AN ABSTRACT OF THE THESIS OF

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Title: SOME BIOLOGICAL EFFECTS OF THE INSECTICIDE SEVIN ON MARINE BIVALVES

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Experiments were performed to study three aspects of Sevin poisoning in marine bivalves: 1) The toxicity of Sevin in the field to indigenous clam populations; 2) The pathological effects of Sevin to adult clams, Macoma nasuta; and 3) The effects of Sevin on early development of embryos of Mytilus edulis.

Sevin significantly reduced numbers of juvenile clams in plots treated with 5 lb (2.3 kg) and 10 lb (4.6 kg)/acre. Pooling of samples taken at 1, 2, 4, 15 and 30 days after treatment showed mean clam numbers per m^2 in untreated plots and those treated with 5 and 10 lb/acre were 364, 283, and 224, respectively, the reductions from the controls being 22 and 38%. Clam species differed in susceptibility to Sevin; for example, numbers of Tresus capax were reduced by 58 and 69% at the low and high application rates in relation to those from the control plots, and Macoma nasuta by only 9 and 28%. There was no reduction in numbers of polychaete and nemertean worms.

A Sevin application of 5 lb/acre was as effective in controlling ghost shrimp (Callianassa californiensis), an oyster pest, as 10 lb/acre.

Toxicity tests of 96-hr duration were done with adult bent-nosed clams, Macoma nasuta. Sevin concentrations of 15, 20, 25, and 30 mg/liter were used in duplicate tests. The criterion of "death" was the inability of clams to retract siphons or to close valves. About half of the animals so affected were removed from the test solutions and returned to clean seawater to observe if recovery occurred, and others were preserved for histological examination.

The 48 hr and 96 hr TL_{50}'s for the clams were estimated to be 27.5 and 17.0 mg/liter, respectively. No "dead" clams recovered within 96 hr after return to clean water. The histopathology consisted primarily of necrosis of epithelial tissue of the gill, mantle, siphon and suprabranchial gland and the severity of damage was directly related to the test concentrations. Vacuolization, rupture, and pyknosis of cells occurred. The gills were the most severely affected organs. Epithelial cells of the gill filaments bearing the frontal, laterofrontal, and lateral cilia were sloughed as early as 24 hr after the beginning of exposure to Sevin. About 50% of the exposed clams had lost one or both siphons and also the epithelia on still attached segments within 96 hr of exposure. There were no deaths of control clams, and their tissues were normal.

Seven developmental stages of Mytilus edulis, from fertilization to 32 hours after fertilization and including still unfertilized eggs, were exposed for 1 hour to concentrations of Sevin and its hydrolytic product, 1-naphthol. After exposure, the larvae or eggs were separated from the pesticide solution and returned to clean water. At 48 hours after fertilization a count of normal vs. abnormal larvae was made and 1-hour EC_{50} values calculated.

The most sensitive stage of development to Sevin occurred shortly after fertilization at the appearance of the first polar body, and susceptibility declined as the age of the larvae increased. The EC_{50} values for the first polar body stage and 32-hour stage were 5.3 and 24.0 mg/liter, respectively. Effects of toxicants on development were characterized by disjunction of blastomeres, a reduction in the rate of development, and unsynchronous and unaligned cleavages.

Some Biological Effects of the Insecticide Sevin on Marine Bivalves

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Typed by Opal Grossnicklaus for David Allen Armstrong

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The time during which this study was performed represents a lot more living than is told of in the prosaic language of this thesis. So in the short space allotted, I will tell of people and events meaningful to me as I did this work.

My wife Jan travelled to the edge of Oregon with me as a scientist, explorer, and bestower of womanly love. She carried mud from my field plots and also carried two fine babies, despite our sophisticated knowledge of contraception.

Raymond Millemann, my major professor, taught me to interject a bit of whimsy into even the most serious of human efforts, and to at least minimize the amphigorical pursuits I sometimes dedicate myself to. As an editor, he reduced an American epic to my thesis.

My excellent friends Freeman Button, David Walker, and Steven Williams rode in the early mornings, past the herons and cormorants, to the field plots where my results lay buried in the mud and helped me dig them out.

Becky Knutson, who, with forbearance, has long typed the genius of mice and men, was forced to read my epic a dozen times as she translated my hieroglyphics into English script. Dr. Jeffersen Gonor was of great assistance, and always shared his ideas with me and listened to the few he thought I had. Dr. Peter Doudoroff used his thorough command of English to transform

the beast into beauty.

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SOME BIOLOGICAL EFFECTS OF THE INSECTICIDE SEVIN ON MARINE BIVALVES

INTRODUCTION

Physical and Biological Properties of Sevin

Sevin, also known as carbaryl, is the registered name for the insecticide 1-naphthyl methylcarbamate (Union Carbide, 1963), and conforms to the general carbamate formula $(CH_3)\ R_1NC(O)OR_2$ (Casida, 1964). In organisms, Sevin is known to inhibit the enzyme acetylcholinesterase (Casida, 1963) thus disrupting nerve transmissions. Sevin enters organisms through either the integument or the alimentary tract. While highly toxic to arthropods, against which it is primarily used, the toxicity of Sevin to vertebrates is low as compared with that of other insecticides. For example, DDT and malathion are 190 and 7 times more toxic, respectively, than Sevin to coho salmon (Macek and MacAllister, 1970), and dieldrin is 40 times more toxic to rats exposed percutaneously than is Sevin (Union Carbide, 1963).

Sevin in sea water is most rapidly degraded at high temperatures and pH's and in the presence of light (Karinen et al., 1967). At 17°C, 44% of Sevin initially present in solution was hydrolyzed in 4 days, whereas hydrolysis of that which accumulated in the underlying mud was much slower. The first product of hydrolysis is

1-naphthol, which is more toxic to marine mollusks and fishes than Sevin, but less toxic to crustaceans than its parent compound (Stewart et al., 1967). The breakdown of 1-naphthol in seawater is accelerated at high temperatures and pH's and by O_2 and light (Lamberton et al., 1970). Microorganisms also aid in the degradation of both Sevin and 1-naphthol in seawater. When Sevin was labeled with C^{14}, up to 30% of the total initial radioactivity was recovered as $^{14}CO_2$ (Karinen et al., 1967). Lamberton et al. (1970) using ^{14}C labeled 1-naphthol recorded evolution of $^{14}CO_2$ only under unsterile conditions, and found that light increased the amount of labeled $^{14}CO_2$ eightfold. These results indicate that Sevin and some of its breakdown products can be utilized as an energy source by microorganisms.

Among compounds resulting from degradation of 1-naphthol in seawater is a reddish precipitate of molecular weight 450 (Lamberton et al., 1970). They found this compound to be 2/3 as toxic as 1-naphthol to bay mussel embryos, and surmised that the compound inhibited acetylchol inesterase activity.

Use of Sevin in the Field

The direct application of Sevin in the marine environment has been on substrates used for oyster rearing, where the suitability of the land has been altered or is threatened by pests and predators (Butler et al., 1968; Chambers, 1970). On the Atlantic coast,

Haven et al. (1966) used Sevin in combination with the pesticide Polystream in an unsuccessful attempt to control oyster drills in Chesapeake Bay. Loosanoff (1960) found that as part of a chemical barrier to prevent movement of molluskan predators on to oyster beds, Sevin caused four species of gastropods to swell and protrude from their shells.

In bays of the Pacific Northwest, Sevin has been used successfully to control two species of burrowing shrimps that inhabit mudflats used for oyster culture. These shrimp, Callianassa californiensis and Upogebia pugettensis, construct extensive burrows leaving the ground in a very porous condition (MacGinitie, 1930, 1934). At typical densities of 2.4×10^5 to 3×10^6 per acre, the activities of the shrimp in maintaining their burrows can prove lethal to the oysters (Snow and Stewart, 1963; Chambers, 1970). Lethality is due to excessive soil turnover that covers young animals, or to sinking of adult oysters into the substrate because of reduced firmness of the soil; in both cases the oysters die of anoxia.

There have been only two reported field studies of Sevin's toxicity to oyster pests and non-target animals (Snow and Stewart, 1963; Washington Department of Fisheries, 1970), and in neither study were quantitative data on the effects of the treatment on indigenous clams given. Among non-target animals usually affected are cockle clams (Clinocardium nuttalli) and, within 24 hours later,

Dungeness crabs (Cancer magister). In a study by Buchanan et al. (1970), 100% of adult Dungeness crabs that had eaten cockle clams just previously exposed to Sevin at 10 mg/liter, were irreversibly paralyzed within 6 hours. I performed a field study to determine: (1) whether application of Sevin to mud flats would reduce the numbers of juvenile clams and other invertebrates; and (2) whether reduction, if any, would be greater at the standard rate of application of 10 lb/acre than at half this amount. Also, a more limited study was conducted to determine if 5 lb/acre was as effective as the higher rate in controlling the ghost shrimp, Callianassa californiensis.

Histopathology Caused by Sevin

There are only two reported studies of histopathology in animals exposed to Sevin. Lowe (1967) described the histopathology in fish (Leiostomus xanthurus) chronically exposed to this pesticide, and Hassanein et al. (1968) reported on the histopathology in spiny bollworm larvae acutely poisoned with several insecticides, including Sevin.

Experiments were performed to study histopathology in the bent-nosed clam, Macoma nasuta, after exposure to Sevin. I chose this mollusk as a test animal because it is abundant in estuarine intertidal mud flats and occurs in close association with oysters and oyster pests such as burrowing shrimp.

Effects of Sevin on Bivalve Larval Development

Stewart et al. (1967) found the 48-hour EC_{50} value (the concentration of pesticide causing a predefined effect in 50% of the test animals in the specified exposure period) of Sevin for mussel larvae, Mytilus edulis, to be 2.3 mg/liter. Butler et al. (1960) found that 1 ppm of Sevin reduced oyster shell deposition by 50%. Davis (1961) reported that 5 ppm of Sevin completely prevented development of oysters (Crassostrea virginica) and clams (Venus mercenaria) to the straight hinge larval stage. Butler et al. (1968) found that concentrations of Sevin greater than 0.8 mg/liter killed all larval cockle clams by 3 days, and yet the 96-hour TL_m for juvenile clams was 3.85 mg/liter, indicating greater susceptibility of larval stages to Sevin.

Use of larvae of the bay mussel, Mytilus edulis, for bioassay purposes was proposed by Dimick and Breese (1965), and such tests with this animal have been performed by Breese et al. (1963), Stewart et al. (1967), and Granmo (1972). The developmental sequence of this animal has been well documented by Field (1922).

