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Effects of Water Soluble Extract and Bacterial Degradation Products of Diesel Oil on Marine Phytoplankton

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EFFECTS OF WATER SOLUBLE EXTRACT AND BACTERIAL DEGRADATION PRODUCTS OF DIESEL OIL ON MARINE PHYTOPLANKTON

By

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Foreward

The author wishes to express her sincere appreciation to Dr. Joseph A. DeMarte for his guidance and encouragement throughout the course of this investigation. Appreciation also goes to Dr. H. David Hammond and Dr. Robert J. McLean for their helpful suggestions.

Special thanks is expressed to Dr. Robert Guillard of the Woods Hole Oceanographic Institute for providing the algal cultures, to Dr. Parmely H. Pritchard and Mr. Roy M. Ventullo for supplying the bacterial degradation products of diesel oil.

To all who have contributed in many ways, I offer my sincere gratitude.

This thesis is dedicated to my mother and father for their unending sources of inspiration throughout my life.

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INTRODUCTION

The biological effects of spillage of hydrocarbon oils into the ocean have been reported since early in the 1920's (Rushton, 1923; Orton, 1925; Roberts, 1926). The wreck of the Torrey Canyon in 1967, greatly stimulated research on oil pollution in the aquatic environment.

Many organism are adversely affected by spillage of oil. The wreck of the Tampico Maru off the coast of Baja California, Mexico in 1957 "created a situation where a completely natural area was almost totally destroyed suddenly on a large scale... Among the dead species were lobsters, abalone, sea urchins, starfish, mussels, clams and hosts of smaller forms." (North, 1967). According to Blumer et al. (1971) crude oil and petroleum products contain many substances that are poisonous to a variety of marine organisms.

Nearly all types of crude and fuel oils are susceptible to microbial oxidation. Seeding of oil polluted areas with suitable microorganisms to clean up oil slicks might be a prospective solution to the oil pollution problem; it is now a subject of considerable research. However, the biological effects of the resulting microbial degradation products are still largely unknown. It has been reported only that some of the intermediate products of oil degradation are toxic to fish (Brown and Tischer, 1969). If the use of microorganisms is to be a feasible solution to the oil pollution problem, the effects of the oil degradation products on plant and animal life

should also be investigated, otherwise we might just be exchanging one toxicant for another.

STATEMENT OF PROBLEM

The primary objective of this research was to provide information on the relative toxicity of diesel oil (No. 2 fuel oil) and the microbial degradation products of this fuel on marine phytoplankton. These organisms comprise an essential element of the marine food web. An assessment of the effects of oil pollution on the growth and photosynthesis of marine phytoplankton was determined in the laboratory.

LITERATURE REVIEW

There has been a good deal of research on the effects of oil and oil products on organisms such as birds (Dennis, 1959; Hartung, 1963; Hartung, 1967), fish (Turnbull *et al.*, 1954; Nelson-Smith, 1970; Kühnhold, 1970) and benthic invertebrates (Galtsoff *et al.*, 1935; Hawkes, 1961; Tegelberg, 1964; Blumer *et al.*, 1970; Wells, 1972). There have also been some reports about the effects of oil pollution on macrophytic algae (George, 1961; North *et al.*, 1964; Ranwell, 1968; Nelson-Smith, 1968a; Craigie and McLachlan, 1970; Foster *et al.*, 1971), but only a few papers have dealt directly with the effects of oil on phytoplankton (Galtsoff *et al.*, 1935; Lacaze, 1969; Nuzzi, 1973; Gordon and Prouse, 1973).

Effects of Oil on Macrophytic Algae:

Unlike all but the simplest animals, seaweeds affected by a large oil spill are likely to show less long-term damage. Many species of marine algae are resistant to oil pollution because of their slimy texture. The observation made by George (1961) following an oil spill in Milford Haven, United Kingdom showed that the intertidal algae *Pelvetia canaliculata* and *Fucus spiralis* were unaffected and had continued to grow at a normal rate. Over a two month period following the large spill of Bunker C oil in Chedabucto Bay, Nova Scotia, no significant changes in the concentration of the littoral algae *Fucus vesiculosus*, *F. serratus*, *F. spiralis* and *Ascophyllum nodosum* were

observed (Craigie and McLachlan, 1970). On Santa Cruz Island in the Santa Barbara Channel, spilt crude oil covering the surface canopy of the kelp Macrocystis pyrifera was easily washed off by water movement (Straughan, 1971, quoted by Butler and Berles, 1972), whereas the smaller alga Hesperophycus harveyanus, which does not have the slimy texture on the outer surface, was heavily oiled and disappeared from the rocks (Anonymous, 1969). Some species in two genera of green algae, Enteromorpha and Ulva, appear to be particularly immune to oil pollution (Foster et al., 1971); the former is also reported to be successful in areas of the Black Sea suffering from chronic oil pollution. In the Torrey Canyon disaster algal flora on rocky shores suffered extensive mortality from oil-smothering (Ranwell, 1968), and the decolorization or development of abnormal tints in Ulva, Porphyra and other red algae was noticed (O'Sullivan and Richardson, 1967; Nelson-Smith, 1968a). After the stranding of the Tampico Maru in 1957 during which 8000 tons of diesel oil was accidentally released into a small cove, nearly all the shore algae were seriously affected (North et al., 1964).

Effects of Oil on Phytoplankton:

Since the distribution of phytoplankton in space and time is patchy, widespread mortality by oil pollution, if any, would be difficult to ascertain. Field observations, unlike laboratory studies, indicated little damage to phytoplankton by oil spills. During the Torrey Canyon incident only slight

phytoplankton mortalities were reported (Smith, 1968). After the Santa Barbara spill phytoplankton studies also failed to reveal any effect of oil pollution (Kolpack, 1970). McCauley (1966) found that many plankters (Lyngby, Gonium, Oscillatoria, Ankistrodesmus, Chlamydomonas, Closterium, Scenedesmus, Asterionella, Cyclotella, Fragilaria, Meridion, Navicula, Tabellaria, Euglena and Trachelomonas) tolerated the pollution even during the period of highest oil concentration in Muddy River, Massachusetts, but he believed that the more sensitive species had been eliminated.

Laboratory research on the effects of oil on phytoplankton dates back to studies conducted by Galtsoff et al. (1935). These investigators were examining the effects of crude oil pollution on oyster culturing. The marine diatom, Nitzschia closterium, was studied quite extensively since it makes up a major portion of the oyster diet. These investigators noted that the presence of oil over cultures of N. closterium inhibited growth of the diatom after a week's time. Water soluble substances in crude oil also exhibited a growth-inhibition effect. It was further noted that water soluble substances at low concentration (25%), accompanied by tremendous bacterial growth, showed a stimulating effect on the growth of Nitzschia.

More recently, experiments by Russian workers confirmed the previous findings that oil in water produced a toxic effect on phytoplankton. The diatom Ditylum brightwelli, Coscinodiscus granii and Chaetoceros curvisetus were found to be

very sensitive to kerosine and fuel oil, which were toxic after 24 hr at 100 ppm or less. Melosira moniliforms and Grammatophora marina tolerated concentrations of oil up to 1% although lower levels suppressed the growth of the cultures (Mironov and Lanskaja, 1967, quoted by Nelson-Smith, 1970).

Lacaze (1969) determined the effects of a water extract of Kuwait crude oil and an anti-petroleum emulsifying agent, Gamosol, on Phaeodactylum tricornutum. Both the extract and the Gamosol were found to inhibit the growth of the diatom under laboratory conditions.

Working in the laboratory, Nuzzi (1973) studied the effects of water soluble extracts of oil on marine phytoplankton. Water soluble substances extracted from No. 2 fuel oil inhibited completely the growth of Chlorella sp., P. tricornutum and Skeletonema costatum. Growth of a Chlamydomonas species was not affected by the extract. He also concluded that extracts from No. 6 fuel oil and outboard motor oil had little effect on these species of marine phytoplankton.

In contrast to the above findings, Crosby et al. (1954) investigated the growth of bacteria, fungi and lower algae in a brackish oil-refinery effluent. They found that 25-50 ppm naphthenic acid, introduced in an attempt to control the slime, stimulated its growth by about 15%.

