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MARINE PETROLEUM POLLUTION; BIOLOGICAL EFFECTS AND CHEMICAL CHARACTERIZATION

PRINCIPAL INVESTIGATORS:

J. A. C. Nicol

C. Van Baalen

University of Texas Marine Science Institute

Port Aransas, Texas 78373

Chemical Characterization of Test Oils

Personnel:

Kenneth Winters
Connie Charlton
Martha Roach

Introduction

Crude and fuel oils are extremely complex mixtures containing hundreds of chemical compounds. Although most of the compounds are probably common to all crude oils, each has a unique composition.

There are many compounds known to occur in petroleum which have been proven toxic at some concentration to plants and/or animals.[1,2] Some compounds are much more toxic than others. Variations in the concentrations of these and other yet unidentified toxic agents probably result in the variations in toxicity observed among oils. Variations in oil toxicity and uncertainties in the determination of environmental concentrations of petroleum make it difficult to evaluate environmental quality.

The goal of the chemical section of this project is to characterize a few of the more toxic agents found in several representative test oils. Our experimental design is to use several physical and chemical methods to fractionate test oils while following toxicity by the bioassays described in the biological sections of this report. Accomplishment of this goal may demonstrate that a few key compounds are responsible for much petroleum toxicity. The concentration (and inferred toxicity) of a few specific compounds could probably be measured more accurately in the environment than the concentration (and inferred toxicity) of "oil".

Materials and Methods

Test oils

The American Petroleum Institute has kindly set aside four oils which may be used by the scientific community as reference oils. The four oils available include two crude oils (Kuwait and Southern Louisiana) and two fuel oils (a #2 fuel oil and a Bunker C). These oils and some associated analytical data are currently available at a modest cost from: Dr. Jack Anderson, Department of Biology, Texas A&M University. In addition to the A.P.I. oils we have studied a sample of a diesel fuel and a Venezuelan crude oil. We have just received and begun work on a sample of an Alaskan crude oil.

Fractionation of test oils

Each of the oils was fractionated on a column of silica gel which had been activated at 175-200°C for at least 12 hours. A 45 cm. x 4 cm. column of silica gel (120-200 mesh) in hexane was prepared. The oil sample (2-7 grams depending on the oil) was diluted with an equal volume of hexane and layered on the column. Column flow rate was adjusted to 2-3 milliliters per minute. The fractions of oil which eluted with hexane, benzene, and chloroform: methanol (1:1) were designated the paraffinic, aromatic, and asphaltic fractions respectively.

Fractions of the #2 fuel oil and Kuwait crude were also prepared by distillation at atmospheric pressure. The oils were distilled through a 50 cm. x 3 cm. column packed with Raschig rings. We are currently experimenting with several methods of vacuum distillation.

Several techniques were examined for the separation of one, two, and multi ring aromatic compounds. The most satisfactory method examined thus far employed a 70 cm. x 2 cm. column of Sephadex LH-20 with isobutyl alcohol as the eluting solvent. A fraction collector equipped with a drop counter was set to collect about 3 milliliters of column eluate per tube. A similar column with acetone gave less resolution.

An Aerograph Model 202 gas chromatograph with thermal conductivity detection has been used for relatively small scale preparative gas chromatography. When a compound(s) was to be collected a 15 cm. x 0.4 cm. O.D. glass tube with a right angle bend was inserted into the exit port of the gas chromatograph. The carrier gas was bubbled through several milliliters of hexane in a small test tube. The glass tubing was removed from the gas chromatograph after the compound(s) had eluted and the process repeated to trap the same compound(s) from several sample injections. A few milliliters of warm hexane were used to transfer any material in the glass tubing to the test tube.

Preparation of a water soluble fraction from the test oil.

One part of the oil to be tested was layered on the surface of eight parts filtered (Gelman glass fiber Type A) sea water in a bottle containing a teflon coated magnetic stir bar. The bottle was sealed and the water stirred at room temperature for 24 hours at a rate which would avoid the formation of an emulsion. The water was allowed to stand undisturbed for several minutes before being removed by means of a stopcock at the base of the bottle.

When less than one hundred milliliters of a water soluble fraction was to be prepared an alternate method was used. The same ratio of oil to water was placed in an Erlenmeyer flask. The flask was sealed and shaken for 24 hours on a reciprocating shaker. The oil and water were transferred to a separatory funnel and the water soluble fraction removed. Water soluble fractions prepared by the two methods appear to be similar in concentration and toxicity.

Extraction of the water soluble fraction.

A continuous liquid-liquid extractor was used to extract the organic compounds contained in the water soluble fractions prepared from the test oils. Extraction of more than 150 milliliters of sea water was carried out in a separatory funnel. The water was extracted three times with 1/10 volume portions of solvent (hexane:benzene, 1:1).

Gas chromatography

All analyses were carried out on a Perkin-Elmer 900 gas chromatograph with a flame ionization detector. Peak areas and retention times determined by an Infotronics 204 integrator were printed and paper tape punched by a Teletype terminal. Routine analyses were usually made on both a 5' x 1/8" stainless steel column packed with 4% Apiezon L on 80/100 mesh Gas Chrom Q and a 6' x 1/8" column of 5% FFAP on the same support. SCOT columns (150' x 0.02") of Apiezon L and FFAP were used when greater resolution was necessary.

Results and Discussion

Since hopefully a rather large number of investigators will be involved in the use of the American Petroleum Institute reference oils

we should probably mention a couple of observations made during our work with these oils. It was observed that there was a fine suspended material in our samples of the #2 fuel oil. Filtration of the fuel oil through Gelman glass fiber type A pads removed most but not all of this material. The material appeared to be inorganic and dark purple-black in color. We have not examined the nature of this material. It has also been observed that our sample of Kuwait crude contains a residue at the bottom of the container which has a consistency which is different from the overlying oil.

The per cent by weight of the paraffinic, aromatic and asphaltic components of the test oils (determined by silica gel fractionation) are given in Table 1. These results demonstrate the difference in composition which exists between oils. These differences in composition probably give rise to the differences in toxicity described in the biological sections of this report. The variations in toxicity observed between oils suggest that to evaluate environmental quality it is as important to know the type of oil as it is to know the concentration. Problems are apparent in the determination of either of these variables in the marine environment. Uncertainties such as weathering of oils, microbial degredation, and input of natural biogenic hydrocarbons make determination of low level petroleum concentrations difficult. Also, petroleum residues measured in the environment are rarely derived from a single specific oil.

In view of these considerations we are attempting to characterize a few of the agents in oils which demonstrate the greatest toxicity to test organisms.

Laboratory toxicity data based on the concentrations of a few specific compounds from petroleum could be quite useful. This data combined with our data on whole oils should enable us to better understand factors which affect toxicity and also allow us to better relate our data to the environmental impact of petroleum. Measurement of the concentrations of a few nonbiogenic compounds in a sample containing an unknown background of biogenic hydrocarbons should be more accurate than the "guesstimated" concentration of total petroleum residue. The total petroleum concentration may also, as mentioned previously, be a poor indicator of toxicity (see biological sections).

A number of physical and chemical fractionation procedures have been considered for the task of characterizing and hopefully isolating toxic materials from petroleum. In addition to its usefulness as a tool to compare the compositions of test oils, silica gel column chromatography provides a quick and simple method for the removal of paraffins from a complex mixture. Paraffins are often present in large quantities in oils and are the least suspect class of compounds. Their removal can therefore result in a substantial concentration of toxicity.

The approximate boiling point ranges of the distillate fractions prepared from the #2 fuel oil are given in Table 2. Gas chromatograms of these fractions and the whole fuel oil are shown in Figures 1-4. In an effort to avoid decomposition of high boiling components and the possible formation of toxic artifacts we have discontinued distillation at atmospheric pressures. We have been experimenting with several methods of distillation under relatively high vacuum. We recently prepared low temperature vacuum distilled fractions of the #2 fuel oil.

Drs. Pulich and Van Baalen are just completing experiments with these new fractions which verify the results obtained with earlier fractions.

Separation of aromatic compounds on Sephadex LH-20 has been previously reported by several workers.[3,4] Separation of aromatic compounds on Sephadex LH-20 is primarily due to adsorption of the aromatic ring to the column material rather than gel permeation normally associated with the use of Sephadex in aqueous solutions. Therefore one ring aromatic compounds generally elute prior to naphthalenes and naphthalenes preceed three ring compounds. Alkyl substitution reduces the absorption of the aromatic ring system, hence methyl naphthalene begins to elute prior to naphthalene. Figures 5-7 illustrate the separation of components from a distillate of the #2 fuel oil which was rich in naphthalenes. The first compounds to elute are paraffins (tube 56, Figure 5). Alkyl benzenes (tube 76) elute prior to naphthalenes. Dimethyl naphthalenes (tube 86) elute before methyl naphthalenes (tube 91). Naphthalene begins to elute in tube 91 and is the major component in tube 96. The three ring compound phenanthrene was found in tubes near 120. Difficulties were encountered when several crude oils were tested on LH-20 columns. A significant amount of many crude oils was insoluble in isobutyl alcohol and an additional amount was bound essentially irreversibly to the column material. The #2 fuel oil was however soluble in and completely removed by isobutyl alcohol.

Thus far our work with preparative gas chromatography has been limited to the examination of several methods of collection. This technique used in conjunction with one or more of the fractionation methods previously described allows the isolation of relatively pure

compounds.. These compounds isolated from the test oils may then be checked for biological activity and analysed by mass spectrometry etc.

Figure 8 illustrates a chromatogram obtained by analysis of the water soluble fraction prepared from the #2 fuel oil. Our data indicate that about 15 mg of organic material per liter of sea water remain after extraction of the water with hexane:benzene (1:1) and the solvent is allowed to evaporate at room temperature. The dramatic difference in per cent composition of compounds in the water soluble fraction (Fig. 8) versus the parent oil (Fig. 1) can be taken as evidence that the water solubles are not simply a dispersion of the fuel oil. Little work has been done on the water soluble fractions of Kuwait or Southern Louisiana crudes at this time.

Summary

Several techniques have been used to fractionate test oils into a series of samples which are more manageable analytically and more informative as to the nature of petroleum toxicity.

Separations based on silica gel column chromatography have been used to characterize gross differences between test oils. The technique has also be used to remove paraffins from samples. Paraffins are often present in large quantities in oils but are the least suspect with regard to toxicity.

Fractionation of test oils by distillation has proved useful and informative. Analysis of lower or higher boiling components of test oils requires special attention and unnecessarily complicates analyses of mid-boiling range components. The biological data indicate that toxicity of some distillate fractions is greater than others. This fractionation technique may therefore be a useful starting point for the isolation of the more toxic components from oils.

The use of Sephadex LH-20 to separate aromatic compounds in petroleum has been effective. With this technique we have proven the ability to simplify a #2 fuel oil to fractions containing only a few major compounds.

Preparative gas chromatography used in combination with these techniques promises to yield milligram quantities of pure compounds and/or simple mixtures from test oils.

Another area of study has been the qualitative and quantitative analysis of components of "water soluble fractions" prepared from test oils. The water soluble components of #2 fuel oil prepared by our method are present at a concentration of about 15 mg per liter of sea water.

References

1. Boylan, D. B. and B. W. Tripp, Determination of hydrocarbons in sea water extracts of crude oil and crude oil fractions. Nature, 230:44 (1971).

2. Kauss, P., et al. The Toxicity of Crude Oil and Its Components to Fresh Water Algae. Proceedings of Joint Conference on Prevention and Control of Oil Spills, 703 (1973).

3. Mair, B. J., P. T. R. Hwang, and R. G. Ruberto, Separation of petroleum hydrocarbons by selective adsorption with Sephadex LH-20. Anal. Chem. 39:838 (1967).

4. Wilk, M., J. Rochlitz, and H. Bende, Column chromatography of polycyclic aromatic hydrocarbons on lipophilic Sephadex LH-20. J. Chromatog. 24:414 (1966).

Figure Legends

Figures 1-7

Chromatographic conditions: 5' x 1/8" stainless steel column, 4% Apiezon L on 80/100 mesh Gas Chrom Q. Temperature was programmed from 80-295° at 6°/minute.

Figure 8

Chromatographic conditions: Same as Figs. 1-7 except the program rate was 4°/minute.

All Figures

Peak identification

10 through 23 = n-paraffin with the corresponding number of carbon atoms (i.e. 10 = n C_{10}, 23 = n C_{23})

DMN = Dimethyl naphthalenes

MN = Methyl naphthalenes

N = Naphthalene

P = Pristane

TMB = Trimethyl benzene

PEHN = Phenanthrene

TMN = Trimethyl naphthalene

Table 1. Paraffinic, Aromatic, and Asphaltic Composition of Test Oils

Test oil	Source	% Paraffinic	% Aromatic	% Asphaltic	% Recovery
#2 fuel oil	A.P.I.	57	35	.5	92.5
Bunker C fuel	A.P.I.	24	60	14	98
Kuwait crude	A.P.I.	37	40	7	84
So. Louisiana crude	A.P.I.	56	24	3.5	83.5
Venezuelan crude	Hess	38	37	7	82
Alaskan crude	Chevron	42	39	5	86
Diesel fuel	U. of T.	64	25	.5	89.5

Partial chemical Characterization

Table 2. ~~Boiling Point Ranges~~ of Fractions, ~~Distilled from #2 Fuel Oil~~ either non-vacuum or vacuum-distilled from #2 Fuel Oil and Southern Louisiana Crude.

A. #2 FUEL OIL, 760 mm.

Fraction	n-Paraffin Range	Boiling Point Range °C
A	C_9-C_{11}	<150-195
B	C_{11}-C_{14}	195-250
C	C_{12}-C_{15}	215-270
D	C_{13}-C_{16}	235-285
E	C_{14}-C_{17}	250-300
F	C_{15}-C_{18}	270-315
G	C_{15}-C_{19}	270-335
H	C_{16}-C_{20}	285-350
I	C_{17}-C_{24}	300-375

B. #2 FUEL OIL, mm

C. SOUTHERN LA., mm

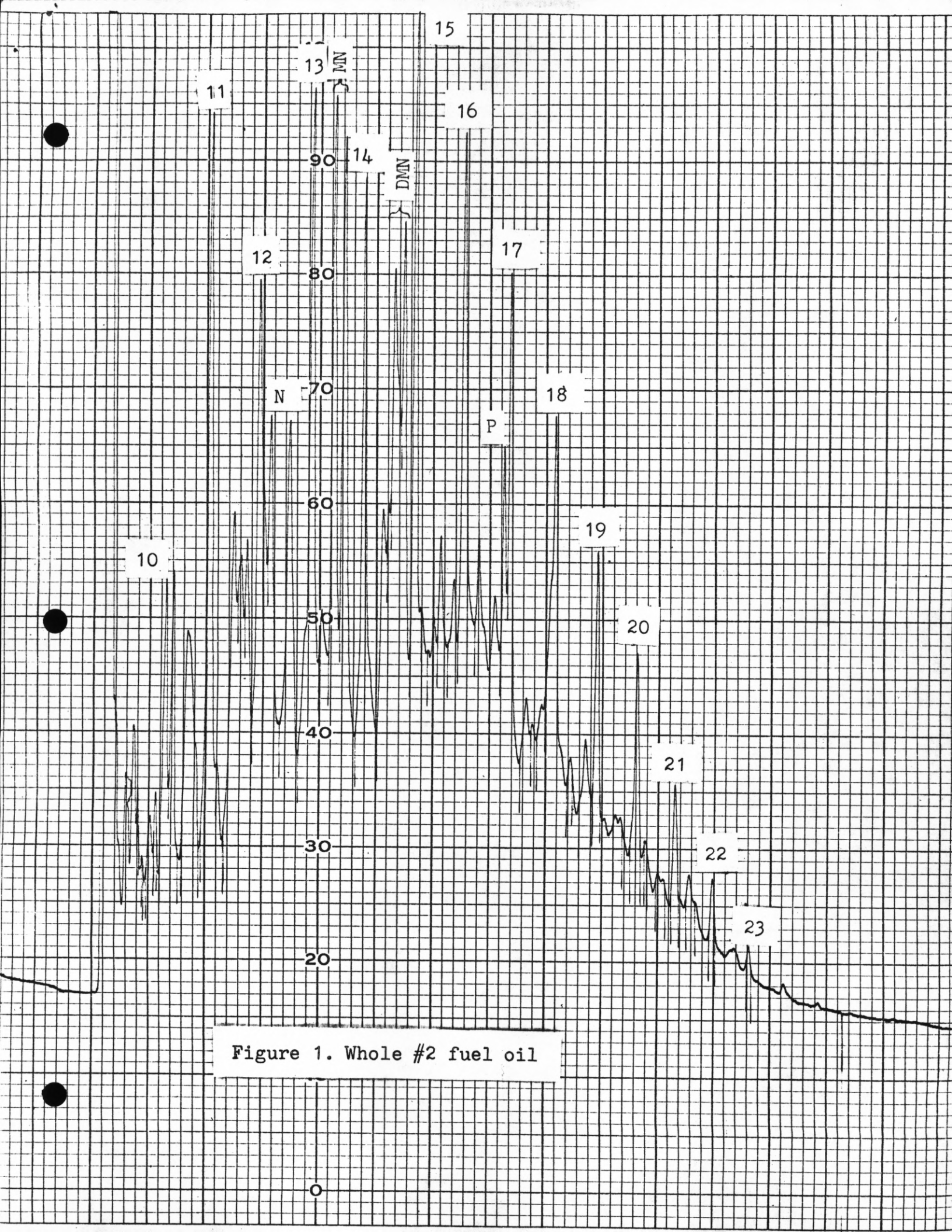


Figure 1. Whole #2 fuel oil

Figure 2. Fractions prepared by distillation of #2 fuel oil

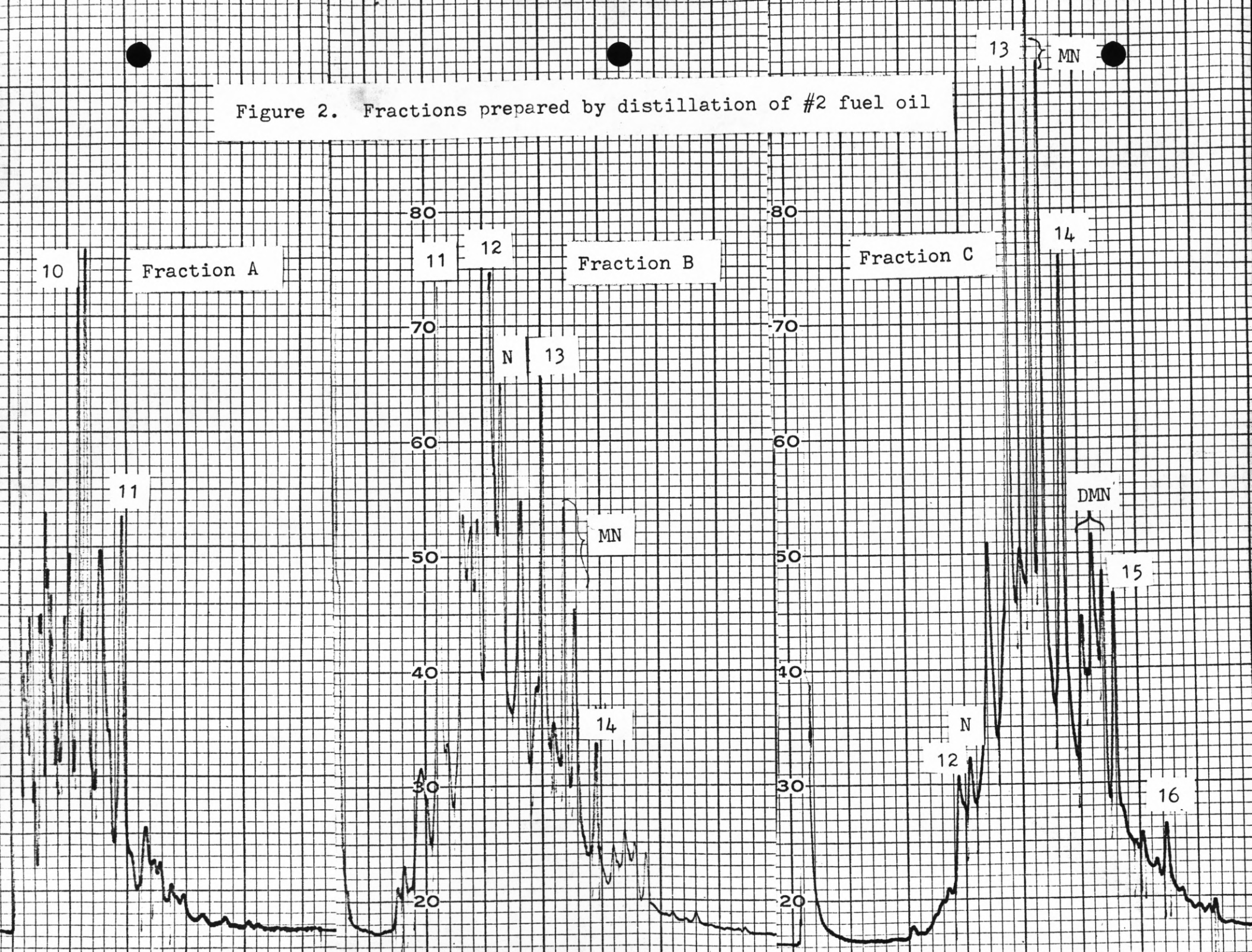


Figure 3. Fractions prepared by distillation of #2 fuel oil(cont.)

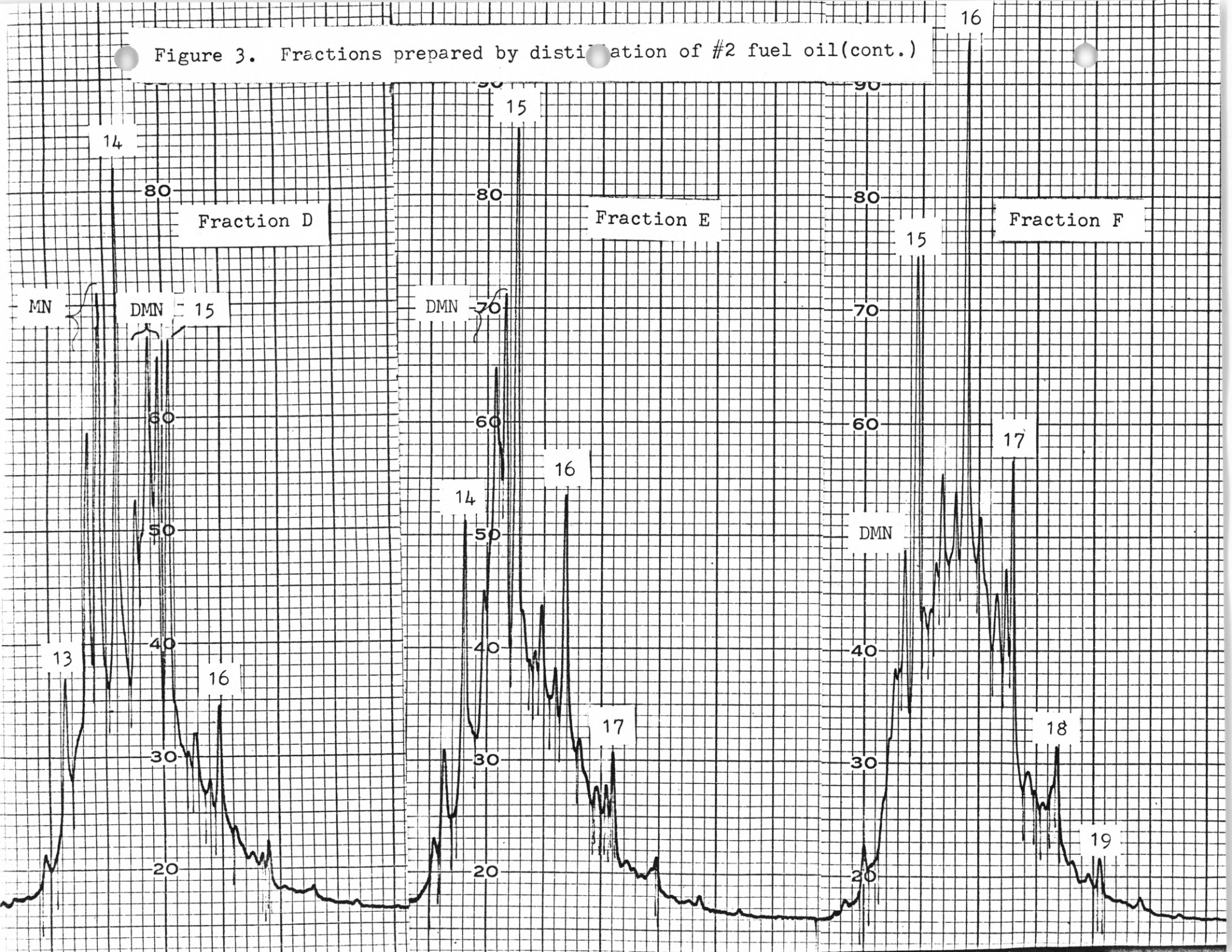
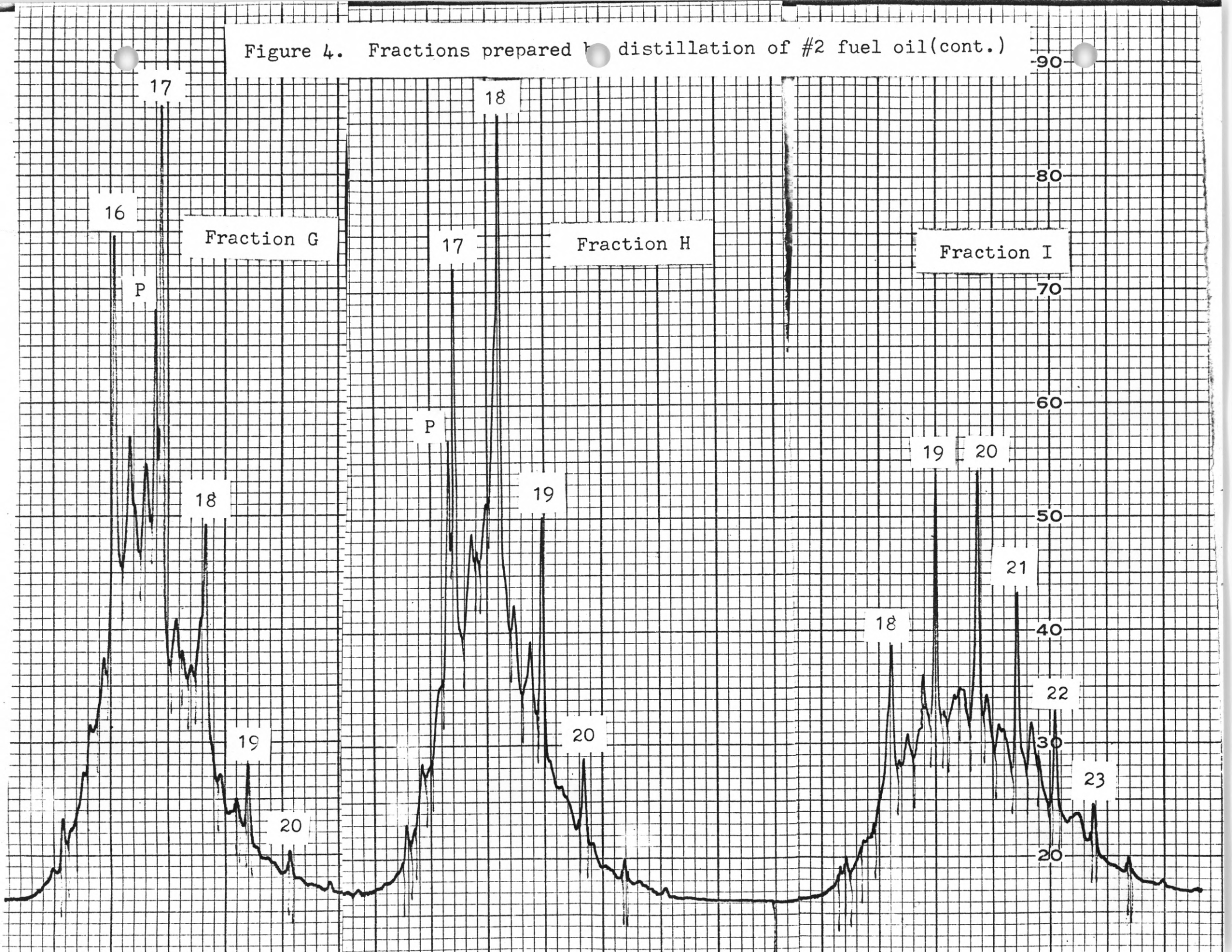


Figure 4. Fractions prepared by distillation of #2 fuel oil(cont.)



