Uptake kinetics and toxicity of diazinon in the American oyster, Crassostrea virginica Gmelin

Ruth L. Williams

College of William and Mary - Virginia Institute of Marine Science

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Uptake kinetics and toxicity of diazinon in the American oyster, 
*Crassostrea virginica* Gmelin

Williams, Ruth L., Ph.D.
The College of William and Mary, 1989

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UPTAKE KINETICS AND TOXICITY OF DIAZINON IN THE
AMERICAN OYSTER, CRASSOSTREA VIRGINICA GMELIN

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

by
Ruth L. Williams
1989
This dissertation is submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Ruth L. Williams

Morris H. Roberts, Jr., Ph.D.
Committee Chairman/Advisor

Michael E. Bender, Ph.D.

Robert J. Huggett, Ph.D.

Evon P. Ruzecki, Ph.D.

J. Ernest Warinner, III, M.A.

Dennis Burton, Ph.D.
Johns Hopkins University
Shady Side, Maryland

Approved, August 1989
DEDICATION

This dissertation is dedicated to the memory of my mother, Lillian M. Williams, with love. Thank you for everything.
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ACKNOWLEDGMENTS

I thank the members of my committee for their support throughout my research. In particular, thanks to my major professor, Dr. Morris H. Roberts, Jr., for his suggestions and to Mr. J. Ernest Warinner for his assistance with radiometric techniques and his constructive review of this document. I am also grateful to Dr. Fu-Lin Chu and Kathleen Greene for their assistance with biochemical assays and to George Vadas for his patient help with gas chromatography.

My sincere thanks to Dr. Homer LeBaron and the Ciba-Geigy Corporation, not only for supplying technical and radiolabelled material, but also for providing information and advice when needed. Research grants from the Sigma Xi Society and the College of William and Mary are acknowledged.

The help of several of my fellow students was invaluable. Thanks especially to Pete DeLisle for patient assistance with the statistical analyses and for helpful discussions during preparation of the manuscript. I am also grateful to Prent Balcom and Dan Sved for their help on the "long" days and for their forebearance during the writing of several drafts.

Finally, I want to thank my parents, Lillian and Robert Williams, for their support and encouragement, and Dory, for her patience and love.
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Knowledge of short- and long-term toxic effects, kinetics and bioaccumulation potential of the organophosphate pesticide diazinon in aquatic animals is important in evaluating its impact on the environment. The American oyster, Crassostrea virginica, an inhabitant of estuarine areas near which diazinon is used, is therefore in danger of accidental exposure. The purpose of this study was to investigate responses of oysters to diazinon exposure.

Uptake of diazinon directly from water was compared with uptake of diazinon sorbed to either fine sediment or algae; the primary route of assimilation seems to be from the water. Concentrations of diazinon that inhibit shell growth during 96 hours of exposure are comparable to concentrations causing mortality in species unable to escape from a contaminated environment; the effect may be an indirect one caused by lack of food during shell closure. Acetylcholinesterase inhibition is the mechanism of action of diazinon and is significant in oyster heart tissue at concentrations of approximately 1 mg/L and greater. Even when inhibition is as great as 72%, however, it is not fatal.

During a six-week exposure at a high temperatures (22-24°C), diazinon accelerated depletion of glycogen stores but had no long-term effect on lipid content. Because of the high temperature and other laboratory stresses, glycogen stores in all animals were depleted by the end of the period and little reproductive conditioning occurred. There is a suggestion of some early gametogenic activity because there was an increase in total lipid content during the first 21 days in the controls and low dose but not in the two highest doses. This increase may have resulted from conversion of some glycogen to lipid for vitellogenesis.

Diazinon is rapidly accumulated and metabolized by oysters. The pesticide and its biotransformation products are quickly eliminated from the animal when exposure ends; more than 90% is removed within two days. A small fraction of the nonpolar byproducts, however, appears to be stored in the lipids. The bioconcentration factor calculated from results of an accelerated uptake test is 56; although this is low compared with that of many compounds, it is higher than that reported for diazinon in other invertebrates.

Diazinon does not appear to pose a significant threat to the aquatic environment. The highest concentrations which have been reported in natural waters are below 0.10 ug/L and the lowest concentration able to produce toxicity in any species has been three times greater than this. In oysters, the lowest concentration causing a significant toxic response is approximately 10,000 times the concentrations reported in natural waters.
UPTAKE KINETICS AND TOXICITY OF DIAZINON IN
THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA GMELIN
GENERAL INTRODUCTION.

Diazinon (0,0-diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate) is an organophosphate insecticide with the chemical structure:

It is used to control a variety of insect pests on fruits, vegetables and crops as well as on lawns and ornamentals and is considered by local and state agencies in Virginia to be one of the more widely used pesticides in the Tidewater area (Robert Baldwin, Va. Truck and Ornamentals Research Station, Accomack, 1981; Charles Elstrodt, Virginia Beach Truck Experimental Station, 1981; and F. B. Goode, Virginia Cooperative Extension Service, Gloucester, 1981; personal communications). Diazinon is also used throughout the world. Its application to rice paddies in India, for example, has effectively controlled pests (Sethunathan and Pathak, 1972).

Diazinon has been found in fresh water at concentrations below 0.1 µg/L - for example, 0.05 µg/L in the Missouri River,
0.02-0.07 µg/L in the Arkansas River, and 0.01-0.04 µg/l in the Gila River in Arizona (Schulze et al, 1973). It has not yet been reported in the marine environment although the potential exists for contamination of estuarine areas from agricultural runoff.

Several physical factors influence the probability of contamination by diazinon. Two weeks after application of diazinon, 50% of it disappears through degradation or runoff and 95% of what is left remains in the top four inches of the soil (Munson, 1970). Ritter et al (1974) found the highest concentrations of diazinon applied to agricultural watersheds in the runoff and sediment collected four to ten days after application, but this was only 0.1% of the applied diazinon. The rest had been degraded or incorporated into the soil.

The persistence of diazinon can be measured by its primary degradation route, its rate of hydrolysis, which is affected by pH. At pH 6.0, more than 50% of the diazinon in water is hydrolyzed in less than two weeks (Cowart et al, 1971) whereas at pH 7.7 its half-life is five to six weeks (Sharom et al, 1980). In the latter study, the significance of biological degradation was apparent because breakdown of diazinon was much slower in distilled or sterilized water than in unsterilized natural water of similar pH. Sethunathan and Pathak (1972) also reported extensive microbial breakdown of diazinon incubated with rice paddy water.

Inhibition of acetylcholinesterase (AChE) activity is the mechanism of action of organophosphate compounds such as diazinon (e.g. O'Brien, 1974; Coppage and Braidech, 1976; Weiss, 1961). The
close relationship between AChE activity and organophosphate or carbamate pesticide exposure in birds allows AChE activity to be used as an indicator of pesticide exposure (Hill and Fleming, 1982). Studies on fish have suggested that AChE inhibition in fish is a sensitive and reliable indicator of pollution by organophosphates or carbamates (Coppage and Braidech, 1976). The effects of pesticide exposure on AChE activity in aquatic invertebrates are largely unknown.

Metabolism of diazinon has been investigated in several organisms. Studies in the rat demonstrate that diazinon is degraded rapidly and completely and that it and its biotransformation products are all eliminated from the body quickly (Mucke et al, 1970). Oxidative desulfuration of diazinon produces diazoxon, a transient intermediate. In fish, the enzyme system responsible for this biotransformation and also for the further breakdown of diazinon and diazoxon requires oxygen and NADPH and is located in the microsomal fraction of fish liver homogenates (Hogan and Knowles, 1972). Diazoxon is the only degradation product with AChE-inhibiting properties (Ciba-Geigy, 1975).

Other transformation products include several water-soluble pyrimidinols produced by hydrolysis of the phosphorus ester bond of diazinon (Mucke et al, 1970). These by-products are non-toxic and also rapidly excreted (Iverson et al, 1975; Mucke et al, 1970).

There have been several studies on the toxicity of diazinon to fish but relatively little work has been done with aquatic
invertebrates. There are few long-term toxicity studies on any aquatic species.

One of the more comprehensive studies of diazinon is that by Allison and Hermanutz (1977). Fathead minnows and brook trout were subjected to long-term (274 days and 6-8 months respectively) exposures to diazinon during growth and reproductive stages in their lives. Exposure concentrations for the fathead minnows were 3.2 to 60.3 µg/L; for the brook trout they were between 0.55 and 9.6 µg/L. Their surviving progeny were also exposed to diazinon for 30 to 60 days after hatching. In both species of fish, diazinon caused neurological symptoms and spinal deformities in the parents. There was an indication in brook trout that exposure during the spawning period caused a reduction in reproductive potential. Further, exposure of mature brook trout for six to eight months resulted in reduced growth rates for their progeny.

Goodman et al (1979) investigated the long-term toxicity of diazinon to the sheepshead minnow as well as its effect on AChE activity, reproduction, survival and growth of progeny. There was no significant diazinon-related mortality of parental fish exposed to diazinon concentrations from 0.47 to 6.50 µg/L over a period of 108 days, but the average number of eggs per female was decreased significantly in all concentrations. Fertility of eggs, survival and growth of surviving progeny were unaffected by diazinon exposure. AChE activity was quickly impaired by diazinon concentrations between 1.8 and 6.5 µg/L and decreased by 71% in the highest concentration. When the fish were returned to clean water, however, AChE activity
returned to normal within 19 days but fecundity remained impaired for three to four weeks.

In aquatic invertebrates, uptake, bioconcentration and metabolism of diazinon have been investigated in the snail (Kanazawa, 1978), the shrimp (Seguchi and Asaka, 1981) and in nematodes (Al-Attar and Knowles, 1982). Arthur et al (1983) studied the effect of diazinon on macroinvertebrate abundance and species diversity in freshwater outdoor channels and found a decrease in numbers of some, but not all, species. The level of acute toxicity of diazinon to the freshwater snail *Gilia altilis* is 3 mg/L (Robertson and Mazzella, 1989). No other types of studies, short- or long-term, have been performed with aquatic invertebrates.

The Test Animal

The American oyster, *Crassostrea virginica*, is often abundant in the intertidal and subtidal zones of estuaries in areas where diazinon is in common use. The oyster is a commercially important species and a food source for many mammals, birds, fish and crustaceans as well as for echinoderms, flatworms and other molluscs (Galtsoff, 1964). Because the adult oyster is sessile, it has no means of escaping long-term contamination although it can close the valves of its shell to avoid unfavorable conditions for a short period of time. Oysters generally thrive in shallow water and are seldom found below 100 feet (Galtsoff, 1964).

One of the endpoints in the present study is shell growth. The valves of the shell are calcareous and are secreted by the underlying
mantle. The rate of secretion of new shell varies at different parts of the mantle edge (Galtsoff, 1964). Generally, if new shell growth is removed, several millimeters of new growth will occur within four days (Butler, 1961).

The mantle adheres somewhat to the shell, and the mantle cavity beneath it is full of sea water which keeps the oyster's body bathed, even when the animal is exposed intermittently to air in an intertidal zone. The gills divide the mantle cavity into an inhalent chamber, by which water enters, and an exhalent chamber by which it leaves. The gills filter almost all suspended particles from this water as well as remove oxygen. The smaller particles are conveyed by cilia to the mouth and the larger ones are moved to the labial palps where they accumulate until released as pseudofeces (Yonge, 1960). In this study, uptake of a chemical in solution was compared with uptake of the chemical sorbed to sediment or algae in order to evaluate the importance of uptake by diffusion versus uptake involving sorting and selection of particles for ingestion.

One of the most frequently used bivalve preparations for the determination of AChE activity is the heart, one of the few organs easily separated from the body. The heart of the oyster consists of one ventricle and two auricles; haemolymph empties into the auricles from large venous sinuses (Galtsoff, 1964). Acetylcholine depresses heart action in a number of mollusc species, including clams and oysters (Galtsoff, 1964; Neuberger and Tatum, 1974). The heart receives inhibitory impulses from the visceral ganglion, one of several ganglia found in the oyster.
The gonad of the oyster is irregular in shape and lies near the surface of the body on both the dorsal and ventral sides. One of the endpoints of this study was reproductive development. The sex of an oyster can be determined by examining gametes scraped from the developed gonad. The maturity of the gametes can be assessed by adding a small amount of salt water to the preparation to determine whether sperm become motile and eggs become round.

**Objectives**

There are great gaps in our understanding of the fate of anthropogenic substances in the aquatic environment. A review of the literature shows that there have been many more studies dealing with short-term effects of compounds than with the more environmentally realistic concentrations that might produce sublethal responses during long-term exposure.

The toxic effect of a chemical contaminant on an aquatic organism depends on its bioavailability and persistence and on the ability of the organism to accumulate and metabolize it (Capuzzo et al, 1988). Biochemical or physiological alterations which might ultimately affect growth, reproduction, adaptive ability or even survival of the organism must be understood to evaluate the toxicity of a compound.

The present study was designed to provide information on the pesticide diazinon in some of these important areas using the American oyster, *Crassostrea virginica*. The main objectives of this study
were:

1. To determine whether diazinon is primarily taken up directly from water or whether uptake of diazinon sorbed to sediment or organic material is significant.