Effects of Oil on Photosynthesis:

Oils consistently reduce the rate of photosynthesis (Baker, 1971). The amount of reduction varies with type and

amount of oil and species of plant. A 0.1% emulsion of diesel oil almost completely inhibited photosynthesis in young blades of Macrocystis pyrifera (North et al., 1964). The effect appeared in three days, although irreversible damage was caused by exposing these plants to the oil for 6-12 hr. A 0.01% emulsion inhibited photosynthesis after a delay of 7 days. Fuel oil was even more toxic. In another study, Clendenning and North (1960) showed that 10-100 ppm of fuel oil caused a 50% inactivation of photosynthesis of M. pyrifera in four days. Similar results were obtained with 5-10 ppm cresol and 10 ppm phenol over the same time period. Gordon and Prouse (1973) studied the effect of oil on the photosynthesis of natural marine phytoplankton communities. They found that No. 2 fuel oil was more toxic to phytoplankton photosynthesis than Venezuelan crude and No. 6 fuel oil. In spite of the inhibitory effect of oil on photosynthesis being reported, green algae coated with Torrey Canyon oil and not treated with an emulsifier remained green and bubbles trapped in the partial oil film indicated that they were photosynthesizing (Nelson-Smith, 1968b).

The cause of inhibition of photosynthesis by oil is not clear. It is suggested from experiments with beet cells (Van Overbeek and Blandeau, 1954) and Amoeba (Marshland, 1933; Goldacre, 1968) that the lower hydrocarbons like hexane, heptane, octane, benzene, toluene, naphthalene and phenol have a narcotic effect probably due to their solubility in the lipid phase of the plasma membrane of the cell. The membrane structure is thus disrupted so that it becomes abnormally permeable;

the cell may then either swell and burst or-in a terrestrial plant-lose its liquid contents, which leads to wilting and death. Similar distruption of the chloroplast may account for disturbance in photosynthesis (Nelson-Smith, 1968a). Schramm (1972) studied the effects of a coating of crude oil on the CO_2 uptake by emersed marine algae. He found the CO_2 uptake was more or less depressed, depending on the thickness of the oil film (0.1 to 0.0001 mm) and suggested that oil might act as a physical barrier to gas exchange. Oil may also act physically absorbing light wavelengths essential for photosynthesis. Roberts (1926) grew the freshwater plants Groenlandia densa, Ranunculus aquatilis and Callitriche playcarpa under a thin film (0.004 cm) of different types of oil. All plants grew luxuriantly, so it seems that a thin film of oil does not absorb enough light to inhibit photosynthesis.

Effects of Microbial Degradation Products of Oil on Wildlife:

An important and almost totally unexplored facet of the oil pollution problem is the effects of oil-oxidation products on wildlife. Several organic acids, lipids, esters, alcohols, ketones, aldehydes and other substances may result from the degradation of oil by microorganisms (Zobell, 1946; Treccani, 1962; McKenna and Kallio, 1965; Davis, 1967). To date, it has been reported by only Brown and Tischer (1969) that some of the intermediate products of microbial oil degradation were toxic to two species of freshwater fish, Mollienesis latipinna and Gambusia affinis.

MATERIALS AND METHODS

Algal Species:

The species assayed in this investigation included *Dunaliella tertiolecta* (WHOI clone "Dun"), a naked green flagellate; *Chlorella autotrophica* (Indiana University #580), a non-motile unicellular green alga; *Phaeodactylum tricornutum* (WHOI clone "Phae"), a pennate diatom. Pure cultures of these organisms were obtained from Dr. Robert Gulliard of the Woods Hole Oceanographic Institute.

Culture Medium:

The algae were grown axenically in half-strength medium "f" (Guillard, 1973). Medium "f/2" was prepared as follows:

$NaNO_3$	75 mg
$NaH_2PO_4 \cdot H_2O$	5 mg
$Na_2SiO_3 \cdot 9H_2O$	30 mg

Trace metals:

$Na_2 \cdot EDTA$	4.36 mg
$FeCl_3 \cdot 6H_2O$	3.15 mg
$CuSO_4 \cdot 5H_2O$	0.01 mg
$ZnSO_4 \cdot 7H_2O$	0.022 mg
$CoCl_2 \cdot 6H_2O$	0.01 mg
$MnCl_2 \cdot 4H_2O$	0.81 mg

Vitamins:

Thiamin·HCl	0.006 mg
Biotin	0.5 ug

B_{12}	0.5 ug
*Seawater	to one liter

*Instant Ocean (salinity 30 ppt) was used in place of natural seawater.

Growth Conditions and Maintenance of Stock Cultures:

The cultures wer kept in a growth chamber (Percival, Model MB54) and illuminated by continuous fluorescent lights (300 ft-c) at $20^{o}\pm2^{o}C$.

Stock cultures of the algae were maintained weekly by transferring 50 ml of old cultures to 50 ml of new growth medium contained in 250 ml Erlenmeyer flasks.

Throughout this investigation, all cultures were agitated daily for one hour on a platform shaker. Agitation prevented the algae from settling in the flask and permitted CO_2 to diffuse into the growth medium. The culture medium and test solutions were sterilized by filtration through a 0.45 micron HA Millipore filter.

Water Soluble Extract of Diesel Oil:

During the initial studies, pure diesel oil was used in experiments and was found to be unsatisfactory for this research. It was determined that pure diesel fuel formed oil globules in the medium and completely or partially coated the cells thereby interfering with the spectrophotometric measurements. To eliminate this problem, a water extract of the diesel fuel was used instead of the pure oil.

The water soluble extract was prepared by stirring to-

gether diesel oil and culture medium in a ratio of 1:100 for 24 hr. The method consisted of stirring with a magnetic stirrer at a rate that produced an oil vortex which was never greater than 50% of the solution depth. Care was taken not to produce an emulsion. The water soluble extract (considered as 100%) was then diluted with culture medium to yield the desired concentrations-0%, 10%, 25%, 50%, 75% and 100%.

A similar extraction method was used by Boylan and Tripp (1971) to study the compounds present in seawater extracts of crude oil.

In order to elucidate the composition of the water extract used in this investigation, subsamples were prepared for analysis by gas-liquid chromatography. For this analysis a Hewlett Packard Model 5750 Research Chromatograph was used.

Bacterial Degradation Products of Diesel Oil:

The bacterial degradation products of diesel oil were supplied by Dr. Parmely Pritchard, SUNY College at Brockport. A continuous culture technique employing aquatic bacteria from Lake Ontario was used to degrade the diesel oil (Pritchard and Starr, 1973). Effluents (considered as 100%) of degradation of this fuel were collected at the end of the following intervals: 0 to 211 hr, 211 to 424 hr, and 424 to 628 hr. These time periods are approximately one week apart. The collected effluents were enriched with Instant Ocean and a composition of minerals used in preparing the original algal culture medium. For each of the collected effluents the following concentrations of bacterial degradation products were prepared:

0%, 10%, 25%, 50%, 75% and 100%.

Growth Studies:

Growth studies, in triplicate, for all three species of algae were carried out in 125 ml Erlenmeyer flasks. An inoculum of 20 ml of 9 day stock cultures (stationary phase) was added to flasks containing 30 ml of test solution. Control cultures entered stationary phase after 7 days. Growth measurements were made at daily intervals over 1 week by determining the optical density (O.D.) at 590 nm (Coleman Junior II Spectrophotometer, Model 6/35).

A pilot study was conducted to determine the wavelength for measuring the growth of the algae. Wavelengths, at 10 nm intervals, between 480 and 630 nm were investigated. This study showed that 590 nm was in the lowest region of the absorption spectrum for all three species of algae.

Monod (1949) was the first to use logarithms to the base 2 to calculate the growth rate of bacterial cultures. Log_2O.D. values plotted against time (day), in this investigation, yielded a curve the slope of which was the growth rate (number of doublings per day) of the algae. Growth rates for each treatment were compared with the control. In this investigation, growth was expressed as a percentage of the control. Growth data were processed with a Monroe Calculator (Model 1860) to determine the line of best fit using the Least-squares equation.

Bacterial contamination of test cultures was checked at the end of each experiment with Bacto nutrient broth (Difco).

During the course of this investigation it became important to determine if the microbial degradation products of diesel fuel could support heterotrophic growth of the algae. Experimental conditions were the same for this study as for the growth studies conducted in the light except that these cultures were maintained in darkness. The culture flasks were wrapped with aluminum foil and placed in a cardboard box contained in the growth chamber. The box was partially covered with black plastic which prevented light penetration but did not prevent air exchange. This experiment was conducted over a 12 day period. Optical density was measured on the 6th and 12th day.

