Progress Report
of the
Bureau of Commercial Fisheries
Center for Estuarine and Menhaden Research,
Pesticide Field Station, Gulf Breeze, Fla.,

Fiscal Year 1969

UNITED STATES DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE
BUREAU OF COMMERCIAL FISHERIES

Progress Report
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Bureau of Commercial Fisheries
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T. R. RICE, Center Director

THOMAS W. DUKE, Station Chief

Circular 335

CONTENTS

Cover illustration: An aerial view of the BCF Pesticide Field Station on Government-owned Sabine Island, an artificial island made of ballast rock in Santa Rosa Sound, 8 miles south of Pensacola, Fla. The Gulf of Mexico is visible in the distance.

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ABSTRACT

Research activities include studies on the fate and effect of pesticides in the estuarine environment.

REPORT OF THE STATION CHIEF

Thomas W. Duke

The BCF (Bureau of Commercial Fisheries) Biological Laboratory at Gulf Breeze, Fla., became a field station of the BCF Radiobiological Laboratory (since part of the Center for Estuarine and Menhaden Research), Beaufort, N.C., May 1, 1968. The consolidation was made to strengthen the Bureau's research program in estuarine pollution because radioactive materials and pesticides have similar effects on some plants and animals. Research on the effects of pesticides on estuarine organisms and the fate of these chemicals in the estuarine environment continues at the Pesticide Field Station at Gulf Breeze.

Pesticides that enter the estuarine environment can be accumulated by the commercial fishery organisms in these fertile waters. Results of laboratory experiments on the acute toxicity of pesticides to these organisms and other data indicate that residues of persistent pesticides are concentrated in the estuarine food web and may become toxic to predator organisms. Also, the evidence is clear that even low levels of pesticides (below those that cause mortality) can adversely affect the animals. In addition, commercial species are affected indirectly when their food organisms are killed or diverted to another habitat because of exposure to pesticides. Thus, man continues to damage the estuarine environment by adding pesticides that insidiously affect estuarine organisms.

We are continuing to study the movement of pesticides through the estuarine environment and to determine acute and chronic effects of pesticides on estuarine organisms. Because of recent interest in applying herbicides to aquatic environments, observations were made on the effect of Dichlobenil on the flow of energy through primary producers in a coastal pond. On a yearly basis, the rate of incorporation of energy through photosynthesis by phytoplankton was nearly twice that in a similar but untreated pond. Other work on populations of organisms includes studies on the effect of DDT on the ciliate Tetrahymena pyriformis. As little as 0.1 p.p.m. (parts per million, = milligrams per liter) DDT reduced a population of these organisms by 14 percent.

Acute (96-hour) toxic effects of several new pesticides on oysters, clams, shrimp, and fish were evaluated in the laboratory. These data were sent to the Pesticide Registration Division of the USDA to enable them to certify pesticides to be used in or near the estuarine environment.

A year-long study was completed on the effects of a mixture of DDT, toxaphene, and parathion on the growth and development of oysters. The mean weight of "control" oysters was consistently greater than that of the experimental oysters after 6 weeks exposure but the difference was not statistically significant until the 22d week. Tests with mirex, an insecticide developed to control fire ants, indicated that this chemical causes delayed toxic effects in blue crabs and shrimp. These experiments emphasize the urgent need for chronic or long-term tests with low levels of pesticides -- the delayed effects of DDT and mirex would not have been detected in a 96-hour bioassay.

We investigated the capacity of estuarine fish to avoid pesticides in water and the effect of these chemicals on their salinity preference. Fish avoided test concentrations of DDT, endrin, Dursban[1], and 2,4-D, but did not avoid malathion or Sevin[1]. Also, fish exposed to DDT preferred a higher salinity than they did before exposure.

Physiological studies were completed with shrimp and fish. Shrimp exposed to 0.1 p.p.b. (parts per billion, = micrograms per liter) DDT exhibited a gradual depression of protein levels in their blood. Also, exposed shrimp had a slight necrosis of the hepatopancreas. Investigations of the relation between the toxicity of organophosphate pesticides and in vivo inhibition of cholinesterase of sheepshead minnows indicated that the degree of inhibition of cholinesterase in brain tissue is not always related to mortality.

Marine Exhibit

The annual estuarine exhibit was held April 28 through May 10. Sessions were held hourly from 9:00 a.m. through 12:00 noon, from Monday through Friday each week. Each session consisted of a short lecture by a staff member on the biology of local estuarine plants and animals. The lecture was augmented by living specimens in flowing water aquariums and a brief question-and-answer period (figs. 1 and 2). The exhibit was organized and presented by Nelson R. Cooley and other staff members. Each member of the Station's staff participated in the exhibit. Total attendance was 9,173, which included school children, Boy Scouts, Girl Scouts, and the general public. Most of those attending were from the Pensacola metropolitan area, but there were also classes from Fort Walton Beach and Niceville, Fla., and Selma, Ala.
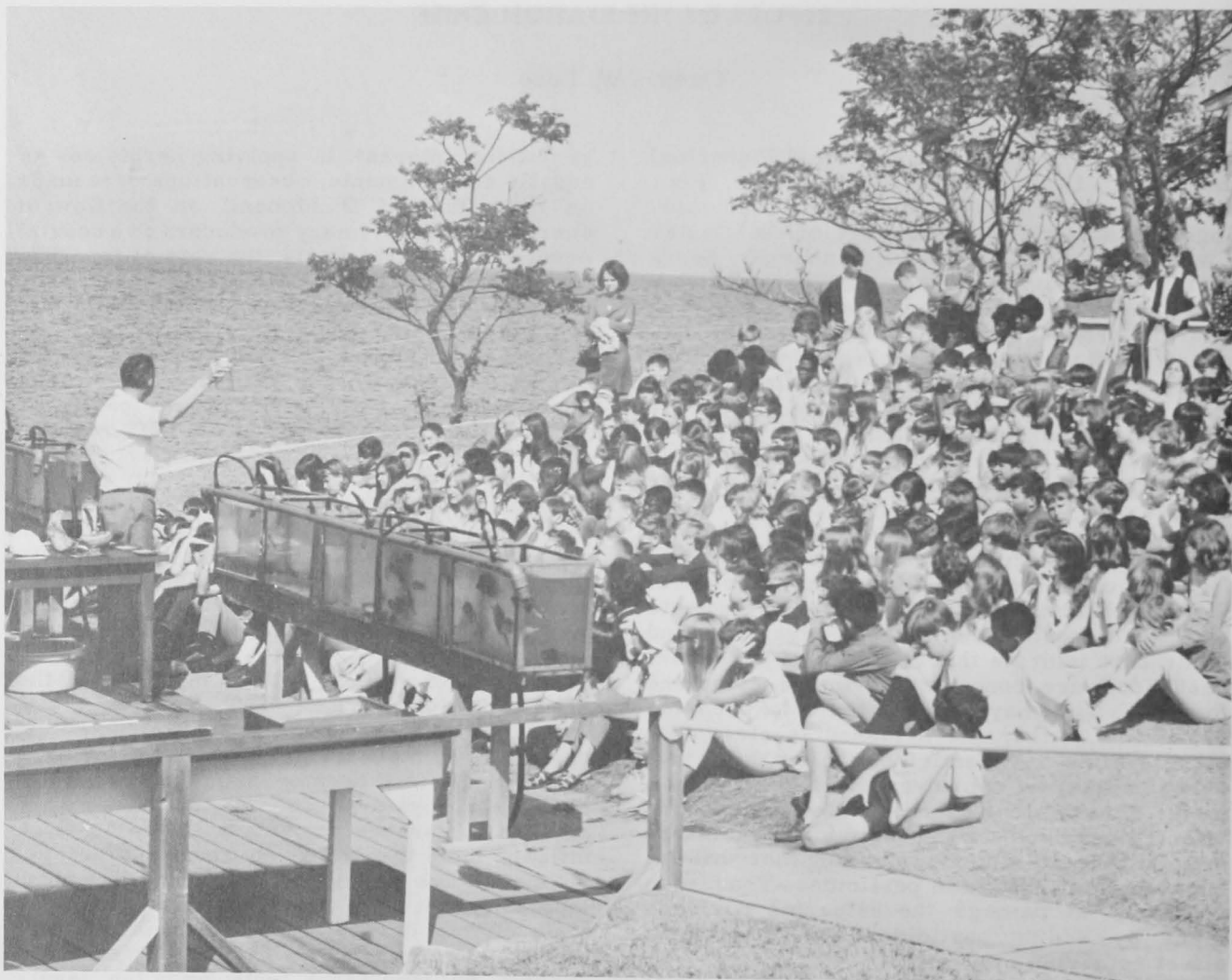
Figure 1.--Staff member presents lecture on biology of local fauna to school classes as part of annual marine exhibit. More than 9,000 guests attended this year's exhibits.

[1] Use of trade names in this publication does not imply endorsement by the Bureau of Commercial Fisheries.

Figure 2.--Students take a close look at spiny burrfish during annual exhibit.

STAFF

Theodore R. Rice, Center Director
Thomas W. Duke, Station Chief

PROJECTS:

Estuarine Productivity

Gerald E. Walsh.	Project Leader
Thomas E. Grow	Biologist (temporary)
Paul T. Heitmuller[1]	Biological Aid
Edward Matthews	Do.

Experimental Environment

Nelson R. Cooley	Project Leader
James M. Keltner, Jr.	Fishery Biologist

Chemical Assays

Alfred J. Wilson, Jr.	Project Leader
Jerrold Forester	Physical Science Technician
Johnnie Knight	Biological Aid

Laboratory Bioassays

Jack I. Lowe	Project Leader
Richard B. Davison.	Physical Science Aid (transferred June 26, 1969)
Paul D. Wilson.	Biological Technician

Behavior of Estuarine Organisms

David J. Hansen.	Project Leader
Stephen L. Nall	Biological Aid (temporary; resigned May 9, 1969)

Physiology of Estuarine Organisms

DelWayne Nimmo.	Project Leader
Robbin R. Blackman	Fishery Biologist

Enzyme Systems of Estuarine Organisms

David L. Coppage.	Project Leader

Kinetics of Pesticides

Charles W. Miller	Project Leader (transferred August 24, 1968)
Thomas W. Duke	Acting Project Leader
Patrick W. Borthwick	Conservation Aid (temporary)
Alan J. Rick	Fishery Biologist
Michael D. Schmitt.	Biological Aid (temporary; co-op student, University of West Florida)

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[1] Granted educational leave.

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STAFF SERVICES:

Hughey L. Jones	Fishery Methods and Equipment Specialist
Lester W. Wolf.	Do.
Anice M. Reynolds.	Administrative Assistant
Edith R. Frazier	Clerk-Typist (part-time)
Cynthia M. Herndon	Clerk (Typing)
Marilyn J. Leggett.	Clerk-Stenographer (temporary)
Kenneth H. Herndon	Maintenance (retired April 14, 1969)
Enrique Pacheco	Do. (temporary; part-time)
Lawrence R. Cannon	Biological Aid (temporary; resigned September 24, 1968)
Wayne J. Hendon	Do. (part-time--President's Back-to-School Program)
James M. Patrick, Jr.	Do. (part-time--President's Back-to-School Program)
Harold T. Sharrett, Jr.	Do. (part-time--President's Back-to-School Program)
Archie Williams	Do. (resigned October 18, 1968)

STAFF ACTIVITIES

MEETINGS ATTENDED AND PAPERS PRESENTED

Blue Crab Mortality Problem Workshop, PL88-309 Project, Bears Bluff Laboratories, Wadmalaw Island, S.C., November 20, 1968.
   T. W. Duke
Gulf and Caribbean Fisheries Institute, Miami, Fla., November 20-27, 1968.
   G. E. Walsh
Southeastern Division Meeting, Entomological Society of America, Biloxi, Miss., January 29, 1969.
   R. R. Blackman
   T. W. Duke
   D. W. Nimmo
Blue Crab Workshop, Brunswick, Ga., February 10, 1969.
   A. J. Wilson, Jr.
Annual Meeting of the Weed Science Society of America, Las Vegas, Nev., February 12, 1969.
   G. E. Walsh - Effects of Dichlobenil upon physical, chemical, and biological factors in a coastal pond.
Estuarine Symposium, Columbia, S.C., April 10-11, 1969.
   N. R. Cooley   D. W. Nimmo
   T. W. Duke   A. J. Wilson, Jr.
   J. I. Lowe
Interagency Pesticide Conference, Tallahassee, Fla., May 22, 1969.
   T. W. Duke - Pesticide research at BCF Biological Field Station, Gulf Breeze, Fla.

APPOINTMENTS, COMMITTEES, CONFERENCES, TRAINING, AND AWARDS

T. W. Duke - Conference, Sport Fisheries Pesticide Laboratory, Columbia, Mo., and BCF, Washington, D.C., July 8-11, 1968.
J. Forester - Training, Analytical Geometry and Calculus, Pensacola Junior College, Pensacola, Fla., August 28 to December 15, 1968.
J. Knight - Training, Introduction to College Chemistry, Pensacola Junior College, Pensacola, Fla., August 28 to December 15, 1968.
D. J. Hansen - Training, Computer Programming, BCF Radiobiological Laboratory, Beaufort, N.C., September 3-6, 1968.
R. R. Blackman - Training, Intermediate Photography, Pensacola Junior College, Pensacola, Fla., October 3 to December 5, 1968.
J. I. Lowe - Training, Technical Report Writing Course, Pensacola Junior College, Pensacola, Fla., November 1968 to March 1969.
N. R. Cooley - Hearing, National Estuarine Pollution Study, sponsored by Federal Water Pollution Control Administration, Mobile, Ala., November 21, 1968.
T. W. Duke - Training, BCF Executive Seminar, Charlottesville, Va., December 1-13, 1968.
L. W. Wolf - Training, Swimming, Skipper's Diving, Inc., Pensacola, Fla., January 6 to March 20, 1969.
R. R. Blackman, L. W. Wolf - Training, Piloting, Seamanship and Small Boat Handling, Pensacola Power Squadron, Pensacola, Fla., January 6 to April 11, 1969.

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P. T. Heitmuller - One-year educational leave, University of West Florida, Pensacola, Fla., January 7, 1969.

N. R. Cooley - Training, Introduction to Data Processing Course, Pensacola Junior College, Pensacola, Fla., February 11 to March 11, 1969.

A. M. Reynolds - Training, Time and Attendance Workshop Session, BCF Regional Office, St. Petersburg, Fla., March 11-13, 1969.

T. W. Duke, J. I. Lowe, and A. J. Wilson, Jr. - Conference, U.S. Department of Agriculture Laboratories, Gulfport, Miss., March 20, 1969.

T. W. Duke - Conference (BCF Master Plan), Acting Regional Director, BCF Regional Office, St. Petersburg, Fla., April 15, 1969.

D. W. Nimmo - Training, Use of Isotopes in Research, Oak Ridge National Laboratory, Oak Ridge, Tenn., April 20 to May 16, 1969.

A. M. Reynolds - Conference, Performance Evaluations, Pensacola, Fla., April 24, 1969.

T. W. Duke - Conference, Laboratory Review, BCF Radiobiological Laboratory, Beaufort, N.C., April 27-30, 1969.

T. W. Duke - Conference (BCF Master Plan), University of Alabama, Bayou La Batre, Ala., May 6, 1969.

T. W. Duke - Conference (BCF Master Plan), University of Georgia, Athens, Ga., May 15, 1969.

T. W. Duke - Conference (BCF Master Plan), Acting Regional Director, BCF Regional Office, St. Petersburg, Fla., May 27, 1969.

R. R. Blackman, L. W. Wolf - Training, Engine Maintenance Course, Pensacola Power Squadron, Pensacola, Fla., June 5 to September 4, 1969.

J. I. Lowe - Training, Course in Marine Botany, Gulf Coast Research Laboratory, Ocean Springs, Miss., June 9 to July 4, 1969.

T. W. Duke, G. E. Walsh - Associate Faculty Members, Faculty of Biology and Marine Sciences, University of West Florida, Pensacola, Fla.

T. W. Duke - Associate Editor, Proceedings of the National Shellfisheries Association.

T. W. Duke - Committee Member of the West Florida Natural Resources Council.

N. R. Cooley - Merit Award, Outstanding Performance as Chairman of the Station Safety Committee.

PUBLIC RELATIONS

N. R. Cooley - Judge, Northwest Florida Regional Science Fair, Pensacola, Fla., March 13, 1969.

T. W. Duke - Guest speaker, Escarosa Woman's Club, Pensacola, Fla., April 23, 1969.

T. W. Duke - Guest speaker, Downtown Kiwanis Club, Pensacola, Fla., April 16, 1969.

M. J. Leggett - Organizational meeting of librarians, University of West Florida, Pensacola, Fla., May 16, 1969.

N. R. Cooley - Presented a lecture on local estuarine fauna to 71 Girl Scouts and counseled two Boy Scouts on the Oceanography Merit Badge.

D. W. Nimmo - Counselling six Boy Scouts on the Oceanography Merit Badge.

STAFF PUBLICATIONS

HAYNE, DON W., T. W. DUKE, and T. J. SHEETS
  1969. Pesticides in estuaries. In H. T. Odum, B. J. Copeland, and Elizabeth A. McMahan (editors), Coastal ecological systems of the United States, Vol. 2, Part 4, E-3B, p. 1075. (A Report to the Federal Water Pollution Control Administration. Institute of Marine Sciences, University of North Carolina, Morehead City, N.C.)

HANSEN, DAVID J.
  In press. Avoidance of pesticides by untrained sheepshead minnows. Trans.
Amer. Fish. Soc. (accepted for publication).
  In press. Vertebral anomaly in Micropogon undulatus. Quart. J. Fla. Acad. Sci. (accepted for publication).

WALSH, GERALD E., and PAUL T. HEITMULLER.
  1969. Effects of Dichlobenil upon physical, chemical, and biological factors in a coastal pond. Abstracts 1969 Meeting of the Weed Science Society of America, p. 92.