Typically, effects of toxicants on embryos and early larvae are measured in bioassays as percentages of test animals exhibiting anomalous development by the D-shaped or straight-hinged veliger stage, about 24 to 48 hours from fertilization for oysters and mussels and clams, respectively. Eggs are either fertilized directly in test solutions or embryos are introduced at the 2-cell stage, and the

cultures are then incubated for the above periods of time. When controls have become shelled veligers, the cultures are killed and the numbers of anomalous and normal larvae are counted at each concentration to derive estimates of median effective concentrations (EC_{50}'s). With this method it is not possible to observe variation of different embryonic and larval stages in their susceptibility to the toxicant tested.

Experiments were performed to determine the most sensitive stage in development to 48 hours of M. edulis larvae exposed to Sevin and its first hydrolytic product, 1-naphthol. The exposure period of 1 hour used in these tests was considered to approximate field exposures to Sevin more closely than a period of 48 hours does. After typical application of Sevin in the field (Washington Department of Fisheries, 1970) tidal action would quickly dilute the pesticide in bays, and it is not likely that larvae would be subject to high concentrations of the pesticide for longer than several hours.

METHODS AND MATERIALS

All the Sevin used in these experiments was an 80% active ingredient wettable formulation, and the 1-naphthol was recrystallized alpha naphthol. The solubility of Sevin in sea water at 20° C is 60 mg/liter (Dale Coulsomb, Stanford Research Institute, personal communication).

Field Study

Six plots 3.7 m (12 ft.) square were established in the Sally's Bend area of Yaquina Bay, Oregon (Figure 1). In the middle of each plot, five subplots adjacent to each other were marked with stakes and rope, and each measured 50 X 150 cm. To minimize variation in substrate type and resident animal species, the four experimental plots were located 4.6 m (15 ft.) apart, and two control plots were 9.1 m (30 ft.) from these. Plots were located at a 0.0 m tidal level in a line at right angles to the flow of ebb and flood tides, so as to reduce the transport of residual Sevin from treated to control plots.

Two plots were treated with Sevin at the rate of 10 lb/acre, two at the rate of 5 lb/acre, and the remaining two served as controls. The insecticide was applied by passing a perforated bucket containing a suspension of Sevin in sea water over the plots until they were covered uniformly. Plots were treated on May 14, 1972 during

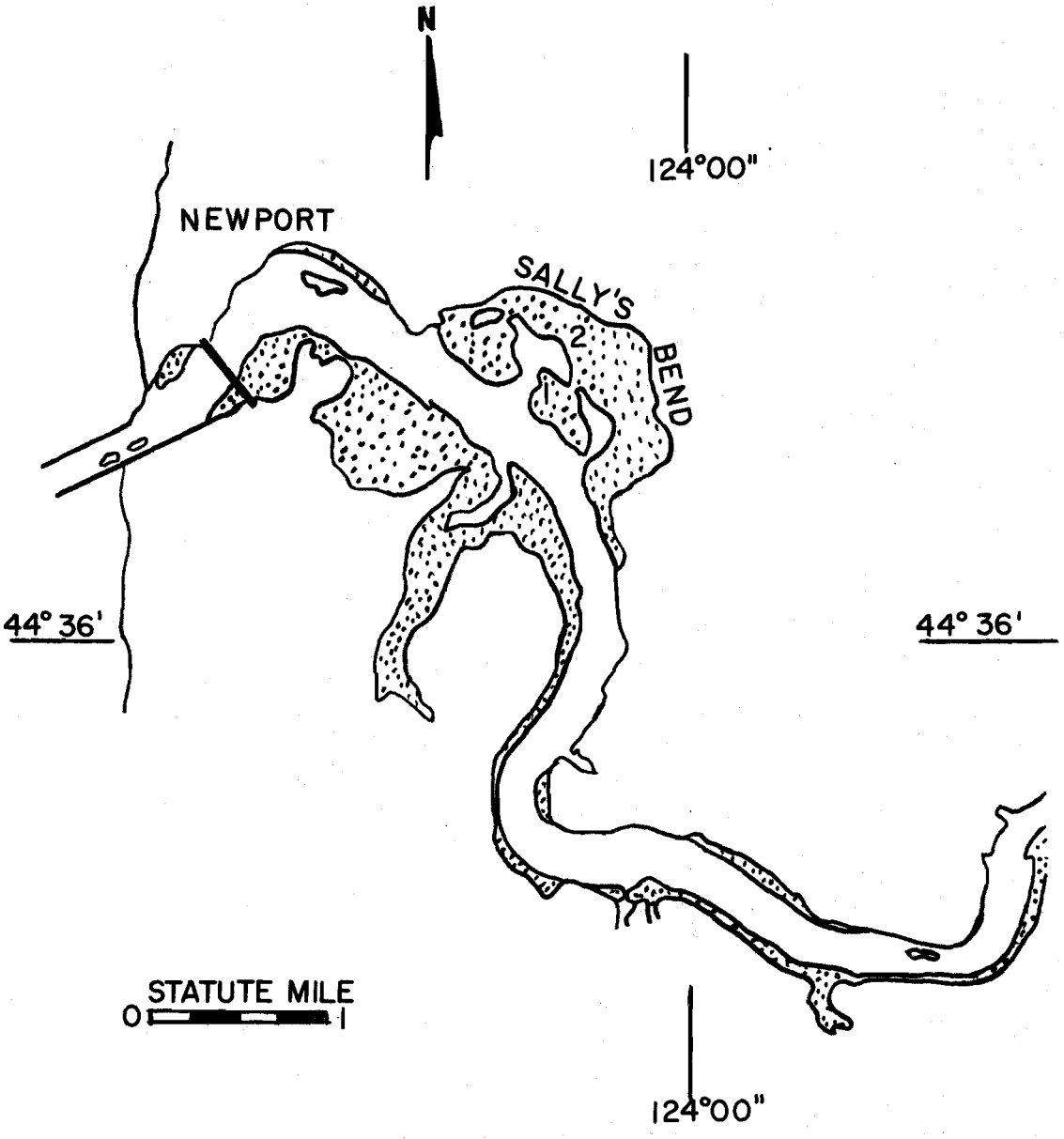


Figure 1. Map of Yaquina Bay, Oregon, showing site of field studies with Sevin. 1 - clam survival site. 2 - shrimp reinfestation.

low tide and remained uncovered, except for water left in depressions, for 2.5 hours. The temperature of the water remaining on the plots was 16° C. The concentration of Sevin in this water immediately after treatment was not determined, but was less than 60 mg/liter.

An entire subplot in each of the six plots was sampled on days 1, 2, 4, 15 and 30 after treatment; the total area sampled for each type of treatment was 7.5 m^2. In sampling, all mud was removed to a depth of 12.7 cm (5 inches), thus retrieving most juvenile clams of the 0-year age group. Samples were washed over a screen of 1.5 mm mesh in the field, and the animals were sorted and numbers of each species recorded in the laboratory. The data were subjected to two-way analysis of variance (Snedecor and Cochran, 1967) to determine the effect of treatment at the two application rates on the survival of animals to 30 days, and treatment means were compared by the method of least significant differences (LSD).

The Japanese littleneck clam, Venerupis japonica, was introduced onto all plots 1 day before treatment. The effect of Sevin on this clam's survival was of interest because of its recent introduction into Oregon bays. Eighty clams were placed on each subplot, and observation of these animals after planting confirmed that they all burrowed into the substrate.

To compare the effectiveness of the two rates of application in

controlling shrimp, three 3.7 m square plots were established in an area of Yaquina Bay that is heavily infested with C. californiensis (Figure 1). On July 9, 1971, two plots were treated at 5 and 10 lb/acre, and the remaining plot was the untreated control. In June 1972 these plots were examined for recolonization by ghost shrimp. Shrimp densities were estimated by counting their burrows in randomly selected areas of about 1 ft. square (0.1 m square) each and effect of treatment was compared using Student's t tests.

Pathological Study

Clams were collected from Yaquina Bay, Oregon, and used in tests within 72 hours. The mean wet weight (without the shell), the length, and the width of 40 clams used were 2.5 gm, 40 mm, and 30 mm, respectively.

The experiments were of 96 hour duration. The test vessels were 5-gallon (19.0-liter) glass jars, each containing 12 liters of pesticide solution. Before use, the clams were acclimated for 24 hours to the test conditions of standing, aerated seawater of 25‰ salinity at 16° C. Eight clams were then placed in each jar, so that the resulting weight/volume ratio of 1.65 gm of tissue/liter of solution approximated the recommended value of 1.0 gm of tissue/liter of test solution for bioassays with fish (American Public Health Association et al., 1971). The seawater used for acclimation and

for the tests was filtered through sand to remove particles larger than about 2.0 μ. This was done to minimize food consumption by the clams.

Two 96-hour bioassays were performed, using Sevin concentrations of 15, 20, 25 and 30 mg/liter, each in duplicate tests, and solutions were renewed every 24 hours. The clams were considered dead when they failed to retract their siphons and close their valves upon mechanical stimulation. However, the heart beat and respiration of these clams probably had not ceased completely, because the animals still showed slight, sporadic movement. Therefore complications from post-mortem changes were not seen in histological sections.

About half of the "dead" clams were returned, as soon as they were observed, to clean, circulating seawater for post-exposure observations, and the others were removed intact from their shells and fixed for 12 to 18 hours in seawater-Bouin's solution. To avoid difficulties in sectioning due to denaturation of the crystalline style proteins, a small dorsal portion of the digestive gland or the end of the foot was severed and the style gently removed. This probably facilitated rapid penetration of the fixative via the style sac. Ten control and 32 experimental clams were embedded in Paraplast, and sagittal or frontal section 8 μ in thickness were cut. They were stained with Mayer's hematoxylin and eosin stain.

The TL_{50} values (the concentrations that caused "death" of 50% of the test animals in the specified exposure period) were calculated as described by the American Public Health Association et al. (1971).

Embryological Study

Tests were carried out between July and October in 1971 and 1972. Adult mussels were spawned in individual finger bowls as described by Dimick and Breese (1965). After spawning, adults were removed from the bowls and water containing the eggs or sperm was poured through a 120 μ or 33 μ sieve, respectively to remove debris and pseudofeces. The segregated gametes were kept at 10° C no longer than 4 hours before use in tests.

Bioassays were performed as 1-hour exposures of seven developmental stages of M. edulis (Table 3) to several concentrations of Sevin or its hydrolytic product, 1-naphthol. All tests were performed in 250 ml beakers with sand-filtered seawater at 17±1° C adjusted to 25‰ salinity. Five duplicated concentrations of pesticide (Table 3) plus a centrifuged and noncentrifuged control were used in tests with each developmental stage. Development was allowed to proceed for 48 hours before a count of normal larvae (D-shaped veligers) and abnormal ones was made. Tests were used only when normal development exceeded 85% in both controls.

