AN ECOLOGICAL STUDY OF HEXACHLOROBENZENE (HCB)

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ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF TOXIC SUBSTANCES
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AN ECOLOGICAL STUDY OF HEXACHLOROBENZENE (HCB)

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I. SUMMARY AND CONCLUSIONS

The purpose of the study summarized in this report was to determine the distribution and toxic effects of hexachlorobenzene (HCB) in selected aquatic systems and organisms. Field collections of soil and water were made in southeastern Louisiana, and were supplemented by samples of aquatic organisms from sites where available. In laboratory experiments, the principal animals used were the red swamp crayfish, Procambarus clarki, the sailfin molly, Poecilia latipinna, and the largemouth black bass, Micropterus salmoides. Both acute and chronic effects of HCB were studied.

Tests aimed at establishing LC_{50}'s for organisms were carried out in flow-through experiments which utilized a modified proportional diluter. HCB, at concentrations near its upper limit of solubility in water (20 ppb), did not produce mortalities in animals exposed for 10 days. In other acute studies the substance, dissolved in oil, was injected directly into animals. No significant acute lethal effects were seen following dosages of 125 µg/g body weight in fishes or crayfish.

Although HCB was not lethally toxic to juvenile bass, HCB concentration reached a maximum of 88 µg/g (ppm) following 15 days' exposure to 2 ppb. This is a concentration factor in excess of 44,000X. Highest levels were found in extracts prepared from the gut, in which some concentration factors exceeded 100,000X. Kidney, gills and liver contained substantial proportions of HCB as well. Light microscope observation of histological slides of tissues from chronically exposed fish and crayfish revealed damage at the cellular and organ level. Changes were observed in kidney, liver and gall bladder of bass exposed to 25 ppb HCB. Hepatopancreas of crayfish was affected by 10 days' exposure to 36 ppb HCB.

Analysis of blood samples taken from freshly-sacrificed fish revealed no differences in hematocrit following chronic exposure to HCB. Serum cortisol levels were observed as indicators of stress. HCB elevated cortisol level, but the differences were not statistically significant (5%, F-test).

Oxygen consumption rates were not significantly altered by HCB exposure within three hours after exposure. Chronic exposures produced complex response patterns which were not resolved satisfactorily in the present experiments with fish and crayfish.

A flow-through system was used to determine uptake of the compound by algae (Oedogonium cardiacum) and sediment. Algae concentrated HCB to a level more than 600 times that measured in aquarium water within seven days. Sediment accumulated 40 times the quantity of HCB in experimental water in one day of exposure. A food-chain study compared the relative accumulation effects on a predator feeding upon HCB-contaminated food fish and a similar predator taking up the compound both through its food and through contaminated water. Bass took up more than 20 times the HCB from water than they did from food. In another feeding experiment with Carbon$_{14}$-labelled HCB, radioautography of thin-layer chromatographic plates showed that recovered material from bass tissue extracts had not been altered by metabolic processes in fish.

Laboratory experiments were compared with patterns of accumulation under field conditions. HCB-free crayfish were caged, placed at a contaminated field site and removed periodically for GC analysis, or for laboratory depuration

and subsequent GC analysis. Crayfish exposed to an average level of 44 ppb HCB had a concentration factor of 1,164X HCB in whole-body tissue. For specimens left in the field site for 10 days, 40 to 60% of the maximum attained level of HCB was lost after 25 days of depuration.

Since HCB has been found as a contaminant in the environment, it is possible that it is degraded or altered by ambient UV light. The environment was simulated in the laboratory and the resultant photoproducts formed following short exposure to UV light were analyzed by GC. Irradiation of HCB resulted in its gradual disappearance and a steady increase of lower molecular weight products.

II. INTRODUCTION

The present study was initiated following the observation, in recent years, of excessive levels of HCB in adipose tissue and milk of cattle being raised in the vicinity of an industrialized region bordering the Mississippi River between Baton Rouge and New Orleans, Louisiana. The field portion of this study was undertaken to measure HCB levels in the environment in southeastern Louisiana. Laboratory experiments were aimed at observing acute and chronic effects of the compound under controlled conditions.

Hexachlorobenzene, in its pure form, is a crystalline powder which has been used as a seed dressing for prevention of fungus disease in wheat. It is both a starting material and by-product of the chemical industry (Gilbertson and Reynolds, 1972). The occurrence and effects of HCB have been reported in many organisms, e.g., birds (Vos et al., 1971, Cromartie et al., 1975) sheep (Avrahami and Steele, 1972), rats (Medline et al., 1973), man (Cam and Nigogosyan, 1963) and fishes (Holden, 1970; Johnson et al., 1974, Zitko, 1971). Magnification in the natural food chain is indicated by Gilbertson and Reynold's (1972) observation of HCB in the eggs of common terns, which had apparently eaten contaminated fish. This compound has also been found in samples of ocean water and its persistence in the environment has been acknowledged (Seltzer, 1975).

In the present study, field work was divided into two distinct phases. First, wide-ranging collections of water, soil and organisms were made to establish levels and distribution of HCB in the southern central portion of the state of Louisiana, extending eastward to Mississippi and following the Mississippi River from Baton Rouge to Port Sulphur. Results from this work provided an overview of environmental reality against which gross exposures could be compared. The study of the extreme in environmental exposure was concentrated in the immediate vicinity of the Vulcan Materials Company at Geismar, La. Here a periodic monitoring of HCB concentrations in soil, water and selected aquatic organisms at various trophic levels was conducted. During the course of field work, localities in this area were contaminated at a fairly constant level and afforded the opportunity for observation in an environment having otherwise relatively natural conditions.

In an effort to define acute and chronic effects of HCB upon local fauna more precisely, laboratory experiments were designed to expose aquatic biota to the test compound. Histological preparations, behavioral observations and gas chromatographic analysis were used to obtain data and gain a greater under-

standing of the significance of the presence of HCB in the environment.

Tests of acute toxicity have been central to studies of contaminants introduced into environmental systems. Tarzwell (1966, 1971) had discussed the use of acute toxicity levels and of application factors setting safe standards for levels of toxic substances in natural waters. It has been obvious for a long time that the concept of application factors is more of a convenient means of dealing with the complicated problems of water quality than an accurate scientifically established criterion. Long term effects of toxic substances are difficult to determine in actual practice. The importance of the problem of water pollution, however, is sufficiently great to justify attempts to approximate a relationship between short term lethal effects relatively simple to measure and long term effects which are far more difficult to determine.

Because of the relative ease with which the experiments can be conducted, there have been numerous experiments designed to determine the level of a lethal factor that can be tolerated by a given percentage of animals for a given period of time. Warren (1971) has discussed this subject and also has reviewed the use of application factors in conjunction with tolerance studies. The rationale for incorporating tolerance studies into the present project is that because of the physiological studies included in the project there is a preliminary basis for suggesting application factors for HCB. The physiological and morphological indications of long term effects of sublethal concentrations of HCB are difficult to establish and such studies are relatively rare. As Warren points out it is extremely important to establish application factors which can be applied with some scientific basis.

Three commonly-used methods of introducing a potential toxicant into an organism are: (1) direct injection, (2) oral feeding, and (3) contaminating its air or water environment. In some experiments during the present study, animals were injected with the test compound and in others they were subjected to a range of concentrations of HCB in aquatic systems. Condition of animals was observed regularly, and any abnormal behavior or appearance was noted for inclusion as possible pathological effects of HCB.

Mortalities give the most positive, visible evidence that a substance is toxic to organisms. In contaminated natural systems, however, concentrations of toxic substances are normally below lethal levels. The variable conditions in nature often preclude positive determination of chronic effects of a given substance upon resident flora and fauna. Controlled laboratory conditions are helpful, therefore, in assessing various responses of an organism to low-level exposures. Results of such work can provide a basis for field observations and augment acute tests in establishing application factors.

Chronic tests and observations reported here dealt with a variety of physiological and morphological parameters. Uncontaminated organisms were brought into the laboratory and adapted to in vitro conditions for periods of from two days to as much as two months before being used in tests. Most animals were subjected to HCB in the flow-through system, the diluter, which is discussed in Section IV. C. Following exposure for specified periods, whole organisms, or excised organs served as material for GC analysis of uptake and differential distribution in chronic studies. Light microscopic examination of histological slides prepared from excised organs provided further information on chronic effects of HCB. Samples of blood taken from fish were used in determinations

of hematocrit and cortisol levels. Cortisol levels were determined by competitive protein binding radioassay (Murphy, 1967, 1971) which has been successfully employed in teleost plasma (Hargreaves and Porthé-Nibelle, 1974). Alterations in these two levels would be considered an indication of stress upon the animals. Significant changes in these parameters might provide a further means of monitoring conditions in a given area of potential contamination.

Rate of oxygen utilization by an organism may vary in response to stress. In order to study possible effects of HCB in ambient water of fish and crayfish, respirometry experiments were carried out in the laboratory.

Accumulation and clearance of HCB in whole-body samples of fish and crayfish and samples of algae and mud are best accomplished in a flow-through system. The modified proportional diluter was used for these exposures and depurations. Concentrations of HCB in diluter tanks were comparable to levels found in contaminated natural systems, except for some experiments, in which higher concentrations were used. Samples of fish or crayfish being tested were removed from all tanks according to schedules outlined in discussions of the various experiments in the text which follows. Handling of specimens and preparation of extracts for GC analysis are described in the Gas Chromatography unit (IV.D.) in the Laboratory Methodology section of this report.

A series of field experiments with crayfish provided comparative information to results from laboratory studies. Uptake and clearance of HCB was determined in animals, initially free of the compound, which were placed in an HCB-contaminated field locality.

Two types of experiments were designed to observe possible breakdown products of HCB. In one, a metabolic fate study, extracts of bass tissue were analyzed following digestion of a $HC^{14}B$-labelled food fish by the bass. Other experiments subjected HCB to UV light in order to determine the resulting photoproducts.

III. FIELD STUDIES, METHODOLOGY

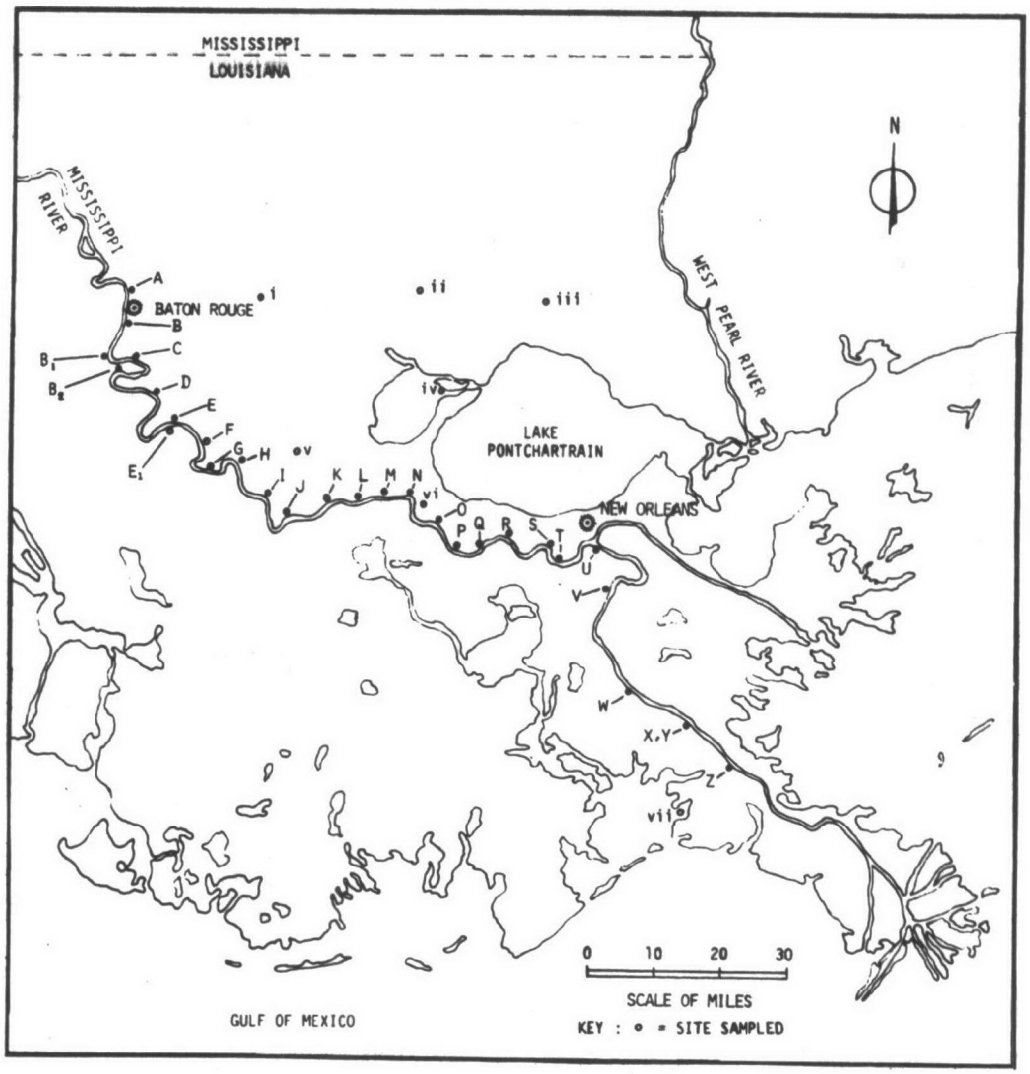
A. Overview

To develop a transect of contamination along the Mississippi River, collections of samples were made at five-mile intervals between Baton Rouge and New Orleans and at greater intervals from New Orleans south to Port Sulphur (Fig. 1). These samples were collected between March and May, 1975. At each site approximately 1 liter of water was taken from about 15 cm beneath the surface of the Mississippi River near the river's edge. A specimen of levee soil was also collected from the river's edge. These samples were taken from beneath the water surface whenever possible. Mud samples were taken from the bottom of ditches running parallel to the levee on the inland side. Fish and aquatic invertebrates were collected from these ditches whenever they were present. All animal specimens were wrapped in aluminum foil and frozen immediately on dry ice for later analysis. The localities for these field sites are shown in Table 1.

In addition to the samples taken along the Mississippi River, collections

were made at several inland sites. These samples were used to provide baseline information on geographic distribution of the compounds. These localities are also shown in Table 1, designated by numbers in italics.

Figure 1. Localities sampled for presence of HCB in soil and water.



Legend to Figure 1.

Sites	Description
A - T	East bank of Mississippi River
$B_1B_2E_1$, U-Z	West bank of Mississippi River
i, ii, iii, v	Inland, east of Mississippi River
iv	Pass Manchac, inland tidal lake
vi	Spillway of Mississippi River, connected to Lake Pontchartrain
vii	Bay

Table 1. Field localities for overview collections.

	Location Description	Type of Sample: Water*	Soil*	Biota**	Type of Organism
A	Hwy. 90, North of Baton Rouge (E)	X	X	X	F
B	Intersect, River Rd., La. Hwy 327 (E)	X	X		
B_1	Addis at La. Hwy. 990 & La. 998 (W)	X	X	X	F
B_2	Plaquemine at La. Hwy. 988 & La. 1148 (w)	X	X	X	C, F
C	5 Mi. South of B (E)	X	X	X	C, S
D	Sunshine (E)	X	X	X	C, F
E	Carville (E)	X	X	X	C, F
E_1	La. 405, 4 mi. South of White Castle (W)	X	X	X	C, F
F	Ashland Plantation (E)	X	X	X	F & C
G	Darrow (E)	X	X	X	C
H	Intersect, La. 44 & La. 942 (E)	X	X	X	C, F
I	Romeville (E)	X	X	X	F & C
J	Convent (E)	X	X	X	C
K	Lutcher (E)	X	X	X	C
L	Garyville (E)	X	X	X	F
M	Reserve (E)	X	X	X	Sh
N	Laplace (E)	X	X	X	C, F, S
O	South of Bonnet Carré Spillway (E)	X	X		
P	Destrehan (E)	X	X	X	C, F
Q	St. Rose (E)	X	X	X	C, Sh
R	River Ridge (E)	X	X	X	C
S	New Orleans, River Rd. at Causeway (E)	X	X	X	F, Sh, S
T	New Orleans, Audubon Park (E)	X	X		
U	Lower Algiers (W)	X	X		
V	Belle Chase (W)	X	X	X	C, F
W	Myrtle Grove (W)	X	X	X	C, F
X	Mile Marker 42 (W)	X	X	X	C
Y	.8 Mi. South of X (W)	X	X		
Z	Port Sulphur (W)	X	X	X	C
i	Walker (I)	X	X	X	C
ii	Hammond (I)	X	X	X	F, Sh, Cl
iii	Covington (I)	X	X	X	C
iv	Pass Manchac (T)	X			
v	Sorrento (I)	X	X	X	C
vi	Spillway (S)	X	X	X	C, F
vii	Lake Grand Ecaille (B)	X	X		

Key

E: East bank of Mississippi River
W: West bank of Mississippi River
I: Inland, east of Mississippi River
T: Tidal lake
S: Spillway
B: Bay

Key

C: Crayfish
Cl: Clam
F: Fish
S: Snail
Sh: Shrimp

* : GC analyses performed on soil and water samples from all locations.
**: GC analyses performed on biota from selected locations (Ref. Tables 3 & 4).

B. Area of Higher Concentration

The site of known contamination was near the Mississippi River, between New Orleans and Baton Rouge, at Geismar, La. (Fig. 2). The locations of samples taken on the property of the Vulcan Materials Company at Geismar are presented in Figure 3. These samples were collected during September and December, 1974, and May, August and October, 1975. Water and mud samples were taken regularly, and aquatic organisms were collected whenever available. These specimens were preserved by freezing for analysis in the laboratory.

The first site, an impoundment referred to as Recreation Pond, covers approximately one-fourth of an acre and is located in the north sector of the property (Fig. 3). The man-made pond is 20 feet deep with steep edges characterized by scattered patches of rooted and floating vascular plants. The ecosystem here more closely resembled a natural one than the other sites sampled at the contaminated locale. Zooplankton were abundant in the water column. Oedogonium sp., a common green alga provided food and substrate for micro-crustacea. Two common aquatic plants support and protect higher organisms. These are Chara and Najas, in whose mats we collected snails, crayfish, dragonfly larvae and small fishes. These animals feed on the plants and their epiphytic and epizoic components. The small fishes, breeding populations of mosquitofish (Gambusia affinis) and sunfish (Lepomis macrochirus), are food for largemouth black bass (Micropterus salmoides) which were stocked as sport fish. At the Recreation Pond, samples of soil, water, aquatic plants, invertebrates and fishes were collected and analyzed to determine HCB content in abiotic and biotic components of this environment.

The second sampling site was adjacent to the hex waste disposal area where waste containing HCB and HCBD are now buried. This sampling site was a newly dug pond measuring approximately 50 by 100 feet, 20 feet in depth. Ground water keeps this pond approximately 2/3 full of water. Eventually this pond is destined for waste disposal and burial. During the study, little vegetation was found in the water, but populations of mosquitofish were common there and were sampled regularly along with soil and water. Clean crayfish were brought in, caged, and left in this pond in a field study of HCB uptake.

The third sampling site was located along a small stream carrying runoff water from the field adjacent to the plant to a small stream in the South ditch. Rainfall determined water level in the south effluent ditch, and the only fishes living there were the mosquitofish and a few mollies (Poecilia latipinna). Crayfish (Procambarus sp.) were taken occasionally, along with regular samples of water, mud and fish.

Organisms were collected on several occasions, and their HCB content determined and tabulated. Concentration factors were computed as a function of HCB level in water at the time they were taken. When organisms from several dates of collection were combined, their concentration factor was computed by taking a weighted average of their HCB content and dividing it by the mean concentration in water samples taken on the same collection dates.

Plankton samples resulted from a tow of a 21 cm plankton net across the Recreation Pond, just below the surface. The material was refrigerated at 3°C in pond water after collection. All organisms settled to the bottom of the holding jar, and were pipetted off. They were then blotted on Whatman #1 filter paper, and subsequently treated with the same methodology as described

for algae samples in the Methodology Section (IV.D.) of this report. Concentration is expressed in terms of wet weight of solid material.

Figure 2. Location of Geismar, Louisiana.