Photosynthesis Studies:

A carbon-14 technique similar to Steemann-Nielsen's (1952) was used in these studies. P. tricornutum was the only species assayed during these studies. Optical density measurements were taken after culturing this alga for 36 hr in test solutions. An inoculum of 1 microcurie of ^{14}C (New England Nuclear Corp. as dissolved $NaH^{14}CO_3$ in 1 ml ampoules) was added to the flasks (125 ml Erlenmeyer). These flasks were stoppered with rubber. The inoculated cultures were then incubated on a shaker for 4 hr at room condition (200 ft-c, 23°C).

The test solutions consisted of different concentrations of water soluble extract (10%, 25%, 50%, 75%, and 100%) and bacterial degradation products (25%, 50% and 100%) of diesel oil. The pH value of the test solutions ranged from 7.8 to 8.2 for these studies.

At the end of 4 hours the algae were fixed with 2 ml of 40% formalin, then duplicate 20 ml aliquots of the cultures were filtered by low suction through a 0.45 micron HA Millipore filter. The filters were washed with 10 ml of $5x10^{-3}N$ HCl, 10 ml of distilled water, dried, and the radioactivity was counted using a low background gas-flow counting system (Nuclear-Chicago, Model 1152).

Throughout these studies light and dark flasks were used. Light flasks were set up in duplicate. The net radioactivity was obtained by subtracting dark fixation from light fixation of ^{14}C. To eliminate the effect resulting from a difference in cell concentration, radioactivity measurements were adjusted for difference in optical density of each culture. Carbon fixation measurements for each treatment were compared with the control. In this investigation, ^{14}C uptake was reported as a percentage of the control.

Statistical Analysis:

Student's paired t-test was used to analyze the data obtained from the water soluble extract studies throughout the observation period. With respect to the experiments involving the bacterial degradation products t-tests were performed on the 3 through 7 day results for *D. tertiolecta* and *C. autotrophica*. Statistical tests were not performed on the O.D. values for the first 3 days because very similar growth rates between the controls and the treatments were obtained. Regarding *P. tricornutum*, several t-tests were performed starting with the 5th or 6th day O.D. values.

For the photosynthesis studies Student's t-test was employed to analyze the radioactivity measurements obtained.

RESULTS

Gas Chromatography:

Figure 1 shows the gas chromatogram of water soluble extract of diesel oil. All straight chain alkanes were identified between C_{16} and C_{26} except for C_{17} and C_{25}.

Growth Studies:

The results of algal growth studies in continuous light are expressed in Figures 2, 3, 4, and 5. Values for growth rate are presented in Tables 1, 2, 3, and 4. Student's paired t-test was employed to analyze the difference in growth between the treatments and the control. Statistical data are presented in Tables 9, 10, 11, and 12.

The water soluble extract of diesel oil showed a significant inhibitory effect on the growth of all three species of phytoplankton tested (Fig. 2 and Table 9). The growth of Dunaliella tertiolecta was reduced by 20 to 30% under the control (Fig. 2 and Table 1) within the concentration range studied. For Chlorella autotrophica, there was 10% retardation in growth in comparison to the control (Fig. 2 and Table 1) throughout the test concentrations. At higher concentrations (75% and 100%) the growth of Phaeodactylum tricornutum was depressed by about 10% with respect to the control (Fig. 2 and Table 1); at lower concentrations (10%, 25%, and 50%) there was only 1 to 2% depression in growth (Fig. 2 and Table 1). Although the inhibition of growth of P. tricornutum caused by low concentrations of the water soluble fraction of diesel oil

was very slight, it was very significant statistically (Table 9).

The effect of bacterial degradation products on the growth of D. tertiolecta, C. autotrophica and P. tricornutum are presented in Figures 3, 4, and 5 respectively. The products collected in the first week showed no significant effect on the growth of D. tertiolecta (Table 2 and 10); the effluents of oil degradation collected at a later time (2nd and 3rd week) exhibited a stimulating effect (Fig. 3). The 2nd week's effluent at 10% concentration only stimulated the growth of D. teriolecta by 3% (Table 2), however, for the concentrations over 10% and for the 3rd week's effluent there was 15 to 30% increase in growth (Fig. 3 and Table 2). The bacterial degradation products promoted the growth of C. autotrophica by 10 to 30% (Fig. 4 and Table 3) within the concentration range tested. The growth of P. tricornutum was increased 10 to 20% (Fig. 5 and Table 4) resulting from the influence of 2nd and 3rd week's bacterial degradation products. For the 1st week's product, there was 5 to 8% increase in growth at 10%, 25% and 100% concentration (Fig. 5 and Table 4), but there was no significant stimulating effect at 50% and 75% concentration (Table 4 and 12). Among the three effluents collected, the effluent obtained after the 1st week showed the least stimulating effect on the growth of algae.

A pilot study was conducted to test the effect of the inorganic nutrients used in continuous culture of oil degrading bacteria on the growth of D. tertiolecta, C. autotrophica

and P. tricornutum in the light. Bacterial medium contained NH_4Cl (100 mg/l), phosphate buffer (100 mg/l, pH 7.2), and $MgSO_4$ (10 mg/l). These mineral nutrients were added to the regular algal culture medium and growth was measured daily for a week. The results of this study showed that growth in the enriched medium was the same as that in the control.

The results of growth studies conducted in darkness are presented in Table 5, 6, and 7. Clearly, the bacterial degradation products did not support growth of the algae in darkness. The optical density continued to decline over the 12 day observation period.

Throughout this investigation bacterial contamination was checked at the end of each experiment. The results were found to be negative in every case.

Photosynthesis Studies:

The effect of water soluble extract of diesel oil on ^{14}C uptake by P. tricornutum is indicated in Figure 6 and Table 8. Although there was a 5 to 10% overall depression in ^{14}C uptake, only the 40% depression occurring at 100% concentration was statistically significant (Table 13).

Figure 7 illustrates the effect of bacterial degradation products on ^{14}C uptake. The data presented shows a 2 to 26% increase in ^{14}C uptake over the control (Fig. 7 and Table 8). However, these results were analyzed statistically using the Student's t-test (Table 13) and no significant difference was determined.

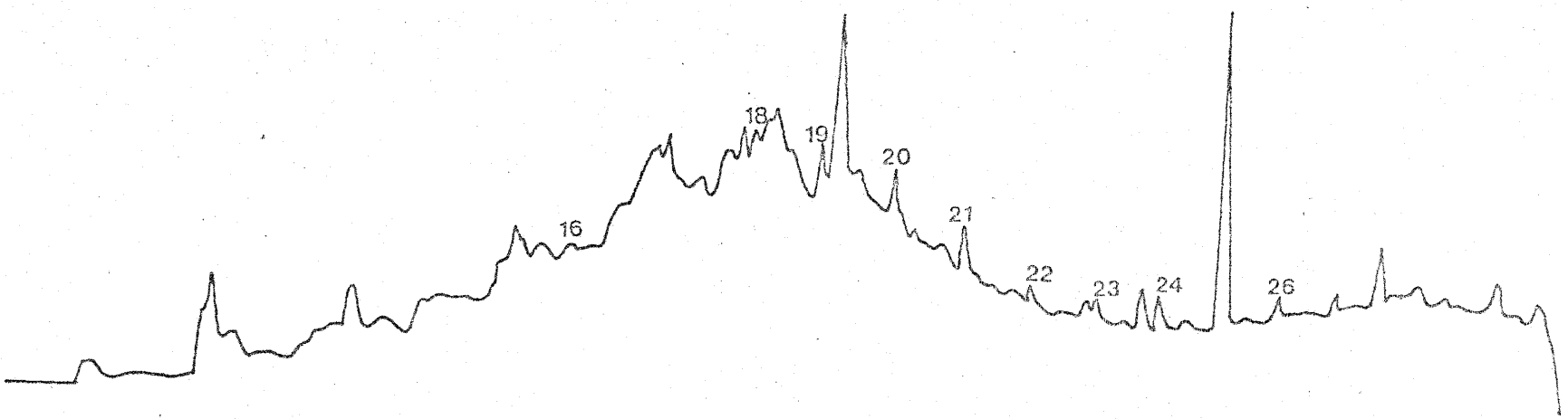

Fig 1. Gas chromatogram of water soluble extract of diesel oil. Normal alkanes are identified by numbered peaks. The numbers correspond to the length of the carbon chain.