For exposure of unfertilized eggs to Sevin and 1-naphthol, about 2,000 eggs were placed in each of two beakers containing 200 ml of pesticide solution of each concentration tested. At the end of one hour all but about 30 ml of the pesticide solution was decanted. The remaining solution with eggs was poured into test tubes and centrifuged by hand for 30 seconds at low speed. The supernatant was poured off and the eggs quickly returned to 200 ml of clean water. It was calculated that no more than 1 ml of the pesticide solution was transferred with the eggs (and all other developmental stages) to the 200 ml of clean water, which resulted in approximately 99.5% elimination of the pesticide. The eggs were immediately fertilized with 1 ml of concentrated sperm suspension. Thirty minutes after fertilization, most of the water in a beaker was decanted to eliminate excess sperm, and the volume was returned to 200 ml. Periodic samples of animals were examined during the 48 hours to compare the development of control and treated groups.

To expose different developmental stages of embryos, 2,000 eggs per beaker were fertilized in 150 ml of clean seawater. At the times indicated in Table 3, 50 ml of pesticide solution, at a concentration 4 times the desired test level, were added to the 150 ml of clean water in each beaker. At the end of 1 hour the pesticide solution was removed as previously described. Animals exposed at 8 hours or more after fertilization were ciliated and swimming.

Therefore, the entire 200 ml of solution in each beaker had to be centrifuged in order not to lose animals. The process of separating animals from the pesticide solutions required 5 to 10 minutes. Embryos and larvae were returned to clean seawater for the duration of the 48 hour developmental period. Data was graphed as the percent normal development on a probit axis and pesticide concentration on a log axis. Straight lines were fitted by eye to determine EC_{50} values, i.e., the concentrations causing 50% abnormal development after given exposure periods.

Several experiments involving exposure of unfertilized eggs and first polar body stage animals to Sevin concentrations as high as 60 mg/l for 1 hour were performed to measure delays of early cleavage. Also, eggs and sperm were added directly to test solutions of concentrations up to 60 mg/l to determine the effect of Sevin on fertilization. At the end of 1 hour, cultures were checked only for fertilization.

RESULTS

Field Study

Sample Analyses

There were significant reductions (α=0.01) in the total numbers of the five common species of clams on the treated plots (Table 1). The mean numbers of these animals per m^2 in the 5 and 10 lb/acre plots were 22 and 38% less (α=0.01) than those in the untreated plots, and the difference between the two rates of application (21%) was also significant (α=0.01). The inverse relationship between the rates of application and the numbers of clams surviving was consistent for the five sampling periods (Figure 2).

The findings of smaller numbers of animals (Figure 2) in the six subsamples taken on day 30 than in those taken on the four previous days in the same plots, may be attributed to the order in which subplots were sampled. Those sampled on day 30 were between subplots previously excavated, so that the sampling areas were elevated strips of ground subject to tidal action, and some of the clams may have been lost by erosion and predation.

Analysis of variance (ANOVA) of the data on the interaction of time with treatment for single species showed that significant interaction occurred only in the case of the gaper clam (Table 2). Thus, levels of Sevin lethal to the other clams did not persist, and most of

Table 1. Species and their total numbers in mud samples from untreated field plots and plots treated with Sevin at rates of 5 and 10 lb/acre. There were two plots for each test condition and each plot was sampled five times during a 30-day period.

	Species	Sevin concentration (lb/acre)[1]			% difference between
		0	5	10	5 and 10 lb/acre
Bivalves					
Macoma nasuta	(Bent-nose clam)	1,744	1,586 (9)	1,262 (28)	20
Macoma incongrua	(Incongruous macoma clam)	154	129 (16)	84 (44)	33
Macoma inconspicua	(Inconspicuous macoma clam)	382	245 (36)	197 (49)	19
Tresus capax	(Gaper clam)	301	125 (58)	94 (69)	25
Venerupis japonica	(Japanese littleneck clam)	186	89 (52)	73 (61)	18
	Subtotal	2,767	2,174 (22)[2]	1,709 (38)[2]	(21)[2]
Transenella tantilla		26	11	12	
Psephidia lordi		5	4	4	
Clinocardium nuttalli	(Basket cockle clam)	9	2	5	
Venerupis staminea	(Pacific littleneck clam)	7	1	1	
Mya arenaria	(Soft-shelled clam)	4	2	2	
Polychaetes					
Nephytys sp.		16	17	15	
Haploscoloplos elongatus		174	148	156	
Goniana brunnea		66	60	64	
Spionid sp.		10	28	20	
Nemerteans					
Cerebratulus sp.		5	4	4	
	Totals	3,089	2,451	1,995	

[1] The numbers in parentheses are the percent reductions based on the numbers of animals in the control plot.

[2] The differences in total animal numbers between the control and treated plots and between the two treated plots are statistically significant (α=0.01).

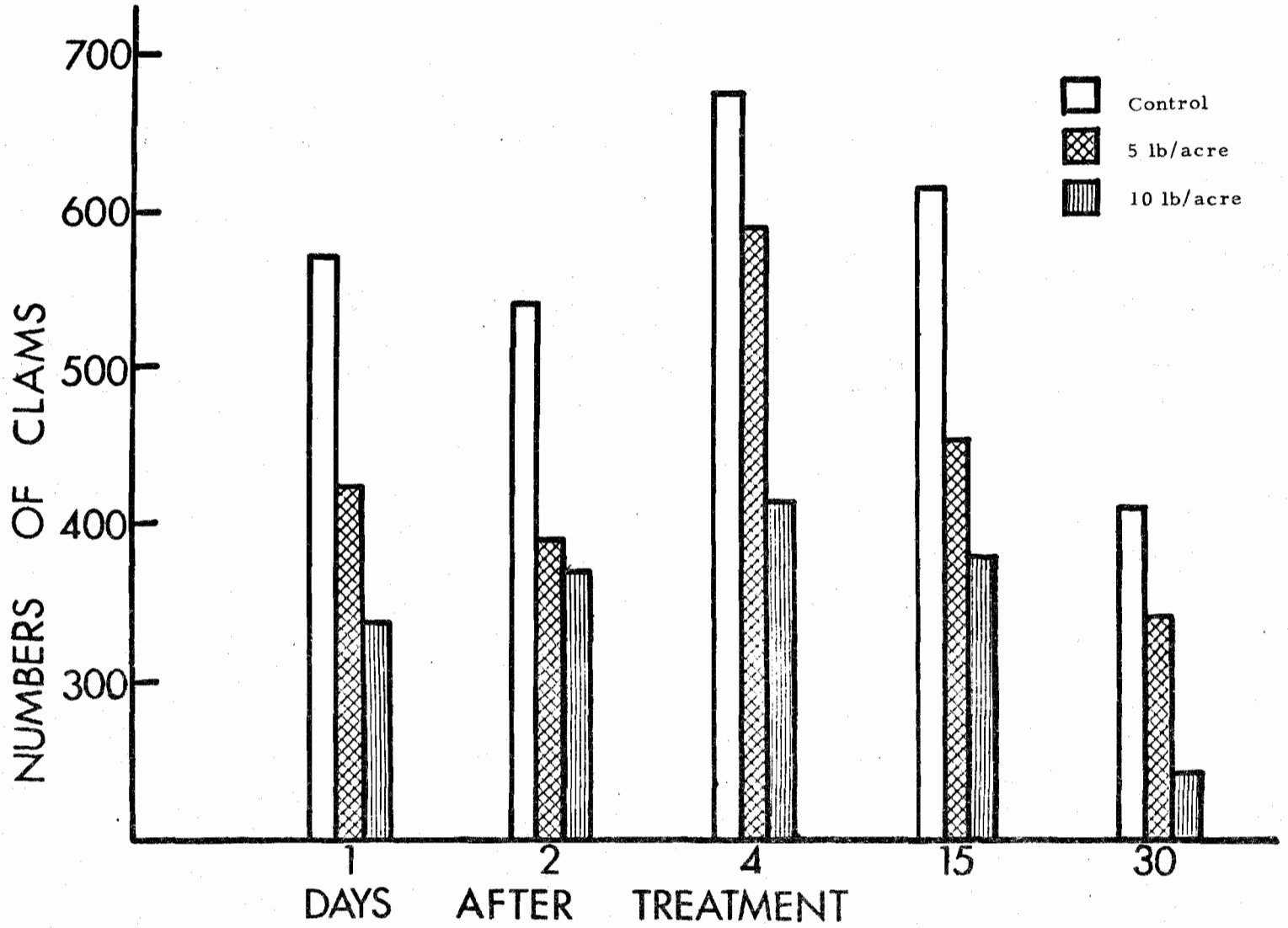


Figure 2. Numbers of clams remaining alive in duplicated field plots treated with Sevin at rates of 5 and 10 lb/acre.

the deaths occurred within the first 24 hours. There was no apparent reduction in numbers of remaining animals in the samples, polychaete worms and a few nemertean worms (Table 1). The significance of "time" in the ANOVA simply indicates variation in the distribution of animals within plots and "time" is not a variable affected by treatments.

The lower rate of Sevin application was about as effective as the higher in controlling ghost shrimp. After 11 months, the mean numbers of shrimp holes/m^2 were 161, 32 and 25 in the control plot and 5 and 10 lb/acre treatment plots. The difference between the control plot and either of the two treated plots was significant (α=0.01), but there was no significant difference between the two treated plots.

Field Observations

As application rates exceeded the water solubility of the pesticide, the inert carrier and undissolved Sevin were visible as a white layer that almost covered the mud. There was no indication of transport of undissolved Sevin beyond the plots before the tide returned. But dissolved Sevin obviously was moved away from the treated plots by subterranean seepage as the tide receded, because dead shrimp, Crago nigricauda, and English sole, Parophrys vetulus, were found as far as 15 m from the treated areas in the direction of tidal recession. The extent of pesticide transport by subsurface routes was not

Table 2. Analysis of variance for various species recovered from field plots treated with Sevin.[1]

Source	Total number of animals from all plots df	SS	MS	F
TOTAL	29	136, 591		
TIME	4	57, 574	14, 393	11.875**
TREATMENT	2	56, 941	28, 470	23. 490**
TIME X TMT	8	3, 902	488	0. 403
ERROR	15	18, 174	1, 212	

Source	Total number of worms, polychaetes and nemertenes df	SS	MS	F
TOTAL	59	2, 052		
TIME	4	815	204	7. 757**
TREATMENT	2	9	4. 5	0. 171
TIME X TMT	8	46	5. 7	0. 217
ERROR	45	1, 182	26. 3	

Source	Total number of bivalves df	SS	MS	F
TOTAL	59	70, 531		
TIME	4	21, 617	5, 404	13. 927**
TREATMENT	2	29, 183	14, 591	37. 606**
TIME X TMT	8	2, 269	284	0. 731
ERROR	45	17, 462	388	

Source	Tresus capax, gaper clam df	SS	MS	F
TOTAL	29	7, 080		
TIME	4	1, 771	443	6. 1527*
TREATMENT	2	2, 508	1, 254	17. 4167**
TIME X TMT	8	1, 722	215	2. 9861*
ERROR	15	1, 079	72	

[1] The difference in total degrees of freedom in the 4 ANOVA arises from the number of samples used in the analysis. A primary total of 6 plots X 5 subplots = 30 samples. In the field, subplots were divided into two halves and studied separately; this gives a secondary total of 2 X 30 = 60 samples.