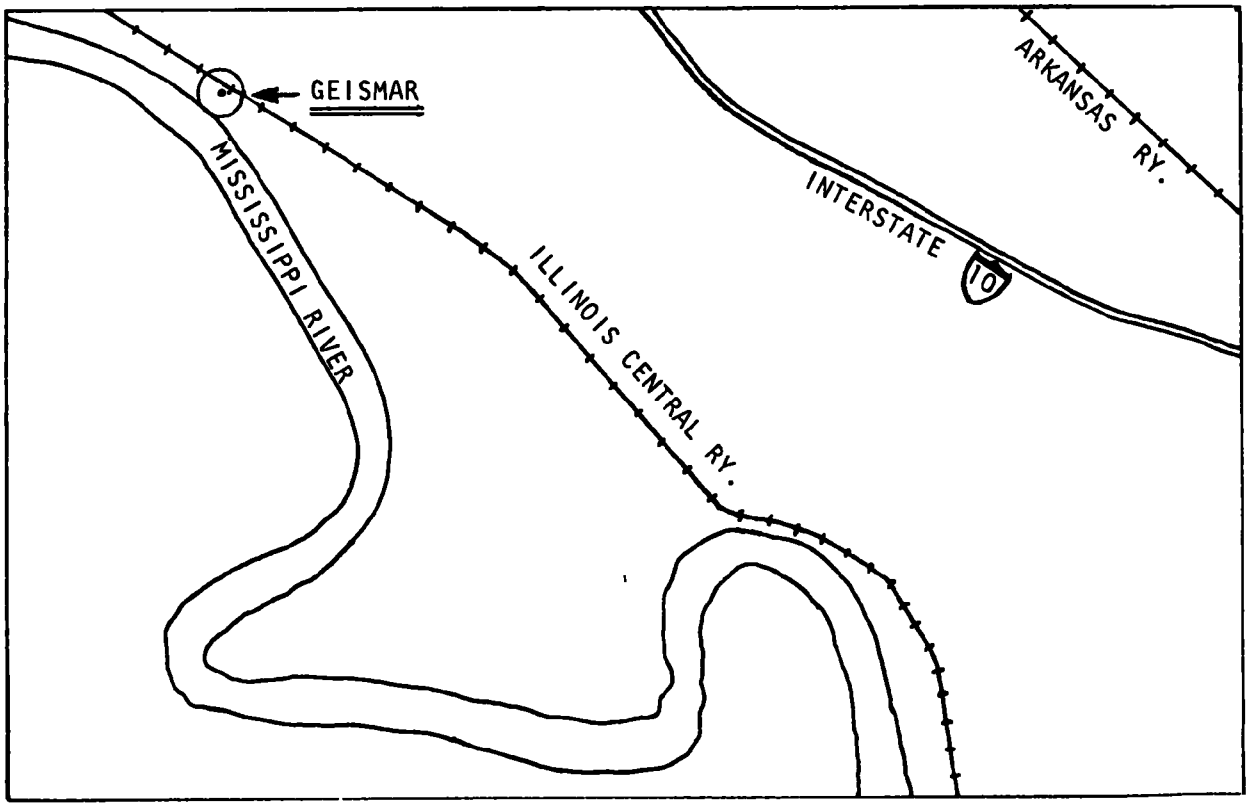
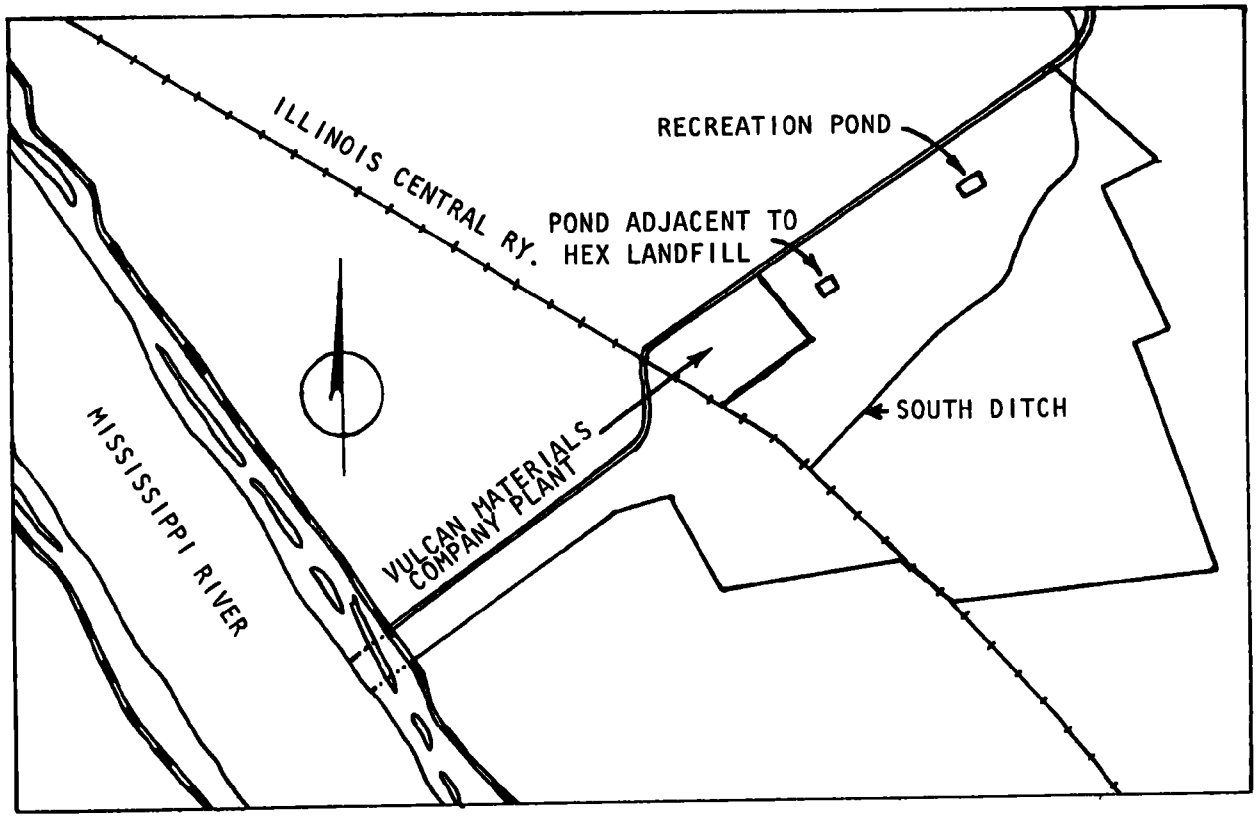


Figure 3. Location of Geismar Field Sites (area of higher concentration).



IV. LABORATORY METHODS AND MATERIALS

A. Test Compound

In order to guarantee the composition of test compounds following their absorption into organisms it is necessary to analyze their purity before application in various test systems. Zone refined hexachlorobenzene (HCB) was obtained from B. Pauric, Philadelphia, Pa. 19120. A purity check via gas chromatography was made using a standard of 0.1 ppm of the pesticide in benzene solvent. The compounds found to be "pure" were used throughout all experiments.

Mass spectra were obtained for the compound using a duPont 21-491 double focusing 90° magnetic sector mass spectrometer (MS) equipped with a Bell and Howell Datagraph 5-134 galvanometer driver recording oscillograph. Hexachlorobenzene was introduced directly into the mass spectrometer via the direct injection probe. All spectra were obtained at 70 eV at a source temperature of 200°C. Computer plots of the spectra were obtained by use of a PDP-12 LDP computer and a D1100 Versatec electrostatic printer plotter. A mass spectrometry plot of HCB starting material is given in Figure 4. Figure 5 represents gas chromatographic traces of the two compounds, HCB and HCBD.

Figure 4. Mass spectrometry plot of HCB.

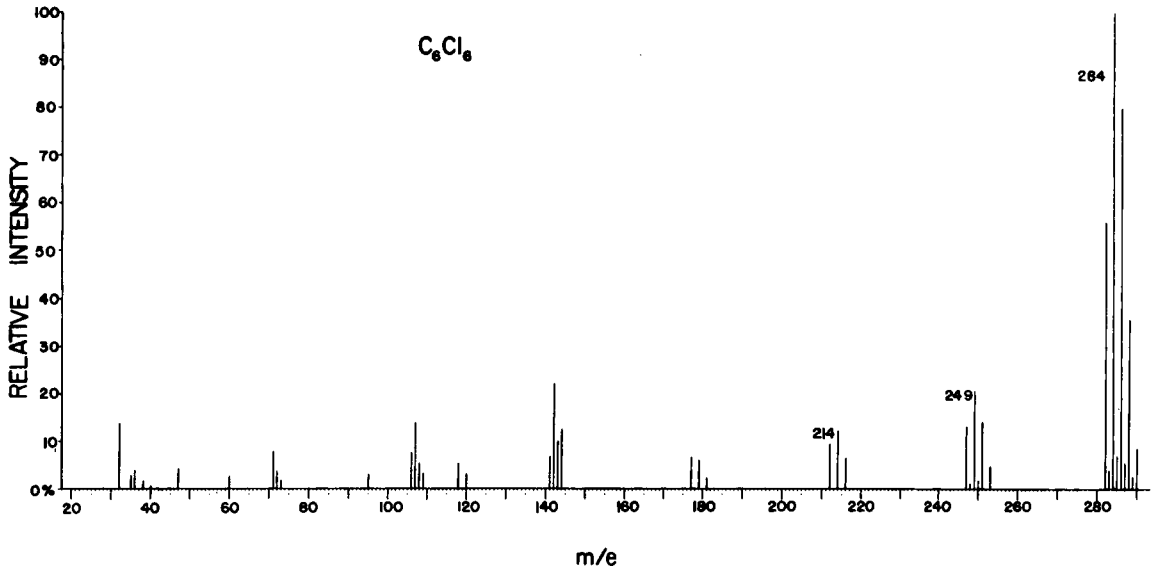
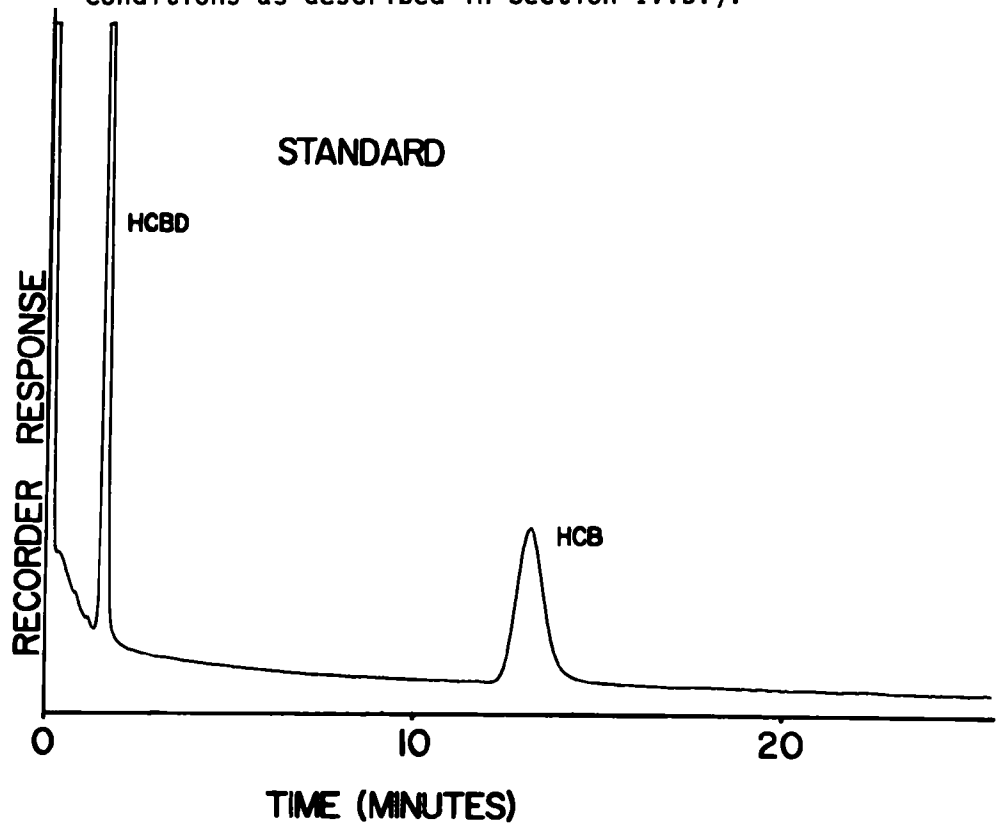


Figure 5. Gas Chromatographic traces of HCB and HCBD (chromatographic conditions as described in Section IV.D.).



B. Culture Techniques

Due to seasonality of some forms or habitat preferences of others, experimental organisms used in laboratory studies were acquired from several sources. In a given experiment, however, organisms were all from the same source.

The original stock of filamentous green algae, Oedogonium cardiacum (IU #39), was received from the Indiana University Culture Collection at Bloomington, Indiana. Subsequent cultures were maintained under controlled conditions in aerated flasks containing Bold's Basal Medium in the algae culture room at the contractor's institution.

Crayfish (Procambarus clarki) were received as available from commercial facilities at The Crayfish Farm, Sorrento, Louisiana. They were maintained in fiberglass tanks filled with enough water to keep gill regions wet. Crayfish were kept at room temperature and fed HCB-free chicken meat and small fish.

Small estuarine fishes, including sailfin mollies (Poecilia latipinna) and sheepshead minnows (Cyprinodon variegatus) were collected by seines and dipnets as needed for experiments and food. These were taken in small canals and ditches at Irish Bayou, inland from the southeastern shore of Lake Pontchartrain, Orleans Parish, Louisiana. Grass shrimp (Palaemonetes sp.) and small crayfish (Procambarus sp.) were also found at this locality. These organisms were maintained in filtered, aerated aquaria at room temperature. Salt levels were held at 4 to 5 ppt with Rila Marine Mix (Rila Products, Teaneck, N.J.) artificial sea salts.

Largemouth bass (Micropterus salmoides) were provided by the Louisiana Wildlife and Fisheries Commission through their hatchery at Alexandria, Louisiana. The fish were held in 300 - liter tanks in filtered, aerated, room-temperature water at a level of .8 to 1 ppt salinity. Initially, bass were fed a commercial fish food (Purina Trout Chow) until it was found to contain HCB residues at levels up to 80 ppb. Small fish from Irish Bayou collections served as bass food during subsequent work.

Experimental animals and the water from which they were taken were analyzed for HCB content prior to acceptance for routine use. All fish and crayfish were maintained in the same quality water, with salinity adjustments as needed. Water for stock holding tanks and experiments was prepared in the following manner. Chemical residues in New Orleans tap water, including chlorine, were reduced by passing water through an activated carbon bed and deionizer tanks. Deionized water was aged approximately 24 hours in holding tanks, diagrammed in Figure 6. Ionic balance was restored and standardized with the addition of Rila Marine Mix to a level of 1 ppt. An automatic salt mix dosing apparatus assured constant salinity level. This device also administered sodium bicarbonate which maintained the pH between 6.65 and 7.9.

C. Static and Flow-Through Assay Systems

Aqueous experiments were of two basic types, static and flow-through. Static tests with mollies (Poecilia latipinna) were carried out in five-gallon glass jugs filled approximately half full with 10ℓ of prepared water at 1 ppt salinity. The test compound, dissolved in nanograde acetone was pipetted into the jugs concurrently with pouring water to effect mixing. Test fish were added and air above the water's surface was saturated with flowing oxygen before the covers were screwed shut. In this type of experiment, water was left unchanged but oxygen was added periodically during the test. Later static tests involved replacement of water and toxicant no more frequently than once daily.

Static tests with crayfish (Procambarus clarki) involved placing the animals in individual finger bowls. Water and toxicant in acetone carrier were replaced daily. The animals were held in environmental chambers with light and temperature controls for the duration of experiments.

The flow-through aqueous system more closely simulated the natural environment because water with a predetermined and constant load of test compound and acetone carrier was flowing at regular intervals into tanks containing the organisms. This system received water from the tank source discussed in Methodology section IV.B. and functioned as a modified proportional diluter. The design for this apparatus was based upon developments by DeFoe (1975) and preceding workers (Benoit and Puglisi, 1973, and Mount and Brungs, 1967). In the modified proportional diluter (Fig. 7), water from a single source fills a series of seven glass chambers in stepwise fashion. Once full, a self-priming siphon in the final chamber initiates a flow of water in the venturi vacuum system. The partial vacuum thus created empties a pre-set, constant volume of water from each of the filled chambers through a siphon whose action is started by the partial vacuum.

Figure 6. Water treatment system.

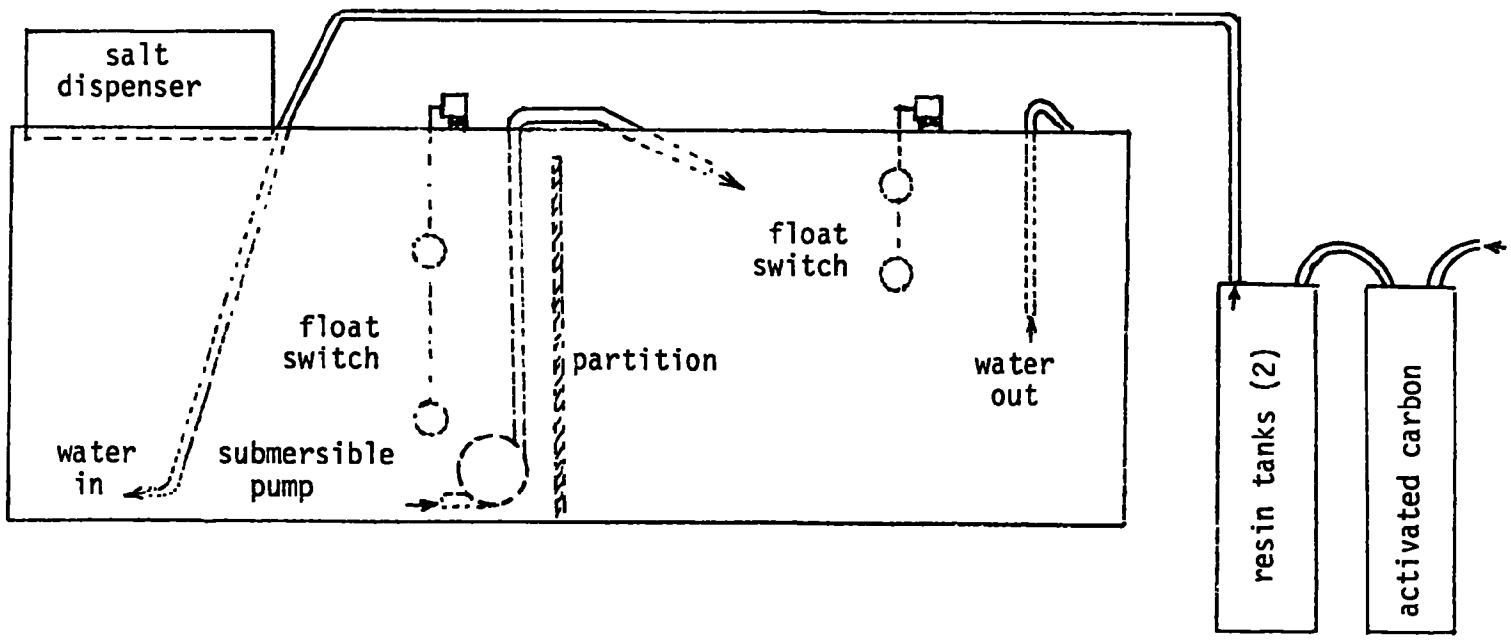
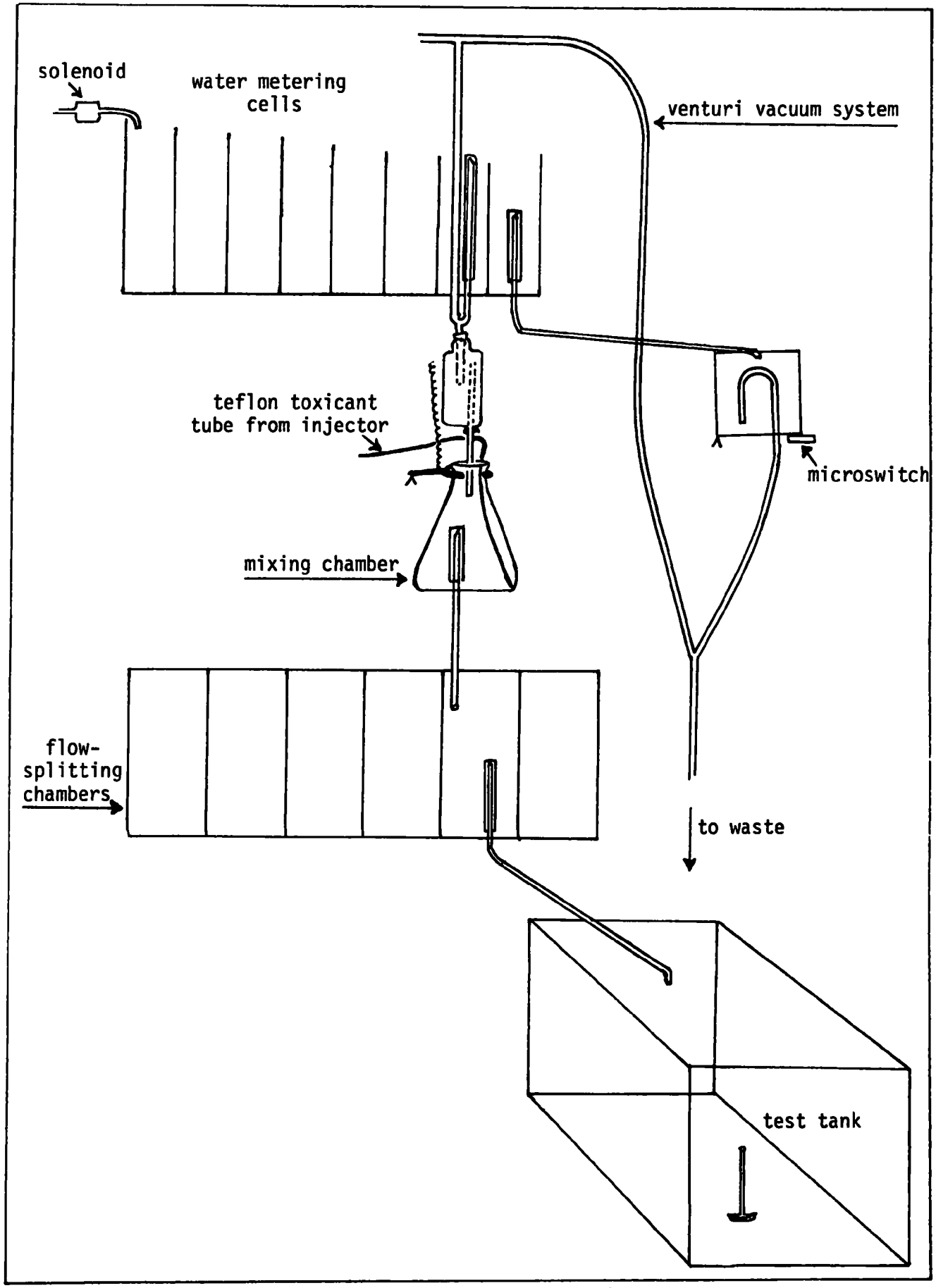


Figure 7. Modified proportional diluter.*



* one of six similar units schematically represented.