RESEARCH PROJECTS

Research projects at the Field Station at Gulf Breeze during the fiscal year investigated many facets of the impact of pesticides on estuarine ecology. They included studies on effects of a herbicide on a coastal pond ecosystem, effects of pesticides on growth of populations of estuarine ciliate protozoans, of exposure to pesticide on the salinity preference of fish and their ability to avoid pesticide pollution, and effects of pesticides on physiology of shrimp and enzyme systems in fish. Other investigations were concerned with chemical assays of pesticide residues and laboratory bioassays that involved studies of effects of acute and chronic exposures of oysters, crabs, shrimp, and fish to pesticides as well as a study of the kinetics of pesticides in estuarine ecosystems. The results of a faunal inventory of the local estuary are summarized.

Estuarine productivity studies reported here are concerned with effects of herbicides on energy incorporation and flow. Studies were made in the field with a view to learning, qualitatively and quantitatively, the normal annual pathways of energy flow in coastal ponds. One pond was treated with herbicide and its energy pattern compared with that of an untreated pond. In the laboratory, studies have recently begun on effects of herbicides on photosynthesis of estuarine unicellular algae.

EFFECTS OF HERBICIDE ON THE BIOTA AND ENERGY BUDGET OF A COASTAL POND ECOSYSTEM

Gerald E. Walsh and Paul T. Heitmuller

Greatly increased use of herbicides in the aquatic environment is a potential threat to the stability of estuarine ecosystems, which support a large number of economically important species of fish, shrimp, and shellfish. Herbicides attack plants, whose ecological functions include: 1) incorporation of solar energy with subsequent use in food by animals; 2) production of oxygen; and 3) provision of substratum for other organisms. Therefore, if estuarine plants were adversely affected by herbicides, many commercial fisheries also could be affected adversely.

Although much is known about the capacity of herbicides to eliminate undesirable plants, very few data are available on how they affect the physical, chemical, and biological properties of aquatic ecosystems. To try to determine some of these effects, we treated a small coastal pond with a commonly used herbicide and compared the ecology of the pond with that of a similar untreated pond. We wanted to learn how an aquatic ecosystem would respond when its rooted plants, the main incorporators of energy from the sun, were eliminated. Three responses seemed likely: 1) radiant energy normally used in photosynthesis by rooted plants would be absorbed by the water and subsequently lost from it, 2) radiant energy would be trapped and diverted along new pathways, and 3) a combination of 1) and 2). Any of the three responses would change the ecology of the pond.

We used pond ecosystems in these studies because they allowed study of isolated populations of several species of plants and animals without tidal effects. At the same time we treated one pond, we sampled an untreated pond to estimate normal conditions.

Methods

Our experiments were designed to test for the three possible responses listed above. To do this, we recorded temperature, water characteristics, fluctuations in numbers and species of planktonic organisms, and meteorological conditions. Because pond ecosystems are always in a dynamic state in respect to incident sunlight, air temperature, wind speed, organism behavior, and many other factors, we sampled the treated and untreated ponds at 8 a.m. and 1 p.m. on the same day at surface, middepth, and bottom at approximately weekly intervals. Factors measured were: dissolved oxygen, pH, nitrate, DCHO (dissoved carbohydrate), alkalinity, air and water temperatures, humidity, conductivity, salinity, and chlorophyll a. We estimated the amount of cloud cover at each sampling. We also measured each week the numbers and kinds of dominant plankters, primary productivity, solar radiation, longwave atmospheric radiation, longwave radiation emitted from the water, back radiation, and energies of evaporation, conduction, and heat. Energy of photosynthesis of algae was calculated from gross primary production data obtained from light- and dark-bottle oxygen studies. We assumed that 8 quanta of light, with an average energy content of 40 Kcal/Einstein, resulted in release of one molecule of oxygen.

To calculate the amount of solar energy that entered the water, we had to know the angle of incidence of the sun's rays at the time of sampling. This angle was calculated from the expression

$$\sin \alpha = \sin \phi \sin \delta + \cos \phi \cos \delta \cos \eta$$

where

α = solar altitude
ϕ = latitude of the ponds
δ = declination of the sun
η = hour angle of the sun.

The following formulas were used to calculate energy budgets. All equations except that for number of calories used to heat the water were taken from the literature.

Effective long wave atmospheric radiation (Q_a):

$$Q_a = 1.66 \times 10^{-7} \ T_a . \beta$$

where

T_a = absolute temperature of the air
β = atmospheric radiation factor

Total radiation entering water (Q_t):

$$Q_t = \text{solar radiation} + Q_a$$

Longwave radiation emitted from water (Q_w):

$$Q_w = 0.97 \, \sigma \, T_w$$

where

σ = Stefan-Boltzmann radiation constant
T_w = absolute temperature of the water

Back radiation (Q_b):

$$Q_b = 0.970 \, \sigma \, (T_w - \beta T_a)$$

Energy used in evaporation (Q_e):

$$Q_e = 12 \, U \, (e_w - e_a)$$

where

U = wind speed
e_w = vapor pressure of saturated air at the temperature of the water surface
e_a = vapor pressure of air

Conducted energy (Q_h):

$$Q_h = 0.00407 \, U \, P \, (t_a - t_w)$$

where

P = atmospheric pressure
t_a = air temperature
t_w = water temperature

Energy used in heating water ($Q\nu$):

$$Q\nu = A_{\Delta h1} \, (\overline{T_1'} - T_B) \Delta hi - A_{\Delta h1} \, (\overline{T_1} - T_B) \Delta hi$$

where

$A_{\Delta hi}$ = horizontal area of the water column
$\overline{T_1}$ = initial average temperature of the water column
$\overline{T_1'}$ = final average temperature of the water column
T_B = an arbitrary base temperature
Δhi = depth of column.

The above methods were applied to two coastal ponds. Although different in size, the ponds were similar chemically and biologically. Both had basins approximately 1 m. deep. Their substrata were composed of sand and fine particulate organic matter. Cattails grew along the shores. The larger pond, 0.91 ha., was chosen to be the untreated pond because fishermen were occasionally seen there. The smaller, 0.15 ha., was treated with 1.0 p.p.m. of Dichlobenil (Casoron, 2,6-dichlorobenzonitrile) on April 3, 1969. We chose Dichlobenil because it is a herbicide used commonly throughout the United States for control of

Chara vulgaris and Potamogeton pectinatus, the dominant hydrophytes of both ponds. About 1 month after treatment, all of the Potamogeton and about 80 percent of the Chara was eliminated. Four months after treatment, the pond appeared as it did before treatment.

Comparison of Treated and Untreated Ponds

Representative physical and chemical data from both ponds show their similarities before treatment (figs. 3 and 4). The significant effect of treatment was a large increase (bloom) in the number of blue-green algae in the plankton as the vascular hydrophytes died. Four genera of filamentous blue-green algae predominated during the bloom (fig. 5). Three genera of zooplankton -- Diaptomus dorsalis (copepod), Keratella cochlearis (rotifer), and Gonyaulax sp. (dinoflagellate) -- also increased greatly in number after application of herbicide (fig. 6). Keratella quadrata was first

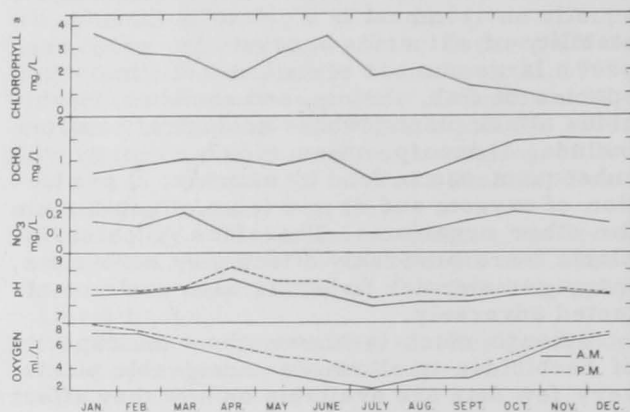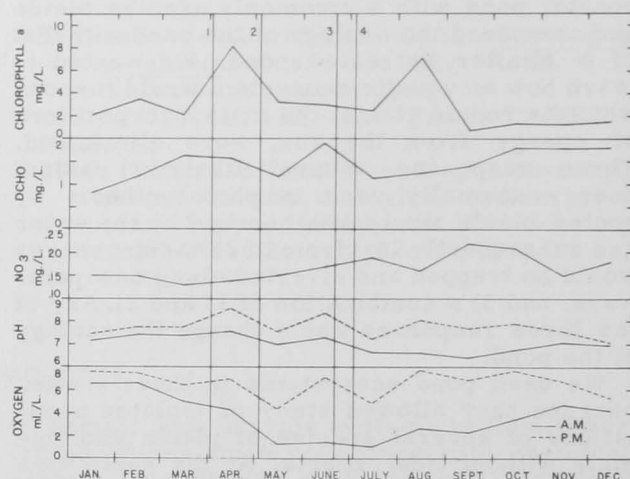Figure 3.--Average monthly values (1968) for selected factors in an untreated coastal pond.

Figure 4.--Average monthly values (1968) for selected factors in a coastal pond treated on April 3 with 1.0 p.p.m. Dichlobenil. 1) Date of treatment; 2) date of maximum kill of vascular hydrophytes; 3) date at which new growth of Chara vulgaris was observed; and 4) date of reappearance of Potamogeton pectinatus.
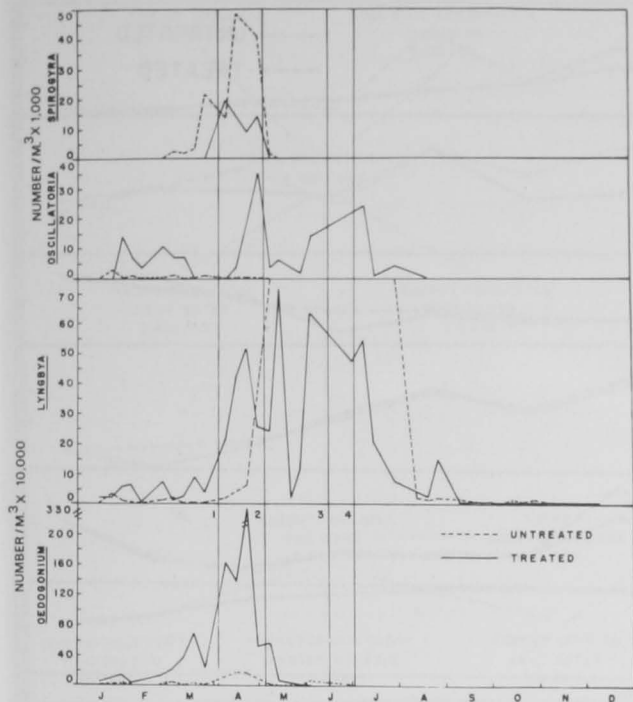
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Figure 5.--Annual variation (1968) in numbers of fila-
mentous algae of treated and untreated ponds. 1) Date
of treatment; 2) date of maximum kill of vascular
hydrophytes; 3) date at which new growth of Chara
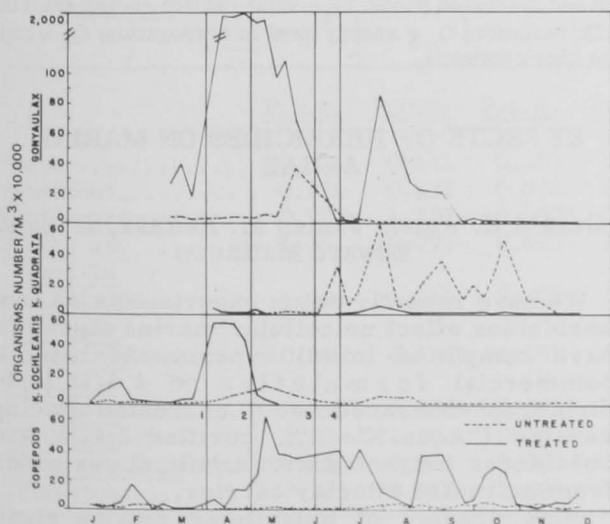vulgaris was observed; and 4) date of reappearance of
Potamogeton pectinatus.

Figure 6.--Annual variation (1968) in numbers of zoo-
plankton of treated and untreated ponds. 1) Date of
treatment; 2) date of maximum kill of vascular hydro-
phytes; 3) date at which new growth of Chara vulgaris
was observed; and 4) date of reappearance of Potamoge-
ton pectinatus.

detected in the treated pond 1 week after treat-
ment and disappeared 6 weeks after treatment.

As vascular hydrophytes died, soluble nu-
trients released from them probably were

utilized for growth by the Dichlobenil-resistant
filamentous algae. The algae were then con-
sumed by zooplankton.

Average monthly energy data for the two
ponds (fig. 7) were similar except for the
greater incorporation of energy by the larger
standing crop of phytoplankton in the treated
one.

Annual energy budgets of the treated and
untreated ponds were similar (figs. 8 and 9).
An appreciable amount of energy not yet ac-
counted for was, most likely, used in heating
sediments and also for photosynthesis by
vascular hydrophytes.

On a yearly basis, rate of energy incorpora-
tion in the treated pond through photosynthesis
by phytoplankton was nearly double that in the
untreated one. Although the increase in energy
of photosynthesis by algae of the treated pond
in April was undoubtedly due to herbicide,
annual incorporation was probably related to
pond morphology and density of plant standing
crop.

In addition to our studies, C. W. Miller
conducted research on persistence of Dichlo-
benil in the water and sediment of the treated
pond and on uptake by several plants and
animals which lived there. His data (table 1)
show that Dichlobenil was lost rapidly from
water, sediment, plants, animals, and plankton.
Almost all herbicide had disappeared 64 days
after treatment.

Ecological Implications

It is common practice to measure effective-
ness of herbicides by their ability to eliminate
unwanted plants in a short time and to main-
tain the weed-free state. Although these are
desirable herbicidal qualities, our data indi-
cate that other criteria must also be applied in
evaluation of herbicides for use in aquatic
ecosystems.

In our experiments, the herbicide used had
little or no detrimental effect on filamentous
blue-green algae. Consequently, as vascular
hydrophytes died and soluble nutrients were
released from them to the water, blue-green
algae absorbed the nutrients, increased in
number, and carried on nearly all of the
photosynthesis in the pond. In effect, the in-
creased numbers of algae maintained normal
pond water chemistry during the period of
herbicide stress. Without such a homeostatic
mechanism, a large amount of life in the pond
might be expected to be lost due to lack of
oxygen. The use of a broad spectrum herbicide
that would destroy blue-green algae might
have resulted in a loss of animals from the
pond.

The most important change brought about by
the herbicide was in the structure of the eco-
system. Normal pathways of energy flow were
diverted because rooted plants were fewer.
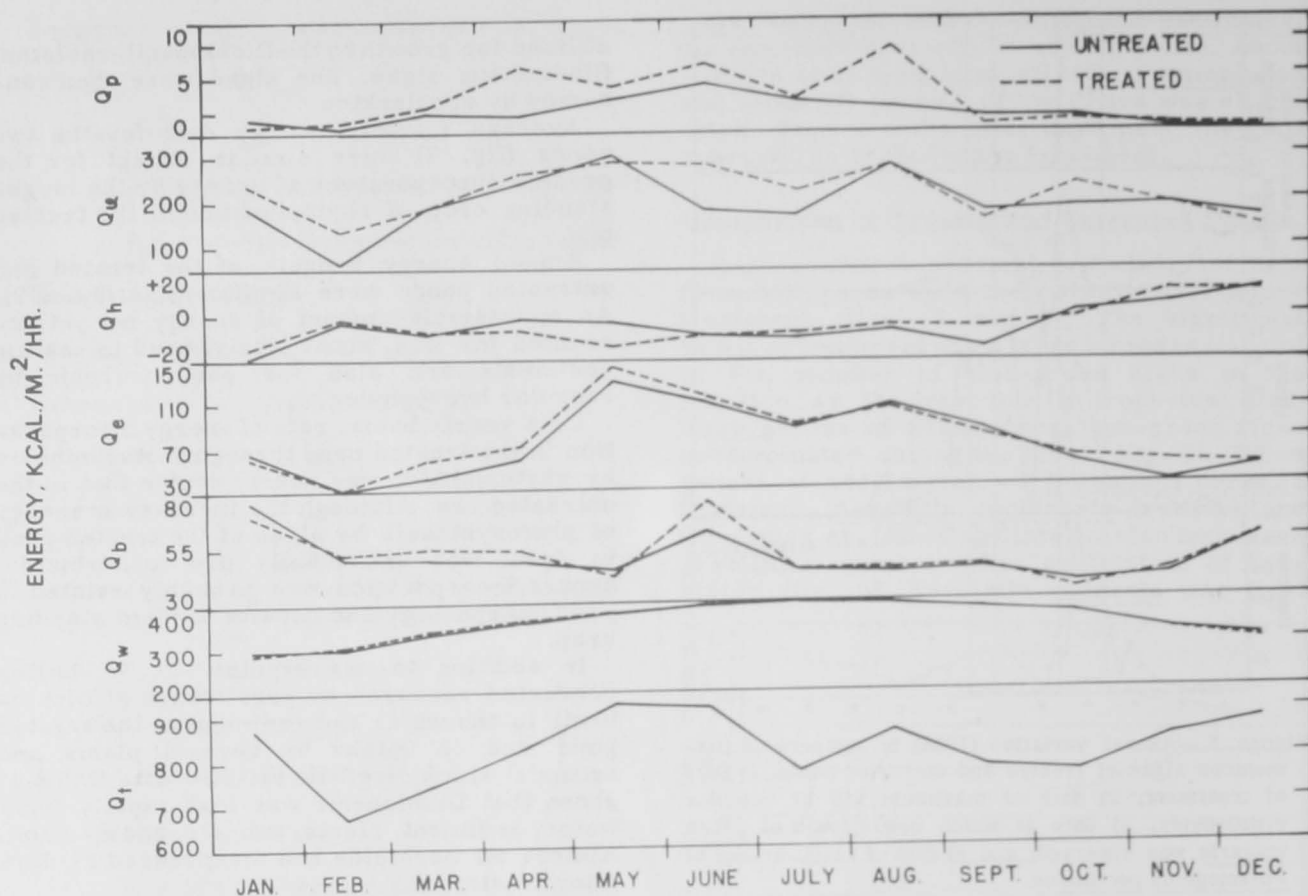Energy that would ordinarily have been used

Figure 7.--Average monthly values (1968) for energy in treated and untreated ponds. Q_t = total radiant energy entering water; Q_w = longwave radiation emitted from water; Q_b = back radiation; Q_e = energy used in evaporation; Q_h = conducted energy; $Q\varphi$ = energy stored as heat; Q_p = energy used in photosynthesis.

and stored in rooted plants was used and stored in algae, thus stopping the flow of energy to animals that feed on bottom plants. The increased amount of energy available as food in the form of algae led to an increase in zooplankton. In an estuary, this situation would lead, theoretically, to larger numbers of plankton-eating animals if herbicide stress were maintained, and could benefit the commercial fishery. It must be realized, however, that the elimination of energy flow to vascular hydrophytes would adversely affect economically important estuarine species which depend upon them for substratum or food.

