ECOLOGICAL RELATIONSHIPS BETWEEN MARINE MICROORGANISMS AND HYDROCARBONS IN THE OEI STUDY AREA, LOUISIANA

by

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ABSTRACT

Seven cruise projects were conducted in association with the Louisiana Offshore Ecological Investigation (OEI) to determine the relationships between microorganisms and hydrocarbons in surface waters. Techniques were developed to take surface samples and to determine bacterial numbers, hydrocarbon content, and the response of indigenous microorganisms to various added hydrocarbon molecules. The numbers of heterotrophic bacteria as determined by dilution techniques varied from 10 to 10,000 per ml. The hydrocarbon oxidizing organisms varied from 10 to 100,000 per liter with maxima in the month of January. Hydrocarbon concentrations varied from 0.03 to 0.6 micrograms per liter. There was no correlation between the numbers of oil degraders and hydrocarbon concentration found. This may be the result of the in situ low concentrations of hydrocarbons which would limit microbial response. The numbers of bacteria and hydrocarbons were similar to control areas in the eastern and western parts of the Gulf. BOD experiments conducted to measure the response of the microorganisms to various low and high molecular weight paraffinic and aromatic hydrocarbons indicated that all hydrocarbons were oxidized by some of the mixed indigenous microorganisms. These data suggest that an adequate inoculum was naturally present to respond to oil contamination in nature.

The high numbers of hydrocarbon oxidizing microorganisms associated with January hydrographic data may indicate that the upland leaching and other contamination of hydrocarbons in the Mississippi River produce the noted microbial response for that month.

The dominant saturated hydrocarbons and isoprenoids ranged from C-15 to C-41. Water from Timbalier Bay generally contained higher concentrations than offshore samples and exhibited a more pronounced odd-even preference in the range C-24 to C-33. GC-MS analysis of the dominant paraffins revealed a characteristic biomodal distribution dominated by C-17 and pristane and C-25 to C-35 with an unresolved envelope. No buildup of specific n-paraffin molecules was found, which data corresponded to BOD results of added hydrocarbons to indigenous microflora.

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The ecological significance of the occurrence and distribution of hydrocarbons in a marine environment cannot be considered without discussing the role of microorganisms. While hydrocarbons as crude oil have been commercially exploited from geological formations since the turn of the century it was not until the advent of the gas chromatograph, that hydrocarbons were found in recent sediments (P.V. Smith, Jr., 1954). ZoBell (1945, 1946, 1950, 1952) discussed the role of microorganisms in the formation and transformation of hydrocarbons in the marine environment and sediments. Stone and ZoBell (1952) first showed that up to 0.25 percent of the dry weight of petroleum was produced by several species of microorganisms. Hydrocarbons found in other organisms such as fungi (Weete, 1972); plants (Stransky et al., 1967); and marine phytoplankton (Blumer et al., 1971), and others clearly established the fact that hydrocarbons are a common constituent of all living organisms at levels from one percent to 10 ppm.

Reviews of the origin of hydrocarbons and the description of the diversity of saturated, unsaturated and aromatic hydrocarbons are given in Meinschein (1959 and 1969). A summary of world distribution of hydrocarbons in marine waters is given in Meyers and Gunnerson (1976).

Up to the time of the Louisiana Offshore Ecological Investigation (OEI), little published work was available on

the distribution of hycrocarbons and hydrocarbon oxidizing microorganisms as related to any given ecological system.

ZoBell (1969) reported on the distribution of hydrocarbon bacteria in water and sediment in Barataria Bay, Louisiana, the Texas coast and California coast. The numbers of bacteria ranged from 0.1 to 10^8 per ml of water or mud. Other reports on oil-microbe distribution are presented in the Symposium on the Microbial Degradation of Oil Pollutants (1973) and Table 10.

The calculated yearly potential of oil production by marine phytoplankton for the world's ocean, using a level concentration of 10 ppb is approximately 8×10^7 bbls. Estimates of man's introduction by all sources to the oceans are 9×10^6 bbls per year. The report by Meyers and Gunnerson (1976) indicate a residual hydrocarbon concentration in the waters of the world's ocean to be an average of 10 parts per billion or 8.6×10^9 bbls and would take more than 100 years to accumulate at the above rates assuming no degradation.

It is generally accepted by microbiologists working in the field that the action of hydrocarbon degrading microorganisms prohibits the accumulation of hydrocarbons in aquatic systems. Davies and Highes (1968), Crow et al. (1974, Byrom and Beastall (1971), and others have demonstrated the mechanism and rate of degradation of crude oils by various microorganisms. Several reviews on the subject have been published including Butler and Berkes (1972), Gholson et al. (1972), McKenna and Kallio (1965).