The flowing water from each chamber fills a flask (mixing chamber) mounted upon a lever extending from an injector apparatus developed by George J. Frazer *. The flasks are suspended by spring tension. Weight of the water filling the flask depresses the lever, actuating an advance rachet whose pressure hub bears upon the plunger of a 50 ml syringe. A known volume of test compound and acetone carrier is injected into the flask (mixing chamber) through an elongate teflon needle. Once filled, the mixing chambers each empty automatically into flow-splitting chambers through self-priming siphons. Other siphons within each flow-splitting chamber deliver the water to respective test tanks through glass tubing.

Each of the six major test tanks (Fig. 8) have a filled capacity of 70ℓ, but were usually filled to a level of 30ℓ as controlled by an adjustable overflow standpipe. The smaller tanks (Fig. 9) are model ecosystems in which water enters a larger section containing an inoculated algae culture. Next, water flows into the smaller space, containing mollies, through a gap at the base of the partition. Eventually water exits at the discharge tube which maintains a volume of 30ℓ in the tanks. Flow rate was fixed such that each experimental tank received 60 to 120ℓ of water daily.

The protocol for most tests provided for two duplicate test tanks of each of two compound concentrations in addition to a water control and an acetone carrier control. The number of organisms per tank was usually determined by the number and type of samples needed for analysis. Availability of animals and their tolerance to conditions were other factors contributing to this figure.

HCB levels in tanks were checked by analysis of water samples siphoned from beneath the surface. In certain experiments, organisms were removed according to a fixed schedule during both phases, uptake and depuration. Each phase usually lasted 10 or 15 days for each test. In other experiments, all animals were sacrificed at the same time, 10 days after initiation of exposure. Variations in routine will be discussed in appropriate sections of this report. Temperature, pH, and dissolved oxygen content of tank water were monitored regularly during flow-through experiments. These ranged as follows: temperature, 22.2 to 23.9°C; pH, 6.5 to 7.9; and oxygen, 7.6 to 8.5 ppm.

The greater portion of acute experiments utilized the flow-through system, with HCB in the test animals' aquatic medium. Some injection experiments, however, were carried out, with HCB dissolved in peanut oil. A dosage of .01 cc/ĝ body weight was injected directly into the hemocoel of each animal, at the base of its second walking leg on the left side. Several concentrations of HCB were utilized.

In an attempt to obtain a more uniform distribution of injected material throughout crayfish bodies, a series of emulsions was prepared with the HCB-peanut oil mixture. Lecithin was added as an emulsifier. In the first of the two emulsion tests reported, the final solution was made up of 40% spring water and 40% 2X saline, by volume. The second solution contained 40% spring water and 40% 2X sucrose, by volume. Prior to injection, each solution had been sonicated while

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*: 4528 Pitt St., Duluth, Minn. 55804

Figure 8. Major test tank.

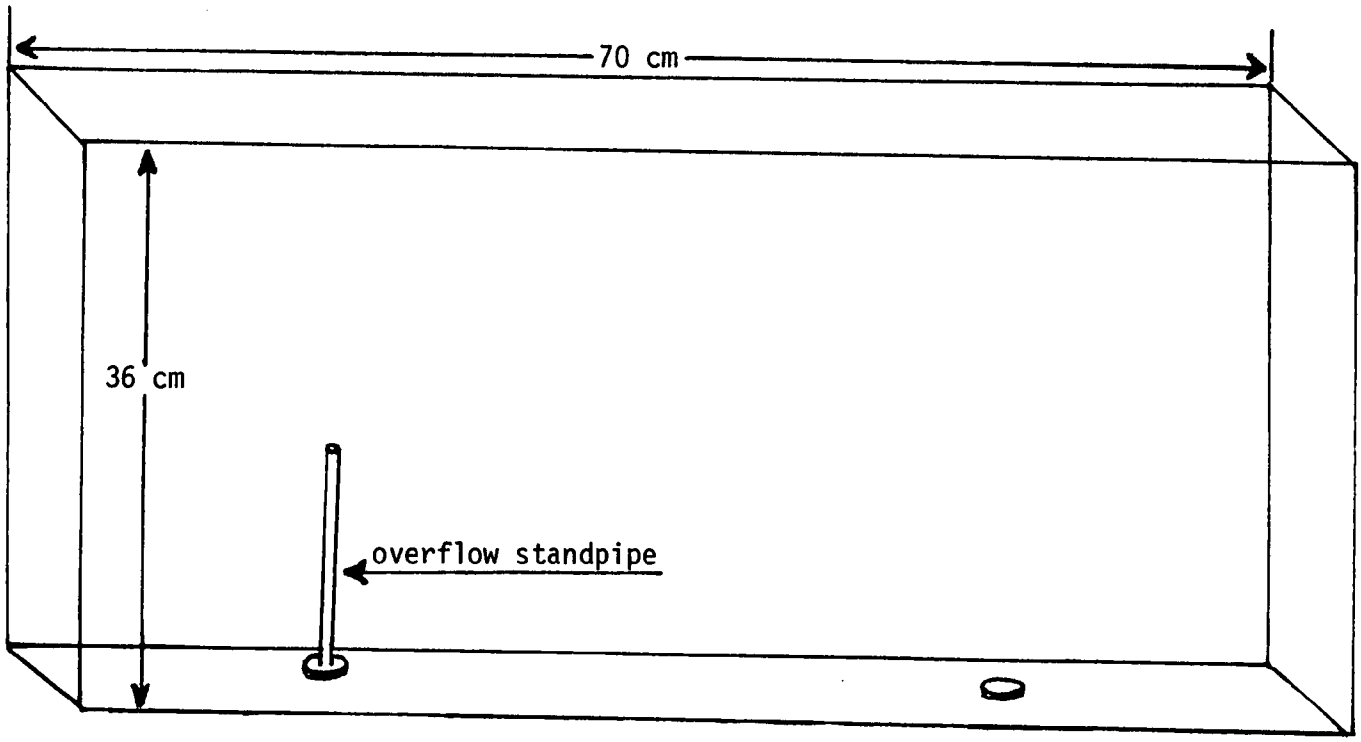
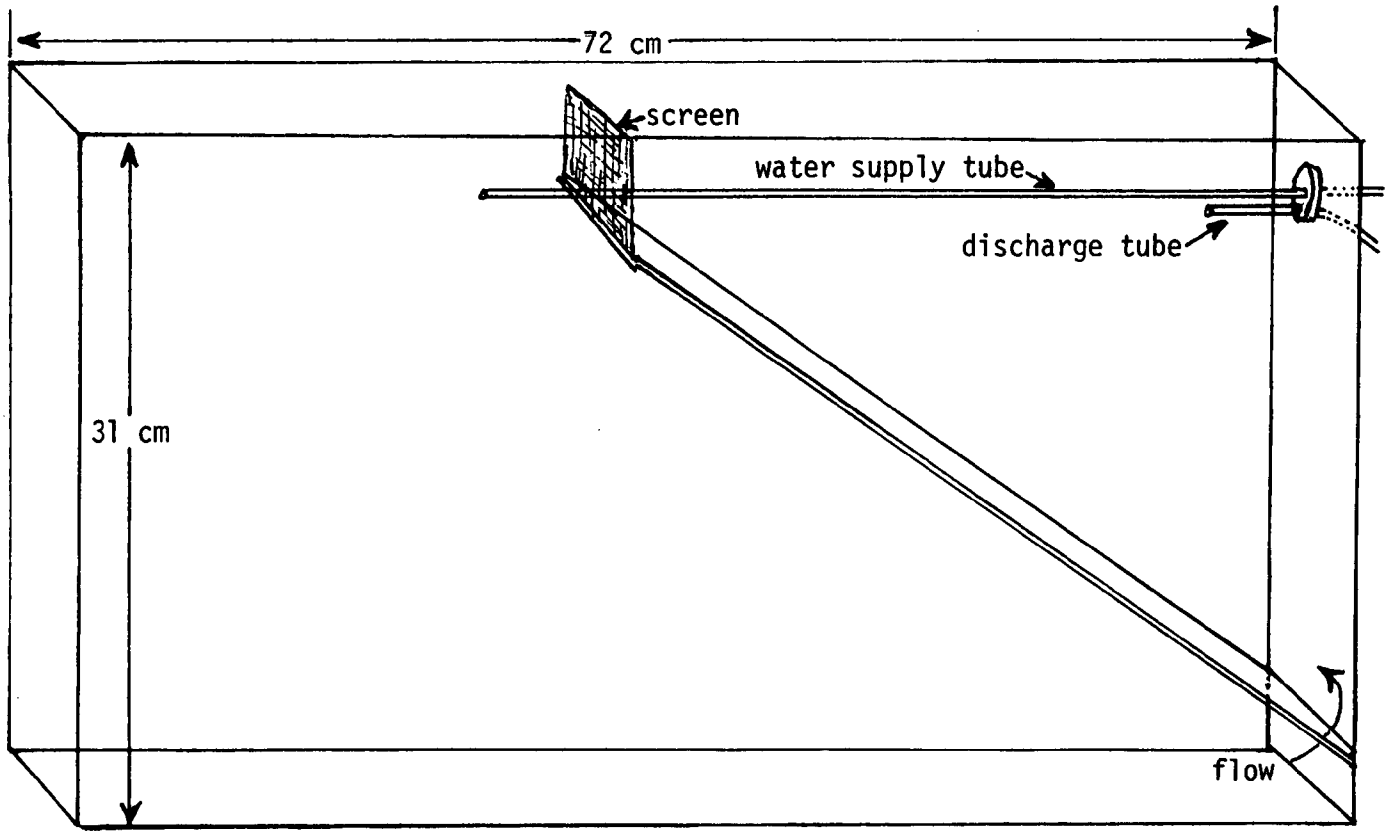


Figure 9. Model ecosystem.



chilled in an ice bath.

Fish were also used in injection experiments. Injections of HCB dissolved in peanut oil were administered intraperitoneally to a series of 10 Gulf killifish (Fundulus grandis). The same volume of oil, .01 cc/gram body weight, was injected into 10 control fish. Fish were held in HCB-free water in diluter tanks for four weeks following injection.

The flow-through conditions of the diluter were useful for two other types of experiments; specifically, those in which algae and sediment were tested.

To test uptake of HCB by a common filamentous green alga, a culture of Oedogonium cardiacum (I.U. #39) was prepared. Original stock was grown in 4ℓ flasks in 3ℓ of 1:1 3N BBM: H_2O solution. Aeration was not provided. Illumination in the culture room was provided by Sylvania lifeline fluorescent bulbs (output 3150 lumens) operating on a 12:12 light: dark photoperiod. Approximately 40 days after inoculation of the stock flask, the algae was divided into four relatively equal portions, three of which were placed into open glass jars and submerged in three tanks (model ecosystems) of the diluter. The fourth was used in an HCBD experiment.

Protocol for diluter tanks consisted of acetone and water controls and 11.5 ppb HCB solution. Illumination to the tanks was provided by three Sylvania lifeline fluorescent bulbs (40 watt; output 3150 lumens) placed at right angles to the tanks' long axes: one lamp located centrally 20 cm above water surface, and two lamps 10 cm behind tanks, +8 cm and -16 cm from water surface. Trace elements present in 1 ppt Rila marine mix-reconstituted deionized water were complemented by metabolic products of four mollies in a separate portion of each tank.

Samples of the algae were collected following one day of exposure and subsequently on every other day through a period of two weeks. Samples were removed from each tank by pipet and placed in a cone of filter paper to drain off excess water. Damp clumps of algae were stored in glass vials and refrigerated at approximately 3°C. Preparation of samples for GC analysis is discussed in Section III.D. of this report.

The sediment experiment was designed to measure uptake of HCB by soil in the form of sediment in the bottom of a test container. For both test and control flasks 200 g samples of dry soil collected at a farm in Talisheek, Louisiana, and determined by GC to be free of HCB, were screened through 1.6 mm mesh aluminum screening and poured into the bottom of 1000 ml aspirator flasks. At the flow rate of 3 l/hr. water originating in the diluter passed through a glass tube into the bottom of the stoppered flasks. Circulated water left through a waste tube attached to the aspirator arm at the neck of the flask. Samples of 40 g (wet wt) of mud were removed from the flasks periodically and stored in glass jars at approximately 3°C until they were extracted for analysis on GC.

Laboratory experiments with crayfish were supplemented with a series of field experiments. Animals initially free of HCB were placed in cages and set on the bottom of a pond adjacent to the hex waste disposal area at the highly elevated field site discussed in Section III.B. HCB and HCBD content of the water was monitored. Periodically, crayfish were removed from the cages

and either frozen for later analysis of uptake or returned to the laboratory and maintained in clean water in environmental chambers. Samples of crayfish were removed at intervals from the environmental chambers, and extracts made from them provided depuration data for animals exposed to both compounds in an environment further complicated by the natural products of the industrial wastes.

D. Gas Chromatography

Preparation methods for samples prior to gas chromatographic (GC) analysis depended upon the substance being extracted. In preparation of water samples, aliquots of 350 ml were shaken with 20 ml of benzene for 3 hr on an Eberback reciprocating action shaker. Following passage through a separatory funnel an aliquot from the benzene layer was ready for injection into the GC. In preparing mud, aliquots of approximately 20 g of mud were shaken with 20 ml of acetone for 20 minutes, after which 20 ml of benzene was added. Following 24 hr of shaking, the benzene-acetone extract was injected into the GC.

Samples of algae were scraped from walls of the tanks and blotted on filter paper. Each sample was weighed, sonicated in acetone in an ice bath to disrupt cell walls, and subjected to the same procedure used for animal tissue. Concentrations of HCB were expressed in terms of wet weight of the algae.

Samples of animal tissue were weighed and homogenized with anhydrous sodium sulfate and acetone. The liquid was filtered into a separatory funnel and the residue homogenized twice with acetone which was then added with filtration to the separatory funnel. After adding sodium chloride to the combined acetone extracts in a separatory funnel, the acetone-sodium chloride mixture was extracted three times with hexane and the hexane evaporated to near dryness on a rotary evaporator. This residue was dissolved in hexane and placed on a Florisil column washed previously with 50 ml of elution solvent (95% hexane, 5% ether). Following elution with 100 ml of elution solvent the eluent was evaporated on a rotary evaporator. The residue was dissolved in 10 ml of benzene and an aliquot was prepared for injection into the GC.

All extracts of water, soil, algae and tissue samples were analyzed by a Hewlett Packard 5710A gas chromatograph (GC) equipped with an electron capture detector utilizing ^{63}Ni foil. Extracts were introduced by a 7671A automatic sampler. This system was attached to a Hewlett Packard 3352B Laboratory Data System. Separation was accomplished with a 91.44 cm X 4 mm I.D. 10% OV-1 column maintained at either 150° or 165°C depending upon the integration method used. Argon-methane 95:5 was employed as the carrier gas at a flow rate of 35 ml/min. The injection port temperature was held at 250°C and the detector was set at 300°C.

Quantification was accomplished using external standards of hexachlorobenzene at concentrations of 1 ppm and 0.1 ppm in a benzene solvent. Concentrations of HCB in water were computed in terms of µg/ℓ of sample, and expressed in parts per billion (ppb). Other samples reported in µg/g are expressed in parts per million (ppm).

E. Mass Spectrometry

A double focusing duPont 21-491 mass spectrometer was employed which was attached to a Hewlett-Packard 5750 gas chromatograph and coupled to a PDP-12-LDP computer. Separations were carried out isothermally on a 9 m X 4mm ID glass column packed with 10% OV-1 stationary phase on acid washed chromosorb. Transfer lines were maintained at 200°C. All spectra were obtained at 70 eV at a source temperature of 200°C.

F. Corticosteroid Analysis

The fish were maintained on a 12:12 (light:dark) photoperiod (light on at 0730) for the entire duration of exposure to HCB. On the final day, blood was collected between 2 and 3 hours after "dawn." Blood was taken from the sinus venosus into heparinized hematocrit tubes which were then sealed with clay and centrifuged. The tubes were cut and the plasma separated and stored in a freezer until used.

Plasma corticosteroid concentration was determined using a modification of the competitive protein-binding radioassay technique described by Murphy (1971) which has been used successfully in teleost plasma (Hargreaves and Porthé-Nibelle, 1974, Meier and Srivastava, 1975). A 10 µl aliquot of plasma was expelled into a centrifuge tube containing 1.0 ml absolute ethanol. The ethanol precipitates plasma proteins which might interfere with the assay (including any endogenous corticosteriod binding globulin) and also extracts the cortisol. The tubes were centrifuged and a 0.5 ml aliquot of the supernatant ethanol containing the cortisol was transfered to a reaction vessel and evaporated to dryness under N_2. Each sample was then incubated for 5 minutes in a water bath at 45°C with 1.0 ml of corticosteroid binding solution. Each 100 ml of this solution contained 0.5 µCi of 1,2H^3-cortisol (obtained from New England Nuclear Corp) and 5 ml of pooled male horse serum (obtained from the LSU Veterinary School) with distilled water to volume. Horse serum was used as a source of corticosteroid binding globulin (CBG) since Ficher *et al*. (1973) have found that it gives greater specificity for cortisol.

After inclubation, the samples were transfered to an ice bath for 30 min. and then 40 mg of Florisil was added to absorb the unbound cortisol. A 0.5 ml aliquot of each sample was counted and compared to a standard curve obtained by measuring known amounts of cortisol.

G. Fish Blood Hematocrit

The animals used in these studies were exposed to various concentrations of toxicant. They were bled on the tenth day of exposure, first being stilled by immersion in ice cold distilled water. Blood was taken from the sinus venosus into heparinized hematocrit tubes which were then sealed with clay and centrifuged. Packed cell volumes were read using a Critocap® micro-hematocrit tube reader.

H. Respirometry

The effect of exposure of juvenile crayfish and mollies to HCB was measured using a Gilson Respirometer (Unbreit, et al., 1972). Each set of tests using juvenile crayfish (average weight = 10 mg) employed animals from one hatching of one female. Juvenile mollies (average weight = .3 g) were collected from uncontaminated environments and used immediately after collection.

Five crayfish were placed in each respirometer vessel in 3 ml of water. In the experiments using mollies a single fish was placed in each vessel in 7 ml of water.

Animals were placed in the respirometer, and following 15 minutes of acclimation, oxygen consumption was monitored. In crayfish studies, respiration rates were determined every 15 minutes for two consecutive 1 hour periods. The system was flushed with air after the first hour.

The rates of respiration of mollies were determined every 10 minutes over two thirty-minute periods. The system was flushed with air following the first thirty-minute period.

Animals were placed in covered 8-inch finger bowls in either control or experimental conditions during exposure periods. The culture solution was changed dialy. Mollies were kept in deionized water reconstituted to 2.5 ppt with artificial sea salt. Crayfish were kept in deionized water reconstituted to 1 ppt. The experimental groups were exposed to water that had been stirred with appropriate amounts of HCB for at least 24 hours before the animals were placed in the solutions.

The solution in each respiration vessel was the same as that to which the individual animal or group of animals had been exposed. Therefore, respiration rates were determined during exposure. Respiration rates for HCB were determined immediately following exposure and after several longer intervals of exposure. The concentrations are given in the "Results" section (V.E.). Oxygen consumption rates were expressed in terms of wet weight of the animals.