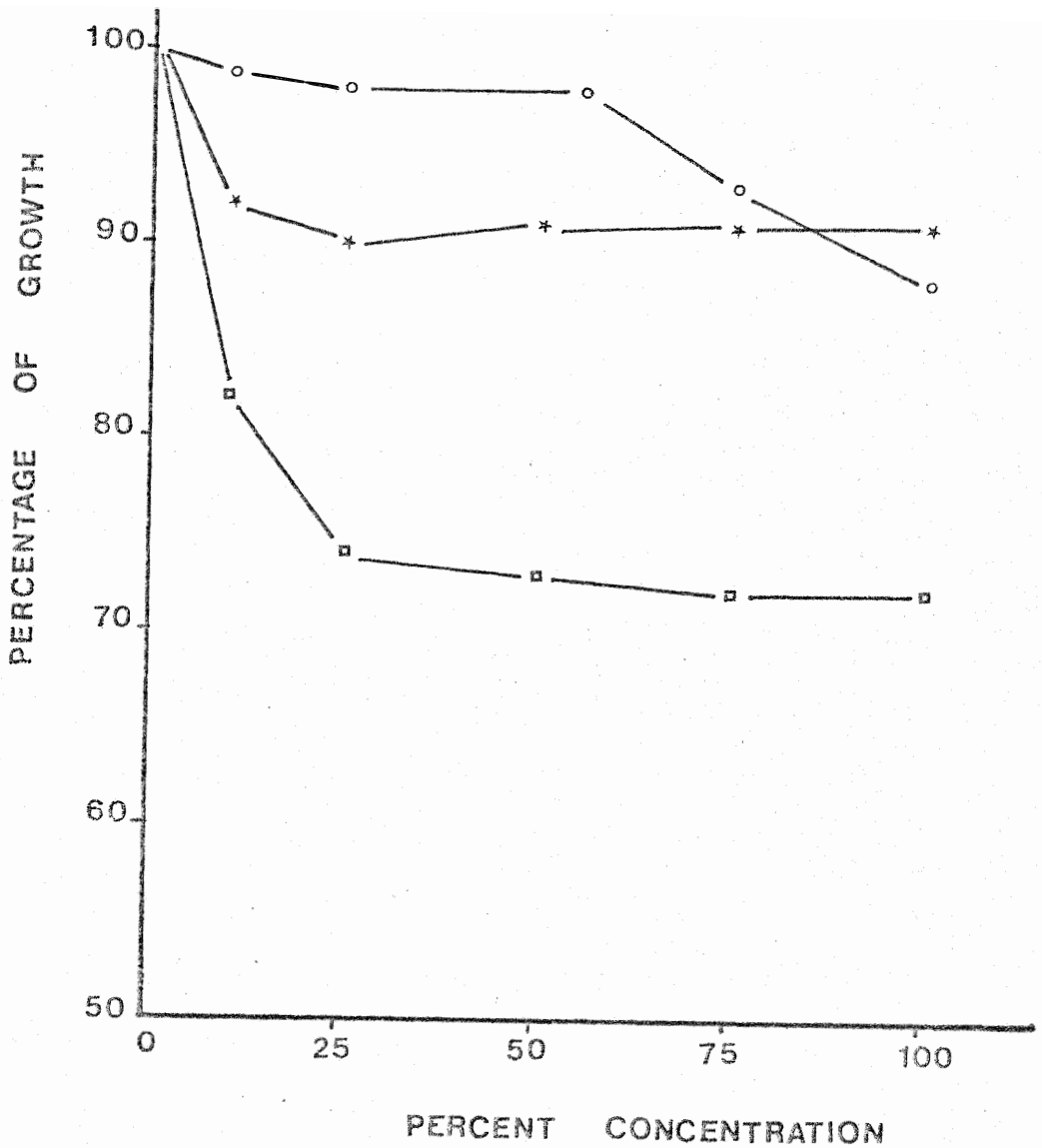

Fig. 2. Effect of different concentrations of water soluble extract of diesel oil on the growth of D. tertiolecta (▫), C. autotrophica (*), P. tricornutum (o). Growth is expressed as a percentage of the control.

Table 1. Effect of different concentrations of water soluble extract of diesel oil on the growth of *D. tertiolecta*, *C. autotrophica*, and *P. tricornutum*.

Algal Species	Con.(%)	Growth Rate[a]	% of Growth
D. tertiolecta	0	0.184	100
	10	0.151	82
	25	0.136	74
	50	0.134	73
	75	0.132	72
	100	0.133	72
C. autotrophica	0	0.226	100
	10	0.208	92
	25	0.204	90
	50	0.206	91
	75	0.206	91
	100	0.206	91
P. tricornutum	0	0.213	100
	10	0.211	99
	25	0.208	98
	50	0.209	98
	75	0.199	93
	100	0.188	88

a. Number of doublings per day (Monod, 1949).

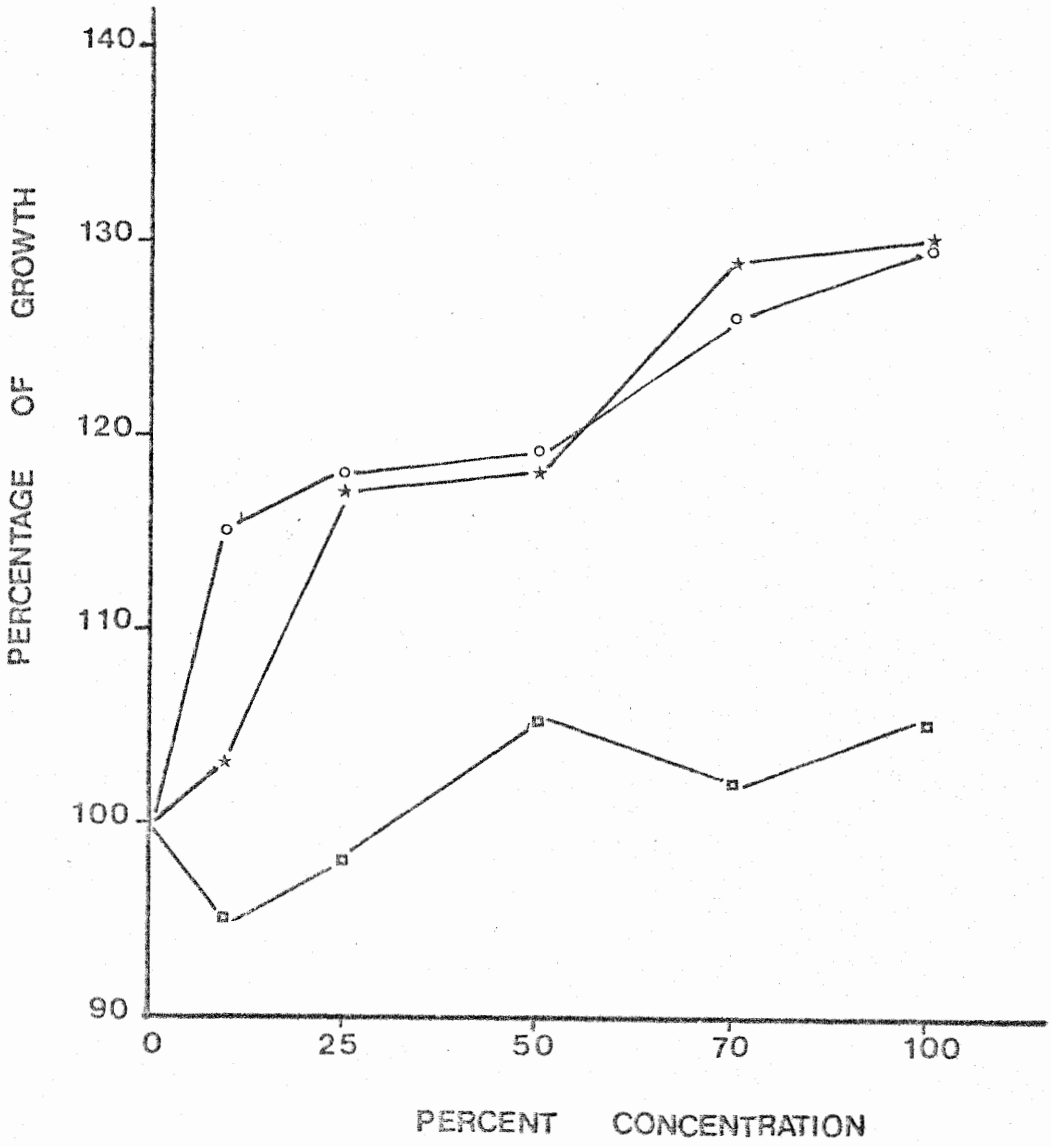

Fig. 3. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of D. tertiolecta. Growth is expressed as a percentage of the control.
▫ = effluent collected after 1 week.
* = effluent collected after 2 weeks.
o = effluent collected after 3 weeks.