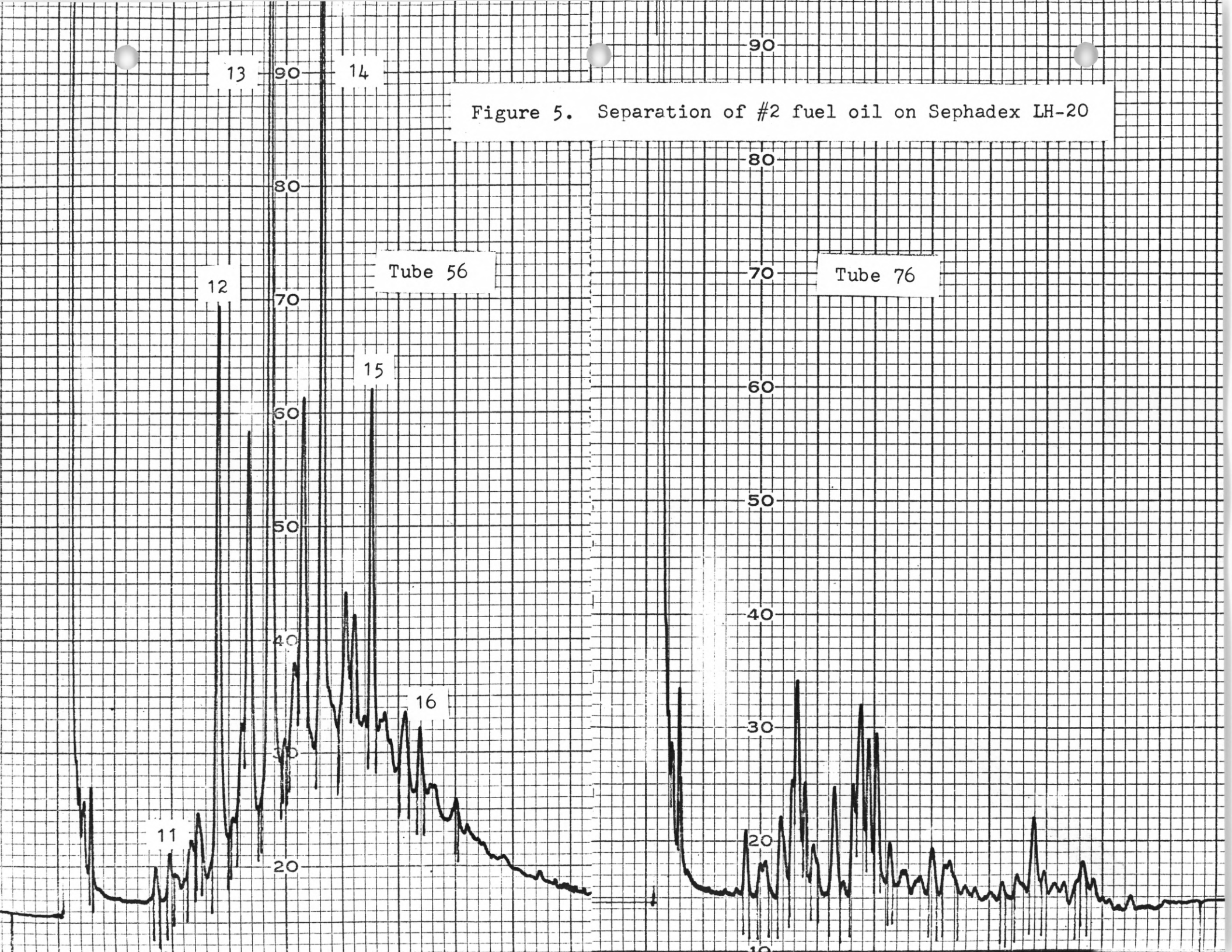
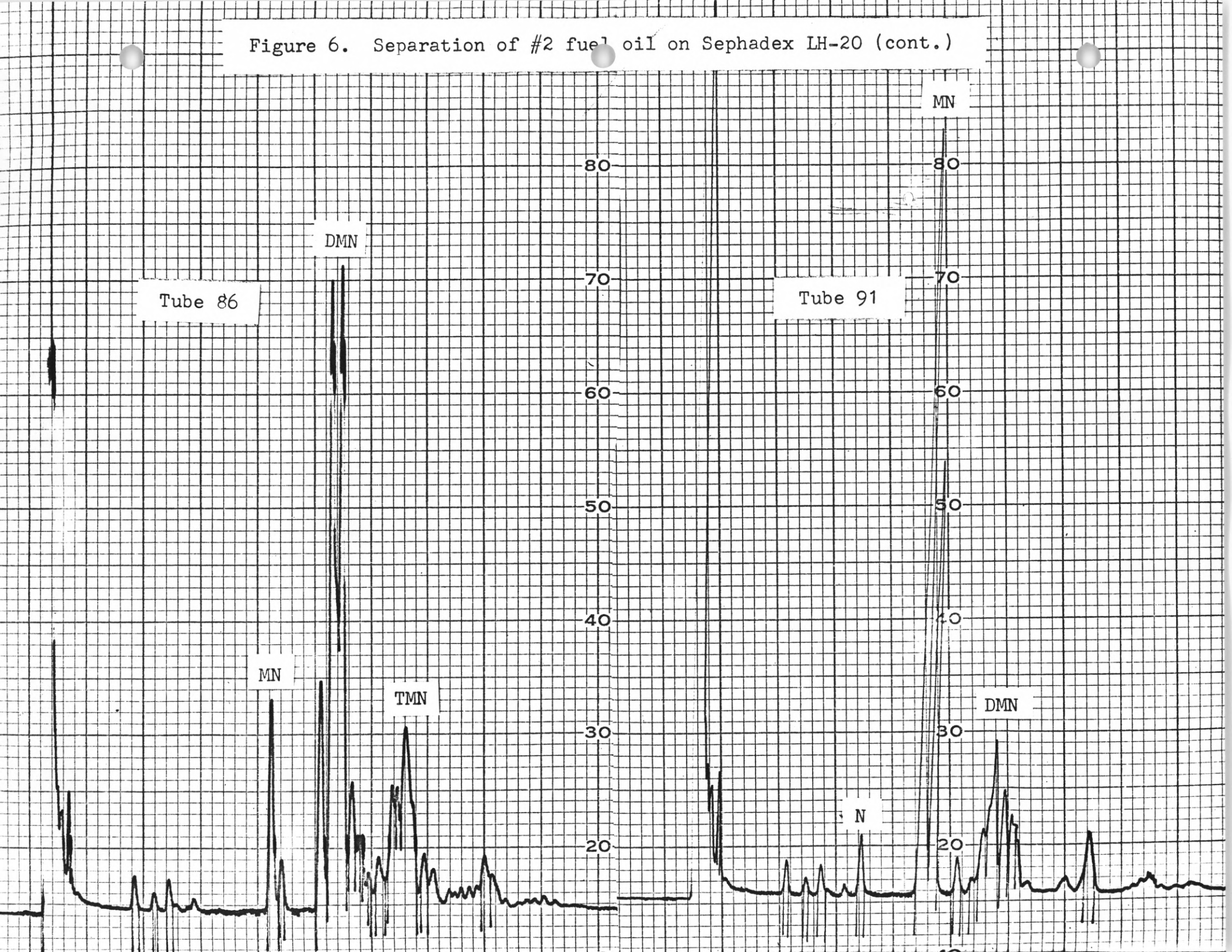


Figure 5. Separation of #2 fuel oil on Sephadex LH-20

Figure 6. Separation of #2 fuel oil on Sephadex LH-20 (cont.)



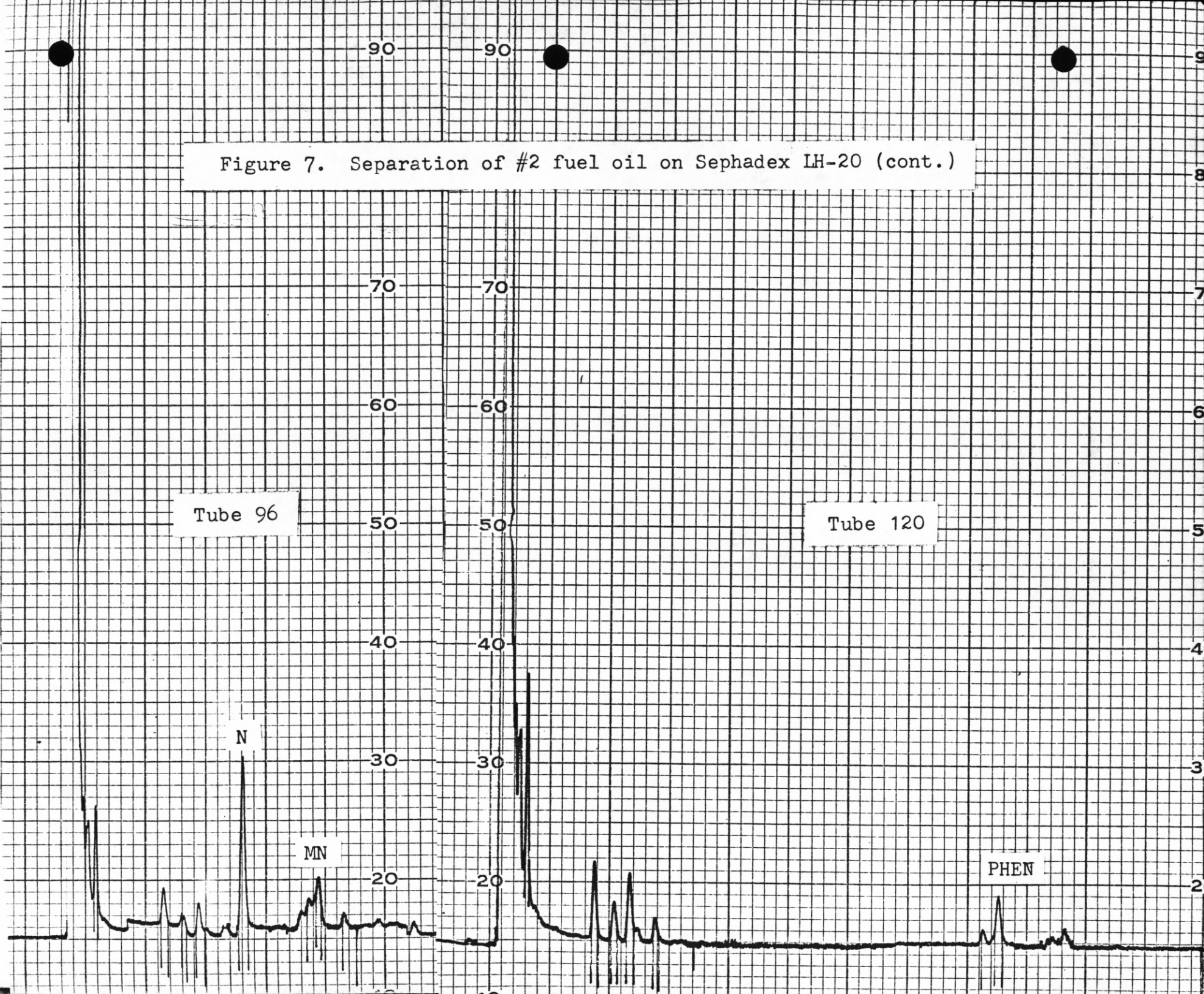
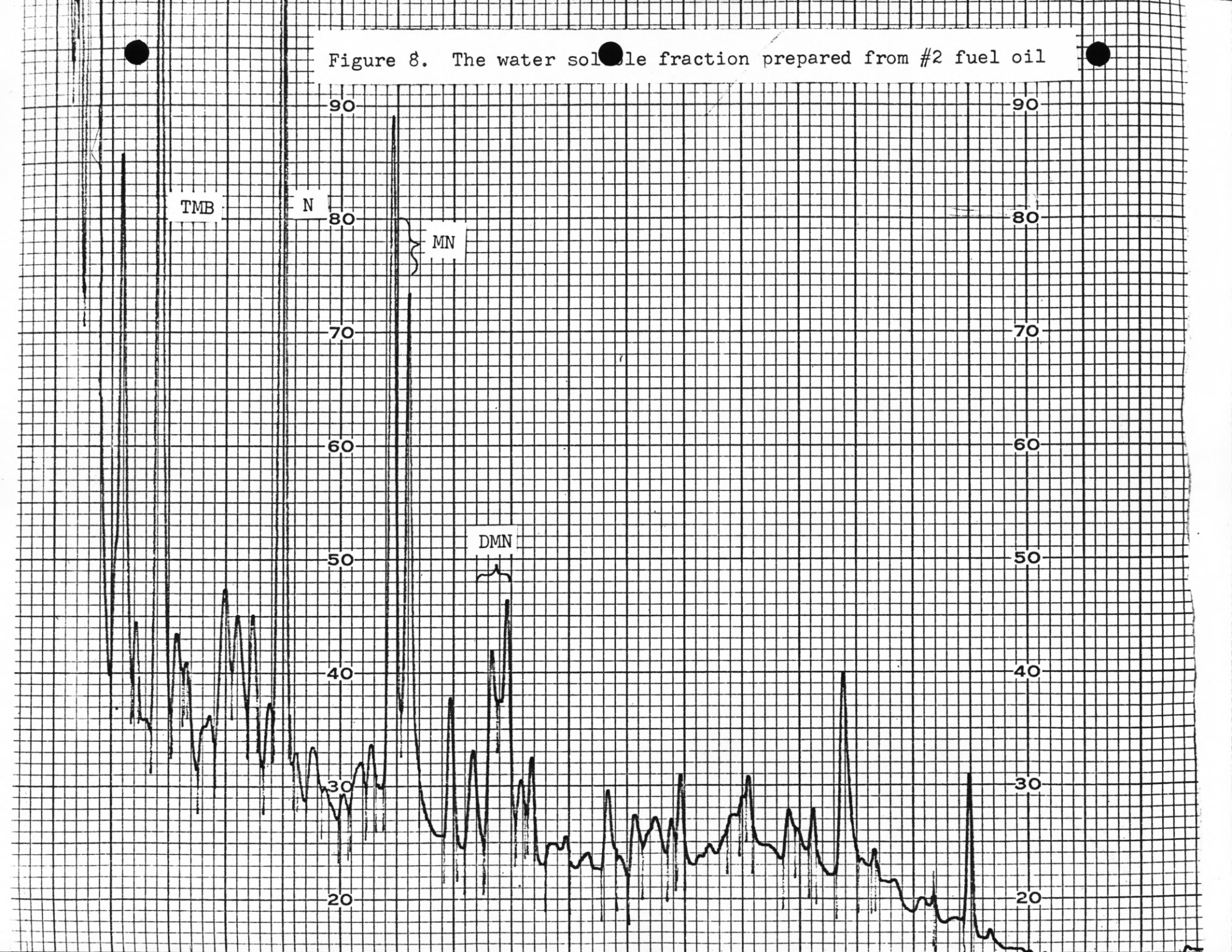


Figure 7. Separation of #2 fuel oil on Sephadex LH-20 (cont.)

Figure 8. The water soluble fraction prepared from #2 fuel oil



International Decade of Ocean Exploration

Project title: Marine Petroleum Pollution. Biological Effects and Chemical Characterization

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Principal investigator: J. A. C. Nicol

Address: The University of Texas Marine Science Institute at Port Aransas, Texas 78373

Telephone: (512) 749-6741

CONTENTS

Abstract

Exploratory studies were made of the possible effects of petroleum oils on marine animals. Materials tested were: No. 2 fuel oil, Kuwait crude, Southern Louisiana crude, Bunker C, ship diesel fuel, five fractions of No. 2 fuel oil, biphenyl, naphthalene, methyl naphthalene, dimethyl naphthalene, durene and fluorene. Oil stock was made up in sea water or Ringer solutions, 1:8; single compounds, 15 mg %. Animals and tissues used for assay were eggs and sperm of sand dollars Melitta quinquesperforata; eggs and larvae of littoral barnacles Chthamalus fragilis and Balanus amphitrite niveus; larvae of crabs, viz. spider crab Libinia dubia, striped hermit crab Menippe mercenaria; porcelain crabs Petrolisthes armatus; sea catfish Arius felis; isolated spiral valve and cornea of stingarees Dasyatis sabina; and isolated gills of pinfish Lagodon rhomboides.

Development of eggs of the barnacle Chthamalus fragilis in vitro was affected adversely by fuel oil at levels of 20% or more. The egg case seemingly afforded the embryos some protection against oil, late embryos hatched at concentrations of 50% oil, but larvae were quickly killed.

Phototactic responses of barnacle larvae (Balanus amphitrite) were weaked and abolished in fuel oil and the single compounds listed above. Concentrations affecting half the larvae were: oil, 10% of stock solution; naphthalene, 20%; methyl naphthalene, 50%; dimethyl naphthalene, 10%.

Hermit crab zoeae survived and developed in 2% fuel oil (11 days). About half the larvae were killed in 8% oil. Spider crab larvae developed

to young crabs (10 days) in 2% oil; development was somewhat retarded in 4%, and greatly retarded in 10% oil. Survival of stone crab zoeae (5 days) was reduced in oil (4 to 50%), the mortality increasing with the concentration and duration of exposure.

Catfish sought to escape from fuel oil (levels of 190 ppm or greater). Fish were killed by oil; half mortality level at 48 h was 140 ppm. Fins and gills were damaged, feeding responses deteriorated, food was regurgitated. The heart rate was slowed temporarily following addition of oil, 100 ppm.

Oxygen consumption of porcelain crabs, gills of pinfish and spiral valve of stingarees was not depressed by oils (2-3 h). But it was reduced in the cornea of the stingaree.

The results are related to findings reported in the literature dealing with oil pollution.

I. Introduction

During this period I recruited two staff, namely a research scientist associate and a technician; set up a laboratory, purchased supplies and equipment; and made exploratory studies of various animals and tissues to test their suitability for oil pollution work. The research scientist was Dr. Richard Tsun-hsiung Wang, who has been organizing and making apparatus. The technician visited the Duke University Marine Laboratory at Beaufort, North Carolina for one week, where she studied methods for culturing crab eggs and larvae under the supervision of Drs. J. D. Costlow, Jr. and C. G. Bookhout; and the Electron Microscopy Laboratory of Texas A&M University at College Station, Texas, for one week, where she learned techniques for EM preparation under the supervision of Dr. E. L. Thurston. During the summer Professor W. H. Donahue of King's College, Wilkes-Barre, Penn., joined the staff temporarily and contributed significantly to the research.

Five lines of research on the effects of petroleum were investigated. One dealt with sperm, eggs, fertilization and larval development; a second, with survival, motility and behavior of larvae in acute experiments; a third, with oxygen consumption of whole animals and tissues; a fourth, with feeding behavior of fish; and a fifth, with cardiac responses under oil stress.

For studies of gametes and development of eggs and larvae we made use of barnacles Chthalamus fragilis, hermit crabs Clibanarius vittatus, stone crabs Menippe mercenaria, spider crabs Libinia dubia, blue crabs Callinectes sapidus, speckled crabs Arenaeus cribrarius, and sand dollars Melitta quinquieoperforata. Acute experiments of

larval survival and behavior were carried out on nauplii of Balanus amphitrite niveus.

For studies of oxygen consumption we employed porcelain crabs Petrolisthes armatus, isolated corneas and spiral valves of stingarees Dasyatis sabina, and gills of pinfish Lagodon rhomboides. Tissues were also examined histologically after exposure to oil.

Sea catfish Arius felis were used to study feeding behavior and to test cardiac responses following exposure to oil.

A. Sand dollar gametes and larvae

It is generally agreed that gametes, embryos and larvae have tolerances to environmental stresses different from those of adults, and that susceptibilities alter during development. Consequently, survival, reproductive capability and colonization may depend more upon the resistance of young stages to external variables than upon adult tolerance (R. I. Smith, 1964). In these studies of oil pollution we have been utilizing some of the commonly available animals of Texas coastal waters and to test developmental stages we have used, inter alia, gametes of the sand dollar. Investigations have dealt with the effects of petroleum on sperm, egg permeability, fertilization, cleavage, and early development of embryos.

Extensive studies of the permeability of animal cells have involved use of marine eggs and mammalian erythrocytes (Lucké and McCutcheon, 1932). Among the marine eggs investigated have been those of sea stars Asterias, urchins Arbacia, polychaetes Chaetopterus, and pelecypods Ostrea and Cumingia (Lucké, Hartline and Ricca, 1939; Lucké and Ricca, 1941). A method frequently used to measure permeability to water has been to expose the egg to a dilute medium and to measure the

change of diameter which ensues with time. Eggs have been exposed to test substances and possible changes of permeability resulting therefrom explored by the same method (Lucké and McCutcheon, 1932).

Marine eggs are invested by semipermeable membranes and behave as good osmometers when transfered to anisosmotic media. In hypotonic solutions water enters the cell owing to the driving force of osmotic pressure. The rate of permeability of the cell to water is calculated from the equation

$$k = \frac{1}{S \cdot \Delta P} \cdot \frac{dV}{dt}$$

where k is the permeability of the cell to water, dV/dt is the rate of change of cell volume, S is the area of cell surface, and ΔP is the difference of osmotic pressure existing between the cell interior and the external medium (Lucké and McCutcheon, 1932).

Experiments with eggs of urchins have involved varying temperature, salinity, ionic composition, etc. of the media; changes were observed in growth rates, size and skeletal formation (Medes, 1917). Eggs and larvae of urchins are very sensitive to slight differences between natural sea waters (Wilson and Armstrong, 1961). Allen (1971) has made a study of the effects of petroleum fractions on early development of the purple urchin Strongylocentrotus purpuratus.

Ripe eggs of sand dollars are easy to secure and fertilize, they develop to the echinopluteus stage quickly, and deviations from normal cleavage and morphogenesis are detected readily.

B. Barnacle eggs and larvae

Development

In the barnacle fertilized eggs released from the oviducts are aggregated into two elongate egg masses or lamellae, containing a

jelly-like matrix. The lamellae are retained in the base of the mantle cavity, and the eggs continue to develop within the parent until they hatch as nauplius larvae, whereupon they are released. Eggs in lamellae removed from the adult barnacle continue to develop *in vitro* (Crisp, 1959), and nauplii have been raised in cultures up to the cypris stage and settlement (Herz, 1933; Sandison, 1954; Costlow and Bookhout, 1957; Tighe-Ford, Power and Vaile, 1970). Even under optimal conditions mortality of larvae is high, especially during the third naupliar stage (Bassindale, 1936; Costlow and Bookhout, 1958).

Barnacle larvae have been used for testing some potentially harmful agents (Spooner, 1968; Tighe-Ford *et al.*, 1970). As part of our studies to investigate the effects of petroleum on development, we made use of the barnacle *Chthamalus fragilis*. Testing experiments were limited to eggs and the initial naupliar periods.

Larval survival and behavior in acute experiments

Nauplius larvae of barnacles are released in enormous numbers. They pass through six naupliar stages and one cypris stage before metamorphosing and settling on the bottom. During the nauplius period they become part of the temporary plankton and depend upon microorganisms for food. This period lasts two to three weeks (Bassindale, 1936; Costlow and Bookhout, 1957). The larvae on hatching are strongly positively phototropic, and the cyprids become negatively phototropic just before settling (Pardi and Papi, 1961).

Apparatuses were designed to test large numbers of early (first and second) nauplii, noting such factors as motility, light-responses, and to measure mortality. The experiments were short term (one hour) and the object was to assess quickly a large body of information about

relative toxicities of many important oils and their constituents. Freshly hatched nauplii of Balanus amphitrite niveus were used.

C. Crab larvae

Effects of natural environmental variables, such as salinity and temperature, on the development of crab larvae have been explored (Ong and Costlow, 1970), and it has been shown that some pollutants influence development and survival (Bookhout et al., 1972). In the present study three species of crabs, two brachyurans and one anomuran, from the Gulf of Mexico, have been utilized to investigate how fuel oil effects larval development and survival. While particular attention was given to the survival of larvae in oil mixtures, observations were also made on the developmental process whenever possible.

D. Catfish: feeding activity and cardiac responses

Catfishes use olfactory and gustatory senses in locating and selecting food. Sensing food by smell, sea catfishes start searching in a frenzy of activity and recognize it on contact with their barbels which bear extraoral taste buds (Kleerekoper, 1968; Hara, 1971). Sea catfish Arius felis were exposed to oil, feeding behavior was observed, and external tissues were preserved for histologic study.

External events affect the activity of the fish heart through cardiac reflexes mediated by the vagus nerve, and bradycardia (slowing of the heart) has been observed in response to a wide variety of external changes, such as salinity and anoxia (Randall, 1968, 1970; Marvin and Burton, 1973). To ascertain sensitivity to petroleum, we recorded cardiac activity of sea catfish Arius felis under normal conditions and during exposure to oil.

II. Materials and Methods

Substances tested and modes of preparation

Oils tested were No. 2 fuel oil, southern Louisiana crude, Bunker C, Kuwait crude, and ship diesel fuel. The first four oils were kindly supplied by the American Petroleum Institute; ship diesel fuel was procured from the Institute's ship storage tank.

Fractions prepared by distillation from No. 2 fuel oil were made available to us. They were:

Fraction I	BP < 150°-250°C	or	C9 to C14
II	195°-270°		C11 to C15
III	215°-300°		C12 to C17
IV	250°-335°		C14 to C19
V	285°-375°		C16 to C24

Known compounds used were biphenyl, naphthalene, methyl naphthalene, dimethyl naphthalene, durene, fluorene, phenanthrene and anthracene. They were obtained from Chem Service, Media, Penn.

Saturated solutions of oils, oil fractions, and putative oil components, as pure compounds, were prepared in several ways.

Method 1. Oil was added to sea water in the ratio 1:8 (by volume), the mixture was continuously agitated for 24h by a magnetic stirrer, and the lower (aqueous) layer was drawn off. This aqueous mixture, termed stock, was stored in a refrigerator and replenished weekly.

Method 2. Oil and oil fractions were added to sea water (30 °/oo) in the ratio 1:8 (by volume) and the mixture was agitated on a mechanical shaker (Ehrbach) at a speed of approximately 260 excursions per min. The aqueous layer was siphoned off and used for testing. Known compounds were added to sea water at a concentration of 15 mg % (weight by volume),

they were shaken for 1 h, and the solutions were filtered.

In studies involving vertebrate tissues, oil stocks were prepared essentially as described above except that appropriate Ringer solutions -- selachian or teleost -- were used in place of sea water; stock solutions were subsequently diluted with Ringer solutions.

Gametes of sand dollars

Sand dollars Melitta quinquesperforata occur along the Gulf side of Mustang Island south of Port Aransas. They were collected from June to September, 1973, by hand or by trawling. To sex the animals and collect the gametes they were electrically shocked (Harvey, 1956); more animals were found to be sexually mature during the latter part of the summer.

Permeability of eggs to water

Eggs were equilibrated in natural sea water (33.1 °/oo) or in sea water the salt content of which had been raised to 35.8 °/oo by the addition of 2.5 g NaCl l^{-1}. The rate of penetration of water was ascertained by transferring unfertilized eggs to 60% sea water and measuring the diameters at short intervals (0.5 to 8 min)(controls). To explore the possible effect of petroleum, eggs were immersed in an oil stock - seawater 35.8 °/oo mixture 1:1 for 2-3h, after which they transferred to oil stock - 60% sea water 1:1, and diameters were measured at regular intervals (experimentals). Measurements were made with a travelling micrometer eyepiece (J. Swift & Son) or a shearing eyepiece (Vickers). Room temperatures were 24-25°C.

Fertilization, cleavage and larval development

In this series of experiments deviations from normality in fertilization, cleavage and larval development were noted in developing

eggs exposed to petroleum oils. Controls were treated identically, except for absence of oil. Dilutions of oil stock are listed in Table I . Offshore water 30 °/oo was used for all experiments. Solutions were placed in 5 cm culture bowls; to each was added 12 drops of eggs drawn by pipette from the small mound of eggs shed by a single sand dollar. The amount of eggs added to each bowl was about equal, forming a discontinuous layer over the bottom of the bowl.

Fresh sperm shed into a small amount of sea water was pipetted into 5 ml of sea water. Twelve drops of this diluted sperm was added to each culture bowl and thoroughly mixed with the eggs. After the eggs had settled, the supernatant was decanted and replaced with fresh solution. Preliminary tests showed that sperm shed into sea water was effective in fertilizing almost 100% of the eggs, if used within 45 min.

For microscopic examination, a sample of eggs from a culture bowl was drawn into a pipette, transferred to a cavity slide and capped with a cover glass. Separate labelled pipettes and microscope slides were used for each solution. Several hundred eggs were present on each slide and could be observed over a period of several hours. To determine whether the confined area of the microslides affected cleavage, the development stage attained on a slide was compared with a specimen freshly drawn from the culture bowl: there was no observable difference.

At intervals of approximately 1/2 h until 1 1/2 h after fertilization, microscopic observations (magnification x80) were made of 100 eggs randomly selected from each culture bowl. The first observation was to determine the presence or absence of a vitelline membrane; the second, the occurrence of first cleavage, and whether or not it were normal; the third, the occurrence of second cleavage, and whether normal or not. In clean sea water usually the first and second cleavages resulted

in cells that were equal in size and clearly separate. Abnormal cleavages resulted in the incomplete separation of cells, or in the production of unequally sized cells; they were easily discernible. A fourth observation was made 24 h after fertilization on the entire culture with a stereomicroscope: at 24 h well developed pluteus larvae were normally present. A fifth observation was made of all cultures at 48 h after fertilization to determine development and survival. Room temperature was 24-25°C.

The experimental procedure for testing fractions of fuel oil No. 2 was identical with that described immediately above. Dilutions of oil fractions are listed in Table II (et seq.).

Experiments with sperm

Fertilizing capability. In this series the same experimental solutions and controls were used. The eggs, which had been shed into a culture bowl of sea water, were agitated and allowed to settle. The supernatant was decanted and replaced with clean sea water; this was repeated twice. Sperm was collected in a 25 mm culture bowl containing a little sea water. Four drops of this concentrate were added to 20 ml of the various stock dilutions (experimentals) and to 20 ml of sea water (the control); dilutions of oil listed in Table II (et seq.). The sperm remained in each solution, experimentals and control, for 30 min. At that time 2 ml of sperm solution was pipetted into 5 cm culture bowls each containing an approximately equal number of eggs in sea water (30 °/oo). Observations were made on fertilization and development in the manner described above. Room temperature was 24-25°C.