*Significant at $\alpha = 0.05$

**Significant at $\alpha = 0.01$

determined. The material in the animal burrows could be moved through the water by resident animals. From MacGinitie's description of shrimp tunnels (1930, 1934), I judge that Sevin may have been transported as much as 76 cm vertically and about a meter horizontally in the extensive burrow network. The pesticide on the surface or in the burrows probably did not penetrate more than 10 cm into the underlying or surrounding mud, because Karinen et al. (1967) found very low levels of Sevin beyond this depth when it was applied to a mud substrate.

No dead or distressed animals were observed on or in the immediate vicinity of control plots. The only animals to die on plots after treatment and before the next flood tide were about 200 opisthobranchs, Ogleija diomedea, and several fish, and the numbers of dead animals on plots treated at the two rates were similar. Most of the moribund and dead animals found at this time were several meters from treated plots in beds of eel grass, Zostera marina. Animals began to die in, on and near the treated plots within 5 minutes after application of Sevin. Usually, the first stress sign in some fish and crustaceans was hyperactivity in the form of rapid and erratic darting movements through the shallow water. This was followed by body rigidity, paralysis and death. Eighty English sole and over one thousand Crago shrimp were counted dead. Most of these animals were observed and counted during hyperactivity, as their protective

coloration effectively concealed them when they were quiescent. Therefore, the observed numbers of affected animals may be considerably less than the true numbers.

The only clams to exhibit stress signs initially following application of Sevin were adult basket cockles, Clinocardium nuttalli, that emerged from the mud, opened their valves, and made digging movements with their feet. No juvenile clams of any species were seen leaving the mud. Many feeding clams, Macoma spp., in the standing water were observed taking in sediment including the particulate Sevin.

One day after treatment, there were 52 dead Dungeness crabs near the plots and 30 had been partially eaten by gulls. There were live English sole on and around all plots, indicating that the levels of Sevin there must have decreased considerably in 24 hours.

Pathological Study

Gross Observations

The feeding movements of the inhalant siphon of all clams were normal during the first 18 hours of the test. During this time the length of their inhalant siphons was about 1.5 times their shell length; this is less than the length observed in the field. After 18 hours, the inhalant siphons of all exposed clams, but not those of

the controls, were extended to about 3 to 4 times their shell lengths, and only animals exposed to the two lowest test concentrations of 15 and 20 mg/liter could retract their siphons when stimulated. After 24 hours, clams exposed to 20, 25, and 30 mg/liter had open valves and extended feet, but most of them could still close their valves when stimulated. Animals in the lowest Sevin concentration had normal, closed shells during the first 48 hours of the test.

Deterioration of siphons, usually the inhalant, of exposed clams was the only gross pathology seen. After 96 hours, about 50% of all exposed clams had lost one or both siphons, and such losses occurred as early as 10 hours after the beginning of exposure. There was no apparent relationship between this tissue damage and toxicant concentration.

The gross pathology of affected siphons before loss was manifested in two ways. First, persistent swellings appeared along the siphon (Figure 3), and this condition was followed by necrosis and sloughing of the external epithelium over the swollen areas; sheaths of the necrotic tissue still attached around the swollen areas could easily be peeled away with a probe. Secondly, lacerations developed perpendicular to the long axis of the siphon and extending into the lumen. Usually, there were swellings on both sides of the point of constriction where a laceration developed, and these features persisted for several hours until the siphon was lost. The siphons of

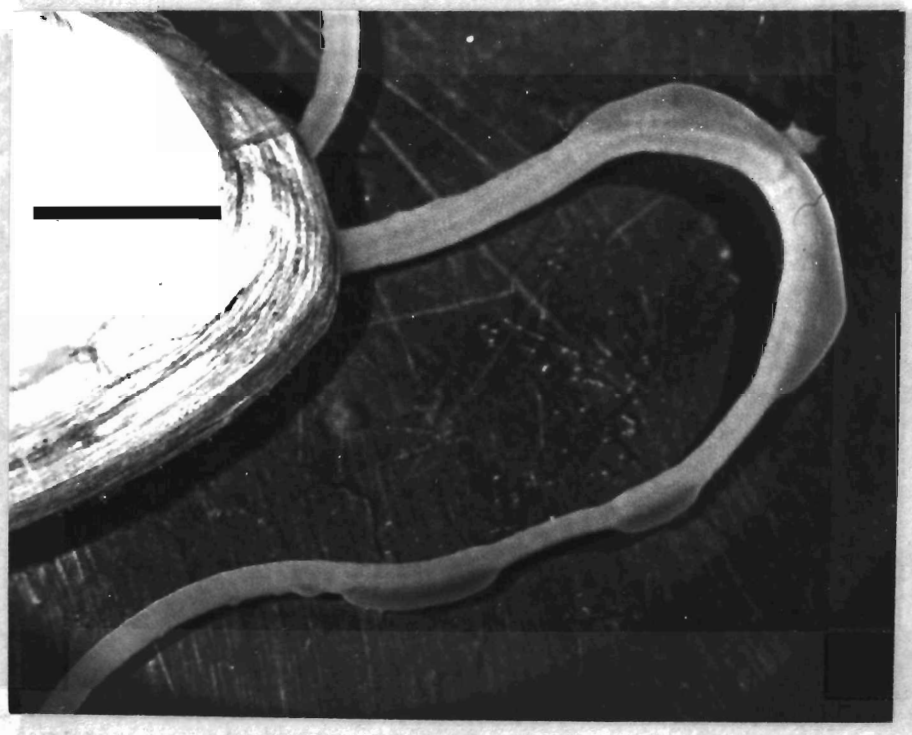


Figure 3. Abnormal swelling of the inhalant siphon of a clam exposed to Sevin at 20 mg/liter for 24 hours. Bar = 10 mm.

control clams were normal.

The 48-hour and 96-hour TL_{50}'s for the clams were estimated to be 27.5 and 17.0 mg/liter, respectively. None of the exposed animals returned to clean water recovered.

Histological Observations

Histological evidence of damage in exposed clams was primarily necrosis of epithelial and other tissue cells. This damage occurred in clams exposed to all Sevin concentrations, but the severity was directly related to the pesticide concentration.

The normal histology of the siphon of the bent-nosed clam (Figures 4, 5) is similar to that of other tellinaceans, which was described by Yonge (1949). In damaged siphons, the cuticular borders of the lumen and the exterior epithelium were usually destroyed (Figure 6). The epithelial cells were vacuolated and the cytoplasm was extruded through ruptures in the cell membranes (Figure 6). The nuclei of these cells frequently were pyknotic. Loss of adhesion between the epithelial cells and underlying stroma (Figure 6) caused portions of the epithelium to separate from the connective tissue, and contributed to extensive sloughing of the exterior epithelium (Figure 7) and less frequently of the lumen epithelium. The swellings of the siphon previously described were caused by dilation of a large blood sinus found within the longitudinal muscle

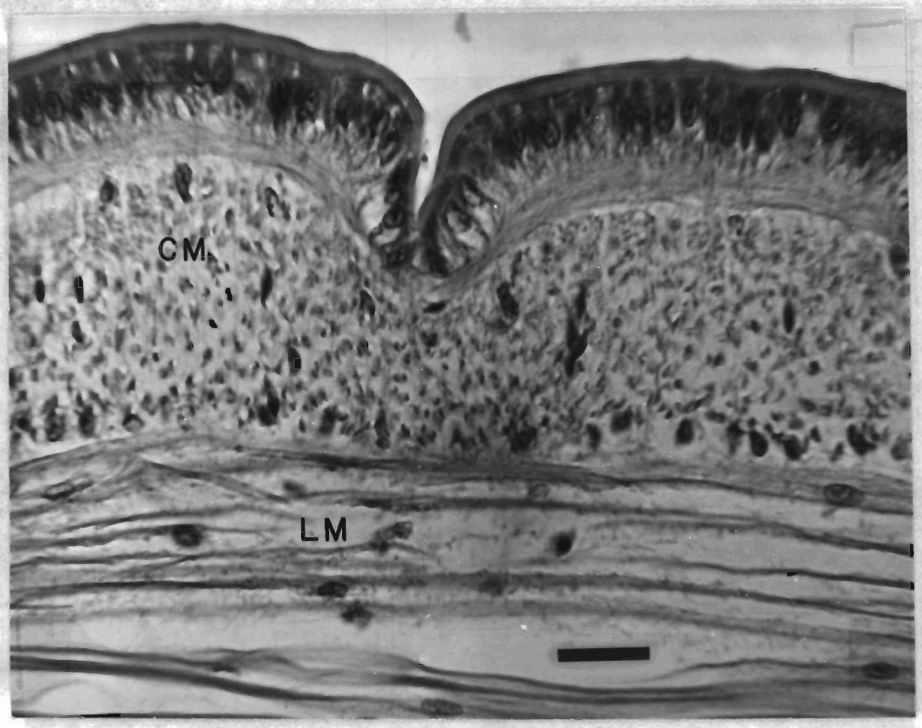


Figure 4. External cuticular epithelium of the siphon of a control clam. CM, LM, circular and longitudinal muscle layers. Bar = 20 μ.