I. Metabolic Fate (^{14}C)

Thin layer chromatography using Kodak Chromogram® silica gel sheets was used to determine if any metabolites of HCB had formed. The HCB used was carbon-14 labelled (New England Nuclear Corp.) and followed by means of autoradiography using Kodak no-screen X-ray film (Estar base).

The $HC^{14}B$ was incorporated into the bass by means of a freshly-killed molly whose peritoneal cavity had been injected with 0.1 ml of $HC^{14}B$ in a peanut oil carrier. This amount of $HC^{14}B$ contained 1.8×10^2 µCi. Injected fish were readily consumed by the bass. The experimental animals were allowed either 24 or 48 hours to digest the $HC^{14}B$-fed bass were then killed and dissected. Five separate body samples were examined for evidence of metabolic products: (1) feces, (2) stomach and intestines, (3) liver, (4) kidney,

and (5) remaining body. These samples were extracted separately and the extracts applied to the thin layer autoradiography system described above.

J. Histology

Fingerling largemouth bass and crayfish for histological examination were selected at random from the inhabitants of each tank of the modified proportional diluter system to represent water control, acetone control, and each nominal level of HCB utilized for GC analysis. Animals were sacrificed on the day of termination of toxicant accumulation. Brain, green gland, hepatopancreas, one gill and a sample of abdominal muscle were excised from each crayfish.

Largemouth bass were stilled by chilling, weighed, and total and standard lengths measured. The liver was excised and weighed as another measure of size and nutritional condition and fixed. The right kidney, the first right gill arch, approximately 5 mm of epaxial muscle caudal to the right operculum, and a segment of intestine immediately caudal to the stomach were excised and fixed. Organs were examined under dissecting microscope magnifications and any grossly damaged areas noted. In one experiment, each animal utilized for histological study was also subjected to GC analysis.

Tissue samples were fixed in neutral buffered formalin, Bouin's or Zenker-acetic, appropriately washed, dehydrated, cleared in toluene, and infiltrated and embedded in 56°C - 58°C paraffin. Tissue blocks were serially sectioned and mounted. Slides representing step sections were stained in Harris' hematoxylin and eosin or Lillie's modification of Weigert's iron hematoxylin and eosin. All mounted sections were kept to permit study of serial sections of any areas found on microscopic examination to be of specific interest.

K. Photochemistry

Solutions of HCB in both hexane and benzene (1 µg/10 ml) were irradiated at varying time intervals. HCB was irradiated for 30 minutes, 65 minutes and 120 minutes. Irradiations at 253.7 nm were conducted in serum capped quartz test tubes employing a Rayonet RPR-100 Chamber Reactor equipped with 16 8-watt lamps and a "merry-go-round" apparatus (The Southern New England Ultraviolet Co., Middletown, Conn.) All samples were degassed by purging with nitrogen for a period of 10-15 minutes prior to irradiation.

V. RESULTS

A. Field Studies

Field work consisted of two distinct phases. The first, a regional overview, dealt with HCB residue determinations in water, mud and aquatic organism samples collected along a Mississippi River transect from Baton Rouge to Port Sulphur, and other parts of southeastern Louisiana. The second phase, at an area of higher concentration, was located on property of the Vulcan Materials Company in Geismar, Louisiana. Specific localities and protocol are discussed in the Field Methodology section of this report.

1. Overview

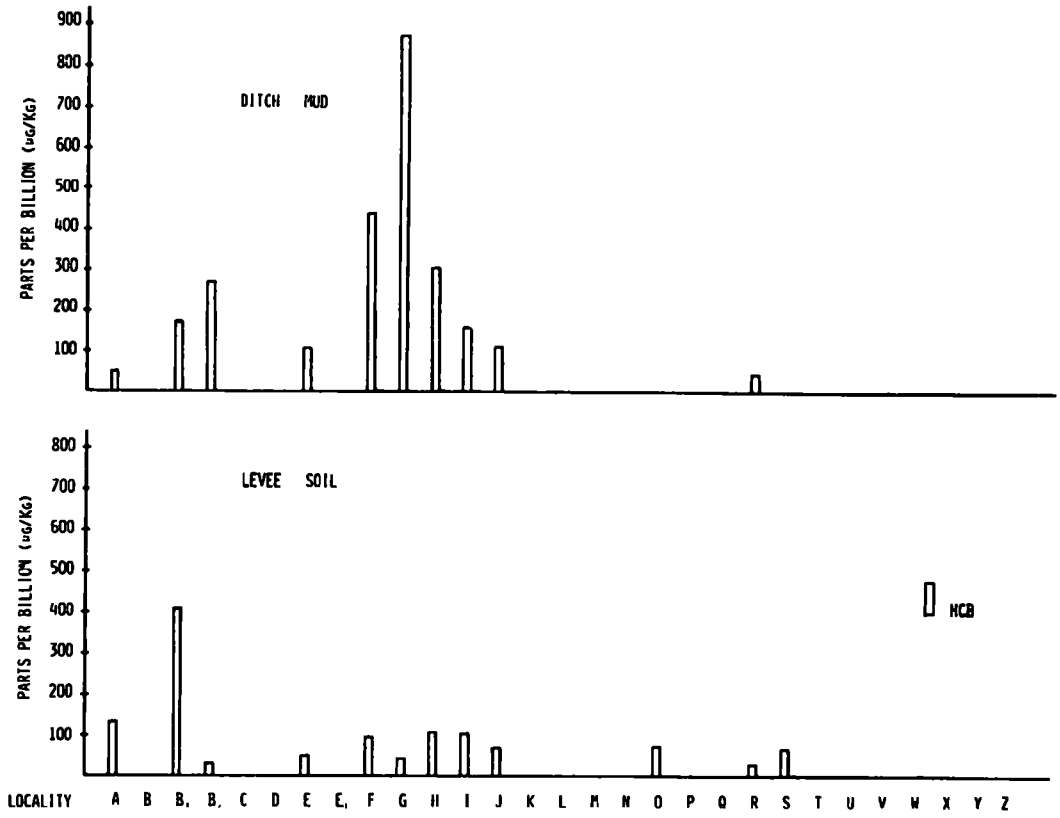
Figure 1 shows localities in the immediate vicinity of the Mississippi River sampled for HCB residues in collections of water, mud, crayfish and fish made during the first phase of field work. Concentrations of HCB in mud and soil are presented in Figure 10. Supplementary sites are outlined in Table 1 in the Field Methodology section. Comparisons of HCB residues in soil, water and organisms from various sites can be seen in Tables 2 through 4.

2. Area of Higher Concentration

The area near the Vulcan Materials Company plant in Geismar, Louisiana, was the site of a series of field collections during 1974 and 1975. Three specific localities were selected for sampling of water, mud and organisms. These sites, designated Recreation Pond, South Effluent, and Landfill Pond (pond adjacent to hex landfill) are discussed in detail in Section III of this report. Table 5 gives concentrations of HCB residues in water, mud, and organism samples taken during several seasons of the year.

Chara and Najas are two plants found in the pond. The former, an alga, is less plentiful, has a high calcium carbonate content and attaches to the bottom. The latter occurs as floating, unattached masses in all parts of the pond. Eleocharis, an emergent plant, was taken in shallow water less than .3 meter deep, near the shoreline. The snail Physa was common in masses of Najas. Anisoptera were represented by dragonfly larvae taken while dipnetting in floating vegetation near the shore. Procambarus sp., the crayfish, was taken periodically during scoops of the dipnet that took up some bottom sediment as well. Gambusia, the mosquitofish, was more frequently found near the surface in shallow water, while juvenile sunfish, Lepomis, less than 4 cm in length, lived in the protective cover of aquatic vegetation. Micropterus, the single bass collected, is a common sport fish in this region.

Figure 10. Distribution of HCB in soil along the Mississippi River, Louisiana. Baton Rouge - Port Sulphur transect.



CODE	LOCALITY
A	Hwy. 90, north of Baton Rouge
B	Intersect River Rd., La. Hwy. 327
B_1	Addis at La Hwy. 990 & La 998
B_2	Plaquemine at La. Hwy. 988 & La. 1148
C	5 mi. south of B
D	Sunshine
E	Carville
E_1	La 405, 4 mi south of White Castle
F	Ashland Plantation
G	Darrow

CODE	LOCALITY
H	Intersect. La. 44 & La 942
I	Romeville
J	Convent
K	Lutcher
L	Garyville
M	Reserve
N	Laplace
O	South of Bonnet Carré Spillway
P	Destrehan
Q	St. Rose

CODE	LOCALITY
R	River Ridge
S	New Orleans, River Rd. at Causeway
T	New Orleans, Audubon Park
U	Lower Algiers
V	Belle Chasse
W	Myrtle Grove
X	Mile marker 42
Y	.8 mi. south of X
Z	Port Sulphur

Table 2. Concentrations of HCB in water and soil samples from sites removed from Mississippi River transect.

Location	Code	Water HCB in µg/l (ppb)	Soil HCB in µg/Kg (ppb)**
Walker	(i)	.8	*
Hammond	(ii)	.9	*
Covington	(iii)	*	*
Pass Manchac	(iv)	*	*
Sorrento	(v)	*	*
Spillway	(vi)	1.5	171.7 (231.2)
Lake Grand Ecaille	(vii)	*	*

*: <.7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 3. Mean concentrations of HCB in Mississippi River mosquitofish (*Gambusia affinis*) in comparison with levels measured in water and soil.

Location	Code	Water HCB in µg/l (ppb)	Soil HCB in µg/Kg (ppb)**	Fish HCB in µg/Kg (ppb)
Garyville	(L)	*	*	71.8
Romeville	(I)	*	107.0 (135.0)	136.8
Baton Rouge	(A)	2.2	134.9 (167.0)	379.8

*: < .7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 4. Mean concentrations of HCB in crayfish (*Procambarus sp.*) from ditches in comparison with levels measured in soil.

Location	Code	Soil HCB in µg/Kg (ppb)**	Crayfish HCB in µg/Kg (ppb)
Walker	(i)	*	*
Romeville	(I)	160.6 (217.7)	22.2
Ashland	(F)	440.3 (884.5)	192.3
Darrow	(G)	874.4 (1676.8)	194.3

*: < .7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 5. HCB residues in water, mud and organism samples from highly elevated sampling area. (Number of organism samples in brackets; figures in parentheses corrected for dry weight of mud samples).

Organism	# of analyses	Mean HCB concentration in µg/Kg (ppb)	Concentration factor	Abiotic Component	Dec. 1974	April 1975	May 1975	Aug 1975	Oct 1975
			Recreation	Pond					
Plankton	1	561.	3,573x	Water(ppb)	*	**	2.2	.04	.16
Chara	1	563.	13,093x	Mud (ppb)	*	290 (410.)	170. (220.)	570. (660)	130. (170.)
Najas	1	147.	66x						
Eleocharis	1	423.	192x						
Physa [3]	1	294.	6,837x						
Gambusia [27]	9	3,291.	2,789x						
Lepomis [45]	15	3,170.	2,690x						
Micropterus [1]	2	liver 23,506.	10,684x						
		muscle 3,362.	1,528x						
			South	Effluent					
Anisoptera	1	4,699.	49,998x	Water(ppb)	3.9	2.8	72.8	.10	9.98 †
Procambarus [9]	3	48,669.	17,382x	Mud (ppb)	11,960 (19,000)	2,520. (4,200)	13,800. (19,000.)	2,150 (3,000)	600 † (1,320.)
Gambusia [9]	3	41,353.	16,408x						
			Landfill	Pond					
Gambusia [24]	9	82,891.	2,060x	Water(ppb)	4.8	12.1	74.9	*	33.72
				Mud (ppb)	*	10,500. (13,000	29,080. (38,800.	*	53,130. (75,000.)

* : no sample taken
**: below detectable levels.
† : site changed due to construction.

B. Acute Toxicity

1. Crayfish Injections

Several experiments were carried out in which HCB, dissolved in peanut oil alone, or prepared as an emulsion, was injected into crayfish. Concentration of HCB in stock emulsion of the first experiment was measured at 1.67 mg/ml (ppt). At the highest concentration, emulsion was injected (full strength) at the rate of .02 cc/g body weight, which resulted in a dose of 33 µg HCB/g body weight, or 33 ppm. The stock emulsion was diluted to .1, .01 and .001 its original concentration, and five crayfish each were injected with these dilutions, at the same rate of .02 cc/g body weight. At the highest concentration, one animal died on day 2, 4 and 56 after injection. The remaining two survived until the experiment was terminated after 61 days. One death occurred after 8 days in the group injected with the lowest concentration and another occurred in the emulsion control group after 9 days. In summary, of the 20 crayfish injected in this experiment, 16, or 80% survived two months after the injections, at which time they were sacrificed. HCB at the concentrations injected did not appear to exert a significant critical toxic effect, with the exception of the most highly concentrated series.

The second emulsion was measured at 423 µg/ml (423 ppm). A total of 20 experimental and control animals was injected. Five of these received .004 cc HCB emulsion/g body weight and another five received .04 cc/g body weight. Sucrose and emulsion controls received .04 cc/g body weight of their respective solutions. During the 24 day period prior to sacrificing only one death was recorded; that of one crayfish injected with .04 cc/g HCB emulsion, which was a dosage of 17 µg HCB/g body weight, or 17 ppm.

A third crayfish injection experiment used only HCB dissolved in peanut oil. One solution was composed of 5 mg HCB/ml oil; the second contained 12.5 mg/ml and was a saturated solution at 35°C. Experimentals and oil control groups were composed of five males and five females each, and were injected with .01 cc oil/g body weight. At this rate, crayfish injected with the HCB-saturated oil were receiving 125 µg HCB/g body weight, or 125 ppm.

Of the 30 crayfish reported in this test, all but two survived the seven days of the experiment in individual finger bowls in the environmental chamber. One specimen, a 23g male, died 24 hours after injection, and the second, a 16.5g female, died 4 1/2 days (103 hours) after injection. Both mortalities were in the group receiving the highest concentration of HCB in oil.

2. Fundulus Injections

HCB, dissolved in peanut oil, was injected intraperitoneally into Gulf Killifish (Fundulus grandis). The dosage of 125 ppm, or 125 µg/g body weight, had no acute lethal effects during the four-week experimental period. During the first 24 hours following injection, both experimentals and controls had blotches of dark pigment dorsally, which disappeared by the second day. These spots may have been in response to the excitation of being handled.

3. Crayfish LC_{50}'s

To determine possible acute toxicity of HCB in aqueous solution to crayfish, both static and flow-through experiments were carried out. Initially, a static test exposed eight juvenile crayfish (Procambarus sp.)(3-6 cm rostrum-telson length) to each of four different conditions. These were 5.2 ppb, 2.6 ppb, acetone control and water control. Animals were kept individually in finger bowls with approximately 50 cc of water. The water was replaced daily. Toxicant solution was prepared by dissolving HCB in acetone to produce a concentrated stock solution. A portion of this stock was further diluted in acetone and added to spring water with vigorous mixing. All crayfish in this test survived the entire eight days of the experiment, with no observable behavioral alterations.

In one experiment in the diluter 32 mature crayfish (Procambarus clarki) were exposed in each of four tanks to 27.3 ppb HCB, 36 ppb HCB, water control and water plus acetone control conditions. HCB was flowing in test tanks for 10 days, followed by 10 days of depuration. A total of four (6%) experimental animals and two controls (3%) died during the entire 20-day period. These deaths in diluter tanks were the result of cannibalism of vulnerable individuals following molt.

4. Fish LC_{50}'s

Two separate experiments were carried out in the diluter in an effort to test acute toxic effects of HCB. In the first test, twenty bass were placed in each of six tanks containing water alone, acetone and water, HCB concentrations of 2, 2, 9 and 10 ppb, respectively. Exposure to HCB lasted 15 days, followed by a 13-day depuration period. No mortalities or abnormal behavior patterns could be attributed to HCB during this experiment.

The second experiment exposed five bass in each of four tanks to water and water plus acetone controls and 21.6 and 25.8 ppb HCB, respectively. All animals survived the 10 days of the test, and showed no overt negative effects due to exposure to HCB. Exposure to HCB in aquatic systems did not result in overt behavioral responses by fishes or crayfish at the concentrations or during the time periods of our tests.

C. Chronic Toxicity

1. Crayfish Tissue Morphology - Normal and Pathological

The hepatopancreas of Procambarus clarki and closely related species has been the subject of histological study since the late nineteenth century. (Huxley, 1880.) Morphological and physiological studies indicate that the hepatopancreas is analogous to vertebrate liver, pancreas and gut. It functions in enzyme secretion, digestion, and absorption of food and in glycogen, lipid and mineral storage (Fingerman et al. 1967.) These facts coupled with reports of liver damage in other species following HCB exposure led to priority being given to histological analysis of this organ.

The hepatopancreas develops as a bilateral evagination of the midgut. Each unilateral component consists of a short common duct which gives rise to longitudinally oriented central canals of the anterior and posterior lobes. The common duct and central canals are lined with tall, simple columnar epithelium with a striated border. From the central canal numerous diverticula of the epithelium are derived. These are surrounded by a "basket" of myoepithelial cells and are bound one to another by fine connective tissue.

The lining of each diverticulum is a simple columnar epithelium which shows a variety of cell types from distal to proximal extent. Four or five distinct cell types have been described; but a discussion of the merits of various classifications is not pertinent to the present report (for references see Loizzi, 1971). There is general agreement that the germinative center for the epithelium lies in the distal-most region of each diverticulum where the epithelium consists of relatively small, slender columnar cells with basophilic cytoplasm (E-cells). These cells apparently give rise to all other cell types, including absorptive, secretory and fibrillar cells which vary cytologically and stain differentially with routine hematoxylin-eosin preparations following Zenker-acetic fixation. Also seen are inclusions which have been described as metals (iron and copper) in the deeply basophilic fibrillar cells (Fe-cells) and in eosinophilic cells (Cu-cells) (Ogura, 1959, Miyawaki et al., 1961). All cell types are found intermingled within the epithelium gradually giving way to large vacuolated columnar cells in the proximal region of each diverticulum. Cells in the proximal region of the diverticulum exfoliate into the lumen and are removed from the gland as a component of the secretion.

Each green gland (antennal gland) is a nephron-like unit consisting of coelomosac, labyrinth, and nephridial tubule. The tubule leads to a bladder and ultimately to an excretory pore. In Procambarus blandingi light and electron microscope studies (Peterson and Loizzi, 1975) reveal that the epithelial cells of the coelomosac are similar to the podocytes of the vertebrate glomerulus. Epithelium with well-defined brush border comprising the labyrinth indicates a reabsorptive function for this region; whereas the nephridial tubule is lined with epithelial cells lacking a brush border but with basal plasmalemmal invaginations (Beams et al., 1956, Peterson and Loizzi, 1975) such as are found in the distal tubule of the mammalian kidney (Pease, 1955). Thus the organ is considered as excretory and osmoregulatory and in many respects analogous to the vertebrate kidney. For this reason the green glands have been preserved for histological analysis from water and acetone controls, and HCB exposed crayfish.

Judicious assessment of tissue health or disease related to toxicant exposure requires consideration of the feeding of the animal, time elapsed between feeding and sacrifice, and possibly the relationship of time of molt. In a diluter experiment in which crayfish were exposed for 10 days to 36.1 ppb, minimal gross pathology was noted as the crayfish were sacrificed; however, occasional, hardened, brown-pigmented areas involving one to several hepatopancreatic diverticula were observed. The color of the organ varied from gray to a bright yellow and this may be related to that cell type predominating in any one gland.

Careful study of numerous serial microscopic sections of hepatopancreas indicates that despite the generally normal gross appearance, change at the cellular and tissue level is taking place in HCB-exposed crayfish. In static experiments with nominal HCB concentrations of 5 ppb and 10 ppb as well as the diluter experiment above (36.1 ppb) there appears to be a heightened exfoliation of epithelium in the central, lobar canals as well as in proximal levels of the diverticula, resulting in empty sacs. The mitotic rate does not appear to be increased, and therefore epithelial loss is not compensated. The various cell types are represented but the majority of the cells are large, with numerous fine vacuoles ("foam cells") unlike the single larger vacuoles in cells of controls. There is additional material in preparation and the analysis of this should lead to a better understanding of the more subtle cytological changes.

2. Bass Tissue Morphology - Normal and Pathological

Cursory histological examination of the various excised organs (see Methodology) has indicated that liver, gall bladder, and kidney were of prime importance for histological study in bass.

In the largemouth bass, (Micropterus salmoides), the liver is elongate, conforming to the shape of the pleuro-peritoneal cavity, and a gall bladder is present. The gland parenchyma is seen to be lobulated with the classical lobule easily discerned. Large hepatic portal veins, hepatic arteries, and bile ducts are seen in the interstices of the lobules (Fig. 11A).