2. To establish the exposure concentration at which diazinon elicits short-term responses in oysters.

3. To investigate the effect of diazinon on glycogen and total lipid content in oysters and to relate any changes to reproductive condition.

4. To determine the tendency for oysters to accumulate diazinon in their tissues by estimating the bioconcentration factor and uptake and depuration rates.

5. To determine the extent of metabolism of diazinon and the persistence of metabolites in the body by estimating formation and depuration rates for the degradation products of diazinon.

The hypotheses to be tested are:

1. That administration to oysters of diazinon sorbed to algae or fine sediment does not increase assimilation efficiency over that when diazinon is administered in water.

2. That diazinon inhibits shell growth in oysters within a 96 hour period.

3. That diazinon decreases acetylcholinesterase activity in the heart muscle of the oyster.

4. That diazinon upsets the energy balance in oysters and depletes glycogen and lipid reserves so that reproductive conditioning is impaired.
5. That diazinon is readily metabolized and that both diazinon and its degradation products are rapidly excreted rather than concentrated in the tissues of the oyster.
BIO-AVAILABILITY AND SHORT-TERM TOXICITY OF DIAZINON

INTRODUCTION

Bio-Availability of Organic Compounds

In aquatic animals, uptake of chemicals may occur either directly from the water through gills and skin or from the diet, with absorption occurring in the digestive tract. Uptake of organic compounds adsorbed to either sediment or food has generally been reported to be negligible compared to uptake from water, although this assessment is far from unanimous (Scura and Theilacker, 1977; Southworth et al, 1979; Muir et al, 1983; Pruell et al, 1986).

In the fathead minnow, Southworth et al (1979) report 98% of the uptake of acridine to be directly from the water with only 1% of the total from food and 1% from sediment. Likewise, Muir and co-workers (1983) found greater accumulation of six organic chemicals when dissolved in water than when sorbed to sediment. Lyes (1979) found uptake from sediment of labelled hydrocarbon very slow compared with uptake directly from water in the marine annelid Arenicola marina.

Zitko (1974) found chlorinated paraffins were also less accumulated by juvenile Atlantic salmon when adsorbed to silica or food than when taken up directly from water. In a study of dietary
accumulation of PCB through a marine food chain ending with anchovy larvae, Scura and Theilacker (1977) concluded that partitioning of PCB directly from the water was the primary determinant of levels of chlorinated hydrocarbons in the anchovy. These observations are not in agreement with the finding by Rubenstein et al (1984) that the dietary contribution of PCB's accounted for 53% of the total body burden in spot. The investigators suggest that the level may be this high because few hydrophobic compounds are available for aqueous uptake compared with the amount associated with organic particulate material. Wyman and Connors (1980) found greater accumulation when contaminated food was present than from water alone. Fisher (1985) also found that dietary accumulation of Kepone by spot and shrimp caused significant increases in total Kepone body burdens in both species compared with the amount taken up from water only.

Bivalve studies have also been contradictory. Langston (1978) found that filtration and ingestion of contaminated particles is an important route of entry of PCB into the tissues of two bivalves, *Cerastoderma edule* and *Macoma balthica*. However, Pruell et al (1986) felt that the dissolved phase was the direct source of the contaminants accumulated by mussels exposed to environmentally contaminated sediment.

Such conflicting information emphasizes the need to evaluate the routes of diazinon uptake in oysters.

**Short-term Effects of Chemicals**

Determination of the acute toxicity of a chemical is generally the first step in studying its effects on an organism. The objective
in measuring acute aquatic toxicity is to determine the correlation between the chemical concentration in the water and the selected response in the group of experimental animals - the concentration-response relationship. Other deleterious factors in the environment (e.g. hypoxic conditions, disease organisms, rapid temperature changes) must be eliminated as much as possible to insure that it is the chemical under study that causes the observed responses. Any concentrations at which there is no effect significantly different from that observed in animals in uncontaminated water (controls) are no-effect concentrations. These lie below the threshold concentration at which the first statistically significant response is produced.

The most commonly studied response in fish and many invertebrates is death. The median lethal concentration (LC50) is the concentration at which 50% of the test organisms die. For some aquatic invertebrates, however, death is not easily determined. With oysters it is not a practical end point in a short study because in an unfavorable environment oysters can close their valves and stop pumping. A response that is sensitive and easily measured must therefore be substituted. The concentration producing this selected response in 50% of the test organisms is the median effective concentration (EC50). In this study, the EC50 defines the concentration at which there is a 50% percent change in the magnitude of a graded response.

The rate of shell deposition has been used as an indicator of toxicity in oysters. Secretion of the shell is a function of the mantle. Butler (1961) determined that within a wide range of
hydrographic conditions, oysters can deposit up to approximately one mm of new shell growth every 24 hours. Since the decrease in growth rate caused by a toxic chemical is immediate, four days is sufficient for a definitive toxicity test. Butler also reported that a ten-fold dilution of the lowest concentration causing a significant decrease in growth produces no toxic effects of any kind, either short- or long-term.

Although a decrease in shell deposition is an indication of stress, it is not necessarily irreversible. With continued exposure, many sublethal effects do not persist because physiological adaptation to the unfavorable environment occurs (Brungs and Mount, 1978). A response to which adaptation is unlikely is the inhibition of AChE activity, the mechanism of action of organophosphate pesticides like diazinon. In other species studied, death occurs after a particular level of inhibition is reached (Hill and Fleming, 1982).

Cholinesterases (ChEs) are enzymes capable of splitting esters of choline. AChE catalyzes the reaction

\[ \text{Acetylcholine} + \text{H}_2\text{O} \rightarrow \text{acetic acid} + \text{choline} \]

in which the ester linkage between choline and the acetyl group is cleaved (Lehninger, 1975). Whereas AChE is always present in vertebrates, it is not always present in invertebrates. However, all invertebrates studied contain at least one ChE (Principato et al., 1988). These ChEs show considerable variation in substrate specificity (Silver, 1974).
AChE occurs as several different isozymes which appear to differ significantly in their sensitivity to inhibition by toxic compounds. Research on mutant flies resistant to the organophosphate Rabon indicates that the precise binding site on AChE for the pesticide is different from that for acetylcholine and that the nearer a binding site is to the substrate binding site, the stronger the inhibition (O'Brien et al, 1974).

Several studies have identified acetylcholine receptors and AChE activity in invertebrates. Calabrese and Maranto (1986) found acetylcholine receptors in heart muscle cells of the leech; they hypothesized receptors might serve in neuromuscular transmission from heart motor neurons. Emson and Kerkut (1971) isolated and purified ChE from the brain of the snail Helix aspersa and concluded from its properties that it was AChE. Cottrell (1966) identified acetylcholine in the nervous tissue of the mollusc Mercenaria. Acetylcholine has been found in the haemolymph, heart and smooth muscles of several other molluscs, including several species of clams, mussels and oysters (Winners et al, 1978). Clearly AChE is present and active in molluscs generally.

Diazinon's mechanism of action is AChE inhibition. There are often differences in the degree of inhibition caused by a compound and by its biotransformation products. Whitmore and Hodges (1978), studying inhibition of several esterases in Gambusia affinis muscle by malathion, found pure malathion incapable of inhibiting esterases unless incubated with the homogenates for an hour. The inhibitory action was thought to be caused by a breakdown product. Likewise,
diazoxon, an early transient intermediate in diazinon's biotransformation, is more toxic to the rat than diazinon itself (Mucke et al, 1970). Its other, more polar, breakdown products produce no AChE inhibition.

Little work has been done on the effect of toxicants on AChE activity in aquatic invertebrates. No studies to date have investigated the effect of diazinon on AChE activity in oysters.

Objectives

The objective of the first experiment was to determine whether water is the primary source from which oysters accumulate diazinon or whether uptake of diazinon sorbed to sediment or organic material is significant. The precise contribution of each source was not the primary concern of the experiment; rather, the study was undertaken to determine the best method of administration of diazinon in subsequent experiments.

The purpose of the shell growth study and the AChE activity studies was to determine concentrations of diazinon able to induce short-term toxic responses in oysters.
MATERIALS AND METHODS

Exposure System

The exposure system used in all studies was a continuous-flow system (Figure 1). Water was pumped from the York River through PVC pipes to the laboratory. It then passed through a polypropylene filter bag into a reservoir beside the wet-table. From there it was sent by a magnetic-drive pump through two Fulfilo sediment-removal type water filters (10 μm followed by 1 μm filtering capacity) up to an 80-liter glass head tank. Water level was maintained in the tank by an overflow pipe which returned excess water to the reservoir. The water temperature was maintained at the desire temperature by a thermostatically-controlled heater.

Dilution water flowed from the head tank to a central distribution box by siphon. This box, as well as the distribution boxes over each test chamber, was made of glass. Flow rate to the central distribution box could be controlled either by a screw clamp on the silicone tubing portion of the siphon or by a needle valve which opened or closed in response to water level changes. Both of these systems operated in conjunction with an overflow pipe in the box.

Water left the central distribution box through pipet tips which were calibrated to produce a flow rate of 200 mL/min each. The water
Figure 1. Schematic representation of the exposure system.
travelled through glass and silicone tubing to the distribution boxes over the flumes.

The flumes were fabricated from glass and a pure silicone sealant. Each flume contained ten individual compartments each designed to hold one oyster off the bottom in approximately 800 mL of water. Water entered each compartment at 20 mL/min from the distribution box situated directly over it through a glass pipet tip; the flushing time of each compartment was approximately 40 min. To insure thorough mixing throughout the compartment, there was a baffle 1/2" from the discharge end where the water left through a small hole approximately 1/4" from the top of the compartment wall.

Toxicant was injected into the dilution water tube at a controlled rate by a Harvard peristaltic pump. The toxicant and water mixed just before entering the flume distribution box. Algae was pumped directly to the central distribution box unless otherwise noted.

**Pre-experimental Period**

Oysters five to ten cm in height were collected from Wreck Shoal in the James River (37°04.0'N, 76°36.2'W) for the availability experiment, from Bowler's Rock in the Rappahannock River (37°49.4'N, 76°43.5'W) for the first study on shell growth and from Deep Water Shoal in the James River (37°08.9'N, 76°38.3'W) for the experiment on AChE activity. They were held in flowing unfiltered York River water for several months prior to the start of the availability study and the shell growth study and for two weeks before the 14-day study. The
first two groups were not fed a supplemental algal diet during this acclimation period; the animals for the AChE experiment were given 20 liters of algae (average $10^6$ cells/mL) per day.

**Availability Study**

**Experimental Design**

Two groups of ten oysters each were exposed for 48 hours to each of three treatments: (1) $^{14}$C-diazinon in solution in deionized water; (2) $^{14}$C-diazinon sorbed to algae in 1 µm-filtered York River water; and (3) $^{14}$C-diazinon sorbed to fine ($\leq$63 µm) sediments in 1 µm-filtered water. Oysters from an initial group of 80 were randomly assigned positions in the flumes.

Each replicate was administered 25 µCi of $^{14}$C-diazinon daily. Toxicant preparations were delivered to each flume at 0.75 mL/min into 1-µm-filtered York River dilution water flowing at 200 mL/min. During the experiment, oysters not in the group receiving diazinon sorbed to algae were fed sufficient algae (Tetraselmis suecica) each day to achieve a flume concentration of approximately $5 \times 10^6$ cells/L.

**Stock Preparation**

To prepare $^{14}$C-diazinon -contaminated algae, 2200 mL of Tetraselmis suecica (approximately $5 \times 10^5$ cells/mL) were incubated on a shaker table for 24 hours with 25µCi of $^{14}$C-diazinon and 25 mg of unlabelled diazinon in 0.5 mL acetone. Diazinon and $^{14}$C-diazinon were supplied for this and all other experiments by the Ciba-Geigy Corporation. The labelled material had a specific activity of 24.5
μCi/mg. Unlabelled diazinon was added to the stock to produce a diazinon concentration similar to concentrations which might be used in later experiments; if the algae could not withstand such a concentration, use of algae as a vehicle for administration of diazinon would be infeasible. During the experimental period the algae were aerated to maintain the cells in suspension. A new stock suspension was prepared each day.

Sediment suspensions were prepared from the top 2-3 cm of York River sediment. This material was wet-sieved to ≤ 63 μm, then stirred into suspension with a magnetic stirring bar. Four 10-ml aliquots were removed by pipet for dry weight determination. The desired flume turbidity was approximately 10 mg/l so the equivalent of 6 g dry weight was incubated on a shaker table for 24 hours with 25 μCi of 14C-diazinon and 25 mg of technical diazinon in 0.5 mL of acetone and 450 mL of 1-μm-filtered York River water. This volume was increased to 2200 mL and maintained in suspension during dosing with a magnetic stirring bar. A new stock solution was prepared each day.

The aqueous stock of diazinon was prepared daily right before use by adding 25 μCi of 14C-diazinon and 25 mg of unlabelled diazinon in 0.5 mL of acetone to 2200 mL of deionized water.

The total diazinon in each stock was approximately 12 mg/L which produced an exposure concentration of 0.045 mg/L in the flumes.
Exposure Period

The thermostat was set to maintain water temperature at $22 \pm 2^\circ C$. Temperature, salinity, dissolved oxygen, and pH were monitored daily as were toxicant flow rate and total flow rate to the flume distribution boxes. *Tetraselmis suecica* was pumped at 0.75 mL/min directly to the flume distribution boxes of the sediment and insolution treatments. No removal of $^{14}$C-diazinon from outgoing water was required for the small quantity of radioactivity being released ($4.26 \times 10^{-5}$ μCi/mL assuming no uptake).