Motility. Freshly shed sperm was suspended in 1 ml of 30 °/oo sea water. Four drops of this sperm suspension were placed in each of five small covered dishes, and an equal amount of oil stock or

diluted oil stock was added to four of them to make the following dilutions, 1/2, 1/5, 1/25, and 1/125 (Table III). The fifth vessel of eggs, retained as control, received an equal amount of sea water. Samples were taken 5, 30 and 60 min after mixing, placed on a microscope slide, and examined by negative phase contrast (magnification x630). A fresh slide was prepared for each observation; room temperature was 25°C. Motility was estimated as an approximate number of sperm that were swimming, or just moving, or dead.

Barnacles

1. Development of eggs and larvae

Chthamalus fragilis was used for these experiments. It is very abundant on the granite boulders of the jetties protecting the ship channel at Port Aransas, Texas, and occurs inside the channel at a fairly high level on the more shoreward stretches (Whitten et al., 1950). Animals were collected from March to June, 1973; they were opened soon after bringing them into the laboratory, the valves were rent and the egg lamellae released. During this period most of the animals contained egg lamellae, some had only eggs in the oviducts, and there seemed to be no synchrony of the reproductive process in the population. Breeding was continuous because a new set of eggs was being formed in the oviducts as the eggs in the lamellae matured. All developmental stages between 5 and 13 (vide infra) were discovered. Egg lamellae were transferred to small polystyrene petri dishes containing artificial sea water ("Instant Ocean", Aquarium Systems, Inc., Eastlake, Ohio), or offshore filtered sea water 30 °/oo. One lamella of a pair from an animal was used as a control, the other as an experimental; eighty-one pairs of lamellae were studied. They were kept at room temperature,

approximately 22°C; the water was changed daily or every second day until the embryos hatched.

Experimental lamellae were cultured in a stock solution of No. 2 fuel oil, and in various dilutions of this stock, viz. 1/2, 1/5, and 1/10 (by volume). Of the 81 experimental lamellae, 38 were cultured in the stock solution, 15 in the 1/2, 15 in the 1/5, and 19 in the 1/10 concentrations. At hatching larvae were fed a small diatom (3H) or a unicellular blue-green alga from culture. The algae were kindly supplied by Dr. C. Van Baalen.

Developmental stages of embryos were designated according to the criteria of Crisp (1954). Although his classification contains 13 stages from the unsegmented egg to hatching of the larvae, only stages 5 to 13 were observed in Chthamalus fragilis. The lamellae measured 1.5 to 2.5 mm long by 0.7 to 1.1 mm wide and contained approximately 200 embryos.

2. Behavior of larvae in acute experiments

Balanus amphitrite nivens is abundant on the seaward side of the outer jetties at low tide levels (Whitten, et al., 1950). Breeding adults were collected during January and February 1974. They were placed in a bowl of sea water; the bowl was partially covered and illuminated unilaterally from a fluorescent lamp placed over the exposed side. Larvae, released in large numbers, were transferred successively to three changes of filtered outside sea water (30 °/oo). The larvae (first nauplii) were drawn upon for 48 h only after hatching.

Various agents, in acute experiments, were tested on the larvae in the apparatus illustrated in Figure 4. It consisted of a test chamber constructed of opaque black acrylic plastic, it contained

four compartments separated by removable gates, and was provided with a detachable lid that spanned the three compartments to the left. At the start of an experiment, 30 ml of fluid (sea water or test solution) were added to the chamber and the gates were closed. A sample of larvae (say 200) in about 1 ml of sea water was added to the left compartment, and the box was left in darkness for 1 h. Then, the gates were removed, the lid was placed over the left three compartments, and the chamber was illuminated by a fluorescent lamp set over the right (and exposed) compartment. At 15 min the gates were replaced, the larvae were removed by pipette from each compartment and stored separately in vials. Activity of the larvae was noted whether they were: lying on the bottom, sporadically moving, or quiescent (and presumed dead). They were preserved by adding a little formalin to each vial. Larvae from each compartment were counted by an operator under a compound microscope.

Controls tests were run in filtered outside sea water (30 °/oo). Agents to be tested were added to the same sea water (Method 2). The temperature was approximately 22°C.

C. Crab larvae

No. 2 fuel oil was used in the experiments about to be described involving crab larvae.

1. The striped hermit crab Clibanarius vittatus

Striped hermit crabs were collected in the bays and channels near Port Aransas during June, 1973, and were maintained in running

filtered sea water. Crabs were removed from their shells in order to determine which were ovigerous females; this was done by directing a stream of hot water at the back of the inhabited shells until the crabs emerged (a method suggested to us by Mr. William Longley). The ovigerous females were allowed to return to their shells and were then placed in 20 cm culture dishes half full of filtered sea water 30 °/oo. On hatching the larvae were collected by pipette and placed in large culture dishes prior to being dispersed into groups for the experiments.

Two different experimental series were studied. In the first there were four groups: controls in sea water, and experimentals in oil stock dilution with sea water 1/10, 1/5, and 1/2 (by volume). One hundred larvae, one day old, were used in each group, and were placed 50 to a culture dish, 14 cm in diameter, containing 200 ml of medium. The dishes were kept in an air conditioned room at approximately 25°C (fluctuating slightly a few degrees). Larvae

were counted daily and mortality was recorded for 11 days. Each day the larvae were transferred to fresh media and were fed freshly hatched Artemia larvae.

The second series likewise consisted of four groups, but the effects of more dilute solutions of the oil stock were studied, namely 1/50, 1/25 and 1/10, plus controls in sea water. Each group again consisted of 100 newly hatched larvae which were placed in 11 cm culture dishes containing 200 ml of medium. The dishes were kept in a dark incubator at 25°C. Observations, changes of solutions and feeding were the same as for the first series.

2. Spider crab Libinia dubia

Specimens of the spider crab were obtained by trawling in the Gulf of Mexico near Port Aransas during June and July 1973. Ovigerous females were placed in glass battery jars supplied with a continuous flow of recycled sea water at 24°-26°C. On hatching the zoeae were pipetted into large (20 cm) culture dishes.

Four hundred larvae were taken for the experiments, they were distributed into four groups of 100 each. Each group was kept in four culture dishes 12 cm in diameter, 25 larvae to a dish containing 100 ml of solution. The groups were maintained in the following media: (1) sea water 30 °/oo (control); (2) oil stock-seawater 1/50; (3) 1/25; and (4) 1:10.

Larvae were counted and transferred to fresh solutions; three drops of concentrated, freshly hatched Artemia nauplii were added as food. Observations were made on the larval stages present, and the bowls were examined for exuviae. All cultures were kept in a dark incubator at 25°C.

The spider crab passes through two zoea and one megalops stage. Durations are: first zoea, 2 days; second zoea, 3-4 days; megalops, 5-7 days.

3. The edible stone crab Menippe mercenaria

Ovigerous stone crabs were obtained from Aransas Bay in June, 1973. They were placed in glass battery jars containing flowing recycled filtered sea water. The jars were tilted slightly so that when the eggs hatched, the larvae were carried in the overflow into plastic pans beneath the battery jars. Larvae were collected by large bore pipettes from the pans and were placed in 20 cm culture dishes.

Day-old zoeae were cultured in oil stock sea water mixtures 1/25, 1/10, 1/5 and 1/2, and in sea water as control. Two hundred larvae were cultured in each group; they were subdivided into groups of 25 and placed in 100 ml of solution in large plastic petri dishes (14 cm diameter). Numbers of living and dead larvae were recorded daily for four days. A larva was considered dead when the heart was not beating. Each day the larvae were transferred to fresh media and fed three drops of Artemia nauplii. Dishes containing oil mixtures were washed with ethanol, rinsed with water and then washed with detergent (Alconox, Alconox, Inc., N.Y.); dishes containing only sea water were washed with Alconox. Cultures were kept in an air conditioned room having a temperature range of 24-26°C. Chi square with Yates correction for continuity was nsed to determine whether a true difference existed between survival of larvae in the experimental group and in the control, with differences considered significant at the $P < 0.05$ level.

Oxygen consumption

Oxygen consumption was measured in an apparatus consisting of a YSI (Yellow Springs Instrument Co.) Clark-type polarographic electrode No. 5331, Biological Monitor Model No. 53, stirrer bath and assembly Model No. 5301; Haake thermostat assembly Model FJ In-A-Wall water cooler Model IW-5A (EBCO) and a potentiometric recorder Sargent Welch Model SRLG or Esterline Angus Model T171B. The probe was cleaned weekly in the early experiments, and daily in the later ones, fresh KCl was added and a new membrane installed. The sensitivity of the probe was calibrated against air saturated sea water or Ringer at the experimental temperature.

Sand dollar sperm

Sperm was collected from 15 male sand dollars over a period of 1 h and was suspended in ~9 ml of sea water 30 °/oo. Two ml of suspension wereadded to 2 ml of sea water or to 2 ml of diluted oil stock-seawater in each of four test chambers to produce a control and three experimentals, viz. oil stock-sea water 1/2, 1/5, and 1/25. Sperm were counted in a Spencer Bright-line hemocytometer (American Optical Co.) at a magnification of 400x. Rates of oxygen consumption were computed as $\mu l\ O_2$ per sperm h.

Porcelain crabs Petrolisthes armatus

Porcelain crabs were collected beneath rocks on the jetty at Port Aransas; they had a carapace length of about 5 mm. The oxygen consumption of seven animals was measured, 1 h in sea water 30 °/oo and 2 h in No. 2 fuel oil water (1/2 and 1/5). After measurement the animals were rinsed in distilled water, dried for 24 h at 102°C and weighed.

Tissues

The oxygen uptake of freshly excised tissues was measured with the apparatus already described. Tissues were submerged in appropriate Ringer solutions (teleost or selachian), and in Ringer solutions containing oil. After the measurements were complete, the tissues were rinsed in distilled water, dried at 102°C for 24 h and weighed.

Rates of oxygen consumption by control and experimental tissues were measured alternately with the same oxygen electrode by moving it back and forth between the experimental and control vials.

Gills of pinfish Lagodon rhomboides and corneas and spiral valves of stingarees Dasyatis sabina were studied. The fish were collected by trawling in the bays, pass, and inshore waters of the Gulf near Port Aransas.

Cornea of stingaree. Ten pairs were collected (20 corneas), one member of each pair was control, the other experimental; controls (10) were pooled, as were experimentals. Oxygen consumption was measured in No. 2 fuel oil 1:1 over 3 h.

Spiral valve of stingaree. The spiral valve was removed from a freshly killed stingaree and submersed in Ringer solution. The tissue was rinsed and two small pieces were removed, one control, the other experimental, which were placed in two vials containing, respectively, Ringer 5 ml and oil stock 5 ml. Oxygen consumption was measured on 5 occasions, each of 5 min duration, and air was bubbled into the medium between readings; the total duration of the experiment was 2 h.

Gills of pinfish. Measurements of oxygen consumption were made on paired pieces of tissue, an experimental and control, one pair from

a fish. Two sections of gills, from the left and the right side were excised, rinsed with Ringer, and clotted blood removed. A small piece of gill tissue, 4 to 7 mm in length was removed from each preparation and placed in two vials: one piece, the control from one side; the other, the experimental, from the opposite side of the fish. To each vial was added Ringer 5 ml and oil-stock 5 ml, respectively. Oxygen uptake was measured as described above for the spiral valve.

Survival and feeding responses of sea catfishes in acute experiments

Sea catfish Arius felis were used for these experiments. This species occurs in the bays and in the Gulf, and migrates out into the Gulf each winter to spawn. Specimens were collected from the Corpus Christi ship channel at Port Aransas. Total body lengths ranged from 80 to 130 mm.

Four aquaria measuring 30 x 40 x 30 cm were used for the experiments. To each aquarium was added 26 l of sea water (25 °/oo) which was recirculated by a pump. Water returning to the aquarium aerated it and caused the formation of an oil-water emulsion when oil was added. About 25 catfish were put into the aquaria at least one day before oil was added.

Two series of experiments were carried out. In the first series, 20, 10, 5, and 2 ml of No. 2 fuel oil were added to the four aquaria, respectively. The fish were observed over four consecutive days, during which time they were not fed and the aquaria were not cleaned.

In the second series, 2, 1, and 0.5 ml of No. 2 fuel oil were added to three aquaria, respectively, while the fourth was used as

control. The aquaria were drained and refilled with sea water each day; before the water was changed they were fed chopped shrimp; unconsumed pieces of shrimp were removed from the aquaria when it was drained. After refilling the aquaria, fuel oil, in the quantities that were used before, were added to the aquaria. The procedure was carried out on four consecutive days. After the fourth day one fish in each aquarium was killed and pieces of barbels, stomach and gills were fixed for histological study. The survivors were fed; their aquaria were cleaned and refilled each day, with sea water only, for an additional seven days. After the seventh day one fish from each aquarium was killed and pieces of barbel, stomach and gills were preserved, as before.

III. Results

Sand Dollars Melitta quinquesperforata

Permeability of eggs to water

The average diameter of 40 eggs in 33.3 °/oo sea water was 106 μm, the range was 94.6 to 114.8 μm, the average volume was 623,763 μm^3. The average diameter of 92 eggs in 35.8 °/oo sea water was 106.7 μm, the range was 95.5 to 120.1 μm, the average volume was 636,022 μm^3. The osmotically inert volume of the cell was 11% and k, the permeability of the cell to water (Lucké, Larrabee and Hartline, 1935; Lucké, Hartline and Ricca, 1939), was 0.103 cubic μm of water per square μm of cell surface per min for a pressure difference of 1 atmos. Values for k of 0.1 to 0.4 for sundry species of urchins, sand dollars and sea stars have been published (Lucké, 1940).

Transferred from full strength (33.3 °/oo) to dilute sea water (60%) the eggs swelled, reaching full equilibrium in 7 to 8 min (Figure 1). The curve for swelling rate is much like those presented

for other marine eggs (sea urchins, etc., Lucké, Hartline and Ricca, 1939), and the sand dollar eggs appeared to be behaving as good osmometers. Two eggs after 8 min in dilute sea water (19.99 °/oo) exhibited protruding blebs of cytoplasm, they seemed to be cytolyzing and were discarded.

In full strength sea water many eggs were slightly ovoid and appeared to have creased surfaces. Placed in dilute sea water some eggs, while swelling, passed through an ovoid phase, finally becoming spherical, and the surface creases disappeared as the eggs became turgid. The deviation from spherical shape introduced some variations into the data for rate of swelling of individual cells, which were mutually conterbalanced in the means. An experiment involved 10 to 12 eggs: with these numbers, mean initial volumes were not identical.

The rate of swelling of eggs in No. 2 fuel oil stock - 60% sea water 1:1 is shown in Figure 2, compared with a control series. It is apparent that the rates of swelling are the same.

Fertilization, cleavage and larval development

Oil stocks.

All the controls in untreated sea water developed normally. Approximately 1/2, 1 and 1 1/2 h after insemination, samples of 100 eggs showed 100% with vitelline membrane, first and second cleavages, respectively. After approximately 24 h the control bowl contained a large swarm of beautifully formed pluteus larvae; there were very few undeveloped eggs on the bottom.

The results of experiments with petroleum oils are summarized in Table I.

Kuwait oil stock-sea water 1:1. In general Kuwait oil, at this high concentration, had the least effect of all mixtures tried on fertilization and development. The reduced number of eggs undergoing first cleavage was probably significant; second cleavage and larval development proceeded normally.

No. 2 fuel oil stock-sea water. At dilution 1/50, fertilization and cleavage proceeded normally. Effects of oil began to show up at a concentration of 1/25, in which only 75% of the eggs showed a vitelline membrane. It was much more difficult to detect the membrane in these eggs than it was in the controls; where present it was raised very little from the surface of the egg and was barely distinguishable. In the 1/10 dilution there were few vitelline membranes and those that were present were difficult to distinguish because of the very small perivitelline space. In the 1/5 dilution no vitelline membrane was clearly distinguishable, except for a suggestion of one around one egg.

The dilutions 1/25, 1/10, 1/5 and 1/2 (Table I) showed some interesting and significant results at first cleavage. In 1/25 a reduced number of cells entered first cleavage, all of them were normal in appearance. In 1/10 not only was there a reduced number of cells going into first cleavage, but many of them doing so clove in an abnormal fashion. In the 1/5 dilution only an insignificant of cleavages appeared normal but a large number showed abnormalities. As might have been expected from the absence of fertilization membranes in the 1:1 dilution, no cleavages occurred in that mixture.

At second cleavage some effects of the oil at 1/25 dilution were seen, the proportion cleaving was slightly reduced and a few were abnormal. Very strong effects of the oil were seen in concentrations

1/10, 1/5 and 1/2 (Table I). In the 1/10 mixture only 35% of the cells reached second cleavage and all but 3% were abnormal. In the 1/5 mixture only 2% of the cells were in the four cell stage and these were abnormal in appearance.

Table I lists the condition of the larvae in the various dilutions of oil and the control after approximately 24 h. Both Kuwait oil 1/2 and No. 2 fuel oil 1/50 permitted development that in all respects could not be distinguished from the control. Larvae were perfectly formed, in great numbers, and there were very few undeveloped eggs in the cultures. In fuel oil 1/25 there were large numbers of actively swimming larvae. However, some developmental retardation was evidenced by the presence of active ciliated larvae still confined within their jelly capsules. In fuel oil 1/10 there was about an equal division between the dead cells on the bottom in various stages of development and lack of development, and living larvae. These larvae were considerably retarded in development compared to the control larvae or even to those in fuel oil 1/25. In fuel oil 1 5 the bottom of the bowl was covered with dead eggs, mostly undeveloped, but many still showing various stages of deformed, aborted cleavages. At first no living larvae were visible; however, a very small number of globular, ciliated larvae were located near the surface of the water at the edges of the culture bowl. There were no larvae in the 1/2 dilution. Fractions of No. 2 fuel oil.

Controls in clean sea water developed normally and well formed pluteus larvae formed in abundance.

Results for the experimental animals are presented in Tables II to VI. All fractions interfered with development, some more than others,

and the adverse influence increased with concentration. No fraction prevented elevation of the vitelline membrane. Fractions I to IV somewhere in the range 1/50 to 1/10 affected cell division; the most deleterious fractions were Nos I, II and III, manifesting themselves in reduced numbers of dividing eggs. Fractions III and V caused many eggs to divide abnormally, either cell division was incomplete or blastomeres were unequal. Larvae were formed in the most dilute mixes of all fractions (1/50 level). Fractions II and III were most deleterious to larval development; at 24 h larvae were retarded and arms were short or wanting. Fractions IV and V were least harmful to larval development of all fractions tested.

Experiments with sperm

Fertilization capability. The results of the second series of experiments in which the sperm were placed in oil-sea water mixtures before being used to fertilize eggs in normal sea water are presented in Table VII. Sperm maintained 1/2 h in sea water and in all the oil dilutions except No. 2 fuel oil 1/2 resulted in excellent fertilization, as shown by the presence of vitelline membranes 1/2 h after the sperm was added to the eggs. Fertilization in the various experimental cultures ranged from 95 to 99%, except for No. 2 fuel oil 1/2 where fertilization was zero. After fertilization the eggs proceeded into first and second cleavages in a perfectly normal fashion.

Observations of all cultures at approximately 24 h after fertilization revealed perfectly developed pluteus larvae in the control and experimental media, and very few undeveloped eggs on the bottom of the culture bowls. The fuel oil 1/2 culture was, of course, again an exception, there were no larvae in this culture. Out of a

very large number of dead cells on the bottom of the culture bowl, a few were detected that seemed to have undergone aborted attempts at first cleavage.

Sperm motility. Kuwait 1:1 and No. 2 fuel oil 1/125 had no observable effect on the motility of sand dollar sperm (Table VIII). At higher concentrations fuel oil was deleterious, motility declining with increase of oil concentration and length of exposure. After 30 min most sperm were quiescent in fuel oil 1:1; after 60 min all were motionless.

Respiration. Oxygen uptake of sperm (control) at the start of the experiment was 0.36×10^{-6} µl per sperm h, falling exponentially to 40% of that value at 100 min (Table IX, Figure 3). Respiratory rate was zero in fuel oil 1/1 and 1/5, and one-tenth of the control in 1/25, falling to zero in 40 min (Table IX). Data in Needham (1931), derived from Warburg and Schearer, give a rate of 0.18×10^{-6} µl oxygen per sperm h for sea urchins (temp. ca 23°C). Gray (in Needham, 1931) found higher and variable rates.

Barnacles

Development of Eggs and Larvae Chthamalus fragilis

Six pairs of lamellae (controls and experimentals) were unsuccessful, and were not included in the data. They were at starting stages 5, 6, 7, 7, 9 and 12. Controls and experimentals failed to develop beyond the starting stages after 8 days.

All other controls, ranging from starting stages 5 to 13, proceeded to hatching and yielded larvae. Not all embryos in one lamella were at the same stage of development and consequently they hatched over several

days. Living nauplii were observed from one to five days after hatching commenced. No. 2 fuel oil was used in the following experiments.

Stock solution. Thirty-five pairs of lamellae provided useful information. Seven at stage 13, that is at hatching, yielded larvae which died within one day. Eggs at stage 12, from 7 lamellae, developed and hatched over several days; the larvae died soon after hatching. In 7 lamellae at stage 11, some eggs developed and hatched and the larvae soon died; other eggs were arrested and died. Eggs at all earlier stages, in 14 lamellae, were arrested in development and died.

Stock - sea water 1/2. Fifteen lamellae were informative. Embryonic development in five lamellae originally at stages 5 to 7 was generally retarded in early stages of growth, with consequent death of the embryos. The effects, however, varied from embryo to embryo: lamellae generally showed embryos in many stages of development, and two lamellae even hatched a few nauplii that quickly died. In two of three lamellae, at intermediate stages 8 to 10, embryonic development ceased at various stages short of hatching. In the third lamella hatching occurred in some abundance, the larvae were active only a short time before dying. Embryos in later stages of development, 11 to 13, were studied in seven lamellae. In all of them hatching proceeded normally and, in two, more rapidly than in the controls. Larvae all died within one day of hatching.

Stock - sea water 1/5. All fifteen pairs of lamellae provided useful information. In five lamellae, originally at stages 5 to 7, development appeared to proceed normally, and survival was good after hatching. In three of the five groups, nauplii of both experimentals and controls were alive four days after hatching and eight days after the culture was begun. Two groups hatched rather small numbers of

larvae, but development was complete for at least some eggs. Four lamellae with embryos in stages 8-10 were studied; three produced living larvae, one did not, the embryos progressed only to stage 11 and died. In one case the experimental hatched before the control, but its larvae also died sooner. Among lamellae with embryos originally at later stages 11 to 13, development to hatching proceeded normally and, in one group, hatching occurred two days before the control. Larval survival was erratic: in each culture, several days after hatching began, there were approximately equal numbers of living and dead larvae. All experimental larvae were dead five days after hatching while the controls were still alive.

Oil stock - sea water 1/10. Sixteen of nineteen pairs of lamellae yielded information; in three, both the experimentals and controls failed to develop. In two of four lamellae in early stages 5 to 7, development, hatching and larval survival were all parallel to the controls. In one group, larval production and survival were less than in the control; in another no hatching took place. In two of four lamellae at starting stages 8 to 10, there was equal embryonic development and hatching in experimentals and controls. Larval survival was as good as that of the controls for five days after hatching. In two of the four groups there was no hatching, but in both cases the controls had few larvae. Eight lamellae starting at stages 11 to 13 were studied: in all of them embryos proceeded to hatching, and larval survival was about the same as in the controls. In a typical instance, experimental and control larvae were surviving six days after hatching had commenced.

The results, in summarized form, are presented in Table X.

2. Behavior of larvae in acute experiments

Balanus amphitrite niveus

When the apparatus (Figure 4) was illuminated for 15 min the control larvae mostly gathered in the illuminated right chamber. The mean number of larvae remaining in the left chamber was 6.2% and the mean number that gathered in the right was 80.1% (Figure 5, Table XI). The experiments carried out showed the feasibility of the method, explored effective concentrations, and tested behavioral responses. The indication of deleterious action of the test agent chosen was larval numbers in the left chamber; and the criterion, 50% of larvae remaining in the left chamber.

All the substances tested (No. 2 fuel oil, biphenyl, naphthalene, methyl naphthalene, dimethyl naphthalene, phenanthrene, anthracene, fluorene, durene), affected the larvae adversely. Using the 50% criterion, No. 2 fuel oil was harmful at a concentration of about 15%, dimethyl naphthalene at about 11% of stock solution (Figures 6 and 7). We noticed in many experiments that although the larvae did not gather in the right (illuminated) chamber, they still were swimming actively. The results shown graphically in the histograms of Figure 5 are interesting in this regard. It is planned to continue this work.

C. Crab Larvae

Tests on crab larvae were made with No. 2 fuel oil.

1. The striped hermit crab Clibanarius vittatus.

Results of the first experimental series are presented in Figure 8. At the end of 11 days the following numbers of larvae were still alive: controls, 37; dilution 1/10, 24; dilution 1/5, 4. In the 1:1 dilution no larvae survived to the third day (Table XII).

Figure 9 presents the results of the second experimental series. At the end of 11 days when the experiment was terminated, the following numbers of larvae were still alive: controls, 90; dilution 1/50, 89; dilution 1/25, 79; and dilution 1/10, 28 (Table XII). Throughout the experiments it was noted that the larvae in dilution 1/10 did not thrive. Molting was greatly inhibited, the zoeae remained small and lagged far behind the other groups in development.

The controls in the two series showed a considerable difference in survival, in the first series there were only 37, whereas in the second there were 90 survivors out of 100. The environmental conditions were not identical for the two groups, an incubator was employed for the second group, and that or some other factor was involved. However, conditions were the same for larvae in dilutions 1/10 as for the corresponding controls, and yet the numbers of survivors in the former were about the same in the two series, namely 24 and 28.

In all ways the zoeae maintained in the 1/50 dilution fared as well as the controls. Their rate of survival was almost identical, the larvae molted as readily and remained as active as the controls at all times. The rate of survival was lower for those zoeae maintained in the 1/25 dilution, and the difference between the experimental and the control (79:90) was highly significant (Table XII). Whilst the 1/10 dilution permitted survival of some zoeae to 11 days, these had not molted and were extremely small and weak. There is every reason to suspect that they would not have reached the glaucothoe stage. In the 1/5 dilution there were still a few survivors at the termination of the experiment, but these were obviously weak and had not molted. The highest concentration tested, 1:1, killed the zoeae very rapidly; there was no survivor on the third day.

The precipitate drop in numbers of survivors which occurred in the 1:1 dilution between days 5 and 6 (Figures 8 and 9) may reflect a critical period associated with molting. We did not make observations on molting; in Pagurus alatus the first zoea has a mean duration of 7.6 days (18°C) (Bookhout, 1972); no such information is available to us for Clibanarius vittatus.

2. Spider crab Libinia dubia

Libinia dubia has two zoeal stages and a megalops stage, in conformity with the developmental pattern found in other spider crabs (Maiidae) (Gurney, 1942). In the second day after hatching, the appearance of exuviae in all the culture dishes revealed that the second zoeal stage had begun. Between the fifth and seventh days molting from the second zoeal to the megalopa stage occurred; young crabs appeared on the tenth day.

Figure 10 presents the survival data and indicates the progress of larval development in the several media. Survival was excellent for the first four days, there was a modest drop on the fifth day, and a dramatic drop between the fifth and sixth days. Disregarding the 1/10 group, it can be seen that the sharp drop between the fifth and sixth days affected the controls as well as the experimentals. At this time the second zoeae were molting to megalops, which cannibalized many of the zoeae. The same predatory phenomenon occurred to a less noticeable degree between the ninth and tenth days in the controls and in the 1/50 dilution when the megalops were molting to the crab stage. Table XIII presents a χ^2 analysis of the survival data.

Second zoeae were found in all groups on the second day after hatching; even those zoeae in the 1/10 dilution had started to molt, indicating that development had not been notably retarded during the first few days after hatching. Between the fifth and seventh days molting was completed from the second zoeal to the megalopa stage except in the 1/10 dilution. In the latter a few megalops appeared on the seventh day, but many larvae failed to progress beyond the second zoeae even at the termination of the experiment. Larval retardation was more subtle in the 1/25 than in the 1/10 dilution, but there was good evidence that it existed. For instance, on the fifth day, the larvae in the 1/25 dilution did not show quite as great a decline in survivors as did those in the 1/50 dilution and the controls. The reason for this would seem to be that molting to the megalopa stage was slightly retarded in the 1/25 dilution, with the result that the destructive effect of cannibalism by the megalopa upon the zoeae lagged slightly in this group. Delayed larval development was even more clearly demonstrated in the 1/25 dilution as evidenced by the fact that early crab stages were abundant in the controls and the 1/50 mixture on the tenth day, but had just begun on the eleventh day in the 1/25 mixture. Development in the 1/50 mixture followed that of the controls almost exactly.

The survival rates in the controls, the 1/50 and 1/25 mixtures were very similar throughout the experiment. The cannibalism operating to reduce the number of survivors in the controls apparently produced the same reduction in the 1/50 mixture. At the termination of the experiment there were 25 survivors in the control, 26 in the 1/50 mixture, 29 in the 1/25 and 13 in the 1/10. The survivors; however,

showed varying degrees of development. There was no significant difference in the number of survivors in the first three groups; there was a highly significant difference between the controls and the 1/10 (Table XIII). The survival data are most meaningful when considered in conjunction with the observations on larval development.

