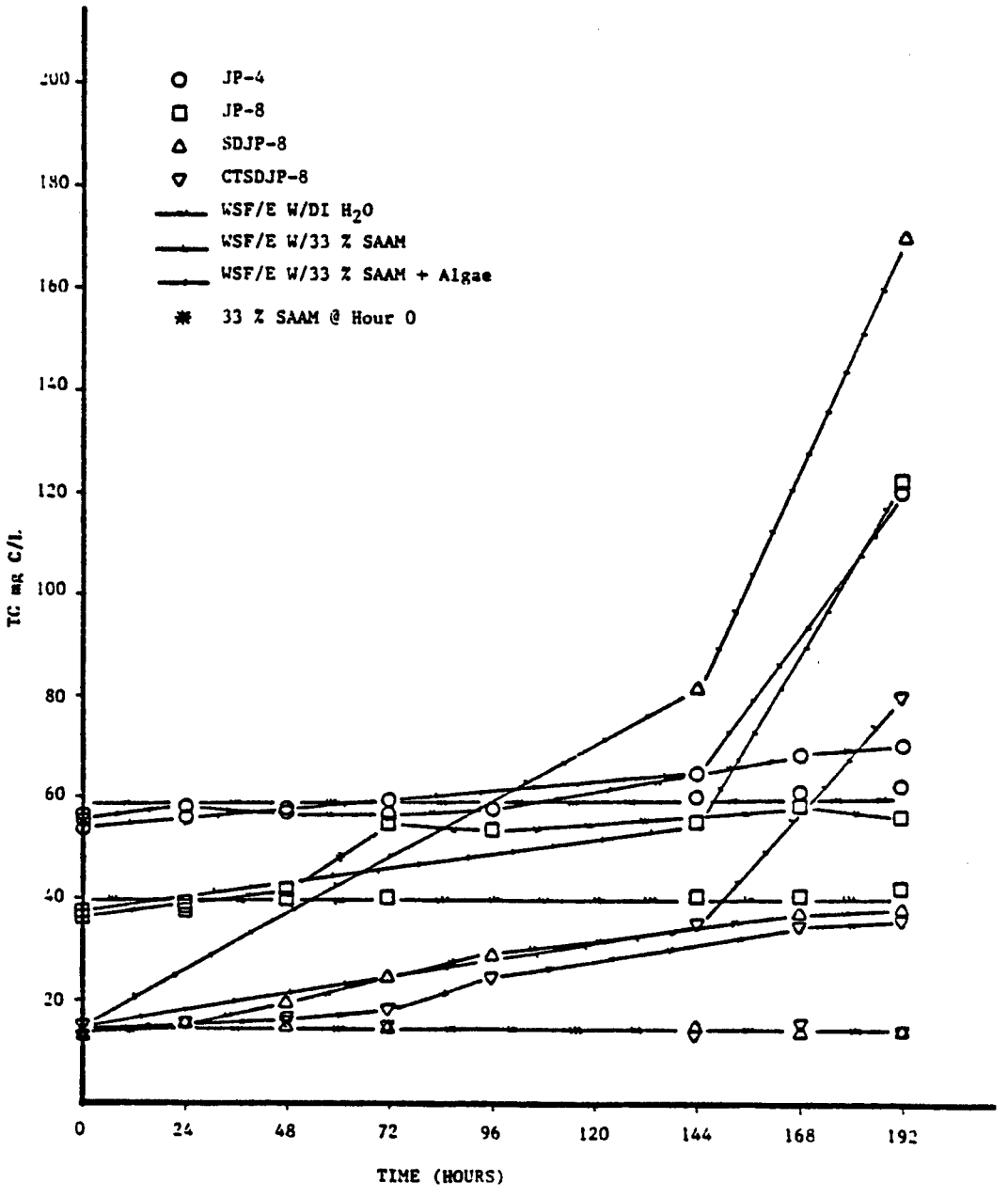


Figure 10 STABILITY COMPARISON OF WSF/E-DEIONIZED WATER, WSF/E-33% SAAM, AND WSF/E-33% SAAM + S. CAPRICORNUTUM (PREPARED WITH AES, 5% FUEL RATIO)

effective cell death and 132 ± 12 hours following initial mixing. All internal structural components released in the medium may react directly as emulsifiers and/or interact with SAAM chemical components generating secondary reactions increasing the fuels' solubility and/or the emulsification process. Further experiments will be conducted to determine the metabolic and/or chemical pathway of such mechanisms.