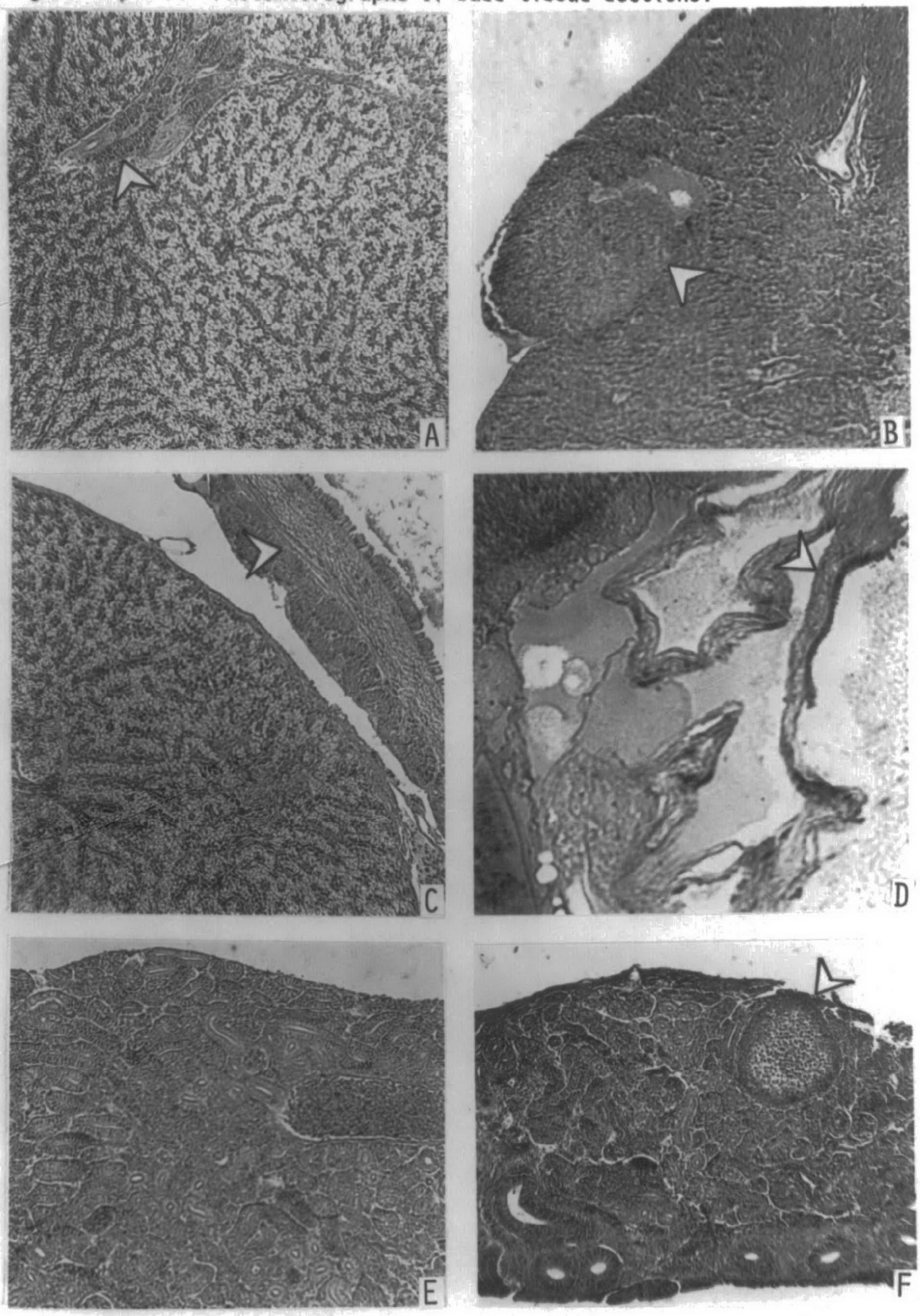
The gall bladder lies cupped by a lobe of the liver. It is lined by a simple columnar epithelium with a rather dense fibrous connective tissue layer beneath. The muscularis is distinct. (Fig. 11C).

The present study reveals that in this species of fish, acini of exocrine pancreas are situated in the connective tissue around the hepatic portal vein and around the major bile ducts in the hilus of the liver and accompany the hepatic portal vein, hepatic artery, and bile ducts in their distribution within the liver parenchyma (Fig. 11A, arrow). Exocrine pancreas also is situated between the muscularis and serosa of the gall bladder (Fig. 11C, arrow). Exocrine and endocrine cells are intermixed to form small nodules of pancreas in the gastro-hepatic ligament. Variability among fish species has been noted previously in the location of endocrine and exocrine pancreas (Bengelsdorf and Elias, 1950, Bertolini, 1965, Falkmer and Windbladh, 1964, Fujita, 1964, Weis, 1972).

The kidney, an opisthonephros, shows relatively large renal corpuscles and short proximal and distal tubules. A thin loop of Henle is not present (Fig. 11E). Other bass tissues (gill, brain, and muscle) have been excised, examined grossly, and scanned histologically. One hundred and ninety blocked specimens are available for further detailed study.

The fish were normal in appearance and behavior at the time of sacrifice; however under dissecting microscope magnifications some abnormalities were noted in the appearance of various organs. Thus, in 9 of 19 HCB-exposed bass in one series, the liver was noted to be pale and the gut white or empty. In one fish the gall bladder was noted to be white and in another, was absent. In the fish in the highest exposures of HCB, (10 ppb) a marked gold irridescence of the parietal peritoneum was also observed.

Figure 11, A-F. Photomicrographs of bass tissue sections.



Explanation of Figures

(All figures are photomicrographs at the same magnification, taken of sections stained with H & E.)

A. A microscopic field of fingerling largemouth bass (Micropterus salmoides) liver shows the normal lobulation pattern. Cells interpreted as exocrine pancreas (arrow) are seen in the connective tissue around a large vessel. (H_2O Control Bass)

B. A microscopic field of the liver removed from a fingerling bass after ten days diluter exposure to HCB. Note the loss of normal liver architecture and the beginning necrosis (arrow). (Bass exposed to 3.5 ppb HCB in diluter; 26.8 µg/g HCB in tissue).

C. Normal liver may be seen on the left and a small segment of the gall bladder wall in section is seen on the right. The arrow indicates the location of pancreatic cells. (H_2O Control Bass)

D. A section through the necrotic liver hilus also includes a portion of the wall of the gall bladder in which almost complete destruction of the epithelium, muscle, and pancreatic acini was noted. (Bass exposed to 25.8 ppb HCB for 10 days in diluter; 69.9 µg/g HCB in tissue. See section V.C4.)

E. A section of fingerling bass kidney illustrates the normal histology. Note the size of the glomeruli and the tubules. (H_2O Control Bass).

F. A microscopic field from the kidney of another HCB-exposed bass in which distended tubules and a large damaged glomerulus (arrow) may be seen. (Bass exposed to 3.5 ppb HCB for 10 days in diluter; 2.4 µg/g HCB in tissue. See section V.C4.)

The minor alterations in gross morphology were in no way indicative of the degree of pathological change, the extent of which could not have been appreciated without thorough microscopic examination. Histopathological change was noted in the liver, gall bladder, and the kidney in HCB-exposed bass.

In the liver, areas of necrosis were found to be most pronounced in the region of the hilus (Fig. 11D), but existed also deeper in the liver parenchyma and just beneath the serosa (Fig. 11B). The necrotic areas seemed to follow the portal canal distribution. Different stages of necrotic change from early hepatocyte death to hepatocyte-barren stroma and phagocytic infiltration were seen. In non-necrotic areas of affected livers, the typical pattern of lobulation was lost and there was an increased intensity of cytoplasmic staining (Fig. 11B).

Gall bladder damage was striking. Both the epithelium and the smooth muscle were destroyed, leaving a dense connective tissue bag (Fig. 11D). Remnants of cords of deeply basophilic pancreatic acini show that this tissue too is affected in its site in the wall of the gall bladder; although it persisted within the liver parenchyma.

In animals with liver damage, kidney damage was also found. Some glomeruli were destroyed, resulting in distended balls of stagnant blood (Fig. 11F). Necrotic areas of tubule destruction were present also. The liver and kidneys from H_2O-controls thus far sectioned were normal histologically. Among acetone controls some liver and kidney necrosis was noted but was not as extensive as that found in HCB-exposed animals.

3. Retention and Distribution of HCB in Crayfish

Crayfish exposed to HCB in a nominal concentration of 5 and 10 ppb (GC values at 5.2 ppb and 2.6 ppb respectively), in aqueous solution in finger bowls, changed daily, showed no readily observable adverse response to the compound. All experimentals and controls survived and were sacrificed after 8 days. Dissected tissue samples were grouped and frozen together. Subsequent GC analysis gave the results seen in Table 6.

Table 6. Concentrations of HCB (in µg/g) in crayfish bodies and tissues of animals exposed to HCB in aqueous solution for 8 days.

	N**	5 ppb HCB	N	10 ppb HCB	N	Acetone Control	N	H_2O Control
Whole body ($\bar{x}$)	2	.45	3	.68	7	*	7	*
Hepatopancreas ($\bar{x}$)	5	*	4	24.59				
Gills ($\bar{x}$)	5	*	4	12.35				
Muscle ($\bar{x}$)	5	1.17	4	2.63				
Remaining body ($\bar{x}$)	5	.89	4	.60				

*: below detectable levels

**: number of specimens

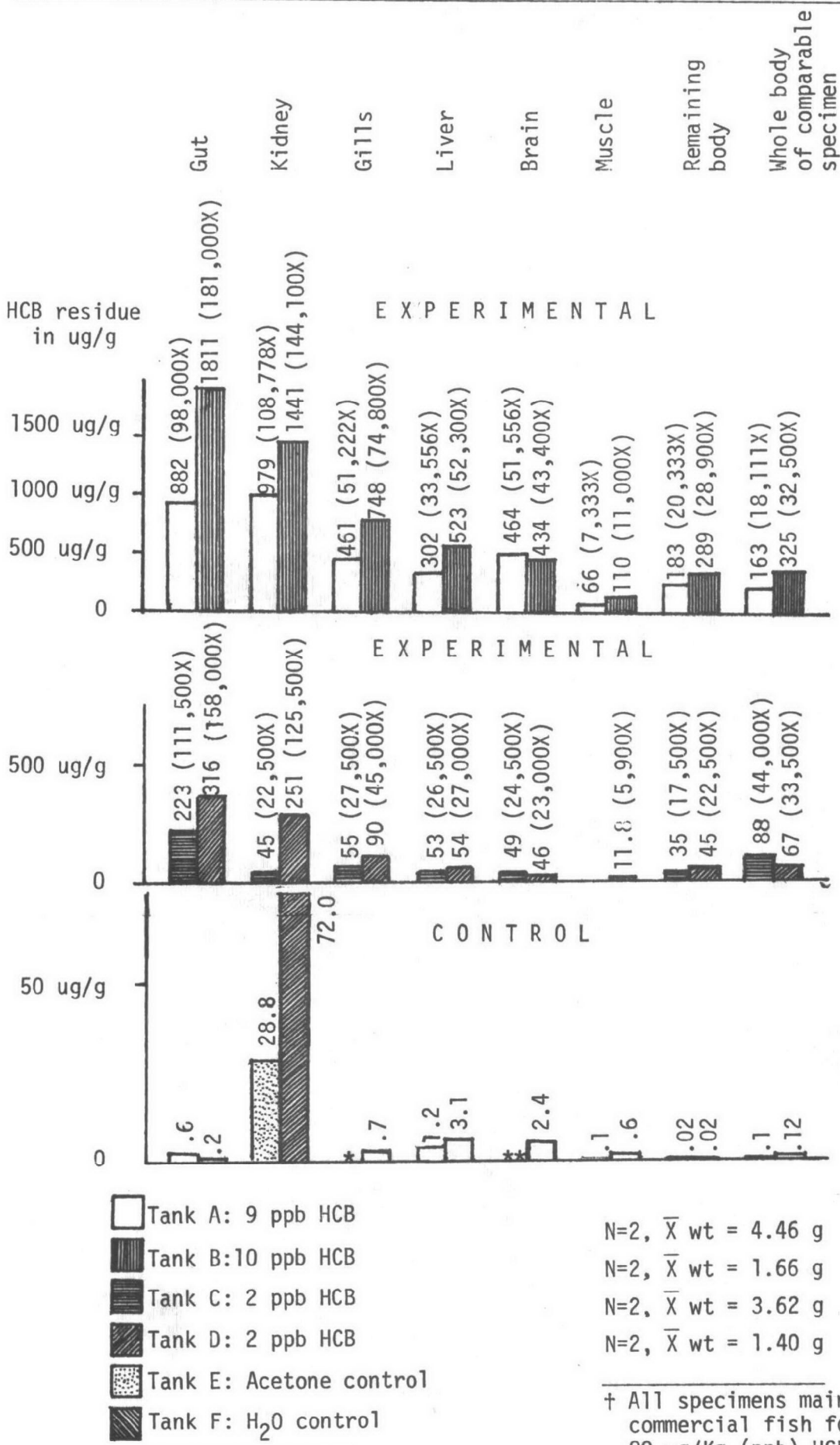
4. Retention and Distribution of HCB in Bass

Two separate tests with bass were conducted to determine the distribution of HCB in organs as a function of dosage administered, size (weight) of specimens, and time of exposure. Figure 12 gives results of an early experiment in which very small fingerling bass were exposed to HCB for 15 days. During this first test, GC analysis of the commercial food revealed a concentration of 80 ppb HCB. Both the controls and experimentals, therefore, were exposed to HCB in their food. It is interesting to note that, in the controls, living in clean water and eating contaminated food, the highest concentration of HCB was found in the kidney. In contrast, the organisms living in a contaminated environment and eating contaminated food had the highest HCB concentration in the gut (the content of the lumen of the gut was not included in the analysis). The relatively high concentration in the kidney of both experimental and control animals may be correlated with its excretory function and the filtration of blood plasma, thus establishing a system which allows the hydrophobic HCB to partition into the lipid-rich components of the kidney cells.

Results of the second experiment are given in Figure 13. The relative importance of various tissue types in concentrating the compound is reasonably consistent with the previous experiment. Lower overall levels in the second experiment are due to larger average size (weight) of specimens and a shorter period of exposure. Concentration factors tend to be considerably higher in fish exposed to lower concentrations of HCB than those in higher test compound concentrations.

In these two experiments, bass showed the highest concentration of HCB in the gut, kidney and gills. After fifteen days' exposure to a mean concentration of 10 ppb HCB, these organs concentrated the compound to levels of 1811, 1441, and 748 µg/g (ppm) respectively. These are concentration factors of 181,100X, 144,100X and 74,800X.

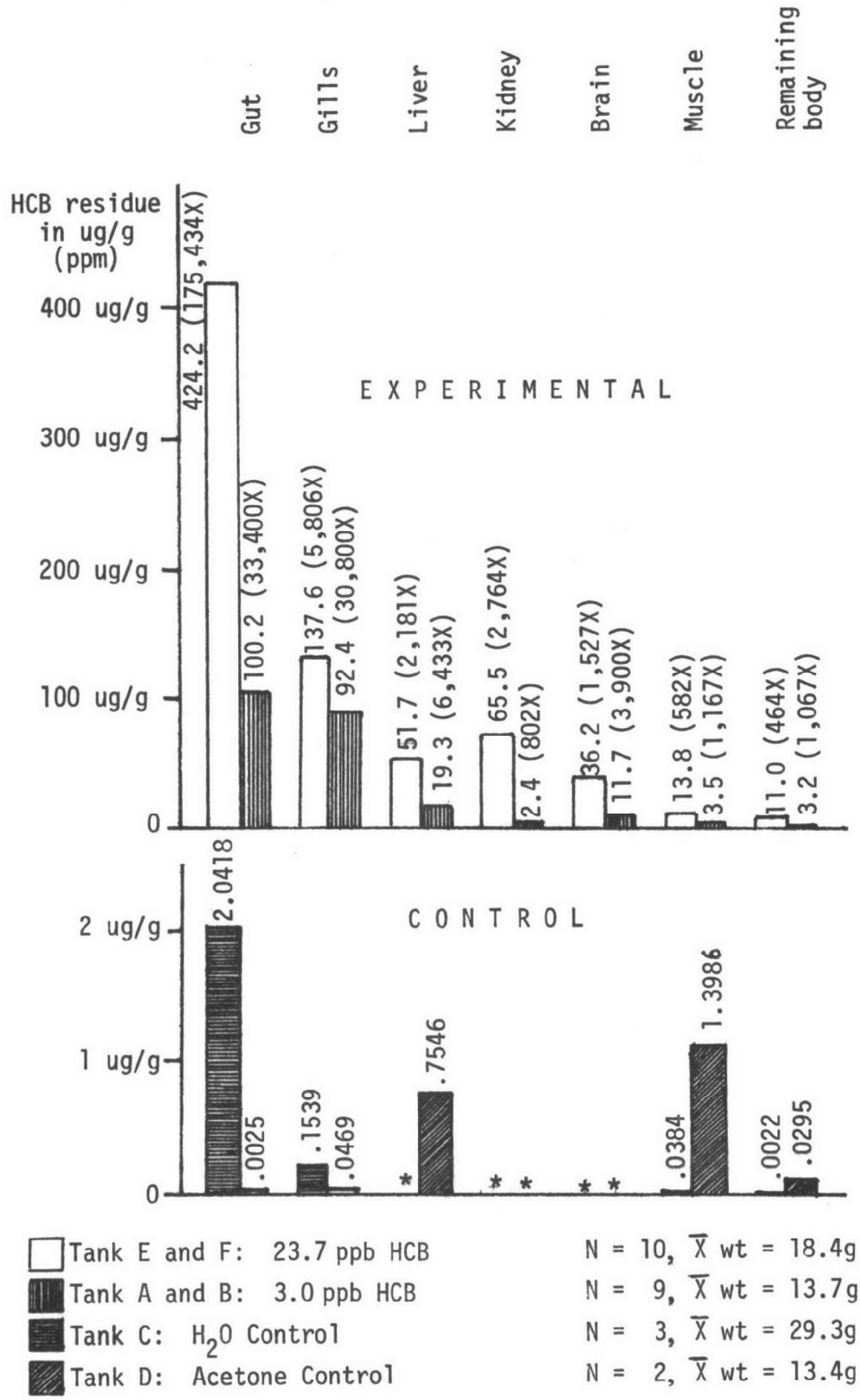
Figure 12. HCB concentrations in organ samples from bass exposed to HCB† for 15 days. Concentration factors in parentheses.



*Sample lost in processing
**Below detectable levels

† All specimens maintained on commercial fish food, containing 80 µg/Kg (ppb) HCB.

Figure 13. HCB concentrations in organ samples from bass exposed to HCB for 10 days. Concentration factors in parentheses.



*below detectable levels

5. Fish Blood Hematocrit

Percent hematocrit in blood is considered an effective means of estimating red blood cell counts in an organism. In the present study, three experiments with three different fish species were carried out in an effort to find differences in hematocrit between experimentals and controls, which could be interpreted as a stressful effect of HCB.

In the first experiment, a total of 24 bass was exposed to control conditions and HCB concentrations up to 25.8 ppb for 10 days. In the second experiment, 6 sunfish (Lepomis macrochirus) were exposed to 2.7 ppb HCB and 6 were held in HCB-free water for 10 days. The third experiment used a total of 75 Gulf killifish, maintained in control conditions and 5.7 ppb HCB for 10 days. In all of the above experiments the individual variation and mean values did not indicate an effect upon hematocrit by HCB at the concentrations tested.

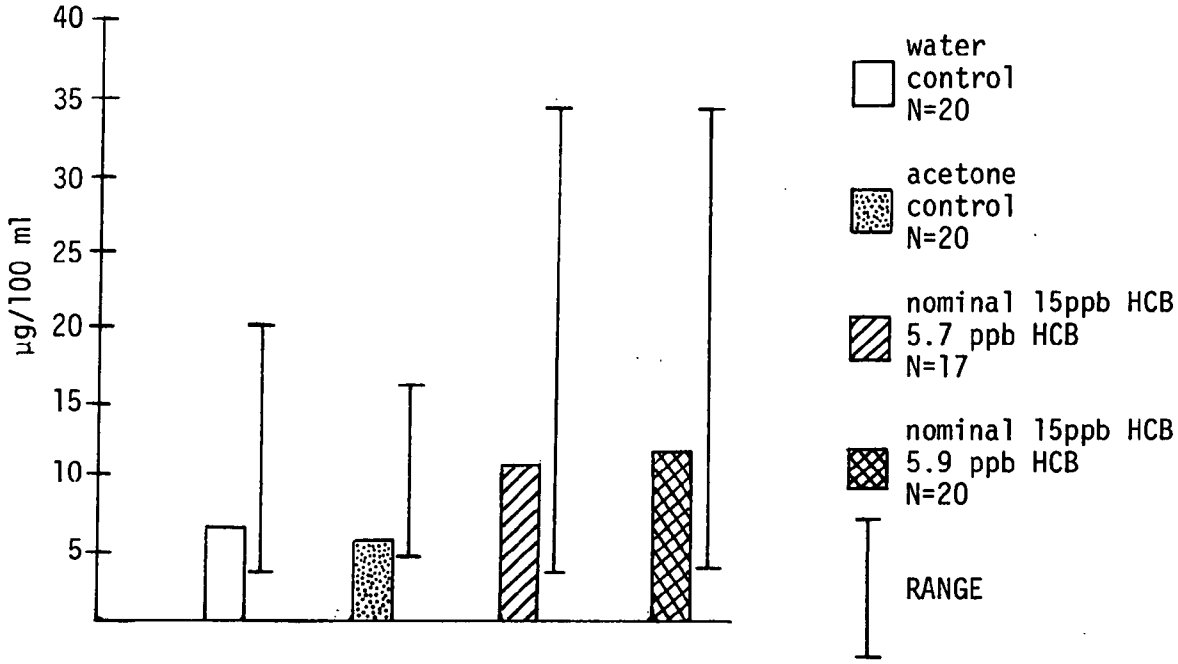
6. Fish Corticosteriod Level

Three experiments were carried out to observe possible cortisol level changes in fish blood as a response to stress imposed by exposure to HCB. In the first two experiments, bass were exposed to HCB for 10 days at the nominal concentrations of 1 and 10 ppb. In neither case did a competitive protein-binding radioassay of plasma cortisol levels show a significant difference between the means of the experimental and control groups. These results were rendered inconclusive for the following reasons: (1) the naturally high variability of any physiological parameter (here plasma cortisol level), (2) small sample sizes necessitated by the limited availability of bass, (3) the tendency to excitability of a predator species, accentuated by capture, (4) inability to match animals by size with consequent variation in cortisol level due to age and not stress endured.