Table 2. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of *D. tertiolecta*.

Effluent	Con.(%)	Growth Rate[a]	% of Growth
I[b]	0	0.191	100
	10	0.181	95
	25	0.187	98
	50	0.200	105
	75	0.195	102
	100	0.204	107
II[b]	0	0.191	100
	10	0.196	103
	25	0.224	117
	50	0.225	118
	75	0.247	129
	100	0.248	130
III[b]	0	0.191	100
	10	0.219	115
	25	0.226	118
	50	0.228	119
	75	0.240	126
	100	0.248	130

a. Number of doublings per day (Monod, 1949).
b. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

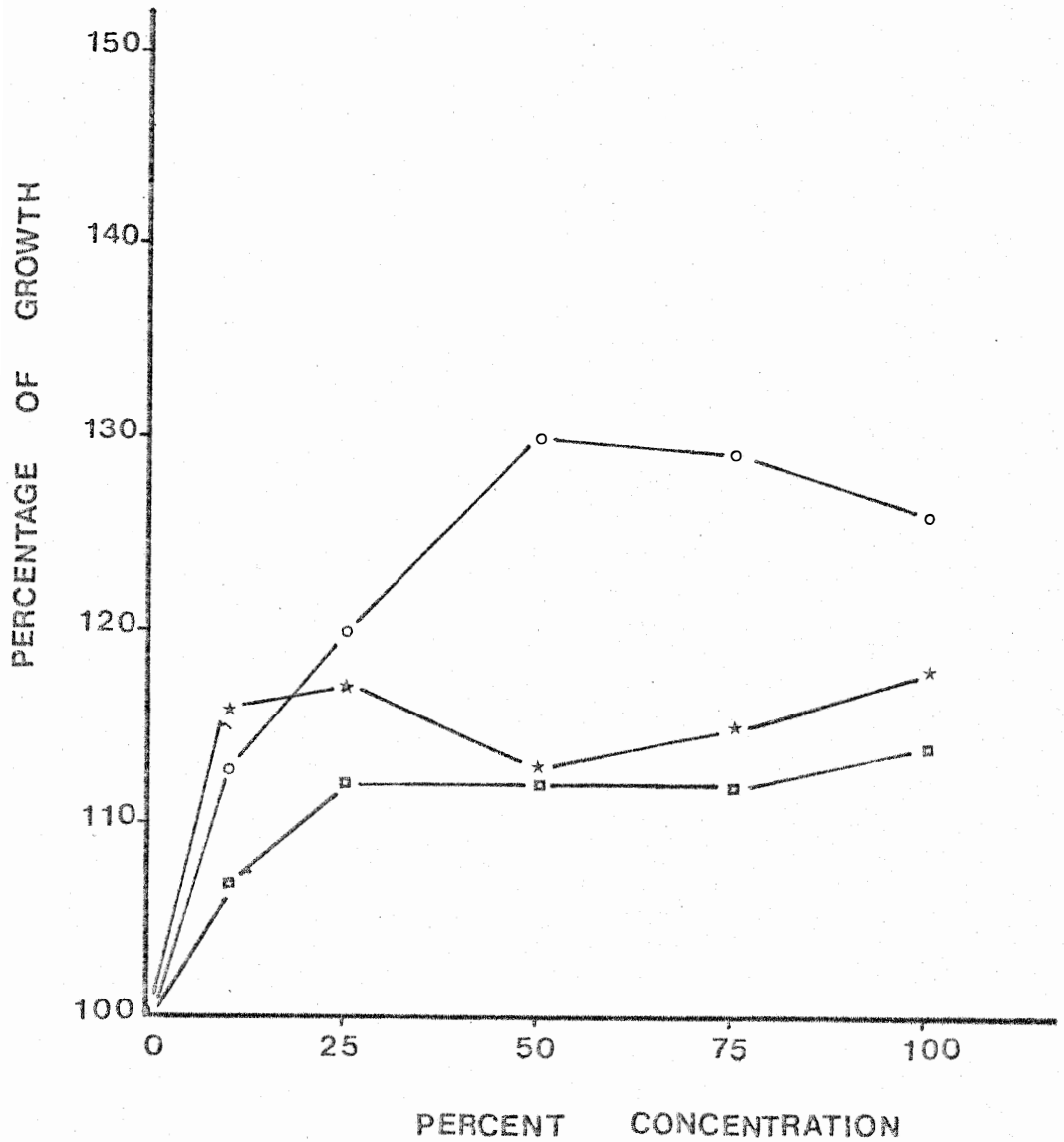

Fig. 4. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of C. autotrophica. Growth is expressed as a percentage of the control.
▫ = effluent collected after 1 week.
* = effluent collected after 2 weeks.
o = effluent collected after 3 weeks.

Table 3. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of *C. autotrophica*.

Effluent	Con.(%)	Growth Rate[a]	% of Growth
I[b]	0	0.209	100
	10	0.224	107
	25	0.235	112
	50	0.234	112
	75	0.234	112
	100	0.238	114
II[b]	0	0.209	100
	10	0.242	116
	25	0.245	117
	50	0.236	113
	75	0.240	115
	100	0.247	118
III[b]	0	0.209	100
	10	0.237	113
	25	0.251	120
	50	0.271	130
	75	0.268	128
	100	0.264	126

a. Number of doublings per day (Monod, 1949).
b. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