3. The edible stone crab Menippe mercenaria

The stone crab has five zoeal and a megalops stage. Development at 25°C lasts on the average 31 days; the first zoeal stage has a mean duration of 3.58 days (Porter, 1960; Ong and Costlow, 1970; Bookhout et al., 1972). Our experiments lasted four days and spanned the first zoeal stage. Survival of larvae in control and experimental groups is presented graphically in Figure 11. Statistically, the daily survival differences between the controls and experimentals were highly significant at the 0.001 level except for the difference between the controls and larvae in the 1/10 mixture on the first day. At the end of the third day all larvae were dead in the 1/5 and 1:1 dilutions.

D. Catfish

1. Behavior and survival in acute experiments

Series 1 (20, 10, 5 and 2 ml of No. 2 fuel oil). Thirty minutes after adding the oil all the fish made an effort to stay near the surface, and some attempted to get out of the aquaria. After 20 hours some fish died, most of the fish exposed to 20, 10 and 5 ml of oil stayed at the surface, while most of the fish in 2 ml of oil stayed at the bottom. Dead fish were removed and counted.

After 48 hours most fish in 20 and 10 ml of oil and about half those in 5 ml of oil were dead, while most fish in 2 ml of oil survived.

It was observed that the tail fins of all the dead fish were damaged, the skin, flesh and rays badly eroded, blood showed at the bases of the dorsal and ventral fins, and the gills were bleeding.

After 96 hours (4 days) the experiment was terminated and the surviving fish released. The results are shown in Figure 12 (fraction of fish killed versus oil concentration (in ppm)); the half lethal dosage was 140 ppm.

Series 2 (2, 1 and 0.5 ml of No. 2 fuel oil). Feeding was observed; responses were classified into five categories.

Excellent. All fish actively sought and ingested food.

Good. All fish sought and ate food.

Fair. Most fish sought and ate food.

Poor. Most fish did not seek food, or failed to locate it, or did not eat it.

Very Poor. There was no response to food.

The results of the experiments are presented in Table XIV.

The fish survived in sea water containing 1 ml (38 ppm) and 2 ml (77 ppm) oil for 4 days, but their feeding responses deteriorated. Recovery of feeding responses after exposure to 2 ml of oil was protracted. Moreover, many pieces of shrimp were found in the aquaria loaded with 2 ml of fuel oil, and likewise after the aquaria had been cleaned and refilled. These shrimp were regurgitated by the fish.

Histological observations of the gills and barbels of catfish treated with 2 ml of oil did not reveal any noticeable damage. On the other hand, in the stomach of a catfish treated with 2 ml of oil, the peripheral mucus layer of the cells lining the lumen was deficient. These are preliminary observations.

2. No. 2 fuel oil and the electrocardiogram (EKG) of the sea catfish

The normal cardiac frequency was 50 to 70 beats per min (20 to 25°C). When fuel oil was added in sufficient quantity to the water, forming an emulsion, bradycardia (slowing of the heart) occurred after a latency of 3 min. The response was clear at an oil concentration of 1000 ppm (frequency reduced to 30-40 beats per min), and barely detectable at 100 ppm. The rate returned to normal after 1/2 h. The records are still being analysed.

E. Oxygen Consumption

1. Porcelain crabs

Oxygen consumption was not reduced in No. 2 fuel oil 1:1 (62,000 ppm). The data await analysis.

2. Spiral valves of stingaree

Nine sets of experiments were carried out involving No. 2 fuel oil stock. Oxygen consumption of the controls was highest initially, the mean being 1.69 μl O_2 mg^{-1} h^{-1}, and decreased to about 93% of the initial rate after 120 min. The nine sets of data were analysed statistically. Rates of consumption between controls and experimentals were not significantly different.

3. Cornea of stingarees

Initially the rate of oxygen consumption of controls was 0.7 μl mg^{-1} h^{-1}. After 3 h it fell to about 80% of the initial level. The rate in No. 2 fuel oil 1:1 (50 %) fell to somewhat less than 50% in the same time (Figure 13). The results have not yet been analysed statistically.

4. Pinfish gills

Seven sets of experiments were carried out, involving No. 2 fuel oil, Southern Louisiana crude, diesel fuel and Bunker C, stock solution or dilution 1:1 (vide Table XV). Oxygen consumption of the controls was high initially, and decreased gradually with time, to about two-thirds of the initial rate in 90 min. The initial rate in the controls was about 2 ul mg^{-1} h^{-1}. The rates in the experimentals showed no obvious and persistent differences from those of the controls. The seven sets of data (Tables XV and XVI) were subjected to statistical analysis by treating all seven sets equally and concentrating on the initial rates and final rates (after 120 min). The results of the analyses are shown in Tables XVII and XVIII. It is concluded from them that the differences between the experimentals and controls are not significant.

IV. Discussion and Conclusions

Experimental solutions were prepared as saturated solutions in aqueous media. Oils and oil fractions were made up as oil:water 1:8; pure substances, 15 mg %. These saturated solutions were designated stock solutions (unity or 100%). Dilutions are expressed as ratios, fractions or percentages of stock solutions, or in ppm of the original amounts of material used.

Materials	Ratio	Fraction	% of stock	ppm of original material
Stock	1:0	1	100	
Oil				111×10^3
Pure substance				150
Dilutions of stock				
Oil	1:9	1/10	10	111×10^2
	1:99	1/100	1	111×10
	1:999	1/1000	0.1	111
Pure substances	1:9	1/10	10	15
	1:99	1/100	1	1.5
	1:999	1/1000	0.1	0.15

Sand dollar eggs and sperm

Permeability of eggs

The experimental results showed that one petroleum material, namely No. 2 fuel oil, did not affect the permeability of the sand dollar egg to water over a period of 4 h. This was well beyond the maximal time during which discharged unfertilized eggs would be awaiting insemination under natural conditions. The concentration of fuel oil was chosen deliberately very high as a first venture in order to determine maximal impact which, in the event, turned out to be negative.

An analogous study (Lucké, Parpot and Ricca, 1941) has been made to test carcinogenic and related hydrocarbons on sea urchin eggs. The test substances used were choleic acids of 10-methylbenzanthrene, 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, 1,2-benzanthracene, phenanthrene, and acenaphthene. Saturated solutions were employed, they had no effect on cell permeability. The first three, carcinogenic

compounds, on the other hand, did interfere with early cleavage (vide infra).

Experiments with sperm

Exposure of sperm cells of the sand dollar to the Kuwait oil 1:1 and to the various concentrations of the No. 2 fuel oil for a half hour prior to fertilization had apparently no effect on the sperm, except in the case of No. 2 fuel oil at a concentration of 1:1 (50%). In the latter mixture the ability of the sperm to fertilize the eggs was completely destroyed. There was no gradual decrease in successful fertilization, as was found when the eggs were subjected to increasing concentrations of oil, but rather a sudden reduction to zero fertilization in fuel oil 1:1 (50%).

The motility of sperm in Kuwait oil 1:1 was not reduced after 60 min, whereas in fuel oil the proportion of sperm actively swimming declined in the higher concentrations, beginning with 1/25 (4%) and after longer exposures (Table VIII). About half the sperm were still motile and swimming in fuel oil 1/5 (20%) at 30 min, none in fuel oil 1:1 (50%). At the former concentration there was still an adequate preponderance of sperm sufficient to fertilize the eggs. It is reasonable to assume that the loss of motility of sperm in fuel oil 1:1 (50%) was a determining factor in the absence of fertilization found in that mixture.

The parallel effects of fuel oil on sperm motility and respiration are brought out in Tables VIII and IX. Movement and respiration of sperm are linked, and factors which enhance movement increase the respiratory rate (Rothschild, 1956).

Experiments with developing eggs and larvae

Sand dollars

It has been demonstrated in these experiments that No. 2 fuel oil at the least concentration employed produced no distinguishable effects on the fertilization and development of sand dollar eggs that had been exposed to it for an hour before the addition of sperm, and that remained in it throughout development. On the other hand, No. 2 fuel oil at the highest concentration employed in the experiments completely destroyed the ability of the eggs to be fertilized and to continue development. The three concentrations between these extremes showed deleterious effects in proportion to the amounts of oil present. These effects became evident first of all in the reduced percentages of fertilization, starting with the 1/25 dilution, viz. 78%, and going down to 0% in the 1:1 dilution. The drastic drop in the last mixture may well have been caused by injury to sperm (vide supra).

Even when fertilization was achieved development was not always normal. Irregular cleavages first appeared in the eggs in the 1/10 and 1/5 dilutions at the time of first cleavage. It might be assumed that those cleaving abnormally at early stages would not give rise to well formed pluteus larvae. Abnormal cleavages were evident in later stages in the 1/5, 1/10 and, to a lesser extent, in the 1/25 dilution.

Of the five fractions of No. 2 fuel oil, Nos I to III were most harmful to larval development; fractions III and V produced more abnormal cell divisions.

The Kuwait crude oil at the 1:1 concentration used in this experiment had almost no discernible effect on motility of sperm and fertilization; there appeared to be retardation of first cleavage but cell division thereafter proceeded normally. Consequently, there

was no incentive to test lower concentrations, as was done with No. 2 fuel oil. The experiments revealed a great difference in the effects of these two types of oil on the sand dollar eggs: the Kuwait, seemingly, was far less toxic, whereas a comparable level of No. 2 fuel oil was lethal.

Analyses of Kuwait and No. 2 fuel oil by Dr. K. Winters gave the following results.

Kuwait:	saturated paraffin hydrocarbons	37%
	aromatics	40
	asphaltics and heterocyclics	7
	amount off column	84
No. 2 fuel oil:	saturated paraffin hydrocarbons	57
	aromatics	35
	asphaltics and heterocyclics	.5
	amount off column	92.5

The different effects of Kuwait and No. 2 fuel oil revealed in our experiments on sand dollar eggs are not to be explained by these general analyses, and they are more probably referable to the presence or absence of specific water-soluble substances. It may be noted that there is evidence in other experiments that dispersions of oils have a greater toxicity (by factors of 10 to 100) than aqueous extracts formed by allowing oil layers to rest on the water (Kühnhold, 1970). Toxic effects of methyl phenanthrene derivatives on sea urchin eggs were noted by Lucké, Parport and Ricca (1941), but they were using saturated aqueous solutions. Kuwait does contain polycyclic aromatic hydrocarbons (Carruthers and Douglas, 1961); any water soluble toxic components must be at low concentrations.

In a study parallel to ours on urchin (Strongylocentrotus) eggs, Allen (1971) tested a series of sixteen crude and refined oils, using aqueous extracts (1/20). Most oils had little or no effect on fertilization; eleven, including crudes and No. 2 fuel oil, affected cleavage at concentrations $\geqslant$ 6.25%. These results may be compared with our findings that No. 2 fuel oil affected cleavage at concentrations $\geqslant$ 1:225.

We regard the experiments on sand dollars as being of an exploratory nature. Eggs and sperm are not regularly available and quantities are limited. Whether it be experimentally advantageous or no, echinoderms are very sensitive to pollution (North, 1967; Allen, 1971). Sand dollars have the advantage that they are typical representatives of an ubiquitous and abundant benthic group, and their larvae equally good representatives of the temporary plankton. Sperm are motile, eggs are easily fertilized, segment regularly, and larvae are formed in abundance. The latter, in rate of development and structural features, are good indicators of normal or abnormal conditions in the media (Wilson and Armstrong, 1961). It is for future work to decide how the various deleterious materials in the oil affect the sperm and eggs, by absorption into the external membrane (Danielli, 1950), by modifying the spindle apparatus, damaging intracellular organelles, curbing enzyme activity, and so forth. Eggs and sperm have the great advantage, shared by erythrocytes, of providing homogeneous suspensions of cells on which the effects of petroleum upon cytological processes can be studied directly.

Barnacles

The development of the barnacle Chthamalus fragilis was modified in several ways by the presence of No. 2 fuel oil mixed with sea water. The type of modification depended upon the concentration of the fuel oil and upon the stage of embryonic development at which exposure commenced. Table X summarizes these findings. When embryos that were at an early stage of development (5 to 7) were placed in the oil-sea water mixtures, the effects were progressively more pronounced in the higher concentration. It was notable that in the 1/10 mix, development, hatching and larval survival were about equal to those of the controls. In the 1/5 mix, development was normal in most of the embryos, and larval survival was good but not equal to the controls. In the 1:1 and stock solutions the effects were clearly disastrous. All embryos died in the oil stock, only a few hatched in the 1:1 mix, and rapid death followed hatching.

When the embryos were exposed to the oil mixes at slightly later stages of development, namely stages 8 to 10, lethal effects again increased with increasing oil concentrations. In the 1/10 mixture, the experimental groups were somewhat erratic: in about half the cases, they were indistinguishable from the controls; in the other half, hatching did not occur in the experimentals, but it occurred only poorly in the corresponding controls. In the 1:1 mixture embryos within a lamella were affected to different degrees. After they had been several days in the solution it was possible to find embryos at different stages, and some dead; yet some embryos completed development and hatched. In the undiluted oil stock death occurred before hatching.

Lamellae exposed to oil at late stages of development, 11 to 13, all hatched out living nauplii. In some instances, at the higher concentrations, a more rapid hatching was observed in the experimentals than in the controls. The oil seemed to have a stimulatory effect on larval hatching, but also a rapid lethal effect on the released nauplii.

These experiments reveal that the nauplii are more sensitive to the presence of oil in the medium than are the developing eggs. It is conceivable that the egg case protects the embryo from the immediate effects of the oil; with time the oil penetrates the case and the embryonic membranes and arrests development. An alternative explanation is that the egg case changes and becomes less permeable during later stages of development. Within the content of these experiments it appears that the embryonic development of Chthamalus fragilis proceeds about as well in the 1/10 mixture as in the controls. It is obvious that in concentrations as high as 1:1, death of nauplii is very rapid and crude oil have given results which pose some interesting questions regarding the physiological effects of the oils and their specific toxic components on egg membranes, cuticle, sperm motility, fertilization, cleavage, respiration, and locomotion. Some effects may be external, others the result of interference with biochemical actions or ultrastructural organization.

Acute experiments of one hour duration with larvae of Balanus amphitrite are in progress. Results on hand indicate that oils and some constituents are deleterious at concentrations 15% and 10%, or less, respectively, of the original stock solutions. A mortality level (50%) to 1 h exposure for larvae of Elminius has been reported

(Spooner, 1968); it is intermediate to those obtained by us.

Our acute (1 h) experiments with barnacle larvae are intended to evaluate the relative toxicities of many oils, their fractions and components. An automated counter is now in use which should render the work less tedious and greatly accelerate it. Already the experiments indicate the need for prolonged exposures to selected substances; and they suggest that profound behavioral changes may intervene before death, thereby gravely altering the opportunity for survival under natural conditions.

Crabs

Larvae of the three crabs tested all proved by various criteria to be about equally sensitive to fuel oil, which was deleterious to development and survival. The most striking effects were found with stone crab zoeae, which were killed in all mixes from 1/25 to 1/2, the mortality increasing with the concentration. Comparable effects were obtained with hermit crab zoeae, but they were somewhat less pronounced and manifested themselves more slowly. In addition to survival, significant differences were observed in the condition of the larvae and rate of development. Higher concentrations of oil retarded growth and inhibited molting of hermit crab and spider crab larvae, the effects being manifest at concentrations of 1/10 and 1/25.

Catfishes

Sea catfish were killed by fuel oil at concentrations $>$ 77 ppm and, prior to death, there was much damage to surface tissue. Lethal limits for fishes in the literature (quoted below) lie in the range 4 to 167 ppm. At a concentration of 35 ppm, feeding responses of catfishes deteriorated, and the animals could not retain their food. A parallel study is, perhaps, that of Foster et al. (1966) on flagfish,

who found that fish in ABS (an alkyl benzene sulfonate mixture) had difficulty feeding and threw out food after seizing it. EKG's showed that the fish sensed oil at levels of at least 100 ppm. Catfish sought to escape from oil and, under natural conditions, it might be expected that they would seize opportunities to remove themselves from the vicinity of an oil spill and thereby avoid the damaging effects. Training and conditioning techniques have revealed a wide range of olfactory sensitivities to various aromatics in selected species, e.g. 500 ppm for phenol and p-chlorophenol in a minnow, 0.9 ppm for phenol and 0.02 ppm for benzene in the roach, and 10^{-5} ppm for morpholine in salmon fry.

General discussion and conclusions

It is generally agreed that all crude oils and oil fractions, except for some highly purified materials, are poisonous to marine animals. Loss of marine life was observed following the wreck of the Torrey Canyon, Tampico Maru and the tank barge Florida (at West Falmouth, Mass.). Mortality from the last two wrecks was especially severe (North et al., 1965; North, 1967; J. E. Smith, 1968; Hampson and Sanders, 1969; Blumer, 1970). The oil from the Torrey Canyon was Kuwait crude; from the Tampico Maru, diesel fuel, four-fifth heavy distillate and one-fifth residual fuel oil; from the tank barge Florida, No. 2 fuel oil.

Toxic levels (or tolerance) depend not only upon the kind of oil pollutant, but also upon the species and life history stage exposed to it. Surveys of the literature (Chipman and Galtsoff, 1949; Kühnhold, 1970; Nelson-Smith, 1971; Lichatowich et al., 1973) reveal a wide range of tolerances among marine (and freshwater) animals tested. Some toxic levels reported for crude and fuel oils were: hydroids

Tabularia 1:20 to 1:2000; barnacle Balanus 1:50; oysters Ostrea 1:100 to 1:250 (Chipman and Galtsoff, 1949); sea urchins Strongylocentrotus 1:100 (North et al., 1965). Fish seem to be more sensitive, and lethal levels in the literature are: 15,400 ppm for carp (crude oils); 4 ppm for dace and ruff (Baku crude); young shad, 167 ppm (diesel fuel), 91 ppm (heavy residual fuel oil) (Veselov, 1948; Chipman and Galtsoff, 1949; Nelson-Smith, 1971).

Concentrations having adverse effects on eggs, embryos and larvae were: toadfish Opsanus embryos 1:200; herring Clupea, cod Gadus and plaice Pleuronectes eggs and larvae 1:50 to 1:25000, turbot Scophthalmus eggs 1:10,000 (Mironov, 1967; Kuhnhold, 1969, 1970); oyster Crassostrea, mussel Mytilus eggs 1:1000, 1:10,000 (Renzon, 1973); barnacle Elminius larvae 1:10,000 (Spooner, 1968).

Toxic levels of oils, between 1.5 and 50% (of added oils), on invertebrate larvae, found in our experiments, are within the ranges discovered by other investigators. Some oils have been found to be more toxic than others, Venezuelan and Iranian crudes more so than Libyan (Kuhnhold, 1970); U.S. navy crude more so than fuel, diesel and lubricating oils (Chipman and Galtsoff, 1949); bunker and heavy crude more so than highly refined petroleums; No. 2 fuel was intermediate in effect between the two latter (Allen, 1971). It is interesting that among the many oils tested by Allen, those that had a strong adverse effect on fertilization had the least toxic effect on cleavage, and vice versa; one was equally harmful to both processes. Young cod eggs were more sensitive than older embryos and larvae (Kuhnhold, 1970). Our results with sand dollar eggs show that aqueous extracts of No. 2 fuel oil are far more toxic than Kuwait crude. It should be noted

that Spooner (1968) found that Kuwait crude was lethal to barnacle larvae at much lower concentrations than we employed. In all such experiments, however, when comparing absolute levels, difficulties of interpretation are introduced by the potently grossly different methods used to prepare the test media.

Components of oils having immediate toxicity are low boiling saturated paraffins and aromatic hydrocarbons, both of which are water soluble in degree. High boiling aromatics are suspected as long term poisons (Nelson-Smith, 1967; Blumer, 1969, 1970). Waters along the Eastern American seaboard contain petroleum at levels of 0.001 to 0.012 ppm. This oil consists largely of nonvolatile or persistent hydrocarbons (Brown et al., 1973; Walker and Colwell, 1973). The results obtained with invertebrate larvae are for chronic exposures to concentrations equivalent to 0.0125 and 0.005 of saturated solutions of the water soluble components of the oil. These concentrations are far in excess ($\times 10^6$) of oil levels now existing in the sea; moreover the toxic fractions have mostly been dissipated from the oceanic milieu (Pelpil, 1968). However, the safety margin for many fishes may be considerably less ($\times 10^6$ to 10^3) and, for impairment of olfactory cells, marginal or even adverse (vide supra).

Oil concentrations in excess of 1000 ppm can be expected to occur for short periods near shore or in enclosed basins after major spills while evaporation and photo-oxidation of volatile and toxic components are taking place. They would harm a limited hatch of larvae; the effect on a year class would be dependent, however, on survival and health of the adults, and duration of the reproductive

season. As we have shown in experiments on sand dollar gametes and barnacle eggs, fuel oil, in increasing concentrations, has deleterious effects on the growth and survival of eggs and embryos. Together, these lines of evidence point to harmful effects throughout embryonic and larval life. Owing to the proliferic reproductive capacity of many marine animals, however, it can be expected that reinvasion of the affected area from neighboring regions will occur once the harmful agents are dissipated or destroyed. In conclusion, it would appear that we lack coherent information about long term effects of oil on larvae and post-larvae under the equivalence of natural conditions; namely, in some key species, information that can predicate at what levels of oil pollution they can successfully complete the entire life cycle and maintain themselves in steady numbers.

V. Summary

1. Eggs and larvae of sand dollars Melitta quinquesperforata.

Kuwait crude, No. 2 fuel oil, and five fractions of No. 2 fuel oil were tested on gametes, eggs and larvae. Criteria employed were: permeability to water of eggs transferred to 60% sea water; motility and oxygen consumption of sperm; fertilizing capability of sperm; and development of eggs (elevation of vitelline membrane, cleavage, formation of echinoplutei). Fuel oil 50% (of oil-stock 1:8) did not alter egg permeability. Kuwait 50%, and fuel oil 20% and higher dilutions did not decrease the fertilizing capability of sperm. However, sperm exposed to fuel oil 50% were rendered immobile, they ceased to respire and they did not fertilize eggs. Kuwait 50% had no effect on cleavage and development. Fuel oil had a marked depressive effect on development, which appeared at a concentration of 4%, and increased at higher

concentrations; practically no fertilization and development took place at a concentration of 20%. Fractions I to III of fuel oil (C_9 to C_{17}) were most deleterious to larval development.

2. Eggs, embryos and larvae of the littoral barnacle Chthamalus fragilis.

The water soluble components of No. 2 fuel oil were tested on the development of eggs in vitro. Concentrations used were saturated stock solution (1:8) (in sea water) and dilutions of stock of 50%, 25% and 10% (in sea water, by volume). Development was arrested in the saturated (stock) solution; in the others, the effect depended on the concentration. The 50% mix was lethal to early embryos (stages 5 to 7); later stages (8 to 10 showed variable development; terminal stages (11 to 13), normal or accelerated development; nauplii soon died. In the 20% mix most embryos hatched, but larval survival was poor. Embryonic development and larval survival in the 10% mix was about equal to the controls. The egg case seemingly afforded the embryos some protection against the oil. The tolerance was high, some development still occurring at a concentration of 2.2%. The resistance of the embryos agrees with that of adult barnacles to environmental stress.

Balanus amphitrite niveus

Tests of the phototactic response of recently hatched nauplii were carried out in the presence of No. 2 fuel oil and petroleum oil constituents, including biphenyl, naphthalene, methyl naphthalene, dimethyl naphthalene, etc. Concentrations used were saturated stock solutions (oil stock-sea water 1:8; pure compounds, 15 mg %), and various dilutions thereof; tests lasted 1 h. The criteria used were positive or negative phototaxis. The larvae are normally positively

phototactic; among the controls, 7.3% failed to migrate towards the light. The oil had an effect by reducing positive phototaxis, at a level of 10%; the effective level for half the larvae was 15%; naphthalene had an effect at 20%, methyl naphthalene at 50%, dimethyl naphthalene at 10%. The materials did not always kill the larvae at the concentrations used within 1 h; however, the loss of positive phototaxis could deprive the larvae of a behavioral response essential to their dispersion, orientation, and survival.

3. Crab larvae were tested in No. 2 fuel oil, namely zoeae of striped hermit crab Clibanarius vittatus, stone crab Menippe mercenaria, and all stages (first, second zoeae, megalops and first crab) of spider crab Libinia dubia.

Hermit crab. Zoeae survived and developed for 11 days almost as well or as well as controls in 2% oil stock. About half the larvae were killed in 8% oil stock. Survival and development were poor in 10% and 20% oil stock; no larvae survived in 100% oil stock.

Spider crab. Development of all stages up to and including metamorphosis into young crabs proceeded as well or almost as well in 2% fuel oil as in controls (10 days). Development to the crab stage took place in 4% fuel oil, but was somewhat retarded. In 10% fuel oil there was gross interference with development. Molting to second zoeae occurred, but most zoeae failed to become megalops, and none of the latter reached first crab.

Stone crab. Experiments involved the first zoeal stage and lasted 5 days. Survival was reduced in all concentrations (4% to 50%) after 5 days; many larvae died in 50% and 20% oil at the end of 1 day, and all

were dead at the end of 3 days. Levels of half mortality were: 1 day, 32% oil; 2 days, 13% oil; 3 days, 6% oil; 4 days, 2% oil.

4. Catfish Arius felis. When fuel oil was added catfish sought to escape; attempts to escape continued in oil at levels of 190 ppm and greater. Fish were killed by oil; at 48 h the concentration of oil causing half mortality was 140 ppm. There was much damage to flesh of the fins and to the gills. Feeding responses deteriorated at oil levels of 38 ppm or greater; food ingested was regurgitated; recovery after exposure to 76 ppm of oil was slow. Electrocardiography showed responses (bradycardia) to oil at a concentration of 100 ppm.

5. Oxygen consumption of porcelain crabs, of gills of pinfish and excised spiral valve of stingaree was not altered from that of controls in various oils (2-3 h). But it was depressed in corneas of stingarees in 50% fuel oil. The cornea is a delicate tissue exposed directly to the environment.

VI. References

Allen, H. 1971. Effects of petroleum fractions on the early development of a sea urchin. Marine Pollution Bull. 2, 138-140.

Bassindale, R. 1936. The developmental stages of three English barnacles, Balanus balanoides (Linn.), Chthamalus stellatus (Poli) and Verruca stroemia (O. F. Müller). Proc. Zool. Soc. Lond. 1936, pp. 57-74.

Blumer, M. 1969. Oil pollution of the ocean. Oceans, 15, 2-7.

Blumer, M. 1970. Oil contamination and the living resources of the sea. FAO Technical Conference on Marine Pollution and its Effects on Living Resources and Fishing. Rome, Italy, 9-18 Dec. 1970.

Bookhout, C. G. 1972. Larval development of the hermit crab Pagurus alatus Fabricius, reared in the development. Crustaceana, 22, 215-238.

Bookhout, C. G., Wilson, A. J., Jr., Duke, T. W. and Lowe, J. I. 1972. Effects of Mirex on the larval development of two crabs. Water, Air and Soil Pollution, 1, 165-180.

Carruthers, W. and Douglas, A. G. 1961. 1,2-Benzanthracene derivatives in a Kuwait mineral oil. Nature, Lond. 192, 256-257.

Chipman, W. A. and Galtsoff, P. S. 1949. Effects of oil mixed with carbonized sand on aquatic animals. Spec. Sci. Rept. Fisheries No. 1, U.S. Dept. Int., Fish and Wildlife Service, 52pp.

Costello, D. P. and Henley, C. 1971. Methods of obtaining and handling marine eggs and embryos. Marine Biological Laboratory, Woods Hole, Mass.

Costlow, J. D., Jr. and Bookhout, C. G. 1957. Larval development of Balanus eburneus in the laboratory. Biol. Bull. Woods Hole 112, 313-324.

Costlow, J. D., Jr. and Bookhout, C. G. 1958. Larval development of Balanus amphitrite var. denticulata Broch reared in the laboratory. Biol. Bull. Woods Hole, 114, 284-295.

Crisp, D. J. 1954. The breeding of Balanus porcatus (da Costa). J. mar. biol. Assoc. U.K. 33, 473-494.

Crisp, D. J. 1959. The rate of development of Balanus balanoides (L.) embryos in vitro. J. Animal Ecol. 28, 119-132.

Danielli, J. F. 1950. Cell physiology and pharmacology. Elsevier Publishing Co., New York, Amsterdam, London, Brussels.

Foster, N. R., Scheier, A. and Cairns, J., Jr. 1966. Effects of ABS on feeding behavior of flagfish, Jordanella floridae. Trans. Amer. Fish. Soc. 95, 109-110.

Gurney, R. 1942. Larvae of decapod crustacea. The Roy. Society, London.

Hampson, G. R. and Sanders, H. L. 1969. Local oil spill. Oceanus, 15, 8-11.

Hara, T. J. 1971. Chemoreception. In Fish physiology (eds. W. S. Hoar and D. J. Randall), Vol. V, pp. 79-120. Academic Press, New York and London.

Harvey, E. B. 1956. The American Arbacia and other sea urchins. Princeton Univ. Press, Princeton, N.J.

Herz, L. E. 1933. The morphology of the later stages of Balanus crenatus Brugniere. Biol. Bull. Woods Hole, 64, 432-442.

Kleerekoper, H. 1969. Olfaction in fishes. Indiana Univ. Press, Bloomington, London.

Kühnhold, W. W. 1969. The influence of water soluble compounds of crude oils and their fractions on the ontogenetic development of herring fry (Clupea harengus). Ber. dt Wiss. Kommn. Meeresforsch. 20, 165-171.

Kühnhold, W. W. 1970. The influence of crude oils on fish fry. FAO Technical Conference on Marine Pollution and its Effects on Living Resources and Fishing. Rome, Italy, 9-18 Dec. 1970, 10pp.