Sample Analysis

After 48 hours of exposure, oysters were rinsed with 1% ammonium formate solution and placed whole in individual flasks for lyophilization. The tissue was lyophilized for 24 hours; each sample was then pulverized with a mortar and pestle. One hundred milligrams of pulverized tissue from each sample was placed in a scintillation vial with 0.2 mL of deionized water and 2 mL of NCS Tissue Solubilizer (Amersham). This mixture was incubated for 24 hours at 50° C and then 50 ul of glacial acetic acid was added. Samples were refrigerated for 24 hours before 10 mL of Handifluor (Mallinkrodt) scintillation solution was added. The samples were analyzed in the Beckman LS-150 Liquid Scintillation Counting System. Counts per minute were converted to disintegration rate by the external standard channels ratio method and a standard quench curve.
96-hour Shell Growth Experiment

Experimental Design

The flumes were randomly positioned on the table and oysters randomly assigned to positions in them from an initial pool of 180 oysters. There were two replicates of ten oysters each for five toxicant concentrations (0.1, 0.32, 0.56, 1.0 and 3.2 mg/L) as well as dilution water and solvent (acetone) controls. Toxicant stock solutions were made up daily as dilutions of the most concentrated stock. Toxicant was delivered to each flume at 1 mL/min into 1 um-filtered York River water flowing at 200 mL/min. During the experiment the oysters were fed sufficient algae (*Tetraselmis suecica* or *Dunaliella tertiolecta*) to produce a concentration of approximately $5 \times 10^6$ cells/L in the flumes.

Experimental Period

The thermostat controlling the heater in the head tank was set to maintain the exposure temperature at $20\pm2^\circ$C. Temperature, salinity, dissolved oxygen and pH of flume compartments were monitored daily as was total flow rate to the flume distribution boxes. Aeration in the central distribution box and the head tank was used to maintain dissolved oxygen levels above 60% of saturation in the flumes.

Sample Analysis

Water samples (200 mL) for gas chromatographic analysis were collected from three individual compartments in both replicates of
each treatment at 96 hours when shell growth was measured. They were
immediately extracted twice with 25 mL of methylene chloride and
concentrated to 5-10 mL in a Roto Vap. An appropriate amount of the
internal standard HHDN (aldrin) was added before this volume was
reduced to the volume desired for GC analysis (Appendix A).

Just before the oysters were placed in the water, the ventral
edges of their shell valves were sanded smooth so that all prior new
shell growth was removed. Any that were sanded so closely that an
opening into the shell cavity was made were not used. At the end of
four days new shell growth was measured with an ocular micrometer. A
small piece of gill tissue was removed from three oysters from each
flume and placed in thioglycolate medium to check for the presence of
Perkinsus marinus (Ray, 1952). Perkinsus marinus, or "dermo", is an
apicomplexan disease common to oysters in the Chesapeake Bay area.

14-day Acetylcholinesterase Activity Experiment

Experimental Design

The flumes were randomly positioned on the table and oysters from
an initial pool of 180 were randomly assigned to them and to an
initial control group. There were two replicates of ten oysters each
of five toxicant concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 mg/L),
the dilution water control and the solvent (acetone) control.
Toxicant solutions were delivered at 0.05 mL/min into dilution water
flowing to each flume at 200 mL/min. During the experiment the
animals were fed 40-50 liters of algae daily (Tetraselmis suecica or
Dunaliella tertiolecta, ca $10^6$ cells/mL density) to satisfy the ration
recommended by Epifanio and Ewart (1977) for optimum maintenance and growth of oysters.

**Experimental Period**

The thermostat was set to maintain the temperature at $20^\circ \pm 2^\circ$ C. Aeration in the head tank and central distribution box was used to maintain oxygen levels at greater than 60% of saturation in the flumes. Temperature, salinity, dissolved oxygen and pH were monitored daily in the flumes, as was total flow rate. Toxicant flow rate and flow to individual compartments were checked at random.

**Sample Analysis**

Water samples (200 mL) were taken on days 1, 7, and 14 and extracted with two 25 mL volumes of methylene chloride. These were then stored for later gas chromatographic analysis.

Half the oysters were sampled on day 14, the other half on day 15. Heart tissue was removed, weighed, and immediately assayed for AChE activity using a modification of the colorimetric method described by Hill and Fleming (1982) which is based on the method developed by Ellman et al (1961) (Appendix B). Enzyme (AChE) activity is measured by following the increase in yellow color produced by thiocholine when it reacts with dithiobisnitrobenzoic acid (DTNB) (Ellman et al, 1961). The rate of color production represents tissue AChE activity as it hydrolyzes the substrate acetylthiocholine iodide (ASCtI) into thiocholine and acetate. Use of ASCtI as the substrate rather than acetylcholine does not alter the results (Ellman et al,
1961). The reactions are:

\[
\text{acetylthiocholine} \xrightarrow{AChE} \text{thiocholine + acetate}
\]

\[
\text{thiocholine + DTNB} \rightarrow \text{yellow anion of DTNB}
\]

A small amount of tissue was also removed from each oyster for the Ray test.

**Statistical Analysis**

Differences in body burden of labelled diazinon between treatments in the availability study were analyzed by a one-way analysis of variance (ANOVA) followed by the Scheffe and Duncan's multiple range tests (Sokal and Rohlf, 1981). The criterion level of significance was 0.05.

Changes in shell growth in the 96-hour study and in AChE activity in the 14-day experiment were analyzed by a one-way ANOVA followed by Williams's isotonic range test (Rand and Petrocelli, 1985). The alpha level for significance was 0.05 unless otherwise noted. Dilution water and solvent controls were initially compared using a t-test and if their means were not significantly different, results for the two groups were pooled for the ANOVA.

Linear regression analysis was used to determine the EC50 for each response and the 95% confidence limits for these regression lines were plotted. For these regression analyses, concentrations were log transformed to produce a straight line. Because of the sharpness of the threshold of effect in the shell growth experiment, only the three
highest concentrations provided useful information for this analysis. These concentrations span the difference between the no-effect level and 100% inhibition of shell growth. All concentrations were analyzed in the AChE activity experiment.
RESULTS

Availability Study
There were significant differences in body burden of diazinon in oysters after 48 hours. The amount of radiolabel in oysters administered the algae-sorbed diazinon (4446 DPM/g wet wt) was significantly less than that in treatments where diazinon was either sorbed to sediment (6991 DPM/g wet wt) or free in solution (7782 DPM/g wet wt). The body burdens in the latter two groups were not significantly different by either multiple range test used. It was noted that, in the treatment receiving diazinon sorbed to algae, a sizeable portion of the algae came out of suspension after reaching the flume distribution boxes.

96-hour Shell Growth Experiment
In a range-finding study covering a nominal range of diazinon concentrations from 0.01 to 10.0 mg/L, there was no shell growth at 10.0 mg/L, a 90% decrease from the control growth level at 5.0 mg/L, and no significant decrease at 1.0 mg/L. Therefore the nominal concentration range in this study was 0.1 to 3.2 mg/L. However, the actual concentrations determined by gas chromatographic analysis ranged from 0.06 to 2.03 mg/L (Table 1). No new shell growth was seen in any animal at the highest concentration and there was a 33%
TABLE 1

SHELL GROWTH IN OYSTERS EXPOSED TO DIAZINON FOR 96 HOURS

<table>
<thead>
<tr>
<th>Nominal Diazinon Conc. (mg/L)</th>
<th>Measured Diazinon Conc. (mg/L)</th>
<th>Growth (mm) Mean (Std. dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>2.7 (1.3)</td>
</tr>
<tr>
<td>0.10</td>
<td>0.06</td>
<td>2.3 (1.3)</td>
</tr>
<tr>
<td>0.32</td>
<td>0.10</td>
<td>2.1 (1.1)</td>
</tr>
<tr>
<td>0.56</td>
<td>0.37</td>
<td>2.2 (1.0)</td>
</tr>
<tr>
<td>1.00</td>
<td>0.91**</td>
<td>1.8 (1.0)</td>
</tr>
<tr>
<td>3.20</td>
<td>2.03**</td>
<td>0.0 (0.0)</td>
</tr>
</tbody>
</table>

** Significantly different from control (p<0.05)
Figure 2. The regression of oyster 96-hour shell growth on diazinon concentrations from 0.37 mg/L to 2.03 mg/L. The EC50 is 1.15 mg/L.
decrease in shell growth at 0.91 mg/L. Both of these concentrations, showed significantly less growth than the dilution control. The control (solvent and dilution water) means were not found to be significantly different by the t-test and were combined in the linear regression analysis which was plotted to show the 95% confidence limits (Figure 2). The 96-hour EC50 was estimated to be 1.15 mg/L.

**14-day Acetylcholinesterase Activity Experiment**

There was great fluctuation in measured aqueous diazinon concentrations. This variability in concentration is closely correlated with the time that the sample was taken after cleaning the apparatus. Because the measured diazinon concentrations were influenced by the presence of excessive algae in the flumes, results of this experiment were analyzed on the basis of nominal concentrations.

Analysis of variance indicates significant differences among treatments. At the lowest concentration AChE activity is only 67% that seen in the control group and at a concentration of 10.0 mg/L activity has decreased to 28% of control level (Table 2). All concentrations showed significantly (p<0.01) lower AChE activity than the control group. The EC50 estimated from linear regression analysis is 8.40 mg/L when AChE activity in the control animals is assumed to be the normal level seen in oyster heart tissue. The regression line and its 95% confidence limits are shown in Figure 3.
### TABLE 2

**ACETYLCHOLINESTERASE ACTIVITY IN OYSTERS EXPOSED TO DIAZINON FOR 14 DAYS**

<table>
<thead>
<tr>
<th>Nominal Diazinon Conc. (mg/L)</th>
<th>Activity (umoles/min/g wet wt) Mean (Std. dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.82 (0.43)</td>
</tr>
<tr>
<td>1.00</td>
<td>0.55 (0.23)**</td>
</tr>
<tr>
<td>2.50</td>
<td>0.48 (0.31)**</td>
</tr>
<tr>
<td>5.00</td>
<td>0.62 (0.31)**</td>
</tr>
<tr>
<td>7.50</td>
<td>0.33 (0.30)**</td>
</tr>
<tr>
<td>10.00</td>
<td>0.23 (0.15)**</td>
</tr>
</tbody>
</table>

**Significantly different from controls (p<0.05)**
Figure 3. The regression of oyster heart acetylcholinesterase activity on diazinon concentrations from 0 (represented as 10^-5) to 10 mg/L. The EC50 is 8.40 mg/L.
Mantle and gill tissue as well as heart were sampled in the initial control group to determine which tissue would be the best to assay at the end of the experiment. Heart was chosen because AChE activity in the mantle was only 52% of that in the heart, and in the gill, activity was less than 50% of that in the heart.

There was only one mortality during the experiment. This animal, in the 10 mg/L treatment, was heavily infected with "dermo". The disease was not found in any of the initial control animals or in over 90% of the experimental group. Those oysters that had it and survived had a light infection (<10% of the tissue examined was covered with spores).
Availability Study

There was no increase in uptake in oysters exposed to diazinon sorbed to either algae or fine (≤ 65 μm) sediment compared to diazinon in solution. Uptake of ^14C-diazinon sorbed to algae was significantly lower than that in solution or sorbed to sediment. The body burden in the group administered diazinon sorbed to sediment, while not significantly lower, was 10% less than that accumulated from solution.

In the group where diazinon was sorbed to algae, the rapid settling of algae after reaching the flume distribution boxes suggests a high rate of mortality. These algae were unavailable for filtration by the oysters. This apparent mortality occurred after only 24 to 48 hours of exposure to what would be a low diazinon concentration in subsequent acute toxicity experiments. This algal response is reason for avoiding this method of administration.

Uptake of sediment-sorbed diazinon is no higher than uptake of diazinon in solution so the presence of sediment did not increase the availability of diazinon. Indeed, diazinon is moderately water-soluble and, after equilibrium is established between diazinon in solution and diazinon sorbed to sediment, may partition rapidly into the water phase as that portion is assimilated by aquatic organisms. It is possible, therefore, that in this treatment much of the
accumulated diazinon may have been taken up directly from the water, as suggested by Pruell (1986).

Because much diazinon appears to be assimilated directly from the water, its environmental impact depends largely on its stability in water. This stability, in turn, is controlled by factors such as the pH of the water. In acidic water (pH 6.0), diazinon is hydrolyzed more rapidly than in water with a pH of 7.7 (Cowart et al, 1971; Sharom et al, 1980). Diazinon is also subject to microbial degradation which decreases its residence time in the water and therefore its bio-availability in the aquatic environment.

96-hour Shell Growth Experiment

In this experiment there was a sharp threshold of effect. A significant effect on growth was observed at the two highest concentrations. At the highest concentration there was absolutely no growth and growth at 1.0 mg/L was 33% lower than that in the controls. At concentrations between 0.10 and 0.56 mg/L, shell growth was 78-85% of the control growth.