The data presented here illustrate the interdependence of all parts of an ecosystem. Man may easily disrupt the pathways of energy flow and the balance among plants, animals, and their environment, and it is clear that change in one part of an ecosystem affects another part. Such changes need not be detrimental, but it is important to know all the effects of herbicide application. Herbicides can be used intelligently to manage ecosystems -- not just to kill weeds.

EFFECTS OF HERBICIDES ON MARINE ALGAE

Gerald E. Walsh, James M. Keltner, Jr., and Edward Matthews

We have recently begun experiments on how herbicides affect unicellular marine algae. We have completed initial experiments using a commercial formulation of 2,4-D (2,4-dichlorophenoxyacetic acid) on treated attaclay carrier ("Aqua-Kleen"), purified 2,4-D, and substances extracted with artificial sea water from untreated attaclay carrier.

Nine species of unicellular marine algae were grown in artificial sea-water medium treated with the above compounds.

A photosynthesis model respirometer measured photosynethic rates during the logarithmic phase of growth. The measurements were expressed as microliters of oxygen evolved per milligram of cells (dry weight). Cells were also harvested on a membrane filter of 0.45μ porosity, dried, and weighed for determination of standing crop.
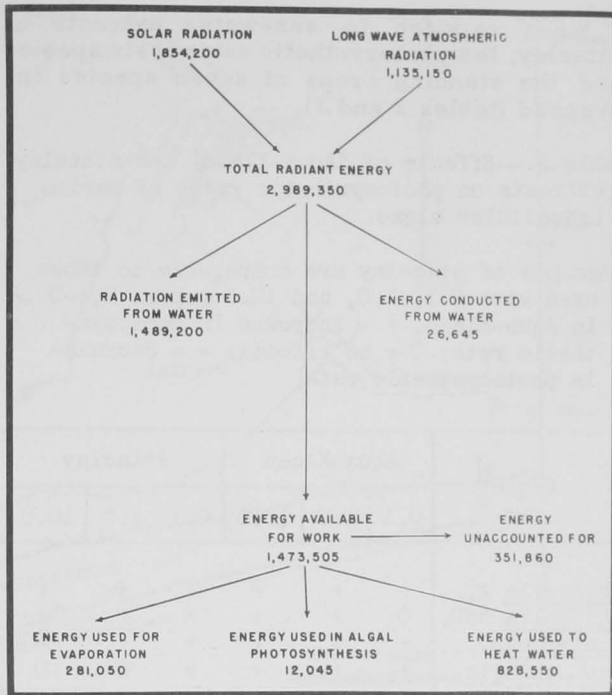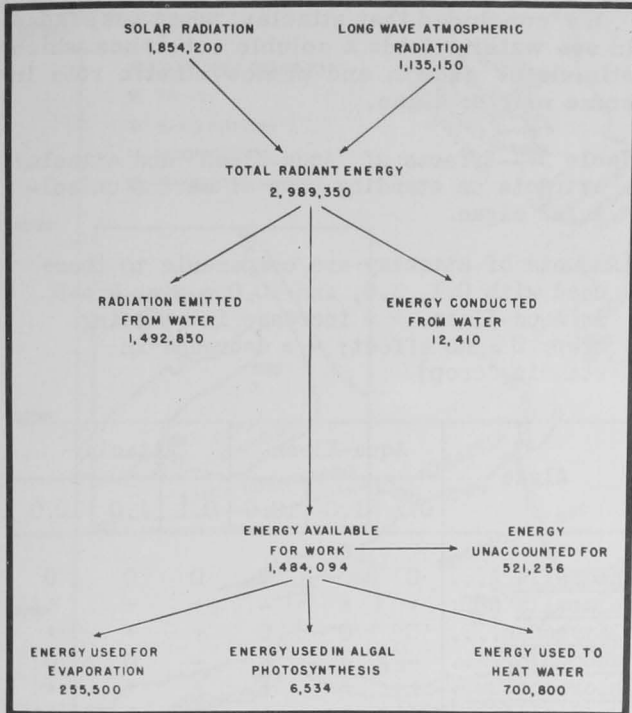
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Figure 8.--Annual energy budget (1968), in Kcal./m.²/ year, for an untreated coastal pond.

Figure 9.--Annual energy budget (1968), in Kcal./m.²/ year, for a coastal pond treated with 1.0 p.p.m. Dichlobenil.

Table 1.--Residues of Dichlobenil in treated pond[1]

Material	Days after treatment									
	1	2	3	7	14	21	28	35	50	64
	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.
Water.............	0.836	0.641	0.403	0.221	0.046	0.017	0.012	0.006	0.002	0.001
Sediment.........	0.526	0.431	0.334	0.212	0.162	0.051	0.022	0.015	0.009	0.004
Potamogeton sp. .	0.953	1.333	0.553	0.634	0.266	(-)	(-)	(-)	(-)	(-)
Chara sp.	1.16	0.771	0.690	0.670	0.191	0.123	0.080	0.050	Neg.	Neg.
Poecilia latipinna......	4.903	4.216	3.407	2.795	0.820	N.S.	0.271	0.071	Neg.	Neg.
Gambusia affinis........	10.957	6.628	4.491	3.061	0.824	0.437	0.343	0.084	0.015	Neg.
Hyallela azteca.........	0.210	0.552	0.691	0.223	0.060	0.021	Neg.	Neg.	Neg.	Neg.
Orthemis sp.	1.36	1.58	0.43	0.53	0.05	Tr.	Neg.	Neg.	Neg.	Neg.
Leuchorrhinia sp.	0.260	0.146	0.067	0.146	0.078	0.035	0.014	Tr.	Neg.	Neg.
Plankton.........	7.231	2.917	1.625	1.250	0.882	0.733	0.312	0.192	0.050	Neg.

[1] N.S. = No sample; (-) = no material available for analyses as plants were eliminated from pond due to treatment; Tr. = trace, less than 0.05 p.p.m.; and Neg. = negative for the presence of the chemical. Unpublished data from C.W. Miller

The first experiments demonstrated that the photosynthetic rates of seven species of algae increased when treated with Aqua-Kleen at 2,4-D concentrations of 0.1, 1.0, and 10.0 p.p.m. (table 2). In addition, the standing crops of five species were greater than in the untreated cultures.

Purified 2,4-D at the above concentrations did not alter rate of photosynthesis of any of the algae.

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When exposed to sea-water extracts of attaclay, the photosynthetic rates of six species and the standing crops of seven species increased (tables 2 and 3).

Table 2.--Effects of "Aqua-Kleen" and attaclay extracts on photosynthetic rates of marine unicellular algae.

[Amounts of attaclay are comparable to those used with 0.1, 1.0, and 10.0 p.p.m. 2,4-D in Aqua-Kleen. + = increase in photosynthetic rate; 0 = no effects; - = decrease in photosynthetic rate]

Algae	Aqua-Kleen			Attaclay		
	0.1	1.0	10.0	0.1	1.0	10.0
Chlorella A...	+	+	+	+	+	+
Chlorella 580.	0	+	+	+	+	+
Platymonas....	+	+	+	+	+	+
Nannochloris..	+	+	+	+	0	0
Chlorococcum..	0	+	+	+	+	+
Dunaliella....	0	0	0	0	0	0
Phaeodactylum.	+	+	+	+	+	+
Isochrysis....	0	+	+	0	0	0
Monochrysis...	-	-	-	-	-	-

We concluded that attaclay, when suspended in sea water, yields a soluble substance which stimulates growth and photosynthetic rate in some marine algae.

Table 3.--Effects of "Aqua-Kleen" and attaclay extracts on standing crop of marine unicellular algae.

[Amounts of attaclay are comparable to those used with 0.1, 1.0, and 10.0 p.p.m. 2,4-D in Aqua-Kleen. + = increase in standing crop; 0 = no effect; - = decrease in standing crop]

Algae	Aqua-Kleen			Attaclay		
	0.1	1.0	10.0	0.1	1.0	10.0
Chlorella A...	0	0	0	0	0	0
Chlorella 580.	+	+	-	+	+	+
Platymonas....	0	0	0	+	+	+
Nannochloris..	-	-	-	-	0	0
Chlorococcum..	+	+	+	+	+	+
Dunaliella....	+	+	+	+	+	+
Phaeodactylum.	0	0	0	+	+	+
Isochrysis....	+	+	+	+	+	+
Monochrysis...	+	+	+	0	+	+

ESTUARINE FAUNAL INVENTORY

Nelson R. Cooley

This study is the first attempt to make a systematic inventory of the fauna of the estuarine waters near the Station at Gulf Breeze. A manuscript that describes this inventory was completed during the past year.

The study area, designated "Pensacola Estuary" for convenience, is located in Escambia and Santa Rosa Counties in extreme northwestern Florida. It is a normal (positive) estuary, i.e., one in which evaporation is less than precipitation and runoff. Five bays (Pensacola, Escambia, East, Blackwater, and Little Sabine) and Santa Rosa Sound form the major part of the estuary (fig. 10). Charted depths in Blackwater, Escambia, and Little Sabine Bays range up to 4.6 m.; most of Pensacola Bay is 6.1 to 9.1 m. deep and increases to about 18.3 m. near its mouth.

The type of bottom varies in the estuary. In shallow water areas, it is chiefly sand, sand plus shell fragments, or muddy sand with or without grass beds. In deep-water areas, the composition ranges from hard sand to muds of various consistencies. In Escambia Bay, the muds are soft and sticky. In Santa Rosa Sound off Town Point, the mud contains sand. In lower Pensacola Bay, the mud varies from a mixture containing sand and fine shell fragments at depths of about 12 m. to sticky mud at greater depths. Escambia and East Bays contain isolated oyster reefs in addition to bottom types found elsewhere in the estuary.

Salinity ranges from nearly that of freshwater, 0.6 to 1.0 p.p.t. (parts per thousand), at the upper end of the estuary to that of almost ocean water, 32 to 33 p.p.t., at the lower end near the mouth of Pensacola Bay. Water temperatures observed during the period of this study were 10.0 to 32.7° C. Figure 11 shows a comparison of seasonal salinity and temperature averages during the study period with the decade averages, 1951-60).

This study established a checklist of 712 benthic and pelagic species belonging to 16 phyla identified from systematic collections made from 1961 through 1963 at six stations located in high-, intermediate-, and low-salinity areas in Escambia, Pensacola, and Little Sabine Bays and Santa Rosa Sound and from casual collections made in other years. Sampling dates in 1961-63 approximated times of annual extremes and midpoints of the spring rise and autumnal decline in water temperature. Biological sampling was centered on
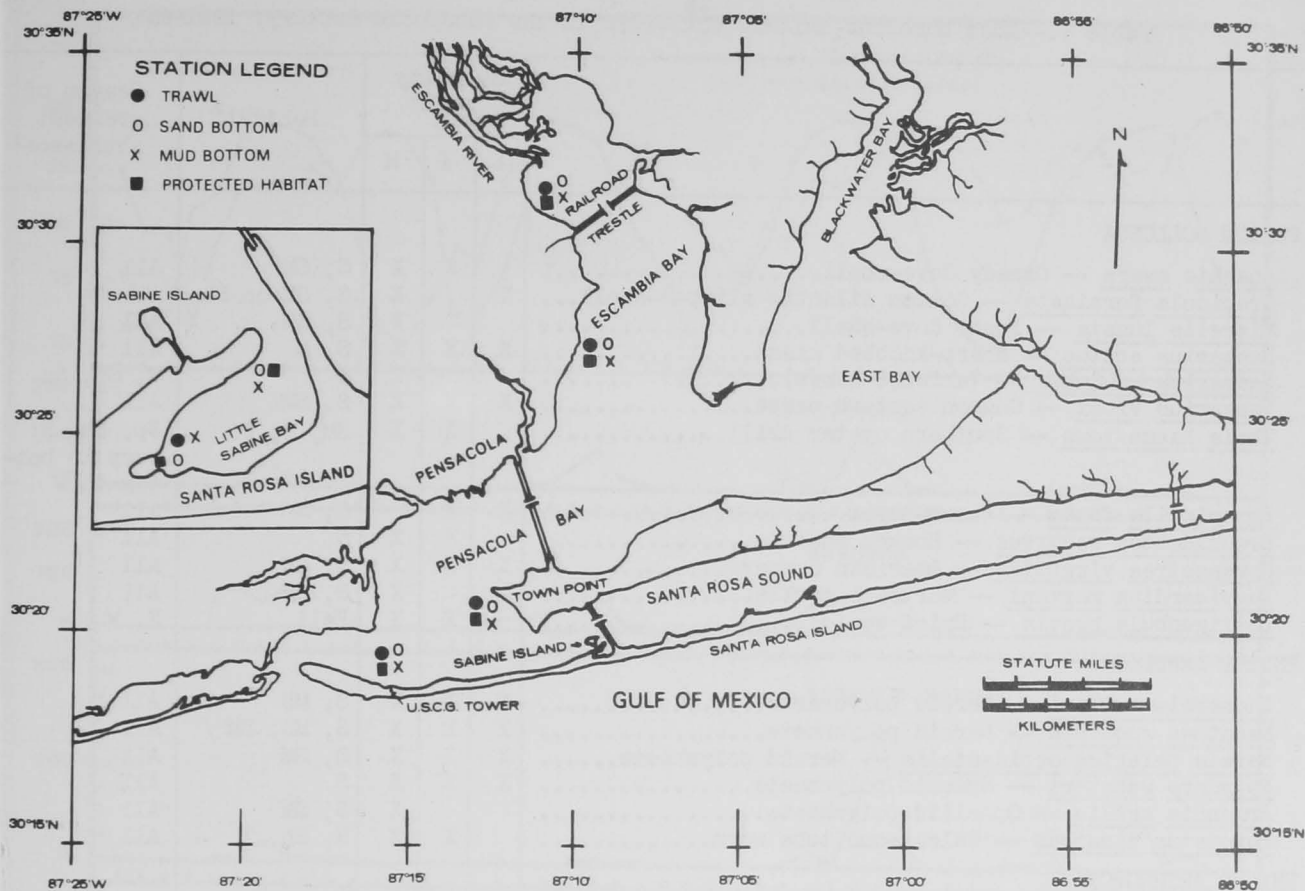
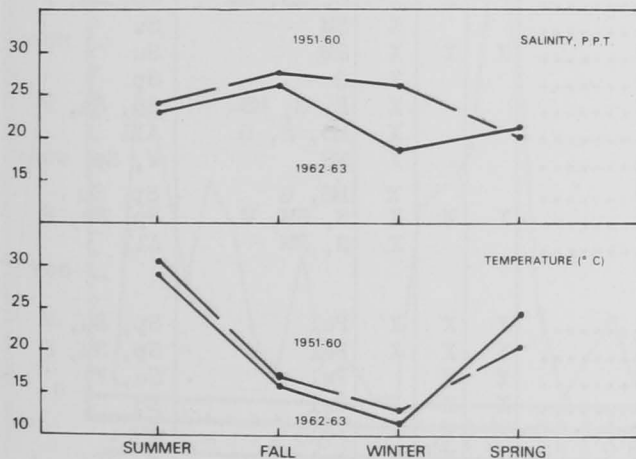Figure 10.--Pensacola Estuary, Fla., showing station locations.

Figure 11.--Comparison of seasonal salinity and temperature averages in the Pensacola Estuary in 1962-63 with decade averages for the same months from 1951-60.

seasonal studies of hourly variation in salinity and water temperature during a single maximum amplitude tidal cycle recorded simultaneously at all stations. Standardized sampling methods included the use of a 4.9-m. otter trawl of 25.4 mm. stretched mesh, an Ekman dredge that covered about 0.1 m.² of deepwater mud bottom, and multiple quadrats

(0.1 m.² by 0.15 m. deep) on shallow-water sand bottoms. Also, wooden "protected habitat" boxes, 0.1 m.² by 0.15 m. deep, covered with 3.2-mm. mesh hardware cloth were placed on the bottom near sand-bottom stations to collect species made rare by predation.

The principal animals collected were mollusks (225 species), annelids (90 species, chiefly polychaetes), arthropods (97 species, chiefly crustaceans), and bony fishes (191 species). Table 4 lists the most numerous of these. The variety of species and numbers of individuals were greatest in spring and summer and least in winter; greatest at high-salinity stations and least at low-salinity stations.

Spawning of 1 turbellarian, 7 species of mollusks, and 25 species of crustaceans, as well as mating of horseshoe crabs, were observed during the study but the limited data do not enable me to define the duration of any of the spawning or breeding seasons.