When bass became unavailable from the hatchery because of the season, the Gulf killifish (Fundulus grandis) was substituted in order to continue studies of possible toxic (stressful) effects of HCB. This animal was chosen because it is readily available in the numbers required for good statistical analysis and also because it is a far more docile fish which is less excited by the process of capture. By minimizing stress of capture, matching the fish closely with regard to size and employing far greater numbers than was possible with the bass, extraneous variations in plasma cortisol determinations were eliminated and a significant stressful effect of HCB was found.

Figure 14 shows the results of an assay of plasma corticosteroid levels (µg/100 ml) in the Gulf killifish. Statistical analysis has shown that the control groups have significantly lower plasma cortisol levels than do the experimental groups, indicating a significant stressful effect of HCB.

Figure 14. Mean plasma cortisol levels (µg/100ml) in the Gulf killifish (Fundulus grandis) exposed to HCB for ten days.



An analysis of variance (F-test) was performed which showed that the experimental groups do not differ significantly from the controls at the 5% level, but do differ significantly at the 6.5% level. Statistical analysis (F-test) of control groups indicated that they were not different from each other. The same results were found between experimental groups.

D. Accumulation and Clearance

It is an established fact that many contaminants accumulate at different levels in different abiotic substances and in organisms at various trophic levels in an ecosystem. Biomagnification has been observed numerous times. With this in mind, the present study was designed to analyze HCB levels from several parts of the natural system. Levels of accumulation were observed in field samples, and laboratory tests were carried out for comparative purposes and to establish rates of accumulation. Components of the simulated ecosystem included sediment, algae, crayfish and fish. The algae, Oedogonium, is a genus found at the contaminated study site, and serves as food for such grazers as snails (Physa), crayfish (Procambarus) mollies (Poecilia latipinna), and mosquitofish (Gambusia affinis). These invertebrates and fishes are common food items of larger predators, including sunfish (Lepomis) and bass (Micropterus). Several organisms named above were used in a number of laboratory experiments discussed here, in an effort to increase available knowledge of accumulation of HCB in the food chain.

1. Crayfish

In an experiment to determine rates of uptake and depuration of HCB by crayfish, 32 test animals were held in each of two experimental diluter tanks. Measured HCB concentrations were 27.3 ppb (µg/ℓ) and 36.1 ppb, respectively. The controls were 32 crayfish in acetone in water and 32 others in uncontaminated water. A low level of contamination was measured in both control systems (.03 ppb and .005 ppb, respectively).

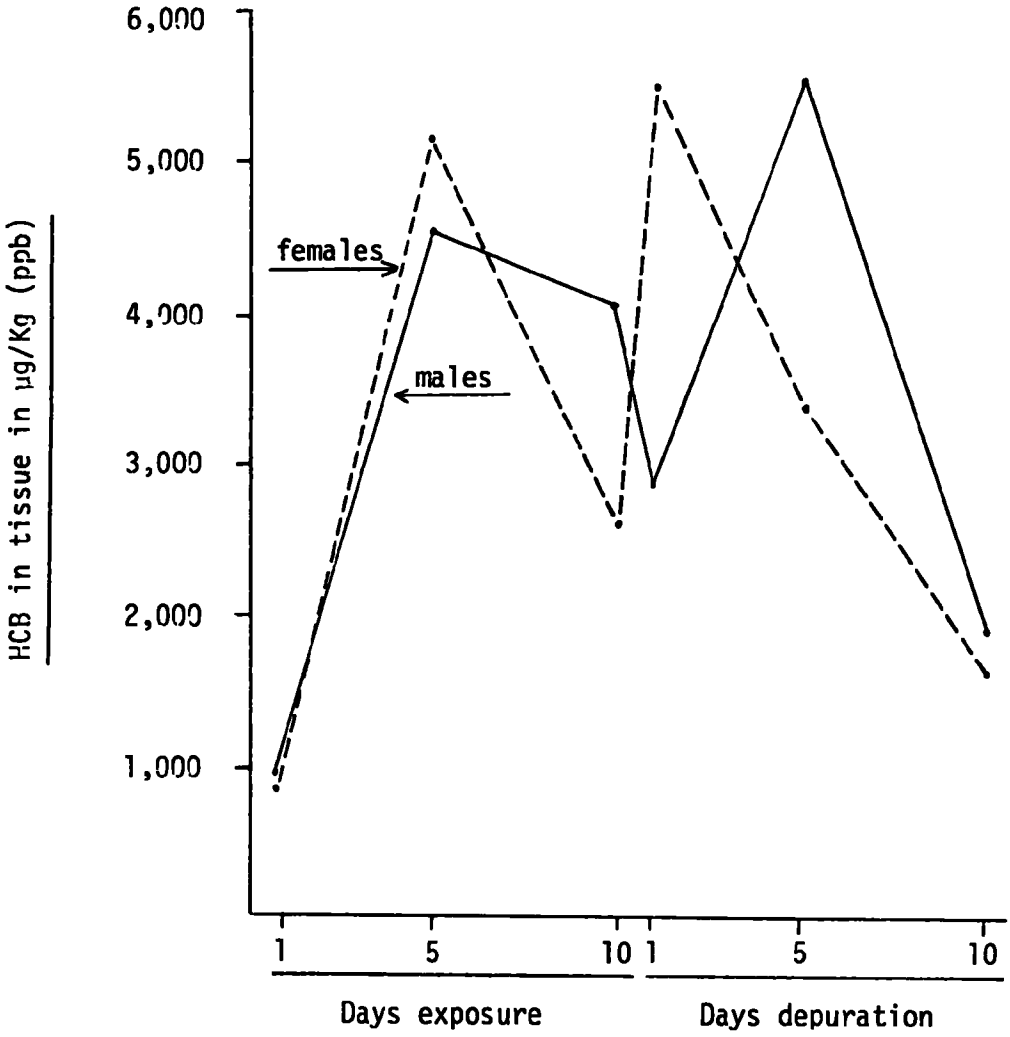
Two males and two females were removed from each tank following 1, 5 and 10 days of exposure and 1, 5 and 10 days of depuration. Whole bodies of males and females were processed and analyzed separately for each removal date. Data from this experiment are presented in Table 7 and Figure 15.

Table 7 Whole-body HCB residue concentrations in µg/Kg (ppb) in male and female crayfish (Procambarus clarki)† during uptake and depuration.

		Days exposure			Days depuration		
		1	5	10	1	5	10
Tank A 27.3 ppb (µg/ℓ) HCB	Males	657.	3,164.	4,056.	2,675.	6,638.	2,347.
	Females	824.	5,467.	2,277.	5,802.	2,219.	1,864.
Tank B 36.1 ppb (µg/ℓ) HCB	Males	1,287.	5,754.	4,038.	3,134.	4,319.	1,483.
	Females	871.	4,810.	2,717.	5,115.	4,484.	1,523.
Tank C .005 ppb (µg/ℓ) HCB H_2O Control	Males	33.	12.	*	11.	32.	38.
	Females	101.	27.	15.	77.	15.	18.
Tank D .03 ppb (µg/ℓ) HCB Acetone Control	Males	21.	17.	29.	13.	20.	*
	Females	*	13.	17.	26.	5.	4.

†: two specimens per data point.
*: below detectable levels.

Figure 15 . Uptake and depuration of HCB in crayfish (Procambarus clarki) exposed to a mean concentration of 31.7 ppb (µg/ℓ). *



*: Average values from Table 7 ; Four specimens per data point.

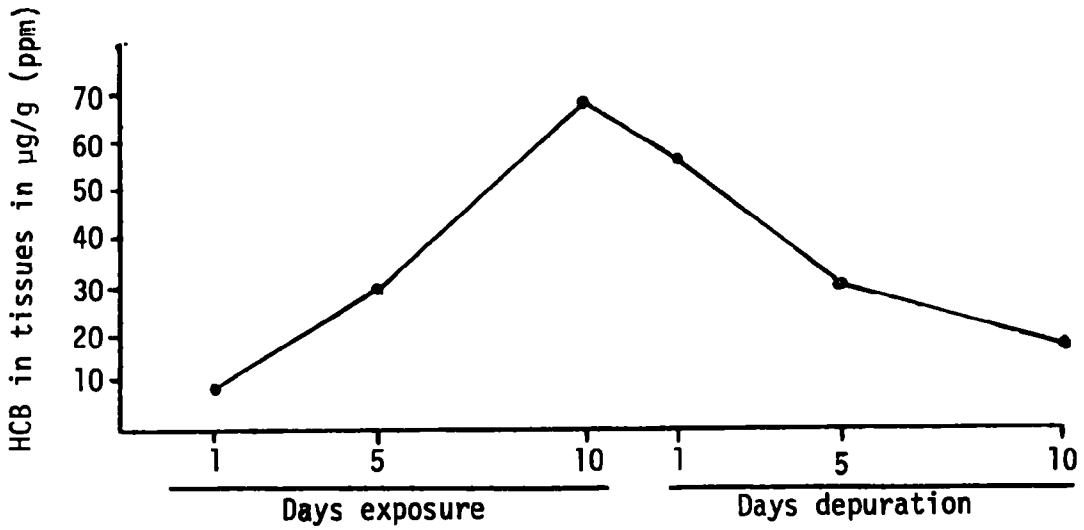
Accumulated levels reached a high point after five days' exposure and tended to show no further increase. Males had an average HCB level of 4,460 µg/Kg (ppb), a maximum concentration factor of 141X. For females, which were measured at 5,140 µg/Kg, a maximum concentration factor was 162X. After 10 days of depuration the factors had been reduced to 60X for males and 53X for females.

2. Mollies

Two static experiments and three diluter experiments were carried out with mollies challenged with HCB. Results from two flow-through experiments are reported here. In each protocol, a set number of male and female sailfin mollies were placed in each test tank. Duplicate tanks received the same concentration of HCB. Groups of three fish per tank were removed at intervals given in the figures below. GC analyses of these groups from individual tanks were done separately and the results combined to form a mean used in tabulation. Data available in this work did not suggest that one sex accumulated a greater proportion of compound than the other. The experiments were carried out well after the season's young had been born.

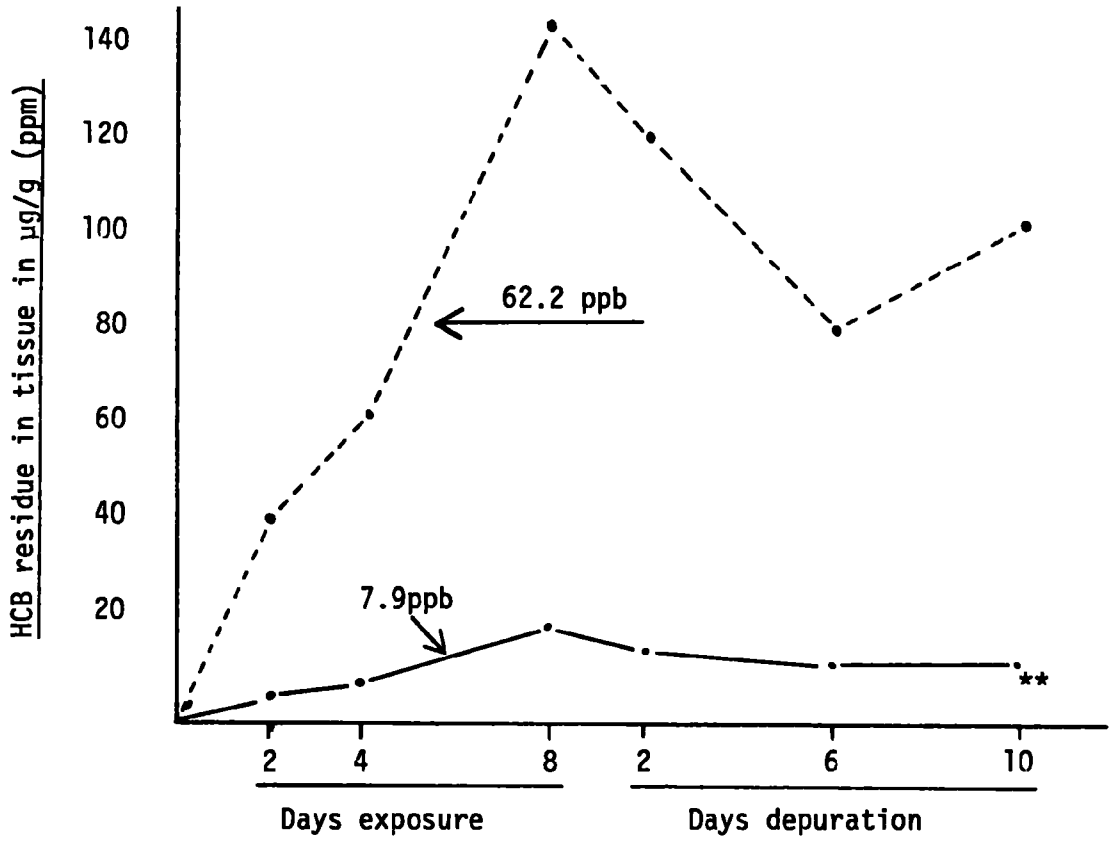
Results of these two experiments are given in Figures 16 and 17. A steady uptake and subsequent loss of HCB are indicated in most of these figures, with the highest concentration factor occurring after eight days' exposure to 62.2 ppb (µg/ℓ), at which point the HCB residue concentration reached 149.1 µg/g; a level 2,397 times that measured in water. The concentration factor for mollies exposed to 7.9 ppb for the same period of time was very close to this, 2241X. The concentration measured in this group was 17.7 µg/g.

Figure 16. Uptake and depuration of HCB in sailfin mollies* (Poecilia latipinna) exposed to 55 ppb (µg/ℓ) HCB.



*: Four males and two females per data point; two separate GC analyses

Figure 17. Uptake and depuration of HCB in sailfin mollies* (Poecilia latipinna) exposed to 7.9 and 62.2 ppb (µg/ℓ) HCB.



* Three males and three females per data point; Two separate GC analyses

** One GC analysis of three females

3. Bass

Rate of HCB uptake and depuration was studied in an experiment with fingerling bass (6-10cm, total length). HCB concentrations in the four experimental diluter tanks were 9, 10, 2 and 2 ppb (µg/ℓ). Twenty fish were placed in each tank at the outset. Three randomly selected animals per aquarium were removed and frozen individually following 5, 9 and 15 days' exposure and after 4, 9 and 13 days' depuration.

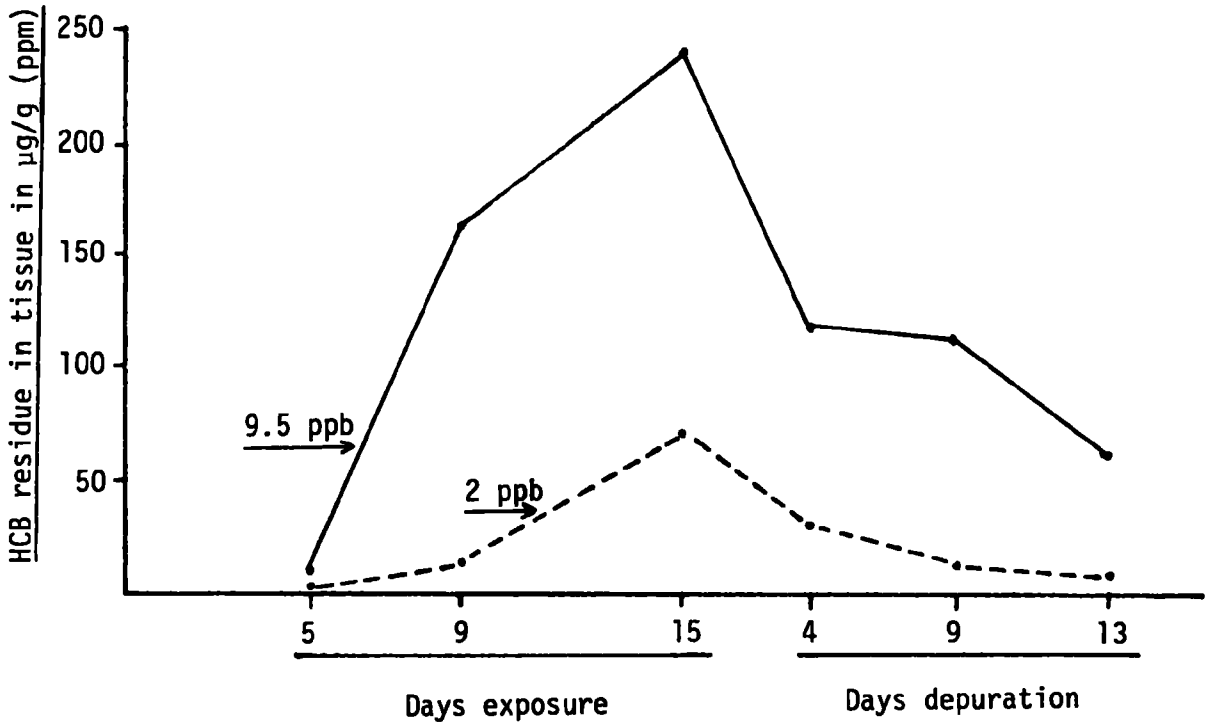
Results of this experiment are shown in Table 8. There was a direct correlation between the length of the period of exposure to HCB and the concentration of HCB in tissue. Highest levels attained in whole body extracts reflect accumulation of more than 44,000 times the concentration measured by GC in test water. These maximum levels were found in fish from all groups, sacrificed on the fifteenth and last day of exposure. No apparent correlations between size of fish and concentration factors were noted in this experiment. Levels of HCB in whole body extracts decreased fairly regularly during depuration, with 8.6 to 26.9% of the residue remaining following 13 days' exposure to HCB-free water (Fig. 18). During this time period, fish initially exposed to lower levels of HCB had succeeded in eliminating a greater proportion of the substance than had those exposed to higher levels.

Table 8. Whole-body HCB residue concentrations in largemouth bass (Micropterus salmoides) during uptake and depuration. Number of fish analyzed in parentheses.

		Days exposure			Days depuration		
		5	9	15	4	9	13
Tank A 9 ppb HCB	HCB (µg/g) in tissue	6.12	160.96	163.92	124.14	54.61	42.42
	Mean wt in g	2.6(4)	2.9(3)	3.9(3)	3.3(2)	4.8(2)	3.3(3)
	Concentration factors	680x	17,885x	18,214x	75.7%[a]	33.3%	25.8%
Tank B 10 ppb HCB	HCB (µg/g) in tissue	17.49	171.49	325.13	116.09	177.14	87.54
	Mean wt in g	.9(4)	.9(3)	1.2(3)	1.4(2)	1.8(2)	2.5(1)
	Concentration factors	1,749x	17,149x	32,513x	35.7%	54.5%	26.9%
Tank C 2 ppb HCB	HCB (µg/g) in tissue	2.72	14.40	88.88	20.14	8.46	7.62
	Mean wt in g	2.4(4)	2.9(3)	4.2(3)	3.2(2)	3.9(2)	3.2(2)
	Concentration factors	1,043x	7,198x	44,437x	22.7%	9.5%	8.6%
Tank D 2 ppb HCB	HCB (µg/g) in tissue	1.00	16.34	67.65	41.00	20.61	13.90
	Mean wt in g	.9(4)	.9(3)	1.3(3)	1.4(2)	1.6(2)	1.5(2)
	Concentration factors	498x	8,170x	33,824x	60.6%	30.5%	20.5%

[a] Percent remaining of maximum measured level.

Figure 18 . Uptake and depuration of HCB in bass (Micropterus salmoides) exposed to 2 ppb and 9.5 ppb (µg/ℓ). Average values from Table 8.