Most of the experimental work on hydrocarbon oxidizing microorganisms has been accomplished in the laboratory using cultures obtained from a variety of environmental areas historically exposed to oil. No known mechanisms have been shown for anaerobic decomposition and this is somewhat supported by the persistence of large quantities of oil in sedimentary environments; i.e., commercial oil deposits.

This natural production and the accidental addition of hydrocarbons in the marine environment has been balanced by the oxidative activities of several species of microorganisms (Davis, 1967). A review of the distribution of hydrocarbons in the water of the world's oceans by Meyers and Gunnerson (1976) suggests persistent levels at the threshold of microbial activity. The low levels of individual molecules; i.e. 1 to 10 parts per billion, suggests that there has been no buildup through the ages of any persistent molecule and that microbial activities have been a continual process that has alleviated the accumulation at high levels except for the stratigraphic accumulation of commercially-available oil in ancient sediments.

The research proposed for this part of the interdisciplinary OEI Project was to determine the relationships of the distribution of microorganisms and hydrocarbons to show the impact of the oil producing area and the responsiveness of the area to biological removal of oil contamination. Emphasis was placed on the water surface. Hydrocarbons, being hydrophobic and insoluble, will remain at the air/water interface until they are physically

absorbed by heavier particles, reduced to more dense asphaltic compounds, emulsified in the water, or ingested by larger organisms. Devices were developed to take surface samples for both hydrocarbons and microorganisms. The analysis for microorganisms was conducted on board the vessel. BOD experiments to determine the response of indigenous microorganisms to added hydrocarbons were also conducted on the ship. Hydrocarbon analysis and the testing of degradation rates and oil changes of mixed cultures from the enumeration studies were conducted at the Port Aransas Laboratory.

The research area selected for the Offshore Ecological Investigation is one of the most prolific oil-producing areas in the world. Development began in the late 1930's, and, as of August 1972, there were nearly 6,000 wells working from 1,900 platforms. Over the years through 1973 approximately 3 billion barrels of oil were produced in the area. In addition, the area has been historically exposed to continuous tanker traffic. During World War II, sinkings in the area from May to July, 1942 released an estimated 700,000 barrels of oil.

The historical picture of oil production and exposure provides a setting to select in the most productive area, the 400 mile square study area to determine the effects of oil production in a coastal environment.

The following report provides data on the distribution and ecology of heterotrophic and hydrocarbon oxidizing microorganisms in the Louisiana coastal environment, and the content and molecular forms of hydrocarbons in the water column.

Methods

Water Sampling Methods and Stations:

The location of stations where water samples were taken for bacteriology and hydrocarbon analysis are presented in Tables 1 and 2 and Figures 1 and 2.

It should be noted that considerable emphasis was placed on obtaining "Gulf" control stations (50 to several hundred miles from the study sites) for comparative purposes. It was felt that the designated control area, being only 5 miles from the platform site, might not accurately reflect possible differences in microbial populations or metabolic activities as a result of oil production activities.

Water samples for hydrocarbons were collected at depths of 1 to 4 meters from the surface. All techniques used were designed to prohibit contamination from the ship or handling. Hydrocarbons in surface films were collected with an apparatus described in Miget et al. (1974). Hydrocarbons in the water were sampled in a clean 5 gallon bottle in a metal frame. The device, attached to hydrographic wire, was dropped into the water over the side of the vessel and allowed to fill. On return to the surface, 100 ml of Benzene was added to preserve the sample and a stopper coated with teflon was used to close the bottle. The sample with Benzene was mixed and not opened until extraction took place.

Surface water for BOD studies and bacterial enumeration was collected using a sterilized sampler developed at this laboratory