Lichatowich, J. A., O'Keefe, P. W., Strand, J. A. and Templeton, W. L. 1973. Development of methodology and apparatus for the bioassay of oil. In Proceedings of Joint Conference for Prevention and Control of Oil Spills, March 13-15, 1973, Wash. D.C., pp. 659-666. American Petroleum Institute, Wash. D.C.

Lucké, B. 1940. The living cell as an osmotic system and its permeability to water. Cold Spring Harbor Symp. Quant. Biol. 8, 123-132.

Lucké, B. and McCutcheon, M. 1932. The living cell as an osmotic system and its permeability to water. Physiol. Rev. 12, 68-139.

Lucké, B., Parport, A. K. and Ricca, R. A. 1941. Failure of choleic acids of carcinogenic hydrocarbons to alter permeability of marine eggs and of mammalian erythrocytes. Cancer Res. 1, 709-713.

Lucké, B., Hartline, H. K. and Ricca, R. A. 1939. Comparative permeability to water and to certain solutes of the egg cells of three marine invertebrates, Arbacia, Cumingia and Chaetopterus. J. cell. comp. Physiol. 14, 237-252.

Lucké, B., Larrabee, M. G. and Hartline, H. K. 1935. Studies on osmotic equilibrium and on the kinetics of osmosis in living cells by a diffraction method. J. gen. Physiol. 19, 1-17.

Marvin, D. E., Jr. and D. T. Burton. 1973. Cardiac and respiratory responses of rainbow trout, blue gills and brown bullhead catfish during rapid hypoxia and recovery under normoxic conditions. Comp. Biochem. Physiol. 46A, 755-565.

Medes, G. 1917. A study of the causes and the extent of variations in the larvae of Arbacia punctulata. J. Morph. 30, 317-432.

Mironov, O. G. 1967. Effects of low concentrations of oil and petroleum products on the development of eggs of the Black Sea turbot. Vop. Ikhtiol. 7, 577-580 (in Russian).

Needham, J. 1931. Chemical embryology. Vol 2, pp. 623-627. Cambridge Univ. Press.

Nelson-Smith, A. 1967. Oil emulsifiers and marine life. J. Devon Trust Nat. Consv., Suppl. July 1967, pp. 29-33.

Nelson-Smith, A. 1971. Effects of oil on marine plants and animals. In Water pollution by oil (ed. P. Hepple), pp. 273-280. Institute of Petroleum. Elsevier Publ. Co., Amsterdam, London, New York.

North, W. J. 1967. Tampico, a study of destruction and restoration. Sea Front. 13, 212-217.

North, W. J., Neushul, M. and Clendenning, K. A. 1965. Successive biological changes observed in a marine cove exposed to a large spillage of mineral oil. Symp. Poll. mar. Micro-org. Prod. petrol., Monaco 1964, pp. 335-354.

Ong, Kah-sin and Costlow, J. D., Jr. 1970. The effect of salinity and temperature on the larval development of the stone crab, Menippe mercenaria (Say) reared in the laboratory. Chesapeake Science, 11, 16-29.

Pardi, L. and Papi, E. 1961. Kinetic and tactic responses. In The physiology of crustacea (ed. T. H. Waterman), Vol. II, pp. 365-399. Academic Press, New York and London.

Porter, H. J. 1960. Zoeal stages of the stone crab, Menippe mercenaria Say. Chesapeake Science, 1, 168-177.

Randall, D. J. 1968. Functional morphology of the heart in fishes. Am. Zool. 8, 179-189.

Randall, D. J. 1970. The circulatory system. In Fish physiology (eds. W. S. Hoar and D. J. Randall), Vol. IV, pp. 133-173. Academic Press, New York and London.

Renzon, A. 1973. Influence of crude oil, derivatives, and dispersants on larvae.

Rothschild, Lord. 1956. Fertilization. Methuen, London.

Sandison, E. E. 1954. The identification of the nauplii of some South American barnacles with notes on their life histories. Trans. Roy. Soc. South Africa, 34 (Pt. I), 69-101.

Smith, J. E., ed. 1968. 'Torrey Canyon,' pollution and marine life. Mar. Biol. Ass. U.K., Cambridge Univ. Press.

Smith, R. I. 1964. On the early development of Nereis diversicolor in different salinities. J. Morph. 114, 437-452.

Spooner, M. F. 1968. Preliminary work on comparative toxicities of some oil dispersants and a few tests with oils and Corexit. Mar. Biol. Ass. U.K., Plymouth, England.

Tighe-Ford, D. J., Power, M. J. D. and Vaile, D. C. 1970. Laboratory reading of barnacle larvae for antifouling research. Helgolander wiss. Meeresunters. 20, 393-405.

Veselov, A. E. 1948. Effect of crude oil pollution on fishes. Ryb. Khoz. 12, 21-22 (in Russian).

Whitten, H. L., Rosene, H. F. and Hedgpeth, J. W. 1950. The invertebrate fauna of Texas coast jetties; a preliminary survey. Publ. Inst. mar. Sci. Univ. Tex. 1, 53-101.

Wilson, D. P. and Armstrong, F. A. J. 1961. Biological differences between sea waters: experiments in 1960. J. Mar. Biol. Ass. U.K. 41, 663-681.

Table I

Effects of petroleum oils on the development of sand dollar eggs. Criteria used were presence of a vitelline membrane at 1/2 h after insemination, normal and abnormal first cleavages at 1 h, and normal and abnormal second cleavages at 1 1/2 h. Samples, each of 100 randomly selected cells, were examined. Estimates of numbers of larvae and states of development were based on examinations of entire cultures 24 h after insemination.

Table I

	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	100	0	0	100	0	0	Excellent	Excellent
Kuwait 1/2	100	0	65	0	35	99	0	1	"	"
No. 2 fuel oil										
1/50	100	0	100	0	0	100	0	0	"	"
1/25	78	22	67	0	33	85	5	10	"	Very good
1/10	5	95	23	31	46	3	32	65	Fair	Fair
1/5	1	99	3	23	74	0	2	98	Extremely poor	Extremely poor
1/1	0	100	0	0	100	0	0	100	None	None

Tables II to VI

Effects of fractions (I to V) of No. 2 fuel oil on the development of sand dollar eggs. Criteria as in Table I. *Less than 100 eggs available.

Table II

Groups	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	97	1	2	99	0	1	Excellent	Excellent
No. 2 fuel oil										
Fraction I										
1/50	100	0	96	0	4	98	0	2	Excellent	Excellent
1/25	100	0	98	0	2	98	0	2	Excellent	Excellent
1/10	100	0	6	1	93	3	0	97	None	None
1/5	100	0	39	0	61	33	0	67	None	None
1/2	34*	0	0	0	34*	0	0	30*	None	None

Table III

Groups	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	99	1	0	100	0	0	Excellent	Excellent
No. 2 fuel oil										
Fraction II										
1/50	100	0	100	0	0	98	0	2	Excellent	Very good
1/25	100	0	96	1	3	99	0	1	Excellent	Good
1/10	100	0	24	7	69	14	1	85	None	None
1/5	100	0	1	1	98	0	0	100	None	None
1/2	65*	0	0	0	63*	0	0	70*	None	None

Table IV

Groups	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	97	0	3	100	0	0	Excellent	Excellent
No. 2 fuel oil										
Fraction III										
1/50	100	0	96	2	2	95	2	3	Excellent	Very good
1/25	100	0	68	28	4	96	4	0	Excellent	Good
1/10	100	0	14	25	61	12	10	78	None	None
1/5	100	0	1	4	95	0	0	100	None	None
1/2	100	0	0	6	94	0	5	95	None	None

Table V

Groups	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	95	1	4	95	1	4	Excellent	Excellent
No. 2 fuel oil										
Fraction IV										
1/50	100	0	99	0	1	100	0	0	Excellent	Excellent
1/25	100	0	100	0	0	100	0	0	Excellent	Excellent
1/10	100	0	91	3	6	86	1	13	Excellent	Excellent
1/5	100	0	84	6	10	84	9	7	Excellent	Very good
1/2	100	0	0	1	99	0	1	99	None	None

Table VI

Groups	Vitelline membrane		First cleavage			Second cleavage			Larvae	
	Present	Absent	Present		Absent	Present		Absent	Number	Development
			Normal	Abnormal		Normal	Abnormal			
Control	100	0	99	0	1	95	2	3	Excellent	Excellent
No. 2 fuel oil										
Fraction V										
1/50	100	0	86	14	0	96	4	0	Excellent	Excellent
1/25	100	0	69	31	0	88	11	1	Excellent	Excellent
1/10	100	0	95	3	2	98	0	2	Excellent	Very good
1/5	100	0	73	21	6	75	8	17	Excellent	Fair
1/2	100	0	0	7	93	0	4	96	None	None

Table VII

Effects of petroleum oils on sand dollar sperm, as revealed by their capacity to fertilize eggs. Numbers of eggs at several stages of development were determined from samples of 100 eggs in each culture bowl. Eggs were examined under a compound microscope (magnification x80) for the presence of a vitteline membrane after insemination, for first cleavage a 1 h, and for 16-32 cell-stages at 2 1/2 h. Culture bowls were examined under a stereomicroscope at 24 h to determine the number and condition of larvae.

Table VII

		Vitelline membrane	First cleavage	16-32 cells	Larvae Number	Larvae Development
Control		99%	99%	99%	Excellent	Excellent
Kuwait 1/2		95	96	96	Excellent	Excellent
No. 2 fuel oil	1/50	95	94	95	Excellent	Excellent
	1/25	99	94	95	Excellent	Excellent
	1/10	99	100	99	Excellent	Excellent
	1/5	97	97	97	Excellent	Excellent
	1/2	0	0	0	None	None

Table VIII

Effects of petroleum oils (No. 2 fuel oil and Kuwait crude) on motility of sand dollar sperm.

Table VIII

Medium	Dilution	Time: 5 min	Time: 30 min	Time: 60 min
Fuel oil	1/2	0.5 swimming " moving	0.1 moving 0.9 immobile	all immobile
Fuel oil	1/5	all swimming	0.5 swimming 0.5 moving	0.1 swimming 0.4 moving 0.5 immobile
Fuel oil	1/25	all swimming	all swimming	0.2 moving 0.8 swimming
Fuel oil	1/125	all swimming	all swimming	all swimming
Kuwait	1/2	all swimming	all swimming	all swimming
Control sea water	--	all swimming	all swimming	all swimming

Table IX

Oxygen uptake of spermatozoa of sand dollars in sea water and oil stock-sea water mixtures at 25°C.

Table IX

Time (min)	Condition	O_2 uptake (µl/sperm-h)
4	Control	0.36×10^{-6}
7	fuel oil 1/2	0
10	fuel oil 1/5	0
15	fuel oil 1/25	0.38
20	Control	0.27
25	fuel oil 1/2	0
30	fuel oil 1/5	0
40	fuel oil 1/25	0
45	Control	0.22
65	Control	0.18
100	Control	0.14

Solubility of oxygen in sea water (30 °/oo) at 25°C is 48 µl ml^{-1}.

Table X

Generalized summary of the effects of petroleum (No. 2 fuel oil) mixtures on embryonic development and larval survival in Chthamalus fragilis

Original Stages	Controls	Oil - sea water mixtures			
		1/10	1/5	1/2	Stock
5-7	Embryos developed, hatched in 4-7 (median 6) days. Larvae lived several days.	Embryos hatched. Larval development about equal to controls	Most embryos hatched. Larval survival good, not equal to controls.	Most embryos died before hatching, a very few hatched. Larvae died rapidly.	All embryos died before hatching.
8-10	Embryos developed, hatched in 3-7 (median 5) days. Larvae lived several days.	Hatching and larval survival equal to controls in half lamellae.	Most embryos hatched. Larval survival poor, not equal to controls.	Variable embryonic development. Some hatching. Rapid larval death.	Embryos developed slightly, died.
11-13	Embryos hatched in 0-6 days (median 1). Larvae survived several days.	Normal hatching. Larval survival about equal to controls.	Normal or rapid hatching. Larval survival erratic, not equal to controls.	Normal or rapid hatching. Rapid larval death.	Rapid hatching. Rapid larval death.

Table XI

Behavior of barnacle larvae in a test chamber
(Figure 4)

Agent	No. of larvae in chambers				Percentages		Observations
	L	LC	RC	R	L	R	
Sea water	55	46	35	465	9.7	82.2	Swimming
Controls	10	18	12	62	9.8	61.1	Swimming
	1	1	10	119	0.7	91.1	Swimming
	8	5	10	150	4.6	86.9	Swimming
No. 2 fuel oil							
First series							
10%	42	19	12	69	29.5	48.6	
20	309	56	13	5	85.0	1.4	
30	368	23	4	2	95.0	0.5	
50	114	22	0	0	84.0	0	
Second series							
1%	9	3	3	176	9.9	92.0	Swimming
2	0	0	1	132	0	98.2	Swimming
4	1	1	2	200	0.5	98.0	Swimming
10	22	4	0	119	15.2	82.0	Swimming
50	141	2	0	0	98.5	0	Mostly dead
Biphenyl							
10%	42	32	42	246	11.6	68.2	
30	59	59	121	415	9.2	61.9	
100	668	29	4	0	95.0	5.0	
Naphthalene							
20%	411	47	199	79	56.1	10.8	Swimming
30	413	8	0	0	98.5	0	Swimming
100	537	6	0	1	98.5	0.2	Swimming
Methyl naphthalene							
1%	16	17	30	326	4.1	83.9	Swimming
2	13	21	23	282	3.8	83.4	Swimming
4	11	17	20	312	3.1	86.4	Swimming
10	12	7	67	335	2.9	79.8	Swimming
50	492	3	0	0	99.4	0	Swimming
Dimethyl naphthalene							
2%	67	14	52	362	13.5	73	Swimming
4	45	7	43	295	11.6	75.8	Swimming
10	130	4	6	106	52.8	43.0	Swimming
50	164	0	0	0	100	0	Mostly dead

Table XI (cont.)

Agent	No. of larvae in chambers				Percentages		Observations
	L	LC	RC	R	L	R	
Fluorene							
1%	14	42	27	243			Swimming
2	0	13	42	309			Swimming
4	16	11	46	276			Swimming
10	75	79	31	148			Swimming
50	48	119	146	0			Swimming
Phenanthrene							
50%	32	102	125	318	5.6	55.2	
78	136	39	4	1	75.8	0.1	
Anthracene							
100%	92	31	180	121	21.7	28.6	
Durene							
30%	43	33	49	121	9	63.5	
100	185	64	42	38	56.2	11.6	

Table XII

Results of two sets of experiments testing No. 2 fuel oil on larvae of the striped hermit crab Clibanarius vittatus. Experiments in both series involved first and subsequent zoeae; they lasted 11 days. Exp. N, Obs. N., expected, observed numbers; NS, not significant.

Table XII

Solution (oil stock-sea water)	Original N	Survivors Exp. N	Survivors Obs. N	χ^2
Series 1				
Sea Water Control	100		37	
1/10	100	37	24	7.24
1/5	100	37	4	46.71
1/2	100	37	0	58.63
Series 2				
Sea Water Control	100		90	
1/50	100	90	89	NS
1/25	100	90	79	13.44
1/10	100	90	28	427.11

Table XIII

Experiments with larvae of the spider crab Libina dubia, testing No. 2 fuel oil. The data show survival of controls and experimentals (exposed to oil dilutions 1/50, 1/25 and 1/10) for 11 days. X^2 analyses. NS, not significant.

Table XIII

Day	Control	1/50	X^2	1/25	X^2	1/10	X^2
1	100	100	NS	100	NS	100	NS
2	100	100	NS	100	NS	99	NS
3	98	100	NS	98	NS	97	NS
4	97	98	NS	97	NS	87	34.36
5	82	78	NS	91	5.48	82	NS
6	44	44	NS	52	2.59	71	29.57
7	41	38	NS	32	3.34	48	2.02
8	39	38	NS	31	2.68	41	NS
9	39	37	NS	29	4.19	30	3.39
10	25	26	NS	29	NS	18	3.32
11	25	26	NS	29	NS	13	8.78

Table XIV

Behavior of catfish exposed to No. 2 fuel oil.

Table XIV

Days in oil or sea water	Oil concentration 2 ml	1 ml	0.5 ml	Control
0	Excellent	Excellent	Excellent	Excellent
1	Excellent	Excellent	Excellent	Excellent
2	Poor	Fair	Excellent	Excellent
3	Poor	Fair	Excellent	Excellent
4	Very Poor	Fair	Excellent	Excellent
Recovery Days				
1	Poor	Fair	Excellent	Excellent
2	Fair	Good	Excellent	Excellent
3	Fair	Good	Excellent	Excellent
4	Good	Excellent	Excellent	Excellent
5	Good	Excellent	Excellent	Excellent
6	Good	Excellent	Excellent	Excellent
7	Good	Excellent	Excellent	Excellent

Table XV

Initial Rate of Oxygen Uptake by Pinfish Gills

Control $\mu l/mg\ h^{-1}$	Experimental $\mu l/mg\ h^{-1}$	Type of oil
1.46	1.53	Diesel fuel
2.34	2.06	Diesel fuel
2.11	1.69	Diesel fuel
2.40	2.05	Southern La. crude 1:1 dilution
1.41	1.25	Southern La. crude
1.89	1.41	Bunker C - 1:1 dil.
1.94	1.97	Bunker C
1.94	1.60	No. 2 fuel oil
1.94	1.53	No. 2 fuel oil
1.93	2.15	No. 2 fuel oil
$n =$ 10	10	
$\bar{x} =$ 1.94	1.72	
$\sigma =$ 0.319 = 0.32	0.31	
S.E. = 0.11	0.11	

$$S.E. = \frac{\sigma}{n-1} = \frac{0.32}{10-1}$$

Table XVI

Final Rate of Oxygen Uptake by Pinfish Gills

Control $\mu l/mg\ h^{-1}$	Time (minutes)	Experimental $\mu l/mg\ h^{-1}$	Time (minutes)	Type of oil
1.09	84	1.13	92	Diesel fuel
1.44	80	2.06	90	Diesel fuel
1.58	97	1.35	104	Diesel fuel
1.15	95	1.25	102	Southern La. crude 1:1 dil.
1.01	96	1.17	102	Southern La. crude
1.15	97	1.03	104	Bunker C - 1:1 dil.
1.51	104	1.56	111	Bunker C
1.36	129	1.28	137	No. 2 fuel oil
1.04	122	1.11	130	No. 2 fuel oil
1.45	127	1.35	119	No. 2 fuel oil
$n = 10$		10		
$\bar{x} = 1.28$		1.33		
$\sigma = 0.21$		0.30		
S.E. = 0.07		0.10		

Table XVII

Oxygen Uptake by Pinfish Gills

Statistical analysis of initial rates

	Control	Experimental
	1.46 µl/mg-h	1.53 µl/mg-h
	2.34	2.06
	2.11	1.69
	2.40	2.05
	1.41	1.25
	1.89	1.41
	1.94	1.97
$n =$	7	7
$\bar{x} =$	1.94 µl/mg-h	1.71 µl/mg-h
$\sigma =$	0.39	0.33
S.E. =	0.15	0.12

Table XVIII

Oxygen Uptake by Pinfish Gills

Statistical analysis of final rates

	Control	Experimental
	1.09 µl/mg-h	1.13 µl/mg-h
	1.44	2.06
	1.58	1.35
	1.15	1.25
	1.01	1.17
	1.15	1.03
	1.51	1.56
$n =$	7	7
$\bar{x} =$	1.28 µl/mg-h	1.36 µl/mg-h
$\sigma =$	0.23	0.35
S.E. =	0.09	0.13

Descriptions of Figures

Figure 1. Result of an experiment showing progressive swelling of sand dollar eggs when transferred from sea water 33.3 °/oo to dilute (60%) sea water. Ten eggs were used and each point is the mean of 9 to 10 measurements. Temp. 25°C.

Figure 2. Time course of swelling of sand dollar eggs when placed in dilute sea water. Left curve, controls, means of 10 cells. Right, experimentals, means of 12 cells. Experimental eggs were treated with No. 2 fuel oil stock-sea water 1:1 for 2 to 3 h. At the start of the experiment, control and experimental eggs were transferred from a medium 35.8 °/oo to 60% sea water, and diameters were measured. Controls: $y = -5.442 + 0.00889\ \bar{x}$. Experimentals: $y = -6.041 + 0.00877\ \bar{x}$. Temp. 24-25°C.

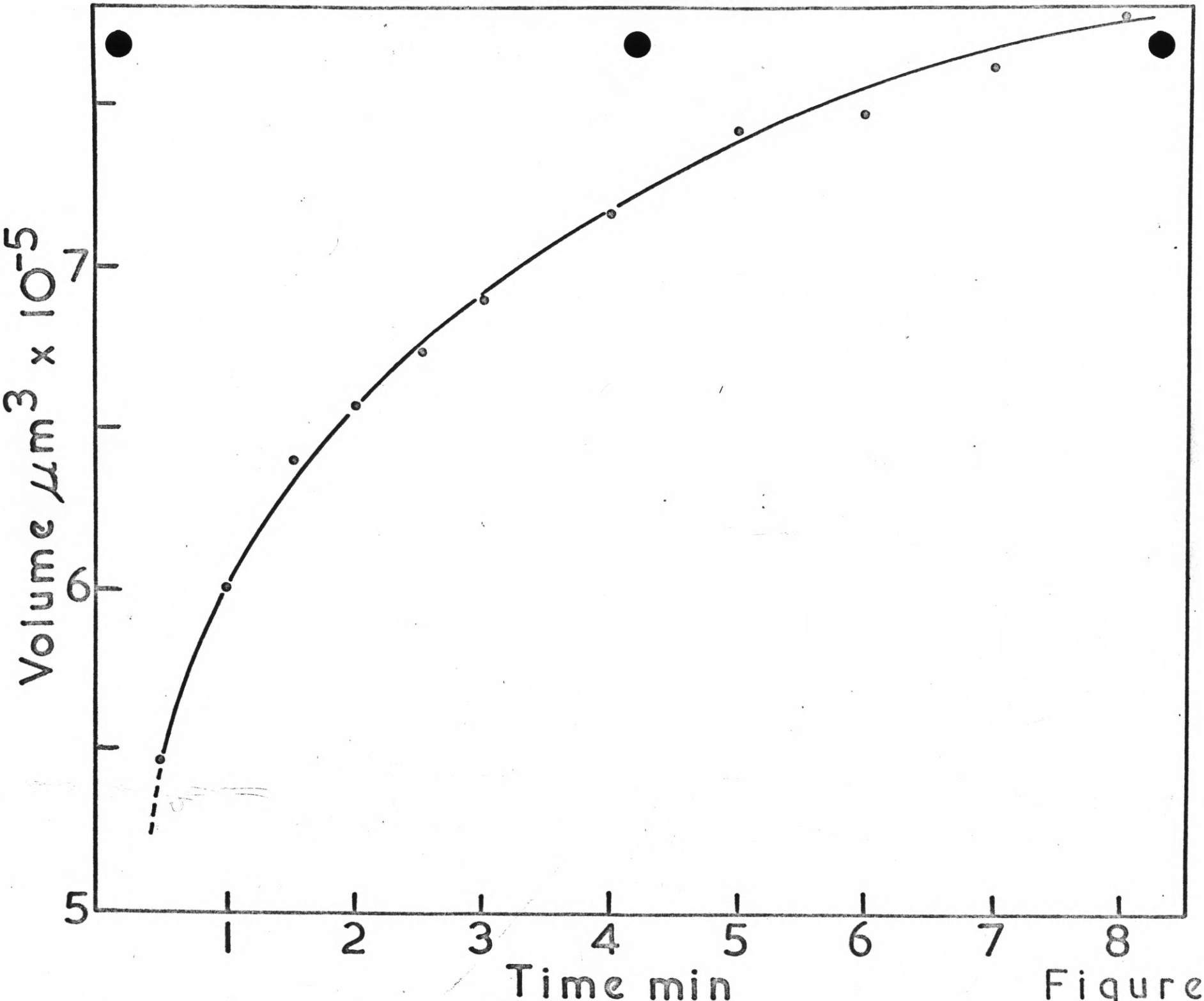


Figure 1

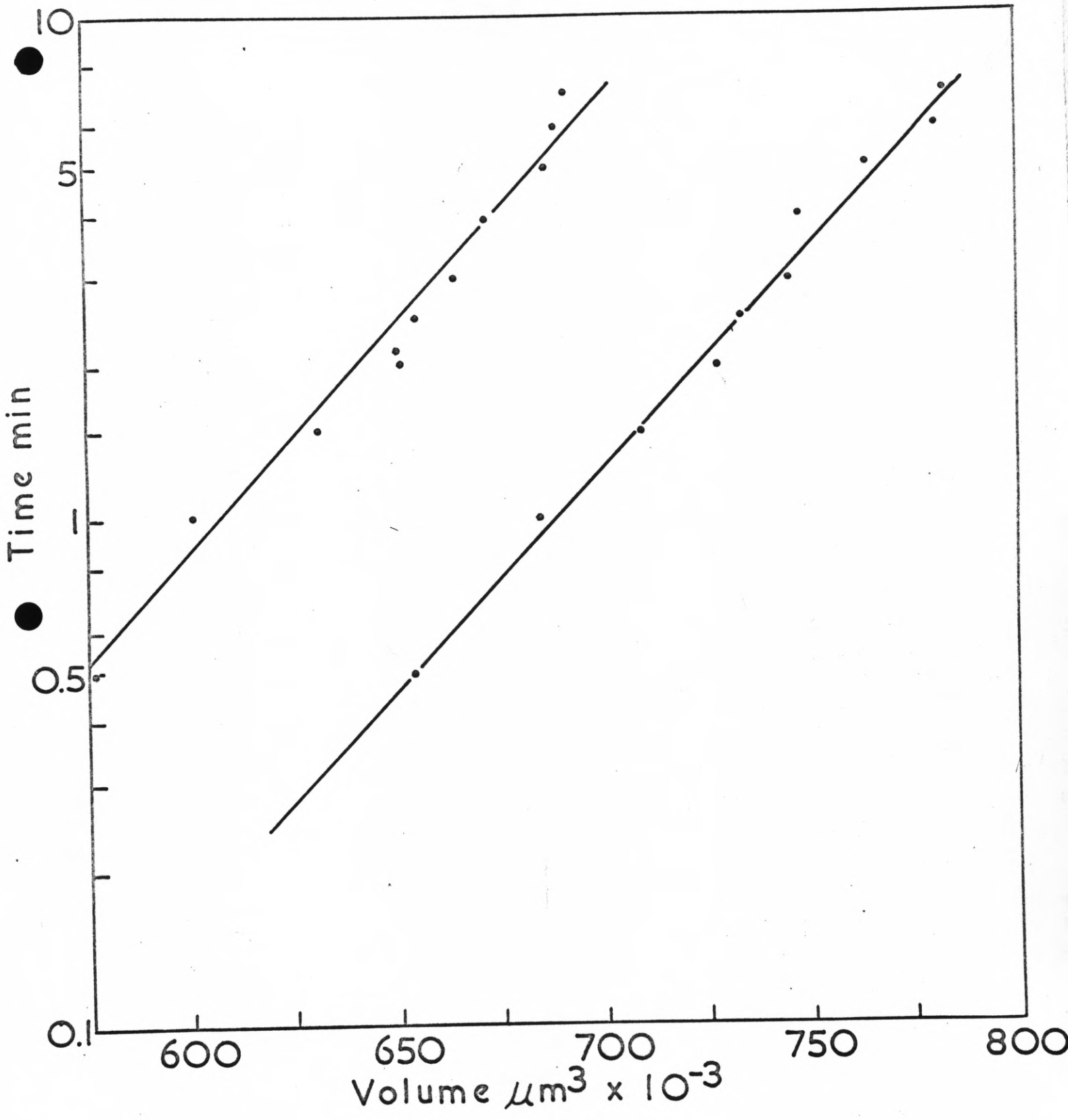


Figure 2

Figure 3. · Rate of oxygen consumption of sand dollar sperm (controls) in sea water 30 °/oo. Temp. 25°C.