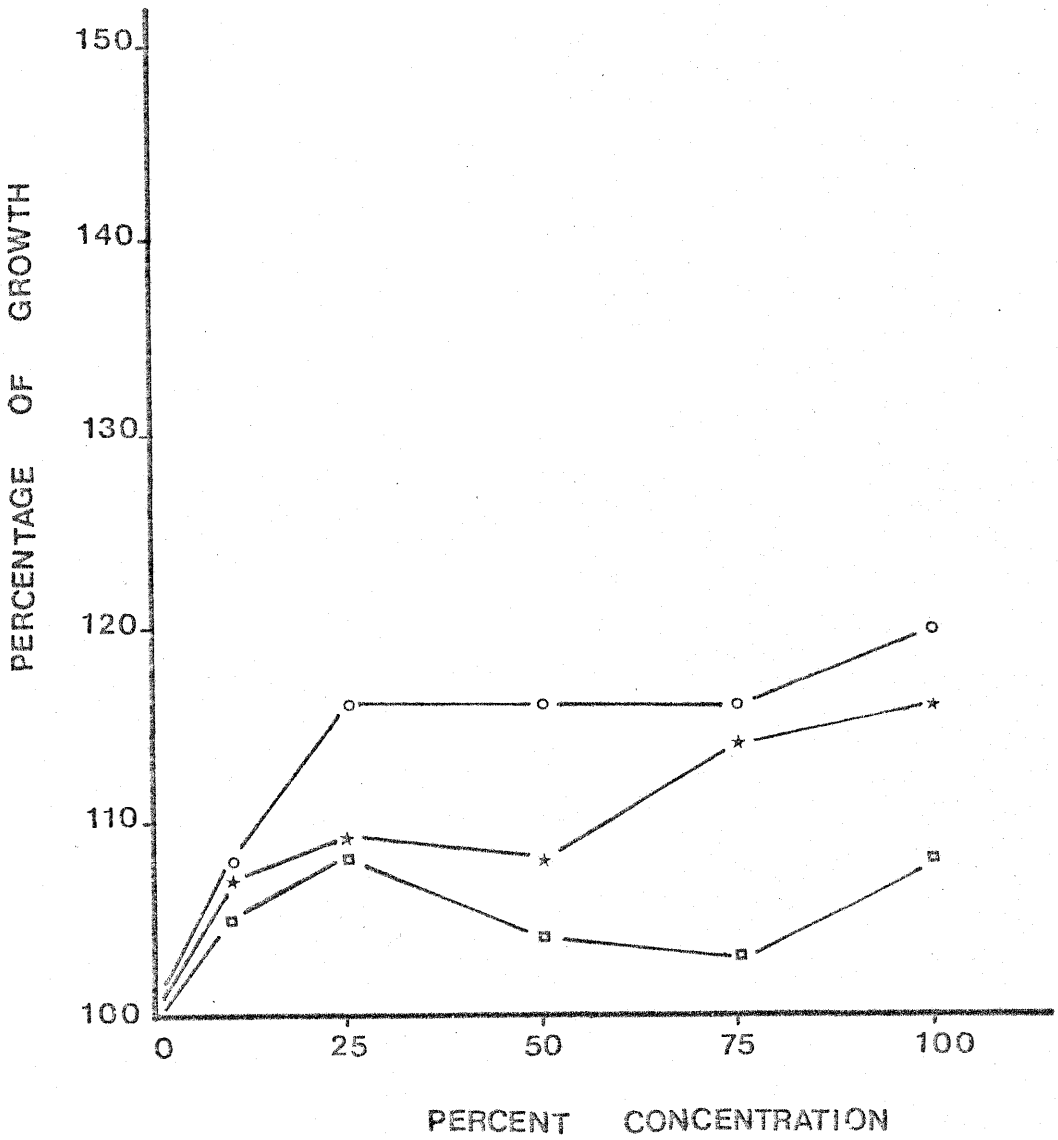

Fig. 5. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of P. tricornutum. Growth is expressed as a percentage of the control.
▫ = effluent collected after 1 week.
⋆ = effluent collected after 2 weeks.
○ = effluent collected after 3 weeks.

Table 4. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of P. tricornutum.

Effluent	Con.(%)	Growth Rate[a]	% of Growth
I[b]	0	0.220	100
	10	0.231	105
	25	0.237	108
	50	0.229	104
	75	0.227	103
	100	0.238	108
II[b]	0	0.220	100
	10	0.236	107
	25	0.240	109
	50	0.238	108
	75	0.250	114
	100	0.255	116
III[b]	0	0.220	100
	10	0.237	108
	25	0.255	116
	50	0.255	116
	75	0.255	116
	100	0.263	120

a. Number of doublings per day (Monod, 1949).
b. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 5. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of *D. tertiolecta* in darkness. Data are expressed as $\bar{O}.D.$ values obtained after 0, 6, and 12 days following inoculation.

Effluent	Con.(%)	Time (day) 0	6	12
I[a]	0	0.1	0.078	0.078
	25	0.1	0.077	0.070
	50	0.1	0.076	0.069
	100	0.1	0.078	0.070
II[a]	0	0.1	0.078	0.078
	25	0.096	0.077	0.068
	50	0.1	0.075	0.069
	100	0.1	0.075	0.067
III[a]	0	0.1	0.078	0.078
	25	0.1	0.075	0.070
	50	0.1	0.077	0.070
	100	0.1	0.073	0.068

a. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 6. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of C. autotrophica in darkness. Data are expressed as O.D. values obtained after 0, 6, and 12 days following inoculation.

Effluent	Con.(%)	Time (day) 0	6	12
I[a]	0	0.071	0.062	0.059
	25	0.071	0.060	0.058
	50	0.071	0.060	0.058
	100	0.070	0.060	0.055
II[a]	0	0.071	0.062	0.059
	25	0.070	0.060	0.057
	50	0.070	0.059	0.057
	100	0.071	0.059	0.056
III[a]	0	0.071	0.062	0.059
	25	0.071	0.059	0.056
	50	0.071	0.058	0.056
	100	0.070	0.059	0.057

a. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 7. Effect of different concentrations of bacterial degradation products of diesel oil on the growth of *P. tricornutum* in darkness. Data are expressed as O.D. values obtained after 0, 6, and 12 days following inoculation.

Effluent	Con.(%)	Time (day) 0	6	12
I[a]	0	0.068	0.060	0.060
	25	0.070	0.058	0.059
	50	0.070	0.062	0.059
	100	0.070	0.059	0.056
II[a]	0	0.068	0.060	0.060
	25	0.069	0.060	0.059
	50	0.071	0.059	0.060
	100	0.068	0.059	0.057
III[a]	0	0.068	0.060	0.060
	25	0.070	0.059	0.060
	50	0.071	0.059	0.058
	100	0.070	0.059	0.058

a. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

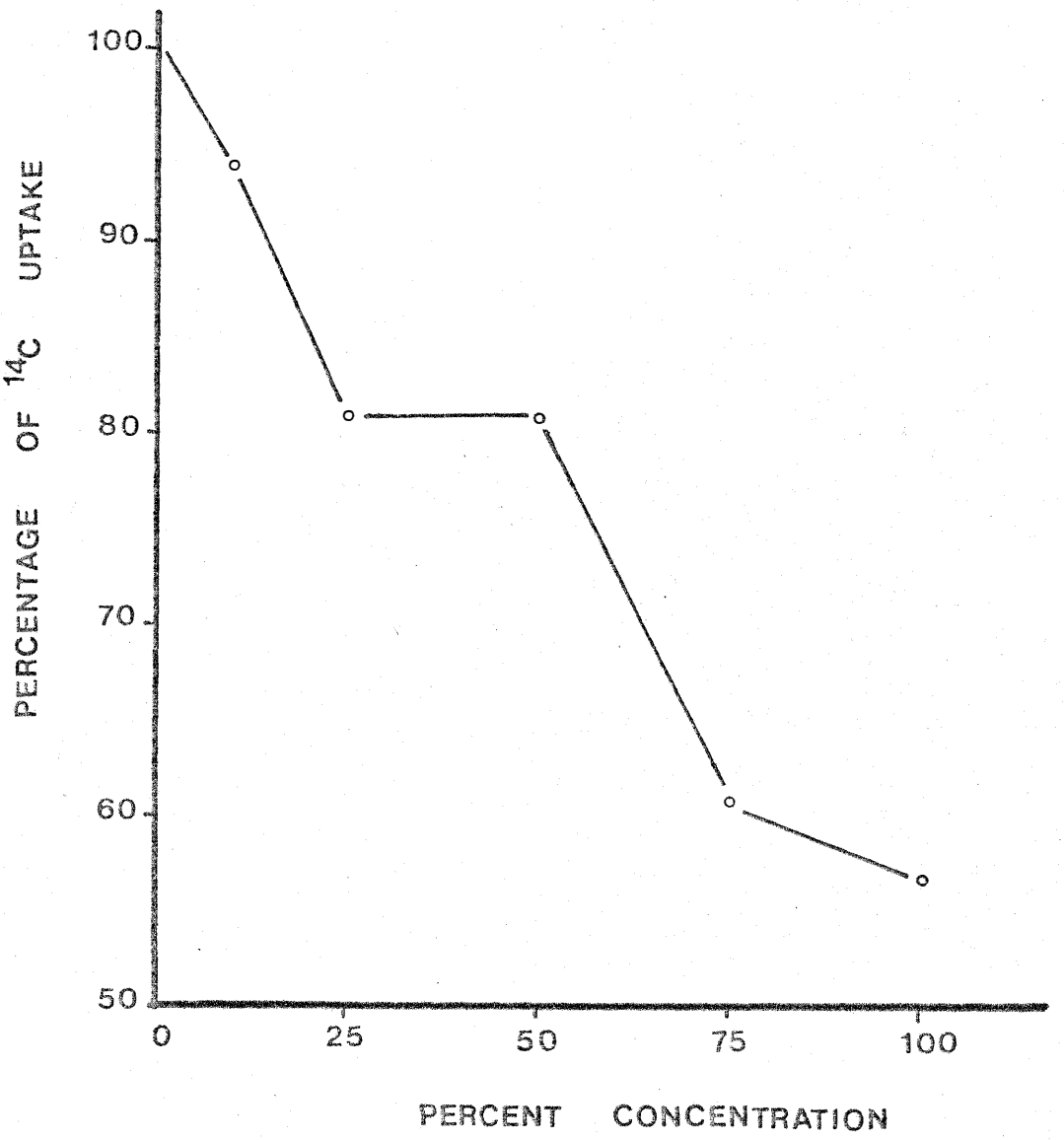

Fig. 6. Effect of different concentrations of water soluble extract of diesel oil on ^{14}C uptake by P. tricornutum. ^{14}C uptake is expressed as a percentage of the control.