4. Algae

In flow-through experiments with green filamentous algae (Oedogonium cardiacum), a lower rate of accumulation was observed in comparison with that of animals. Results of one such experiment are given in Table 9.

Table 9 . Concentration and depuration of HCB by green alga, Oedogonium cardiacum exposed to a flowing solution of 11.5 ppb (µg/ℓ) HCB in water.

Days exposure	HCB in µg/Kg (ppb) wet wt	Concentration factor
1	1,973.	172x
3	3,472.	302x
7	7,161.	623x
Days depuration		
4	6,314.	
8	682.	

5. Bottom sediment

Results of a brief experiment with sediment showed soil accumulated proportionately less HCB than did organisms, but retained it longer. Following one day of exposure to a regular flow of 8.3 ppb (µg/ℓ) HCB, a soil sample contained a concentration of 332 µg/Kg (332 ppb) HCB, a concentration factor of 40x. Four days after initiation of the test, soil contained 269 µg/Kg HCB, a concentration factor of 32x. Depuration began on the fourth day. After four days of depuration the sample of sediment removed and analyzed on GC still contained 303 µg/Kg HCB, a level nearly as high as that of the first day's sample.

6. Effect of Food Chain

An experiment was carried out to determine the difference in HCB uptake between bass fed HCB-contaminated mollies in a clean environment and others fed similar mollies in an HCB-contaminated environment. Mollies were exposed to HCB until a sample tested contained a substantial amount of the substance; in this instance, 64 µg/g (ppm). The entire test with bass lasted 8 days, during which time numerous attempts to feed the bass were made under constant observation. Results of the test are included in Table 10.

Table 10. HCB concentrations in largemouth bass (Micropterus salmoides)* feeding on contaminated sailfin mollies (Poecilia latipinna).

Bass held in 37.6 ppb HCB				Bass held in HCB-free water			
Bass spec. #	wt in g	# mollies eaten	HCB in µg/g	Bass spec. #	wt in g	# mollies eaten	HCB in µg/g
1	10.24	4	95.24	1	8.58	5	3.33
2	13.56	5	78.53	2	10.59	0	0.43*
3	18.28	4	56.96	3	16.33	5	3.42

*The diet initially fed these test animals contained .08 µg/g HCB.

These data illustrate the effect size has upon concentration factors of fish in a contaminated medium. The smallest bass in the HCB-contaminated water of Table 10 had an HCB concentration factor of 2,533x, while the largest had a comparable value of 1,515x. Bass accumulated a much greater proportion of the compound from the ambient water than from ingestion of HCB included in mollies.

Since a body is not likely to retain all of the compound ingested, calculations were made to determine the approximate proportion excreted or otherwise eliminated by the bass.

Of the approximately 106 µg of HCB estimated to have been contained in five mollies and taken in by bass #1 in clean water, approximately 25 µg remaining in the whole body of the bass can be attributed to this source, indicating that one-fourth of the HCB ingested was retained. A longer period of ad libitum feeding would be expected to have given a greater concentration of HCB through ingested contaminated food fish.

The seasonality of bass juveniles made it impossible to repeat this test with a larger number of the same species. However, two groups of sunfish, Lepomis macrochirus, also in the family Centrarchidae, were tested in a similar way. In this experiment, HCB concentration in the contaminated tank was lowered to a mean level of 2.7 ppb, and the six sunfish in each tank were fed contaminated mollies twice daily ad libitum. Mollies contained approximately 16 µg/g HCB. It was ascertained that all fish had eaten daily for the seven days of feeding. All fish were fasted for one day prior to sacrifice to eliminate remains of solid food from the gut. Results of this experiment are given in Table 11.

Table 11 HCB concentrations in sunfish (Lepomis macrochirus) feeding on contaminated mollies (Poecilia latipinna).

Sunfish held in 2.7 ppb (µg/ℓ) HCB			Sunfish held in HCB-free water		
Sunfish spec. #	wt in g	HCB in µg/Kg	Sunfish spec. #	wt in g	HCB in µg/Kg
1	21.0	4,248.	1	17.0	576.
2	24.6	3,965.	2	26.2	665.
3	27.2	4,782.	3	26.3	799.
4	32.2	3,186.	4	28.7	504.
5	38.1	3,084.	5	31.6	780.
6	42.4	2,205.	6	51.4	258.

The mean concentration of HCB in the two groups is 3,578. and 594. µg/Kg respectively. For an experiment of this duration, it appears that HCB in water contributes a greater proportion of HCB to sunfish than does HCB taken in with contaminated food, since sunfish in control water accumulated less than 800 µg/Kg HCB through contaminated food while those in HCB-contaminated water had levels of 2,200 to 4,700 µg/Kg HCB. Sunfish ate approximately 7 grams of mollies which would have contributed a maximum of 112 µg of HCB to each of them. Assuming ingestion of 112µg of HCB, a 30 g sunfish would have contained 3,733 µg HCB per Kg body weight, if all HCB were retained. Since HCB content in sunfish of this size was approximately 640 µg/Kg, it appears that sunfish assimilated approximately 6% of the HCB from food taken in. Specimens in the contaminated tank show an inverse relationship between fish size (weight) and concentration of HCB.

7. Crayfish uptake of HCB in Field Environment

A series of field studies was carried out to demonstrate uptake of HCB in clean crayfish (Procambarus clarki) under contaminated field conditions at the Geismar site. In the first experiment, crayfish were placed in cages and submerged in the pond adjacent to the hex landfill. HCB levels in water measured 74.9 ppb and in mud were 29.08 ppm. Animals had access to the mud bottom.

Following 10 days' exposure all specimens were removed from the site. Six were frozen immediately for analysis and the remainder were transferred to trays of clean water, which was changed regularly. These latter specimens were sacrificed periodically. Figure 19 shows mean levels of HCB measured in whole body extracts of crayfish, before and during depuration. Males usually weighed more than females, but females tended to accumulate and retain greater proportions of HCB.

At the end of the initial ten days' exposure whole bodies of males contained an average of 90 µg/g (ppm) HCB, which is a concentration factor of 1,200x the amount measured in water. Females contained 129.5 µg/g HCB; a concentration factor of 1,729x. Following 3 days of depuration, males retained 82% and females held 87% of initial HCB. At the end of 25 days, this figure was reduced to 53% in males and 36% in females.

A short-term experiment in the same waste pond consisted of introducing clean crayfish on one day and removing a sample at daily intervals for seven days. The increase in HCB levels of whole-body extracts is given in Figure 20.

Depuration rates following shorter-term exposures appear to be more rapid. Table 12 gives the mean HCB concentrations attained after one to six days' exposure, and the subsequent level reached at the end of one to nine days' depuration. In this abbreviated experiment, more than half of the samples lost more than 50% of their HCB content if depurated for a period of five to nine days.

Table 12. Short-term exposure and depuration of crayfish introduced into a contaminated environment (43.5 ppb HCB).

Days exposed	Mean HCB concentration in µg/g (ppm)	Days depuration	Mean HCB concentration in µg/g (ppm)
1	14.04	9	3.47
2	21.74	5	8.79
		6	5.60
		8	13.22
3	44.28	7	15.15
4	39.37	1	53.34
		4	33.13
		7	16.21
5	46.76	7	59.38
6	50.64	7	24.70

Figure 19. Levels of HCB in crayfish during depuration following 10 days' exposure to a contaminated environment (74.9 ppb HCB).

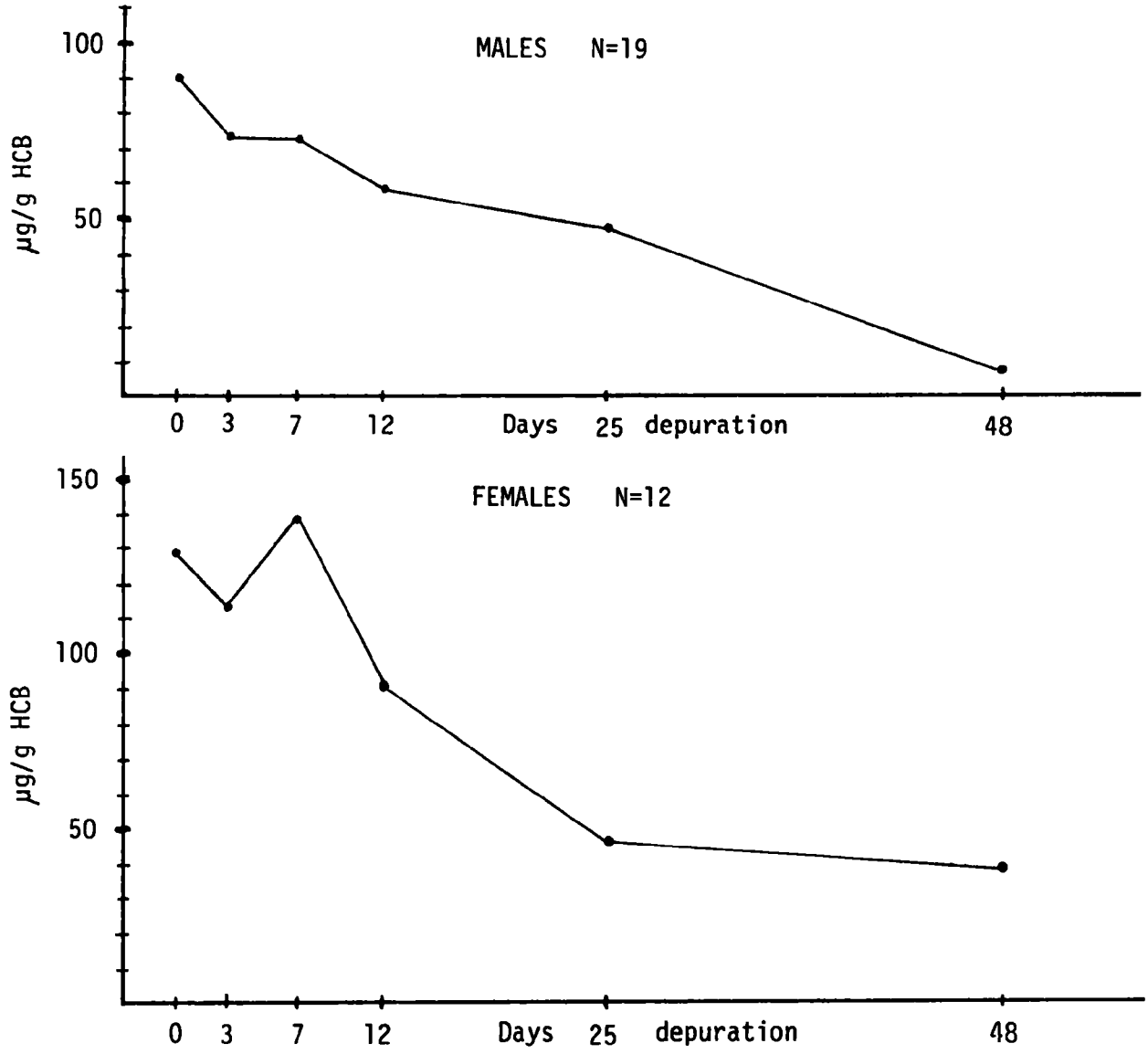
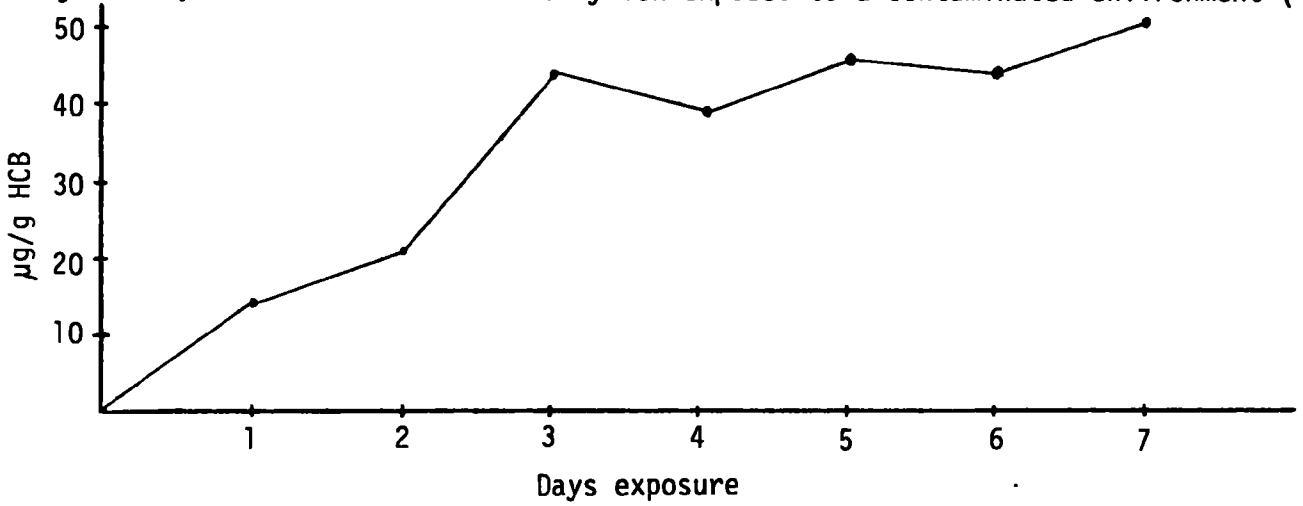


Figure 20. HCB concentrations in crayfish exposed to a contaminated environment (43.5 ppb)



E. Respirometry

1. Crayfish

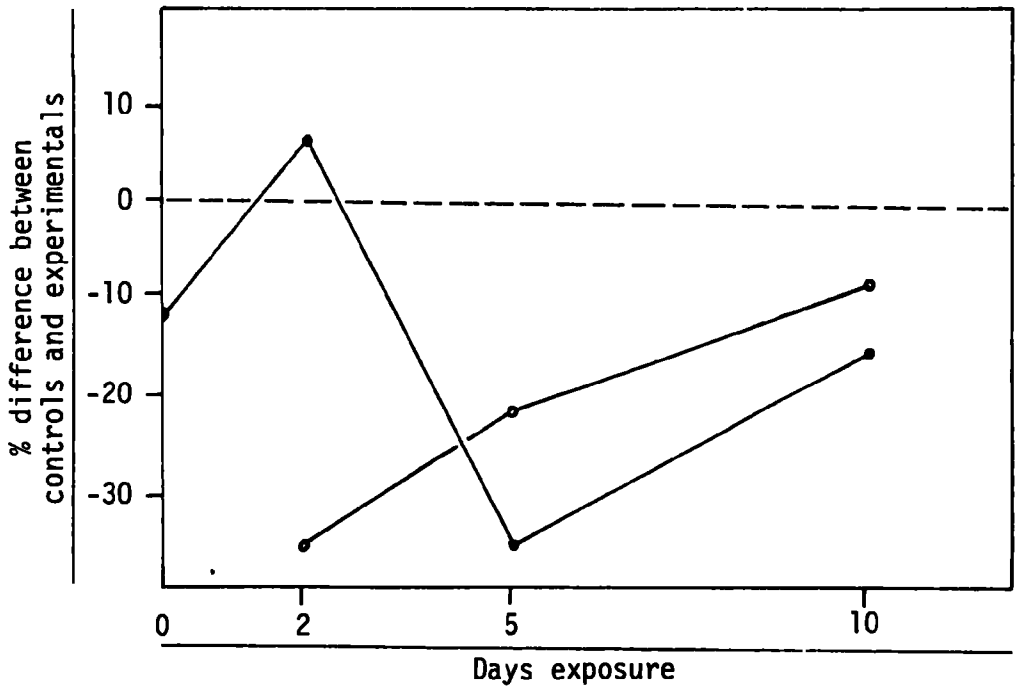
Exposure of juvenile crayfish to 1903 ppb HCB resulted in variable oxygen consumption patterns. The average oxygen consumption rates ($\mu\ell$ O_2/mg wt/ hr) for HCB-exposed and for control animals are shown in Table 13. Each value is based upon an average of 20 animals whose oxygen consumption rates were measured for a total time period of two hours. For Figure 21 the percentage deviations of the experimental groups from control groups are plotted against time (in days). Time "zero" represents the initial exposure period. By establishing the control values as a base line, any trends in the experimental values should become evident through this method of plotting the results.

There were no significant differences between experimentals and controls during the first three hours of exposure. Determinations on days 2, 4 and 10 gave variable results among repeated experiments. The trend, which was not consistent, indicated an initial decline in rate for the first four days followed by an increase to control levels after a period of ten days. These observations must be considered tentative.

Table 13 Effect of time of exposure to HCB on respiration of juvenile crayfish ($\mu\ell$ O_2/ mg/ hr).

Experiment #	Group	Duration of exposure (days)			
		0	2	5	10
1	Control	0.4425	0.2844	0.7078	0.5243
	Exp. (1903 ppb)	0.3874	0.2996	0.4470	0.4495
2	Control		0.4080	0.4984	0.5233
	Exp. (2437 ppb)		0.2600	0.3942	0.4812

Figure 21. The effect of time of exposure to HCB (1903 ppb, • ; 2437 ppb, ○) on respiration rate of juvenile crayfish (Procambarus clarki).



2. Mollies

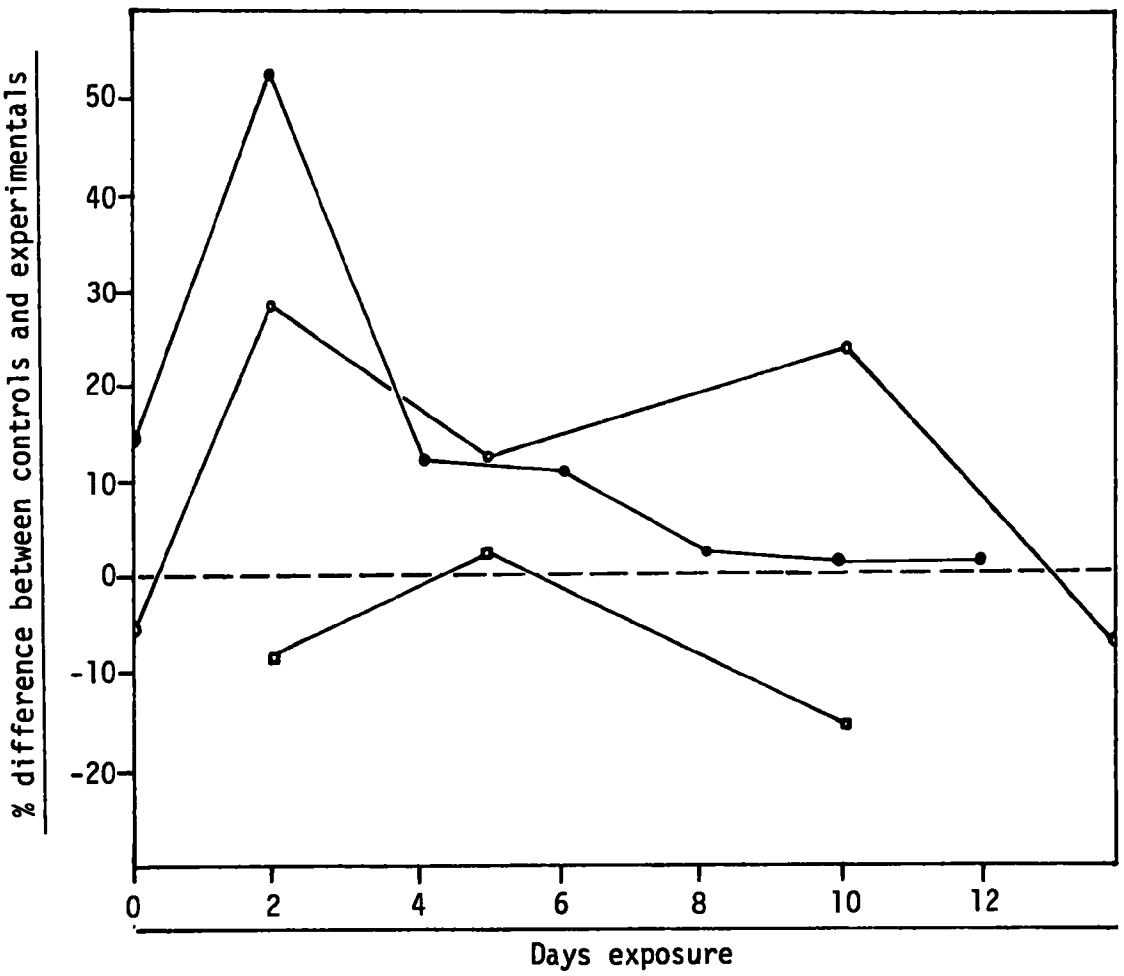
Three sets of experiments were carried out to determine the possible effect of HCB upon oxygen consumption in mollies (Poecilia latipinna). The average oxygen consumption rates ($\mu\ell$ O_2/mg wt/ hr) for HCB-exposed and for control animals are shown in Table 14. Each value is based upon an average of 4 animals whose oxygen consumption rates were measured for a total time period of one hour. In Figure 22 the percentage deviations of the experimental groups from control groups are plotted against time (in days). Time "zero" represents the initial exposure period. By establishing the control values as a base line, any trends in the experimental values should become evident through this method of plotting the results.

