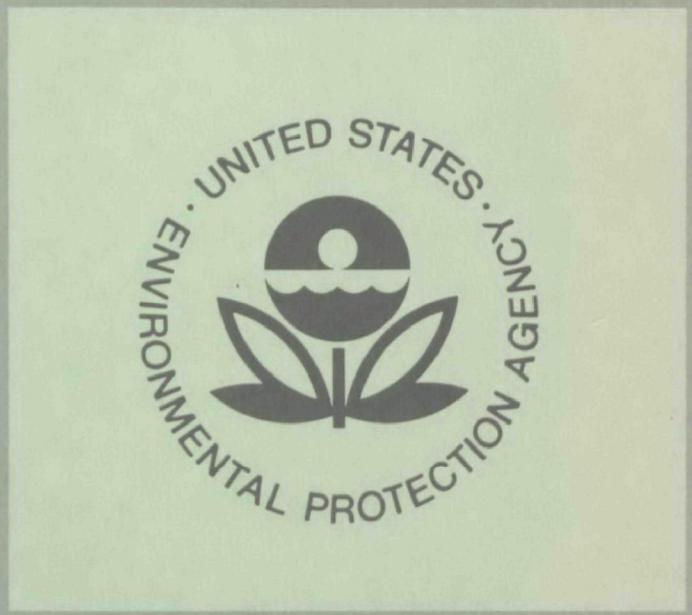
EPA-600/3-76-048
June 1976

Ecological Research Series

EFFECTS, UPTAKE, AND METABOLISM OF METHOXYCHLOR, MIREX, AND 2,4-D IN SEAWEEDS



Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, Florida 32561

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into five series. These five broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The five series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies

This report has been assigned to the ECOLOGICAL RESEARCH series. This series describes research on the effects of pollution on humans, plant and animal species, and materials. Problems are assessed for their long- and short-term influences. Investigations include formation, transport, and pathway studies to determine the fate of pollutants and their effects. This work provides the technical basis for setting standards to minimize undesirable changes in living organisms in the aquatic, terrestrial, and atmospheric environments.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

EPA-600/3-76-048
June 1976

EFFECTS, UPTAKE, AND METABOLISM OF METHOXYCHLOR, MIREX, AND 2,4-D IN SEAWEEDS

by

Harish C. Sikka, Gary L. Butler, and Clifford P. Rice

Syracuse University Research Corporation
Syracuse, New York 13210

Contract No. 68-03-0271

Project Officer

Gerald E. Walsh

Environmental Research Laboratory
Gulf Breeze, Florida 32561

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
ENVIRONMENTAL RESEARCH LABORATORY
GULF BREEZE, FLORIDA 32561

DISCLAIMER

This report has been reviewed by the Office of Research and Development, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

ABSTRACT

This report presents the results of a study concerning effects, uptake, and metabolism of mirex, methoxychlor, and 2,4-D in the seaweeds Ulva sp., Enteromorpha sp., and Rhodymenia sp. None of the pesticides, at concentrations corresponding to their maximum solubility in seawater, had any significant effect on photosynthesis, protein, carbohydrate, lipid, chlorophyll, carotenoid, or trace metal content of the algae. All three algae removed substantial amounts of mirex and methoxychlor from the medium, but uptake of 2,4-D was extremely low. The rate of uptake of methoxychlor was considerably greater than that of mirex. Bioaccumulation of methoxychlor was greater than that of mirex. Enteromorpha accumulated considerably more mirex and methoxychlor than Ulva or Rhodymenia.

Both Ulva and Enteromorpha failed to metabolize either mirex or 2,4-D. Enteromorpha metabolized methoxychlor to a limited extent. After 7 days of incubation with ^{14}C-methoxychlor, a major portion of the ^{14}C in the tissue and medium was present in unchanged methoxychlor. A small amount of a ^{14}C-metabolite, 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene, was detected in both the tissue and medium. In addition, medium contained 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane and four unidentified minor ^{14}C-metabolites. Unlike Enteromorpha, Ulva did not metabolize methoxychlor.

This report was submitted in fulfillment of Contract No. 68-03-0271 by the Syracuse University Research Corporation under the sponsorship of the U.S. Environmental Protection Agency. Work was completed as of August 31, 1975.

CONTENTS

TABLES

ACKNOWLEDGMENT

The technical assistance of Robert Lynch is gratefully acknowledged.

SECTION I

INTRODUCTION

GENERAL

Mirex (dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta [cd] pentalene), methoxychlor [2,2-bis(p-methoxyphenyl)-1,1,1-trichloroethane] and 2,4-D (2,4-dichlorophenoxyacetic acid) are three commonly used pesticides. Mirex has been used for many years to control imported fire ants in pasture lands in the southeastern United States (Alley, 1973). Methoxychlor, a broad-spectrum insecticide, is a very likely biodegradable replacement for DDT. Though chemically similar, it is less toxic to fish, birds and mammals than DDT (Pimental, 1971). Recently, methoxychlor has replaced DDT for several predominantly environmental uses, such as controlling blackfly larvae in streams, elm bark beetles, and fruit and garden pests (Burdick et al, 1968). 2,4-D is widely used to control broadleaf weeds. Formulations of 2,4-D are also extensively used to control aquatic plants, such as water hyacinth and Eurasian watermilfoil (Lawrence, 1962).

These pesticides may enter the estuarine environment in a variety of ways, including application to control objectionable flora and/or fauna, disposal of wastes from pesticide manufacturing and formulation plants, run-off from treated lands adjacent to estuaries, and drift from pesticide application. The contamination of the estuarine environment with pesticides and their metabolites is of great environmental concern because of their potential toxicity to estuarine fauna and flora. To evaluate the impact of pesticides in the estuarine environment, it is important that we have a knowledge of their fate, and the effects that they and their metabolites have on the biota.

Among the estuarine biota, seaweeds play a vital role in marine ecology. They contribute to the oxygen supply in the marine environment through

photosynthesis and also play an important role in nutrient regeneration. If pesticides adversely affect the growth of these algae, significant changes may occur in the estuarine ecosystem.

The seaweeds may play a role in determining the fate of pesticides in the estuarine environment. They may remove pesticides by adsorption and/or absorption and may degrade them. The pesticides and their metabolites which are accumulated by the seaweeds may be transferred to other trophic levels and may constitute a health hazard. Therefore, identification and measurement of residues of pesticides and their metabolites are necessary to fully evaluate their impact on the marine environment.

A number of studies have been done to examine effects and metabolism of pesticides in marine phytoplankton, but no information is available on effects, uptake, and metabolism of pesticides in seaweeds. This project was undertaken to investigate effects and fate of 2,4-D, mirex, and methoxychlor in three species of commonly occurring seaweeds, Ulva lactuca (Chlorophyta), Enteromorpha linza (Chlorophyta), and Rhodymenia pseudopalmata (Rhodophyta). The overall objective of this investigation was to provide information needed for establishing marine water-quality standards for the above chemicals.

SPECIFIC OBJECTIVES

1. To study effects, if any, that 2,4-D, mirex, and methoxychlor have on photosynthesis of Ulva lactuca, Enteromorpha linza and Rhodymenia pseudopalmata at concentrations not exceeding pesticide solubility in the growth medium.

2. To establish whether pesticides affect the chemical composition (carbohydrate, protein, pigment, lipid and trace metal content) of seaweeds.

3. To determine uptake and metabolism of the pesticides by the seaweeds.

SECTION II

MATERIALS AND METHODS

PESTICIDES

Technical-grade pesticides were used to evaluate effects on seaweeds. The sources and the purity of the chemicals were: 2,4-D, purity 97% (The Dow Chemical Company); methoxychlor, purity 98% (E.E. duPont de Nemours and Co.); and mirex, purity 98% (Allied Chemical Compnay).

Pesticides labeled with ^{14}C were used to study uptake and metabolism by the seaweeds. Uniformly ring-labeled ^{14}C-2,4-D was purchased from the California Bionuclear Corporation, Sun Valley, California. ^{14}C-Uniformly ring-labeled methoxychlor and ^{14}C-uniformly labeled mirex were purchased from the Mallinckrodt Chemical Company, St. Louis, Missouri.

PESTICIDE SOLUBILITY DETERMINATION

An excess of each pesticide was added to artificial seawater medium having a salinity of 33-34 ppt. (For medium composition, refer to page 6.) After 24 hours of shaking on a wrist shaker, the mixture was filtered through a 0.45 μ porosity glass fiber filter. The concentration of mirex or methoxychlor in the filtrate was determined by gas-chromatography, whereas the 2,4-D concentration was determined spectrophotometrically.