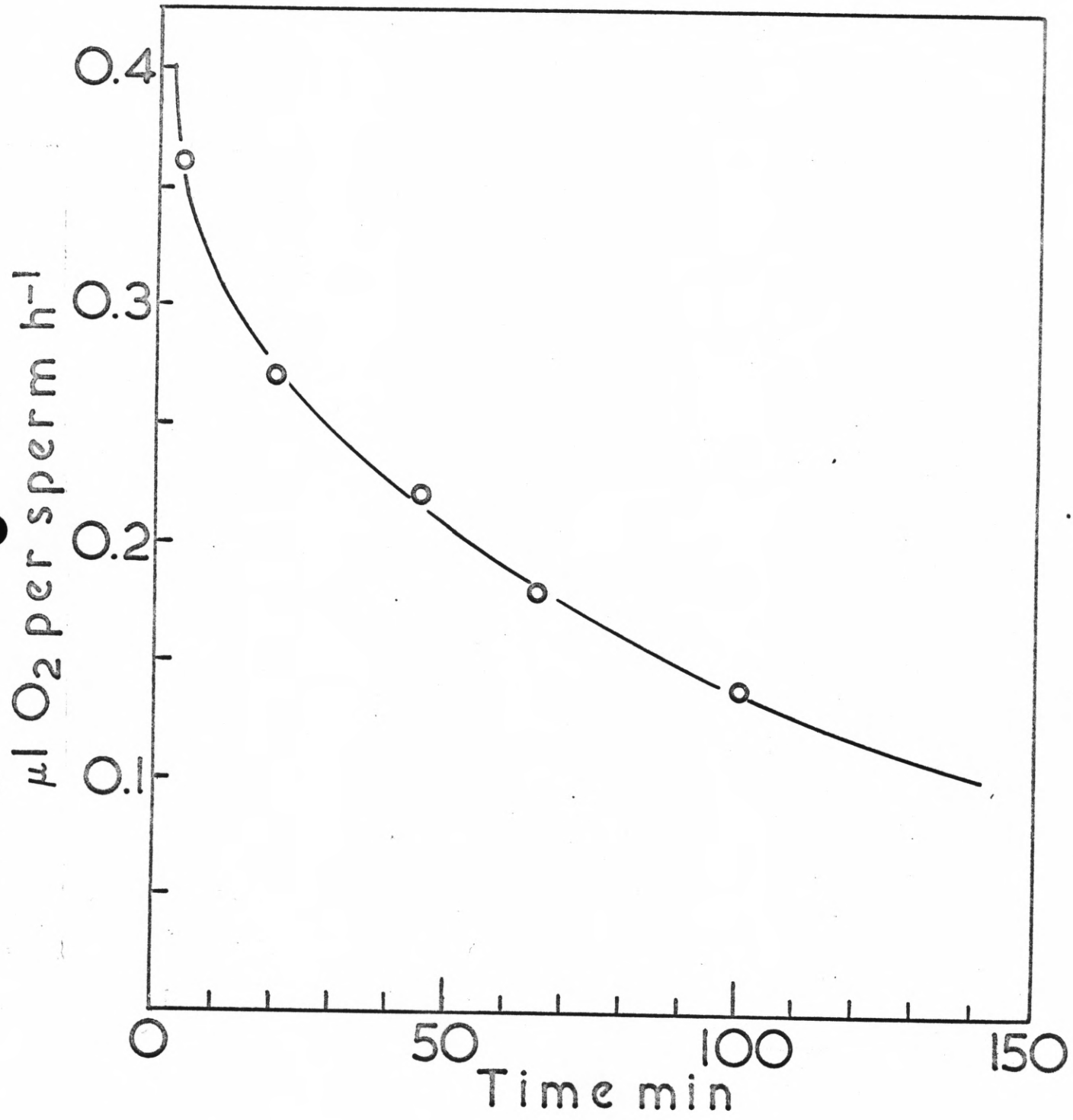


Figure 3

Figure 4. Drawing of the apparatus used for testing the behavior of barnacle larvae in acute experiments. A, side view. B, top view. Scale X2.

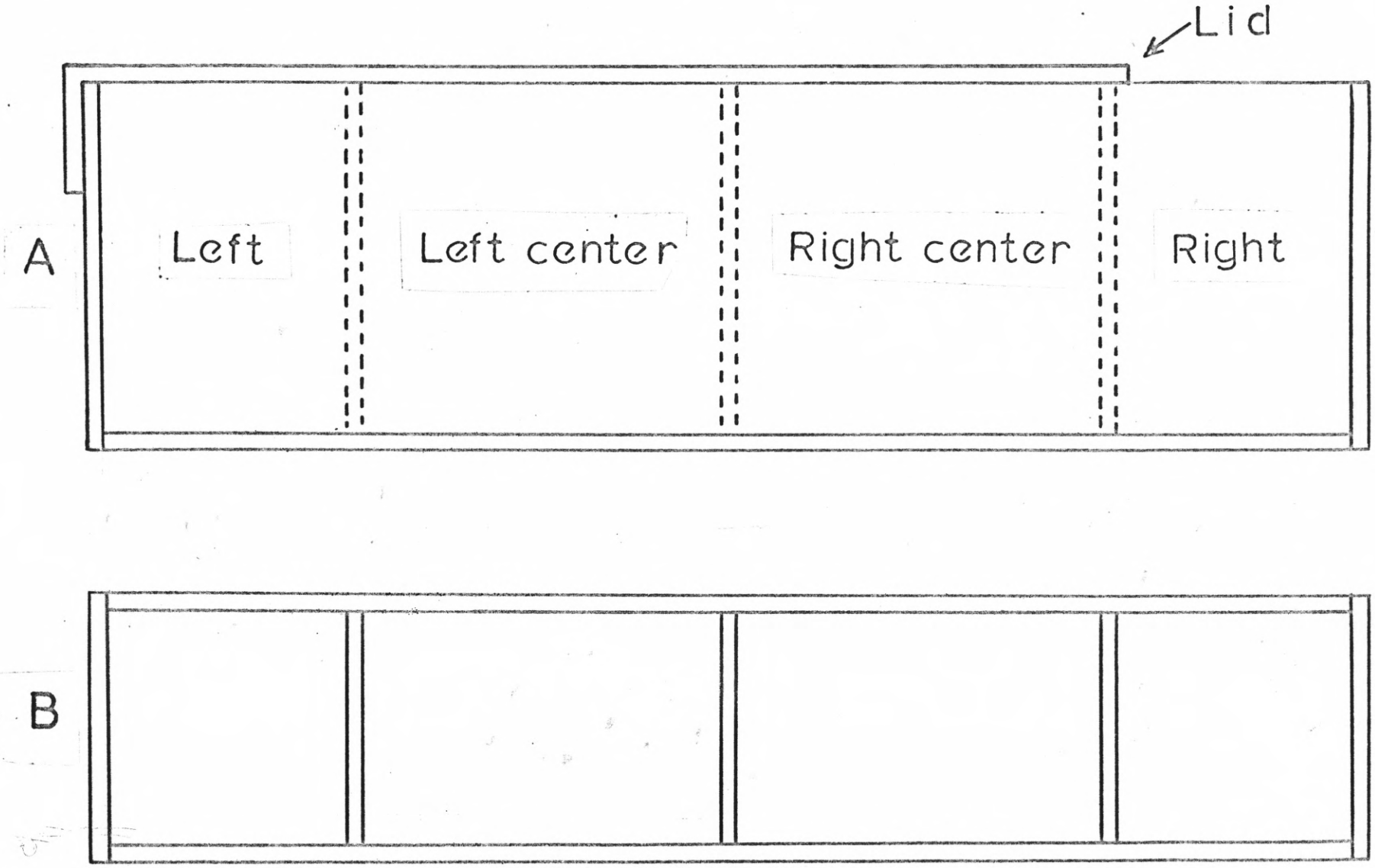


Figure 4

Figures 5 to 7. Behavior of barnacle larvae in the apparatus illustrated in Figure 4. The apparatus is designed to test the ability of larvae to migrate towards the light (positive phototaxis). Larvae are added to the left chamber and the right chamber is illuminated for 15 min.

Figure 5. Distribution of larvae in the four chambers after 15 min illumination. Control and an experiment involving durene at the concentrations (%) shown.

Figure 6. Experimental larvae, in No. 2 fuel oil for 1 h. Proportion remaining in the left chamber.

Figure 7. Experimental larvae, in dimethyl naphthalene for 1 h. Proportion remaining in the left chamber.

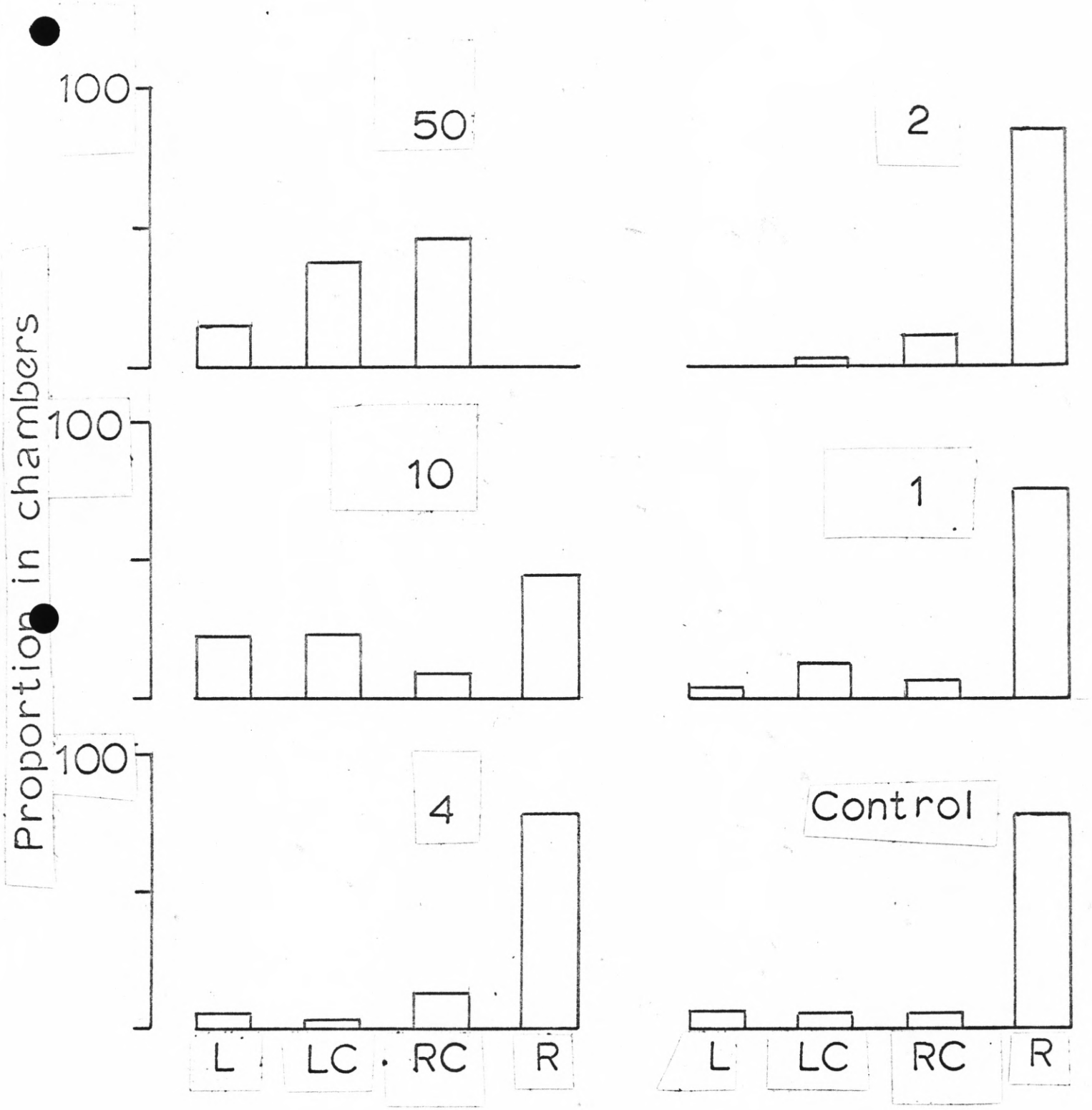

Proportion in chambers
100
50
2
100
10
1
100
4
Control
L
LC
RC
R
L
LC
RC
R

Figure 5

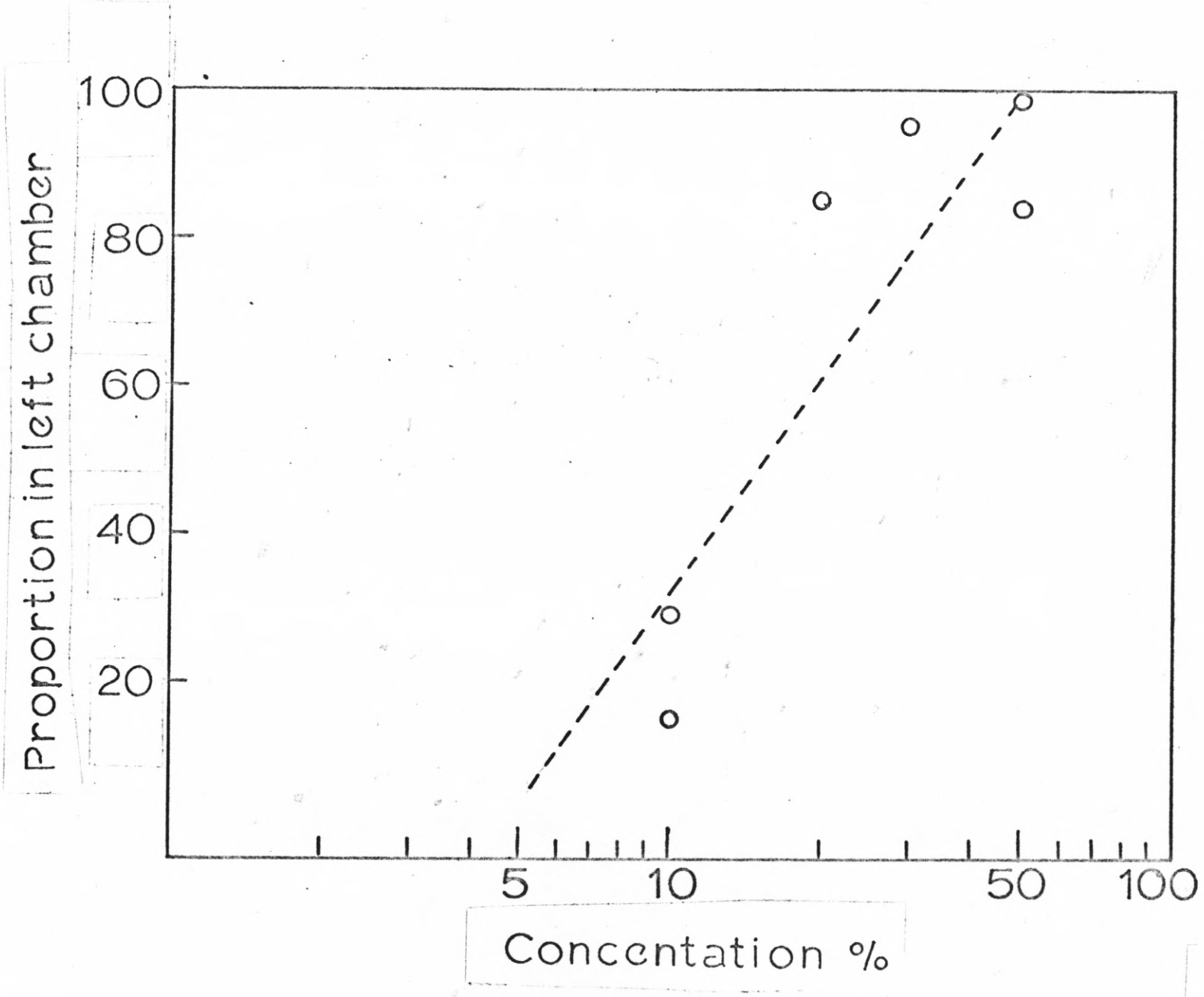

Proportion in left chamber
100
80
60
40
20
5
10
50
100
Concentation %

Figure 6

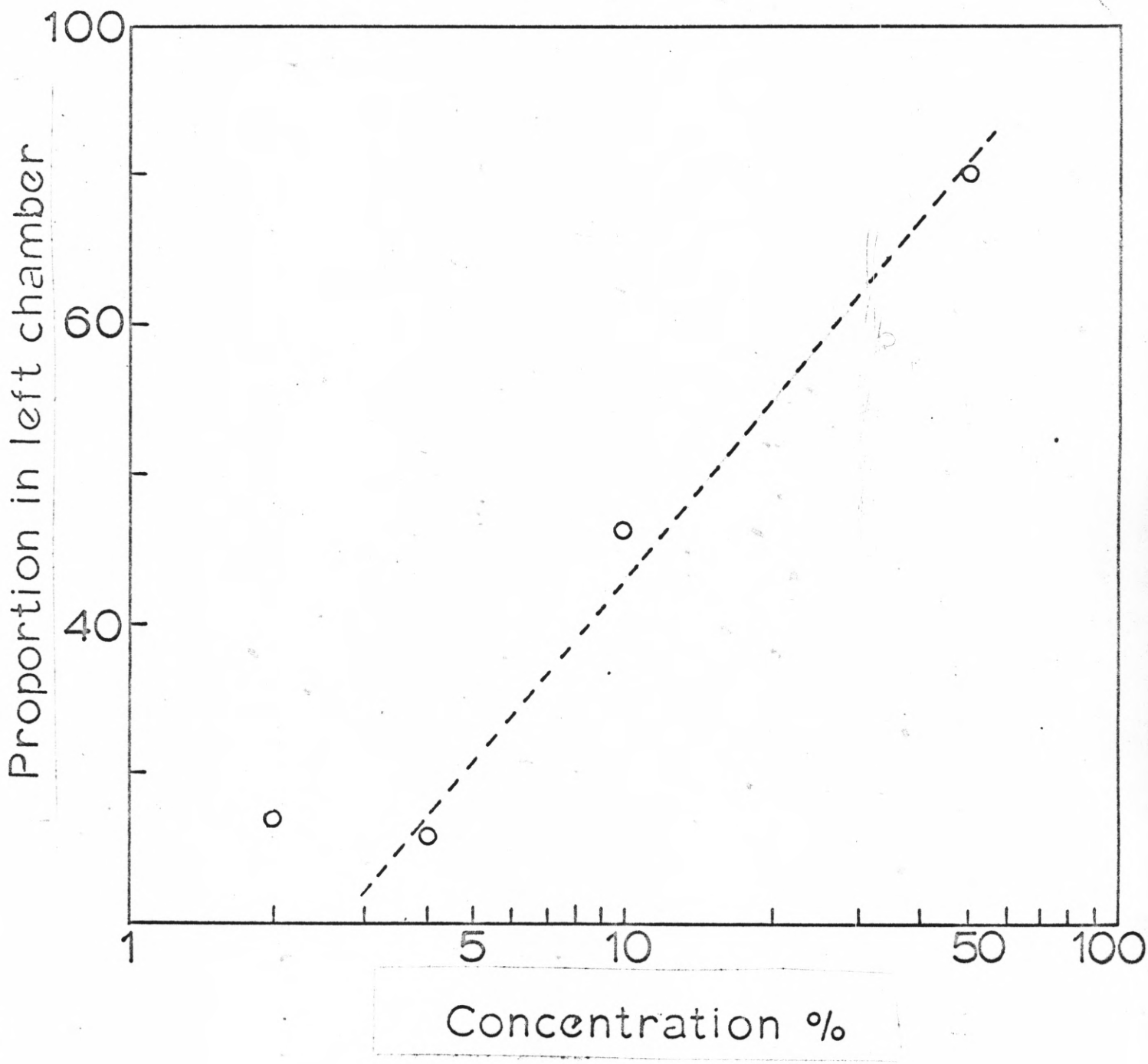

100
60
40
Proportion in left chamber
1
5
10
50
100
Concentration %

Figure 7

Figures 8 and 9. Larvae (zoeae) of the striped hermit crab Clibanarius vittatus. Survival in No. 2 fuel at the concentrations shown on the curves.

Figure 8, first experimental series.

Figure 9, second experimental series.

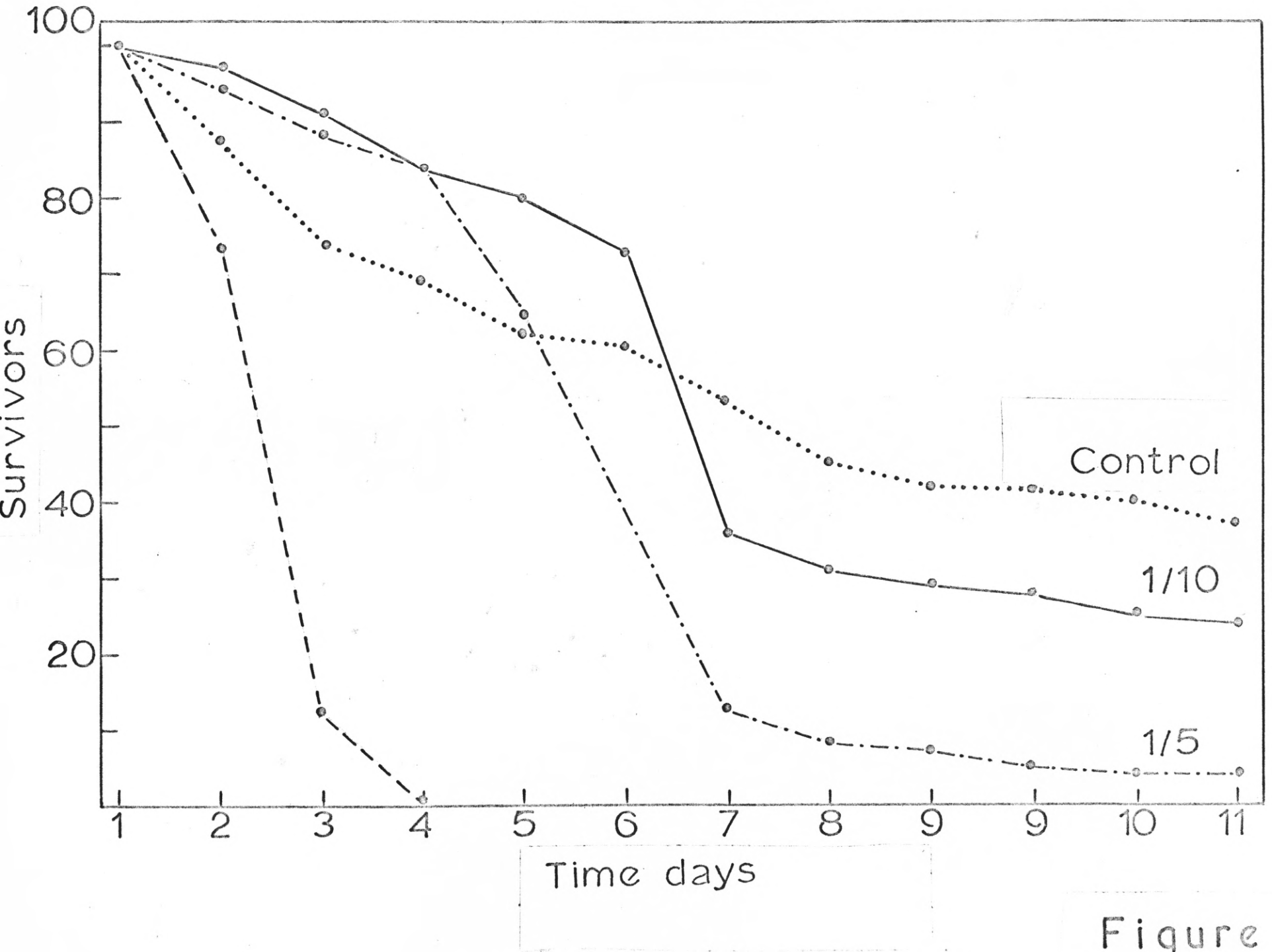

100
80
60
40
20
Survivors
Control
1/10
1/5
1
2
3
4
5
6
7
8
9
9
10
11
Time days

Figure 8

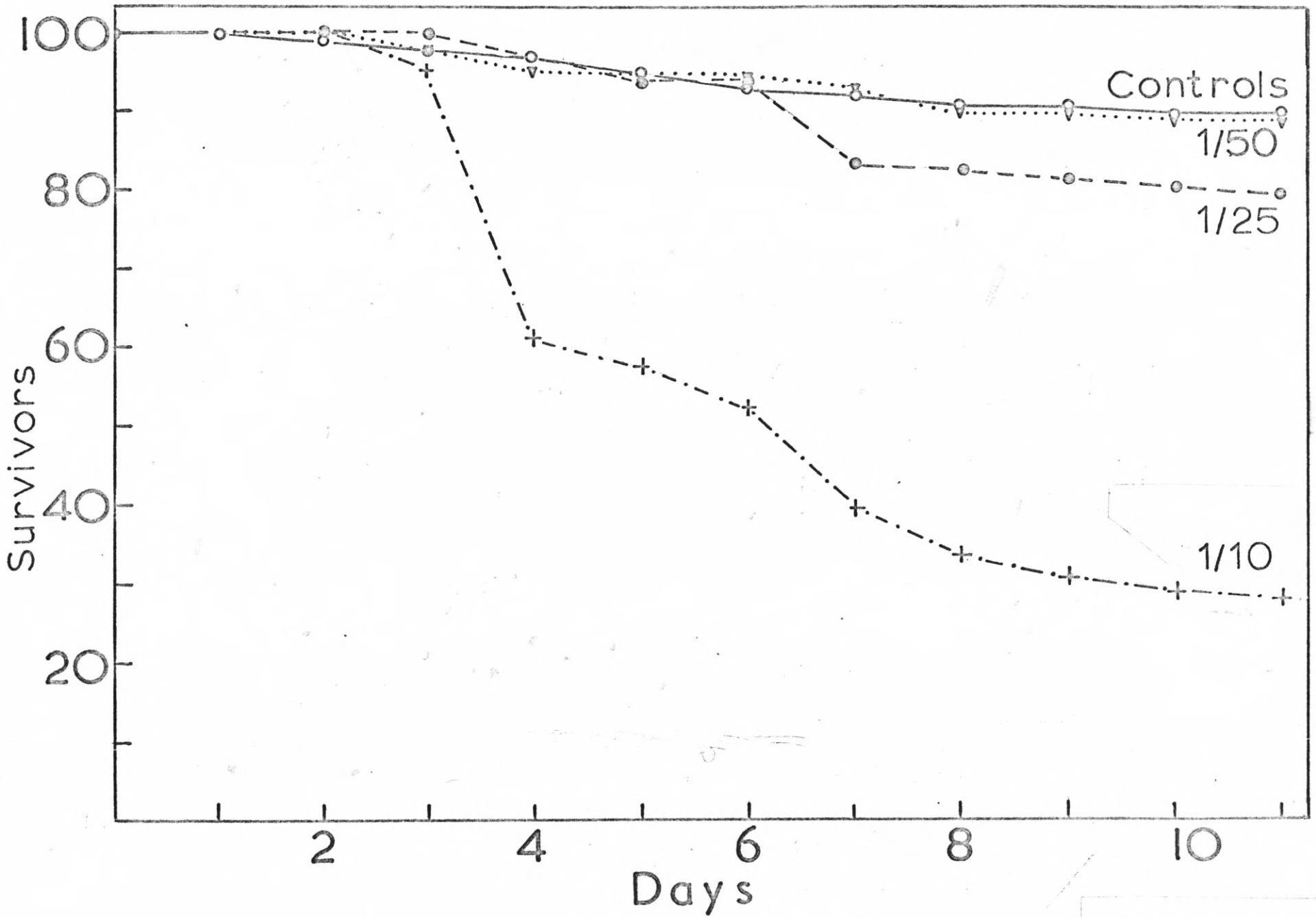


Figure 9

Figure 10.. Histograms showing the survival of larvae of the spider crab Libinia dubia in No. 2 fuel oil at the concentrations shown. Duration of experiment, 11 days. Z1, Z2, first and second zoeae; M, megalops; C1, first crab.

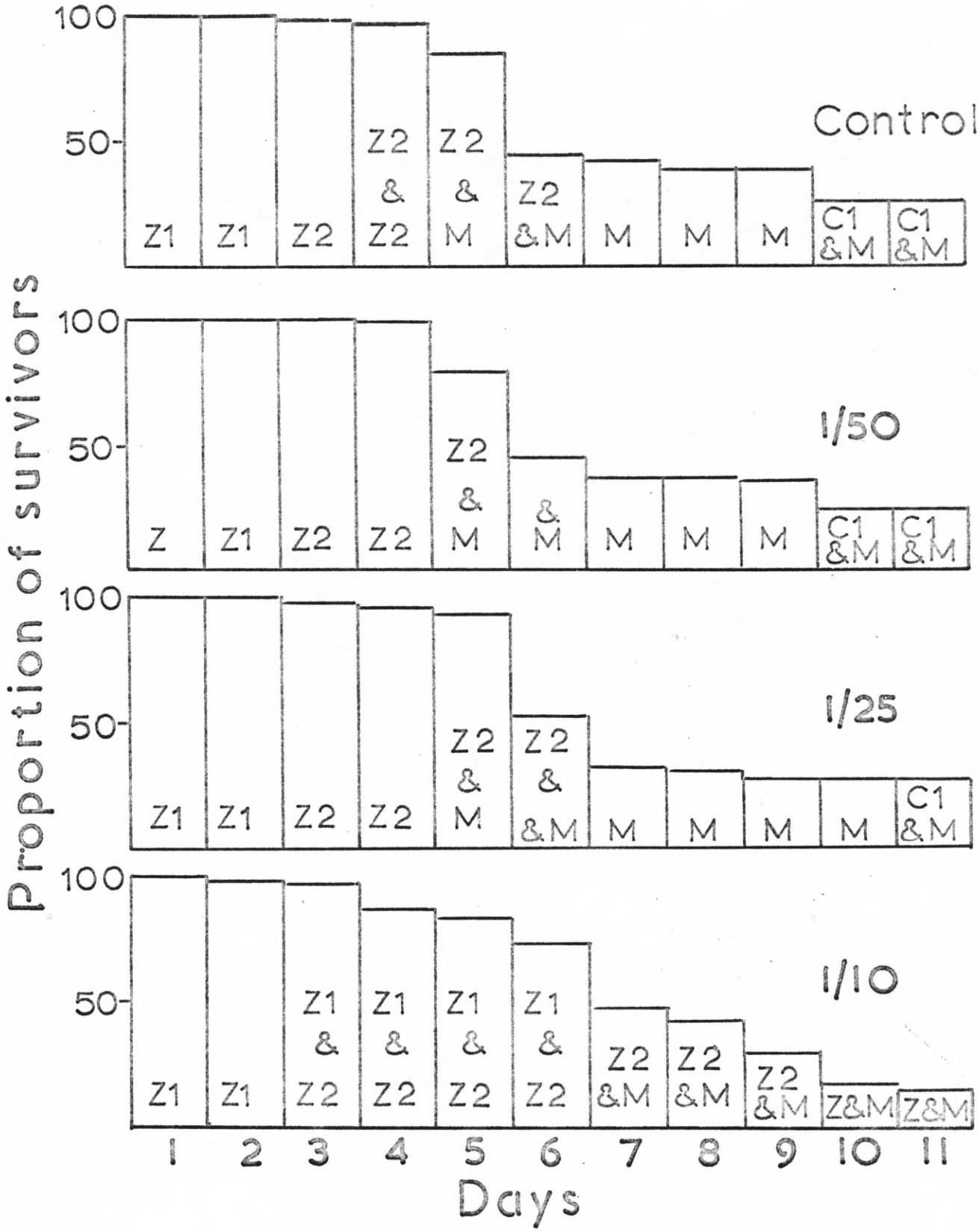

Proportion of survivors
100
50
Control
Z1 Z1 Z2 Z2 & Z2 Z2 & M Z2 & M M M M C1 & M C1 & M
1/50
Z Z1 Z2 Z2 Z2 & M & M M M M C1 & M C1 & M
1/25
Z1 Z1 Z2 Z2 Z2 & M Z2 & & M M M M M C1 & M
1/10
Z1 Z1 Z1 & Z2 Z1 & Z2 Z1 & Z2 Z1 & Z2 Z2 & M Z2 & M Z2 & M Z&M Z&M
1 2 3 4 5 6 7 8 9 10 11
Days

Figure 10

Figure 11.. Survival of stone crab larvae (zoeae) exposed to No. 2 fuel oil. Controls and experimentals ($x10^{-1}$). Concentrations of oil shown on the curves.