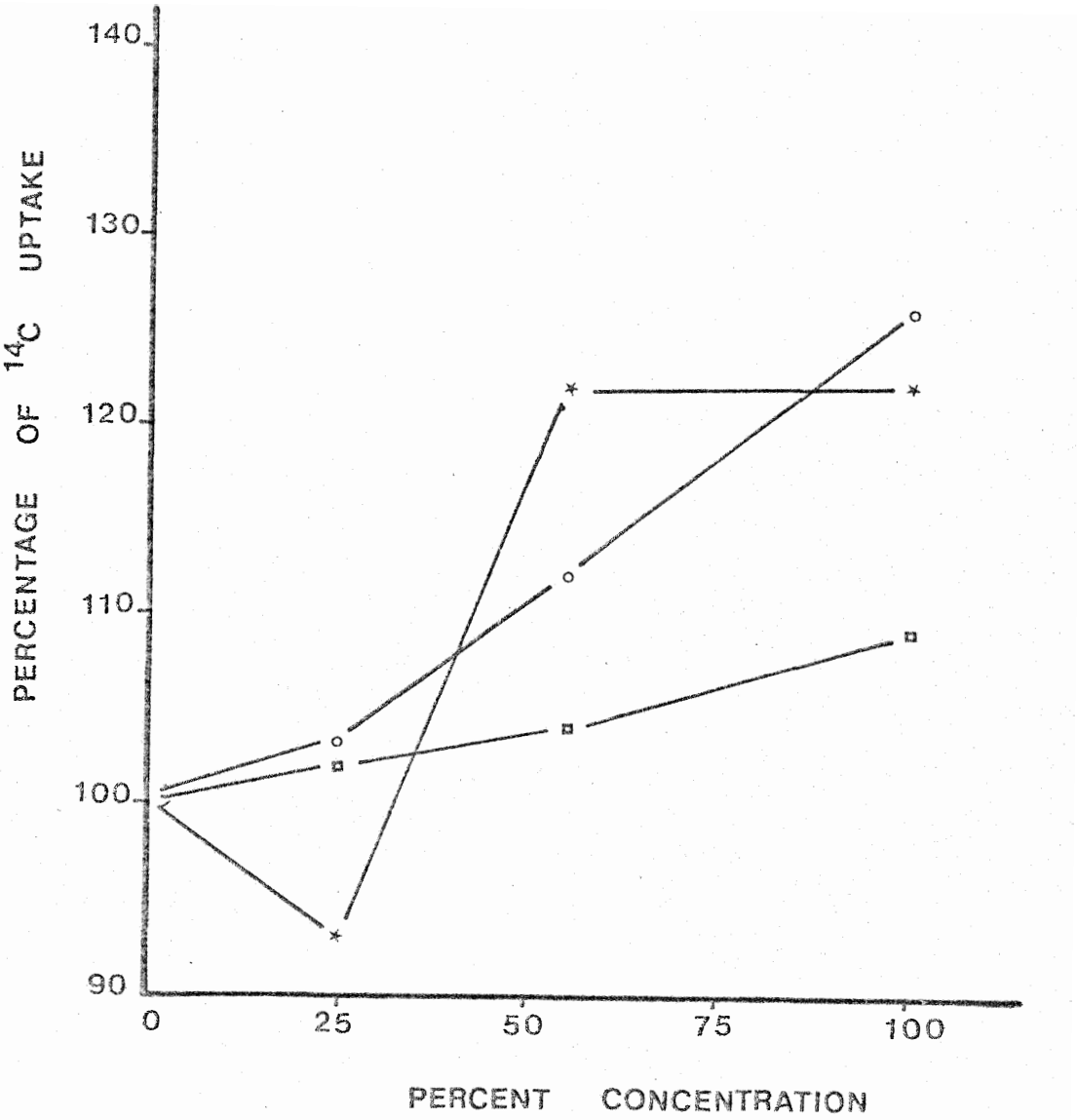

Fig. 7. Effect of different concentrations of bacterial degradation products of diesel oil on ^{14}C uptake by P. tricornutum. ^{14}C uptake is expressed as a percentage of the control.
▫ = effluent collected after 1 week.
* = effluent collected after 2 weeks.
○ = effluent collected after 3 weeks.

Table 8. Effect of different concentrations of water soluble extract and bacterial degradation products of diesel oil on ^{14}C uptake by P. tricornutum.

Treatment	Con.(%)	^{14}C Uptake[a] (count/min-O.D.)	% of ^{14}C Uptake
WSE[b]	0	311800	100
	10	293567	94
	25	254644	81
	50	254887	81
	75	192349	61
	100	179442	57
I[b]	0	311800	100
	25	319752	102
	50	326177	104
	100	340032	109
II[b]	0	311800	100
	25	291517	93
	50	382963	122
	100	381254	122
III[b]	0	311800	100
	25	320922	103
	50	350582	112
	100	393139	126

a. Radioactivity counts have been corrected for differences in O.D.

b. WSE = water soluble extract.
I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 9. Results of Student's paired t-test for the effect of water soluble extract of diesel oil on growth rates. Data expressed are computed t values. The table t value for 23 df is 1.714 for the one tailed 5% probability level.

Con. (%)	Algal Species		
	D. tertiolecta	C. autotrophica	P. tricornutum
10	6.509**	5.320**	8.056**
25	6.314**	4.111**	8.221**
50	6.266**	4.969**	10.117**
75	6.228**	5.243**	10.357**
100	6.204**	5.282**	10.414**

Table 10. Results of Student's paired t-test for the effect of bacterial degradation products of diesel oil on growth rates of D. tertiolecta. Data expressed are computed t values. The table t value for 14 df is 1.761 for the one tailed 5% probability level.

Effluent	Con.(%)				
	10	25	50	75	100
I[a]	-0.456	-0.970	-1.424	-0.500	1.468
II[a]	2.411	3.070**	3.799**	4.397**	6.783**
III[a]	2.749	2.632**	2.283**	3.851**	5.920**

** = highly significant.
a. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 11. Results of Student's paired t-test for the effect of bacterial degradation products of diesel oil on growth rates of C. autotrophica. Data expressed are computed t values. The table t value for 14 df is 1.761 for the one tailed 5% probability level.

Effluent	Con.(%) 10	25	50	75	100
I[a]	15.735**	17.363**	22.020**	24.516**	31.619**
II[a]	19.349**	18.800**	15.369**	20.258**	15.074**
III[a]	12.651**	12.089**	13.286**	18.570**	25.393**

Table 12. Results of Student's paired t-test for the effect of bacterial degradation products of diesel oil on growth rates of P. tricornutum. Data expressed are computed t values. The table t value for 14 df is 1.761, 8 df is 1.860, and 5 df is 2.015 for the one tailed 5% probability level.

Effluent	Con.(%) 10	25	50	75	100
I[a]	4.753**	2.233*	-0.298+	0.942#	1.948+*
II[a]	10.990**	6.221**	2.889+*	5.109#**	5.810#**
III[a]	7.679**	6.404**	8.149**	2.822+*	3.067**

* = significant.
** = highly significant
\+ = 8 df.
\# = 5 df.
a. I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

Table 13. Results of Student's t-test for the effects of water soluble extract and bacterial degradation products of diesel oil on ^{14}C uptake by P. tricornutum. Data expressed are computed t values. The table t value for 2 df is 2.920 for the one tailed 5% probability level.

Treatment	Con.(%)				
	10	25	50	75	100
WSE[a]	-0.435	-1.374	-1.288	-2.827	-3.111*
I[a]	--	0.160	0.303	--	0.617
II[a]	--	0.438	1.687	--	1.688
III[a]	--	0.220	0.898	--	1.851

* = significant.
a. WSE = water soluble extract.
I = effluent collected after 1 week.
II = effluent collected after 2 weeks.
III = effluent collected after 3 weeks.

DISCUSSION

The inhibitory effect of the water soluble fraction of diesel oil on marine phytoplankton growth found in this study agreed with the major biological research that has been done with different types of oil and oil fractions (Galtsoff et al., 1935; Lacaze, 1969; Nuzzi, 1973).

Galtsoff et al. (1935) noted that the water soluble components of Pelto crude oil extract exerted a retarding effect on the growth of Nitzschia closterium at a concentration of 25% and higher. Lacaze (1969) tested 1% water extracts of Kuwait crude oil on P. tricornutum and obtained a 10% reduction in cell growth. Nuzzi (1973) determined the effect of water soluble extracts of No. 2 and No. 6 fuel oil and outboard motor oil on the growth of Chlorella sp., Clamydomonas sp., P. tricornutum and Skeletonema costatum. Water soluble substances extracted from No. 2 fuel oil inhibited completely the growth of all the test organisms, except Chlamydomonas sp., while extracts from No. 6 fuel oil and outboard motor oil had little effect on the growth of these marine phytoplankters.