Juvenile mollies exposed to 2,783 ppb HCB showed no significant change in respiration rate initially (first 3 hours) during exposure. Rates were compared in three sets of experiments at 2, 4, 8 and 12 days of exposure. Differences as great as 50% increase of experimentals over controls were observed. The patterns resulting from each experiment were not clearly repeatable and therefore definitive conclusions based upon the present data are not possible. Both increased and decreased rates in experimentals were observed. As a preliminary pattern there was a tendency for rates in experimentals to rise between the first and fourth days and to decline to control levels around days ten to twelve.

Table 14. Effect of time of exposure to HCB on respiration rate of juvenile mollies ($\mu\ell$ O_2/mg/ hr).

Experiment #	Group	Duration of exposure (in days)								
		0	2	4	5	6	8	10	12	14
1	Control		0.5397		0.4704			0.3577		
	Exp. (2783 ppb)		0.4919		0.4789			0.2935		
2	Control	0.3308	0.3314		0.3568			0.3568		0.3685
	Exp. (2783 ppb)	0.3130	0.4282		0.3619			0.4404		0.3383
3	Control	0.3244	0.2654	0.2272		0.4088	0.2859	0.2596	0.2391	
	Exp. (1587 ppb)	0.3578	0.3742	0.2548		0.4524	0.2915	0.2622	0.2429	

Figure 22 The effect of time of exposure to HCB (2783 ppb, • and ∘; 1587 ppb, ▫) on respiration rate in juvenile mollies (Poecilia latipinna).



F. Metabolic Fate

Autoradiograms of $HC^{14}B$ extracts run on thin layer plates were developed and showed only one labelled component in the following samples: feces, stomach and intestines, liver, and remaining body. Since the label from each of these extracts migrated on the chromatograms with the same relative mobility as $HC^{14}B$ standards, there was no indication that HCB had been metabolized. Had metabolites been produced, thin layer chromatography/autoradiography may have shown more than one labelled spot. The relative mobility of HCB in this experiment was 0.91.

Extracts of kidney, the only other organ studied in this experiment, contained low radioactivity of only twice the background level as measured by liquid scintillation counting. Autoradiographs of chromatograms of kidney extract did not develop spots even after two months of exposure because of the low level of radioactivity applied to the origin.

The relative distribution of labelled HCB in the extracts as determined by liquid scintillation counting is shown in Table 15.

Table 15 . Activity (cpm/g) of extracts of bass (Micropterus salmoides) fed C^{14}-HCB

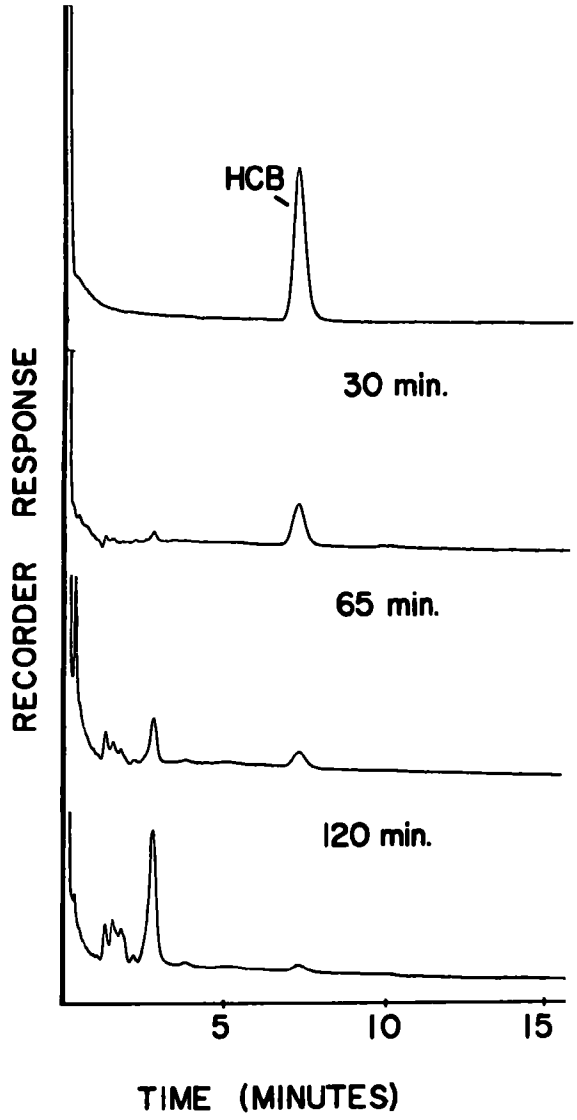
Sample	BASS #1	BASS #2
feces	10,677	5,951
gut	1.494	1,840
liver	253	165
kidney	160	135
remaining body	44	*

* sample lost in processing

G. Photochemistry

Irradiation at a wavelength of 2735 Å causes the disappearance of HCB. After 60 minutes of irradiation in benzene, less than 10% of the original HCB remained. Lower molecular weight compounds appear after 30 minutes of irradiation and continue to increase proportionately with time of exposure. GC traces of resultant products taken at intervals are shown in Figure 23.

Figure 23. Gas chromatographic separation of photoproducts resulting from ultraviolet irradiation of HCB.



VI. DISCUSSION OF RESULTS

Analytical support for the studies contained in this report included GC analyses of more than 1,300 separate water, soil and organism samples. Laboratory specimens accounted for more than 1,000 of these prepared extracts, while the remaining analyses included samples collected during more than 40 field trips.

Results of GC analysis of HCB extracts from samples taken from the environment in southeastern Louisiana revealed a consistent association of HCB with the Mississippi River. The principal source of contamination is very likely to be petrochemical industries that border the river. Three major areas having considereble HCB concentrations are immediately north of Baton Rouge, Addis and Plaquemine on the west bank and a stretch of river between Ashland Plantation and Darrow along the east bank.

Samples taken at the most contaminated sites on property of the Vulcan Materials Company in Geismar had substantially more HCB residues than any taken in the regional survey. Mud samples at Geismar had as much as 45 times the highest level found elsewhere during the Mississippi River survey. In water samples from the river survey, HCB contamination rarely exceeded 2 µg/ℓ (ppb), but at Geismar, levels up to 30 times this concentration were found at a site where wastes were being properly disposed. A pond, less than 500 meters from the disposal site, called the Recreation Pond, had very low levels of HCB, exceeding 2 ppb on only one occasion. The highest concentration factor was found in a naturally-occurring organisms at this site, however. The snail, Physa, was found to have a concentration of 561 µg/Kg (ppb); a concentration factor of nearly 7000X at the time of water sampling. The mosquitofish, Gambusia, had a mean of 3,291 µg/Kg HCB residues, which in terms of HCB-content in water at the sampling times, was a concentration factor of 2,789X. These results are consistent with data from a model ecosystem study by Metcalf et al. (1973) in which Physa had accumulated 4,099 µg/Kg HCB, a level proportionately higher than the 3,154 µg/Kg found in mosquitofish at the same exposure of 6.44 µg/ℓ (ppb). A comparison between mosquitofish from the Baton Rouge site and the Recreation Pond at Geismar reveals an order of magnitude higher HCB level in fish from the latter site, while HCB levels in water were close to the same for the two sites. This is probably a function of the static nature of the Geismar pond in comparison with the dynamic situation in the Mississippi River. Fish in the river are probably not exposed to a constant dosage of HCB.

Injections of HCB in oil were not lethal to animals in this study. Dosages were given to a maximum of 125 µg/g body weight (125 ppm). Since the injected solution was saturated with HCB, the only means to increase dosage would have been to increase volume injected, which, in the case of our specimens might have resulted in physical damage due to the volume entering the organisms. HCB did not produce acutely toxic results in experiments reported by Davis et al. (1959). In those tests, rats were injected intraperitoneally with 500 µg/g (ppm) HCB which was a fourfold multiple of concentration used in the experiments reported here. Parke and Williams (1960) noted that much of the HCB injected subcutaneously remained near the injection site. We did not analyze the distribution in our organisms following injection.

No HCB toxicity studies have been published to date using bass as the experimental animal. However, in an experiment with pinfish (Lagodon rhomboides) in a sea-water system, Parrish et al. (1975) reported a maximum concentration factor of HCB in muscle of 34,000 times the tank level (maintained at 5.2 µg HCB/ℓ for 42 days). Following 14 days' exposure to a mean concentration of 1.87 µg/ℓ , they measured 63.5 µg/g (ppm) of HCB in liver tissue, 36.9 µg/g in muscle and 67.4 µg/g in the entire remaining bodies. At the end of 15 days' exposure to 2 µg/ℓ HCB in our experiments, the average concentrations for each tissue were: liver, 535; muscle, 11.8; and remaining body, 39.5 µg/g.

In general, the highest concentrations were found in the gut, kidney and gills. Each of these organs has an abundant supply of blood and relatively high metabolic rate. Since HCB is lipophyllic, it would be expected to partition from the blood into the hydrophobic lipid-rich cell membranes of these organs. This transfer would result in the passive accumulation by the tissues. The higher concentrations in the brain as compared with muscle tissue may be related to the relatively high lipid content of the brain. The accumulation of HCB in these essential organs, the gut, kidney, gills, liver and brain would suggest that if HCB is a toxic substance, these tissues may be the ones most adversely affected.

Reference to examination of crayfish or largemouth bass tissues following exposure to HCB has not been seen in the literature. The present findings of tissue damage to liver of fingerling bass exposed to 3.5 ppb HCB add to those findings thus far noted in HCB-exposed rats (Ockner and Schmid, 1961 and Medline et al. 1973) and Japanese quail (Vos et al., 1971). Hepatomegaly reported for man and rat (Ockner and Schmid, 1961), and the quail (Vos et al., 1971), was not observed in the bass in which the liver weight relative to body weight remained within normal range in those animals utilized for histological examination. Kidney damage was noted in bass following HCB exposure (3.5 ppb) and has been reported also in quail (Vos et al., 1971). Gall bladder necrosis, severe in the fingerling bass after 10 days' exposure to 25.8 ppb HCB, has not been mentioned in the literature relative to this or other species exposed to this pesticide.

Although to date there are relatively few animals in each category that have been submitted to a thorough histological scanning, the findings are so consistent that continued examination of the accumulated tissue blocks is warranted. Furthermore, repetition of diluter exposure experiments and subsequent depuration with attention to obtaining additional histological sampling of those organs now known to be affected would lend further significance to the findings. Also pertinent in such a repeat run would be histological examination of liver, gall bladder and kidney after depuration to determine the extent of organ recovery.

In addition to histological preparations of bass tissue, a considerable volume of material has been prepared from a second fish species, the sailfin molly (Poecilia latipinna). Mollies were used in several experiments during the present study, and substantial information on their response to the test compounds has been included in this report. Observations of gross pathology of the liver and gall bladder have been noted and warrant histological examination of the accumulated material.

Useful correlations can be made between histological observations of tissue changes and the concentration of a substance in the various tissues in chronically-exposed bass. As a part of a metabolic fate experiment, another means of determining distribution used radiolabeled HCB. Extracts made from tissues of bass exposed to a single acute oral dose of C^{14}HCB were analyzed with a scintillation counter.

The results of feeding experiments with C^{14}HCB (See Table 15, Section V F) provide a means of following the distribution of ingested HCB in bass living in clean water but given only one feeding with contaminated food. Our other studies (section V C4) were done either by exposing the animals to a contaminated environment for varying periods and then analyzing tissues for HCB content by gas chromatography or by feeding contaminated food in a clean environment. Under these latter conditions, bass showed very high concentrations of HCB in gut and kidney. These two tissues have abundant interphases which would allow the passive accumulation of HCB because of its lipid:water partition coefficient.

The C^{14}HCB study has slightly different results. Kidney tissue in both cases had one of the lowest activities measured, only the remaining body being less active in proportion to weight. It must be remembered, however, that the previous studies involved much longer term exposure and feeding (15 days) which would allow for much greater accumulation of HCB in an excretory organ. In this experiment the exposure was acute (only one feeding) rather than chronic and this may account for the difference observed in kidney tissue.

Values for feces were not obtained in previous studies. The results here show the highest concentration in feces after acute exposure indicate that much of the HCB is defecated soon after ingestion. This is in agreement with inferences made from data from food chain experiments, reported in Section V D6. Total HCB content of fish used as food for bass was compared with the final whole-body HCB content in bass sacrificed after feeding and digestion. It was concluded that three-fourths of the HCB ingested was lost through defecation by the predator. Mehendale (1975) noted an elimination of only 16% of a single oral dose of HCB in the feces of rats and 1% in urine. He reported that the majority of HCB was retained in fat tissue.

Parke and Williams (1960) stated that much of the ingested HCB remained in the gut of rabbits. Work with bass in the present study agrees with this observation (Section V F, Table 15). Since any solid waste remaining in the gut was removed prior to extraction, the high concentration of radiolabelled HCB in the gut indicates a high retention of HCB by the digestive system. Passive accumulation by partitioning of hydrophobic HCB into lipid phases seems the most likely mechanism. Liver showed more accumulation than kidney by weight but is still 5 to 10 times lower than the gut.

Tissue extracts from C^{14}HCB-fed bass discussed above were part of an experiment to determine whether or not HCB was being metabolized. Results of autoradiography of thin-layer chromatograms were negative, with only a single spot migrating at the same rate as HCB. Metcalf et al. (1973) reported a biodegradability index (BI) of .46 for HCB in mosquitofish in a model ecosystem study. They found little indication of products of degradation, but noted the presence of "highly polar materials and conjugates." They noted no metabolites of HCB in the saltmarsh caterpillar, Estigmene acrea.

In uptake and depuration experiments with bass during the present study, levels of HCB in whole body extracts decreased fairly linearly during depuration, with 8.6 to 26.9% of the residue remaining following 13 days' exposure to HCB-free water. This is a substantially higher rate of loss than that observed by Parrish et al. (1975), who reported maximum loss of 50% from tissue following 28 days' depuration. Their period of exposure (42 days) however, was nearly 3 times that of the present study. Time of exposure may be very important in relationship to time of depuration. Since HCB probably partitions passively in the organism, it may gradually accumulate in lipid deposits with slow turnover rates. Depuration of that HCB component might be much less rapid than the HCB in gut, liver and kidney.

The significance of contamination along the Mississippi River might be put in better perspective if HCB levels in fish from this area are compared with HCB levels in fish reported by authors working in other locations. Zitko (1971) reported .002 to .006 µg/g (ppm) HCB in herring from Nova Scotia and .006 to .019 µg/g in American eel from freshwater sites in New Brunswick. Johnson et al. (1974) reported .016 to .34 µg/g in white perch from New York and New Jersey. A high point of 62 µg/g was found in carp from near a source of industrial pollution, but most other fish samples from North America sources had less than .1 µg/g HCB in whole-body extracts (Johnson et al. 1974). In Australia, Best (1973) reported .01 µg/g HCB from fatty tissue of freshwater catfish. She noted 102 µg/g HCB in blacktip shark. In our laboratory, an analysis of ocean perch intended for feeding yielded .064 to .262 µg/g HCB (unpublished). The perch had been purchased locally, already cleaned and frozen.

Mosquitofish collected during our Mississippi River transect had a maximum level of .379 µg/g HCB. These specimens were from a site near Baton Rouge, immediately downstream from a heavily industrialized area. While this concentration is higher than most others reported above, the effect of size of specimens must not be overlooked. Mosquitofish weighed less than 3 grams each, while individuals of most species analyzed by other authors weighed more than 30g and usually exceeded 100 g. Observations in the present study supported Murphy's (1971) statement of an inverse correlation between fish size and capacity for concentration of test compound.

Experiments with crayfish in field and laboratory situations provided useful comparative information. Females accumulated more HCB than did males in both field and laboratory. Net concentration of HCB and concentration factors were much higher in the field than the laboratory.

HCB exposure appears to exert some physiological stress upon bass in the form of elevated plasma cortisol levels. Experimental groups differed significantly from controls at the 6.5% level (F-test). Further experimentation must be done to clearly establish the relationship between HCB exposure and corticosteriod level in the fish.

Oxygen consumption studies utilized the Gilson Differential Respirometer instead of a flow-through respirometer which we designed, because of several advantages which became evident during our experiments. During the season of the year when the flow-through system was completed we had available only small juvenile animals which could not be used in the flow-through respirometer. The Gilson Respirometer was more sensitive. Significant differences between control and experimental animals did not appear within 24 hours. The flow-through system was designed to measure oxygen consumption during periods up to 12 hours.

Bass did not survive in the confined chambers of the flow-through system. These circumstances, among others, made the Gilson unit a more useful instrument.

The results of respiration measurements both in mollies and crayfish did not produce repeatable patterns from one experiment to another. It must be concluded that, in the case of HCB, oxygen consumption data from whole animals was not particularly useful as an indication of chronic stress.

The data, presented as percentage variation of the experimental from control rates, in Figures 21 and 22, often indicated high percentage differences. HCB is a hydrophobic substance which is probably not metabolized or actively transported in the system; the distribution may be through passive partitioning through lipid phases in the organism. Any effects on respiration may be a result of the accumulation of HCB in membranes. Many enzymes depend upon the integrity of the membrane elements in the immediate vicinity of the enzyme (Coleman, 1973). Steric distortion of membranes by hydrophobic substances which partition into membranes may be an important and chronically cumulative problem in toxicology. The effects on a central metabolic process, such as oxidative phosyphorylation, may be complex. For example, if steric alteration of membranes in the vicinity of the electron transport system in mitochondria occurs, the transport system may be inhibited. Oxygen utilization would be decreased. If, on the other hand, oxidative phosphorylation were uncoupled from electron transport by the steric alteration, oxygen utilization would increase. The absence of rate changes upon initial exposure may reflect the time required for transfer into the organism. Variations in exposure time and concentrations of hydrophobic substances such as HCB for longer exposure periods may result in highly complex effects when the whole organism is studied. Respiration studies utilizing mitochondria and specific experiments utilizing membrane-bound enzymes may produce much more useful results. Such experiments are particularly recommended in view of the tissue alterations which we have reported in the present study.

An intensive investigation of cellular and subcellular damage in critical organs utilizing electron microscopy in combination with subcellular physiological and enzymatic studies would be an important direction in the toxicology of hydrophobic substances. Establishment of detail syndromes of toxicological effects which are legally defensible are much more likely at the subcellular level, than at the organismic level. It is expected that early warnings of toxic effects would first be observed at the levels of enzymatic activity and subcellular structure. We recommend this direction as potentially important.

Table 16 presents an overview of the critical results from this project. The table includes only those experimental results which clearly showed some alteration in experimental animals. From each of those experiments only the minimum concentration resulting in the effect is included in the table. Maximum observed concentration in the environment at Geismar and along the Mississippi River are included for comparison purposes.

Table 16. Minimum concentrations of HCB tested that resulted in an observed response in organisms.

Method	Lowest tested level (in ppb) at which changes were observed	Observations	Section containing discussion
a) Histology	5.	Heightened exfoliation of hepatopancreas epithelium in crayfish	V C1
	3.5	Liver and kidney necrosis, and gall bladder epithelium damage in bass	V C2
b) Cortisol level in blood plasma	6.	Cortisol level in blood elevated (significant at 6.5% level) in bass	V C6

Maximum HCB concentrations found in environmental survey

	Water	Mud *
Area of High Concentration (Geismar)	74.9 ppb (µg/ℓ)	53,130 (74,970) ppb (µg/Kg)
Overview	90.3 ppb (µg/ℓ) (Plaquemine)	870. (1,950) ppb (µg/Kg) (Darrow)

*: Figures corrected for dry weight in parentheses.

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16 ABSTRACT

Hexachlorobenzene (HCB) has been found in the environment in southeastern Louisiana in addition to other parts of the world. In this region it is a byproduct of the petrochemical industry. HCB is a fungicide and has been found to accumulate in fatty tissue of wild and domestic animals. It has had toxic effects upon humans. A number of cases of porphyria cutanea tarda were traced to ingestion of treated grain. In this study, soil, water and organism samples were collected periodically in 1974 and 1975 from sites in southeastern Louisiana, with emphasis along the Mississippi River and an industrial region of known contamination of HCB near Geismar, Louisiana. Maximum HCB concentrations in water from the two areas were 90.3 and 74.9 µg/ℓ (ppb). Maximum HCB concentrations in soil from the two areas were 874 and 53,130 µg/Kg (ppb). Laboratory experiments with the compound included acute toxicity studies in aquatic systems and through injection in fish and crayfish. Accumulation and depuration rates were determined and observations made with histological slides of tissue. Other potential measures of stress were made, including blood cortisol levels and oxygen uptake rate. HCB was not lethally toxic during our experiments, but its chronic effects were noted in histology and cortisol levels. Damage to kidney, liver and gall bladder in fish was observed and serum cortisol levels were elevated in response to exposure to HCB in ambient water. Highest levels of uptake were measured in gut, kidney and gills, attaining a maximum concentration factor of 181,000X in gut of bass.

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