Because of this sharp threshold, regression analysis to determine the EC50 for shell growth was done using only the three highest concentrations. Only these points determine accurately the actual steepness of the slope of the central part of the sigmoidal concentration-response curve which they define and within which the EC50 is located.

The 96-hour EC50 for shell growth in this study, estimated to be 1.15 mg/L, is comparable to the LC50 for other species. Allison
and Hermanutz (1977) reported LC50's of 7.8 mg/L for fathead minnows, 0.46 mg/L for bluegills, 0.77 mg/L for brook trout, and 1.6 mg/L for flagfish (Allison and Hermanutz, 1977). Goodman et al (1979) calculated an LC50 of 1.47 mg/L for sheepshead minnows.

If an oyster's environment becomes contaminated with a pesticide such as diazinon, its first response is to close up and stop pumping activity during the short term. This response might be modified at relatively lower concentrations of the pesticide but, after a threshold concentration is reached, the oyster might remain totally incommunicado for a period of several days. During this time, of course, no food could be ingested.

A significant portion of absorbed energy in bivalves is incorporated into the matrix of the shell (Newell, 1982). If there is no incoming food supply because pumping has slowed or ceased because of a contaminant, decreased growth results while the animal uses its energy reserves for maintenance metabolism. Secretion of new shell material by the mantle would therefore decrease or, in the presence of high enough levels of the contaminant, cease altogether.

14-day Acetylcholinesterase Activity Experiment

The fluctuations in diazinon concentrations during this experiment appear to have been the result of the cleaning of the exposure system. This relationship suggests that the presence in the system of the large amount of algae required to satisfy the ration recommended by Epifanio and Ewart (1977) may interfere with dosing. Algae in this high a concentration may have taken up the diazinon
quickly enough to prevent it from reaching the oysters and metabolized it themselves. The large GC peak with a longer retention time than diazinon in the day 7 samples may represent a diazinon breakdown product such as diazoxon, produced by the substitution of a sulfur atom for an oxygen atom.

Although more frequent cleaning would have produced diazinon concentrations closer to nominal, the increased disturbance to the oysters would have been counterproductive since oysters often remained closed for several hours after cleaning (personal observation). Oysters, because they are able to close themselves off from their environment, are not continuously dosed in any situation.

Mean AChE activity in control oysters was 0.82 μmoles/min/g wet wt; this activity is low compared with that found in many other species. Goodman et al (1979) found AChE activity in sheephead minnow brain to be 31 μmoles/min/g wet wt; Finlayson and Rudnicki (1982) reported activity of 32 μmoles/min/g in channel catfish; for Japanese quail brain Hill and Fleming (1982) report AChE activity to be 13.6 μmoles/min/g. Ellman et al (1961), who pioneered the colorimetric method of determining AChE activity, found a ten-fold variation in activity in the organs of the rat, from 10.31 μmoles/min/g in the brain to 1.07 μmoles/min/g in the liver. Emson and Kerkut (1971) report activity of 16 μmoles/g in the brain of the snail *Helix aspersa* but no unit time is given. Other activities discussed in that paper relate to time periods of 5 minutes. Assuming the unit time was the same for this AChE activity, the rate was 3.2 μmoles/min/g.
The relatively low level of AChE activity in the sessile oyster is not unexpected since the amount of its nervous tissue in comparison with birds, mammals, fish and many other invertebrates is low. It is difficult to isolate a sufficient mass of ganglia to perform the assay. Although the heart has a low level of AChE activity, this organ shows higher activity than do either mantle or gill, two other easily isolated organs.

Inhibition of AChE activity has been used as evidence of organophosphate or carbamate poisoning, primarily in fish and birds (Ludke et al, 1979; Hill and Fleming, 1982; Haines, 1981). Generally, a 60-80% inhibition level must be reached before death occurs in fish (Finlayson and Rudnicki, 1985) and more than 50% in birds (Hill and Fleming, 1982). There is a lack of information on the level of inhibition causing death in aquatic invertebrates. In this study there is 60% inhibition at 7.5 mg/L and 72% inhibition at 10.0 mg/L. Despite the degree of inhibition at a diazinon concentration of 10 mg/L, the single death among the twenty oysters at this dose is attributed to disease, i.e. the heavy infestation of "dermo". Therefore, at concentrations of 10 mg/L or less, the only direct effects of diazinon on the oyster appear to occur at a suborganismic level.
LONG-TERM TOXICITY OF DIAZINON

INTRODUCTION

Long-term toxicity studies are more sensitive indicators of the toxicity of a chemical than are acute studies because lower concentrations are generally required to produce a response. A chemical that produces no toxic effect in a 96 hour study may yet be harmful to a species if the responses it elicits require more than 96 hours to be manifested. The end points in long-term studies include changes at several levels of biological organization including biochemical, cytological, physiological and organismal responses (Stebbing, 1985). Changes of these types may reduce growth rate and fecundity, affect survival of eggs and embryos, disrupt metabolism or cause behavioral changes (Murty and Devi, 1982).

Unfortunately, studies of the effects of pesticides on aquatic animals have often been limited to reporting the LC50. Usually, however, aquatic animals are not exposed to acutely toxic concentrations of anthropogenic materials unless they are in the vicinity of an oil spill or unable to leave an area of chemical release.
A few studies with fish and zooplankton have investigated effects on growth and reproduction (Brungs and Mount, 1978). Much less work has been carried out on biochemical or physiological changes in any aquatic animals.

**Glycogen**

Glycogen is the main storage polysaccharide in animal cells and is a multi-branched compound composed of linked glucose molecules (Lehninger, 1974). When there is surplus glucose available, glycogen is formed by linkage of D-glucose molecules to glycogen chains by glycogen synthetase. In times of metabolic need, these glucose units can be released enzymatically by glycogen phosphorylase.

Seasonal variation in tissue glycogen content has been observed in several animal phyla but most markedly in bivalves (Swift et al, 1988). A definite repeatable annual cycle has been observed in *Crassostrea virginica* (Galtsoff, 1964), *Mytilus edulis* (de Zwaan and Zandee, 1972; Bayne et al, 1982), *Ostrea edulis* (Gabbott and Stephenson, 1974) and *Placopecten magellanicus* (Thompson, 1977) among others.

In *C. virginica*, glycogen is rapidly accumulated during the spring and is then used in the development of gametes so that by the end of the reproductive cycle the amount of glycogen is at a minimum (Galtsoff, 1964). Soon after spawning, however, the oysters begin to synthesize and store glycogen again. Some of this reserve is used as food during the winter, but levels remain high until gamete production occurs. The exact timing of this cycle varies with the geographical
location of the oysters (e.g. New England vs. the Chesapeake Bay).

In bivalves, glycogen is stored in various tissues when surplus food is available and is later used for energy production or converted to lipid in developing gametes (Bayne et al., 1982). In *M. edulis*, this storage occurs primarily in the mantle, the organ that shows the greatest seasonal variation in percent glycogen (de Zwaan and Zandee, 1972). In *C. virginica*, glycogen is stored primarily in the connective tissue of the mantle and labial palps (Galtsoff, 1964). Depending on the season, glycogen can vary from under 20 to more than 50% of the dry weight of an oyster (Walne, 1970).

**Lipids**

Lipids are water-insoluble organic molecules which serve several important biological functions. The hydrocarbon nature of the major portion of their structure results in their distinctive properties (Lehninger, 1975). Among their most important functions, lipids serve as structural components of membranes and as a form of metabolic fuel.

The polar lipid fraction, or phospholipid, functions chiefly as a structural unit in membranes (Gardner and Riley, 1972) and the neutral lipid fraction is accumulated as an energy reserve (Holland and Spencer, 1973). In feral oysters, sampled monthly for 16 months, polar lipids remained at a constant level, approximately 0.5% of the live tissue weight of the animal (Trider and Castell, 1980). The neutral lipid content changed dramatically over the year, with the sharpest drop in level, greater than 75%, occurring at the time of spawning. Seasonal changes in the lipids of the mollusc *Chlamys*
Tehuelcha also occur mainly in the neutral lipid fraction (Pollero et al, 1979).

Seasonal changes in tissue weight and lipid content occur in the bivalve Nucula sulcata (Ansell, 1974). Both increase as the gonad develops and after spawning, tissue weight and lipid content decrease. The female gonad contains significantly more lipid than the male gonad in these animals.

An annual cycle in lipid levels has also been observed in a freshwater crustacean, Macrobrachium borellii, which appears to be related to the sexual cycle (Gonzales-Baro and Pollero, 1988). In these shrimp, changes in the lipid content of the hepatopancreas were inversely correlated with those in the female gonads, suggesting a transfer of lipids from the hepatopancreas to the gonads before gametogenesis.

The Role of Glycogen and Lipid

Gametogenesis is supported by reserves of glycogen and protein stored in the adductor muscle of the scallop and by lipid stored in the digestive gland (Taylor and Venn, 1979). Lipid is an important constituent of bivalve gametes and is an important energy source for oyster larvae; endogenous lipid provided by the parent during vitellogenesis is rapidly metabolized during embryogenesis (Gallagher et al, 1986). Gabbott (1975) has proposed a metabolic pathway for conversion of glycogen in mussels into lipid reserves in developing eggs.
In developing larvae, during short periods of starvation there was a greater loss of neutral lipid than of glycogen (Holland and Spencer, 1973). When mussels underwent stress caused by high temperature and low food ration, there was some resorption of ripe gametes and in those produced there was an 80% decrease in lipid, both neutral and phospholipid (Bayne et al, 1978).

There have been a few studies investigating the effects of pesticides on the lipid and glycogen content of aquatic animals. In the freshwater crab Oziotelphusa senex senex, there was a decrease in glycogen levels in the hepatopancreas and muscle after exposure to methylparathion (Reddy et al, 1986). In Channa punctata, endosulfan significantly decreased glycogen and lipid in the liver and glycogen in the muscle but increased glycogen levels in the kidney (Murty and Devi, 1982). Malathion did not alter total lipid in the freshwater catfish, Clarias batrachus, but apparently mobilization of lipids from the liver during vitellogenesis was inhibited by these pesticides (Lal and Singh, 1987). In brook trout, PCB appears to interfere with the reproductive process by reducing hatching success of eggs (Freeman and Idler, 1975).

Objectives

The primary objective of the present study was to investigate the effect of the pesticide diazinon on glycogen and lipid pools in oysters. Secondarily, the somatic condition index of the oysters was determined to evaluate whether this index serves as an indicator of metabolic storage pools.
MATERIALS AND METHODS

Pre-Experimental Period

Oysters five to ten cm in height were collected from Deep Water Shoal (37°08.9'N, 76°38.3'W) in the James River. They were acclimated at ambient temperature (ca 15°) for five days and over the course of the next two days the temperature was raised to 21°C. During this holding period the oysters were fed 15-20 liters of mixed species of algae (density $10^5$-$10^6$ cells/mL) per day. Prior to starting the experiment, gill tissue from six oysters was checked for *Perkinsus marinus* infestations using the Ray test (Ray, 1952).

Diluent York River water heated to 22-24°C was pumped through the system for a week prior to the start of the experiment. Toxicant flow was started 12 hours before the oysters were placed in the flumes.

Experimental Design

There were three replicates of ten oysters each at three concentrations of diazinon, a solvent (acetone) control and a dilution water (York River water) control. The nominal diazinon concentrations were 0.68, 3.4, and 6.8 mg/L. York River water was pumped to each flume distribution box at 200 mL/min, 20 mL/min to each oyster. The flumes and set-up of the wet table were as described in the previous
chapter.

Oysters from an initial pool of approximately 200 were randomly assigned to positions in the flumes or to an initial control group. The flumes were randomly positioned on the table. During the experiment the animals were fed sufficient algae (density ca $10^6$ cells/mL) daily to produce a density of approximately $10^7$ cells/L in the flumes. The algae fed individually or as a mixture included Isochrysis galbana, Tetraselmis succica, and Dunaliella tertiolecta.

Experimental Period

On day 0 the initial control group was sampled as described below under Sample Analysis.

A heater in the head tank was set to maintain the experimental temperature between 22 and 24°C to induce conditioning but not spawning. Temperature, salinity, dissolved oxygen and pH were monitored daily. The dissolved oxygen level was adjusted by aeration in the central distribution box if necessary to maintain it at greater than 60% of saturation. The flow rate to each flume distribution box was checked daily; toxicant flow rates and flow to individual compartments were monitored at random twice a week.

New stock solutions were made up every two days. The entire system, including flumes, tubing and distribution boxes, was cleaned twice a week to prevent excess build-up of algae and bacteria on surfaces.
Sample Analysis

Half the oysters were sampled on day 21 of the experiment to determine whether conditioning was occurring and whether any changes were yet detectable in lipid or glycogen levels. The remaining oysters were sampled on day 42. Water samples were taken weekly from one replicate of each treatment.

After an oyster was removed from the flume, its total displacement was determined (Fig. 4). The oyster was then shucked and its wet meat weight obtained. The displacement of the shell was determined. The oyster was then examined for reproductive activity. The gonad was scraped several times with a razor blade, then gently squeezed. Any gametes present were transferred by pipet to a slide and examined microscopically to determine whether they were active. A small piece of gill tissue was then excised for the Ray test for "dermo" (Ray, 1952) before the oyster was homogenized in its palial fluids.