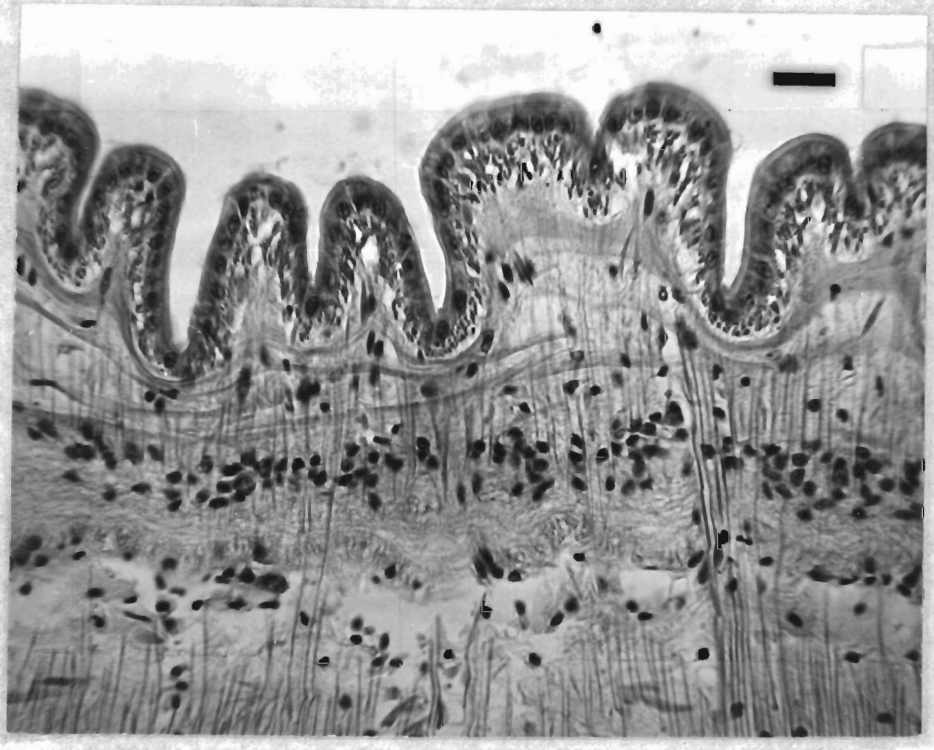


Figure 5. Lumen epithelium with a cuticular border from the siphon of a control clam. Bar = 20 μ.

Figure 6. Necrotic lumen epithelium of the siphon of a clam exposed to Sevin at 30 mg/liter for 24 hours. Note the vacuolization (V) and rupture of cells, the disappearance of the cuticular border, and the lack of adhesion (LA) between the epithelium and stroma. Bar = 20 μ.

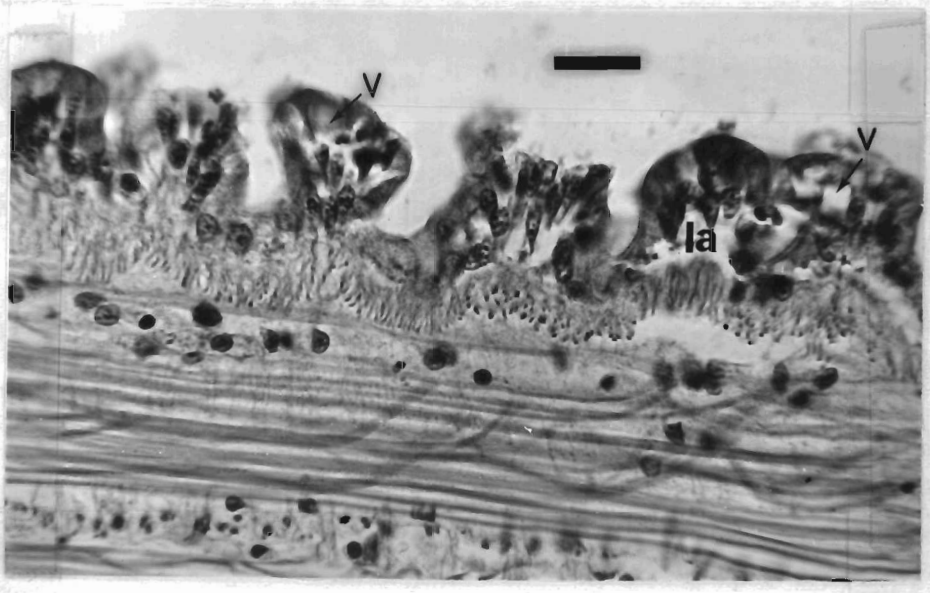
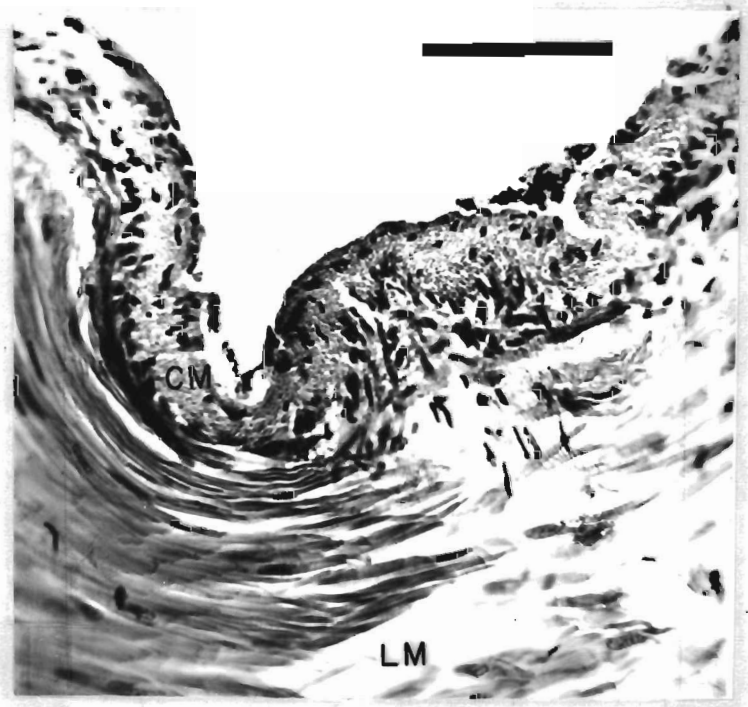


Figure 7. The exterior portion of the siphon of a clam exposed to Sevin at 30 mg/liter for 48 hours. The entire epithelium has been sloughed. CM, LM, circular and longitudinal muscle layers. Bar = 100 μ.



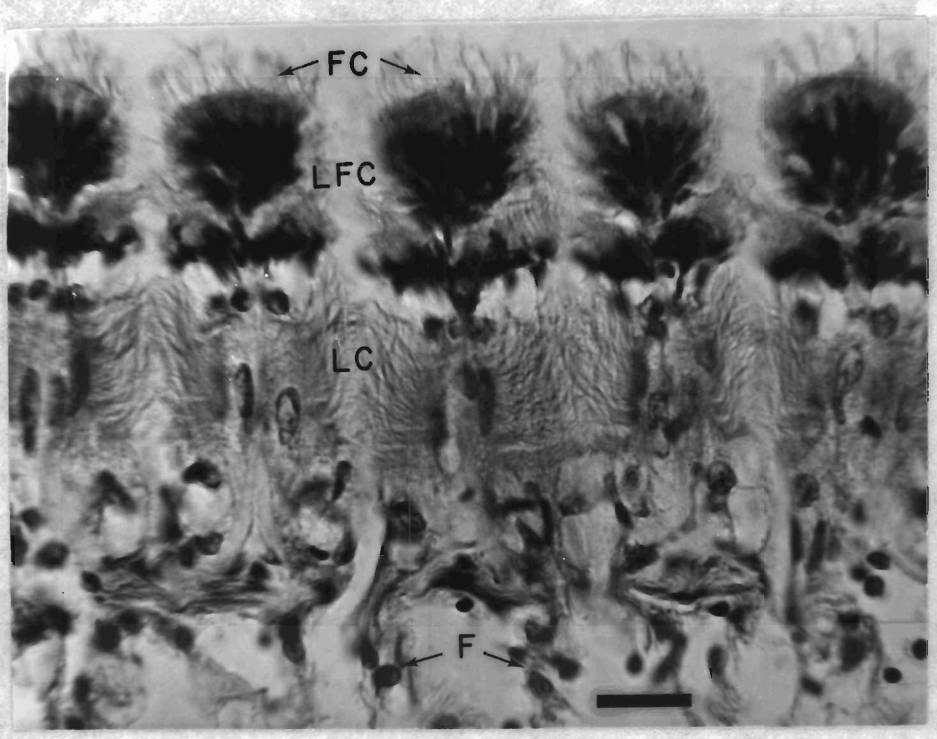


Figure 8. Gill border of a control clam. FC, frontal cilia, LFC, laterofrontal cilia. LC, lateral cilia. F, filaments. Bar = 20 μ.

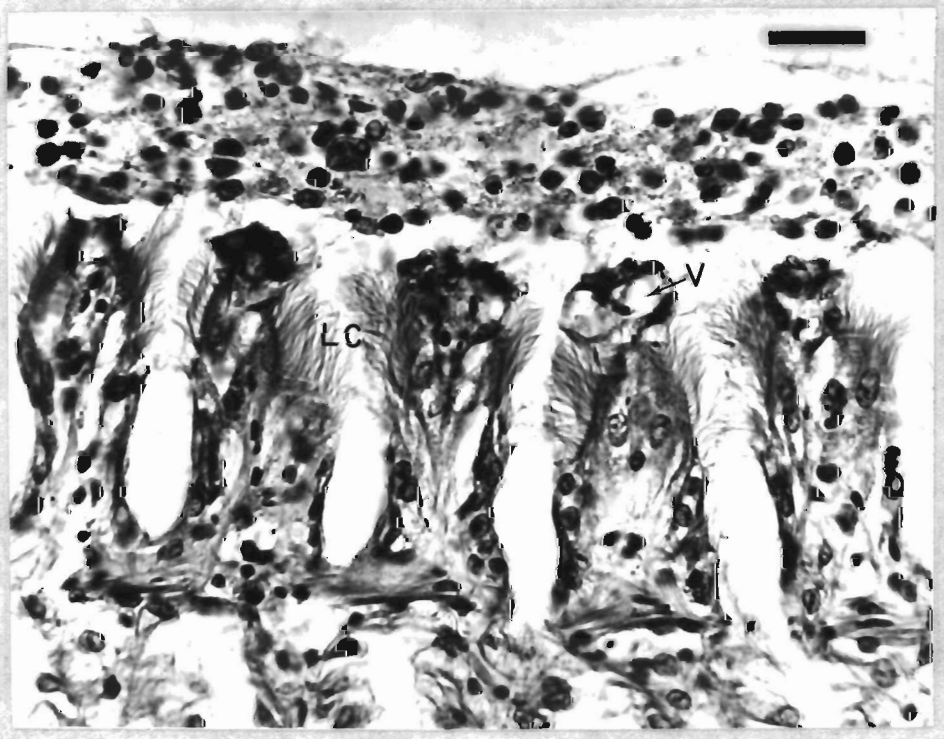


Figure 9. Damaged gill border of a clam exposed to Sevin at 25 mg/liter for 60 hours. Note the loss of cells bearing the frontal cilia, held in mucus above, and the vacuolization (V) of the remaining cilia-bearing cells. LC, lateral cilia. Bar = 20 μ.