To analyze the filtrate for mirex and methoxychlor, aliquots were extracted successively three times with equal volumes of pesticide-grade petroleum ether. The first two extracts were combined, and the third was analyzed separately. The extracts were concentrated by being evaporated in a Kuderna-Danish apparatus and analyzed for pesticides using a Microtek-Model MT-220 gas-chromatograph equipped with a ^{63}Ni electron capture detector. Operating temperatures for the various components were: inlet, 210°C; oven, 205°C; and detector, 290°C. The chromatographic column, an 800 cm x 4 mm (i.d.) glass U-tube, was packed

with an equal-weight mixture of 1.5% OV-17 and 1.95% QF-1 on 80-100 mesh size Supelcoport. The carrier gas (nitrogen) flowed at a rate of 50 ml/min. The amount of pesticide in the extract was determined from peak area measurements which were compared with those of standard mirex or methoxychlor solutions. Mirex or methoxychlor could not be detected in the third extract of each set.

The concentration of 2,4-D in the filtrate was determined spectrophotometrically by measuring absorbance at 271 nm in a Cary 14 spectrophotometer. A standard curve relating optical density at 271 nm to the concentration of 2,4-D was used to determine the concentration of the herbicide in the unknown sample.

SOURCE OF SEAWEEDS

It was considered desirable to use seaweeds grown in the laboratory because their age and culture conditions were known. Such organisms were expected to be more uniform in their physiological response than field-collected seaweed. When it was not possible to culture a sufficient amount of algal material in the laboratory, seaweeds collected from the field and maintained in the laboratory were used for experimental work. It may be mentioned that algae collected from the field at different times of the year may vary in their physiological conditions, which may alter their response to toxicants. Also, seaweeds obtained from estuarine areas may contain pollutants present where the plants were collected. In such cases, problems arising from the interaction of the test chemicals with other pollutants may arise.

Cultures of Ulva lactuca, Enteromorpha linza, and Rhodymenia pseudopalmata were obtained from the University of Indiana Culture Collection of Algae. Field-collected Ulva sp., Enteromorpha sp. and Rhodymenia sp. were purchased from the Northeast Marine Specimens Company, Inc., Woods Hole, Massachusetts. The latter were maintained in aerated seawater in a cold room at 4°C under a light intensity of about 150 lux.

CULTURE CONDITIONS

Although the culture of unicellular marine phytoplankton has been studied in detail (Guillard and Ryther, 1962; McLachlan, 1964; Mclachlan, 1973; Provasoli et al, 1957) there is little information available on methods for culturing seaweeds. Since the proposed studies required the use of a relatively large amount of algal tissue, techniques for culturing seaweeds were developed to obtain sufficient plant material. Favorable conditions for growing or maintaining the organisms were determined by varying the factors most likely to affect the culturing process: (1) size of inoculum; (2) composition of the growth medium; and (3) light and temperature.

Inoculum

Vegetative Propagation - The algae used were obtained from the field or grown from stock cultures. Prior to being used, field-collected plants were maintained in aerated seawater in a cold room at 4°C under a light intensity of approximately 150 lux. To culture the organisms, sections of the algae were transferred to the appropriate growth medium contained in 80 x 100 mm Pyrex dishes and grown in a growth chamber under conditions of light and temperature described on page 6.

Propagation From Zoospores - Under laboratory conditions, Ulva and Enteromorpha may be induced to form reproductive structures (zoospores) which may then be used for establishing cultures. Zoospores were obtained by filtering one-month-old cultures of the algae through a double layer of cheesecloth. The number of zoospores per ml of culture was estimated using a hemocytometer. Approximately 2,000 spores were added to each culture dish (80 x 100 mm Pyrex dish) containing a sterilized growth medium and were incubated in the growth chamber. Approximately 5% of the added zoospores germinated.

Composition of the Growth Medium

Both synthetic and natural seawater media were tested for their ability to support adequate growth of the alge. The media were those of Erdschreiber (Føyn, 1934), Guillard and Rhyther (1962), ASP-6 (Provasoli, 1963; Provasoli et al, 1957), and an enriched synthetic seawater medium. Synthetic seawater was prepared by dissolving 40 gm of Rila Marine Mix (Rila Products, Teaneck, N.J.) in a liter of distilled water. The fresh synthetic seawter was aged in a glass aquarium with a metal frame containing thalli of field-collected Ulva. After one week, the seawater was removed and stirred with activated charcoal to remove dissolved organic matter. The medium was filtered, the salinity was adjusted to 33-34 ppt and the pH to 7.8. After autoclaving, each liter of the medium was supplemented with the following (Ott, 1973): $NaNO_3$, 200 mg; sodium glycerophosphate, 25 mg; $FeSO_4.7H_2O$, 4.98 mg; $ZnSO_4.7H_2O$, 8.82 mg; $MnCl_2.4H_2O$, 1.44 mg; MoO_3, 0.71 mg; $Co(NO_3)_2 \cdot 6H_2O$, 0.49 mg; H_3BO_3, 11.4 mg; Na_2EDTA, 56.6 mg; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 7.0 mg; biotin, 1 µg; vitamin B_{12}, 1 µg; thiamin HCl, 200 µg.

Light and Temperature

Ulva and Enteromorpha were grown under controlled conditions in a growth chamber, i.e., 14 hrs of light per day at 2150 lux and at 18°C ± 1°. These conditions proved more favorable than a light intensity of 5400 lux and a temperature of 20°C. Rhodymenia was cultured under a light intensity of 540 lux.

TREATMENT OF SEAWEEDS

Ulva

Only field-collected Ulva was used and experiments were begun less than 48 hrs after the tissue was received. Discs (5 cm in diameter) were cut from the Ulva thalli, rinsed in the growth medium and suspended in

a pesticide-saturated medium. At the end of the treatment period, smaller discs were cut from the larger discs for measuring physiological effects.

Enteromorpha

Preliminary experiments were conducted using individual germlings that were obtained by scraping them from the walls of a culture dish and transferring them to the growth medium. Attached germlings were used, however, in later studies because they grew faster and remained healthy longer. Enteromorpha germlings 3-4 weeks old were used for experimental work. Culture dishes containing germlings were removed from the growth chamber and the medium was replaced by one containing the pesticides. The treated cultures were then returned to the growth chamber.

Rhodymenia

Culture dishes containing Rhodymenia thalli were removed from the growth chamber. The thalli were rinsed with sterile growth medium and resuspended in a pesticide-saturated medium.

PHYSIOLOGICAL EFFECTS OF PESTICIDES

In these studies, the tissue was incubated in saturated solutions of pesticides prepared as described above.

Photosynthesis

Photosynthetic activities of the algae were determined by measuring $^{14}CO_2$ fixation by the tissue. To make this measurement, algal tissue was incubated in 25 ml of growth medium containing 0.90 μ moles of $NaH^{14}CO_3$ (1,200,000 dpm) for 30 min at 20°C under a light intensity of 2150 lux. The tissue was then removed from the medium, washed free of ^{14}C-sodium bicarbonate with medium, blotted dry, and analyzed for the amount of ^{14}C fixed. To analyze for ^{14}C the tissue was homogenized in a glass tissue-homogenizer with a liquid-scintillation solution

containing PPO, POPOP, Triton-X, and toluene. The radioactivity of the homogenate was measured in a Nuclear Chicago liquid scintillation counter.

In preliminary studies, photosynthesis was also measured by determining oxygen evolution by the tissue. Small sections of algal tissue were incubated in the growth medium containing 0.025M sodium bicarbonate and oxygen evolution was measured polarographically at 20°C at a light intensity of 2150 lux using a Yellow Springs Instrument Co. oxygen electrode.

Proteins

Protein content was determined according to the method of Strickland and Parsons (1965). The tissue was boiled for 1 min in 1 ml of distilled water and homogenized with 80% ethanol in a glass tissue-homogenizer. The homogenate was centrifuged, the supernatant was removed, and the pellet was extracted again with 80% ethanol. After the second extraction, the residue was suspended in 4 ml of 6N HCl and heated at 100°C for 4 hrs. The acid extract was centrifuged, the supernatant adjusted to a pH of 1 with NaOH, and the final volume made up to 10 ml with distilled water. One ml of this protein extract was mixed with 1 ml of 2,4-hexanedione reagent (1 ml of 2,5-hexanedione in 50 ml of 0.5M $Na_2CO_3 \cdot H_2O$) and the mixture was heated for 40 min at 100°C. The solution was then cooled rapidly, made up to 9 ml with 95% ethanol, and one ml of Ehrlich's reagent (800 mg dimethylaminobenzaldehyde dissolved in 30 ml of 95% ethanol and 30 ml of concentrated HCl) was added. After the reaction proceeded for 30 min at room temperature, the absorption was read at 530 nm. The concentration of proteins was calculated from a standard curve prepared from bovine serum albumin.