Season, duration, and intensity of setting of larvae of oysters, barnacles, bryozoans, and serpulid worms were studied in Little Sabine Bay during 1960-63 (fig. 12) and in Santa Rosa Sound during 1962-63.

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Table 4.--Most numerous animals collected in the Pensacola Estuary, 1961-63

Animal	Salinity area[1]			Habitat[2]	Season of greatest abundance[3]
	L	I	H		
PHYLUM MOLLUSCA					
Anachis avara -- Greedy dove-shell.....................		X	X	S, SM	All
Crepidula fornicata -- Common Atlantic slipper-shell...	X		X	S, SM on Sh	All
Mitrella lunata -- Lunar dove-shell...................			X	S, SM	All
Nassarius acutus -- Sharp-knobbed nassa...............	X	X	X	S, M	All
Nassarius ambiguus -- Variable nassa..................			X	S	W, Sp, Su
Nassarius vibex -- Common eastern nassa...............	X	X	X	S, SM	All
Thais haemastoma -- Southern oyster drill.............		X	X	SM, O, P	Sp, Su, F; bury in bottom in W
Pyramidella fusca -- Brown pyram......................	X	X		S, M	All
Brachidontes recurvus -- Hooked mussel................		X	X	S	All
Crassostrea virginica -- American oyster..............	X	X	X	S, SM	All
Laevicardium mortoni -- Morton's cockle...............			X	S, SM	All
Lolliguncula brevis -- Brief squid...................		X	X	Pell	F, W
PHYLUM ANNELIDA					
Laonereis culveri -- Nereid polychaete................	X	X	X	S, MS	All
Neanthes succinea -- Nereid polychaete................	X	X	X	S, MS, SM	All
Nereis pelagica occidentalis -- Nereid polychaete......	X	X	X	S, SM	All
Polydora websteri -- Spionid polychaete...............	X	X	X	S	All
Armandia agilis -- Opheliid polychaete................		X	X	S, SM	All
Eupomatus dianthus -- Calcareous tube worm............		X	X	R, Sh, P	All
PHYLUM ARTHROPODA					
Balanus eburneus -- Ivory barnacle....................	X	X	X	R, Sh, P	All
Haustorius sp. -- Amphipod............................	X		X	S, SM	W, Sp
Penaeus aztecus -- Brown shrimp.......................		X	X	S, MS, SM	Sp, Su, F
Penaeus duorarum -- Pink shrimp.......................			X	SM	Su
Penaeus setiferus -- White shrimp.....................	X	X	X	SM	Su
Palaemonetes pugio -- Grass shrimp....................			X	G	Sp
Clibanarius vittatus -- Striped hermit crab...........			X	R, S, MS	Sp, Su, F
Clibanarius tricolor -- Hermit crab...................			X	MS, S, G	All
Pagurus pollicaris -- Hermit crab....................			X	MS	W, Sp
Pagurus bonairensis -- Hermit crab...................			X	MS, G	Sp, Su
Callinectes sapidus -- Blue crab.....................	X	X	X	S, SM, M	Sp, Su, F
Neopanope t. texana -- Mud crab......................			X	S, SM	All
PHYLUM CHORDATA					
Brevoortia patronus -- Largescale menhaden............	X	X	X	Pel	Sp, Su, F
Anchoa hepsetus -- Striped anchovy....................	X	X	X	Pel	Sp, Su, F
Anchoa mitchilli -- Bay anchovy......................	X	X		Pel	Su, F
Synodus foetens -- Inshore lizard-fish................	X	X	X	Pel	Su
Bagre marinus -- Gafftopsail catfish.................	X	X	X	Pel	Su
Urophycis floridanus -- Florida hake.................	X	X	X	Pel	W, Sp
Urophycis regius -- Spotted hake.....................			X	Pel	Sp
Bairdiella chrysura -- Silver perch..................	X	X	X	Pel	Su
Cynoscion arenarius -- Sand seatrout.................	X	X	X	Pel	Su
Leiostomus xanthurus -- Spot........................			X	Pel	W, Sp, Su
Micropogon undulatus -- Atlantic croaker.............	X	X	X	Pel	Sp, Su
Lagodon rhomboides -- Pinfish.......................		X	X	Pel	Sp, Su
Stenotomus caprinus -- Longspined porgy..............			X	Pel	Sp

[1]L = Low, 10 p.p.t.; I = Intermediate, 10-20 p.p.t.; H = High, 20 p.p.t.
[2]G = grass beds, M = mud, MS = muddy sand, O = oysters, P = pilings, Pel = pelagic, R = rocks, S = sand, Sh = shells, and SM = sandy mud.
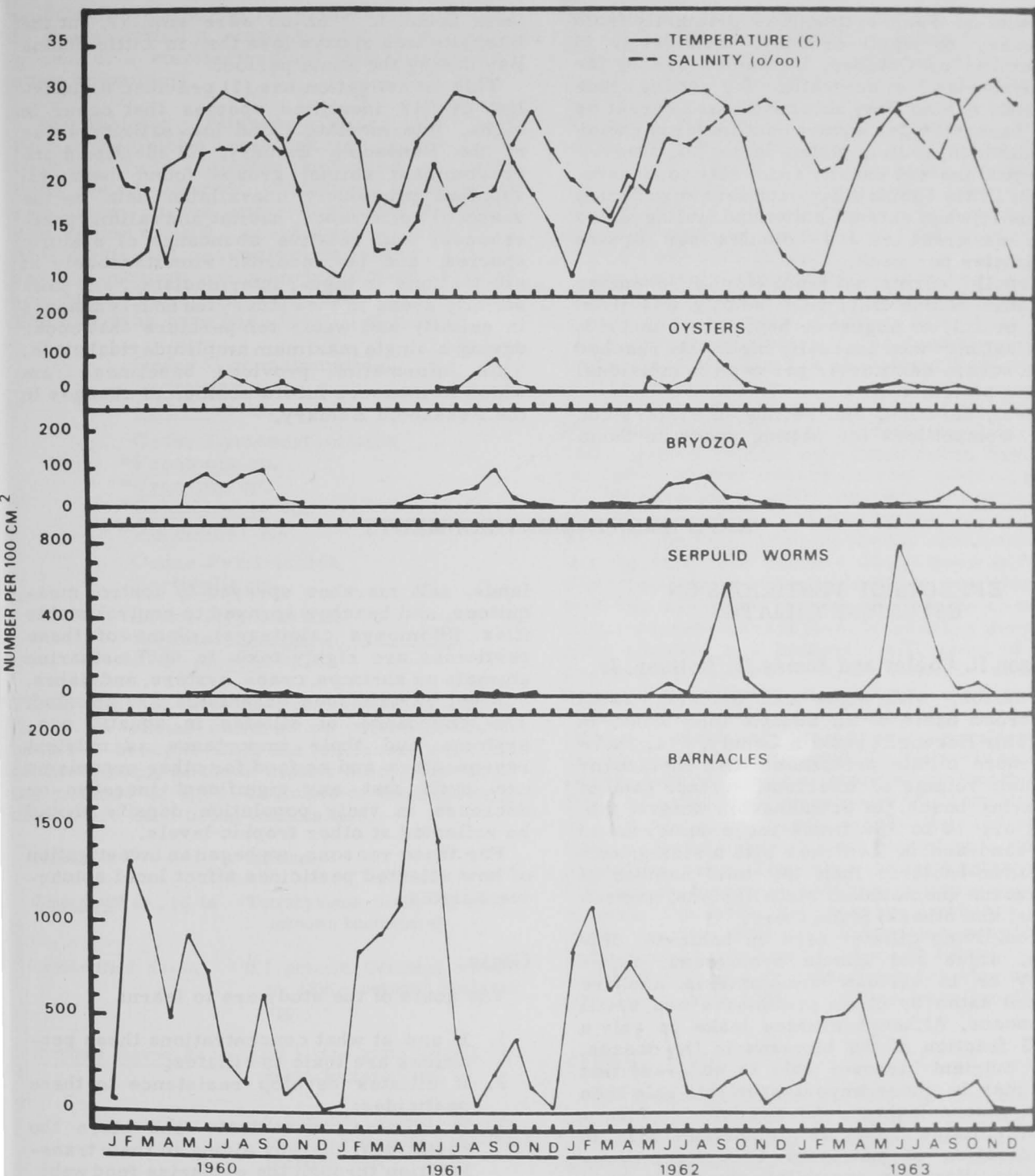[3]Sp = Spring, Su = Summer, F = Fall, W = Winter, and All = Year-round.

Figure 12.--Trends in setting of oysters and other sedentary organisms in Little Sabine Bay, 1960-63.

Setting of oysters in Little Sabine Bay usually begins in May or June and ceases in October or November; peak setting period is usually in June. Sets as great as 4.6 per square centimeter per week were observed on individual fouling plates. Patterns of setting may be markedly altered by low salinity, such

as that caused in 1961 by unusually heavy and prolonged spring rains, or by high salinity, such as that caused by subnormal rainfall during most of 1963.

Barnacle setting in Little Sabine Bay was essentially continuous from 1960 to 1963, halting only very briefly in the coldest part of

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the winter. Peak setting was primarily from February to April or May, secondarily in September or October. Barnacles are by far the oyster's chief competitor for setting space in Little Sabine Bay; sets have been as great as 45.8 barnacles per square centimeter per week on individual fouling plates during the study.

Bryozoans set usually from May to November in Little Sabine Bay; peak sets were during summer, when sets on individual fouling plates were as great as 6.4 colonies per square centimeter per week.

Serpulid worms set from May to November in Little Sabine Bay; peak setting was from June or July to August or September. In 1963, when salinity was unusually high, sets reached 8 per square centimeter per week on individual fouling plates.

Onset, duration, and setting of oysters and their competitors for setting space in Santa Rosa Sound in 1962-63 were similar, but the intensity was always less than in Little Sabine Bay during the same period.

This investigation has (1) provided a checklist of 712 identified species that occur in high-, intermediate-, and low-salinity areas of the Pensacola Estuary, (2) identified the predominant animal groups found there, (3) supplied previously unavailable data on the seasonal occurrence, habitat and salinity preferences, and relative abundance of specific species, and (4) recorded simultaneously at six stations in high-, intermediate-, and low-salinity areas in the estuary the hourly changes in salinity and water temperature that occur during a single maximum amplitude tidal cycle. This information provides baselines from which to measure future ecological changes in the Pensacola Estuary.

EXPERIMENTAL ENVIRONMENTS

EFFECTS OF PESTICIDES ON ESTUARINE CILIATES

Nelson R. Cooley and James M. Keltner, Jr.

Protozoa, with algae and bacteria, form the broad basis of all aquatic food webs. In Alligator Harbor, Franklin County, Fla., there are more ciliate protozoans than metazoans per unit volume of intertidal surface sand of a marine beach. In Scandinavian waters, ciliates are 10 to 100 times more numerous in fine sand and in localities with a rich growth of sulfur-bacteria than the total number of metazoans (nematodes, turbellarians, gastrotrichs, and others) found there.

Free-living ciliates feed on bacteria, diatoms, algae and minute protozoans, either singly or in various combinations, and are in turn eaten by other protozoans and small metazoans. Although ciliates make up only a small fraction of the biomass in the oceans, their nutrient turnover rate is so great that they may be more important in this role than some macroplankton. As predators of bacteria, ciliates have an important role in regeneration of nutrients in aquatic ecosystems. Bacteria assimilate the nutrients in the large amounts of organic detritus that occur in estuaries, thus making them available to bacteria-feeders. Ciliates feed on the bacteria and excrete large quantities of dissolved nitrogen and phosphorus compounds which are utilized as nutrients by other bacteria, thereby aiding bacterial decomposition or organic detritus.

Increasing evidence shows that pesticides are entering estuaries via runoff from farm lands, salt marshes sprayed to control mosquitoes, and beaches sprayed to control stable flies (Stomoxys calcitrans). Some of these pesticides are highly toxic to such estuarine animals as shrimps, crabs, oysters, and fishes.

Also, certain food organisms are affected. The abundance of ciliates in aquatic ecosystems and their importance as nutrient regenerators and as food for other organisms are such that any significant increase or decrease in their population density should be reflected at other trophic levels.

For these reasons, we began an investigation of how selected pesticides affect local estuarine ciliates.

Goals

The goals of the study are to learn:

1. If and at what concentrations these pesticides are toxic to ciliates;
2. If ciliates develop resistance to these pesticides;
3. If ciliates concentrate and store the pesticides, thereby aiding in their translocation through the estuarine food web;
4. How toxic effects of pesticides are produced and which metabolic processes are affected.

Methods

We have studied both agnotobiotic and axenic cultures of ciliates. An agnotobiotic culture is one in which one or more identified species is grown in a medium that contains a mixed

unidentified bacterial flora. An axenic culture is one in which a single identified species is grown in a sterile medium that is free of all other organisms.

Agnotobiotic cultures.--During the past year, agnotobiotic mass cultures derived from enriched field samples collected in Santa Rosa Sound adjacent to Sabine Island were established in sea water containing uncooked rice grains or in 0.1 percent (w/v) Cerophyl - ASW (artificial sea water) infusion[1]. Ciliate species encountered in enriched field samples are being identified as quickly as possible. The following have been identified so far:

> Order Gymnostomatida
> Lacrymaria sp.
> Litonotus sp.
>
> Order Hymenostomatida
> *Frontonia sp.
> *Uronema sp.
> *Cohnilembus sp.
> *Glaucoma? sp.
>
> Order Peritrichida
> Vorticella sp.
>
> Order Hypotrichida
> *Euplotes charon
> *Uroleptopsis sp.

Those marked with an asterisk were established in clonal cultures on mixed unknown bacteria brought into the cultures with the ciliates. Many of these clones died after varying periods of time. Cultures of the following clones are still being maintained:

Ciliate	Medium
Glaucoma? sp., #8.1a	Tetrahymena broth[2] in ASW+ unknown bacterial sp.
Unidentified, #2-4A	0.1 percent Cerophyl infusion in ASW + unknown bacterial spp.
Euplotes charon, #AA	ASW + rice grain + mixed unknown bacterial spp.
	0.1 percent Cerophyl infusion in ASW + at least 2 unknown bacterial spp.

Axenic cultures.-- Attempts to axenize clonal cultures have so far been unsuccessful. Bacterial populations were reduced greatly by

[1]Cerophyl = a vitamin-rich powder prepared from dehydrated cereal leaves. RILA salts, 24.0 p.p.t. salinity in Pyrex-distilled water.

[2]Proteose peptone, 2 percent; yeast extract, 0.1 percent; dextrose, 0.5 percent; RILA salts, 24.0 p.p.t. salinity in distilled water.

washing the ciliates repeatedly in sterile ASW before transferring to test tubes of sterile Tetrahymena broth in ASW of 24.0 p.p.t. salinity plus 1,000 units potassium penicillin-G and 0.1 mg. streptomycin sulfate per milliliter. Bacterial growth was inhibited and not apparent until the fifth day, when it reappeared as a film on the wall of the test tube. Growth of ciliates in this medium was slow but increased rapidly as the bacteria multiplied, indicating that the medium alone is less nutritious than the medium plus bacteria.

We are now attempting to axenize our cultures by means of an experimental bactericide, 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4 thiadiazole. When the unidentified mixed bacterial flora found with unidentified ciliate strain #2-4A grown in 0.1 percent Cerophyl infusion in ASW is transferred to fluid thioglycollate medium used to test sterility, the flora grows rapidly and luxuriantly. Similar bacterial growth occurs in this medium plus the bactericide at 0.001 mg./ml., but bacterial growth is greatly inhibited by the bactericide at 0.01 mg./ml. and completely eliminated at 0.1 mg./ml. The ciliates do not grow in fluid thioglycollate medium; therefore, we plan to test the herbicide for toxicity to the ciliates in 0.1 percent Cerophyl-ASW infusion in which both ciliates and bacteria will grow. If the bactericide eliminates the bacteria without harming the ciliates, it will be a useful tool.

Because of difficulty in axenizing local ciliates, a stock of Tetrahymena pyriformis, axenic strain W, was obtained for use in establishing and refining our experimental methods. The animals were grown at 26° C. on 10 ml. of Tetrahymena broth in 18- by 150-mm. bacteriological test tubes closed with polypropylene caps. The tubes were slanted at a 60° angle during incubation to increase the surface available for gaseous exchange between medium and air. Population growth is determined turbidimetrically with a Bausch and Lomb Spectronic 20 spectrophotometer using monochromatic light at 540 mμ wave length.

Toxicity Tests

The main effort was directed toward learning whether selected pesticides are toxic to ciliates. Since many pesticides are slightly soluble or insoluble in sea water, it is necessary to dissolve them in a solvent that is miscible with water before adding the pesticide to the culture medium. Suitable solvents for the pesticides themselves must be tested for toxicity against the test organism.

Solvents.--Acetone and Carbowax 200[3] (polyethylene glycol 200) have been used routinely in this laboratory as solvents. For example,

[3]Carbowax 200 = polyethylene glycol, average molecular weight 200.

1,000 p.p.m. p,p'-DDT can be dissolved in Carbowax 200 with mild heat.

In cultures of Tetrahymena grown on Tetrahymena medium at 26° C. from log-phase inocula, 0.1 percent (v/v) Carbowax 200 appears to have little effect on final population size attained at 96 hours, and growth rates of control and experimental cultures are similar (fig. 13). Concentrations of Carbowax 200 greater than 1 percent cause osmotic stress, and marked shrinking of individual ciliates is rapidly apparent at 10 percent.

Pesticide.--DDT is long-lived and has been widely used for nearly 30 years; its residues occur widely in animals and are worldwide in distribution. Also, it has become more or less a "standard" for relative toxicity effects or a reference insecticide. We have, therefore, begun testing the effect of DDT on ciliate population growth. Tests with Tetrahymena pyriformis, strain W, grown at 26° C. on Tetrahymena medium are in progress.