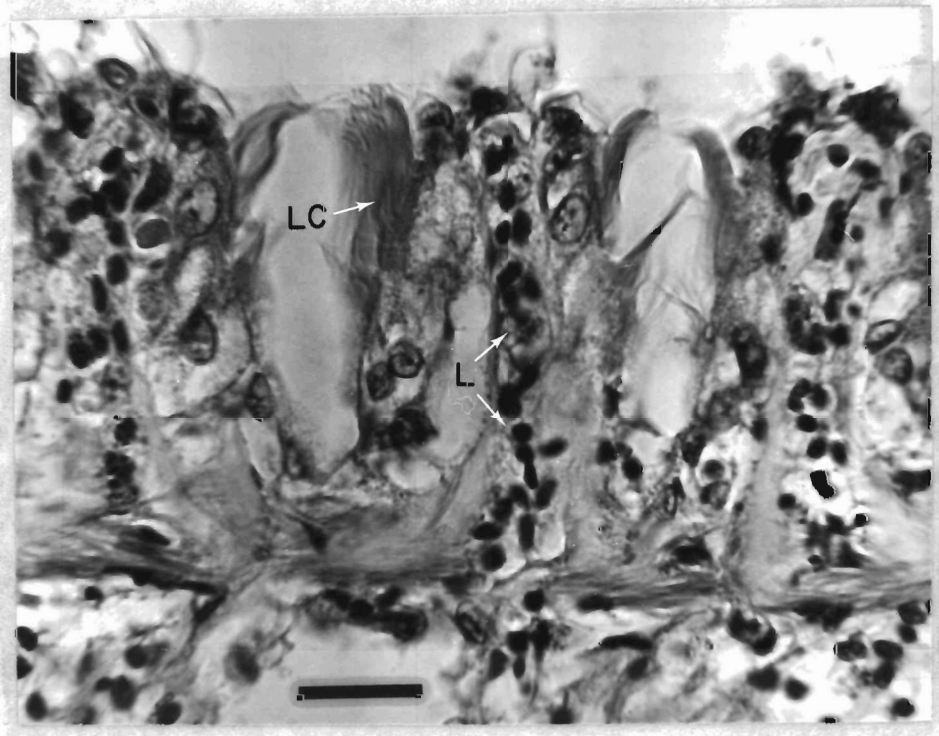


Figure 10, Damaged gill border of a clam exposed to Sevin at 30 mg/liter for 24 hours, The frontal and laterofrontal cilia-bearing cells are sloughed and a portion of the lateral cilia is gone. Note the leukocytes (L) in the center of the filament borders. LC, lateral cilia. Bar = 20 μ.

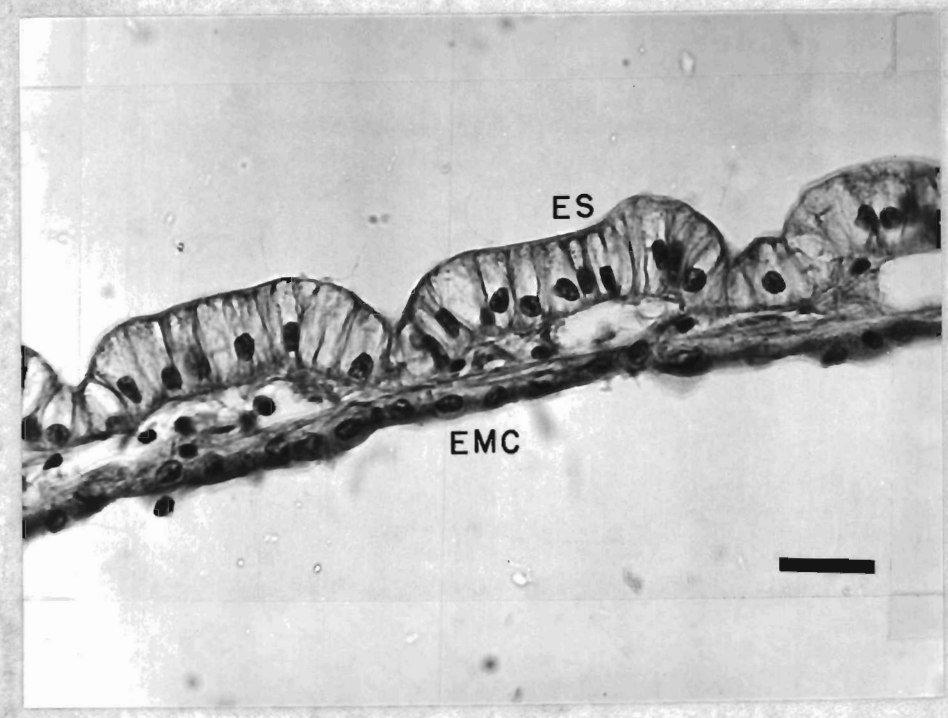


Figure 11. Mantle over the viscera of a control clam. ES, epithelium bordering the shell. EMC, epithelium bordering the mantle cavity. Bar = 40 μ.

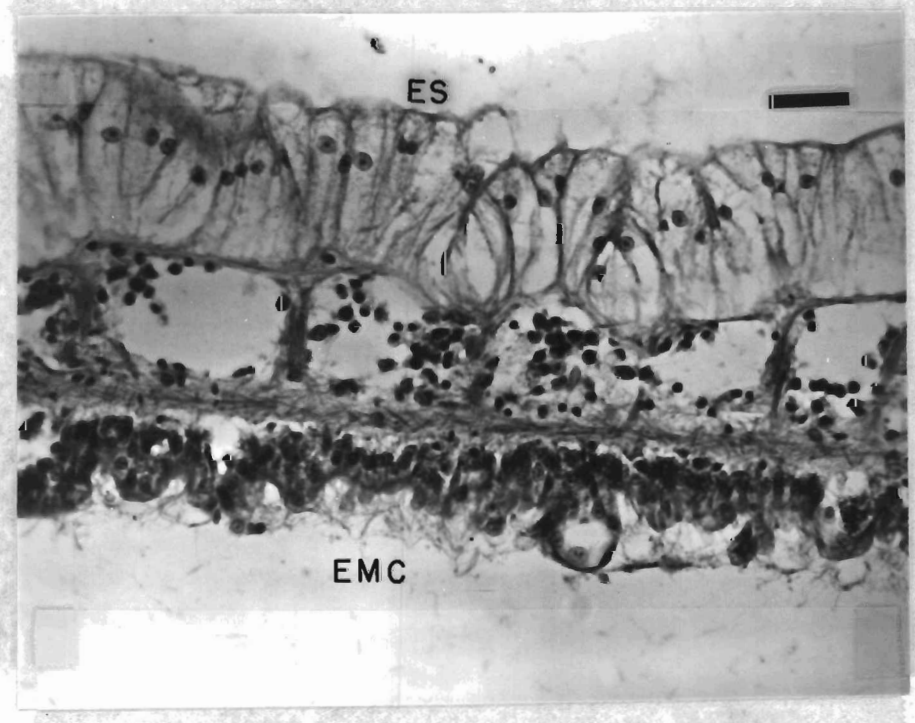


Figure 12. Necrosis of the mantle of a clam exposed to Sevin at 20 mg/liter for 96 hours. Note increased leukocytes in the sinus between the epithelia. Lettering as before. Bar = 20 μ.

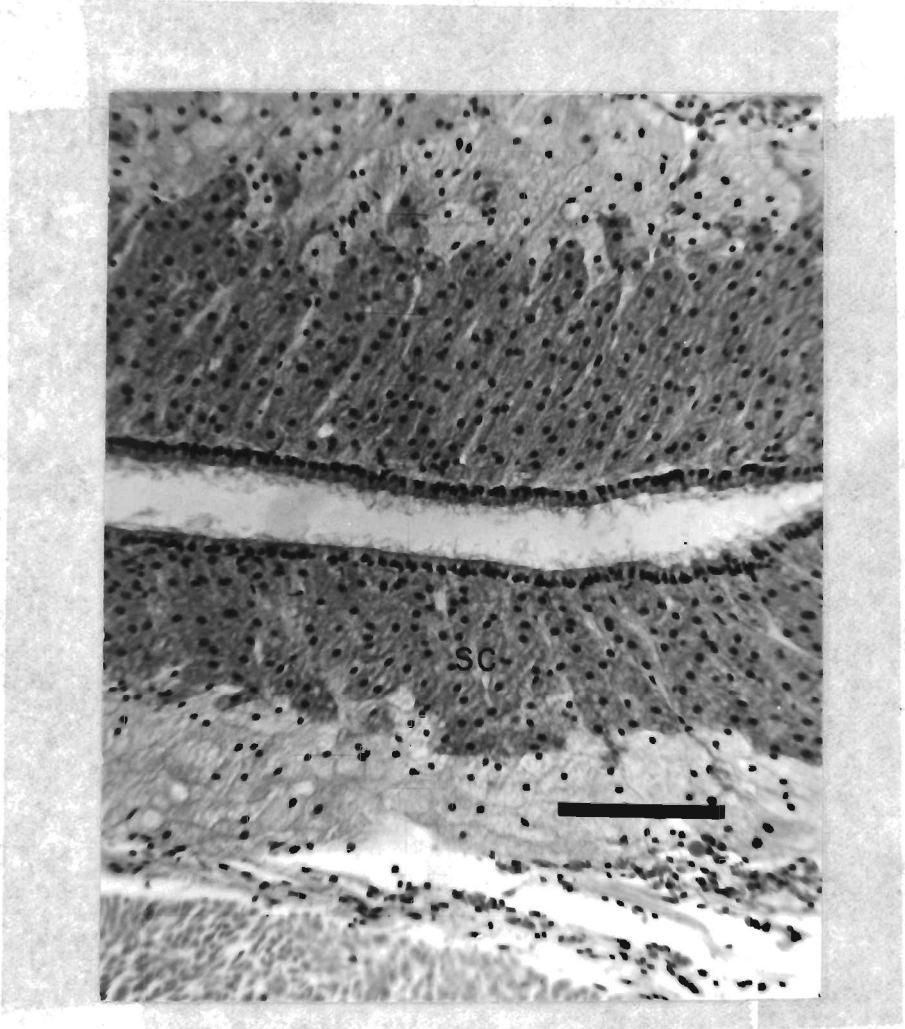


Figure 13. Suprabranchial gland of a control clam. Note the cord-like arrangement of the secretory cells (SC) and the distinct epithelium. Bar = 100 μ.

layer. The dilation caused the tissue to bulge either internally or externally, and in the former case the siphon lumen was almost occluded.