It is supposed that the fuel layer maintained in each experimental flask was responsible for most of the fuel WSF/E toxicity. The fuel layer creates an airtight film, even at 1.5% fuel concentrations, generating four types of toxic synergistic effects:

- partial volatilization of the fuel film into the atmosphere (adverse effects not measured in the scope of this investigation).
- air-tight seal preventing any gaseous exchanges between air/water phases.
- increased fuel solubility and toxicity due to trapped volatile compounds in the WSF/E.
- semi-equilibrium state reached between dissolved versus emulsified fuel fractions.

Partial evidence supporting these hypotheses was documented in the following preliminary experiment:

1.5 and 5% JP-4 WSF/E was prepared with the AES. Portions of the water phase were drawn from the bottom of the separatory funnels into open beakers without any fuel layer. The loss rate of hydrocarbons was monitored over a period of 5 days. Figure 11 indicates substantial loss for both fuel concentrations. Although these preliminary results were obtained from experiments testing only JP-4 and deionized water, a comparison of Figures 3 and 11 indicates the influence of the jet fuel film.

CONCLUSIONS

Results from these studies supported the following conclusions:

1. A preliminary screening experiment was conducted to determine the range of toxic effects of RJ-4, RJ-5, regular JP-8, shale-derived JP-8 (with clay treatment), JP-9, and JP-10. The screening was conducted by exposing bioassay media (simulating typical lake waters) to 5, 4, 3, 2, and 1 percent of each fuel, mixing briefly by hand, separating the fuel and media, and exposing the test organisms to the media now containing dissolved and emulsified compounds from the fuels. The results showed no effect of RJ-4, JP-9, and JP-10 in this range.