So far, data from cultures of Tetrahymena pyriformis, strain W, grown in test tubes at 26° C. on Tetrahymena medium containing final concentrations of 0.1, 1.0, and 10.0 p.p.m. p,p'-DDT and 0.1 percent Carbowax 200 (fig. 14) show that population growth decreases with increasing concentration of DDT. As measured by absorbance, populations at 96 hours are reduced 13.8 percent by 0.1 p.p.m. DDT, 20.2 percent by 1.0 p.p.m., and 25.7 percent by 10.0 p.p.m. The data suggest that T. pyriformis, strain W, may be more sensitive to DDT than are Paramecium multimicronucleatum and P. bursaria, which have been reported to show "no adverse effects" when grown for 7 days in medium containing 1 p.p.m. DDT and 0.1 percent acetone, although DDT accumulated in the cells of P. multimicronucleatum was 264 times, and in P. busaria 964 times, greater than in the medium.
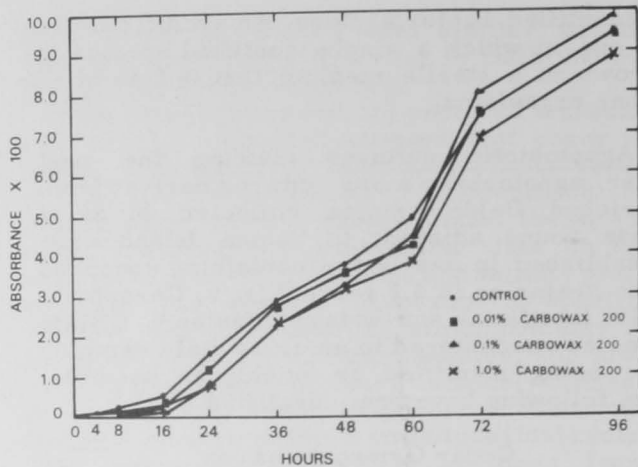

Figure 13.--Effect of Carbowax 200® on growth of a population of Tetrahymena pyriformis, strain W, at 26° C. For each datum, n = 10.
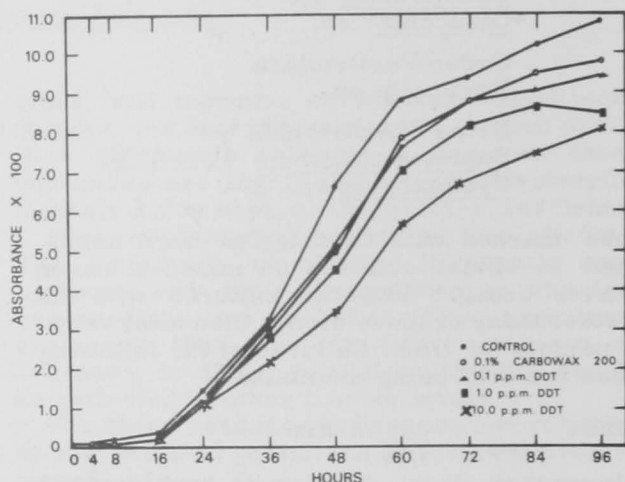

Figure 14.--Effect of p,p'-DDT on growth of a population Tetrahymena pyriformis, strain W, at 26° C. For each datum, n = 10.

CHEMICAL ASSAYS

Alfred J. Wilson, Jr., Jerrold Forester, and Johnnie Knight

The purpose of the chemical assay project is to provide pesticide residue analyses for all research projects at the Field Station in Gulf Breeze and the Bureau's National Estuarine Pesticide Monitoring Program. In addition, we cooperate with Federal and State agencies in the analysis of samples for pesticide residues and provide consultation for pesticide residue laboratories that are being established.

In pesticide residue analysis, we have to pay careful attention to detail to ensure that the sample does not become contaminated before and during the analysis, and that the pesticide is not lost. Tissue samples are usually extracted on a Soxhlet apparatus (fig. 15) and the extract is purified to remove interfering material. The purified extract is then injected into a gas chromatograph (fig. 16) for qualitative and quantitative evaluation.
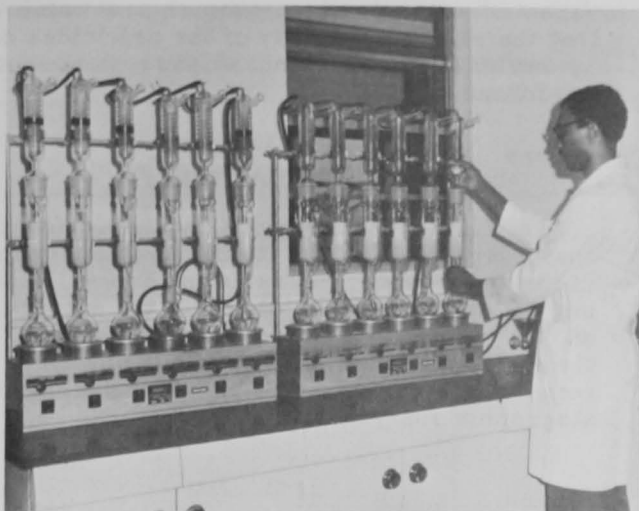
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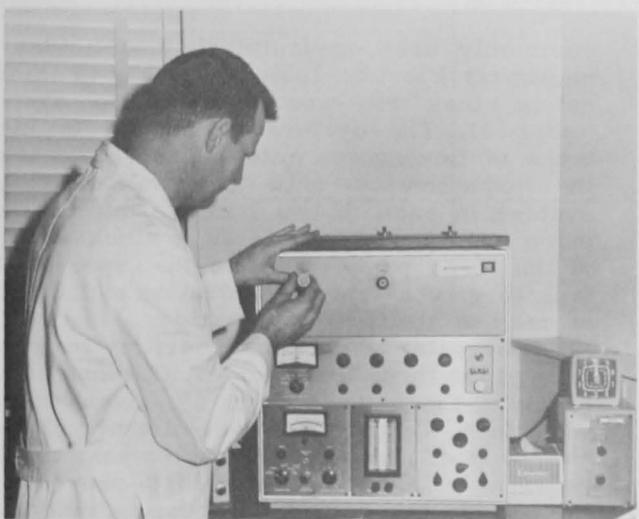Figure 15.--Soxhlet extraction of pesticide residues.

Figure 16.--Injection of pesticide sample into gas chromatograph.

Because the techniques of residue analysis are difficult to learn, a manual has been prepared for the analysis of chlorinated pesticides in the marine environment. This manual has been distributed to the Bureau's contracting agencies to standardize the methods of pesticide residue analysis for the National Estuarine Pesticide Monitoring Program.

During the year, we analyzed 988 samples for research projects at the Field Station in Gulf Breeze and 1,642 samples from monitor stations along the Atlantic, Gulf, and Pacific coasts. Also, we analyzed pesticide residues in samples from the following Federal and State agencies: BCF Biological Laboratories at Oxford, Md., Seattle, Wash., Milford, Conn., and Galveston, Tex.; U.S. Army Corps of

Engineers, Jacksonville, Fla.; U.S. Army, Fort Amador, C.Z.; U.S. Geological Survey, Miami, Fla.; Florida Game and Freshwater Fish Commission, Tallahassee, Fla.; Manatee County Health Department, Bradenton, Fla.; Marineland, St. Augustine, Fla.; and University of Texas Marine Science Institute, Port Aransas, Tex. We also participated in the analysis of samples for the Cooperative Blue Crab Mortality Study of the South Atlantic States. Locally, the laboratory provided assistance in the analysis of samples from fish kills in the Pensacola estuary. Thus, the samples analyzed varied widely in geographic source and in the kinds of organisms (included, for example, were seagrasses, sponges, sooty terns, fur seals, and whales).

In addition, we cooperated with the U.S. Public Health Service in a study to determine the effect of pesticides on trace metal content of oysters. We began preliminary studies to determine the stability of pesticide in sea water. We investigated analytical methods for the analysis of 2,4-D in seagrasses. Discussions of these studies follow.

EFFECT OF PESTICIDES ON TRACE METAL CONTENT OF OYSTERS

A cooperative study was completed with the U.S. Public Health Service, Gulf Coast Marine Health Sciences Laboratory, Dauphin Island, Ala., to determine the effects of pesticides on uptake of trace metals by oysters. Oysters were exposed to 0.1 and 10.0 p.p.b. of DDT and dieldrin in four flowing water aquariums for 12 days. Analyses for trace metals and residues were made on tissues of the test animals before and after exposure to pesticide. Although pesticide residues in the tissues increased more than 900-fold, the concentrations of cadmium, chromium, copper, iron, lead, magnesium, manganese, and zinc did not change significantly.

STABILITY OF PESTICIDES IN SEA WATER

We began preliminary studies to determine the stability of pesticides in sea water. Three p.p.b. of aldrin, p,p'-DDT, malathion, and parathion in acetone were added separately to four clear glass, one-gallon bottles containing sea water (salinity 29.8 p.p.t.; pH 8.1), one chemical per bottle. After an initial sample of the water was analyzed, the bottles were sealed and completely immersed in an outdoor flowing sea-water tank. Table 5 shows the concentration of the chemical at the indicated time interval.

Although we used natural sea water in these preliminary experiments, the tests will be

Pesticide	Days after start of experiment					
	0	6	17	24	31	38
	P.p.b.	P.p.b.	P.p.b.	P.p.b.	P.p.b.	P.p.b.
p,p'-DDT.....	2.9	.75	1.0	.27	.18	.16
p,p'-DDE*..		.096	.95	.065	.034	.037
p,p'-DDD*..			.081	.041	.038	.037
Aldrin**.....	2.6	.58	.096	<0.01	<0.01	<0.01
Dieldrin*..		.74	1.0	1.0	.75	.56
Malathion....	3.0	<0.2	<0.2	--	—	—
Parathion....	2.9	1.9	1.25	1.0	.71	.37

*Metabolites of parent compound.
**From the seventeenth day onward, 2 unidentified peaks appeared on the chromatographic charts after aldrin had eluted.

repeated with sterile artificial sea water so that the relative stability of the pesticides can be evaluated under standardized experimental conditions.

2,4-D ANALYSIS

Analytical methods were evaluated for the analysis of 2,4-D and its butoxyethanol ester in the seagrasses Thalassia and Ruppia. Existing methods were suitable for the extraction of the herbicides from the grasses. The cleanup technique was modified, however, to remove interfering material before gas chromatographic analysis.

LABORATORY BIOASSAYS

Jack I. Lowe, Paul D. Wilson, and Richard B. Davison

New pesticides are continually being developed for commercial use, and some of these compounds will be used in or near estuaries. Therefore, there is continuing need for determining both acute and chronic toxicity of these chemical pesticides to commercially valuable marine species. Laboratory bioassays have been made at this station since 1961. Several new chemicals received during the year were evaluated on shrimp, fish, and oysters. We devoted much of our effort to long-term bioassays involving the chronic exposure of selected marine species to low-level pesticide pollution.

ACUTE TOXICITY STUDIES

Short-term bioassays do not provide conclusive data, but they do provide information on the relative toxicity of pesticides to different species of marine organisms. Table 6 shows median toxicity values of selected compounds screened during the year. The order of toxicity varies greatly with shrimp, fish, and oysters. As a group, insecticides are more toxic to all forms other than pesticides, but there are exceptions. The fungicide, Delan, and the experimental antifouling agent, ET-546, were extremely toxic to oysters.

CHRONIC TOXICITY STUDIES OF OYSTERS TO DDT, TOXAPHENE, AND PARATHION

A year-long laboratory study was completed on the effects of a mixture of three insecticides (DDT, toxaphene, and paration) on the growth and development of American oysters. These three compounds are the most

commonly used agricultural insecticides in northwest Florida. Individually and in combination they are acutely toxic to oysters (table 6). The oysters were held in large tanks of flowing sea water (fig. 17). We began the experiments with 100 two-month-old oysters in each of two tanks. A multichannel pump continuously metered a stock solution of the three pesticides into the experimental tank to give a test concentration of 1.0 p.p.b. of each of the three insecticides. Analyses of the test water each month verified the presence of the three insecticides. The control tank received the same amount of solvent without pesticides. Test oysters were carefully cleaned and weighed individually each week; the in-water weighing technique (fig. 18) proved to be a sensitive method for measuring individual variations in growth. Random samples of control and experimental oysters were made at 3-, 6-, 9-, and 12-months intervals for pesticide residue analyses and 6-, 9-, and 12-month intervals for pathological examination.

Experimental oysters were exposed continuously for 9 months to the pesticide mixture and then held in clean flowing sea water for an additional 3 months. The mean weight of the control oysters was consistently greater than that of the experimentals after 6 weeks of exposure, but the difference was not statistically significant (P <0.05) until the 22d week (fig. 19). After 9 months, the control oysters outweighed (mean in-water weight) the experimentals by about 3 g. This difference represents 12 percent of the total body weight of the experimental oysters. At the end of 12 months, the mean weight difference was still about the same.

Experimental oysters concentrated the chlorinated hydrocarbons, DDT and toxaphene,

Table 6.--Relative toxicity of selected pesticides to shrimp, fish, and oysters

[Rank in toxicity is shown in parentheses; (1) = most toxic and (11) or (12) = least toxic]

Pesticide	Shrimp 48-hour EC_{50}[1]		Fish 48-hour EC_{50}[2]		Oysters 96-hour EC_{50}[3]	
	P.p.m. (mg./liter)		P.p.m. (mg./liter)		P.p.m. (mg./liter)	
Insecticides:						
Dursban..........................	0.0002	(1)	0.0032	(2)	0.27	(8)
Parathion........................	0.0002	(2)	0.015	(4)	22% decrease at 1.0 p.p.m.	(10)
DDT..............................	0.0006	(3)	0.0028	(1)	0.010	(3)
Toxaphene........................	0.0042	(4)	0.028	(5)	0.038	(4)
DDT + Toxaphene + Parathion.....	---		--		0.05	(5)
Landrin..........................	0.0042	(5)	3.2	(7)	No effect at 1.0 p.p.m	(11)
Abate 4-E........................	0.020	(6)	No effect at 1.0 p.p.m.	(9)	0.17	(6)
Herbicides:						
Igran............................	No effect at 1.0 p.p.m.	(9)	20% mortality at 1.0 p.p.m.	(8)	1.0	(9)
Weed-B-Gon (2,4-D + 2,4,5-T formulation)..................	No effect at 1.0 p.p.m.	(10)	Irritated at 100.0 p.p.m.	(10)	0.19	(7)
Fungicide:						
Delan............................	0.15	(7)	0.010	(3)	0.0086	(2)
Miscellaneous compounds:						
Corexit 7664 (oil-spill remover)	No effect at 1000.0 p.p.m.	(11)	No effect at 1000.0 p.p.m.	(11)	26% decrease at 32.0 p.p.m.	(12)
ET-546 (arsenical antifouling agent)........................	10% mortality at 10.0 p.p.m.	(8)	0.15	(6)	0.0021	(1)

[1]48-hour EC_{50} = Concentration of pesticide in sea water causing 50% mortality or loss of equilibrium to juvenile penaeid shrimp.

[2]48-hour EC_{50} = Concentration of pesticide in sea water causing 50% mortality to juvenile killifish (_Fundulus similis_).

[3]96-hour EC_{50} = Concentration of pesticide in sea water causing 50% decrease in oyster shell growth.
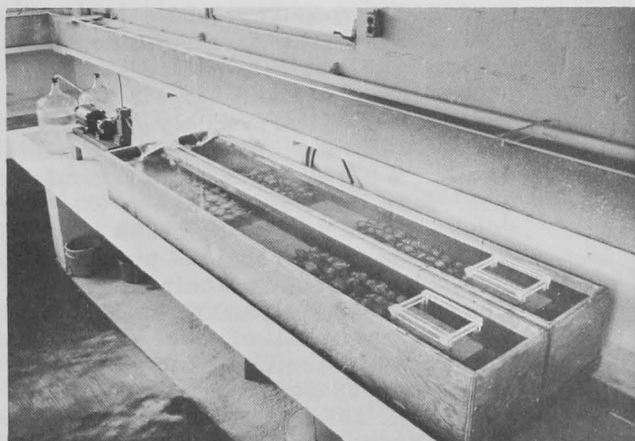

Figure 17.--Chronic exposure of oysters to low-level pesticide pollution.

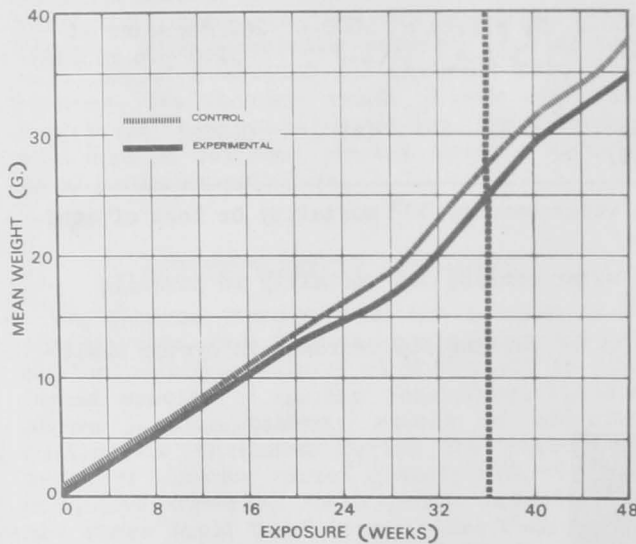Figure 18.--Oysters are weighed suspended in water.