The normal histology of the gill filament epithelium (Figure 8) conforms to descriptions by Yonge (1949) and Owens (1966). Gill pathology was extensive in clams exposed to all concentrations of Sevin and varied only in the time of onset of signs. Damage was evidenced by necrosis and sloughing of the filament epithelium, which resulted in general loss of cilia-bearing cells on both sets of demibranchs. These changes occurred within 24 hours in some clams exposed to 30 mg/liter, and within 46 hours in some exposed to the other concentrations.

The basophilic cells bearing the frontal and laterofrontal cilia, which are responsible for particle transport, were the first to be lost (Figure 9). Usually these cells were vacuolated and had pyknotic nuclei. Some demibranchs had lost the entire basophilic border consisting of these ciliated cells. Sloughed cells were often found embedded in mucus and in extruded cytoplasmic material in the mantle cavity (Figure 9).

When the pathology of the filament epithelium was more extensive, there was necrosis and sloughing of the large acidophilic cells bearing the lateral cilia (Figure 10), which produce the inhalent water currents. Concurrently with this necrosis, leukocytes and

larger granulated, phagocytic cells in the filaments increased in numbers. Packing of leukocytes in the sinus within each filament tip (Figure 10) suggested thrombus formation to prevent fluid loss after cells were sloughed. Loss of leukocytes from necrotic filament tips was not observed. Vacuolization, rupture, and disjunction of filament cells was seen.

The mantle overlying the viscera consists of two cell layers. A blood sinus separates the two epithelia, which differ considerably in appearance (Figure 11). In exposed clams the mantle was damaged, exhibiting vacuolization and rupture of cell membranes (Figure 12). The leukocytes in the sinus and granulated cells in the squamous interior epithelium increased in numbers.

Pathology of the suprabranchial gland was also extensive. This paired organ lies posterodorsally and is attached to the adductor muscle and gills. It is a secretory gland supplying mucus to the gills (Owen, 1966). The cells form cord-like bands that extend to the outer margin of the organ (Figures 13, 14). Exposure to Sevin always caused increased mucus secretion and resulted in disorganization of the well-defined epithelium. More severe damage included vacuolization of the cells in the interior of the organ, accompanied by pyknosis and loss of cellular organization (Figure 15).

Several exposed clams had portions of siphon and gill epithelia in thir esophagus and dorsal pouch.

Figure 14. Higher magnification of a suprabranchial gland from a control clam showing distinct epithelium and mucus secretion. Bar = 45 μ.

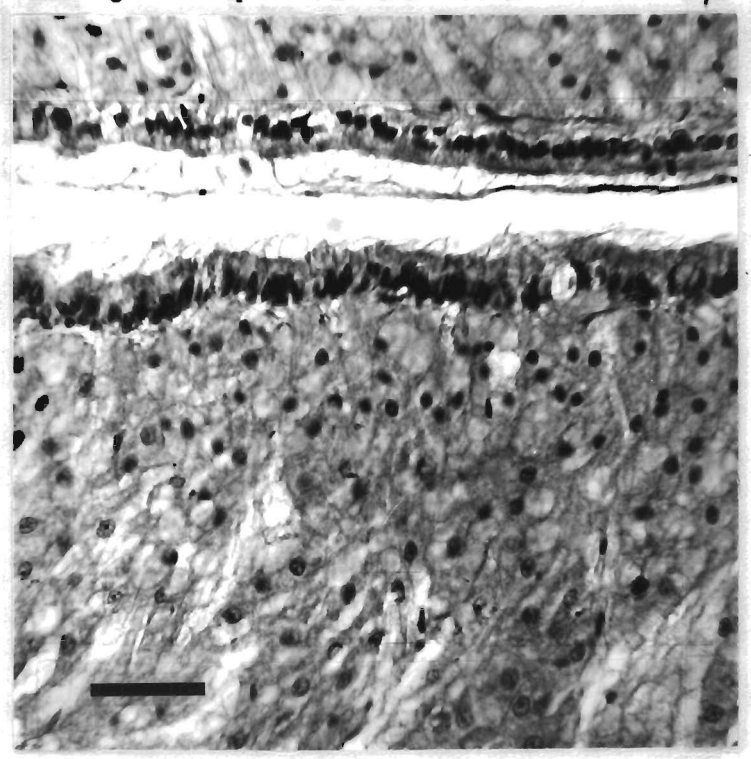
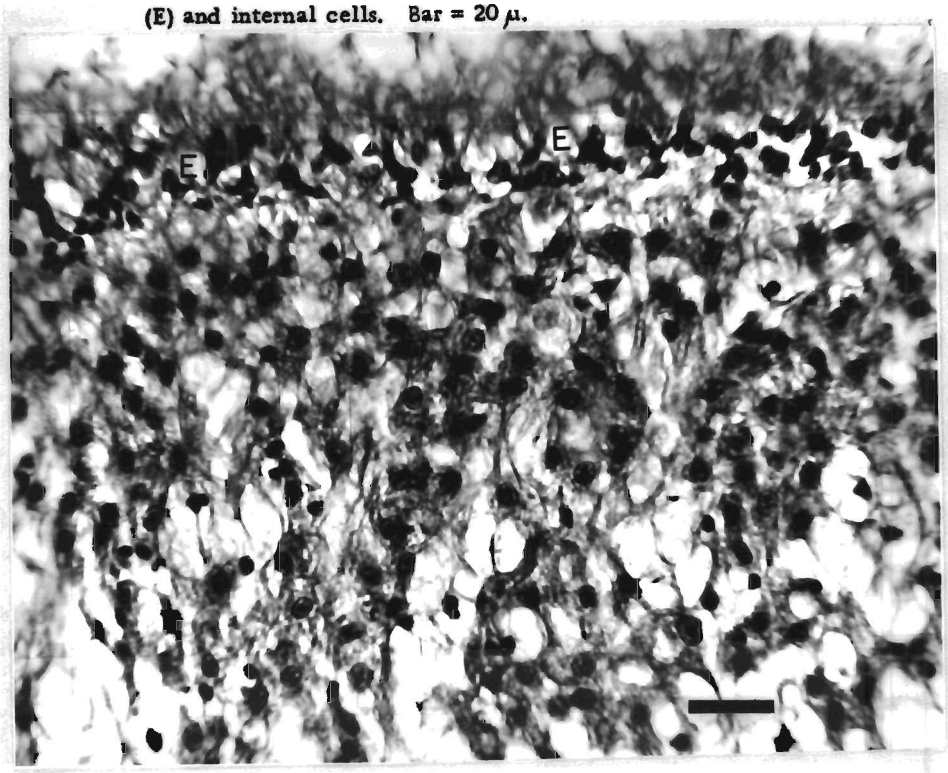


Figure 15. Necrosis of a suprabranchial gland of a clam exposed to Sevin at 20 mg/liter for 60 hours. Note the disorganization of the epithelium (E) and internal cells. Bar = 20 μ.



Embryological Study

Toxic Concentrations

The EC_{50} values for 1-hour exposures of several developmental stages of M. edulis to Sevin and 1-naphthol are listed in Table 3. These compounds had similar toxicity values for the two stages against which both were tested. Sevin was slightly more toxic than 1-naphthol to unfertilized eggs, the EC_{50} values being 20.7 and 24.5 mg/liter, respectively. The stage of development most sensitive to Sevin occurred shortly after fertilization, at the appearance of the first polar body (1-PB), and susceptibility declined as the age of the larvae increased. The EC_{50} values for the 1-PB and 32-hour stages were 5.3 and 24.0 mg/liter, respectively.

Developmental Anomalies

It is difficult to state exactly at what stage, and by what time after fertilization, development became anomalous at the different concentrations used. A generalized pattern of development is given for larvae exposed at two stages, and is compared with controls in Table 4. Fertilization occurred at all concentrations of Sevin, including 60 mg/liter. Development and time to appearance of the two polar bodies and first polar lobe were also normal (Table 4). Retardation of development was often apparent at the first cleavage which failed

Table 3. Toxicity of Sevin and 1-naphthol to several early developmental stages of Mytilus edulis.

Developmental stage	Approximate time after fertilization	No. of parental crosses	Range of five concentrations tested (mg/l)	1 hr EC_{50} values (mg/l)[1]			
				Sevin		1-naphthol	
				Mean	Range	Mean	Range
Unfertilized egg	Pre-fertilization	7	10-40	20.7	18.0-23.8	24.5	22.8-28.3
1st Polar body	20 minutes	6	1-10	5.3	4.4- 6.6	5.2	4.8- 5.7
2 cell	1 hour	6	1-10	7.0	4.8- 8.5	Not determined	
64 cells to blastulae	4 hours	4	1-16	8.3	5.2-10.0	"	"
Ciliated blastulae	5 hours	4	7.5-24	16.0	14.0-20.0	"	"
Trochophore	20 hours	4	10-32	19.0	15.0-22.5	"	"
Early veliger	32 hours	4	10-40	24.0	18.0-34.0	"	"

[1] The 1-hour EC_{50} value is the concentration causing 50% abnormal development of larvae exposed for 1 hour to a pesticide during 48 hours of development.

Table 4. Effect of Sevin on the rate of development of M. edulis larvae exposed for one hour as unfertilized eggs or at the first polar body stage.

Developmental stage	Developmental times Exposed stages		
	Control[1]	Unfertilized eggs (25 mg/l)[1]	First polar body (10 mg/l)[1]
Fertilization	--	--	--
1st polar body	20 min	20 min	20 min
2nd polar body and polal lobe	40 min	40 min	40 min
2-cell	65 min	110 min	130 min
4-cell	90 min	150 min[2]	150 min[2]
8-cell	120 min	180 min	200 min
16-cell	150 min	--	--
32-cell	180 min	--	--
64-cell	220 min	--	--
Ciliated blastulae	300 min	600-650 min	600-700 min
Trochophore	16-19 hr	Not reached	Not reached
Veliger	40 hr	Not reached	Not reached

[1] Animals were exposed to the indicated concentrations at 17°C and 25‰ salinity.