This homogenate was divided and prepared for three separate analyses: (1) For determination of dry weight, necessary to calculate condition index, a portion of homogenate of known wet weight was placed in a tared pan; (2) for total lipid analysis, a 3-ml volume of chloroform and methanol was added to a known wet weight of homogenate and immediately frozen at -10° C; (3) for glycogen analysis, a portion of the homogenate of known wet weight was immediately frozen.

Water samples (200 ml) were extracted immediately after collection with methylene chloride for later GC analysis. The
Figure 4. Sampling procedure.
extracts were later concentrated with a Roto Vap to 5ml and then to the appropriate volume for GC analysis under a stream of nitrogen at 40°C after the internal standard had been added (Appendix A).

**Condition Index**

The condition index (CI) was calculated from the total oyster displacement (TD) in ml, shell displacement (SD) in ml, and dry meat weight (W_D) in g. Dry meat weight was obtained by drying a portion of homogenate of known wet weight at 90°C for 24 hours and then weighing it. Based on the dry weight to wet weight ratio of the homogenate, the total dry weight of each oyster was determined from the total wet weight. The following formula describes the condition index (Walne, 1970):

\[
C.I. = \frac{W_D}{TD - SD} \times 1000
\]

**Total Lipid Analysis**

Total lipid in the tissues was determined by a modification of the method of Bligh and Dyer (1959) (Appendix C). After the initial extraction in a methanol:chloroform (2:1) solution prior to storage at -10°C, the sample was thawed and extraction continued by stirring twice for 30 minutes in mixtures of methanol:chloroform:distilled water (2:1:0.4). After each 30-minute extraction, the mixture was vacuum filtered. The combined filtrates were extracted with methanol:chloroform:distilled water and then allowed to separate for
45 minutes or more. The chloroform containing the lipid was separated from the methanol and water layers. Triplicate known volumes of chloroform were removed from each sample, allowed to evaporate to dryness and then analyzed gravimetrically.

**Glycogen Analysis**

Glycogen was extracted from the tissue by a modification of the methods described by van Handel (1965) and Harleston (1971). The estimation of glycogen was based on a photometric method using anthrone reagent also described by van Handel (1965) (Appendix D).

Glycogen was isolated from the tissue by digestion with hot KOH in which the non-reducing linkages of the glycogen molecule are stable (Lehninger, 1974). Because glycogen is extremely insoluble in ethanol, and KOH and Na$_2$SO$_4$ both cause precipitation of glycogen, the tissue was incubated overnight in a mixture of KOH, distilled water, saturated Na$_2$SO$_4$ and ethanol. After centrifugation and removal of the supernatant, the glycogen precipitate was redissolved in water.

An aliquot of this dissolved precipitate was removed for reaction with the anthrone reagent. Because the maximum blue-green color produced by the reaction of glycogen with anthrone is produced rapidly and begins to diminish soon afterward at room temperature, samples were chilled until absorbances were read on a Bausch & Lomb Spectronic 20 spectrophotometer at 620 nm. The blue-green color is the result of the reaction of the enol tautomer of anthrone with the glycogen furfural derivative (Ashwell, 1957).
Statistical Analysis

Differences in glycogen levels and total lipid were analyzed with respect to time and diazinon concentration by analysis of covariance. Condition index and dry weight percentage were analyzed in the same way. The alpha level for significance was 0.05 unless otherwise indicated.
RESULTS

Measured diazinon concentrations were between 66 and 109% of the nominal levels in the different treatments. The low concentration was 0.74 mg/L, the middle was 2.40 mg/L, and the high was 4.49 mg/L. No diazinon was detected in control water.

For the initial sample of control oysters, the mean glycogen level was 40.68 mg/g wet weight (Table 3). Analysis of covariance, including the day 0 control oysters, reveals statistically significant changes in glycogen levels with respect to both time and treatment. At the first sampling time, 21 days into the experiment, the oysters exposed to the highest concentration had a significantly lower level of glycogen than that in any other treatment. These oysters contained 33% less glycogen than in those in the control group, 42% less than those in the middle dose and 37% less than those in the low dose.

When sampled at the end of the experiment, the glycogen level in the oysters exposed to the lowest concentration was 18% less than that in control oysters; in the middle dose the level was 27% less and in the highest dose it was 18% lower than in the control oysters. None of these relatively low levels was significantly different from control level. In all concentrations except the highest, however, there had been a statistically significant decrease in level since day 21.
## Table 3

**Glycogen Content in Oysters after Long-Term Exposure to Diazinon**

<table>
<thead>
<tr>
<th>Measured Diazinon Conc. (mg/L)</th>
<th>Time (days)</th>
<th>Mean (Std. Dev.) (mg/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>0</td>
<td>40.68 (10.58)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>28.87 (14.06)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>21.47 (7.72)</td>
</tr>
<tr>
<td>0.74</td>
<td>21</td>
<td>30.68 (13.51)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>17.71 (8.54)</td>
</tr>
<tr>
<td>2.40</td>
<td>21</td>
<td>33.29 (14.91)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>15.75 (10.23)</td>
</tr>
<tr>
<td>4.49</td>
<td>21</td>
<td>19.32 (11.02)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>17.52 (7.60)</td>
</tr>
</tbody>
</table>
If glycogen content at these two sampling times is compared with that in the initial control sample, it is significantly lower at both times in all concentrations, including controls, except in the group exposed to 2.40 mg/L on day 21. Between day 21 and day 42 there was a significant decrease in the glycogen content of all oysters except those in the 4.49 mg/L treatment which had reached their minimum glycogen level by day 21. Although the mean glycogen content in control oysters was higher than that in animals in any other treatments, the differences among treatments on day 42 are not statistically significant. The amount of glycogen found in the dosed oysters at this time was 44% of that in initial controls in the lowest concentration, 39% in the middle concentration, and 43% at the highest concentration.

Diazinon had little effect on total lipid content at the two higher concentrations (Table 4). Total lipid in oysters in the initial control group was 13.92 mg/g wet weight. Over the course of the experiment, this level had risen significantly by day 21 but then decreased again by day 42 in animals exposed to no diazinon. The same trend was observed in oysters at the lowest exposure concentration, whereas no increase took place in animals at the two higher doses.

The ratio of dry weight to wet weight did not change significantly over the course of the experiment despite the decline in glycogen content. There was a significant decrease in the condition index of oysters in the middle concentration on day 21 and in those in the high concentration group on day 42 compared with oysters examined on day 0 (Table 5).
<table>
<thead>
<tr>
<th>Measured Diazinon Conc. (mg/L)</th>
<th>Time (days)</th>
<th>Mean (Std. dev.) (mg/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>0</td>
<td>13.92 (2.10)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>18.06 (4.09)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>16.00 (3.55)</td>
</tr>
<tr>
<td>0.74</td>
<td>21</td>
<td>18.12 (3.42)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>17.14 (3.33)</td>
</tr>
<tr>
<td>2.40</td>
<td>21</td>
<td>16.19 (2.97)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>17.55 (9.21)</td>
</tr>
<tr>
<td>4.49</td>
<td>21</td>
<td>16.69 (3.84)</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>16.45 (5.31)</td>
</tr>
<tr>
<td>Diazinon Concentration (mg/L)</td>
<td>Day 0</td>
<td>Day 21</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Control</td>
<td>102 (40)</td>
<td>79 (25)</td>
</tr>
<tr>
<td>0.74</td>
<td>89 (36)</td>
<td>94 (41)</td>
</tr>
<tr>
<td>2.40</td>
<td>66 (16)</td>
<td>92 (52)</td>
</tr>
<tr>
<td>4.49</td>
<td>75 (20)</td>
<td>60 (18)</td>
</tr>
</tbody>
</table>
Few oysters were reproductively developed on either day 21 or 42 and those that were had no active gametes. Many of the oysters in all treatments appeared "watery" with thin undeveloped gonads, particularly on day 42.
DISCUSSION

The mean glycogen content in the initial control group of oysters was 40.68 mg/g wet weight, or 21.5% of dry weight. This amount corresponds well with those that have been reported for oysters and other bivalves. Mean glycogen content of C. virginica varies between 21.2 and 33.6% in different geographical locations (Lee et al, 1960). Over a 22 month period, glycogen content of oysters fluctuated between 4% of dry weight and more than 75% (Galtsoff, 1964). In an uncontaminated environment, the glycogen content of oysters has been reported to be approximately 4% of wet weight (Galtsoff et al, 1947). In the present study, glycogen was 4.07% of wet weight on day 0.

In Mytilus edulis, glycogen levels have been found to fluctuate between 10 and 35% of dry weight (de Zwaan and Zandee, 1972). Dare and Edwards (1975) determined glycogen content of M. edulis to have a mean value of 22.5% of dry weight.

At the time of year when this experiment was conducted (November-December), glycogen stores in oysters are normally being replenished. Although this increase may be modified if glycogen reserves are mobilized to meet metabolic needs of the oysters, levels generally remain high until gametogenesis begins in the spring (Galtsoff, 1964). The significant reduction in glycogen stores observed in all animals including the controls suggests that another
factor besides diazinon was involved in the response. Gabbott and Walker (1971) noted that, although there was an increase in glycogen in oysters in the field during the period April to June, there was a decrease in glycogen in oysters maintained in a hatchery during the same period. They hypothesize that the higher water temperatures in the hatchery increased metabolic rates and therefore energy demand which caused this depletion of glycogen stores.

In the present study, oysters were brought into the laboratory when ambient water temperature was 12-15°C and were then maintained at 22-24°C for six weeks. Over this period, although they were fed, the condition index of oysters in all groups declined significantly. It is likely that this temperature elevation and other stresses of laboratory conditions increased energy demand to such an extent that glycogen reserves had to be mobilized. Widdows and Bayne (1971) reported that mussels collected in November and acclimated to 20°C in the laboratory experienced a disruption in their energy balance which forced them to utilize energy reserves. Quick (1971) also reported a loss of glycogen in C. virginica when taken from the field and exposed to higher temperatures in the laboratory.

Despite the decrease in glycogen content in all treatments, it appears that diazinon did have an effect on glycogen stores at the highest concentration. Glycogen content in oysters exposed to 4.49 mg/L of diazinon was significantly lower than in the other concentrations on day 21. Although in all treatments and the controls glycogen stores were equally depleted by day 42, oysters in the highest concentration of diazinon evinced a more rapid response. The
faster decrease in this group was later reflected in the significantly lower condition index of oysters in the high dose on day 42.

In the initial group of control oysters, the mean total lipid was 13.92 mg/g wet weight or 7.39% of dry weight. The mean total lipid content in C. virginica was reported to be 16 mg/g wet weight by Watanabe and Ackman (1974). Trider and Castell (1980) found that it fluctuated between 0.5 and 2.0% of wet weight. Total lipid is present at a mean level of 10.4% of dry weight in Mytilus edulis and 7.1% of dry weight in Balanus balanus (Barnes and Blackstock, 1973). Total lipid comprises 7.0% of the dry weight of scallops (Dare and Edwards 1975).

The less dramatic change in total lipid content in this study reflects the role of glycogen as the primary energy reserve in oysters (Gabbott and Bayne, 1973). The increase in total lipid in control animals and those in the lowest concentration may reflect the conversion of some glycogen into lipid during the early stages of the study, since the oysters were kept at an elevated temperature. Glycogen reserves were moderate at the beginning of the experiment and some decline in glycogen would be expected because of its mobilization for conversion to lipid for vitellogenesis (Gabbott, 1975). If there were some activity of this type, conversion to lipid would not only deplete glycogen stores but also further increase the energy demand (Gabbott and Bayne, 1973).

Although the ultimate response was similar in all treatments, including controls, the data suggest accelerated depletion of energy
reserves at high concentrations of diazinon. The rapid decline in
glycogen in oysters exposed to the highest diazinon concentration was
not accompanied by any increase in total lipid. The glycogen was
being mobilized solely for maintenance metabolism of the oysters.

In the middle concentration, glycogen was depleted more slowly
and again with no significant increase in lipid at any time. It has
been suggested that exposure to pesticides may inhibit mobilization of
lipids for vitellogenesis, thus not significantly changing total lipid
content (Lal and Singh, 1987). In the low dose, as in animals exposed
to no diazinon, there was slower depletion of glycogen than in the
high dose and a significant increase in lipid content on day 21. The
failure of most oysters to develop gametes indicates that glycogen
stores were not high enough at the beginning of the experiment to
support both an increased maintenance energy demand and conversion to
lipid for successful gametogenesis.

Seasonal changes in the glycogen and lipid content of oysters
and other bivalves are related to gonad development and spawning.
Differences within a species are usually associated with the longer
reproductive periods in warmer climates. Patterns may vary, however,
in response to abnormalities in temperature, salinity, abundance of
food or other environmental factors (Galtsoff, 1964). It appears that
an extended period of unseasonable high water temperature, after the
normal seasonal decrease had begun, combined with other stresses of
laboratory conditions, upsets the energy balance and depletes glycogen
stores.
At high concentrations, diazinon exacerbates the effect and accelerates the depletion of glycogen. The concentration required, however, near 5 mg/L, is far higher than any concentration that has so far been reported in natural waters.