TABLE 1

GURC-OEI MICROBIOLOGICAL PROGRAM
SAMPLING STATIONS

YEAR	MONTH	DAY	STA.#	LOCATION	AREA	GURC LOC.
1972	June		1	28.34 N 93.00 W	N.W. Gulf coast	None
			2	28.32 N 90.18 W	OEI study area	PO72
			3	27.37 N 88.30 W	N. Central Gulf	None
			4	27.11 N 85.40 W	N.E. Gulf	None
			5	27.20 N 84.11 W	E. Central Gulf	None
			6	24.46 N 83.16 W	Dry Tortugas area	None
			7	23.53 N 84.08 W	S.E. Gulf	None
			8	24.49 N 88.00 W	Central Gulf	None
1972	Sept.	19	1	28.51 N 92.10 W	S. Marsh Is. area	None
		20	3	28.49.53 N 90.23.18W	S. Tim. 54A	KO07
		20	4	28.53.13 N 90.19.30W	Desig. control	HO62
		20	6	28.48.30 N 90.27.30W	S.W. Plat. 54A	KO23
		21	8	28.49.53 N 90.23.18W	S. Tim. 54A	K007
		21	9	28.53.13 N 90.19.30W	Desig. control	HO62
1973	Jan.	9	1	28.49.53 N 90.23.18W	S. Tim. 54A	K007
		9	4	28.53.13 N 90.19:30W	Desig. control	HO62
		10	5	29.12.20 N 90.21.30W	Tim. Bay	A079
		11	6	28.54 N 90.45 W	Ship Shoal area	None
		12	7	29.39 N 93.11 W	East of Sabine R.	None
		12	8	29.30 N 94.01 W	West of Sabine R.	None
1973	April	2	1	29.06 N 93.59 W	West of Sabine R.	None
		2	2	28.50 N 91.20 W	Ship Shoal area	None
		3	3	28.49.53 N 90.23.18W	S. Tim 54A (Upstm)	K007
		3	5	" "	" (Dwnstm)	K007
		4	6	29.13 N 90.00 W	11 mi off Grande Is	C070
		5	7	28.49.53 N 90.23.18W	S.T. 54A (Dwmstm)	K007
		5	8	28.53.13 N 90.19.30W	Desig. control	HO62
		6	16	29.12.20 N 90.21.30W	Tim. Bay	A079
1973	July	7	1	28.55 N 95.05 W	Off Galveston, TX	None
		8	2	29.13 N 93.42 W	West of Sabine R.	None
		8	4	28.30 N 91.10 W	Ship shoal area	None
		9	5	28.49.53 N 90.23.18W	S. Tim. 54A	K007
		11	7	28.53.13 N 90.19.30W	Desig. control	H062
		13	8	29.12.20 N 90.21.30W	Tim. Bay	A079
1973	Oct.	14	1	29.07 N 92.52 W	East Cameron area	None
		15	4	28.49.53 N 90.23.18W	S. Tim. 54A	K007
		16	7	29.12.20 N 90.21.30W	Tim. Bay	A079
		17	6	28.53.13 N 90.19.30W	Desig. control	H069
1974	Jan.	13	1	28.53 N 94.06 W	N.W. Gulf	None
		14	4	28.49.53 N 90.23.18W	S. Tim. 54A	K007
		15	5	28.53.13 N 90.19.30W	Desig. control	H062
		16	6	29.12.20 N 90.21.30W	Tim. Bay	A079

TABLE 2

Sample Locations

FOR HYDROCARBON ANALYSIS

Seawater

Date	Location	Designation
9-20-72	28 50, 90 30	K040, S.E. 54A Platform
9-20-72	28 53, 90 19	H062, Control
9-20-72	28 49, 90 23	K007, 54A Platform
1-10-73	29 12, 90 21	Timbalier Bay
1-9-73	28 53, 90 19	H062, Control
1-12-73	29 40, 93 11	E. Sabine River
1-12-73	29 30, 94 01	W. Sabine River
1-11-73	28 54, 90 45	Ship Shoal
1-9-73	28 50, 90 30	K014, S.W. 54A Platform
1-9-73	28 49, 90 23	K007, 54A Platform
4-6-73	29 12, 90 21	Timbalier Bay
5-5-73	27 46, 92 52	Port Aransas
7-11-73	28 53, 90 19	H062, Control
7-8-73	29 03, 95 52	Control
7-8-73	29 13, 93 42	W. Sabine River
7-8-73	29 00, 92 20	S. Timbalier Isle
7-21-73	26 10, 96 40	Brownsville
10-16-73	29 12, 90 21	Timbalier Bay
1-15-74	29 12, 90 21	Timbalier Bay
1-14-74	28 49, 90 23	K007, 54A Platform
1-16-74	28 53, 90 13	H062, Control
4-15-73	27 46, 92 52	Port Aransas