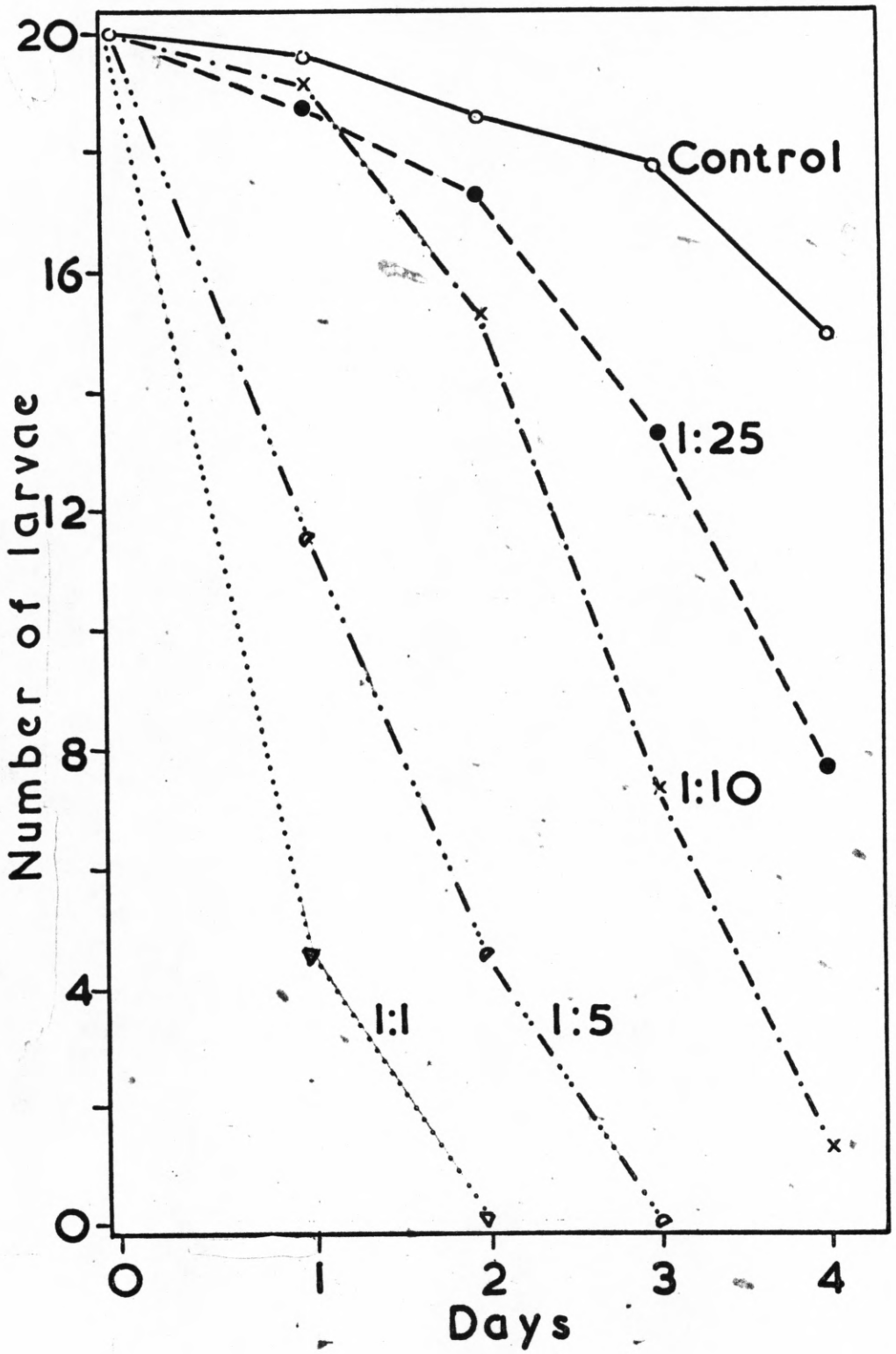


Figure 11

Figure 12.· Survival of catfish in No. 2 fuel oil.

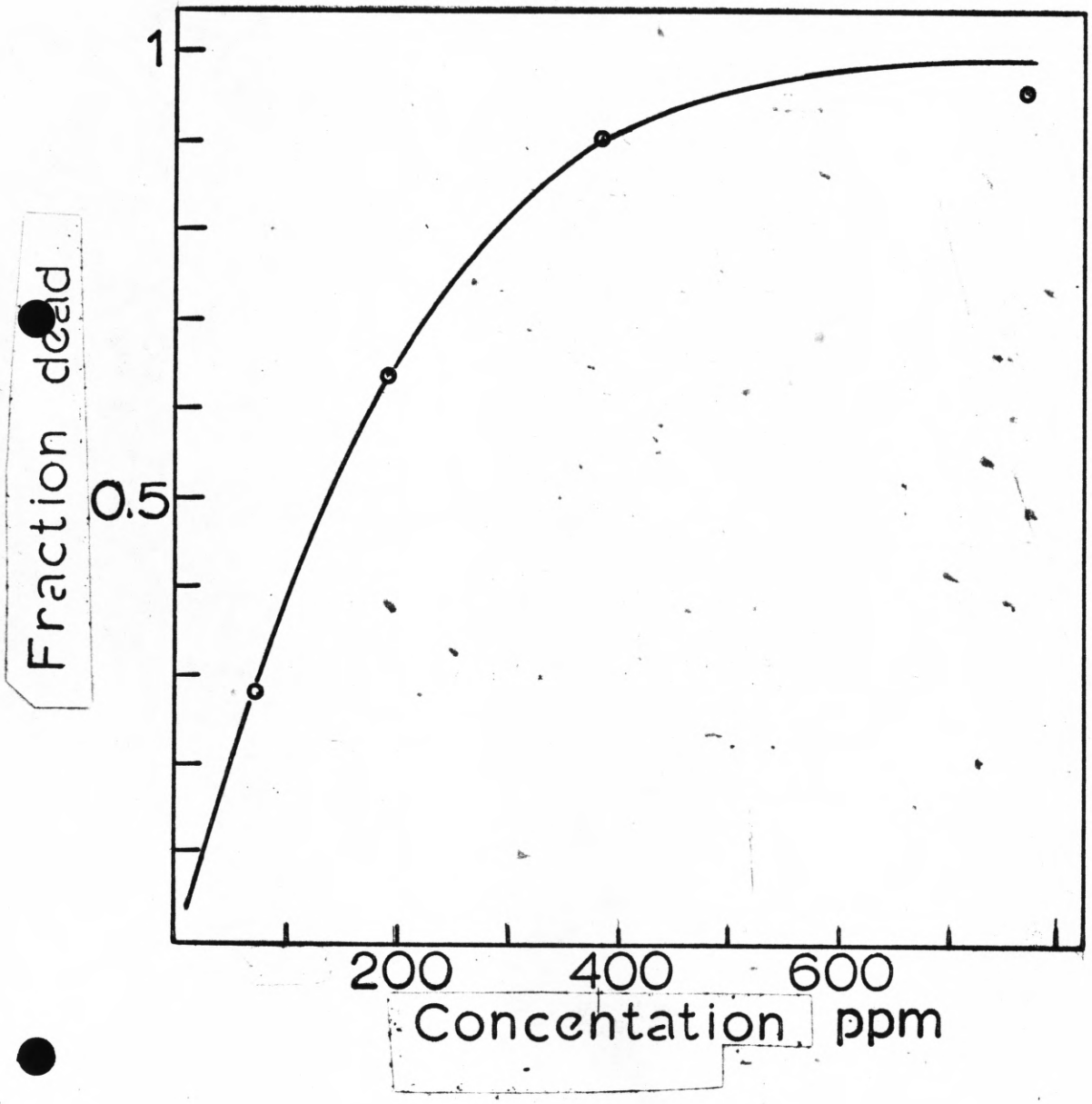


Figure 12

Figure 13.. Respiration of corneas of stingarees Dasyatis sabina (oxygen consumption in $\mu l\ mg^{-1}$ (dry weight) h^{-1}. Controls ⊙ and experimentals △ , the latter in No. 2 fuel oil (50%).

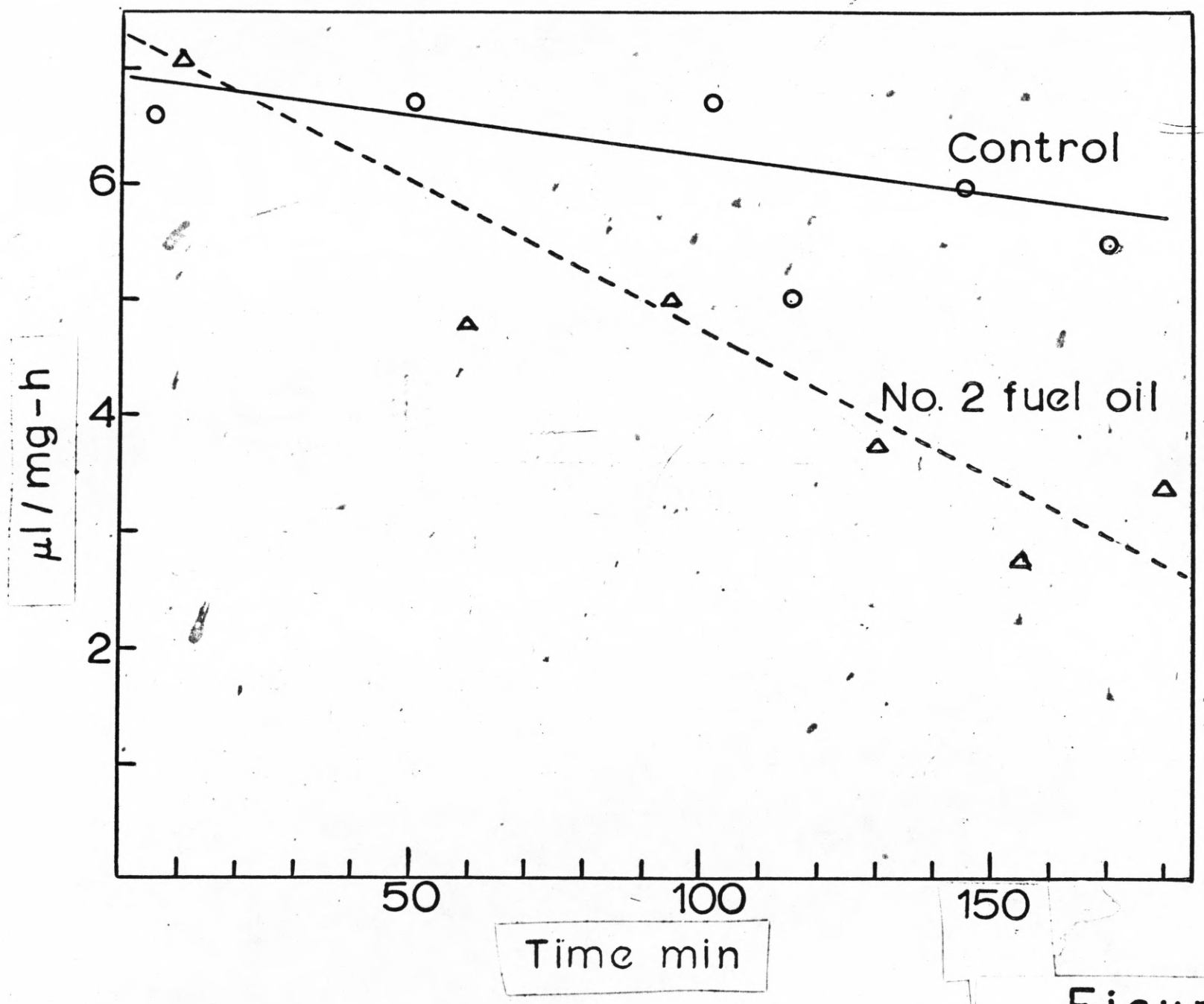


Figure 13

NSF Grant GX 37345: Marine Petroleum Pollution: Biological Effects and Chemical Characterization

Report covering period February 1, 1973 to January 31, 1974

Algal Section

Personnel

C. Van Baalen
Warren Pulich
James Armstrong
Joe Morgan
Rita O'Donnell

SUMMARY

Sea water when equilibrated with a sample of #2 fuel oil becomes toxic in varying degrees to growth of representative types of microalgae, two blue-greens, a diatom, two greens, and a dinoflagellate. For a sensitive organism such as Chlorella autotrophica, strain 580, 2 ml of sea water equilibrated with oil (15 µg of organics ml^{-1}) in 20 ml of growth medium is lethal, or roughly in the range of 15-150 ppb if the toxic material(s) constitute 1-10% of the sample. This fuel oil-equilibrated sea water also immediately stops photosynthesis in organism 580. For the other microalgae tested similar effects on growth and photosynthesis were found but required higher concentrations of the oil-equilibrated sea water.

Water solubles from Kuwait or Southern Louisiana crudes were not toxic; however, growth experiments in open or closed growth systems showed that organism 580 would not grow above 5 µl of Southern Louisiana/25 ml of medium, or 10 µl of Kuwait/25 ml of medium (oil in direct contact with algae).

With both the sea water equilibrated with fuel oil and the crudes, the toxic activity is mainly localized in higher boiling fractions derived from distillation cuts from these materials.

To date, it is clear, and not surprising, that water solubles and whole oils were toxic to the microalgae. We plan to extend this survey work to more petroleum samples. In the case of the #2 fuel oil sample we have begun attempts at chemical separation which together with bioassays may give us information on the amount and types of toxic material(s) present.

Introduction

We herein provide a summary of the results obtained so far on this grant. This section deals with inhibitory effects of petroleums and derived products on growth and photosynthesis of representative types of pure cultures of microalgae, two blue-greens, a diatom, two greens, and a dinoflagellate.

It was and still is our rationale to search for effects on growth rate as the experimental endpoint. In addition the measurements of effects on photosynthesis were begun to localize the effects seen in the growth experiments. Obviously, the more refinement we make in the chemical characterization of the so far found toxic fractions and the more refined our observations on the effects of the materials on discrete metabolic processes, the more we will come to understand the potential that petroleum and petroleum products may or may not have for damaging the environment.

Materials and Methods

The organisms, their source, and the media used to grow them are given in Table 1.

Three types of growth systems were used to examine the crude oils, #2 fuel oil and fractions derived from them, for inhibitory effects on growth of the microalgae.

The first system which is termed an "open system" is the commonly used test tube culture method patterned after the original design of Myers (1). In this system the algae are grown in 22.5 x 175mm Pyrex test tubes with 1% CO_2 in air continuously bubbled (5-7 cc sec^{-1}) through the tubes. Light intensity and temperature are

controlled as desired. Growth was measured turbidimetrically, using a Lumetron Model 402-E. The measured optical density (OD) is proportional to cell number over the range used. A plot of $\log_{10}$ OD versus time allows evaluation of the specific growth rate constant, k, in $\log_{10}$ units day^{-1} (see Fig. 1). For simplicity the growth rate data reported herein are in terms of doubling time. A lag in initiation of growth, so-called lag time, was measured by comparing the time that growth in the tube under question reached a certain point on the growth curve with that of a control. A lag of one generation time, 3 to 8 hours depending upon the organism, is indicative of a severe but temporary depression in growth rate.

The second type of growth system, a "closed system", consisted of Bellco Glass 500 ml Nephelo-culture flasks (254-S0005) with 22.5 x 175mm side arms and screw cap closures. In this system growth rate or lag effects were mainly judged by visual observation in comparison to controls. However, in the case of borderline effects the side arm allowed turbidimetric measure of growth rate as in the open system. The sterilized flasks were filled with 25 ml of appropriate medium, vigorously gassed with 2% CO_2 in air for 30 minutes, then inoculum and test material added and the flasks sealed with aluminum foil and the screw caps. The flasks were clamped onto a linear rotating bar with a 5 cm throw and shaken at 40 rpm. The bottom 3 cm of the flasks rested in a constant temperature circulating water bath. Illumination was provided by a bank of six F48T12 CW/HO fluorescent lamps beneath the water bath. Intensity was controlled by the number of lamps turned on and by the distance from the lamps to the flasks. In this type of growth condition the

limited amount of CO_2 originally present limits algal yield; however, reasonable densities of 0.2 - 0.25mg dry wt ml^{-1} were reached by the controls and the growth rates were the same as observed in the open system. This flask system was also run as an open system with 1% CO_2 in air continuously bubbled through the flasks while they were shaking.

The third type of growth system consisted of a light-temperature gradient plate (2). With this device, small petri dishes (50mm), filled with appropriate medium (10ml) and inoculated with an organism, were arranged on the plate such that each dish was at a different light intensity and temperature. The top edge of the bottom of the petri dish was ground with carborundum. This gave a fair seal between the top and bottom dishes and minimized evaporation and volatilization at the high temperature end of the plate. The petri dishes were allowed to incubate for a time period commensurate with the growth rate of the organism, usually 5-7 days. They were then read turbidimetrically or visually, or by a combination of both methods.

The petri dish-light temperature-gradient plate system was also used to judge the inhibitory effects of petroleums or derived products on the ability of "seed" populations to grow out in mildly enriched, freshly collected sea water. In this case the freshly collected sea water was supplemented with K_2HPO_4, 5mg/l; $NaNO_3$, 10mg/l; and $FeCl_3$, 50µg/l; and 10ml were distributed to sterile petri dishes. Our experience with this technique shows that a number of different organisms will grow out, depending upon the original seed population, and usually in different regions of the plate. Corresponding plates containing crude oils were incubated next to the controls in an effort to judge effects in this

continuous enrichment culture situation. Since we need to follow this type of experiment over a period of at least a year we have not chosen to enter the data in this report.

The pure compounds were tested by the conventional algal lawn technique. A given concentration of algal cells, usually (5-10,000 cells/ ml) of the organism, was added to agarized medium held at 42°; 20 ml was then immediately distributed to plastic petri dishes. The test materials are presented to the algal cells embedded in the agar by absorbing them in antibiotic sensitivity discs (12.7 mm) and placing the discs directly on the agar surface. The plates were then sealed with Scotch tape and incubated in the light for 5-8 days. The experimental endpoint is the zone size of inhibition around the pad, judged visually and microscopically.

Photosynthesis was measured as O_2 output using a Gilson Medical Electronics Clark-type electrode (OX7000 and water jacketed cell OX705) (3). The temperature was controlled by a circulating water bath, with illumination provided by a Standard projector with a 500w DAY projection bulb. Intensity was controlled by a Variac, screens, and neutral filters. A Baird-Atomic hot mirror limited the transmission of energies in the system to 350-800nm. Intensity measurements were made with a calibrated Kipp and Zonen CA-1 thermopile with readout on a Keithley 150A Microvolt-Ammeter.

When directly added, the crude oils were measured and dispensed with disposable microliter pipettes. The oil/sea water equilibration mixtures were made by gently stirring 8 parts of sea water, collected and filtered through Gelman Type A filters immediately after collection and stored in a Pyrex carboy, with 1 part of fuel oil or

crude oil. The whole mixture was stirred for 24 hours and then gently separated. Before use the sea water equilibrated with oil was filtered through a 0.45nm Millipore filter. The approximate concentration of organics introduced into the sea water by this method and partial characterization via gas chromatography were done by Dr. Ken Winters. Where pertinent, his data are pointed out in our section of the results. The fractions of #2 fuel oil were separated by distillation, again by Dr. Winters, and the equilibrations with sea water done in the same manner as above.

Results

Table 1, summary growth data obtained using open test tube growth systems, shows the effects of various concentrations of a water soluble fraction derived from a sample (API, kindly obtained from Dr. Anderson, Texas A&M) of #2 fuel oil. Of these representative types of microalgae, all are inhibited either fully or partially (lags) although the concentration response of individual organisms varies. The same results as in Table 1 for PR-6 were also found in open or closed flask culture growth systems at 30°.

Figures 1, 2, and 3 show that sea water solubles from fractions of #2 fuel oil, obtained by distillation by Dr. Winters (see chemical section), have variable effects on three microalgae, PR-6, 3H and 580. In both organisms PR-6 and 3H it is the later, high boiling, fractions that contain the toxic materials. For the green alga, strain 580, fractions B and C were most toxic, although all other fractions except I caused severe lags in initiation of growth. Clearly this sample of #2 fuel oil is of considerable interest; it is toxic and the material(s) is non-volatile. The question may be raised if this sample is typical

or atypical. We have determined that a sample of #2 Diesel fuel from the Institute storage tanks has no effect on the growth or PR-6 or 580 (a sensitive organism) when the water solubles from it are examined in the same way and at the same concentration as #2 fuel oil. (Obviously, more samples of fuel oils and related cuts should be examined.)

A modest foray into the bacterial world, using 3 marine isolates (two gram- rods and one gram- coccus tested against the water soluble fraction of #2 fuel oil), has shown no particular toxicity. At most only small lags in initiation of growth were evident.

Kuwait and Southern Louisiana crudes

Water solubles from both oils, when tested on PR-6 and 3H (the latter a sensitive organism) in the same open growth systems and at the same concentration as #2 fuel oil, show no effects. However, when tested in either open or closed flask growth systems, Kuwait and Southern Louisiana crude oil added directly to the culture medium were toxic to varying degrees. For PR-6, 150 µl of oil added to 25 ml medium allowed growth after a lag time of 1.5 days (9 doubling times). With 75 µl, a 10-12 hour lag was found. In contrast, growth of organism 3H is completely inhibited by less than 10 µl of Kuwait or less than 5 µl of Southern Louisiana, each in 25 ml of medium. This occurred in both open and closed flasks and also with attention to possible pH effects. Bacterial growth, judged via microscopic examination of the cultures at the end of the growth run, was nil; hence this appears to be an effect of direct contact between the algae and the oil. It is interesting that some emulsification of the oil was seen with organism PR-6, as with other microbial systems.

Fractions of Kuwait crude oil have been obtained by distillation (see chemical section); these have been tested for toxicity in the closed flask growth system. Table 3 shows the effect of small amounts of nine fractions on growth of 3H and 580. Toxicity was found in the higher boiling fractions (E and above).

Light-Temperature Gradient Plate

There is a distinct possibility of synergism between the nutritional or physical conditions imposed on the algae and petroleum toxicity. It should be noted, that, in the open and closed quantitative types of growth systems, the algae are growing at some fixed point of light intensity and temperature. In general, this point is chosen so that growth rate is close to k_{max}. For this reason it seems necessary to examine in one experiment the growth responses to an oil over a range of growth conditions. Such a situation is provided by the light-temperature gradient plate, although it is recognized that in this growth system the exponential growth rate is soon limited primarily by diffusion of CO_2.

Tables 4 and 5 show the results with two crude oils and two organisms when grown on the gradient plate. For organism PR-6 exposed to Kuwait crude at low or intermediate temperature, high or low light, the higher the concentration of Kuwait crude, the less growth. At the highest temperature tested (near optimum) under high light, PR-6 grew well even at 80 µl of oil, but at the same temperature and at the lower light intensity, 80 µl of Kuwait crude was lethal. Southern Louisiana crude was more toxic than Kuwait, volume for volume, and

at high temperature-high light, PR-6 did not fare as well as with Kuwait crude.

Organism Dun again suffers less inhibition of growth from Kuwait crude in a high light-medium temperature (judged near optimum) condition, similar to the results with PR-6. Southern Louisiana crude is again much more toxic than Kuwait, but the organism shows some ability to grow at the high temperature end of the plate and in both high and low light.

These experiments show the gradations of results which may occur with one organism and suggest that results from any single point growth experiment (one light intensity-one temperature) should be viewed with caution, except in the clear cut case of lethality.

Testing of Pure Compounds

Ten pure compounds were tested for their effect on growth of the organism PR-6, using the algal lawn procedure. Table 6 demonstrates that with several of these compounds (e.g. substituted naphthalenes, phenanthrene, biphenyl) which are present in most crude oils, a zone of inhibition was produced by as little as 1 mg of the compound on the Petri plate.

Discussion and Criticisms

We consider the data so far obtained only a start in answering the question of the potential damage that petroleum pollution may or may not do to the environment. There are obviously a number of questions that can be raised. Does the manner of presentation of a crude oil make a difference in growth rate, e.g. stationary or shake culture, emulsified (physically or through bacterial action)? Are any of the microalgae

slowly able to adapt or develop detoxification mechanisms, which enable them to grow in otherwise lethal concentrations of crude oils or fractions therefrom? What is the concentration range (tolerance) of different organisms, and will this lead to an enrichment situation in nature for selected species or groups? What is the influence of the presence of a bacterial and fungal population on the chemical composition of the oil, will they alter any toxic materials and thereby allow algal growth? On this latter point, aromatics are apparently only degraded slowly and with difficulty (4) and if they form the basis for any of the toxicity we are finding, then they may with chronic spills accumulate to significant levels in the environment.

The immediate sensitivity of photosynthesis to the sea water soluble fraction from #2 fuel oil was not anticipated (Fig. 4). It is a rapid effect and indicates a major interruption of total through-put photosynthesis. Sea water extracts of fractions A to I (Table 2, Chemical section) showed that A, D, and I had little toxicity, B, E, F, G, and H slightly more, while fraction C was the worst. However, this is a short time measurement and must be viewed against the growth work in deciding if this damage is repairable or not. Gordon and Prouse (5) on the basis of <u>in situ</u> measurements of rate of $^{14}CO_2$ uptake with natural populations have also found inhibition of photosynthesis. Interestingly, a sample of #2 fuel oil they tested was more toxic than a Venezuelan crude or a #6 fuel oil. In addition, photosynthesis is only one of a number of cell processes that could lead to cell death. We have no information on other potential sites of damage such as transcription, protein synthesis, essential biosynthesis, cell division mechanisms, general membrane effects, electron transport (ATP), etc.

From the work so far done we feel that continuation of the survey growth work with more crudes and derived products is mandatory. Certainly this work forms the basis for any more sophisticated efforts in cell physiology or biochemistry. It may also provide a firm and predictive background of information to logically test the responses of a community to petroleum.

Of equal importance is the direct isolation and characterization of those compounds from an oil that are demonstrably toxic via a bioassay. To this end we have begun with the help of Dr. Winters to slowly delineate wherein lies the toxicity of water solubles such as derived from #2 fuel oil. We prefer this approach over that provided by the exclusive testing of pure compounds known to occur in petroleums. We are not convinced that the pure compound approach will give a satisfactory or complete answer. It is in essence a guessing game and some or many of toxic compounds in oils may be missed if it is the only avenue pursued. The conundrum of "concentration in nature" is a toxic compound present and in sufficient concentration to inhibit can only be answered by identification, quantification of the amount present (input rate and stability), and a detailed knowledge of its biological effects on a range of microalgae.

References

1. Myers, J. 1950. The culture of algae for physiological research. In The Culturing of Algae. C. F. Kettering Foundation, Yellow Springs, Ohio. pp. 45-51.

2. Van Baalen, C. and P. Edwards. 1973. Light-temperature gradient plate. In Handbook of Phycological Methods. Cambridge University Press, Cambridge. pp. 267-273.

3. Van Baalen, C. 1968. Effects of ultraviolet light on a coccoid blue-green alga: survival, photosynthesis, and photoreactivation. Plant Physiology, 43, 1689-1695.

4. Perry, J. J. and C. E. Cerniglia. 1973. Studies on the degradation of petroleum by filamentous fungi. Center for Wetlands Resources, LSU-SG-73-01, 89-94.

5. Gordon, D. C. and N. J. Prouse. 1973. The effects of three oils on marine phytoplankton photosynthesis. Marine Biology, 22, 329-333.

6. Van Baalen, C. 1962. Studies on marine blue-green algae. Botanica Marina, 4, 129-139.

7. Van Baalen, C. 1967. Further observations on growth of single cells of coccoid blue-green algae. Journal of Phycology, 3, 154-57.

8. Personal communication.

Table 1. Doubling times (hours) of various microalgae grown in Sea H_2O equilibrated with #2 Fuel Oil. Numbers in parentheses are lag times in hours.