Carbohydrates

The algae were analyzed for their water-soluble and total carbohydrates content by the method of Ashwell (1957). The soluble carbohydrates were extracted by homogenizing fresh tissue in 1-2 ml of distilled water. The homogenate was boiled for one hour, centrifuged, and the supernatant

made up to 10 ml with distilled water. One ml of this extract was added to 9 ml of a 0.2% solution of anthrone dissolved in concentrated sulfuric acid, and the mixture heated for 15 min at 90°C. After cooling the solution to room temperature, its absorbance was measured at 625 nm, and the concentration of soluble carbohydrates was calculated from a standard curve perpared from glucose. For determination of total carbohydrates, the tissue was digested with 60% H_2SO_4 for 30 min. It was then centrifuged, and the supernatant was made up to 10 ml with 60% H_2SO_4. One ml of this was added to 9 ml of anthrone reagent, and the absorbance was measured at 625 nm.

Pigments

The procedure described by Strain et al (1971) was used to extract pigments from the algae. The tissue was softened by placing it in boiling distilled water for 1 min. It was then ground in a small volume of acetone and made up to a volume of 5-10 ml with 80% acetone. The homogenate was centrifuged, and the absorbance of the clear supernatant was measured spectrophotometrically in a Cary Model 14 spectrophotometer at 645, 663 and 480 nm. The methods of Kirk and Allen (1965), Kirk (1968), and Davies (1965) were used to calculate the concentrations of chlorophyll (a + b) and carotenoids, respectively.

Lipids

The amount of total lipids in the tissue was determined as described by Radin (1969). The tissue was lyopholyzed, weighed, and homogenized in a glass tissue homogenizer in chloroform:methanol (2:1). The homogenate was passed through a glass-fiber filter, the filter was washed with chloroform, and the residue was discarded. The filtrate was placed in a previously weighed aluminum weighing pan, the extract was evaporated almost to dryness on a hot plate and dried for about 18 hrs in an oven at 100°C. The pans were cooled in a dessicator, and the weight of the lipid residue was determined.

Trace Metals

The algal tissue was lyopholized and ground to a fine powder in a mortar and pestle. One gram of this tissue was digested with 5 ml of HNO_3 and 2 ml of 70% $HClO_4$. The digestate was evaporated to 3 ml, diluted to 20 ml with deionized, distilled water and passed through acid-washed filter paper. The filter paper was washed with water, and the filtrate was diluted to 50 ml with deionized water. A reagent blank was also prepared using the procedures described above, except that the tissue sample was excluded. A Perkin Elmer Model 303 atomic absorption spectrophotometer was used to analyze the extracts. To analyze for magnesium, a portion of the extract was diluted with a 1% (w/v) lanthanum solution, and the determinations were made against standard blanks containing a similar concentration of lanthanum. The other trace metals (Cu, Mn, Zn and Fe) were analyzed using an undiluted extract. Concentrations of the trace metals in the test solutions were determined from standard curves prepared for each metal.

UPTAKE AND METABOLISM OF MIREX, METHOXYCHLOR AND 2,4-D BY SEAWEEDS

These studies were done using ^{14}C-uniformly ring-labeled methoxychlor (sp. activity 4.03 mCi/mM), ^{14}C-uniformly ring-labeled 2,4-D (sp. activity 10.5 mCi/mM) and ^{14}C-uniformly labeled mirex (sp. activity 5.76 mCi/mM).

Uptake Studies

The algal tissue was incubated in the Rila medium containing ^{14}C-labeled methoxychlor, mirex, or 2,4-D. ^{14}C-pesticides were added as ethanolic solutions so that the final concentration of ethanol in the medium was 0.005%. The cultures were incubated on a reciprocating shaker under controlled environmental conditions described previously. At intervals over a 6-day period following treatment, the tissue from the cultures was sampled, washed, weighed and analyzed for total radioactivity. To

determine the amount of ^{14}C in the tissue, it was homogenized with scintillation fluid in a glass tissue homogenizer; the homogenate was then transferred to scintillation vials and counted for radioactivity.

Metabolism Studies

To study transformation of pesticides by the algae, both medium and tissue were analyzed for the parent compound and possible metabolites.

Mirex - The tissue was homogenized with acetone, the homogenate was centrifuged, and the extract was decanted. The residue was re-extracted with acetone, and the extracts were combined. The combination was concentrated under a stream of nitrogen and chromatographed on thin-layer silica-gel plates in the following solvent system: (i) hexane-acetone (9:1), and (ii) heptane (Mehendle et al, 1972; Jones and Hodges, 1974). The chromatograms were scanned for radioactivity in a Nuclear Chicago Actigraph.

The incubation medium was extracted three times with hexane. The three extracts were combined, and the amount of radioactivity in the aqueous and organic phases was determined. The hexane extract was concentrated, and an aliquot was chromatographed on a thin-layer silica gel plate as described above.

Methoxychlor - The tissue was homogenized with acetone in a glass tissue homogenizer, the homogenate was centrifuged, and the extract was decanted. The residue was then extracted with 80% acetone in the same manner. The extracts were combined, and acetone in the extract was removed under vacuum to give an aqueous solution. The latter was acidified to a pH of 2, extracted with ethyl ether, and the amounts of ^{14}C in the ether and aqueous phases were determined. The ether layer was concentrated and chromatographed on thin-layer silica gel plates in the following solvent systems: (i) benzene-acetic acid (9:1), (ii) petroleum ether-ethyl ether (9:1), (iii) hexane-acetone (8:2) and (iv) petroleum ether-chloroform-methanol (3:2:1). The chromatograms were scanned for radioactivity on a Nuclear Chicago Actigraph.

To analyze the medium for methoxychlor and its metabolites, it was acidified to a pH of approximately 2 and then extracted three times with ethyl ether. The ether extracts were combined, and the amounts of ^{14}C in the ether extract and the remaining aqueous fraction were determined. The ether extract was subjected to thin-layer chromatography as described above.

Synthesis of model metabolites of methoxychlor - The following model metabolites were synthesized as described by Kapoor et al (1970) for comparison with unknown metabolites produced by Enteromorpha: (i) 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene was prepared by refluxing 3.45 g technical analytical grade methoxychlor (99.2%) in 100 ml of ethanol that contained 0.75 g KOH; it was then recrystallized from ethanol; (ii) 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane was prepared by condensing 10.8 g of phenol with 7.4 g of anhydrous chloral in 250 ml of chloroform to which was added 7.4 g of anhydrous aluminum chloride at 4°C. Stirring continued at 20°C for 30 min and for a further 8 hrs at room temperature. The product was crystallized from methylene chloride with a trace of ethanol.

2,4-D - The tissue was homogenized with methanol, the homogenate was centrifuged, and the extract was decanted. The residue was then extracted with 80% methanol. The extracts were combined, and methanol in the extract was removed under vacuum. The remaining aqueous solution was acidified to a pH of approximately 2 and extracted with ethyl ether. The ether extract was concentrated in a rotary evaporator under reduced pressure and chromatographed on thin-layer silica gel plates in the following solvent systems described by Hamilton et al (1971): (i) chloroform, and (ii) n-butyl alcohol-benzene-water (1:9:10). The chromatograms were then scanned for radioactivity on a Nuclear Chicago Actigraph.

The medium was acidified to a pH of approximately 2 and extracted twice with ether. The ether extracts were pooled and the amounts of ^{14}C in the ether extract and the aqueous phase were determined. The ether extract was concentrated under a stream of nitrogen and chromatographed on thin-layer silica gel plates as described above.

SECTION III

RESULTS AND DISCUSSION

CULTURE CONDITIONS

Major problems encountered in these studies were: (i) an extremely slow growth rate of seaweeds cultured under laboratory conditions, and (ii) maintenance of field-collected algae in a healthy state in the laboratory for longer than one week. A considerable amount of time was spent determining culture conditions favorable for growing or maintaining the algae. The findings of these exploratory studies are described below.

Medium

Sections of thalli from the three algae appeared to grow better in the artificial seawater supplemented with trace elements and vitamins than on Guillard and Ryther's medium 'f' or on Provasoli's ASP-6 medium. Therefore, the artificial seawater medium was used in all subsequent studies. We believe that a synthetic medium with a well-defined composition is preferable for studies on effects of pollutants because seasonal and geographical variations in natural seawater affect certain physiological processes which may alter the response of organisms to pollutants. Furthermore, artificial seawater medium permits one to study only the effect of the pollutant under consideration, since under these conditions the possibility of interaction with other pollutants in natural waters is excluded.