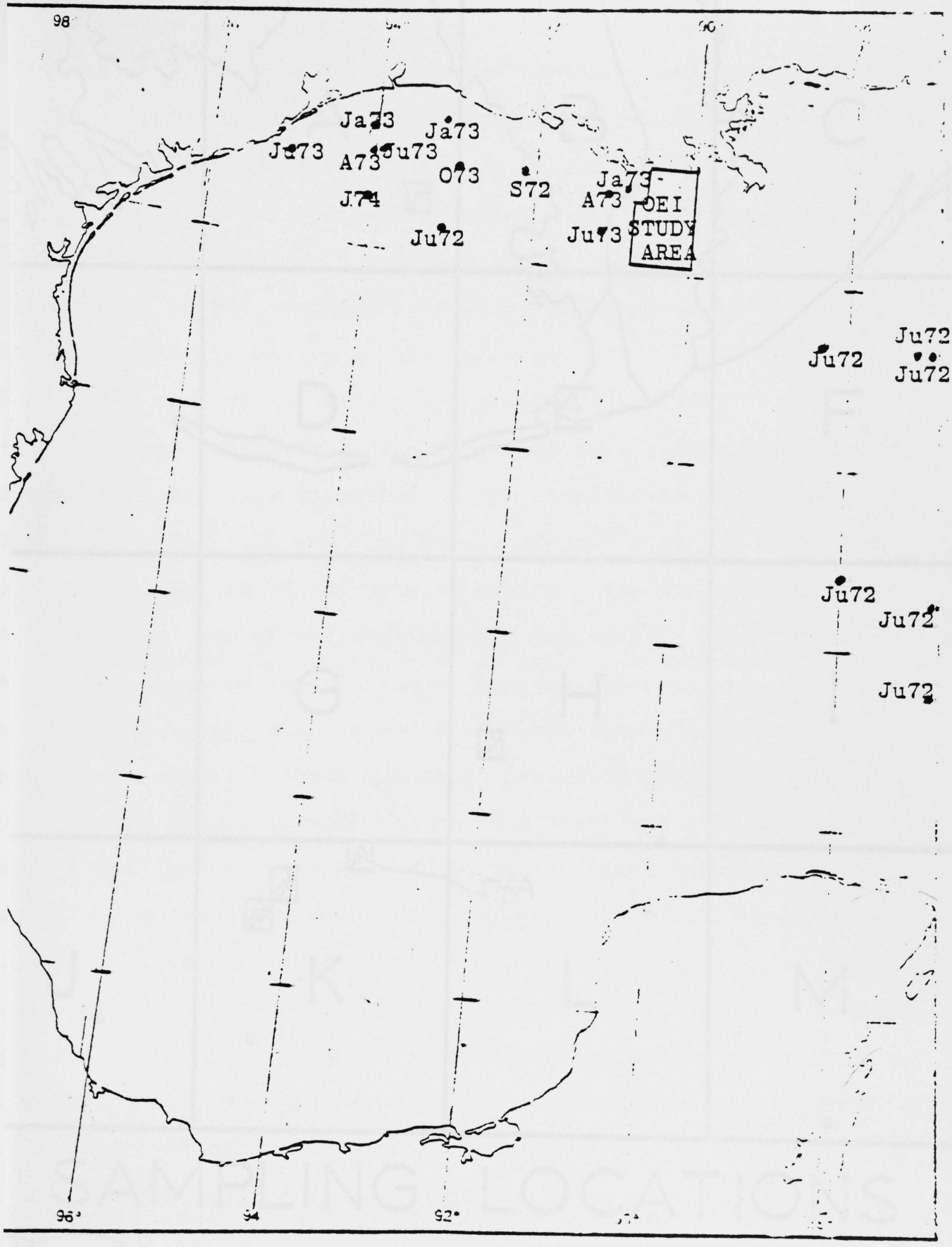