Galtsoff et al. (1935) reported a stimulating effect on the growth of phytoplankton by low concentrations of water soluble extract of crude oil. This effect was found in algal cultures contaminated with large growths of bacteria. This bacterial contamination may have been responsible for the stimulating effect observed by these investigators.

Observations made by researchers working in the field

normally reported little damage to marine phytoplankton by an oil spill (Smith, 1968; Kolpack, 1970). Butler and Berkes (1972) noted that the lack of evidence of cell damage to phytoplankton may be partly explained by the logistical and technical difficulties associated with plankton research in the field. The present investigation done entirely in the laboratory demonstrates that water extract of diesel oil is capable of inhibiting the growth of selected marine phytoplankton. Whether this happens in the environment at any particular time or place is not within the realm of this study, but it indicates the potential for inhibition under certain conditions. According to Straughan (1972) biological damage caused by an oil spill is governed by a combination of several factors including:

> "(1) the type of oil spilled, (2) the dose of oil, (3) the physiography of the area of the spill, (4) weather conditions at the time of the spill, (5) the biota of the area, (6) the season of the spill, (7) previous exposure of the area to oil, (8) exposure to other pollutants, and (9) the treatment of the spill."

Diesel oil contains four types of hydrocarbon molecules: paraffins, naphthenes, olefins, and aromatics (Blumer et al., 1971). Each has a different solubility in water. In this study, some paraffins present in water extract of diesel oil were identified. Additional hydrocarbons existing in the extract were not identified because of the lack of known comparative substances. Boylan and Tripp (1971) found that seawater extract of Kuwait crude oil and kerosene contained mostly benzenes and naphthalenes in the low boiling range of 80-262°C. By spraying pure hydrocarbons on plants, Havis

(1950) showed that aromatics ranked highest in toxicity, naphthenes and olefins were next, while straight paraffins were found least toxic to plants.

To the knowledge of this investigator, no research exists on the effects of the bacterial degradation products of oil on marine phytoplankton. In the present study, bacterial degradation products of diesel oil stimulated the growth of phytoplankton. The growth of Dunaliella tertiolecta, Chlorella autotrophica, and Phaeodactylum tricornutum in the light was enhanced by the oil degradation products. The effluents of oil degradation collected at one, two and three weeks caused different degrees of growth stimulation. Among the three effluents collected, the effluent obtained after the 1st week showed the least stimulating effect on the growth of the algae. However, cultures grown in the dark were not similarly affected.

There are a number of explanations for the growth stimulation mentioned above. The possibility exists that the increased growth may have resulted from the algal uptake of the inorganic nutrients used for the continuous culture of oil degrading bacteria. This was ruled out by the results of a pilot study which showed that the inorganic nutrients of the bacterial medium did not stimulate algal growth.

Nutrients could have also been contributed to the algal medium by the living and dead oil degrading bacteria. Since nitrogen and phosphorus were present in the bacterial medium the quantities of these elements released by the living bacteria themselves should have been negligible. Furthermore,

throughout this study the bacterial population size utilized in the degradation of the diesel oil was kept as constant as possible (Pritchard and Starr, 1973). Since the bacterial population size remained constant, the contribution of nutrients to the effluents by living and dead bacteria remained the same over the 3 week period. If algal growth were stimulated by the nutrients released from the bacteria, then there should not have been a difference in the extent of increased growth resulting from oil degradation products collected at different times.

Still another possibility exists that these algae may be able to utilize the alkane residues from the decomposed oil. It has been reported that Scenedesmus quadricauda and two isolates of Scenedesmus brevicauda showed improved growth in the light in a medium containing n-heptadecane (Masters and Zajic, 1971). These investigators also suggested that many natural algal populations contain individuals capable of metabolizing hydrocarbons.

A further possibility is that the algae can grow photoheterotrophically on the microbial degradation products of oil, most probably the fatty acids. Barker (1935) found that the colorless alga, Prototheca zopfii grew readily on all the straight chain fatty acids from C_2 (acetate) to C_{10}. Samejima and Myers (1958) reported that acetate can support continued growth of Chlorella pyrenoidosa and Chlorella ellipsodea in darkness. Acetate also supported the heterotrophic growth of Chlamydomonas dysosmos (Lewin, 1954). P. tricornutum and some other species of marine phytoplankton have been observed to

grow photoheterotrophically on glycerol (Cheng and Antia, 1970).

Additional work is needed to ascertain specific algal utilization of hydrocarbons and oil degradation products, particularly the fatty acids. If algae have the ability to metabolize hydrocarbons or the microbial degradation products, there is a possibility that they can assist microorganisms and provide some relief to oil pollution in the aquatic environment.

Strand et al. (1971) stated that the in situ algal bioassay utilizing the ^{14}C technique represented a rapid sensitive way to assess the effect of discharged oil on phytoplankton. These investigators used the ^{14}C method to study the effect of an emulsion of crude oil and chemical despersant on the photosynthesis of natural marine phytoplankton communities. They found an inhibitory effect on photosynthesis at high concentration levels of the emulsion, and a stimulating effect at low concentration levels. Gordon and Prouse (1973) also found that high concentrations of Venezuelan crude oil inhibited photosynthesis of marine phytoplankton, while low concentration levels stimulated the uptake of ^{14}C by natural phytoplankton communities. These investigators suggested that the increased photosynthesis might be due to the presence of possible growth-regulating compounds in the crude oil. They noted that under the condition investigated No. 2 fuel oil was more toxic to marine phytoplankton than was No. 6 fuel oil or Venezuelan crude.

In the present study, a high concentration of water sol-

uble extract of diesel oil caused a significant decrease in the uptake of ^{14}C by P. tricornutum. It has been suggested that hydrocarbon molecules may damage cells through intercalation in cell membranes (Marshland, 1933; Van Overbeek and Blondeau, 1954; Goldacre, 1968). A similar disruption of the chloroplast may account for disturbance in photosynthesis (Nelson-Smith, 1968a).

The inhibitory effect of the water soluble extract of oil on the photosynthesis of P. tricornutum gives additional support to the idea that oil is a potential toxicant to phytoplankton.

Failure to show statistical significance for the remaining ^{14}C uptake studies, in this research, was due primarily to the great within-group variance of the replicates tested. The ^{14}C uptake study was conducted once in duplicate, and to obtain a meaningful statistical evaluation the number of replicates should have been greater.

SUMMARY

Preliminary laboratory studies revealed that pure diesel oil interfered with the spectrophotometric measurements required for this investigation. To solve this problem a water extract of the diesel fuel was used in place of the oil itself.

To determine the relative toxicity of the water extract and the microbial degradation products of the diesel fuel, an assessment of the effects of oil pollution on the growth and photosynthesis of marine phytoplankton was conducted in the laboratory. Three species of marine algae (Dunaliella tertiolecta, Chlorella autotrophica and Phaeodactylum tricornutum) were employed for the growth studies. The common marine diatom, P. tricornutum was the only species assayed during the photosynthetic studies.

Laboratory data provided evidence that the water soluble extract of diesel oil inhibited the growth of all three species of marine phytoplankton tested. D. tertiolecta, a flagellated green alga, was the most affected species. Within the concentration range studied, growth of this species was reduced as much as 30% below the control. Though field observations usually indicate little damage to phytoplankton by an oil spill, the present findings demonstrated that the chemical components of diesel oil were toxic to some species of marine phytoplankton.

The effect of water soluble extract on ^{14}C uptake by P. tricornutum was analyzed in the laboratory. A statistically significant depression in photosynthesis occurred when water

extract at 100% concentration was used in this study.

Gas-liquid chromatography was employed to determine the chemical components present in the water soluble extract of diesel oil. This chromatographic analysis, though incomplete, did reveal some paraffins were present in the extract.

The biological effects of microbial degradation products of fuel oil is still largely unknown. This study revealed that the bacterial degradation products of diesel fuel stimulated the growth of marine algae in the light. Effluents of the bacterial degradation were collected weekly for a period of three weeks. The 2nd and 3rd week effluents exerted 3 to 30% increase in algal growth within the concentration range tested. Growth studies conducted in darkness clearly showed that these effluents could not support D. tertiolecta, C. autotrophic and P. tricornutum heterotrophically. It may be speculated that growth stimulation observed in the light may have resulted from algal utilization of alkane residues from decomposed oil or the bacterial degradation products, most possibly the fatty acids. Additional research is needed to ascertain this possibility.

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