Source of Inoculum

Although we were able to propagate both Ulva and Enteromorpha vegetatively from sections of thalli, growth was quite slow and did not yield sufficient plant material to study effects and metabolism of the pesticides. In an attempt to obtain a larger amount of tissue, we investigated the possibility of culturing the algae from zoospores. In the case of Enteromorpha, we were consistently able to obtain sufficient plant material when it was propagated from zoospores.

It was possible to induce Ulva to produce zoospores, but the germlings did not grow well. Since growth of Ulva, when propagated vegetatively or from zoospores, was not satisfactory, thalli collected from the field were used.

We were able to culture Rhodymenia vegetatively from sections of thalli obtained from the Indiana Culture Collection, but growth of the algae thus propagated was extremely slow. Rhodymenia collected from the ocean was not satisfactory for these studies since the tissue deteriorated rapidly, even when maintained in aerated seawater in a cold room at 4°C. Loss of pigments was noticed 24-48 hrs after the tissue was transferred to fresh growth medium in the growth chamber.

Based on the results of exploratory studies, the experiments were done under the following culture conditions:

Culture Medium: Artificially enriched seawater supplemented with trace elements and vitamins.

Temperature: 18°C $\pm$ 1°

Experimental Plant Material:

Enteromorpha: Germlings from zoospores

Ulva: Sections of field-collected thalli

Rhodymenia: Sections of laboratory-grown thalli

ANALYSIS OF SYNTHETIC SEAWATER MEDIUM FOR 2,4-D, MIREX, METHOXYCHLOR, DDT, DDD, DDE, DIELDRIN, TRACE METALS, AND ORGANIC MATTER

Prior to addition of trace elements and vitamins, the medium was analyzed for pesticides, trace metals, and organic matter. The concentrations of pesticides in the growth medium were below the limits of detection. Since the medium consisted of synthetic seawater and was filtered through charcoal before it was used, the amount of organic matter in the medium was expected to be negligible. Therefore, we did not analyze the medium for organic matter content.

PESTICIDE SOLUBILITY IN THE CULTURE MEDIUM

The maximum solubility of methoxychlor, mirex, and 2,4-D in synthetic seawater (33-34 ppt salinity) at 20°C was 22.8 ppb, 10.2 ppb, and 220 ppm, respectively. In these studies, we examined the effect of 2,4-D on seaweeds at a concentration equivalent to its maximum solubility in the medium, although it is unlikely that 2,4-D will be present in the marine environment at such a high concentration. However, lack of effect of pesticide at a concentration many times that occurring in the environment establishes the safety of a compound with regard to an organism.

PHYSIOLOGICAL EFFECTS OF METHOXYCHLOR, MIREX, AND 2,4-D

In these studies, the algae were exposed to the pesticides for an appropriate period after which the photosynthetic activity and concentrations of tissue constituents were measured. Enteromorpha and Rhodymenia were incubated with the pesticide for three weeks. Ulva was exposed to the pesticide for one week because the tissue disintegrated if maintained in the laboratory for a longer period. In studies concerning physiological responses of algae to pesticides, it would be desirable to measure response to a chemical at different intervals during the incubation period. However, since only a limited amount of algal tissue was available and a relatively long period was required to culture it, effects of pesticides were assessed only at the end of the treatment periods.

In the toxicity studies, the amount of tissue in 100 ml of medium ranged from 350-450 mg of fresh weight in the case of Ulva and Rhodymenia and from 450-650 mg for Enteromorpha.

Photosynthesis

In preliminary studies, effect of the pesticides on photosynthesis was determined by measuring oxygen evolution from algal tissue. The polarographic method for determining oxygen evolution permitted use of a very

small amount of tissue. Though it was possible to obtain a measurable rate of oxygen evolution, an inherent error in the weighing of small quantities of tissue produced variable results. Therefore, we studied effects of 2,4-D, mirex, and methoxychlor on photosynthesis by measuring $^{14}CO_2$-fixation, which has the following advantages: (1) this method permits use of larger amounts of plant tissue, thus reducing the magnitude of error encountered when weighing smaller amounts of tissue used in polarographic determinations; (2) the variations among replications in CO_2-fixation were much smaller than those for O_2 determinations; (3) the $^{14}CO_2$-fixation method is less time-consuming than that used to make O_2 determinations; and (4) the rapid stirring required in measuring O_2 exchanged appears to physically damage algal tissue. The $^{14}CO_2$-fixation method requires only gentle agitation to achieve adequate gas exchange.

Mirex, methoxychlor, and 2,4-D failed to inhibit photosynthesis in Ulva, Enteromorpha, or Rhodymenia when the algae were incubated with saturating concentrations of the pesticides (Table 1).

Lack of effect of 2,4-D on photosynthesis by the seaweeds can be explained by the fact that the herbicide does not enter the tissue in sufficient amounts, as shown by uptake studies (Table 9). In our experiments, 2,4-D in the growth medium (pH 7.8) was present mostly in the ionized form. Since only the undissociated 2,4-D molecule penetrates the cell membrane readily, the concentration of 2,4-D in the algae under the conditions of these experiments is expected to be low. Wedding et al (1954) and Erickson et al (1955) observed that inhibition of photosynthesis by 2,4-D in Chlorella was related to concentration of undissociated 2,4-D acid molecules in the bathing medium. With a decrease in pH, the same concentration of 2,4-D caused a greater inhibition of photosynthesis. The inhibition by 2×10^{-3} M 2,4-D (440 ppm) was complete at a pH of 3-4 while at pH values above 6 there was no inhibition. Their results suggest that the undissociated 2,4-D molecule is the effective agent in inhibiting photosynthesis.

Table 1. PHOTOSYNTHESIS IN SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D [a]

Pesticide	Organism	Photosynthetic $^{14}CO_2$-Fixation % Control[b]
Methoxychlor	Enteromorpha	105.5
	Ulva	111.7
	Rhodymenia	108.0
Mirex	Enteromorpha	99.0
	Ulva	102.0
	Rhodymenia	94.0
2,4,-D	Enteromorpha	101.0
	Ulva	98.7
	Rhodymenia	96.0

a. The seaweeds were exposed to pesticide concentrations representing their maximum solubility in seawater. Treatment times for Enteromorpha, Rhodymenia, and Ulva were 3, 3 and 1 weeks, respectively.
b. Mean of two experiments.

The effects of mirex, methoxychlor, and 2,4-D on growth and photosynthesis in phytoplankton have been reported by other workers. However, in most studies, effects were examined at concentrations exceeding the maximum solubility of the pesticides in water. Sikka and Rice (1974) observed that methoxychlor at a concentration of 100 ppb significantly reduced growth of three marine algae, Skeletonema, Tetraselmis, and Thalassiosira, but had no effect on Dunaliella and Porphrydium. At 50 ppb, the pesticide was toxic only to Skeletonema. Butler (1963) reported a slight decrease in carbon fixation by estuarine phytoplankton following a 4-hr exposure to 1 ppm of methoxychlor. de la Cruz and Naqvi (1973) examined the effect of mirex on photosynthesis and respiration in fresh-water phytoplankton. Photosynthesis was reduced by 16, 10, 33, and 19% in a

naturally occurring phytoplankton population after 5, 10, 15, and 20 days exposure to 1 ppb of mirex. Treatment of a pure culture of Chlamydomonas with 1 ppm of mirex for 7 days reduced photosynthesis by about 55%. Moore (1972) reported that mirex was not toxic to marine phytoplankton populations exposed for 24-48 hrs to concentrations ranging from 0.5 ppb to 1 ppm. Butler (1963), on the other hand, reported a 42% decrease in $^{14}CO_2$-fixation by estuarine phytoplankton following a 4-hr exposure to 1 ppm of mirex. Hollister et al (1975) observed that 0.2 ppb of mirex did not affect either population growth or oxygen evolution of six species of marine unicellular algae.

The available information on toxicity of 2,4-D indicates that the pesticide has little or no effect on phytoplankton even at very high concentrations. Elder et al (1970) reported that 2,4-D had no effect on the growth of fresh-water and marine algae tested at a concentration representing the maximum solubility of the herbicide in water (240 ppm). In another study, 2,4-D up to a concentration of 400 ppm did not inhibit growth of the algae Chlorella vulgaris, Chlorococcum sp., and Cylindrospermum lichenforme (Arvik et al, 1971). Wedding et al (1954) observed that 2,4-D at a concentration of 2×10^{-3}M (440 ppm) in the culture medium at pH above 6 did not inhibit photosynthesis in Chlorella. Treatment of estuarine phytoplankton with 1 ppm of 2,4-D for 4 hrs did not reduce photosynthesis (Butler, 1963). However, Walsh (1972) reported that treatment of four species of marine phytoplankton with 50-75 ppm of the herbicide for 10 days reduced growth by about 50%.