Figure 19.--Comparison of the growth rate (mean in-water weight of 100 young oysters) of control oysters and oysters chronically exposed to 1.0 p.p.b. each of DDT, toxaphene, and parathion. The vertical dotted line represents the point at which the pesticide exposure was halted and the oysters were placed in clean water.

to relatively high levels, but apparently metabolized parathion, an organophosphorous compound, very rapidly. At the end of 6 months exposure, a homogenate of five experimental oysters contained residues of 91.0 p.p.m. DDT (DDE + DDD + DDT), 30.0 p.p.m. toxaphene, and 0.36 p.p.m. parathion. After 9 months, a homogenate of 10 oysters contained 42.0 p.p.m. DDT (DDE + DDD + DDT), 9.0 p.p.m. toxaphene, and 0.07 p.p.m. parathion. After 3 months in clean water, the remaining oysters contained no toxaphene or parathion and only background levels (same as controls) of DDT.

Histopathological examination of the oysters by a consulting pathologist revealed considerable structural change that was apparently due to the pesticides. Oysters exposed to the pesticides had pathological conditions in the kidney, visceral ganglion, tissues beneath the gut, gills, and digestive tubules. These tissue changes were present after 6 and 9 months. Each of 10 experimental oysters examined from the 9-month exposure had a mycelial fungus that was not present in the control oysters. The exposure to pesticide appeared to cause a breakdown in the oyster's natural defense against this parasite. In general, experimental oysters held in clean water for 3 months showed less structural change than those examined at the end of the 9-month exposure period. Therefore, under these experimental conditions, oysters subjected to low levels of DDT, toxaphene, and parathion are capable of repairing certain pesticide-damaged tissues when placed in nonpolluted water. We were unable to make an intensive study of how exposure to pesticide affects reproduction, but both control and experimental oysters had viable eggs and spermatozoa at the end of the 9-month exposure. Eggs of both groups of oysters developed to the 24-hour trochophore stage when fertilized artificially.

We are now studying the effects of DDT, toxaphene, and parathion separately on populations of oysters, using the techniques described above.

EFFECTS OF MIREX ON CRABS, SHRIMP, AND FISH

The use of mirex (Dodecachlorooctahydro-1, 3, 4-metheno-2H-cyclobuta [cd] pentalene) to control imported fire ants in coastal areas of the southeastern United States is causing some concern about its effects on estuarine organisms. We previously reported this compound to have a relatively low acute toxicity to marine crustaceans. Experiments completed this year, however, show that mirex has delayed toxic effects on crabs and shrimp.

Juvenile blue crabs and pink shrimp showed no symptoms of poisoning during a 96-hour exposure to 0.1 p.p.m. technical mirex in flowing sea water. All of these crustaceans, however, became irritated and paralyzed, and then died within 18 days after being placed in clean water (fig. 20). About 30 percent of the shrimp and 20 percent of the crabs were dead or paralyzed after 10 days, and 80 percent of the
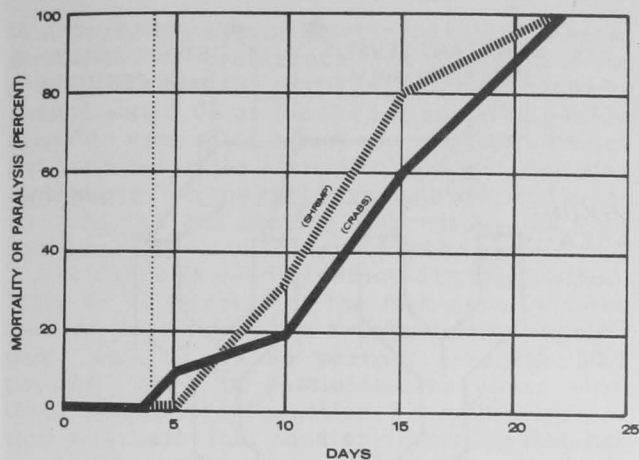
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Figure 20.--Delayed toxic action of mirex to juvenile penaeid shrimp and blue crabs. The shrimp and crabs showed no symptoms of poisoning at the end of a 4-day exposure (vertical dotted line) to 0.1 p.p.m. technical mirex, but became paralyzed and died within 22 days.

shrimp and 60 percent of the crabs after 15 days. This was our first encounter with delayed toxicity of a pesticide to marine crustaceans.

Mirex bait (the actual formulation used in the field to control fire ants) also causes delayed toxicity to juvenile blue crabs. Mirex may act as both a contact poison (as shown in the experiment above) and a stomach poison to small crabs. In preliminary experiments, small crabs placed in tanks containing several particles of Mirex Granulated Bait 4X (84.5

percent corn cob grits, 15.0 percent soybean oil, and 0.3 percent mirex) became paralyzed within 7 days. In a simulated field application (1.25 pounds per acre) of the bait, individual crabs (20-30 mm. carapace width) were paralyzed within 3 to 14 days by a single particle of the bait. The crabs were held in compartments in flowing-water (400 liters per hour) aquariums containing one crab and one particle (average weight 1.5 mg.) of bait per compartment. In several instances, crabs picked up the particle of bait and were observed "chewing" with their mandibles. Seventy-six percent (19 of 25) of the crabs were dead or paralyzed 2 weeks after receiving the mirex bait. No mortality or paralysis occurred in 25 control crabs. The availability of fire ant bait to feeding crabs should be considered in evaluating the effect of field application of this material on estuarine organisms.

Pinfish do not appear to be affected by mirex either in their food or in the water in which they live. Test pinfish lived 5 months on a diet containing about 20 p.p.m. technical mirex. Mirex bait was also generously applied to the bottom of their holding tank. The fish had no symptoms of pesticide poisoning during the experiment, but they concentrated high residues (30-40 p.p.m.) of mirex in body tissues. Eight weeks after the exposure, fish still contained an average of 18.0 p.p.m. mirex in their tissues--indicating that mirex is not easily metabolized.

These experiments with mirex further emphasize the need for studies to evaluate the long-term effects of pesticides.

BEHAVIOR OF ESTUARINE ORGANISMS

David J. Hansen

Behavioral responses of estuarine organisms influence their abundance, distribution, and survival. Changes in these responses induced by pesticides could be detrimental to a commercial fishery. Temperature preference, learning, and other behavioral characteristics of fish are known to be affected by exposure to pesticides. We are collecting information that is needed to answer two questions: Whether fish can avoid pesticides in water and whether their salinity preference is altered after exposure to pesticides.

PESTICIDE AVOIDANCE STUDIES

The probability of survival of estuarine fish would be improved if they could avoid pesticides in their environment. This capacity has been demonstrated for some fresh-water fishes but not for estuarine fish.

The purpose of this study was to evaluate the capacity of untrained sheepshead minnows, Cyprinodon variegatus, to avoid six pesticides (DDT, endrin, Dursban, malathion, Sevin, and 2,4-D) that are likely to be found in estuaries.

Experimental Procedure

Sheepshead minnows, 20 to 40 mm. total length, were seined from brackish-water marsh ditches on Santa Rosa Island. To eliminate weak or injured individuals, we maintained the test fish in the laboratory in water of 20 p.p.t. salinity at 20° C. for at least 10 days before they were used. The fish were fed pieces of fish flesh daily until 24 hours before an experiment.

One herbicide and five insecticides (two organochlorines, two organophosphates, and a carbamate) were used in the tests (table 7).

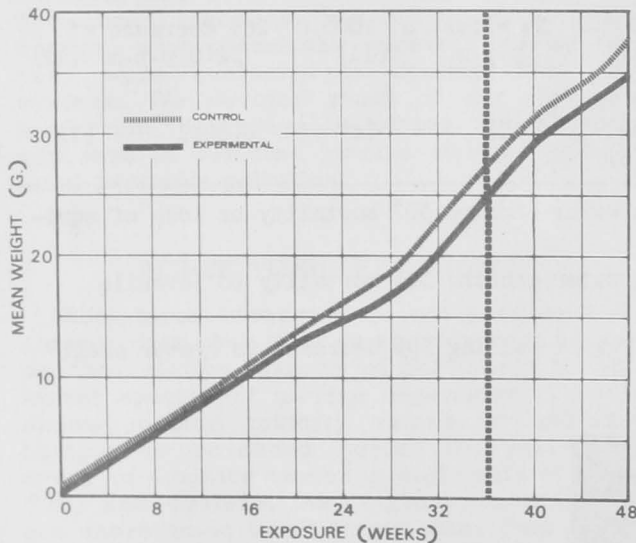Figure 18.--Oysters are weighed suspended in water.

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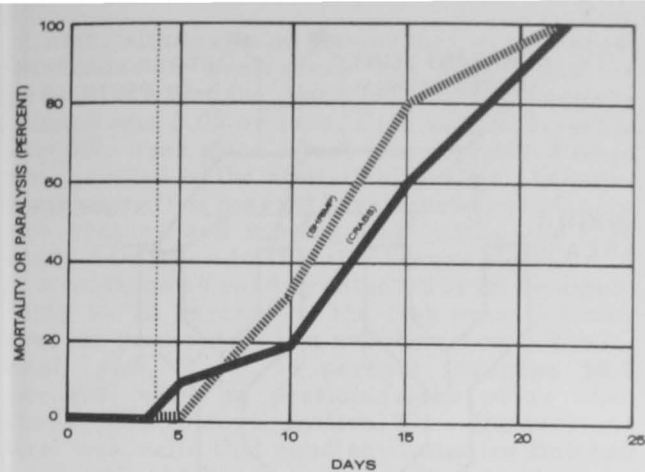
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One herbicide and five insecticides (two organochlorines, two organophosphates, and a carbamate) were used in the tests (table 7).

Pesticide	Type	Active ingredient	24-hour LC_{50}
		Percent	P.p.m.
DDT.............	Organochlorine	99	0.006
Endrin...........	do.	97	0.003
Dursban.........	Organophosphate	99	([2])
Malathion........	do.	95	0.3
Sevin (carbaryl).	Carbamate	98	2.8
2,4-D (butoyx-ethanol ester).	Herbicide	70 (acid equivalent)	7.0

[1]Personal communication, Jack I. Lowe, Fishery Biologist, Bureau of Commercial Fisheries Biological Field Station, Gulf Breeze, Fla. 32561, March 10,1967.

[2]Showed signs of pesticide poisoning after 24 hours at a concentration of 1.0 p.p.m., but were not dead.

Initially, three concentrations of each pesticide were used--one higher and two lower than the concentration that would kill 50 percent of the fish in 24 hours (24-hour LC_{50}). If avoidance was observed, other concentrations were tested to determine the upper and lower limits that elicited a response in the fish.

Fish were tested in an apparatus designed to allow them to move from a holding area into either water containing pesticide or "clean" water. The apparatus, constructed of black plastic, had two Y-shaped "arms" 7.6 cm. wide, and a circular holding area 30.5 cm. in diameter (fig. 21). A gate at the intersection of the arms was lowered to trap the fish. When a test was in progress, the apparatus was covered with black plastic to exclude light. Filtered sea water diluted with aerated tap water to 20 p.p.t. salinity and maintained at 20° C. entered each of the four upper arms at a rate of 200 ml. per minute and flowed to the drain in the center of the circular section. The water depth in the apparatus was maintained at about 50 mm. Exchange of water between different sections of the apparatus was negligible. Pesticides in an acetone stock solution were metered through stopcocks into the rectangular area of the upper arms where pesticides mixed with the water before they reached the testing area.

This investigation was accomplished in two sets of experiments. In the first set, we tested the ability of sheepshead minnows to choose between water with pesticide and water without pesticide. Fifty fish were tested at each concentration at least four times. The distributions of the fish in holding and test apparatus indicated that they are not gregarious and that each individual should respond to the pesticide rather than to other test fish. In preliminary tests, 63 percent of the fish preferred the right side of the apparatus, compared to 37 percent in the left side; therefore, half of the tests were made with the two stock bottles delivering pesticide in the right side
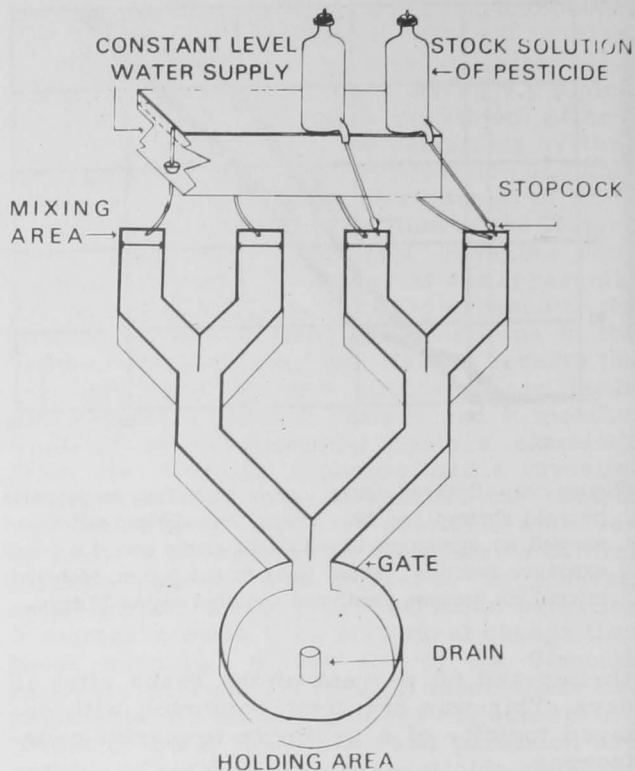

Figure 21.--Apparatus used to test the ability of sheepshead minnows to avoid pesticides.

of the apparatus and half with two stock bottles delivering pesticide to the left side. The two upper "Y's" served no function in these tests. An acetone control was not used because sheepshead minnows did not avoid acetone at the concentration tested (0.25 percent). Fish were placed in the circular area with the gate lowered into position for 1/2-hour to permit them to acclimate and to reduce the fright response. The gate was then raised to give the fish access to the arms. After 1 hour, the gate was closed and the number of fish in each arm was recorded. Test fish were used once and then discarded. After each test, the apparatus was rinsed at least three times with a 50 percent solution of acetone in water. After tests in which 10 p.p.m. of 2,4-D or Dursban were used, the apparatus was washed with detergent to remove the visible film.

In the second set of tests, we investigated the capacity of test fish to discriminate between "high" and "low" concentrations of the pesticides. We tested the concentrations previously avoided and a concentration below the threshold of avoidance. The testing procedure was the same in this set as in the first, except that four stock bottles were used and two concentrations were tested simultaneously.

Avoidance of pesticides by the fish was evaluated statistically by the chi-square test on the assumption that if fish could not discriminate they would have an equal opportunity

to enter either arm on leaving the circular area. Avoidance or preference was accepted if the probability that the distribution could occur by chance was 0.05 or less. Fish remaining in the circular area after a test was completed were not included in the statistical analysis because stationary fish may not have been exposed to the two choices and moving fish within this area could have been in transit between arms.

Avoidance is readily detected by this method. Sixty to 92 percent of the fish were in water free of pesticide when avoidance was significant, and 42 to 55 percent (average 50.1 percent) were in pesticide-free water when there was no discrimination. Thus, the assumption was valid that nondiscriminating fish had an equal chance to enter water in the arm with pesticide or the arm without the pesticide.

Capacity of Fish to Seek Water Free of Pesticides

Sheepshead minnows avoided four of the six pesticides at two or more of the concentrations tested--DDT, endrin, Dursban, and 2,4-D (table 8). The lowest concentration that the fish avoided presumably approximates the lower limit of perception, within an order of magnitude in some tests, and the highest concentration avoided is less than the amount that affects the fish's capacity to avoid the pollutant. All of the concentrations of each pesticide avoided, except Dursban, covered a range of at least an order of magnitude and many were near the 24-hour LC_{50}. The highest concentration of Dursban avoided was only 2.5 times greater than the lowest (excluding the avoidance of 10 p.p.m., which may have been a reaction to crystals).

Except for 2,4-D, a concentration response-- avoidance in relation to concentration of the pesticide--was not observed in these experiments within the tested concentrations. Evidently sheepshead minnows were not able to sense an increase in concentrations of the pesticides tested. The avoidance of 10 p.p.m. 2,4-D by these fish may not have been true concentration response because avoidance was probably a reaction to crystals of the pesticide.

Sheepshead minnows did not avoid malathion or Sevin at the concentrations tested (table 8); therefore, we made no further tests of these pesticides.

Table 8.--Tendency of fish to seek water free of pesticides

N.S. = Not significant. x^2 = P(3.84 = 0.05; 6.63 = 0.01; 10.83 = 0.001)

Pesticide and concentration	Tests	Fish in pesticide	Fish in water		x^2 value if significant
P.p.m.	Number	Number	Number	Percent	
DDT:					
0.1........	6	75	69	47.9	N.S.
0.05.......	4	46	70	60.3	4.96
0.01.......	8	67	109	61.9	10.02
0.005......	4	41	66	61.7	5.84
0.001......	4	46	45	49.4	N.S.
0.0001.....	4	49	44	47.3	N.S.
Endrin:					
0.01.......	4	48	43	47.2	N.S.
0.001......	4	30	60	66.7	10.00
0.0001.....	4	34	58	63.0	6.26
0.00001....	4	25	31	55.4	N.S.
Dursban:					
10.0........	8	46	86	65.2	12.12
1.0........	10	83	79	48.8	N.S.
0.5........	4	38	27	41.5	N.S.
0.25.......	4	24	85	78.0	34.14
0.1........	8	73	132	64.4	16.98
0.05.......	8	100	82	45.0	N.S.
0.01.......	4	47	41	46.6	N.S.
Malathion:					
1.0........	4	36	32	47.0	N.S.
0.1........	4	33	41	55.4	N.S.
0.01.......	4	51	59	53.6	N.S.
Sevin:					
10.0........	4	41	50	54.9	N.S.
1.0........	4	53	54	50.5	N.S.
0.1........	4	47	58	55.2	N.S.
2,4-D:					
10.0........	4	7	86	92.5	67.11
1.0........	4	33	67	67.0	11.56
0.1........	4	37	68	64.8	9.15
0.01.......	8	86	102	54.2	N.S.