Total lipid does not seem to be affected by an abnormal temperature pattern. The response usually induced by high temperature, an increase in lipid pools in preparation for vitellogenesis, failed to occur because of the depletion of glycogen stores and the requirement that all food energy be utilized for maintenance metabolism. Diazinon appears to have little direct effect on lipid content. The failure of lipid reserves to increase initially in oysters exposed to diazinon was probably the result of diazinon's effect on glycogen stores.
INTRODUCTION

Bioaccumulation

Bioaccumulation is the process by which a substance enters and accumulates in an aquatic organism. It is possible only if the uptake rate is greater than the rate of elimination of that substance. Uptake may occur either directly from the water through gills or skin or from food, with absorption occurring in the digestive tract. Clearance takes place through the skin, gills and normal routes of excretion, or by biotransformation to other compounds. A steady state exists when the rates of uptake and elimination are equal so that the concentration of the chemical in the tissues remains constant during continuous exposure.

The bioconcentration factor (BCF) of a chemical is a measure of its tendency to concentrate in the tissues of aquatic animals at a higher level than that found in the surrounding water. Compartment models are often used in kinetic studies to describe bioconcentration. The most commonly used is the one-compartment model which assumes uptake is directly from the water and therefore proportional to the
concentration of the chemical in the water. Elimination is assumed to be directly proportional to the concentration of the compound in the tissues. The organism is a single "compartment" and all the chemical within it is equally available for depuration. At any given time during exposure, the animal's tissue concentration ($C_a$) depends on uptake rate ($k_1$), clearance rate ($k_2$), and water concentration ($C_w$), and changes over time can be described by the following equation:

$$\frac{dC_a}{dt} = k_1 C_w - k_2 C_a$$  \hspace{2cm} (1)$$

Both the uptake and clearance rate constants have been shown to be constant over a wide range of concentrations (Branson et al, 1975; Mayer, 1976). A low uptake rate constant at high water concentrations indicates a toxic effect on physiological processes (Bishop and Maki, 1980).

At steady state:

$$C_a = \frac{k_1}{k_2} C_w$$  \hspace{2cm} (2)$$

so the BCF in this model is described by:

$$BCF = \frac{C_a}{C_w} = \frac{k_1}{k_2}$$  \hspace{2cm} (3)$$

The initial increase in tissue concentration followed by a leveling
off at a steady-state plateau produces a time curve equivalent to the pharmacological infusion model (Goldstein et al., 1974) and is described by the integration of equation (1) with respect to time (Spacie and Hamelink, 1982):

\[
C_a = \frac{k_1}{k_2} (C_w)(1-e^{-k_2 t})
\]  

(4)

where \( k_1, k_2, \) and \( C_w \) are considered constant. This model is the most frequently used but does not adequately describe chemicals which are biotransformed to substances which are eliminated more slowly than the parent compound. In order to determine the BCF for such a chemical, it is necessary to distinguish parent material from biotransformed material and to evaluate their individual rates of elimination (Spacie and Hamelink, 1982).

Branson et al. (1975) described an accelerated uptake procedure to determine uptake and depuration. This "kinetic" method requires an uptake period as short as five days and then a depuration phase like that in the plateau method. The BCF and rate constants can then be estimated from the pharmacokinetic one-compartment model (eq. 4) using, for example, the BIOFAC computer program (Blau and Agin, 1978). Maximum likelihood estimates of the BCF calculated this way has been found to be in good agreement with BCF values measured directly in tests with the same species and chemical using the plateau method (Branson et al., 1975; Bishop and Maki, 1980). The limitations of the one-compartment model are that it fails to account for changes in
biotransformation rate, saturation kinetics and other nonideal processes (Rand and Petrocelli, 1985).

Bioaccumulation of diazinon by aquatic organisms has previously been studied in only a few aquatic species. Kanazawa (1978) examined bioconcentration of diazinon by freshwater fish, crayfish, and snails exposed to diazinon for seven days and found the BCF for fish generally higher than that for invertebrates, although the animals did not appear to be at saturation. Seguchi and Asaka (1981) found that diazinon levels in several species of freshwater fish and a shrimp increased to a maximum on day three of uptake, then decreased slightly and remained at equilibrium during the rest of the 14 day uptake period. Again, the BCF for fish was higher than that for the invertebrate. Kanazawa (1975) also studied the fate of diazinon in the freshwater fish *Pseudorasbora parva* and found a maximum body burden at three days which then decreased gradually over the rest of the 30 day uptake period because of excretion and biotransformation.

**Biotransformation**

Biotransformation is the biologically catalyzed conversion of one chemical compound to another. Such conversions may make a chemical more toxic or less toxic; they often make them more polar and therefore easier to excrete. This is often accomplished through two types of reactions: reactions which introduce polar groups into the molecule by oxidation, hydrolysis, and reduction, and others which involve conjugation of a chemical or metabolite with polar cellular
constituents such as glucuronic acid or glutathione to form easily-
excreted highly water-soluble molecules (Buhler and Williams, 1988).

Biotransformation of diazinon has been studied by several
investigators, particularly in mammals (Mucke et al, 1970; Yang et
al, 1971) and insects (Ahmad and Forgash, 1975; Riskallah et al,
1986). The primary route of metabolism is the hydrolysis of the
phosphorus ester bond to produce 2-isopropyl-6-methyl-4-hydroxy
pyrimidine (pyrimidino.l) and oxidation at the primary and tertiary
carbon atoms of the isopropyl side chain. The oxygen analog,
diazoxon, is a transient intermediate and the only one that retains
the AChE-inhibiting properties of diazinon (Ciba-Geigy, 1975).

Additional work on biotransformation has been done with some
aquatic species. Hogan and Knowles (1972) determined that it is
mainly the microsomal fraction of fish liver homogenates that
metabolizes diazinon. In nematodes (Panagrellus redivivus and
Bursaphelenchus xylophilus) incubated with 14C-diazinon for 96 hours
(Al-Attar and Knowles, 1982), there was a rapid decrease in diazinon
levels in P. redivivus accompanied by an increase in diazoxon
concentration that peaked at 48 hours. Pyrimidinol, a more polar
metabolite, increased throughout the exposure period. B. xylophilus
showed a similar response but the diazinon degradation was less
extensive. Seguchi and Asaka (1981) observed that not all diazinon
metabolites could be found in carp and rainbow trout, including
diazoxon. Metabolite concentration reached a maximum after three to
seven days exposure to diazinon in a flow-through system and after
transfer to clean water, diazinon and its metabolites were rapidly excreted.

Objectives

One of the objectives of this study was to determine the extent to which diazinon is accumulated in the tissues of the oysters at a higher concentration than that in the water. Another objective was to examine the rate and extent of biotransformation of diazinon by the oyster. The rates of elimination of diazinon as well as its degradation products were estimated to determine whether any were retained in the lipid compartment of the oyster.
MATERIALS AND METHODS

Pre-experimental Period

Oysters six to ten cm in height were collected from Bowler's Rock in the Rappahannock River (37°49.4'N, 76°43.5'W). These were held in flowing seawater at 22-24°C and fed 30 liters of algae per day for 30 days prior to the start of the experiment. Dilution water was pumped through the system for a week prior to the start of the experiment, and toxicant flow was started two days before the oysters were added.

Experimental Design

There were four replicates of ten oysters each at two concentrations of 14C-diazinon, 0.10 and 0.05 mg/L and a solvent (acetone) control. Twenty-five uCi of 14C-diazinon were added to the non-radiolabelled stock solution for each diazinon-dosed treatment daily during the uptake phase. Dilution water, 1 um-filtered York River water, was pumped to each flume distribution box at 200 mL/min, 20 mL/min to each oyster. Toxicant was mixed with the dilution water just before it entered the distribution box.

Oysters from the initial pool of 150 were randomly assigned to the different treatments and placed in the appropriate flumes. The locations of the flumes on the table were randomly assigned. During the experiment the animals were provided sufficient algae.
(Tetraselmis suecica or Dunaliella tertiolecta) to produce an algal concentration of approximately $5 \times 10^6$ cells/L in the flume compartments.

**Experimental Period**

The thermostat was set to maintain the temperature at $22 \pm 2^\circ C$; temperature, salinity, dissolved oxygen and pH were monitored daily in the flumes. Dissolved oxygen levels were maintained at greater than 60% of saturation in flume compartments by aeration in the distribution box as necessary. The flow rate to each flume distribution box was checked daily; toxicant flow rates and flow to individual compartments were checked at random twice a week.

**Uptake Phase**

During the uptake phase, four oysters from each treatment were sampled at 7.5, 15, 30, 60, and 120 hours. Water samples from the diazinon-dosed treatments were also taken at each of these times while control water samples were taken only at 7.5 and 120 hours. The water was siphoned from the individual compartment of the oyster being sampled at that time. Sample size was 750 mL.

Water samples were extracted with two 50 mL volumes of methylene chloride and the combined extracts concentrated to approximately 5 mL with a Roto-Vap and transferred to graduated centrifuge tubes. This extract was evaporated to 0.1 mL in a 40°C water bath under a stream of nitrogen and then placed in a scintillation vial with 15 mL of Scinti Verse E Liquid Scintillation Counting Cocktail (Fisher) and
counted using the Beckman LS 5000TD Liquid Scintillation Counting System.

Oysters sampled were shucked, weighed, and immediately placed in a lyophilizer for 24-48 hours. The tissue was then pulverized using a mortar and pestle and the dry weight obtained. A 500 mg portion of this powdered tissue was extracted with three 10 mL volumes of a methylene chloride: methanol (50:50) solution using a PT 10-ST Willems Polytron. Each extract was centrifuged at 2000 rpm (800 RCF) for 15 minutes in a Beckman Model TJ-6 centrifuge. The supernatants were combined in a clean tube and concentrated to 1 mL in a 50°C water bath under nitrogen. A 100 μL aliquot of this extract was spotted on a TLC plate and the plate developed in a benzene:acetone (80:20) solvent (Ahmad and Forgash, 1975). A diazinon standard was also spotted on each plate so that the parent compound could be distinguished from its polar and nonpolar metabolites.

Under long wave UV light, the locations of diazinon and its polar and nonpolar metabolites were identified. Because of the properties of the TLC plates, polar metabolites did not migrate from the origin. Diazinon was identified by the location of the diazinon standard spotted on each plate. All other materials present were considered to be nonpolar metabolites. Each area was scraped into a scintillation vial to be analyzed separately. Fifteen mL of the scintillation cocktail were added to each vial which was then analyzed in a liquid scintillation counter.
Clearance Phase

After 120 hours, toxicant flow was discontinued, all flumes were cleaned and the animals replaced in them. Twenty-four hours later the flumes were again cleaned to remove any diazinon or metabolites that might have been cleared by the oysters in that time. During this phase, water quality and flow rates were monitored as during uptake.

Four water and oyster samples were collected from each diazinon treatment on days 2, 4, 8, 12, and 16 of depuration. Tissue samples from control oysters were taken at these times but water samples only on day 2. The length of depuration was based on data from a preliminary experiment that indicated that the time required to eliminate 90% of labelled material was 10 to 12 days. Water and tissue samples were processed as described above for samples taken during the uptake phase.

Data Analysis

The BIOFAC program was used to estimate uptake and clearance rate constants and the BCF of diazinon. Analysis of covariance was performed to determine if there were significant response differences between concentrations at the different sampling times and whether there was a concentration effect on either mean tissue levels of diazinon or changes over time. Because of large variations in the amount of total radioactivity among oysters, the proportion of label present as diazinon was used for this analysis. Since proportions are not normally distributed, the square root of each proportion was
transformed to its arcsine to produce a nearly normal distribution (Sokal and Rohlf, 1981).

Formation and clearance rates for nonpolar and polar biotransformation products were also estimated and an analysis of covariance performed on each to determine statistically significant differences with respect to time and exposure concentration. Linear regression lines were also plotted for each depuration curve
RESULTS

Uptake of diazinon is rapid as is the formation of both polar and nonpolar derivatives (Table 6). The uptake rate constant calculated by BIOFAC is 21.30 day\(^{-1}\) and the clearance rate constant is 0.38 day\(^{-1}\). Formation rates for the polar and nonpolar biotransformation products (represented by \(k_1\) for these compounds) are greater than the uptake rate for diazinon. After only 7.5 hours of exposure to diazinon, the metabolites accounted for nearly 90% of the radiolabel in the tissues.

The BCF calculated for diazinon is 56. If it had been calculated based on mean total radiolabel in the tissue, the apparent BCF would have been 421.

Total diazinon was administered at two concentrations to determine whether concentration has any effect on its accumulation in the animal. The absolute concentration of radiolabelled diazinon was the same in both treatments. Mean body burdens of the radiolabelled diazinon at the different sampling times are shown in Table 7. Analysis of covariance reveals that during the uptake phase there was no significant effect of exposure concentration on body burden of radiolabelled material. During depuration there was again no significant effect of diazinon concentration. Therefore, in plotting the uptake/depuration curve for radiolabelled diazinon (Figure 5),
TABLE 6

BIOFAC-ESTIMATED PHARMACOKINETIC PARAMETERS
(Std. Dev.)