On the basis of our findings and those of other workers, it appears that mirex, methoxychlor, and 2,4-D do not adversely affect growth and photosynthesis in phytoplankton and seaweeds at concentrations several times those found in natural waters.

Effect of Mirex, Methoxychlor, and 2,4-D on Tissue Composition

To learn if 2,4-D, mirex, or methoxychlor induce changes in algal composition, the tissues were analyzed for protein, carbohydrates, total lipids,

pigments, and trace elements following incubation with saturating concentrations of the pesticides. As in the photosynthesis studies, both Enteromorpha and Rhodymenia were exposed to the pesticides for three weeks, whereas Ulva was treated for one week. Unless otherwise indicated, the data are the average of two experiments.

Protein - The data in Table 2 show that saturating concentrations of 2,4-D, mirex, or methoxychlor did not significantly influence the protein content of Ulva, Enteromorpha, and Rhodymenia.

Table 2. PROTEIN CONTENT OF SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D

Pesticide	Organism	Protein % Control
Methoxychlor	Enteromorpha	113.3
	Ulva	93.0
	Rhodymenia	113.5
Mirex	Enteromorpha	87.0
	Ulva	95.0
	Rhodymenia	110.7
2,4-D	Enteromorpha	110.7
	Ulva	97.3
	Rhodymenia	88.0

Carbohydrates - Treatment with 2,4-D, mirex, or methoxychlor failed to alter the total carbohydrate content in the three algae (Table 3). However, mirex and 2,4-D increased the amount of soluble carbohydrates present in Enteromorpha by 34 and 44%, respectively. The changes in the concentrations of soluble carbohydrates would seem to be indicative of

either a mobilization of polysaccharides or a reduction in rate of incorporation of soluble carbohydrates into polysaccharides.

Table 3. TOTAL AND SOLUBLE CARBOHYDRATE CONTENT OF SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D

Pesticide	Organism	Total Carbohydrate % Control	Soluble Carbohydrate % Control
Methoxychlor	Enteromorpha	97.5	110.5
	Ulva	99.3	91.7
	Rhodymenia	93.0	99.5
Mirex	Enteromorpha	97.5	134.5
	Ulva	102.0	92.0
	Rhodymenia	89.5	112.0
2,4-D	Enteromorpha	89.0	144.5
	Ulva	98.7	93.0
	Rhodymenia	84.0	104.5

Pigments - The data in Table 4 show that saturating concentrations of 2,4-D, mirex, or methoxychlor did not change chlorophyll concentration in Ulva, Enteromorpha, or Rhodymenia. The carotenoid content of the algae was unaffected by mirex or methoxychlor. However, 2,4-D increased the concentration of carotenoids in Enteromorpha and Rhodymenia but had little effect on the pigment content in Ulva.

Lipids - The lipid content of the three algae was essentially unaffected by saturating concentrations of 2,4-D, mirex, or methoxychlor (Table 5). Sumida and Ueda (1973) reported that treatment of Chlorella elipsoidea with 5 ppm of 2,4-D produced no change in lipid content of the alga.

Table 4. PIGMENT CONTENT OF SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D

Pesticide	Organism	Chlorophylls % Control	Carotenoids % Control
Methoxychlor	Enteromorpha	118.7	115.0
	Ulva	106.3	91.0
	Rhodymenia	99.0	95.0
Mirex	Enteromorpha	106.3	108.7
	Ulva	96.0	89.5
	Rhodymenia	93.5	109.5
2,4-D	Enteromorpha	89.3	129.0
	Ulva	88.3	91.3
	Rhodymenia	106.5	124.0

Table 5. TOTAL LIPID CONTENT OF SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D

Pesticide	Organism	Lipids % Control
Methoxychlor	Enteromorpha	98.0
	Ulva	104.0
Mirex	Enteromorpha	104.0
	Ulva	99.0
2,4-D	Enteromorpha	100.0
	Ulva	117.0

Trace Metals Content - Table 6 shows the Mg, Cu, Mn, Fe, and Zn content of Enteromorpha and Ulva treated with methoxychlor, mirex, and 2,4-D. Methoxychlor at saturating levels did not affect the trace metal content of Enteromorpha or Ulva. Treatment with mirex increased the concentrations of Fe and Mn in Ulva and of Cu in Enteromorpha, but did not change those of the other metals in the two algae. Incubation with 2,4-D had no effect on the concentration of trace metals in Ulva. However, in Enteromorpha, saturating concentrations of this herbicide decreased the Mg and Zn content and slightly increased the Cu content. The pesticide showed no effect on content of Fe and Mn. The findings suggest that the three pesticides have no marked effect on the concentration of the above-mentioned trace metals in Ulva and Enteromorpha.

Table 6. TRACE METAL CONTENT OF SEAWEEDS TREATED WITH METHOXYCHLOR, MIREX, AND 2,4-D

Pesticide	Organism	Mg	Cu	Mn	Fe	Zn
		% Control				
Methoxychlor	Enteromorpha	96.5	95.5	103.0	94.5	105.0
	Ulva	99.0	109.0	100.0	91.0	109.0
Mirex	Enteromorpha	102.0	139.0	92.0	85.5	86.5
	Ulva	113.0	86.0	164.0	134.0	81.0
2,4-D	Enteromorpha	67.0	129.0	98.0	103.5	61.0

Effects of the pesticides on the trace-metal content of Rhodymenia was not examined since it could not be grown in sufficient quantities. The amount of boron and cobalt in Ulva and Enteromorpha could not be determined since their concentrations in the algae were below the limit of detection.

UPTAKE OF METHOXYCHLOR, MIREX, AND 2,4-D

Methoxychlor

The uptake (absorption and/or adsorption) of ^{14}C-methoxychlor by all three species was rapid. More than 50% of the maximum methoxychlor uptake by a given species occurred within three hrs of treatment. In the cases of Ulva and Enteromorpha, uptake of pesticide reached its maximum 24 hrs after treatment, whereas the maximum uptake was observed 48 hrs after treatment of Rhodymenia (Table 7). In all three organisms, there was a gradual decline in concentration of radioactivity once maximum accumulation had been reached. The three algae varied in their ability to accumulate methoxychlor from the medium, and they accumulated the pesticide in the following order: Enteromorpha > Ulva > Rhodymenia.

Table 7. UPTAKE OF ^{14}C-METHOXYCHLOR BY SEAWEEDS[a]

Organism	Concentration of ^{14}C-Residue in the Organism-ppm (expressed as Methoxychlor equivalent [b])				
	Hours After Treatment				
	3	6	24	48	72
Enteromorpha	106	117	165	120	102
Ulva	25.7	29.2	36.1	21.3	24.8
Rhodymenia	3.9	7.2	8.7	9.0	8.0

[a]Concentration of ^{14}C-methoxychlor in the medium at the time of treatment was 25 ppb.
[b]Calculated from the specific activity of ^{14}C-methoxychlor.

Mirex

The three species readily removed ^{14}C mirex from the medium. However, uptake was relatively slow as compared to that of methoxychlor. The algae continued to remove mirex up to 144 hrs after treatment, when the experiment was terminated (Table 8), whereas the concentration of

methoxychlor in the algae reached its maximum within 24-48 hrs (Table 7). Enteromorpha was more effective in removing mirex from the medium than Ulva or Rhodymenia, which did not differ greatly in their ability to accumulate the insecticide.

Table 8. UPTAKE OF ^{14}C-MIREX BY SEAWEEDS[a]

Organism	Mirex Concentration in the Organism (ppm)					
	Hours After Treatment					
	3	6	24	48	72	144
Enteromorpha	1.0	1.0	1.6	4.3	8.8	15.7
Ulva	1.0	1.5	2.6	3.1	4.3	4.8
Rhodymenia	0.9	1.6	3.0	4.3	4.9	6.3

[a]Concentration of ^{14}C-mirex in the medium at the time of treatment was 15 ppb.

Although mirex is more lipid-soluble than methoxychlor, its uptake by the seaweeds was less than that of methoxychlor. It is possible that uptake of mirex was limited by its molecular weight (546) which is higher than that of methoxychlor (345).