Strain[1] Designation	Basal medium[1]	Sea H_2O control	Concentration of Sea H_2O equilibrated with #2 Fuel Oil (8:1 v/v)[2]					
			0.05%	0.05%	5%	10%	25%	50%
PR-6	3.9	3.9	3.9	3.9	3.9(4)	3.9(7)	N.D.(72)	∞
MAC	6	9	9	9	9	9	9(10)	N.D.(96)
3H	7.8	7.8	7.8	7.8	7.8(24)	7.8(60)	∞	∞
Dun	6.3	6.3	6.3	6.3	6.3	6.3	6.3	N.D.(96)
Ind. 580	7.2	8.3	8.3	8.3	9.5(168)	∞	∞	∞
OBB	50	50	50	50	50(120)	50(170)	∞	∞

[1] PR-6 = Agmenellum quadruplicatum (blue-green), medium ASP-2 + B_{12} (6), temp. 39°, this laboratory.

MAC = Nostoc sp. (blue-green), medium Cg10 (7), temp. 39°, source (D.S. Hoare, U.T. Austin).

3H = Thalassiosira pseudonana (diatom), medium ASP-2 + B_{12} + B_1 + $Na_2SiO_3 \cdot 5H_2O$, temp. 30°, source (R. Guillard, Woods Hole).

Dun = Dunaliella tertiolecta (green), medium ASP-2 + B_{12} + B_1, temp. 32°, source (R. Guillard, Woods Hole).

Ind. 580 = Chlorella autotrophica (green), medium ASP-2 + B_{12} + B_1, temp. 32°, source (R. Guillard, Woods Hole).

OBB = Gymnodinium halli (dinoflagellate), modified medium NH15 (8), temp. 30°, source (B. Wilson, Texas A&M, Galveston).

[2] Forty ml of #2 Fuel Oil layered on 320 ml filtered (gelman type A) off-shore sea water, stirred gently for 24 hours at room temperature, sea water layer removed and refiltered through 0.45 nm Millipore filter. This Sea H_2O equilibrated has 15 mg total extractables/liter.

Table 2. Highest concentration (μ liters oil in 25 ml medium) of Kuwait and Southern Louisiana Crude Oil which allows growth of various microalgae, when in direct contact with oil. Closed growth system. Temp. 30°.

Strain	Kuwait	Southern Louisiana
PR-6	150	Not tested
Ba-1	100	100
3H	10	5
Dun	200	40
Ind. 580	10	5

Organism Ba-1 = Microcoleus chthonoplastes, medium ASP-2 + B_{12}

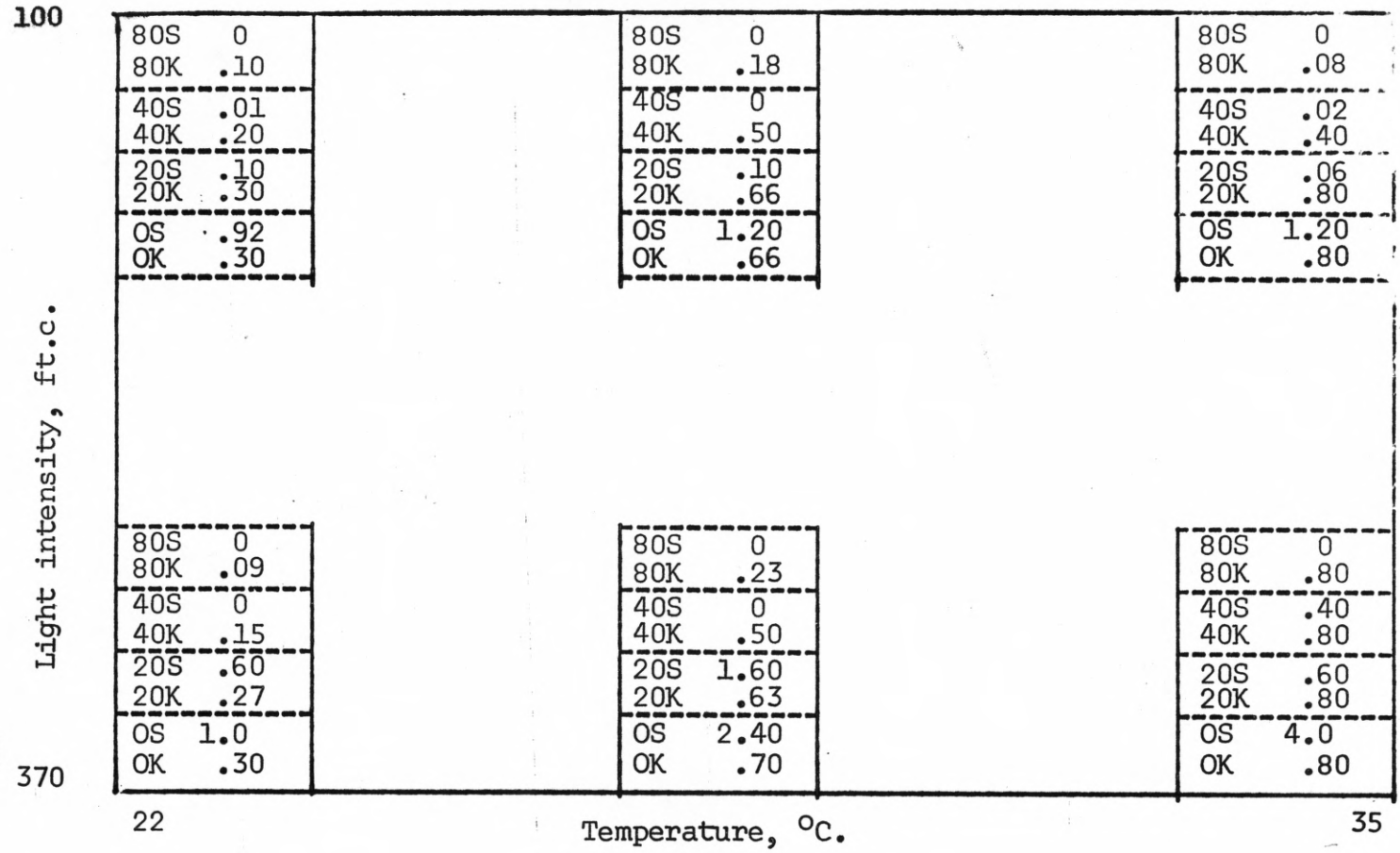
Table 3. Effect of fractions of Kuwait Crude Oil (made by distillation) on growth of diatom strain 3H and green alga, Chlorella autotrophica strain 580. Closed growth system, temp. 30°C. (+) = no growth; (-) = growth with only slight lag or no lag.

Fraction	3H[1]	580[2]
A	-	-
B	-	-
C	-	-
D	-	-
E	+	-
F	+	+
G	+	+
H	+	+
I	+	+

[1] 5 µl of a fraction in 25 ml. medium

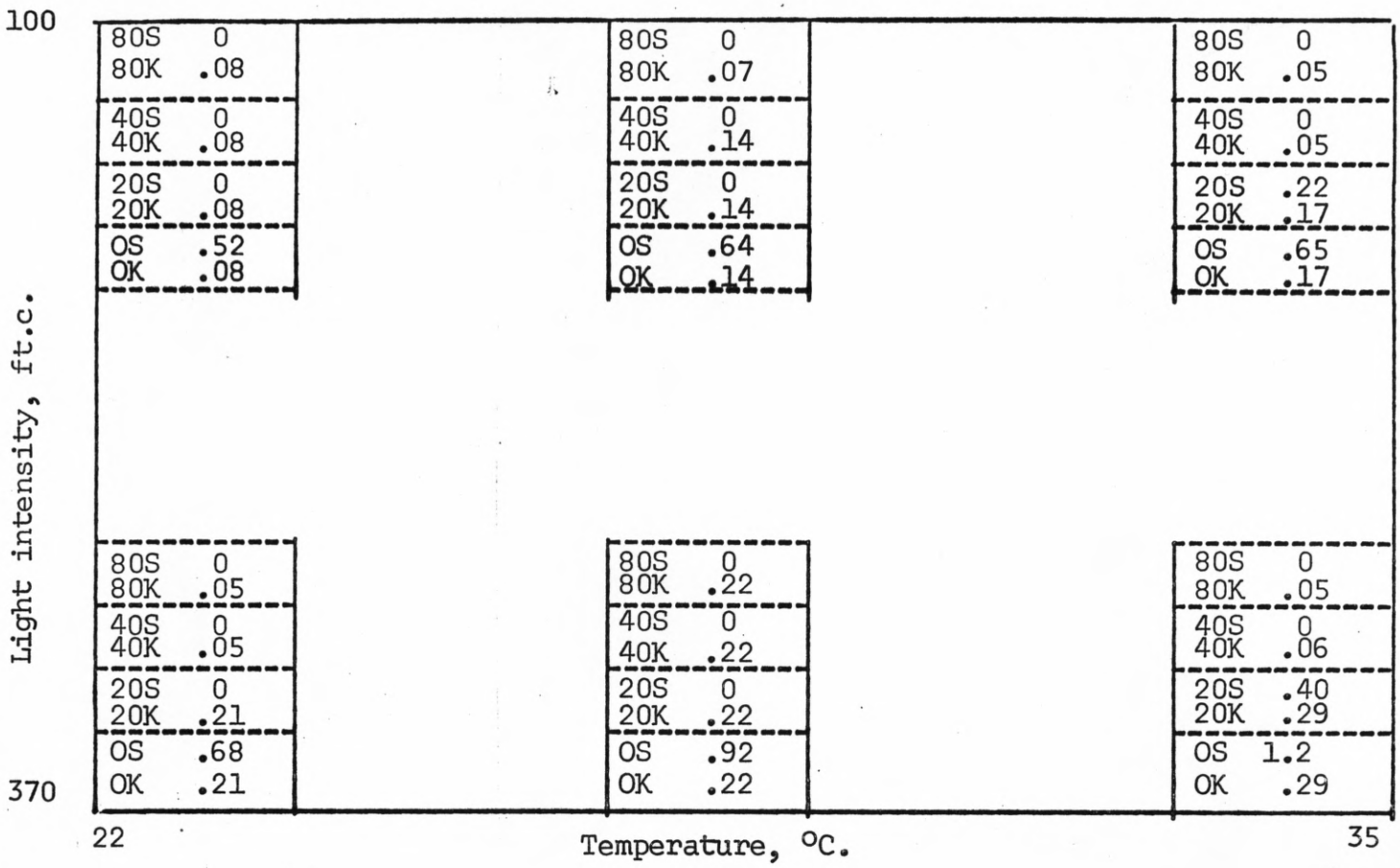
[2] 20 µl of a fraction in 25 ml. medium

Table 4 Light-temperature gradient plate growth of organism PR-6 to crude oils.



First number = µl of oil/10 ml; following letter identifies crude oil sample K = Kuwait, S = Southern Louisiana; right hand number = OD measured or visually estimated in comparison to a control. Medium was ASP-2 + B_{12}.

Table 5 Light-temperature gradient plate growth of organism DUN to crude oils.



First number = µl of oil/10 ml; following letter identifies crude oil sample K = Kuwait, S = Southern Louisiana; right hand number = OD measured or visually estimated in comparison to a control. Medium was ASP-2 + B_{12} + B_1.

Table 6 Effect of pure compounds on growth of organism PR-6 using algal lawn technique. Numbers represent zone of inhibition (in mms) out from edge of filter pad. Complete killing results in a zone of inhibition of 36 mm on the plate. Dilutions were made of a stock solution containing 100 mg/0.1 ml of the specified compound.

Dilutions	TEB[1]	TMB[2]	cresol[3]	MN[4]	DMN[5]	NAP[6]	PHEN[7]	Durene[8]	Fluorene	Mesitylene[9]	Biphenyl
1:10	3	20-22	36	36	36	36	5(15^{s})	1	0	15^{s}	36
1:50						1	4(9^{s})	0	0	0	6-7
1:100	0	6	1(10^{s})	2	3-4	0	4	0	0	0	2
1:500						0	1	0	0	0	0

1. Triethyl benzene
2. 1,2,4-trimethyl benzene
3. m-cresol
4. methyl naphthalene
5. Dimethyl naphthalene
6. Naphthalene
7. Phenanthrene
8. 1,2,4,5-tetramethyl benzene
9. 1,3,5-trimethyl benzene

Figure Legends

Fig. 1. Growth rate in units of $\log_{10}$-(ODx100) of diatom 3H on fractions (water solubles) of #2 fuel oil derived by distillation. A indicates lowest boiling, I the residue. Water solubles tested at 10% concentration (2 ml + 18 ml basal medium), Temp. 30° C, 100 ft-c, open system. For a further description of these fractions of #2 fuel oil see the data of Dr. Winters on chemical characterization (Table 2, Chemical Section). Fraction H did not grow (7 days).

Fig. 2. Same as figure 1 but done with organism PR-6, a coccoid blue-green alga. Water solubles at 50% concentration, temp. 39°C, 350 ft-c, open system. Dashed line indicates organism filamentous, that is, cell division not normal during this time. Fractions F and H did not grow (6 days).

Fig. 3. Same as figure 1 but done with organism 580, a green alga. Water solubles at 10% concentration, temp. 32°C, 300 ft-c, open system. Fractions B and C did not grow (12 days).

Fig. 4. The effect of water solubles from #2 fuel oil on the photosynthesis of three microalgae.
Conditions.
#2 fuel oil equilibrated for 24 hours with stirring with filtered sea water, sea H_2O separated and filtered through 0.45nm Millipore filter and added to the algal suspension 8 minutes before light was turned on. The concentration of this water soluble fraction is given in percent, v/v, thus 18% means 1.4ml of algal suspension plus 0.2ml of sea water-oil solubles, controls sea H_2O + medium ASP-2. The algal strains are

identified in Table 1. The algal concentrations are: for PR-6, 3H and for 580 approximately 1 x 10^7 cells/ml in medium ASP-2. Electrode current at air saturation, medium ASP-2 = 3.4μa. Light intensity, limited by Baird-Atomic Hot Mirror (34-01-2) to 350-800nm was 5800μw/cm^2 at level of electrode chamber.

FIG.

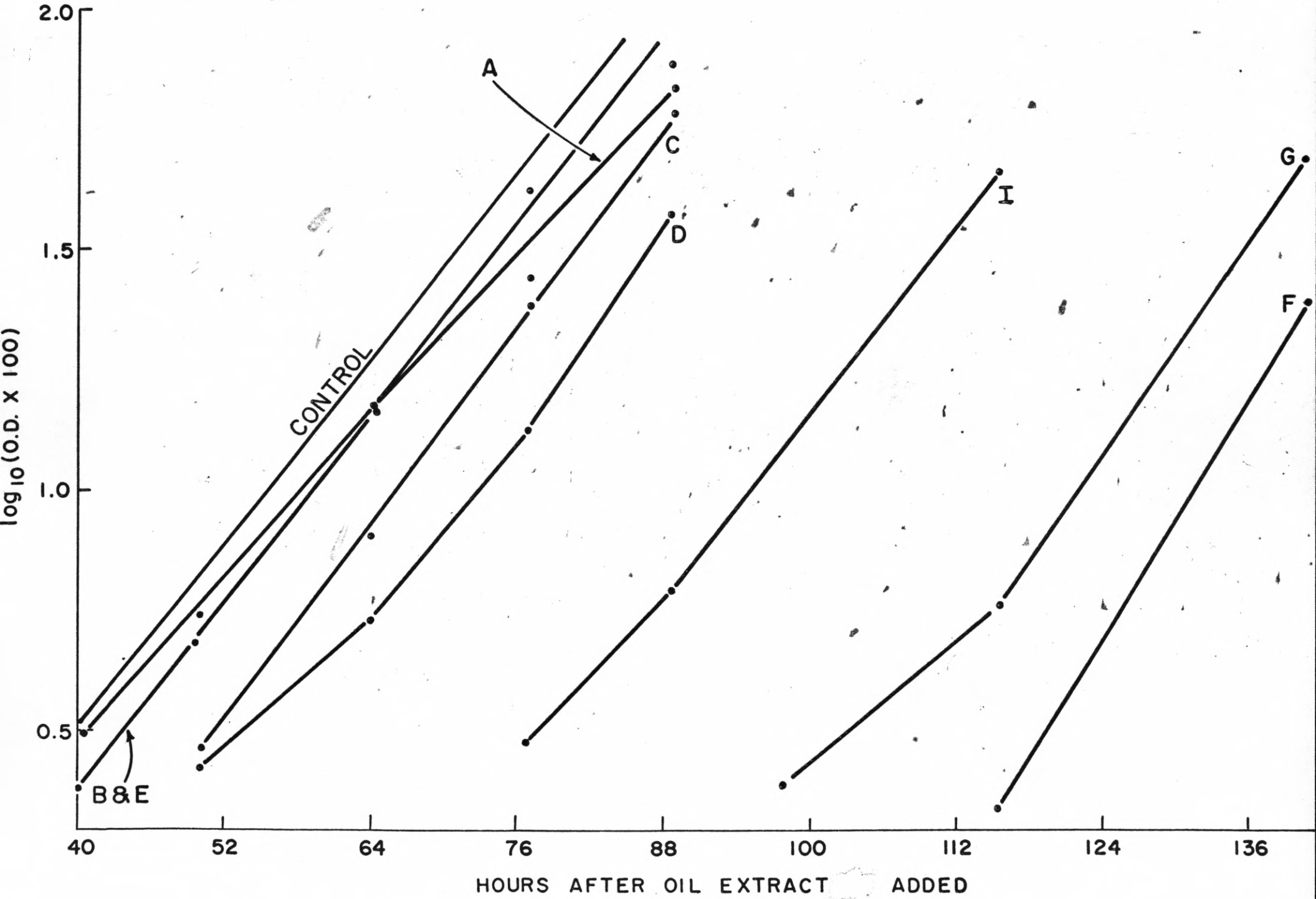

2.0
1.5
1.0
0.5
log10(O.D. x 100)
A
C
D
I
G
F
CONTROL
B&E
40
52
64
76
88
100
112
124
136
HOURS AFTER OIL EXTRACT ADDED

FIG. 2

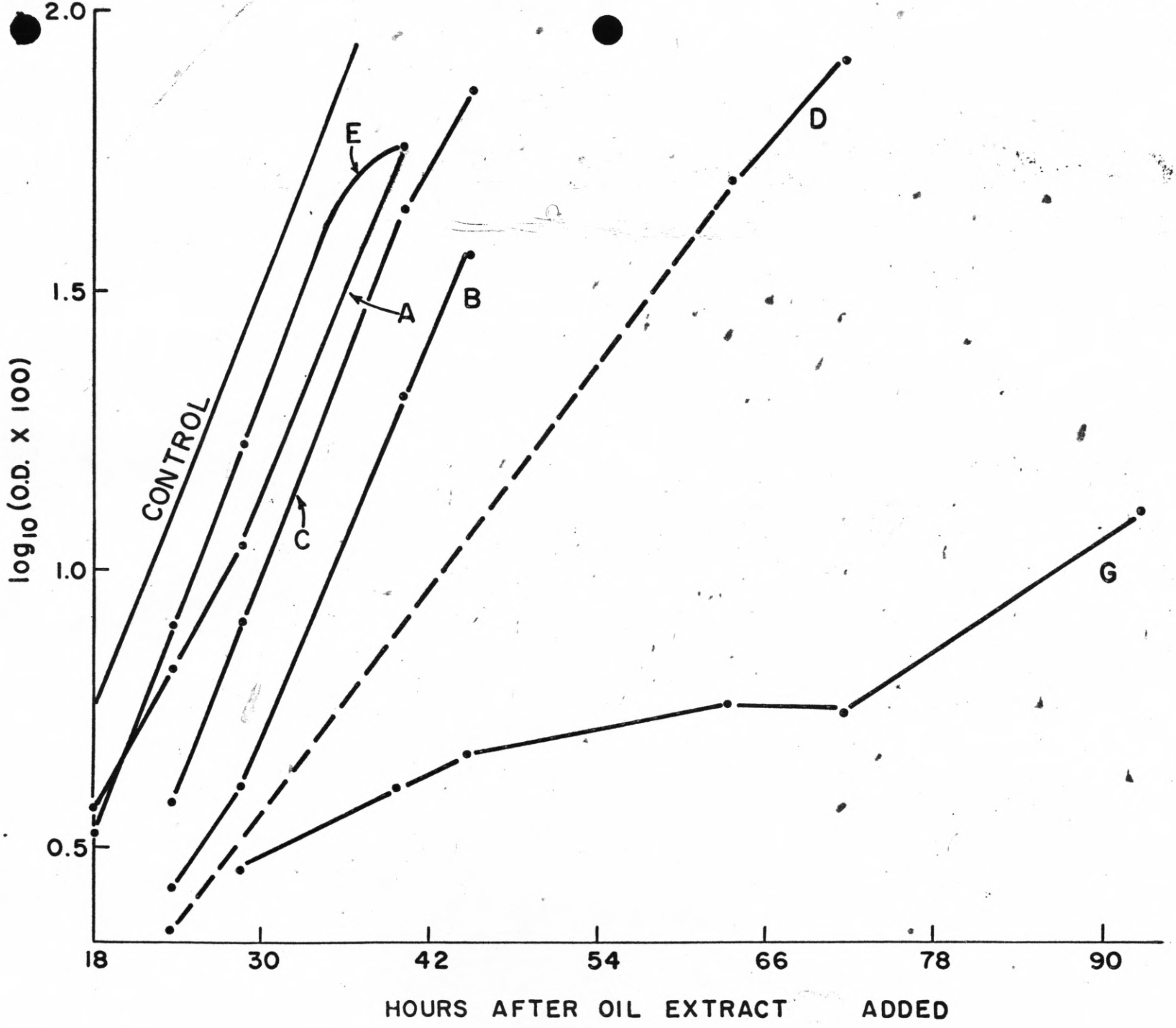

2.0
1.5
1.0
0.5
log10 (O.D. X 100)
CONTROL
E
A
B
C
D
G
18
30
42
54
66
78
90
HOURS AFTER OIL EXTRACT ADDED

FIG. 3

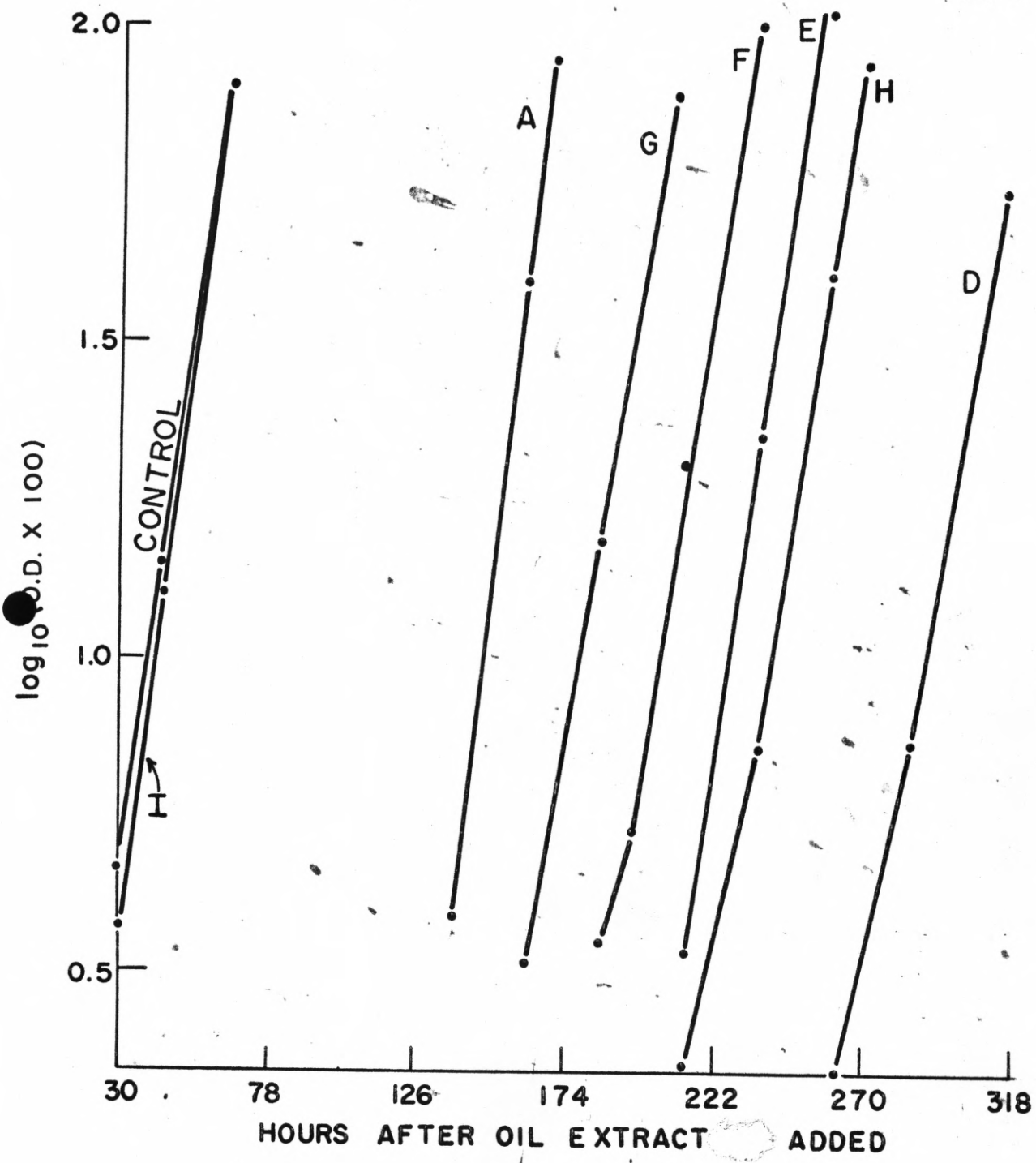
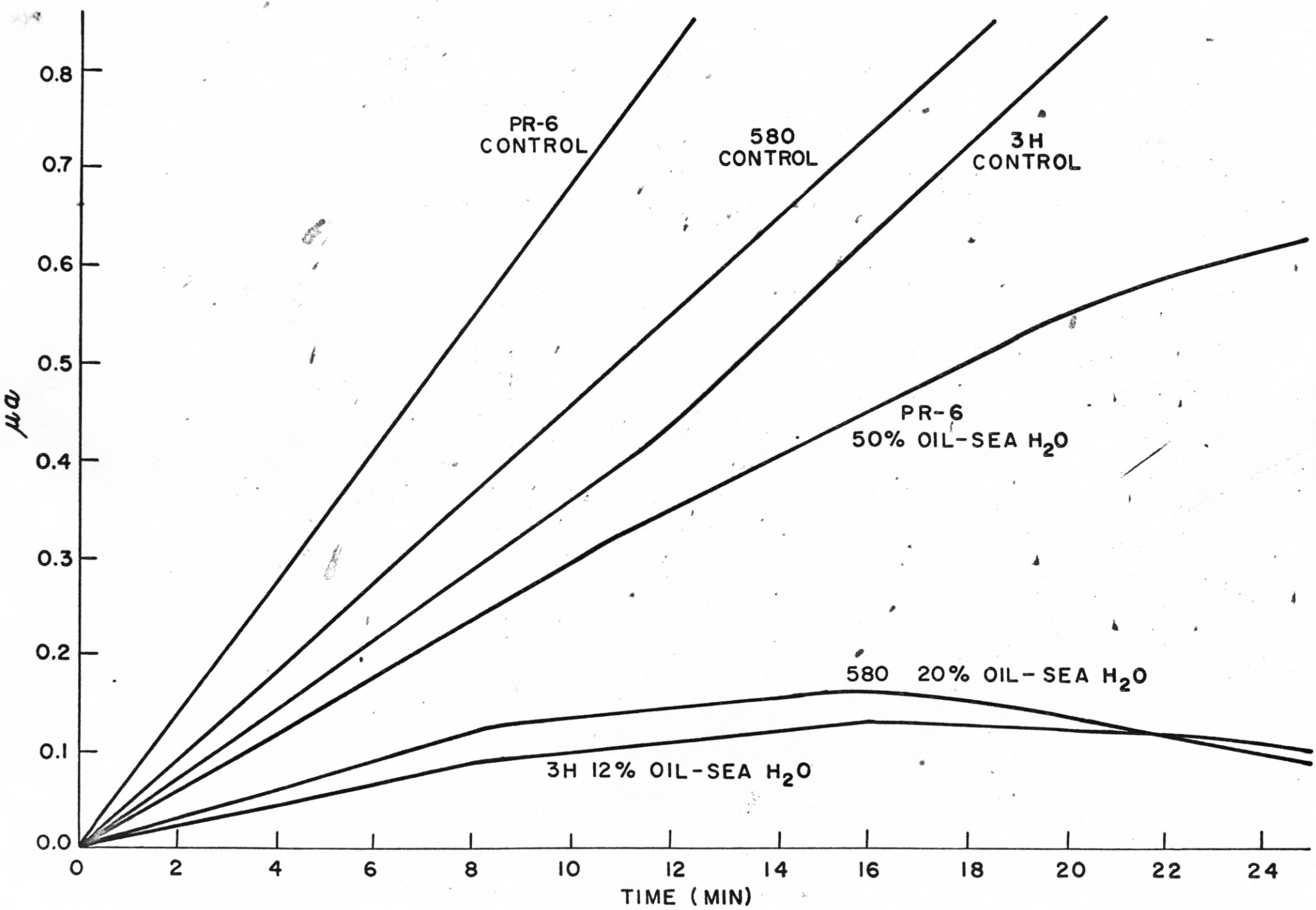

2.0
1.5
1.0
0.5
$\log_{10}$ (O.D. X 100)
CONTROL
H
A
G
F
E
H
D
30
78
126
174
222
270
318
HOURS AFTER OIL EXTRACT ADDED

FIG. 4


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
μa
PR-6
CONTROL
580
CONTROL
3H
CONTROL
PR-6
50% OIL-SEA H2O
580 20% OIL-SEA H2O
3H 12% OIL-SEA H2O
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (MIN)