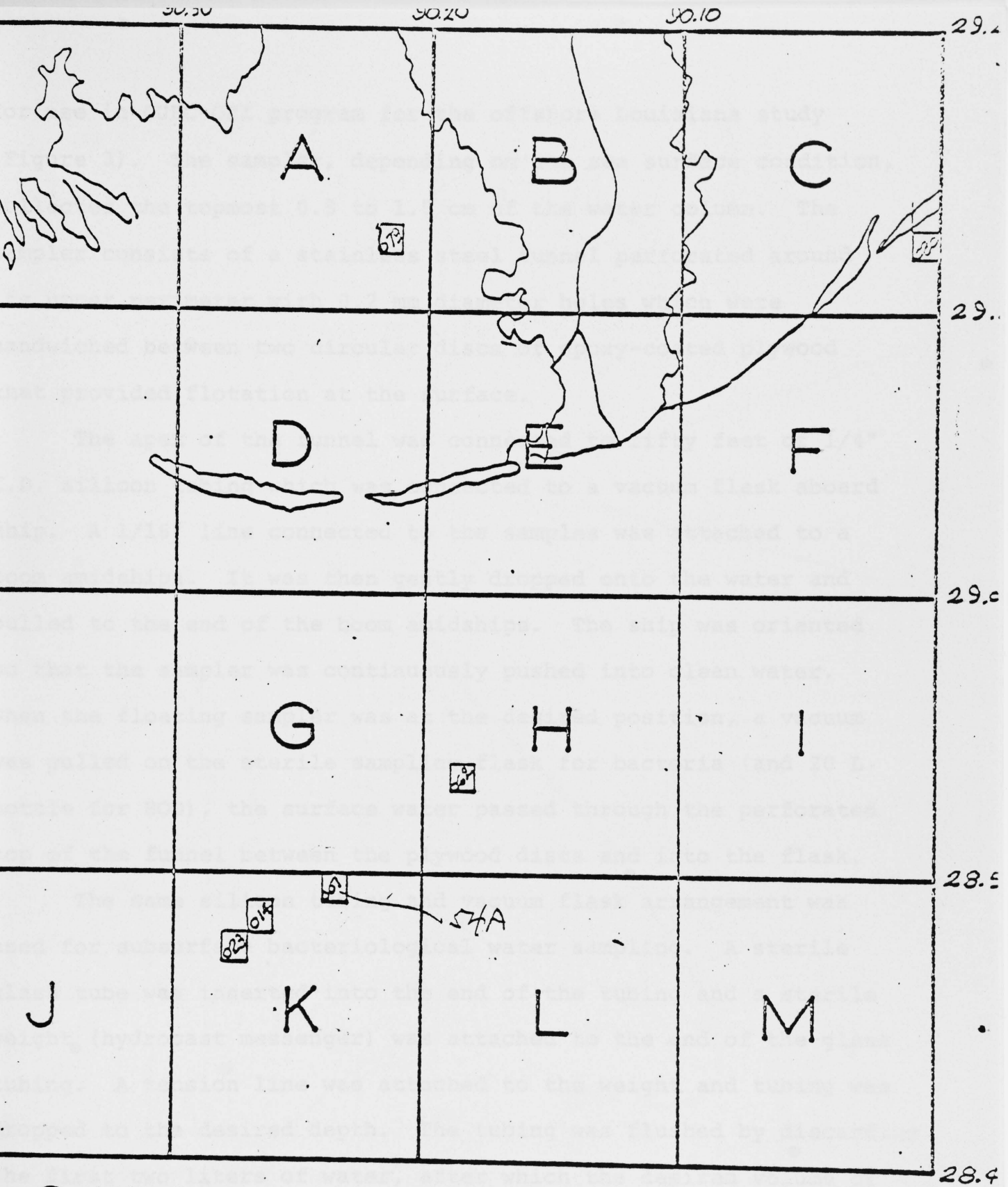