2,4-D

Although maximum solubility of 2,4-D in the growth medium used was about 200 ppm, uptake of the pesticide was studied at a concentration of 25 ppb. We chose this concentration because it approximates the concentration of 2,4-D in water in the estuarine environment. Since this is close to the concentration at which uptake of mirex and methoxychlor was determined, the results would also permit us to compare the relative uptake of the three pesticides by the algae. Maximum uptake of 2,4-D by the algae occurred within 24 hrs of treatment, after which the concentration of radioactivity in the tissue did not change (Table 9). Uptake of 2,4-D

was low, the total amount of 2,4-D absorbed varying from 0.01 to 0.03% of the pesticide added to the culture medium. Rhodymenia and Ulva appeared to remove somewhat greater amounts of 2,4-D from the medium than Enteromorpha.

Table 9. UPTAKE OF ^{14}C-2,4-D by SEAWEEDS[a]

Organism	2,4-D Concentration in the Organism (ppb)			
	Hours After Treatment			
	6	24	48	72
Enteromorpha	0.01	0.01	0.01	0.01
Ulva	0.13	0.44	0.42	0.34
Rhodymenia	0.11	0.28	0.20	0.19

[a]Concentration of ^{14}C-2,4-D in the medium at the time of treatment was 25 ppb.

Our results are similar to those reported by Valentine and Bingham (1974) on uptake of ^{14}C-2,4-D by the fresh water algae Chlorella pyrenoidosa, Scenedesmus quadricauda, Chlamydomonas reinhartii, and Euglena gracilis. Only S. quadricauda removed a measurable amount of 2,4-D from the medium but only at a pH below 6. A greater uptake at low pH was presumably caused by a relatively high concentration of the undissociated 2,4-D molecule which may enter the cell more readily than the ionized form. In the marine environment, where the average pH is 8.3, 2,4-D would be expected to be present in an ionized form (pK of about 3) and consequently its uptake by the cells may not be high, possibly because of an interaction between charged groups on the cell surface and the ionized carboxyl group of 2,4-D.

A separate experiment was conducted to study uptake of 2,4-D by Ulva as a function of pesticide concentration in the medium. Since uptake of

2,4-D by the alga was extremely low, the biomass of Ulva in the medium was increased to 2.75 g/100 ml to give a greater amount of 2,4-D in the tissue. Uptake increased linearly with increase in concentration of the pesticide from 2 to 200 ppm. When Ulva was incubated in a medium containing 2, 10, and 200 ppm of 2,4-D, the concentration of pesticide in the tissue was 12.4, 58.9, and 1150 ppb, respectively, 96 hrs after treatment.

BIOACCUMULATION OF METHOXYCHLOR, MIREX, AND 2,4-D

All three algae accumulated both mirex and methoxychlor from the medium (Table 10). In the case of Enteromorpha treated with methoxychlor, a small percentage of the total ^{14}C in the tissue was present as ^{14}C-

Table 10. BIOACCUMULATION OF METHOXYCHLOR, MIREX, AND 2,4-D BY SEAWEEDS[a]

Pesticide	Organism	Bioaccumulation Factor[b]
Methoxychlor	Enteromorpha	5375
	Ulva	1174
	Rhodymenia	289
Mirex	Enteromorpha	1112
	Ulva	332
	Rhodymenia	419
2,4-D	Enteromorpha	.001
	Ulva	.001
	Rhodymenia	.003

[a] ^{14}C-Methoxychlor, mirex and 2,4-D were added at initial concentration of 25, 10, and 25 ppb in the medium, respectively.

[b] Concentration of pesticide in the tissue/concentration of pesticide in the medium; calculated at the time of maximum pesticide uptake by the tissue.

metabolites. Enteromorpha accumulated considerably more mirex and methoxychlor than Ulva or Rhodymenia. Although the algae accumulated both methoxychlor and mirex from the medium, bioaccumulation of methoxychlor was greater than that of mirex except in Rhodymenia. The concentration of ^{14}C-methoxychlor and its metabolites in the algae three days after treatment ranged from about 300 to 5400 times the concentration in the medium. In the case of algae treated with ^{14}C-mirex, the bioaccumulation factor ranged from about 350 to 1100.

Like other chlorinated hydrocarbon pesticides, mirex and methoxychlor have been reported to undergo bioaccumulation in various organisms. Sikka and Rice (1974) observed that five species of marine phytoplankton accumulated methoxychlor from a medium containing 20 ppb of the pesticide. Concentrations of methoxychlor in the algal species were 710 to about 8200 times greater than that in the medium. In a fresh-water model ecosystem, methoxychlor was found in fish as a concentration 1500 times that of the water (Kapoor et al, 1970). Bioaccumulation of mirex by various species of aquatic organisms has been reported (Butler, 1969; Cooley et al, 1972; Borthwick et al, 1973; Wolfe and Norment, 1973; Hollister et al, 1975). In a fresh-water model ecosystem, mirex was concentrated 214- and 1165-fold in fish and snails, respectively (Metcalf et al, 1973).

Although both mirex and methoxychlor appear to have no adverse effects on the seaweeds at the concentrations tested, their bioaccumulation is of significance. Pesticide accumulated by algae may be transferred to the higher trophic levels and thereby may have an impact on the estuarine ecosystem. In contrast to mirex and methoxychlor, 2,4-D did not accumulate in the seaweeds. Wojtalik et al (1971) reported no harmful effects or accumulation in zooplankton, phytoplankton, or microinvertebrates in water treated at 20 or 50 lb of 2,4-D/acre. Hence, unlike the chlorinated hydrocarbon insecticides, there is little danger of biomagnification of 2,4-D.

METABOLISM OF METHOXYCHLOR, MIREX, AND 2,4-D

These studies were done using Ulva and Enteromorpha. Metabolism of the pesticides by Rhodymenia was not investigated because the algal tissue was not available in sufficient quantities. To learn if the pesticides were metabolized, tissues and the culture media were analyzed for the parent compounds and their possible metabolites after the organisms had absorbed the maximum amounts of pesticides as determined from the results of the uptake studies.

Methoxychlor

Enteromorpha - To obtain relatively large amounts of the methoxychlor metabolites produced by Enteromorpha, 50 culture dishes, each containing approximately 500 mg of germling tissue, were incubated with 500 ppb of ^{14}C-methoxychlor. After 7 days, both the tissue and medium were extracted separately as described in the Methods section, and the extracts were concentrated. To remove pigments and other extraneous material coextracted from the tissue and the medium, the concentrated extract was applied in a narrow band on several 1-mm preparative thin-layer silica gel plates, which were developed in chloroform. This procedure separated essentially all the ^{14}C-compounds from the pigments, which were found between 5 and 6 cm from the origin. The area of silica gel containing the radioactive material was scraped off the plate and extracted twice with chloroform; the extract was then filtered to remove the silica gel. The extract was concentrated by flash evaporation, and about 96% of the ^{14}C material was recovered. Aliquots of the extracts were spotted on thin-layer silica gel plates, which were then developed in the following solvent systems.

I. Petroleum ether:ethyl ether (9:1)
II. Hexane:acetone (8:2)
III. Petroleum ether: chloroform:methanol (3:2:1)
IV. Benzene:acetic acid (9:1)

The chromatograms were scanned for radioactivity on a Nuclear Chicago Actigraph. The ^{14}C-containing areas were scraped off the plate into liquid scintillation vials and counted for radioactivity.

^{14}C-Analysis of tissue - Two successive extractions with acetone and 80% acetone removed more than 95% of the radioactivity from the tissue. Thin-layer chromatography of the purified extract in solvent systems I and II revealed the presence of methoxychlor and a minor metabolite which co-chromatographed with authentic 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene (MPDE). Solvent systems III and IV did not effectively separate the metabolite from methoxychlor. The relative amounts of ^{14}C-methoxychlor and the metabolite in the tissue 7 days after treatment are shown in Table 11 along with their respective Rf values.

Table 11. DISTRIBUTION OF ^{14}C-METHOXYCHLOR AND ^{14}C-MPDE[a] IN ENTEROMORPHA

Compound	% of ^{14}C in Acetone Extract	Rf Value in Solvent System I	Rf Value in Solvent System II
Methoxychlor	96.8	0.51	0.62
MPDE	3.2	0.60	0.68

[a]2,2-bis (p-methoxyphenyl)-1,1,1-dichloroethylene

^{14}C-Analysis of culture medium - Extraction of the culture medium with ether following acidification removed more than 90% of the total radioactivity in the medium. Thin-layer chromatography of the ether extract in the solvent systems revealed that radioactivity in the extract was present in the form of ^{14}C-methoxychlor and six minor metabolites. Two of the latter were identified as 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene and 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane by

co-chromatography with authentic standards. System IV gave the best resolution of the various ^{14}C-compounds, but it did not effectively separate methoxychlor and MPDE. Systems I and II separated the two compounds. The relative amounts of ^{14}C-methoxychlor and various metabolites in the ether extract of the medium are given in Table 12. Because only extremely small amounts of the metabolites were present, they could not be identified by spectral methods.