<table>
<thead>
<tr>
<th></th>
<th>Diazinon (Std. Dev.)</th>
<th>Nonpolar (Std. Dev.)</th>
<th>Polar (Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (day$^{-1}$)</td>
<td>21.30 (3.43)</td>
<td>166.0 (19.0)</td>
<td>33.13 (3.83)</td>
</tr>
<tr>
<td>$k_2$ (day$^{-1}$)</td>
<td>.38 (.06)</td>
<td>.50 (.03)</td>
<td>.40 (.05)</td>
</tr>
<tr>
<td>BCF</td>
<td>56 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearance $t_{1/2}$</td>
<td>1.84 (.28)</td>
<td>1.38 (.08)</td>
<td>1.73 (.22)</td>
</tr>
</tbody>
</table>
TABLE 7

BODY BURDEN\(^1\) (DPM/G) OF \(^{14}\)C-DIAZINON AND ITS BIOTRANSFORMATION PRODUCTS DURING UPTAKE PERIOD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hrs.)</th>
<th>Diazinon</th>
<th>Nonpolar</th>
<th>Polar</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 mg/L</td>
<td>7.5</td>
<td>197 (38)</td>
<td>1669 (528)</td>
<td>243 (77)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>148 (113)</td>
<td>955 (67)</td>
<td>255 (97)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>90 (47)</td>
<td>1174 (254)</td>
<td>254 (61)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>178 (82)</td>
<td>1545 (617)</td>
<td>359 (124)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>124 (50)</td>
<td>2051 (984)</td>
<td>367 (97)</td>
</tr>
<tr>
<td>0.10 mg/L</td>
<td>7.5</td>
<td>84 (7)</td>
<td>876 (428)</td>
<td>172 (53)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>388 (287)</td>
<td>805 (202)</td>
<td>264 (66)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1156 (112)</td>
<td>1156 (112)</td>
<td>221 (48)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>141 (91)</td>
<td>1752 (287)</td>
<td>356 (150)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>193 (177)</td>
<td>824 (181)</td>
<td>246 (49)</td>
</tr>
<tr>
<td>Combined</td>
<td>7.5</td>
<td>141 (65)</td>
<td>1273 (615)</td>
<td>207 (72)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>268 (239)</td>
<td>880 (161)</td>
<td>260 (77)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>103 (40)</td>
<td>1165 (182)</td>
<td>238 (54)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>160 (83)</td>
<td>1649 (459)</td>
<td>357 (128)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>158 (126)</td>
<td>1437 (927)</td>
<td>307 (97)</td>
</tr>
</tbody>
</table>

1. Mean (Std. dev.)
Figure 5. BIOFAC-generated uptake/depuration curve for diazinon.
results from the two treatments were combined and analyzed as one group over the uptake and depuration periods. Diazinon was completely cleared from the tissues by day 8 of depuration (day 13 overall) as indicated in the BIOFAC plot.

Results from the two diazinon treatments were also combined to plot curves for the formation and clearance of metabolites. Although the BIOFAC plot for the nonpolar breakdown products (Figure 6) shows complete clearance before day 3 of depuration, the data points indicate rapid elimination of nonpolar materials during the first two days of the clearance phase followed by equilibrium at approximately 10% of their steady-state concentration during uptake. This steady state persisted through the end of the clearance phase. The BIOFAC plot for the polar degradation products (Figure 7) indicates complete clearance by day 8 of depuration, but the data indicate that polar materials reached an equilibrium between days 2 and 12 of depuration before being completely eliminated.

Concentrations of diazinon and its breakdown products in tissue show no significant change during the uptake phase. There was a large amount of variability among individual oysters but Cochran's and Bartlett-Box tests indicate homogeneity of variance at the different sampling times for all three groups.

In a preliminary static experiment without animals there was little breakdown of $^{14}$C-diazinon until at 24 hours 32% of the radioactive material was in the form of nonpolar metabolites. No polar degradation products were detected. At earlier sampling times (0, 1.5, and 4 hours) 94% of the radiolabelled material was diazinon.
Figure 6. BIOFAC-generated uptake/depuration curve for nonpolar biotransformation products.
Figure 7. BIOFAC-generated uptake/depuration curve for polar biotransformation products.
DISCUSSION

Accumulation Kinetics

There was rapid initial accumulation of diazinon during the uptake phase of this experiment and the level of diazinon and its biotransformation products did not change significantly between 7.5 and 120 hours. The amount of biotransformation products formed was large compared with the amount of unchanged diazinon in these oysters. Much of the elimination of diazinon occurred through degradation; diazinon disappeared from the tissues by day 8 of the clearance phase, whereas degradation products were present for several more days. All polar metabolites were eliminated by day 16 of depuration, but some nonpolar degradation products were still present at that time.

Ninety percent of nonpolar degradation products in the tissues were eliminated in the first two days after exposure ceased. After this time, however, they reached steady-state level and remained at equilibrium through day 16 of depuration despite the complete disappearance of diazinon and polar breakdown products. Formation of nonpolar metabolites from diazinon still in the tissues may account for some of this material, but it was present at a higher concentration than was the remaining diazinon available for degradation. Such biphasic kinetics, where a "fast" stage of elimination is followed by a "slow" stage, indicate the presence of a
second compartment in which a small portion of the nonpolar breakdown material was stored. In a multicompartment system, both elimination and transfer between compartments are assumed to occur by first-order processes and, unless there is contradictory evidence, elimination is assumed to occur exclusively from the "central", or non-storage, compartment (Gibaldi and Perrier, 1982). Material in the peripheral compartment, the nonpolar material in this case, is sequestered there and eliminated more slowly than material in the central compartment.

Polar biotransformation products remained in the tissues of the oyster for several days after the complete disappearance of diazinon. Degradation from diazinon remaining after exposure ended could easily account for the amount present and its duration in the tissues for four days after the parent diazinon disappeared.

Few studies have been done to determine the BCF of diazinon in aquatic animals. In those studies available (Kanazawa, 1978; Seguchi and Asaka, 1981), reported BCF values for aquatic invertebrates (shrimp, snails, crayfish) range from as low as 2 to as high as 17 in these studies; BCF's for fish lie between 10 and 150. These BCF's were calculated directly from levels of diazinon in the water and in the tissues after either 7 or 14 days of exposure. In the study by Kanazawa (1978), diazinon levels in the topmouth gudgeon fluctuated from 1450 µg/L on day 1 to 2008 µg/L on day 3 to 1359 µg/L on day 7 and 1725 µg/L on day 14. The fish were assumed to be at equilibrium by day 3 and the BCF was calculated on this basis. In animals in the study by Seguchi and Asaka (1981), diazinon did appear to have reached equilibrium by day 7. The shrimp *Panaeopsis joyneri*
was reported to have a BCF of 3.

The BCF for oysters in this study is much higher than that reported for other invertebrates. This may be because bivalves are generally more efficient accumulators than other taxa. Bivalves are known to be able to accumulate contaminants in their tissues to levels considerably higher than in surrounding water (Bayne, 1978; APHA, 1976; Phelps et al, 1985). Studies have shown that PCB's, for example, are accumulated more extensively in mussel tissue than in the tissues of the worm Nereis virens or the shrimp Crangon septemspinosa (Goldberg et al, 1978; McLeese et al, 1980).

There is fluctuation in the level of diazinon in the tissues as well as considerable variation within treatments. In view of the rapid initial uptake and biotransformation, neither type of variation is surprising. The variability could be caused by differences in the amount of pumping activity during the hours just preceding sampling or by individual differences in metabolic rate. Such variability in bioaccumulation has been reported for other compounds and species (Blanchard et al, 1977; Gunkel and Streit, 1980; Morales-Alamo and Haven, 1983; Laughlin and French, 1988; Cuvin and Furness, 1988). Fluctuation in level would be expected before compounds attain an equilibrium level in the tissues.

**Biotransformation**

Biotransformation generally tends to produce more polar compounds. Although nonpolar metabolites of diazinon accounted for 76% of the radiolabelled material extracted over the course of the uptake phase,
these compounds are all more polar than diazinon itself. Since the radiolabelled material in the water was not separated by TLC before counting, it is not known whether any nonpolar material was excreted unchanged.

There have been a limited number of studies of diazinon metabolism. Ahmad and Forgash (1975) found that diazinon is so rapidly metabolized in gypsy moth larvae that by 16 hours after ingestion almost none remains unmetabolized. Similarly, in the present study, little unmetabolized diazinon was present just 7.5 hours after dosing began. In the study with gypsy moth larvae, diazoxon, the nonpolar oxygen analog of diazinon, was found to reach its maximum level seven hours after ingestion; it declined to 0.3% of radiolabelled material by 16 hours. Pyrimidinol was the major metabolite, reaching a maximum at five hours. This predominance of polar metabolites was not observed in oysters in the present study, and the levels of polar compounds remained statistically unchanged over the 120-hour period.

In a study of degradation of diazinon in the rat, Mucke and coworkers (1970) found that 50% of the radioactivity administered was excreted in 12 hours. They also determined that there was no accumulation in essential organs of the rat of either diazinon or its metabolites. In this study, diazinon was also seen to undergo rapid biotransformation and elimination; although there appears to be some storage in a peripheral compartment, a relatively small fraction of the total radiolabelled material is involved.
The relative slowness of diazinon degradation in water without animals suggests that the rapid breakdown in oysters was enzymatic. Al-Attar and Knowles (1982) observed the same phenomenon when diazinon was incubated in medium without nematodes; 95% of the administered diazinon was unmetabolized after 96 hours. This is nearly identical to the diazinon levels found during the first four hours among radiolabelled compounds in the water in the preliminary study, although by 24 hours 32% of this material was present as nonpolar degradation products. Since the turnover time of water in each flume compartment is less than one hour, changes in diazinon to other compounds would not seem to be due to microbial or other degradation unrelated to the oysters.

Diazinon may be accumulated in moderate amounts by animals in the aquatic environment. Unless exposure is continuous, however, the great majority of the pesticide and its metabolites are rapidly eliminated from the tissue. The BCF for other aquatic species may not be as high as it is for oysters, which are especially efficient bioaccumulators. At the highest concentrations at which diazinon has been reported in natural waters and based on its BCF in oysters, diazinon would not be accumulated sufficiently to be acutely toxic in any species (Goodman et al, 1979; Allison and Hermanutz, 1977).
Diazinon is acutely toxic to oysters at a concentration of 1 mg/L; at this level there is fifty percent less shell growth than in oysters in uncontaminated water and AChE activity is significantly depressed. This concentration is comparable to the LC50 reported in several species of fish (Goodman et al, 1979; Allison and Hermanutz, 1977) and slightly lower than that in others (Sastry and Malik, 1982). In contrast, two crustacean species are more sensitive: e.g. the 96-hour LC50 is 4 µg/L for Mysidopsis bahia and 2 µg/L for Daphnia magna (Eisler, 1986). Certain life stages respond to lower levels of diazinon than others; the 96-hour LC50 in the postlarval and juvenile Japanese knifejaw, Oplegnathus fasciatus, is 25-28 µg/L but it is 5.5 mg/L in the prelarval stage (Seikai, 1982).

Although diazinon concentrations much lower than 1 mg/L may severely inhibit AChE activity in fish, recovery is possible if exposure is not prolonged (Goodman et al, 1979; Weiss, 1961). Recovery of AChE from poisoning by diazinon appears to occur more readily than recovery of AChE inhibited by other organophosphates (Fleming and Bradbury, 1981).

Although exposure concentrations of diazinon as low as 1 mg/L produced a statistically significant decrease in AChE activity in oysters, no concentration up to 10 mg/L was fatal. In another
mollusc, the freshwater snail *Gillia altilis*, the 96-hour LC50 was 3 mg/L (Robertson and Mazzella, 1989). The higher tolerance of oysters reflects the protective value of shell closure during short-term exposure.

A major route of assimilation of diazinon is directly from the water. If diazinon entering a natural body of water becomes sorbed to sediment or organic material, much of it may remain less available for uptake before it is degraded and its toxicity eliminated. Although an equilibrium may be established between sorbed diazinon and that in solution, the amount available for uptake at any particular time would be reduced. Rapid assimilation and degradation by plankton and microorganisms present at high concentrations in the water could also reduce available diazinon rapidly.

Long-term exposure to much lower (by a factor of 1000) diazinon concentrations than those that are acutely toxic affects reproduction, growth, health and sometimes survival in various fishes (Goodman et al, 1979; Allison and Hermanutz, 1977). Spinal deformities were among the most harmful effects. An hypothesis of the present study was that long-term exposure of oysters to diazinon would upset their energy balance and stimulate depletion of energy reserves of glycogen and lipid; this depletion would be reflected in the overall condition index and in reproductive development. Short-term exposure had produced a decrease in shell growth at 0.91 mg/L and eliminated it at 2.03 mg/L. Such inhibited growth is an indication of metabolic stress to which these oysters may have responded with decreased pumping activity. During a prolonged period of metabolic stress, energy
supplied by food is insufficient to maintain metabolism and allow growth to continue (Murty and Devi, 1982; Gabbott, 1976; Gabbott and Walker, 1971). During a long-term exposure to diazinon, both glycogen and lipid energy stores could be called upon to fulfill the oyster's needs.