Response of Fish to Simultaneous Exposure to Two Different Concentrations of the Same Pesticide

Sheepshead minnows that were exposed simultaneously to two different concentrations of a specific pesticide avoided 2,4-D at the highest concentration tested, preferred the higher concentration of DDT and did not discriminate between different concentrations of endrin or Dursban, or other concentrations of 2,4-D (table 9). The failure of fish to discriminate may be explained by the lack of a response to different concentrations as observed in the first set of tests. Fish that avoided 10.0 p.p.m. 2,4-D may have been reacting to the high concentration or to crystals of the chemical, which were absent at other test concentrations.

The preference of sheepshead minnows for the higher concentration of DDT is difficult to explain because they avoided it in the first set of experiments. Also, they did not discriminate between concentrations of Dursban, endrin, and lower levels of 2,4-D in the second set of experiments. When there was no area free of DDT the higher concentration either was preferred or interfered with the capacity of the fish to avoid it.

Ecological Implications

It would undoubtedly be advantageous to fish if they could avoid pesticides. In these tests, fish did not, in general, differentiate between concentrations of the same pesticide, but did

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Table 9.--Response of sheepshead minnows exposed to two different concentrations of four pesticides

[N.S. = Not significant. χ^2 = P(3.84 = 0.05; 6.63 = 0.01; 10.83 = 0.001)]

Pesticide and concentrations		Tests	Fish in high concentration	Fish in low concentration		χ^2 value if significant
High	Low					
P.p.m.		Number	Number	Number	Percent	
DDT:						
0.05	0.005	8	117	81	40.9	6.54
0.05	0.001	4	80	31	27.9	21.63
0.01	0.001	8	119	74	38.2	10.49
0.005	0.001	8	143	84	37.0	15.33
Endrin:						
0.001	0.0001	4	39	44	53.0	N.S.
0.001	0.00001	4	54	54	50.0	N.S.
0.0001	0.00001	4	62	48	43.6	N.S.
Dursban:						
0.25	0.05	8	82	71	46.4	N.S.
0.10	0.05	4	27	28	50.9	N.S.
2,4-D:						
10.0	1.0	4	8	139	94.6	116.74
10.0	0.1	4	9	118	92.9	93.55
10.0	0.01	4	7	133	95.0	113.40
1.0	0.1	4	49	38	43.7	N.S.
1.0	0.01	4	48	44	47.8	N.S.
0.1	0.01	4	48	42	46.7	N.S.

have the ability to seek water free of pesticides. Therefore, a prerequisite for avoidance in nature would be a reasonably distinct boundary between clean water and water containing a pesticide.

Estuaries often have conditions that cause boundaries or interfaces which would give fish an opportunity to avoid contaminated water. One such boundary, the pycnocline, is formed when fresh water from rivers flows over denser salt water in an estuary. In the deeper estuaries, mixing across the pycnocline is limited. Thus, pesticides entering the estuary in river water would not contaminate water in the salt wedge below the pycnocline. A second type of boundary is the transient boundary that may exist between pesticide-carrying and other estuarine water, depending upon the type of carrier, solubility of pesticide, and methods of application. By using these boundaries, some fish may be able to avoid harmful concentrations of pesticides.

To obtain a better understanding of the phenomenon of pesticide avoidance, we are studying the mosquitofish, Gambusia affinis.

Preliminary tests show that this fish, unlike the sheepshead minnow, does not avoid DDT or endrin at concentrations near the 24-hour LC_{50}. Thus, not all fishes have the ability to avoid these pesticides.

EFFECT OF PESTICIDES ON THE SALINITY PREFERENCE OF FISH

The salinity gradient in an estuary is undoubtedly one of the most important stimuli that direct movements and determine the distributions of fish and other estuarine organisms. We are therefore investigating how the pesticides DDT and malathion affect the movement of mosquitofish in salinity gradients produced in the laboratory.

Experimental Procedure

Mosquitofish, 18 to 55 mm. total length, were seined from ponds and ditches near the laboratory, and acclimated to water of 15 p.p.t. salinity and 20° C. for at least 2 weeks
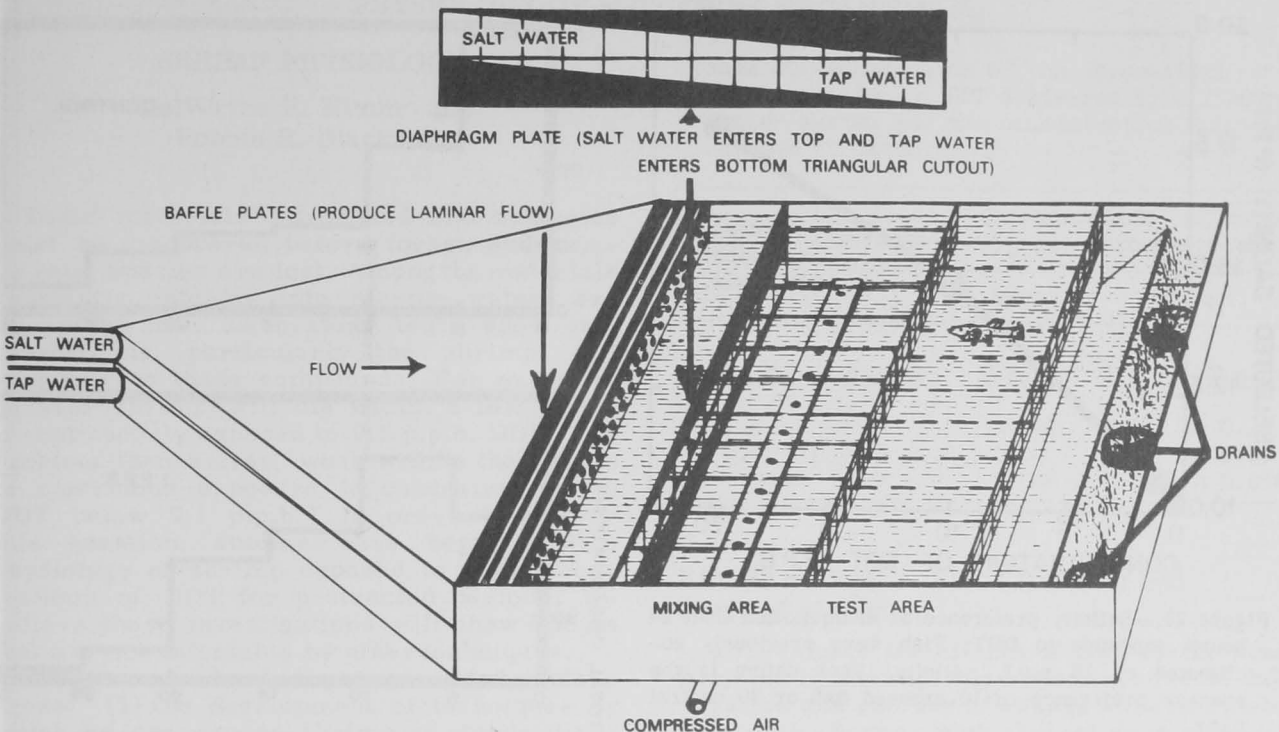
Figure 22.--Apparatus (fluviarium) used to produce a horizontal gradient of salinities for investigating how pesticides affect the salinity preference of fish.

before testing. Acclimated fish were healthy, fed readily, and bore young.

Five fish were exposed for 24 hours to DDT or malathion. The pesticide was dissolved in polyethylene glycol (average molecular weight 200) and metered into flowing water to achieve the desired concentration in the test chamber. Salinity and water temperature in the test chamber were identical with those in the acclimation tank. Control fish were treated similarly except that polyethylene glycol without pesticide was metered into the water.

Movements of control and exposed fish were tested in a "fluviarium" where flowing tap and salt water are mixed to produce a horizontal salinity gradient (fig. 22). Salinities typically ranged from 0 to 30 p.p.t. Individual fish were acclimated to fluviarium conditions for 5 minutes before testing, and their movements were observed either for an additional 5 minutes, or until they traversed one-half the distance across a salinity gradient. Then, the position of the fish in the gradient was noted every 15 seconds for 10 minutes and

those positions were used to indicate salinity preference. Nontraversing fish were not included in the analysis. This technique is suitable for determining the salinity preference of fish because fish do react to a salinity gradient by seeking a particular salinity rather than being evenly distributed in the test area as occurs in the absence of a gradient. Fish were not retested.

DDT

DDT affects the salinity preference of mosquitofish as indicated by the average salinity selected (fig. 23) and by the distribution of fish within the 0 to 30 p.p.t. salinity gradient (fig. 24). The average, as well as modal position of fish in the gradient, increased with an increase in concentration of DDT.

The salinity selected by exposed fish in the fluviarium is probably the combined result of the effect of the pesticide, the salinity preference of the fish in nature, and the
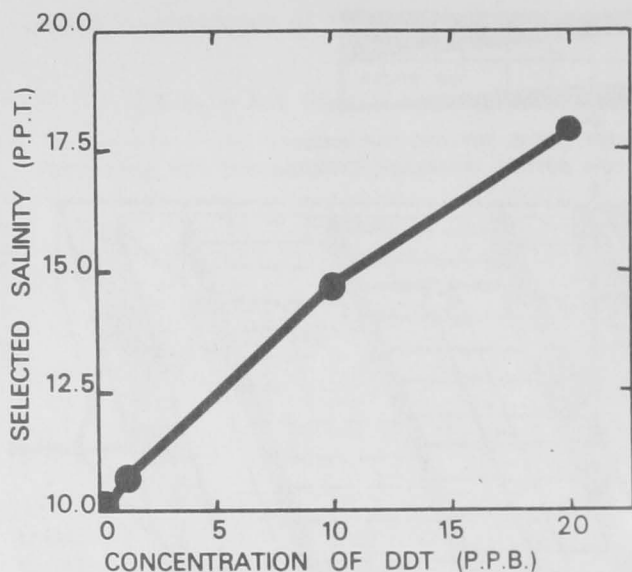
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Figure 23.--Salinity preference of mosquitofish after 24 hours exposure to DDT. Fish were previously acclimated to 15 p.p.t. salinity. Each datum is the average preference of 10 exposed fish or 30 control fish.

acclimation conditions before testing. Preference by control fish of a salinity that is lower than acclimation salinity probably reflects their typical distributions in fresh or occasionally brackish waters. The salinity selected by test fish increased to near that of acclimation, 15 p.p.t., as the concentration of DDT increased.

DDT can affect the lateral movement of fish in the fluviarium. Both control and exposed fish swam against the 5-cm.-per-second current of water entering the test area. Fish exposed to DDT, however, tended to remain in a limited portion of the salinity gradient, whereas controls frequently moved laterally across the gradient and then returned to their original position.

Malathion

The movements in a salinity gradient of mosquitofish exposed to 1.0 and 0.1 p.p.m. malathion were compared to movements of unexposed fish. There was no apparent alteration in the salinity selection or swimming behavior of exposed fish. Control fish, on the average, preferred 8.8 p.p.t. salinity, whereas fish exposed to 0.1 and 1.0 p.p.m. malathion preferred 8.0 and 9.9 p.p.t., respectively.
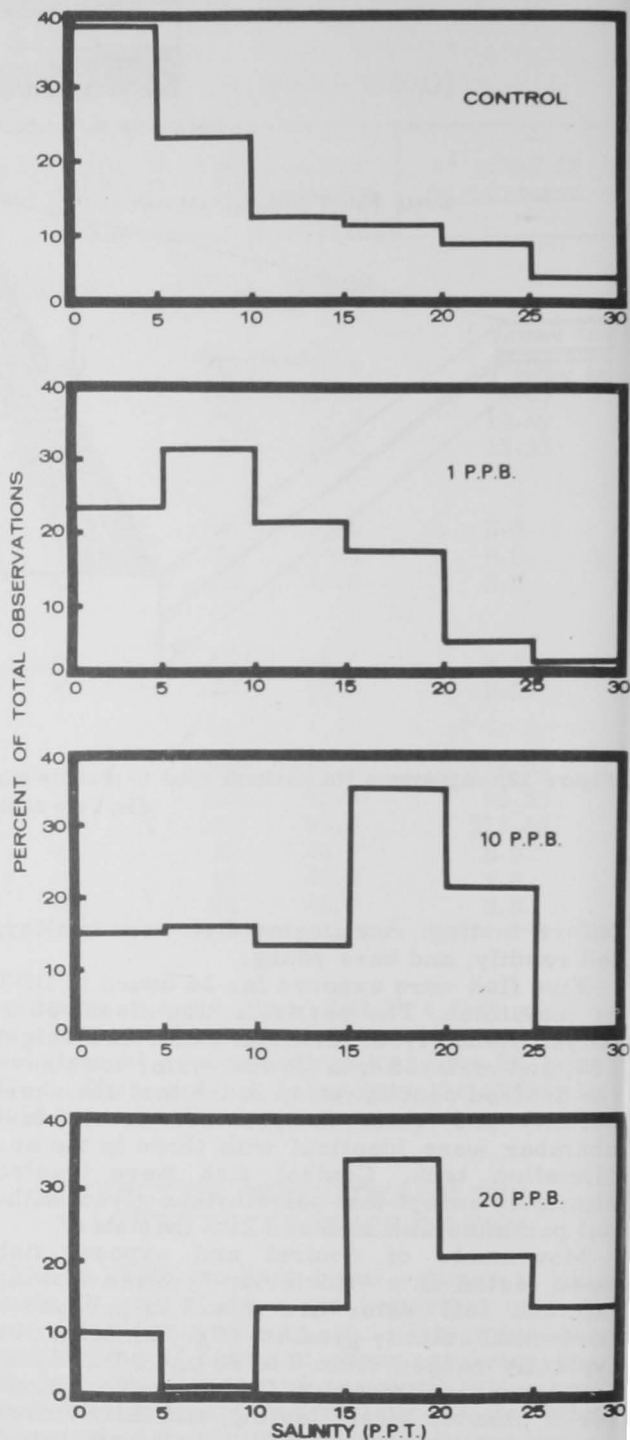

Figure 24.--Distribution of mosquitofish in a salinity gradient after 24 hours exposure to DDT. Fish were previously acclimated to 15 p.p.t. salinity. Distributions are those of 30 control fish and 10 fish exposed to each concentration of DDT.

SHRIMP PHYSIOLOGY

DelWayne R. Nimmo and
Robbin R. Blackman

Toxic materials introduced into estuaries must be discovered before forage and commercial species are lost. Among the materials constantly entering into marine waters are the pesticides. Laboratory tests show that crustaceans, particularly the shrimp, are sensitive to these compounds. For example, penaeid shrimp will die within a few weeks if continuously exposed to 0.1 p.p.b. DDT. The problem then arises, what will be the effects of continuous exposure to concentrations of DDT below 0.1 p.p.b.? In order to answer this question, studies were begun on the physiology of shrimp exposed to low concentrations of DDT for protracted periods. We believe these investigations will show effects that are not detectable by other techniques.

Experiments have been completed in four areas: (1) the development of techniques by which we can expose shrimp to standardized concentrations of DDT; (2) the effects of DDT on blood proteins and survival of shrimp; (3) the localization of DDT in shrimp tissues; and (4) investigations into the effects of DDT on shrimp tissues.

Maintaining DDT at Standardized Concentrations

A method was devised for constant delivery of minute amounts of DDT so that shrimp were chronically exposed to low concentrations of this chemical. Technical grade DDT was added to polyethylene glycol (200 molecular weight). The mixture was warmed to 60° C. and stirred until the DDT dissolved. This stock solution was refrigerated and kept out of direct sunlight to prevent degradation. It was warmed to room temperature before use to prevent condensation in the container. A syringe pump was used to infuse the stock solution into a flowing sea-water system through small polyethylene tubes inserted directly into the inflow. Three concentrations (0.05, 0.08, and 0.1 p.p.b.) were prepared and delivered over several months, and the concentrations were checked each week by gas chromatography (table 10). In addition to the accuracy of delivery, this method has three advantages over methods previously used: (1) polyethylene glycol is not toxic to any species tested to date; (2) one preparation of about 100 ml. suffices for long-term experiments; and (3) the pump-and-delivery apparatus is reliable, compact, and corrosion-free.

Table 10.--Comparison of the theoretical concentration of DDT delivered to a flowing water system and the concentration indicated by gas chromatography

Desired concentration	Determinations	Concentration indicated by gas chromatography	
		Mean	Range
P.p.b.	Number	P.p.b.	P.p.b. P.p.b.
0.1	6	0.097	0.07 to 0.11
0.08	8	0.08	0.067 to 0.09
0.05	9	0.046	0.027 to 0.058

Effects of DDT on Blood Proteins and Survival of Shrimp

To evaluate the effects of DDT on the blood proteins of shrimp, one experiment of 24 days duration and another of 45 days were completed this year. Sixteen pink shrimp were placed in each of two tanks: one group was exposed to pesticide, the other was not. Each shrimp was tested at 2- or 3-day intervals to determine if chronic exposure to 0.1 p.p.b. would alter the proteins in the blood. Proteins in whole blood of shrimp were separated by acetate electrophoresis, analyzed for qualitative changes, and measured. The only difference appeared to be the quantity of the fraction believed to be hemocyanin, as measured with an optical densitometer. This measurement is relative but is useful for detection of day-to-day changes. The procedure was especially applicable to this study because only a small volume of blood was obtained from a single shrimp. Student's "t" values were calculated to determine significant difference between control and experimental animals.

Shrimp exposed to DDT exhibited a gradual decrease in protein levels in their blood. In the 24-day experiment the average optical density of the controls decreased from 13.6 to 8.4 (38 percent) while that of the experimental shrimp decreased from 13.0 to 4.8 (63 percent). A difference between the two groups at the 95 percent confidence level existed on the 24th day (fig. 25). In the experiment that lasted for 45 days, the proteins in the controls decreased from 14.3 to 8.4 (41 percent) while those in the treated animals decreased from 16.8 to 6.4 (62 percent). The difference was not significant until the 24th day in the first experiment and the 39th day in the second; however, the trend of depressed protein was evident (fig. 26).