Figure 1 OEI MICROBIOLOGICAL "GULF" CONTROL STATIONS



SAMPLING LOCATIONS

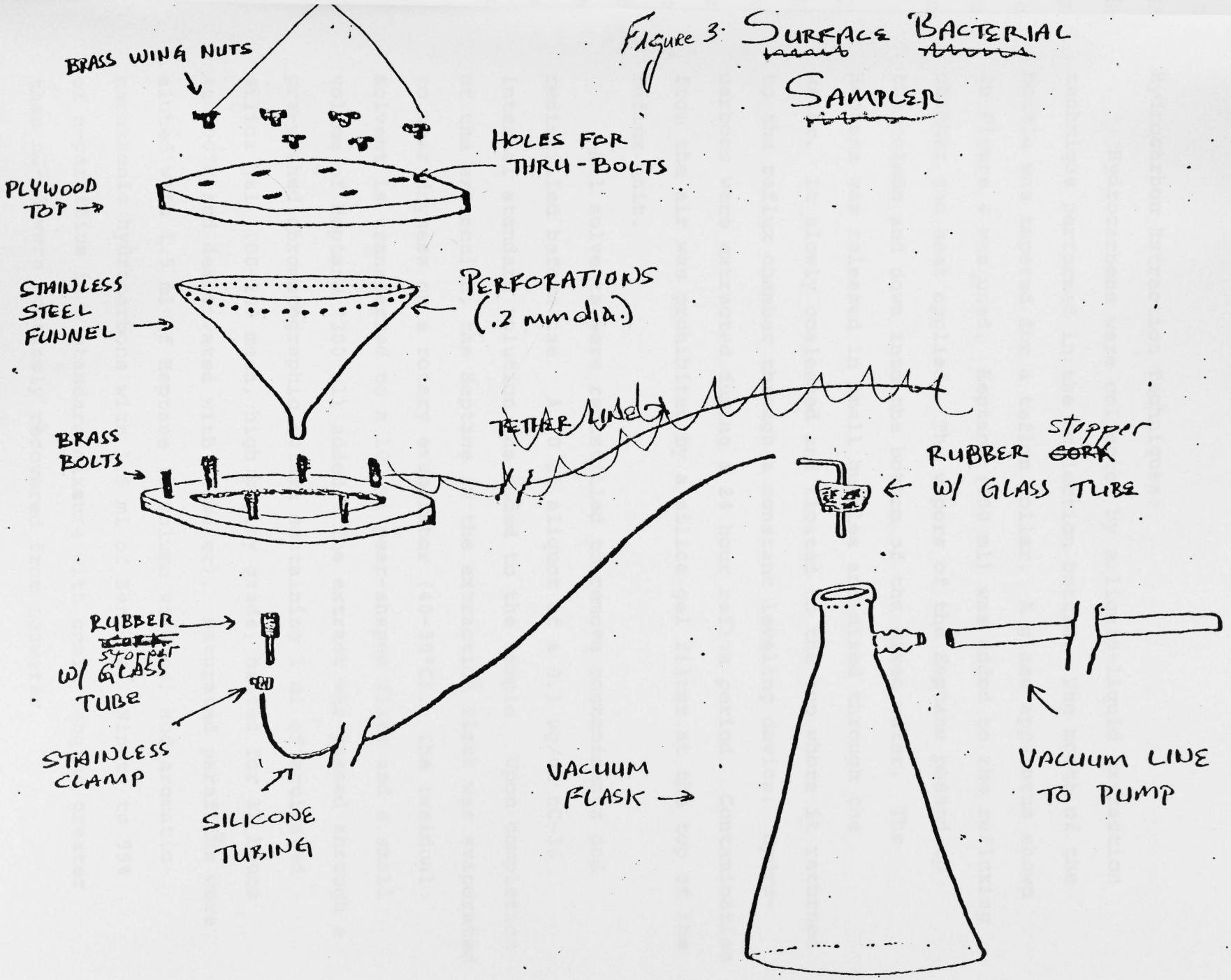
Figure 2

for use in GURC-OEI program for the offshore Louisiana study (Figure 3). The sampler, depending on the sea surface condition, collected the topmost 0.5 to 1.5 cm of the water column. The sampler consists of a stainless steel funnel perforated around its upper perimeter with 0.2 mm diameter holes which were sandwiched between two circular discs of epoxy-coated plywood that provided flotation at the surface.

The apex of the funnel was connected to fifty feet of 1/4" I.D. silicon tubing which was connected to a vacuum flask aboard ship. A 1/16" line connected to the samples was attached to a boom amidships. It was then gently dropped onto the water and pulled to the end of the boom amidships. The ship was oriented so that the sampler was continuously pushed into clean water. When the floating sampler was at the desired position, a vacuum was pulled on the sterile sampling flask for bacteria (and 20 L bottle for BOD), the surface water passed through the perforated top of the funnel between the plywood discs and into the flask.

The same silicon tubing and vacuum flask arrangement was used for subsurface bacteriological water sampling. A sterile glass tube was inserted into the end of the tubing and a sterile weight (hydrocast messenger) was attached to the end of the glass tubing. A tension line was attached to the weight and tubing was dropped to the desired depth. The tubing was flushed by discarding the first two liters of water, after which the desired volume of sample water was drawn into a sterile vacuum flask.

Figure 3: Surface Bacterial Sampler



Hydrocarbon Extraction Techniques:

Hydrocarbons were collected by a liquid-liquid extraction technique performed in the collection bottle. The mouth of the bottle was tapered for a teflon collar. A glass apparatus shown in Figure 4 was used. Heptane (300 ml) was added to the refluxing chamber and heat applied. The vapors of the Heptane passed up the column and down into the bottom of the mixed water. The Heptane was released in small bubbles and mixed through the water. It slowly coalesced and floated to the top where it returned to the reflux chamber through a constant leveling device. Hydrocarbons were extracted during a 24 hour reflux period. Contamination from the air was prohibited by a silica gel filter at the top of the reflux unit.