Table 12. DISTRIBUTION OF ^{14}C-METHOXYCHLOR AND ITS METABOLITES IN THE MEDIUM

Compound	% of ^{14}C in Ether Extract	Rf Value in Solvent Systems		
		I	II	IV
Methoxychlor	84.0	0.51	0.62	0.90
MPDE[a]	4.6	0.60	0.68	0.90
HPTE[b]	1.0			0.29
Unknown 1	0.7			0.15
" 2	2.1			0.40
" 3	4.3			0.52
" 4	3.3			0.64

[a]2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene

[b]2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane

Enteromorpha metabolized methoxychlor although only to a limited extent. The presence of 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene and 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane suggests that methoxychlor undergoes dehydrochlorination and O-demethylation in cultures of Enteromorpha. Another chlorinated hydrocarbon pesticide, DDT, is also dehydrochlorinated by marine algae to produce DDE (Rice and Sikka, 1973). O-demethylation of methoxychlor has also been reported in other

biological systems. Kapoor et al (1970) observed that methoxychlor was converted by O-demethylation into 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane in mice. However, they found no evidence of dehydrochlorination of the pesticide.

Since the Enteromorpha plants were not axenic, metabolism of the pesticide may have resulted from the action of the algae or microorganisms present in the medium, or a combination of both. To ascertain the role of the microorganisms and non-biological factors in methoxychlor metabolism, the transformation of the pesticide was studied in the following systems: (1) ^{14}C-methoxychlor was incubated in the culture medium after 3-week old germlings had been removed (hereafter referred to as old medium); the purpose was to determine if microorganisms in the medium were capable of metabolizing the pesticide; (2) the pesticide was incubated in the old medium without algae, but supplemented with glucose and nutrient broth to enhance the growth of microorganisms; (3) methoxychlor was incubated in a sterile fresh medium to account for any non-biological conversion. In each treatment the pesticide was incubated for 7 days, after which the algae, microorganisms, and the medium were extracted separately and analyzed for ^{14}C-methoxychlor and its possible metabolites by chromatography on thin-layer silica gel plates and radiochromatographic scanning.

No transformation of ^{14}C-methoxychlor was observed in the old medium with or without an exogenous source of carbon. In these treatments, all of the ^{14}C was present in the form of a single compound which co-chromatographed with authentic methoxychlor. These findings suggest that microorganisms in the culture medium did not metabolize methoxhchlor. No transformation of pesticide was observed in fresh, sterilized growth medium. These findings demonstrate that the metabolism of methoxychlor in the cultures of Enteromorpha resulted primarily from the action of algae, although the role of microorganisms associated with the plant surface cannot be ruled out.

Ulva - The tissue and the incubation medium were analyzed after 7 days of incubation with ^{14}C-methoxychlor. Thin-layer chromatographic analysis of extracts of the tissue and medium showed that all radioactivity in these extracts was present as unchanged methoxychlor. Our findings show that Ulva and Enteromorpha differ in their ability to metabolize methoxychlor.

Mirex

Thin-layer chromatographic analysis of the extracts of Ulva, Rhodymenia, and Enteromorpha incubated with ^{14}C-mirex for 7 days revealed the presence of one compound which co-chromatographed with authentic mirex. When the hexane extract of the culture medium was chromatographed, only one spot with an Rf value of 0.93 in hexane:acetone (8:2) and 0.79 in heptane could be detected. This compound co-chromatographed with authentic mirex in both solvent systems. The results show that neither organism was able to metabolize mirex. Our findings support the observations of other workers that mirex is resistant to transformation by plants and microorganisms (Mehendle et al, 1972; Jones and Hodges, 1974).

2,4-D

Extremely low uptake of 2,4-D by the seaweeds made it necessary to use a relatively large amount of algal tissue in the metabolism studies so that the pesticide or its metabolites might be extracted in quantities sufficient for their characterization by conventional methods. To study the metabolism of 2,4-D by Ulva and Enteromorpha, the algae were incubated with 2 ppm of ^{14}C-2,4-D for 4 days. Both the tissues and medium were then analyzed separately for 2,4-D and its metabolites.

All of the ^{14}C in the methanol extract of Ulva and Enteromorpha incubated with 2,4-D for 4 days was present in the form of a single compound which co-chromatographed with 2,4-D in the following solvent systems: (1) butanol:benzene:water (1:9:1), Rf 0.04; and (2) chloroform, Rf 0.11.

Thin-layer chromatography of the ether extract of the medium did not reveal the presence of any ^{14}C-compound other than 2,4-D. These findings indicate that neither Ulva nor Enteromorpha is able to transform 2,4-D. It may be pointed out that because of extremely low uptake of 2,4-D by the algae, it would be difficult to detect herbicide metabolites which may have been formed in small amounts by these organisms.

SECTION IV

CONCLUSIONS

1. Methoxychlor, mirex, and 2,4-D at concentrations corresponding to their maximum solubility in water do not adversely affect photosynthesis and composition of Ulva sp., Enteromorpha sp., and Rhodymenia sp.

2. The seaweeds accumulate mirex and methoxychlor but not 2,4-D, which is taken up by the organisms in extremely small amounts.

3. Although the seaweeds accumulate mirex and methoxychlor, they vary in their ability to concentrate the pesticides.

4. Ulva and Enteromorpha do not metabolize mirex and 2,4-D. Enteromorpha, however, can transform methoxychlor to a minor extent.

5. We conclude that methoxychlor, mirex, and 2,4-D, at concentrations approaching their maximum solubility in seawtater, do not adversely affect the seaweeds. However, the ability of the algae to accumulate mirex and methoxychlor suggests that these organisms may act as physical agents in transporting the pesticides in an estuarine ecosystem.

SECTION V

RECOMMENDATIONS

1. In the present studies, mirex, methoxychlor, and 2,4-D were used in highly purified forms. It is recommended that for purposes of comparison, effects of the pesticides in the form of commercial formulations be examined. It is possible that the pesticide formulation may contain by-products and other impurities which may alter toxicity of the pesticide to algae.

2. It is likely that in an estuarine environment, algae will be exposed to more than one pollutant at a time and that problems arising from the interaction of a pesticide with other toxicants may result. Therefore, it is recommended that the effects and fate of mirex, methoxychlor, and 2,4-D in algae be studied in the presence of other pesticides and environmental contaminants.

3. The pesticides may be converted into other products as a result of biological and/or non-biological transformation in an estuarine environment. To fully assess the effects of mirex, methoxychlor, and 2,4-D on estuarine algae, we suggest that effects of known metabolites of these pesticides be examined.

4. Algae growing in estuaries are subjected to changes in environmental factors, such as temperature, salinity, and nutrients. Since an algal species may become more susceptible to an outside stress under environmental conditions which are not optimal for that species, it is recommended that the effects and metabolism of mirex, methoxychlor, and 2,4-D be examined in algae growing under varying environmental conditions.

SECTION VI

REFERENCES

Alley, E. G. The Use of Mirex in Control of the Imported Fire Ant. J. Environ. Quality, 2:52-54, 1973.

Arvik, J. H., D. L. Willson and L. C. Darlington. Response of Soil Algae to Picloram-2,4-D Mixtures. Weed Sci. 19:276-278, 1971.

Ashwell, G. Colorimetric Analysis of Sugars. In: Methods of Enzymology, Colowick, S. P. and N. O. Kaplan (ed.). New York, Academic Press, 1957. p. 73-105.

Borthwick, P. W., T. W. Duke, A. J. Wilson, J. I. Lowe, J. M. Patrick, and J. C. Oberheu. Accumulation and Movement of Mirex in Selected Estuaries of South Carolina. Pestic. Monit. J. 7:6-14, 1973.

Burdick, G. E., H. J. Dean, E. J. Harris, J. Skea, C. Frissa and C. Sweeney. Methoxychlor as a Black Fly Larvicide, Persistence of its Residues in Fish and its Effect on Stream arthropods. J. N. Y. Fish Game, 15:121-142, 1968.

Butler, P. A. Commercial Fisheries Investigations. In: Pesticide-Wildlife Studies, Fish and Wildlife Service, Department of the Interior Cir. 167, Washington, D.C., 1963. p. 11-25.

Butler, P. A. Monitoring Pesticide Pollution. Bioscience, 19:889-891, 1969.

Cooley, N. R., J. M. Keltner and J. Forester. Mirex and Archlor 1254 - Effect on and Accumulation by Tetrahymena pyroformis. J. Protozool. 19:636-638, 1972.

de la Cruz, A. A. and S. M. Naqvi. Mirex Incorporation in the Environment: Uptake in Aquatic Organisms and Effects on the Rates of Photosynthesis and Respiration. Arch. Environ. Contam. Toxicol. 1:255-265, 1973.