Although these data are not conclusive, further study is warranted. In both experiments
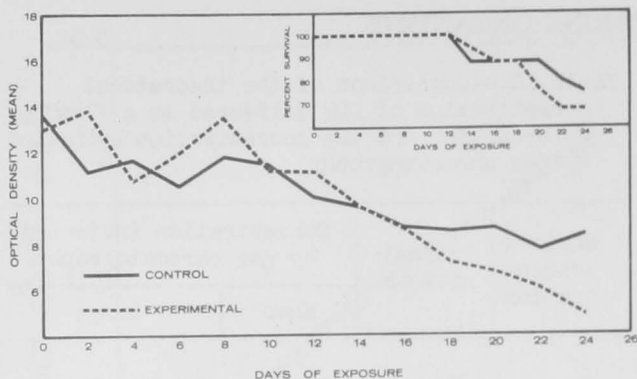
Figure 25.--Mean optical density of protein levels in the blood and percentage survival with time. The experimental group of pink shrimp was exposed to 0.1 p.p.b. DDT. Significance at the 95 percent level occurred on the 24th day.
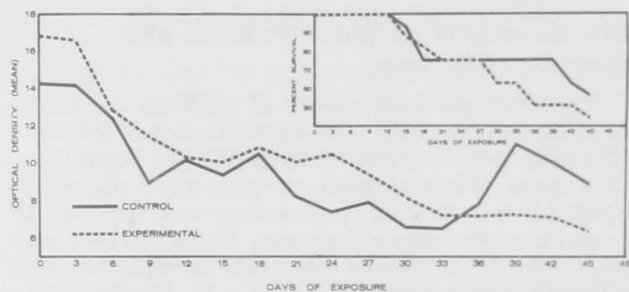

Figure 26.--Mean optical density of protein levels in blood and percentage survival with time. The experimental group of pink shrimp was exposed to 0.1 p.p.b. DDT. The difference between experimental and control shrimp was significant at the 95 percent level on the 39th day and at the 90 percent level on the 45th day.

protein levels of test animals were depressed and the percentage decrease was similar. In a recent experiment, shrimp exposed to 0.05 p.p.b. DDT showed no significant change in protein concentrations compared to the untreated shrimp. This lack of change suggests that a "threshold" concentration is necessary before an observable effect is reflected in blood proteins. This concentration might be 0.05 to 0.1 p.p.b.

Many variables such as temperature fluctuations, food availability, presence of substrate (sand), and handling were inherent in our procedures and appeared to mask the effect caused by pesticide. Some of these variables have been controlled, and food availability is being studied, but some cannot be eliminated. The level of protein in the blood of shrimp is currently measured by biuret and Folin-phenol methods. Specific tests are being made for various isoenzymes, lipoproteins, glyco-proteins, and hemocyanin.

The survival of shrimp exposed to 0.1 p.p.b. DDT indicates the need for an expansion of our

bioassay program. For several years bioassay for acute effects has been useful in estimating the relative toxicity of chemicals to various species of animals. Usually these tests are short, from 24 to 96 hours. One of the first discoveries in the long-term experiments was the increased death rate of the treated animals (figs. 25 and 26). No doubt the influence of many environmental factors in the laboratory contributed to the mortality. Some factors could be synergistic, but evidence indicates that trace amounts of DDT in a chronic exposure could affect populations. It appears necessary to include long-term tests at low concentrations as well as short-term tests when evaluating toxicities of new chemicals.

Localization of Pesticide in Shrimp Tissues

Little information is available concerning the localization of DDT in the tissues of shrimp or the residues that accumulate in whole populations. This information must be known if we are to answer such pertinent questions as: (1) Do commercial species accumulate pesticides? (2) What are the levels of DDT in the tissues and organs of apparently healthy shrimp as compared to dying shrimp? (3) What is the rate of accumulation, the rate of degradation, and what are the metabolites? (4) Are postlarvae or juveniles more susceptible than adults? (5) Do susceptibilities differ with season or species?

Two experiments compared the residues of DDT in various organs with those of the entire body. In the first experiment, we isolated eight large white shrimp in each of two flowing-water aquaria. One group was in water containing 0.2 p.p.b. DDT in polyethylene glycol and the other group (controls) was in water containing polyethylene glycol. Each dead treated shrimp was removed and frozen. Untreated shrimp were killed on the 19th day. Table 11 shows weights and residues in various tissues and organs of each group.

In the second experiment, accumulation of DDT in shrimp was measured by analyzing

Table 11.--Localization of DDT in the tissues of white shrimp poisoned with DDT

Tissue	Experimental		Control	
	Weight	DDT	Weight	DDT
	G.	P.p.m.	G.	P.p.m.
Hepatopancreas (liver).	4.6	11.0	4.5	0.10
Exoskeleton............	7.0	0.86	6.4	0.01
Ventral nerve..........	0.7	0.55	0.5	0.01
Heart..................	0.6	0.46	0.4	0.01
Gills..................	3.5	0.36	1.9	0.01
Gut (stomach and intestine)...........	1.0	0.04	1.3	0.01
Muscle (tail)..........	38.6	0.02	35.3	0.01

Table 12.--Pesticide residues in whole pink
shrimp exposed to 0.1 p.p.b. DDT

Days	DDE	DDD	DDT	Total
	P.p.m.	P.p.m.	P.p.m.	P.p.m.
1........	--	--	--	--
4........	--	--	0.02	0.02
7........	--	--	0.06	0.06
10.......	0.02	--	0.17	0.19
13.......	--	--	0.21	0.21
16.......	--	--	0.16	0.16
19.......	0.01	--	0.14	0.15
22.......	0.02	0.01	0.12	0.15

Note.--Four shrimp that died during the ex-
permiment had 0.13 p.p.m. DDT in the entire
body.

whole-body residues. Residues in dead and
dying shrimp were also compared. Two groups
of juvenile pink shrimp were placed in two
large plastic aquariums with sand substrate.
The level of DDT in flowing sea water was
maintained at 0.1 p.p.b. Every third day,
eight treated and eight untreated shrimp were
removed and immediately frozen. Dead treated
shrimp and those showing symptoms of poison-
ing were also removed and frozen. Table 12
shows data for treated shrimp. The un-
treated group had no detectable DDT in the
residues.

Four aspects of these studies are:

1. DDT is concentrated most in the hepato-
pancreas and least in the muscle.
2. Shrimp do not accumulate large concen-
trations of DDT; however, the body
residues have 2,000 times as much
pesticide as the water.
3. Some individuals in the population appear
to be more resistant than others as
reflected in the residues of dead shrimp.
4. In the tissues DDT appears to degrade
to the metabolites DDE and DDD.

Effects of DDT on Shrimp Tissues

Two experiments have been completed to
determine if histological changes occur in
shrimp tissues after chronic exposure to DDT.
Juvenile pink shrimp were subjected to 0.1
p.p.b. DDT for 35 days. Untreated animals
were maintained under similar conditions.
Every other day, a treated and an untreated
specimen were killed and fixed in preservative.
They were embedded in paraffin, sectioned, and
stained with hematoxylin and eosin. Gross
microscopic examination of the sections re-
vealed no pathological conditions except for
slight necrosis of hepatopancreas in the treated
shrimp. Some individuals had severe infesta-
tions of parasites; therefore, the experiment
was repeated with grass shrimp, which were
free of parasites. Although the exposure period
was longer, we observed no abnormalities in
the tissues of grass shrimp.

ENZYME SYSTEMS OF ESTUARINE ORGANISMS

David L. Coppage

Metabolic activity in fish, as in other ani-
mals, is the result of enzyme activity; toxic
chemicals, such as pesticides, can interfere
with this activity. Specifically, organophos-
phate pesticides inhibit cholinesterases, which
are enzymes that function in nerve-impulse
transmission and ion transport. To evaluate
how these insecticides affect estuarine or-
ganisms, we have to understand cholinesterase
inhibition by organophosphates. To evaluate
this inhibition intelligently, we need to know
the following relations: toxicity of the pesticide
to in vivo (living organism) inhibition of
cholinesterase; rate and duration of exposure
of the organism to the pesticide to in vivo
inhibition; and toxicity to in vitro (isolated
tissue components) inhibition. Also, since
cholinesterases differ with kinds of tissue
and species, we must determine the proper-
ties of these enzymes in selected estuarine
fish.

TOXICITY AND IN VIVO INHIBITION

Tests were made to determine the relation
of toxicity of several organophosphate pesti-
cides to in vivo inhibition of cholinesterase
of sheepshead minnows. Adult fish were ex-
posed to acute doses of diazinon, guthion,
malathion, parathion, and phorate that killed
40 to 70 percent of the fish in 24 and 48 hours.
Five male and five female fish per test were
exposed to the pesticides in 20 liters of
artificial saltwater (salinity, 4 p.p.t.) main-
tained at a temperature of $21 \pm 1°$ C. The
brains of exposed fish were removed after
each test and frozen until assayed for
cholinesterase activity. The enzyme activity of
exposed fish was compared with normal enzyme
activity previously determined from unexposed
fish. We determined the inhibited and normal
enzyme activities photometrically by observing
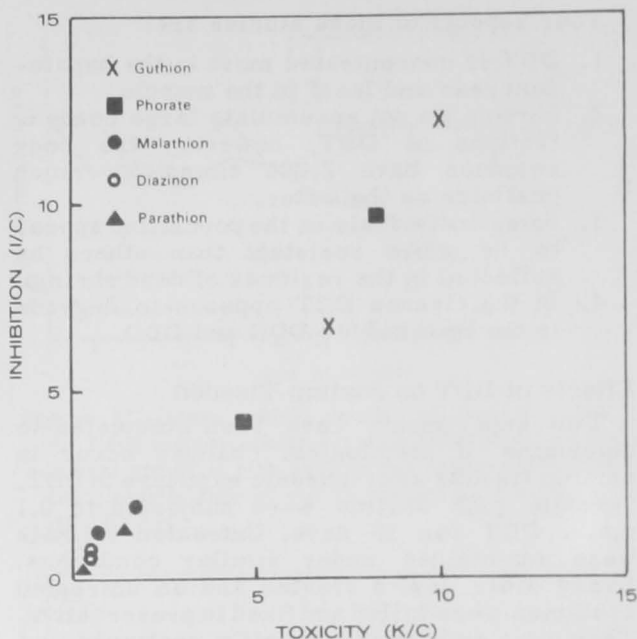the disappearance of acetylcholine incubated

Figure 27.--Potency of five organophosphate pesticides in relation to in vivo inhibitions of fish brain-cholinesterase and acute doses that killed 40 to 70 percent of test fish in 24 and 48 hours. The lower of the pair of points for each toxicant is the 24-hour datum; the higher, the 48-hour datum. K/C is percent killed/dose in p.p.b.; I/C is average percent inhibition/dose in p.p.b.

with brain homogenates. Because the brain cholinesterase inhibition of fish surviving a test was similar to that of the fish killed, the average percentage inhibition was computed from all fish exposed in a test.

The number of fish killed by each of the five organophosphates was proportional to the inhibition of cholinesterase in the brains of the fish. Each pesticide was ranked as a toxicant in terms of two ratios (fig. 27)--percentage of fish killed to the concentration of the pesticide in the water (K/C) and average percentage inhibition to concentration of pesticide in the water (I/C). The potency of the pesticides increased with time i.e., each was more potent after 48 hours than after 24 hours (fig. 27).

The average percentage of cholinesterase inhibition in the brain of fish exposed in the acute tests was 56 to 77 percent, but these levels of inhibition do not always indicate the percentage of the population that will be killed. For example, chronic exposures of fish to guthion, phorate, and diazinon showed that these chemicals could cause 100 percent inhibition of cholinesterase activity (but kill fewer fish) in the same period of time as acute exposures. This observation suggests that "threshold levels" of inhibition indicating mortality do not exist. The 100 percent cholinesterase inhibition in the chronic exposures should not be interpreted as complete disruption of brain cholinergic mechanisms. Complete disruption would kill all the fish, but in

our experiments 30 percent or more survived each chronic exposure. In this situation, the photometric assay method probably measures the amount of pesticide in the tissue but not necessarily that interfering with cholinergic mechanisms in vivo. If a "threshold" exists, it may be masked by limitations of the method.

STUDIES OF ENZYME PROPERTIES

An automated pH-stat was acquired during the year for enzyme studies. In the pH-stat cholinesterase assay the acid liberated during hydrolysis of a substrate (choline ester) is titrated with NaOH; the pH is held constant with a potentiometer. This method is better suited for studies of enzyme kinetics than other methods because it allows a greater range of pH, enzyme concentration, and substrate concentration.

Several conditions should be studied before a satisfactory quantitative assay of cholinesterases can be performed with the pH-stat. Animal tissues contain two general types of cholinesterase, true or acetylcholinesterase and pseudocholinesterase. These types must be differentiated. Both are assayed by measuring the rate of hydrolysis of acetylcholine or other suitable esters, and the reaction rate must be proportional to the concentration of enzyme. Special properties are used to distinguish each type of enzyme. In general, acetylcholinesterase requires a lower concentration of acetylcholine for maximal hydrolysis than pseudocholinesterase, and is inhibited by excess acetylcholine. Thus, the assay of acetylcholinesterase requires determination of acetylcholine concentrations that give a straight line relation between rate of hydrolysis and time. The type of enzyme may also be determined by choline ester specificity; acetyl beta methylcholine is specific for acetylcholinesterase, and butyrylcholine is specific for pseudocholinesterase.

The function of enzyme and choline ester concentration, and the action of enzymes on specific choline esters were studied with the pH-stat to better define brain cholinesterase of sheepshead minnows. Reactions were carried out in 4 ml. of distilled water at pH 6 and 22° C. Acetylcholine iodide (15 mM) was used as the substrate in the enzyme (brain homogenate) concentration study. Brain homogenate concentration was 10 mg. wet weight in the studies of choline ester concentration and specificity.

The primary brain enzyme that hydrolyzed choline ester exhibited the properties of acetylcholinesterase. The rate of hydrolysis of acetylcholine iodide (15 mM) was proportional to the concentration of brain homogenate (fig. 28). A bell-shaped curve typical of acetylcholinesterase activity was obtained when hydrolytic activity of the brain homogenate
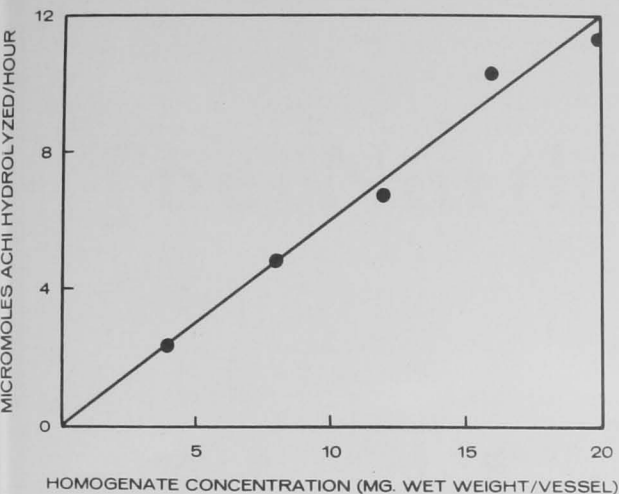
Figure 28.--Hydrolysis of AChI (acetylcholine iodide--15 mM) as a function of the concentration of brain homogenate from sheepshead minnows.
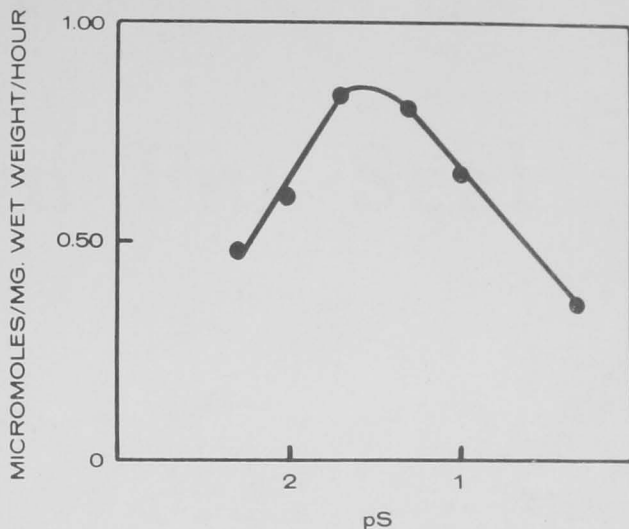

Figure 29.--Effect of acetylcholine iodide concentration on cholinesterase activity of the brains of sheepshead minnows. pS represents the negative logarithm of the molar substrate concentration.

(micromoles of acetylcholine iodide hydrolyzed per mg. wet weight per hour) was plotted against the negative logarithm of various molar substrate concentrations (pS) (fig. 29). The optimum acetylcholine iodide concentration was near 20 mM. Titragraphs at optimum and near-optimum acetylcholine concentrations were straight lines. Brain homogenate hydrolyzed the acetylcholinesterase-specific substrate, acetyl beta methylcholine chloride, but not the pseudo-cholinesterase-specific substrate, butyrylcholine iodide.

KINETICS OF PESTICIDES

Thomas W. Duke, Patrick W. Borthwick, Alan J. Rick, and Michael D. Schmitt

A former residence was renovated during the year to provide 1,600 square feet of air-conditioned laboratory space for work with radioactive materials. We installed a liquid scintillation spectrometer with three channels and automatic printout and added other equipment for tracer work, such as a lead vault and chemical hood.

We have started experiments to study the rates at which plants and animals accumulate and excrete pesticides labelled with radioactive elements and the rates of movement of these chemicals through estuarine ecosystems. For example, DDT with labelled radioactive carbon was transferred through three trophic levels of a "laboratory" food chain consisting of phytoplankton and two species of fish. Also, experiments are underway to determine the rate of movement of pesticides between water and sediments.

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