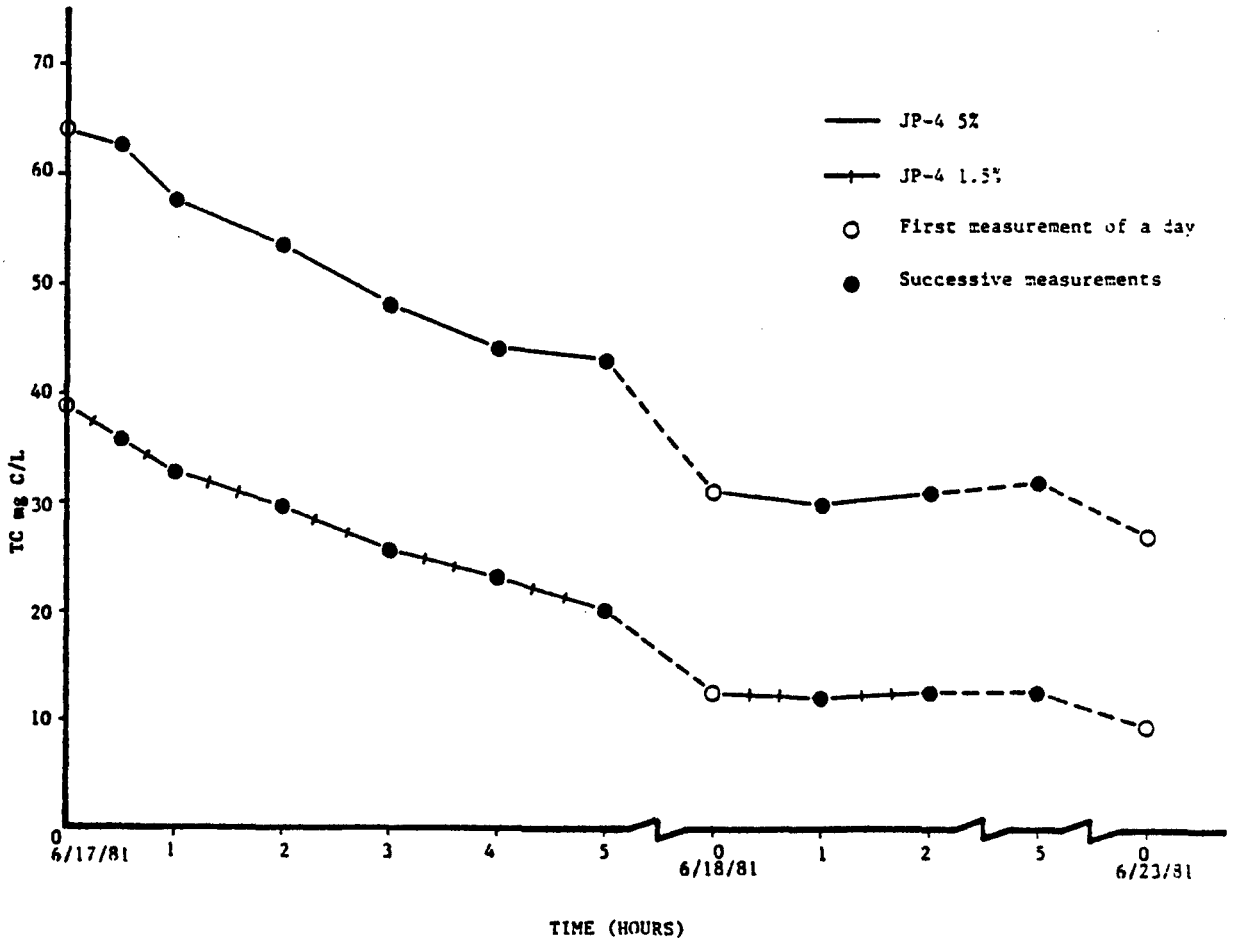


Figure 11 WSF/E TOTAL CARBON LOSS AS A FUNCTION OF TIME (PREPARED WITH AES)

2. RJ-5 and JP-8 showed measurable growth reductions at initial mixtures of greater than 2 percent fuel.

3. Shale-derived JP-8 (clay treated) showed a very strong effect with essentially no growth occurring in the media exposed to 5% fuel and significant reductions in growth in the media exposed to 4 and 3% fuel, respectively.

4. Particle counting and optical microscopy evaluations of water algal growth media after contact with the fuels revealed the presence of emulsified droplets in addition to organic compounds in true solution. Results indicated that as time elapsed fuel composition and mixing procedure influenced the semi-equilibrium status reached between the water-soluble fraction emulsion and the portions of the fuels in the emulsion state.

5. A standard Automated Extraction System (AES) was developed to obtain reproducible extractions/emulsions of JP-4, JP-8, shale-derived JP-8, and clay-treated shale-derived JP-8 over various fuel/deionized water ratios. Total carbon in solution and emulsion combined remained constant over 192 hours as compared to substantial variations over time with manual extraction.

6. Results of the exposure of S. capricornutum to JP-8, monitored through total carbon and GC analysis, indicated that the toxic effects were manifested by the algal test organisms even after the purgeable volatiles leave the solution. Apparently, non-purgeable components were responsible for the growth limiting properties of the fuels.

7. The effect of fuel additives was determined by comparison of JP-4 with and without additives. The presence of additives increased the amount of organic compounds into solution/emulsion by 26.6 mg TC/1 for JP-4, 1.5%, and 19.5 mg TC/1 for JP-4, 5% (measured 192 hours after initial fuel layering).

8. Following exposure of S. capricornutum to the WSF of various fuels, a sudden total carbon increase occurs 132 $\pm$ 12 hours after initial mixing and cell seeding. Since cell growth was obstructed by the toxicity of several of the fuels, it is possible that emulsifiers may be released into the medium by cell lysing and increase the fuels' solubility significantly.

RECOMMENDATIONS

Based on the conclusions derived in this investigation, the following recommendations are made:

1. Additional investigations should be conducted to improve the GC/purge and trap procedure in order to extend the range of recovery from C_4 to C_{19}.

2. Qualitative and quantitative evaluations should be emphasized to determine the relative toxicity and significance of emulsified versus dissolved fractions of

the fuels, and evaluate changes in "effect levels" as a result of propellant aging/decay rates.

3. Current batch algal assays should be completed and continuous culture investigated in order to establish NOEL, EC_{50}, and non-lethal cell structure modifications, if any.

4. Larger-scale experiments should be conducted to verify results obtained with the experimental protocols in simulated natural conditions, and provide basis for models to be developed in years 3, 4, and 5 of the contract.

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