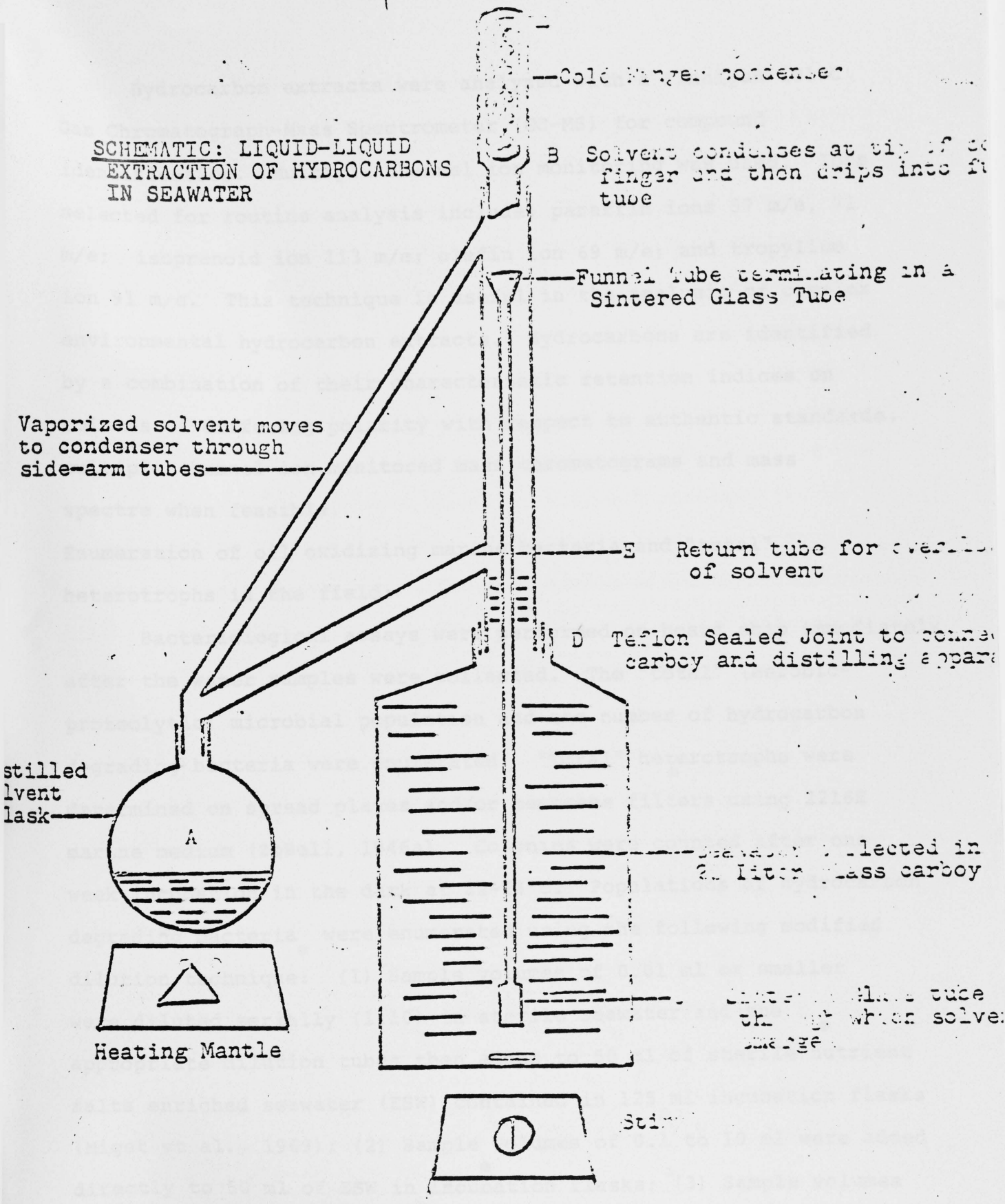
All solvents were redistilled to remove contaminants and redistilled before use. A 20 µl aliquot of a 0.1 µg/µl nC-36 internal standard solution was added to the sample. Upon completion of the extraction, the Heptane in the extraction flask was evaporated to near dryness on a rotary evaporator (40-50°C). The residual solvent is transferred to a 100 ml pear-shaped flask and a small volume of Heptane (300 µl) added. The extract was passed through a pre-washed chromatographic column containing 1 ml of activated silica gel (100-200 mesh, high purity grade, heated for 12 hours at 200°C and deactivated with 5% water). Saturated paraffins were eluted with 1.5 ml of Heptane (3 column volumes) and aromatic-naphthenic hydrocarbons with 1.5 ml of Benzene. Ninety to 95% of n-paraffins in a standard mixture with chain lengths greater than nC-18 were routinely recovered from seawater.

Figure 4. Liquid-liquid extraction apparatus designed to extract hydrocarbons from seawater in the container in which it was collected. Redistilled solvent is heated at the boiling point in the Distilled Solvent Flask at A and distills off and condenses at B. Solvent then drips from B into the funnel tube which termintes in a sintered glass tube C. With sufficient head to overcome the water hydrostatus pressure the solvent emerges from the sintered glass in the form of small bubbles. These bubbles possess enormous surface area for extraction and revolve in the water sample due to the action of a stir bar at bottom of the bottle. Because of the density difference between Heptane and water, these bubbles of solvent rise past D until sufficient solvent accumulates to return to the distilling flask at E. In this fashion the seawater is continuously extracted with fresh solvent.

~~Figure 1-~~

Figure 4-- Liquid-Liquid extraction apparatus designed to extract hydrocarbons from seawater in the container in which it was collected. Redistilled solvent is heated at the boiling point in the Distilled Solvent Flask at A and distills off and condenses at B. Solvent then drips from B into the funnel tube which terminates in a sintered glass tube C. With sufficient head to overcome the water hydrostatus pressure the solvent emerges from the sintered glass in the form of small bubbles. These bubbles possess enormous surface area for extraction and revolve in the water sample due to the action of a stir bar at bottom of the bottle. Because of the density difference between Heptane and water, these bubbles of solvent rise past D until sufficient solvent accumulates to return to the distilling flask at E. In this fashion the seawater is continuously extracted with fresh solvent.