Exposure to diazinon for 42 days prevented an initial increase in lipid stores at 2.40 and 4.49 mg/L, and in the high concentration, accelerated depletion of glycogen reserves. The condition index in animals exposed to this concentration (4.49 mg/L) decreased below the control level after 42 days, but no similar response was observed at 0.74 or 2.40 mg/L. Long-term depletion of glycogen reserves took place in all animals, including controls, and little reproductive conditioning occurred. The stress of laboratory conditions may have masked the effect of diazinon on these parameters; much lower exposure concentrations than 2.40 mg/L might have impacted glycogen and total lipids significantly over a 42 day period if the animals had not been subjected to a stress which had a more immediate and overwhelming effect on oysters not even exposed to diazinon.

The EC50 in the AChE activity experiment was 8.40 mg/L; in the long-term experiment, the lowest concentration to alter glycogen or lipid content significantly was 2.40 mg/L. This response became insignificant when laboratory stress in all animals caused depletion of glycogen stores resulting in failure of reproductive conditioning and failure of total lipid content to increase for vitellogenesis. In the 96-hour study with shell growth as the endpoint, the EC50 was 1.15 mg/L. The EC50 in this study, which would normally be expected to be
greater than that in the two longer studies, suggests that diazinon in the water caused oysters to respond by merely closing their shells more and more in successively higher concentrations, thus avoiding the diazinon rather than assimilating it.

In the two longer studies, the animals were unable to remain closed during the entire period and therefore did respond to the presence of diazinon with inhibition of AChE activity and some changes in glycogen and lipid content. The amount of actual pumping activity may not, however, have been the same in different diazinon concentrations. The water transport rate of an individual oyster can vary tremendously depending on its environment; Galtsoff (1964) reported that one oyster transported 77 L during one day and 457 L during the next; another oyster pumped 8.6 L on one day and 239 on the next. A decrease in pumping activity with increasing diazinon concentration has frequently been observed (personal observation). If the oysters did indeed respond to increases in diazinon concentration with corresponding decreases in pumping activity, then an oyster in a high exposure concentration could encounter a much smaller percentage of the nominal dose than an oyster exposed to a low diazinon concentration.

A related factor is the rapid degradation of diazinon in the tissues to non-toxic biotransformation products. Diazinon is broken down so rapidly by the oyster that within eight hours more than 90% of it has disappeared. During periods when the oyster is closed, therefore, the concentration in the tissues, and hence the effective
exposure concentration, can rapidly decrease to well below what it would be if the animal were forced to remain open.

The toxicity of diazinon to the oyster may thus depend on the relationship between the amount of time spent open and actively pumping and the amount of time spent closed and degrading diazinon to relatively harmless breakdown products. Further, it is possible that, along with the reduced assimilation rate resulting from avoidance behavior, there could have been activation of the detoxication mechanism of diazinon so that it would be degraded faster as exposure continued (Stebbing, 1985). In a long-term experiment it is possible that a physiological adaptation, resulting in rapid detoxication, along with prolonged periods of isolation, allowed oysters to withstand surprisingly high water concentrations of diazinon. The actual exposure concentrations that produced long-term responses may therefore have been much lower than they appear to be. The avoidance mechanism of oysters may be one reason fish and other animals unable to escape constant exposure to diazinon in a laboratory environment often show greater sensitivity to the pesticide during long exposures.

The BCF values reported for diazinon in fish are usually significantly higher than the BCF value found here for oysters (Kanazawa, 1975, 1978; Seguchi and Asaka, 1981; Goodman et al, 1979). An organism responds ultimately to the tissue concentration of a chemical; therefore a relatively low BCF is another factor which must be considered in interpreting the high exposure concentrations required to cause long-term effects in oysters.
As predicted, diazinon is degraded rapidly and completely and the great majority of its biotransformation products are also eliminated quickly in oysters. This rapid breakdown is also reported in other species (Al-Attar and Knowles, 1982; Ahmad and Forgash, 1975; Mucke et al, 1970). The relative stability of diazinon in water without animals, where degradation occurs over a period of days rather than hours, provides evidence that the rapid breakdown observed in oysters and other species is enzymatically catalyzed. In the channel catfish Ictalurus punctatus, the maximum metabolic activity was isolated in the microsomal fraction of the liver (Hogan and Knowles, 1972).

Although diazinon's solubility in water is 40 mg/L (Ciba-Geigy, 1975), hydrolysis to non-toxic metabolites begins within 24 hours. Microbial breakdown enhances total degradation in natural water although chemical degradation is predominant (Sharom et al, 1980; Sethunathan and Pathak, 1972). Although the half-life of diazinon in water normally varies from two to six weeks depending on pH (Sharom et al, 1980; Cowart et al, 1971), the concentration available for uptake by animals may be limited by the fraction in solution rather than sorbed to sediment or organic matter.

Most applied diazinon is retained or degraded in the soil so that, after a short time, little is found in runoff (Ritter et al, 1974; Munson, 1970). Even though field runoff concentrations of diazinon up to 82 µg/L have been measured, concentrations observed in natural water have not exceeded 0.10 µg/L (Schulze et al, 1973). Diazinon usually does not find its way into the ground water because, under normal conditions, it is retained in the top four inches of soil.
(Munson, 1970). If diazinon enters a body of water through inadvertent spraying, this short-term localized exposure is one from which mobile animals can escape and against which organisms like bivalves can close their valves if the concentration attained is high.

Diazinon limits for the protection of aquatic life have been recommended by two advisory groups. The International Joint Commission set a value of 0.08 µg/L in 1975 and the National Academies of Science and Engineering (1973) suggested a limit of 0.009 µg/L. The lowest diazinon concentration that appears to have any adverse effect on oysters is more than 10,000 times greater than the higher recommended limit. An additional safety factor in comparing laboratory findings with possible field concentrations is that technical grade diazinon, the formulation usually employed in laboratory experiments, is generally more toxic than the emulsifiable concentrates, dusts and oil solutions used in agriculture (Eisler, 1986).

The long-term effects on other species which have been reported at diazinon concentrations considerably lower than those detrimental to oysters have been elicited at concentrations at least four times the higher suggested limit (Arthur et al, 1983; Goodman et al, 1979; Allison and Hermanutz, 1977). Few studies investigating long-term effects of fluctuating and intermittent exposures of fish and invertebrates to diazinon, which might reflect a more realistic environmental situation, have been done (Allison and Hermanutz, 1977).
Conclusions

The potential hazard of diazinon exposure in the aquatic environment is limited by both its bioavailability and its persistence. Diazinon's persistence is a factor of its physical characteristics. It is not volatile and has a low enough water solubility that under normal conditions diazinon tends to remain in the soil rather than be carried away in runoff. At concentrations reported in soil and runoff water, the concentrations finally attained in ponds and lakes, streams and estuaries, and in the sea would not reach levels that have ever been shown to be either acutely or chronically toxic to any species. Long-term effects on oysters are extremely unlikely at these concentrations or even concentrations ten-fold higher, because the oyster has the ability to isolate itself from the environment and thus decrease the exposure concentration. Other species may be more susceptible to long-term effects but apparently require continuous exposure to a uniform concentration for at least 40 to 60 days; this type of exposure could occur only in unusual circumstances, such as a point discharge situation. Data on the effects of intermittent exposures of the type more likely to occur in nature are needed to establish realistic safe concentration limits.

The environmental threat of diazinon is further mitigated by the completeness and speed of its degradation by organisms which assimilate it. Although it may be accumulated in moderate amounts by aquatic animals, very little is actually stored in the lipids. Recovery of AChE from inhibition by diazinon is possible. Within 48
hours after exposure ends, more than 90% of the diazinon accumulated by oysters had been either degraded or eliminated and its only toxic metabolite, diazoxon, is degraded equally rapidly. Most of diazinon's biotransformation products, both polar and nonpolar, are as rapidly depurated as the parent compound.

The highest concentrations of diazinon that have been found in natural water are less than 0.10 μg/L (Schulze et al, 1973). Exposure concentrations 1000 times higher have produced no toxic response in oysters. Diazinon does not appear to be an environmentally threatening compound if used properly because it persists in water only two to six weeks (Cowart et al, 1971; Sharom et al, 1980) and long-term effects of diazinon on any aquatic species studied to date require much longer exposure (e.g. Goodman et al, 1979; Allison and Hermanutz, 1977).
APPENDIX A

GAS CHROMATOGRAPHIC METHOD FOR DIAZINON
(modified from Goodman et al, 1979)

1. Water extraction
   a. Extract water with two volumes of methylene chloride (25 ml each for 200 ml water sample, 75 ml each for 750 ml sample).
   b. Combine extracts and concentrate to 10-20 ml in Roto Vap.
   c. Add internal standard HHDN (aldrin) to produce peak of approximately same size as that of the diazinon.
   d. Concentrate to desired volume (0.2 to 4.0 ml depending on diazinon concentration).

2. GC Operating Conditions (FID detector):
   - Column temperature: 175°C
   - Injection temperature: 310°C
   - Detector temperature: 330°C
   - Carrier gas: Helium
APPENDIX B

ACETYLCHOLINESTERASE ACTIVITY ASSAY PROCEDURE
(modified from the methods of Hill and Fleming, 1982
and Ellman et al, 1961)

1. Weigh and homogenize the whole heart in 0.05M, pH 8.0 Tris
buffer in the ratio of 100 mg/ml.

2. To a photocell with a 1 cm lightpath add:
   3.0 ml dithiodinitrobenzoic acid in Tris buffer (DTNB)
   200 µl of heart homogenate
   100 µl of the substrate, acetylthiocholine iodide in
deionized water (ASChI)

3. Mix final solution with vortex. Allow reaction to stabilize
   for 20-30 seconds.

4. Read the absorbance (A) at exactly 30-second intervals for 3
   minutes with a Beckman Spectronic 20 spectrophotometer set at
   a wavelength of 405 nm.

5. Calculate theΔA/min.

6. Calculate rate of yellow color production as follows:

\[
R = \frac{\Delta A/min}{1.36 \times 10^4} \times \frac{1}{(200/3300)C_0}
\]

where R = rate, in moles substrate hydrolyzed per min per
   g of tissue
ΔA = change in absorbance
   C_0 = original concentration of tissue (mg/ml)

The extinction coefficient of the yellow anion of DTNB
at 405 nm is 1.36 \times 10^4, the volume of homogenate is 200
ul and the total reaction volume is 3300 ul.

...
APPENDIX C

Determination of Total Lipid
(modified from the method of Bligh and Dyer, 1959)


2. Filter the above by vacuum into a flask. Use only a small amount of vacuum. After filtration, cut up filter paper, place in original beaker, add MeOH:CHCl₃:DH₂O (2:1:0.8) and stir for 30 min. Refrigerate filtrate while stirring.

3. Filter second extract and combine with first. Add CHCl₃ and DH₂O so that final ratio is 1:1:0.9 (MeOH:CHCl₃:DH₂O).

4. Swirl the mixture and pour into a separatory funnel. Let it stand for at least 45 minutes, refrigerated, so the layers can separate.

5. Draw off the lower CHCl₃ lipid layer into a centrifuge tube and save. Discard the rest.

6. Centrifuge the CHCl₃ layer at 3000 rpm in a refrigerated centrifuge for 10 min. Pipet off any excess DH₂O and MeOH that have risen to the top. Add a small amount of anhydrous Na₂SO₄ to the tube and centrifuge again for 10 min at 3000 rpm.

7. Pour CHCl₃ through phase separating paper into a graduated cylinder. Record volume of CHCl₃ layer. Measure out three 8-mL samples into tared weighing pans; place these in a hood to evaporate to dryness overnight.

8. Weigh samples. Calculate total lipid as:

\[
\text{Total lipid (mg)} = \frac{\text{dry sample wt (mg)} \times \text{total lipid vol (mL)}}{\text{sample vol (mL)}}
\]
APPENDIX D

Determination of Glycogen
(modified from methods of van Handel, 1965, and Harleston, 1971)

1. Homogenize meat. Place 100-150 mg in a tared centrifuge tube and weigh on an analytical balance.

2. Add 3 ml of 30% KOH to the homogenized sample and heat for 30 min in a boiling water bath. Be sure sides of tube are free of meat.

3. Remove from water bath and dilute with 5 ml hot distilled water.

4. Refrigerate until completely cool, then add 0.05 ml Na$_2$SO$_4$ (saturated solution) and 5 ml 95% EtOH. Cover securely with parafilm and invert centrifuge tube several times to mix thoroughly.

5. Place in refrigerator for 12-24 h.

6. Centrifuge at 2000 rpm for 5 min; discard supernatent and wash twice with 2-ml portions of 95% EtOH. Discard washings and invert in test tube rack for 10 min.

7. Dissolve glycogen precipitate by adding 10 ml hot dissolved water and mixing on Vortex. Be sure mixing is complete.

8. Pipet 0.1 ml into a test tube. Add 4 ml anthrone reagent.

9. Mix on Vortex and heat for 10-12 min at 90°C in a water bath. Be sure tubes are covered.

10. Cool to room temperature and read absorbance on a spectrophotometer at 620 nm.

NOTES:

1. Glycogen standards that will produce a standard curve with concentrations of glycogen from 0.005 to 0.075 mg are prepared and read with each set of samples.

2. Anthrone reagent: add 7.6 ml concentrated sulfuric acid to 30 ml distilled water while stirring and cooling. Add 0.15 g of recrystallized anthrone to this, stirring until anthrone dissolves. Make reagent fresh on the day samples are run.

3. Determine the amount of glycogen in each sample from the standard curve. The dilution factor for each sample is 100.