Davies, B. H. Analysis of Carotenoid Pigments. In: Chemistry and Biochemistry of Plant Pigments, Goodwin, T. W. (ed.). New York, Academic Press, 1965. p. 489-532.

Elder, J. H., C. A. Lembi and D. J. Morre. Toxicity of 2,4-D and Picloram to Fresh Water Algae. National Tech. Inform. Service, Report No. PB 199114, 1970, 13 p.

Erickson, L.C., R.T. Wedding and B.L. Brannaman. Influence of pH on 2,4-dichlorophenoxyacetic Acid and Acetic Acid Activity in Chlorella. Plant Physiol. 30:69-74, 1955.

Føyn, B. Lebenzyklus, Cytologie und Sexualitat der Chlorophycee Cladophora suhriana. Kutz. Arch. Protistenk. 83:1-56, 1934.

Guillard, R.R.L. and J.H. Ryther. Studies of Marine Planktonic Diatoms. Can. J. Microbiol. 8:229-239, 1962.

Hamilton, R.H., J. Hurter, J.K. Hall and C.D. Ercegovich. Metabolism of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic Acid by Bean Plants. J. Agr. Food Chem. 19:480-483, 1971.

Hollister, T.A., G.E. Walsh and J. Forester. Mirex and Marine Unicellular Algae: Accumulation, Population Growth and Oxygen Evolution. Bull. Environ. Contam. Toxicol. 14:753-759, 1975.

Jones, A.S. and C.S. Hodges. Persistence of Mirex and its Effects on Soil Microorganisms. J. Agr. Food Chem. 22:435-439, 1974.

Kirk, J.T.O. Studies of the Dependence of Chlorophyll Synthesis on Protein Synthesis in Euglena gracilis, Together with a Nomogram for Determination of Chlorophyll Concentration. Planta (Berlin) 78:200-207, 1968.

Kirk, J.T.O. and R.L. Allen. Dependence of Chloroplast Pigment Synthesis on Protein Synthesis: Effect of Actidione. Biochem. Biophys. Res. Comm. 21:523-530, 1965.

Kapoor, I.P., R.L. Metcalf, R.F. Nystrom and G.K. Sangha. Comparative Metabolism of Methoxychlor, Methiochlor, and DDT in Mouse, Insects and in a Model Ecosystem. J. Agr. Food Chem. 18:1145-1152, 1970.

Lawrence, J.M. Aquatic Herbicide Data. USDA Agric. Handbook No. 231. 1962.

McLachlan, J. Some Considerations of the Growth of Marine Algae in Artificial Media. Canad. J. Microbiol. 10:769-782, 1964.

McLachlan, J. Growth Media-Marine. In: Phycological Methods (Janet R. Stein, ed.) Cambridge University Press, 1973, p. 25-51.

Mehendle, H.M., L. Fishbein, M. Fields and H.B. Matthews. Fate of Mirex-^{14}C in the Rat and Plants. Bull. Environ. Contam. Toxicol. 8:200-207, 1972.

Metcalf, R.L., I.P. Kapoor, P.Y. Lu, C.K. Schuth and P. Sherman. Model Ecosystem Studies of the Environmental Fate of Six Organochlorine Pesticides. Environ. Health Perspect. 4:35-44, 1973.

Moore, S. A. Some Effects of Selected Organochlorine Compounds on Radiocarbon Uptake by Marine Phytoplankton Populations in St. George Sound, Florida. Abstracts, 35th Annual Meeting Amer. Soc. Limnol. and Oceanogr., Tallahassee, Fla., 1972.

Ott, F.D.. Personal Communication, 1973.

Pimental, D. Ecological Effects of Pesticides on Non-target Species. U.S. Govt. Printing Office, Washington, D.C., 1971.

Provasoli, L. Growing Marine Seaweeds. In: Proceedings 4th International Seaweed Symposium. 1963. p. 9-17.

Provasoli, L., J. A. McLaughlin and M. R. Droop. The Development of Artificial Media for Marine Algae. Arch. Microbiol. 25:392-428, 1957.

Radin, N. S. Preparation of Lipid Extracts. In: Methods in Enzymology. J. M. Lowenstein (ed.) 14:245-254, 1969.

Rice, C. P. and H. C. Sikka. Uptake and Metabolism of DDT by Six Species of Marine Algae. J. Agr. Food Chem. 21:148-152, 1973.

Sikka, H. C. and C. P. Rice. Effects, Uptake and Metabolism of Methoxychlor in Marine Algae. In: Interaction of Selected Pesticides With Marine Microorganisms. Research Report to the Office of Naval Research. 1974. p. 33-47.

Strain, H. H., B. T. Cope and W. A. Suec. Analytical Procedures for the Isolation, Identification, Estimation and Investigation of the Chlorophylls. In: Methods in Enzymology, San Pietro, A. (ed.). 14:452-476, 1971.

Strickland, J. D. H. and T. R. Parsons. A Manual of Sea Water Analysis (2nd ed.). Fisheries Research Board of Canada Bulletin, No. 125. Ottawa, 1965. 203 p.

Sumida, S. and M. Ueda. Studies of Pesticide Effects on Chlorella Metabolism. I. Effect of Herbicides on Complex Lipid Biosynthesis. Plant. Cell Physiol. 14:781-785, 1973.

Valentine, J. P. and S. W. Bingham. Influence of Several Algae on 2,4-D Residues in Water. Weed Sci. 22:358-363, 1974.

Walsh, G. Effects of Herbicides on Photosynthesis and Growth of Marine Unicellular Algae. Hyacinth Control J. 10:45-48, 1972.

Wedding, R. T., L. C. Erickson and B. L. Brannaman. Effect of 2,4-Dichlorophenoxyacetic Acid on Photosynthesis and Respiration. Plant Physiol. 29:64-69, 1954.

Wojtalik, T. A., T. F. Hall and L. L. Hill. Monitoring Ecological Conditions Associated with Wide-scale Applications of DMA-2,4-D to Aquatic Environments. Pestic. Monit. J. 4:184-203, 1971.

Wolfe, J. L. and B. R. Norment. 1973. Accumulation of Mirex Residues in Selected Organisms After an Aerial Treatment, Mississippi 1971-1972. Pestic. Monit. J. 7:112-116, 1973.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/3-76-048		

4. TITLE AND SUBTITLE	5. REPORT DATE
Effects, Uptake, and Metabolism of Methoxychlor, Mirex, and 2,4-D in Seaweeds	June 1976 (Issuing Date)
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO
Harish C. Sikka, Gary L. Butler and Clifford P. Rice	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Life Sciences Division Syracuse University Research Corporation Syracuse, New York 13210	1EA077
	11. CONTRACT/GRANT NO.
	Contract No. 68-03-0271
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Gulf Breeze, Florida 32561	Final (5/73-10/75)
	14. SPONSORING AGENCY CODE
	EPA-ORD

15. SUPPLEMENTARY NOTES

16. ABSTRACT

This report presents the results of a study concerning effects, uptake, and metabolism of mirex, methoxychlor, and 2,4-D in the seaweeds Ulva sp., Enteromorpha sp. and Rhodymenia sp. None of the pesticides, at concentrations corresponding to their maximum solubility in seawater, had any significant effect on photosynthesis, protein, carbohydrate, lipid, chlorophyll, carotenoid or trace metal content of the algae. All three algae removed substantial amounts of mirex and methoxychlor from the medium, but uptake of 2,4-D was extremely low. The rate of uptake of methoxychlor was considerably greater than that of mirex. Enteromorpha accumulated considerably more mirex and methoxychlor than Ulva or Rhodymenia. Both Ulva and Enteromorpha failed to metabolize either mirex or 2,4-D. Enteromorpha metabolized methoxychlor to a limited extent. After 7 days of incubation with carbon-labelled methoxychlor, a major portion of the label in the tissue and medium was present in unchanged methoxychlor. A small amount of radioactive metabolite, 2,2-bis (p-methoxyphenyl)-1,1-dichloroethylene, was detected in both the tissue and medium. In addition, medium contained 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane and four unidentified minor radioactive metabolites. Unlike Enteromorpha, Ulva did not metabolize methoxychlor.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Algae, insecticides, herbicides		6F

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release unlimited	None	48
	20. SECURITY CLASS *(This page)*	22. PRICE
	None	

EPA Form 2220-1 (9-73)

☆ U.S. GOVERNMENT PRINTING OFFICE: 1976-657-695/5445 Region No. 5-11