FIGURE 4


SCHEMATIC: LIQUID-LIQUID EXTRACTION OF HYDROCARBONS IN SEAWATER
Cold Finger condenser
B Solvent condenses at tip of cold finger and then drips into funnel tube
Funnel Tube terminating in a Sintered Glass Tube
Vaporized solvent moves to condenser through side-arm tubes
E Return tube for overflow of solvent
D Teflon Sealed Joint to connect carboy and distilling apparatus
stilled lvent lask
A
Heating Mantle
Sintered Glass tube through which solvent emerges
Stirrer

Hydrocarbon extracts were analyzed with a Finnigan 1015C Gas Chromatograph-Mass Spectrometer (GC-MS) for compound identification. Multiple channel ion monitoring was used. Ions selected for routine analysis include: paraffin ions 57 m/e, 71 m/e; isoprenoid ion 113 m/e; olefin ion 69 m/e; and tropylium ion 91 m/e. This technique is useful in the analysis of complex environmental hydrocarbon extracts. Hydrocarbons are identified by a combination of their characteristic retention indices on columns of differing polarity with respect to authentic standards, multiple channel ion monitored mass chromatograms and mass spectra when feasible.

Enumeration of oil oxidizing marine bacteria and "total" heterotrophs in the field:

Bacteriological assays were performed on board ship immediately after the water samples were collected. The "total" (aerobic-proteolytic) microbial population and the number of hydrocarbon degrading bacteria were enumerated. "Total" heterotrophs were determined on spread plates and/or membrane filters using 2216E marine medium (ZoBell, 1946a). Colonies were counted after one week incubation in the dark at 22-24°C. Populations of hydrocarbon degrading bacteria were enumerated using the following modified dilution technique: (1) Sample volumes of 0.01 ml or smaller were diluted serially (1:10) in sterile seawater and the appropriate dilution tubes then added to 50 ml of sterile nutrient salts enriched seawater (ESW) contained in 125 ml incubation flasks (Miget et al., 1969); (2) Sample volumes of 0.1 to 10 ml were added directly to 50 ml of ESW in incubation flasks; (3) Sample volumes

greater than 10 ml were filtered through sterile 0.45 micron membrane filters and the filters cut in small pieces with sterile scissors and added to the incubation flasks containing 50 ml ESW. All flasks, including uninoculated controls, then received 0.5 ml of sterilized crude oil (obtained from a wellhead in ST 54), and were incubated on a gyrotary shaker at ships' temperature (22-24°C) for four days. Positive growth (scored minus to plus 4) was indicated by the presence of a visible oil emulsification, or an increase in turbidity of the water accompanied by a physical change in the oil sheen -- all relative to the appearance of the oil and water in the control flasks. Although positive flasks were scored on the completeness of emulsification, the number of oil degrading microorganisms for each sample was determined by dilution technique based on replicate dilution flasks showing any change in appearance of the oil or water compared with control flasks.

Evaluation of bacterial hydrocarbon degradation using the Biochemical Oxygen Demand (BOD) technique:

BOD studies were carried out as follows: sterile 19 liter glass carboys were filled with sample water from the surface at a given station, shaken vigorously to insure uniform oxygen dispersion, then carefully siphoned into sterile 300 ml standard BOD bottles. Nutrient salts [$(NH_4)_2SO_4$ @ 1 gm/l and K_2HPO_4 @ 0.01 gm/l] were added to each bottle from a sterile concentrated stock solution. Hydrocarbon substrates (either pure hydrocarbons or crude oils) were added to the replicate bottles in one of the

following two ways: A. Hydrocarbons which were solid at 20°C were dissolved in benzene and added to approximately 0.1 gm of clean, silicon coated, sterile micro glass beads (590-840 micron diameter) contained in small pieces of solvent-cleaned aluminum foil. The solvent was allowed to evaporate in a fume hood and the coated beads were placed in respective BOD bottles immediately prior to filling with seawater. B. Hydrocarbons which were liquid at 20°C were added directly to filled bottles using a micro-liter syringe by rapidly injecting the substrate into the nutrient enriched water near the bottom of the bottle then withdrawing the syringe and stoppering the bottle before the hydrocarbon floated to the surface. The bottles were incubated upside down. All ground glass stoppers were coated with a thin film of silicon grease to insure an airtight seal. Replicate BOD bottles of water from each carboy were immediately preserved and the oxygen concentration referred to as T=O values.

The oxygen concentration in each sample was determined after incubation in the dark by the Winkler method described in Standard Methods.

Results and Discussion

Hydrocarbons in Seawater:

The concentrations and several salient features describing n-paraffins and selected isoprenoid hydrocarbons for the study period are listed in Tables 3, 4, 5, 6 and 7 and Figures 5 and 6. N-paraffins are generally the predominant hydrocarbons in these extracts. The isoprenoid hydrocarbons, pristane and phytane,