[2] Animals reaching this and subsequent stages were abnormal as described in the text.

to occur only after exposure to concentrations greater than 40 mg/liter. The resulting 2-cell embryo was usually normal in appearance. Initiation of the second cleavage, and all subsequent stages were also delayed (Table 2), and anatomical anomalies were first seen in the 4-cell embryo. Blastomeres contained very large, refractile vacuoles not seen in controls, and cells were lobulated and bore several to many polar-body size protrusions on their surfaces. Also, by the 4-cell stage or shortly thereafter, blastomeres would sometimes disjoin and continue development to ciliated but abnormal blastulae. Cleavages, subsequent to the 4-cell stage, were unsynchronous and unaligned from the normal pattern of this spirally-cleaving animal (Wada, 1968) and resulted in larvae bearing little resemblance to control animals (Figures 16, 17).

Retardation of development did not result in anomalous larvae when the embryos were exposed to concentrations well below the EC_{50} values for the exposed stages (Table 3). In these cases, time lags during the first three cleavages were similar to those listed in Table 4; however, the embryos were always normal and continued development to the veliger stage.

Eggs or embryos from a single female varied in their sensitivity to Sevin and some would continue to develop longer than others. For example, of embryos exposed to a Sevin concentration of 50 mg/liter at the 1-PB stage, 62% were undivided with the polar lobe

formed, 15% were 2-cell, 17% 4-cell, and 5% were at the 8-cell stage 8 hours after fertilization; controls by this time were ciliated blastulae.

There was no observable effect of centrifugation on subsequent development of larvae, and there were no more anomalies in a centrifuged group than in unspun controls.

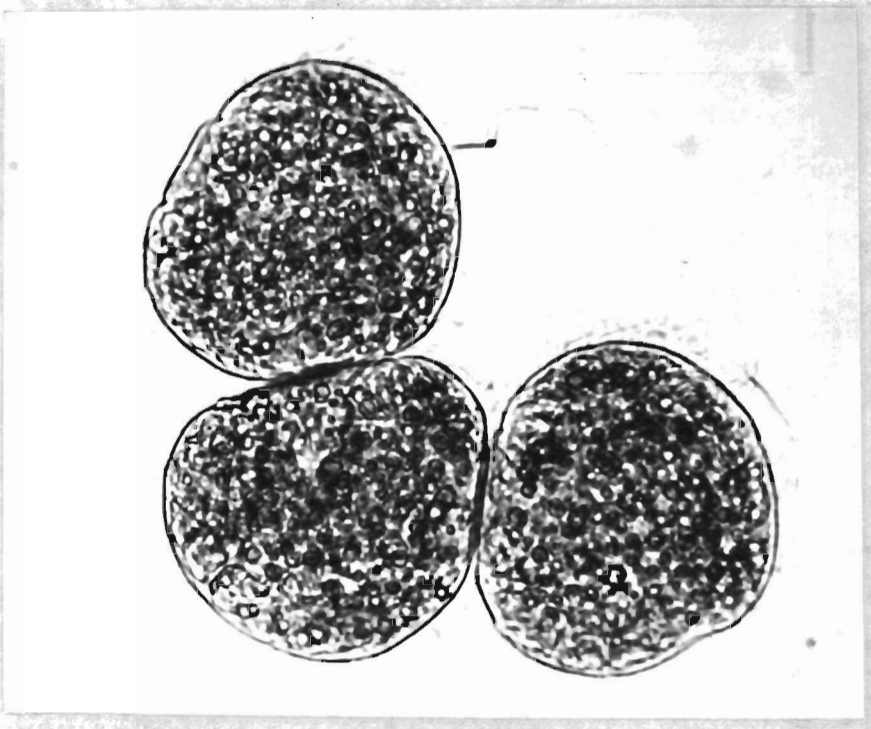


Figure 16. Three control larvae at the trochophore stage 21 hours after fertilization. The larvae were ciliated and had a prominent flagellum.

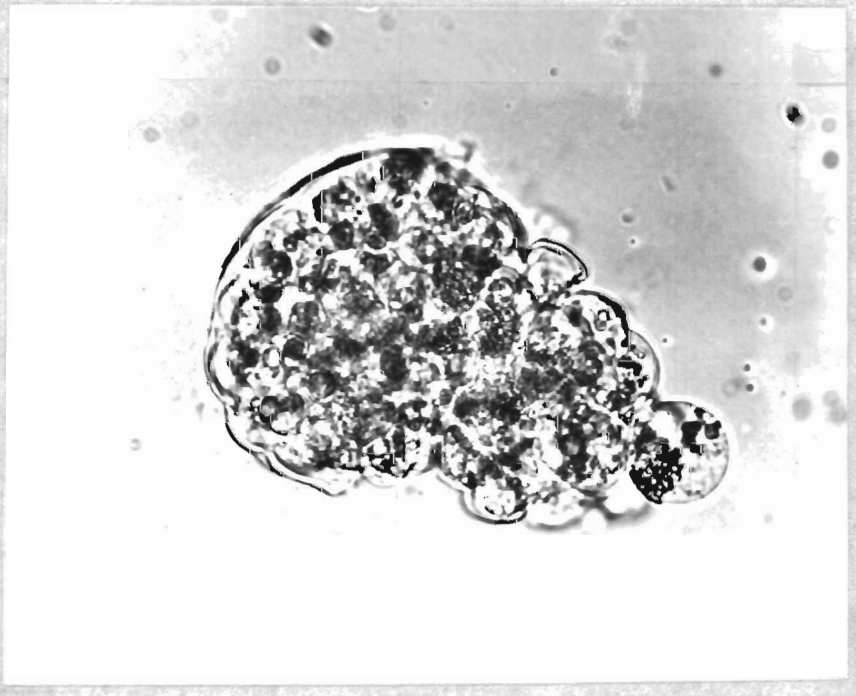


Figure 17. Abnormal larva exposed at the first polar body stage for 1 hour to Sevin at 10 mg/liter. This larva was also 21 hours old and rotating slowly with a small number of cilia.

DISCUSSION

Field Study

The results of this study provide the first demonstration that juvenile clams are significantly reduced in numbers by application of Sevin to estuarine mud flats. Lossanoff (1960), Haydock (1964) and Haven et al. (1966), using Sevin in conjunction with the pesticide Polystream, noted adverse but not always lethal effects of Sevin on gastropods, and Lindsay (1961) and Chambers (1970) reported deaths of clams after treatment of a mud flat with Sevin, but the number of dead animals was not determined.

There are no published data to support the view that a Sevin application rate of 10 lb/acre is the best one for controlling shrimp. Chambers (1970) stated that this level was the most efficient concentration for eradication of shrimp and that 1.75 lb (0.79 kg)/acre was inadequate, but he presented no data to support this claim. Snow and Stewart (1963) believed that 10 lb/acre should be considered the maximum concentration and suggested the use of lower concentrations for shrimp control. Clams surviving on plots treated with Sevin concentrations of 5 and 10 lb/acre were fewer by 22 and 38%, respectively, than those in the control plots, and survival was less by 21% at the higher application rate relative to the lower one.

It is not likely that T. capax or other commercial species of

clams would be abundant on mud flats heavily infested with C. californiensis and the burrowing shrimp Upogebia pugettensis, but Macoma spp. are as plentiful there as at lower, uninfested elevations in the intertidal zone (personal observation). Reductions in their numbers may affect other animals through food chain relationships. Treatment of a 30-acre commercial plot at 10 lb/acre would kill about 12.3 million Macoma clams based on extrapolation from my data (30 acres is 16, 700 times larger than my sample area treated with 10 lb/acre). Macomas are a direct food source for crabs, fish, and birds (Quayle and Bourne, 1972; personal observations). Miller (1967) reported that clams, including Macoma spp., were important food items for adult starry flounders, Platichthys stellatus, during the months from July to October. Also, clam gametes and larvae which enter the planktonic community probably serve as food items for other zooplankton and for sedentary filter feeders (Gonor, 1972). Edwards and Steele (1968) and Edwards et al. (1969) stated that siphons of small tellinid clams were the major food items of plaice and to a lesser extent of sanddabs from their metamorphosis to the age of 2 months. Pacific Northwest bays are nursery grounds for English sole and speckled sanddabs, Citharichthys stigmaeus (Olson and Pratt, 1973). I found no information on the food habits of these fish less than 2 months old, but it is not unlikely that siphons from clams of the size killed in my study are a major food for them also.

For the above reasons and because Sevin at 5 lb/acre was as effective as 10 lb/acre in controlling burrowing shrimp, I recommend the use of the lower application rate for controlling shrimp on oyster grounds.

Stewart et al. (1967) and Butler et al. (1968) showed with laboratory studies that 1-naphthol is more toxice to mollusks than is the parent compound. In my study, I believe that 1-naphthol had little if any toxic effect on the animals for the following reasons. The first returning tide diluted and removed most of the Sevin from the surface of the mud flats. Karinen et al. (1967) reported that only 3.3 to 0.46 ppm of Sevin remained in the top 1 to 3 inches of mud 24 hours after it was sprayed at the rate of 10 lb/acre. They found that after 24 hours the 1-naphthol levels at the same mud depths ranged from 0.30 ppm to undetectable levels; this observation indicated slow hydrolysis of Sevin in mud. Butler et al. (1968) reported that all of the juvenile basket cockle clams used in their tests survived after a 24 hour exposure to 5.60 mg/liter of 1-naphthol in the laboratory, which is a concentration 18 times higher than that found in the treated mud analyzed by Karinen et al. (1967). Finally, my statistical analyses showed that there was no significant effect of treatment beyond 24 hours over the 30-day sampling period. Therefore, it can be assumed that most deaths occurred within 24 to 48 hours, and very little 1-naphthol would have been formed by this time.

In my study, Sevin did not significantly reduce the number of worms in ground treated with 5 or 10 lb/acre. Haven et al. (1966) and Haydock (1964) both reported high mortalities of polychaetes following treatment of mud flats with Sevin and chlorinated benzenes, and perhaps the latter chemicals would have caused these mortalities. Chambers (1970), in his field study, found that some nereid worms left their burrows after Sevin treatment of an oyster ground and noticed a subsequent decline in their numbers in treated areas, and Snow and Stewart (1963) reported annelid worms were adversely affected following application of Sevin to mud flats. Apparently the toxic effects of Sevin to marine worms can vary, depending on the species involved and the physical factors operative at the time of application. In this study, the fine particle size of mud flat substrate, which resulted in poor interstitial circulation, and the removal of Sevin by the first returning tide probably prevented toxic levels from reaching most of the worms.

Pathological Study

The information on the histopathology of pesticide poisoning in invertebrates, reviewed by Sparks (1972), is meager. This is the first report of histological damage in a bivalve associated with acute poisoning by a pesticide. The extensive epithelial damage is similar to that reported for chronic poisoning. Lowe et al. (1971)

described epithelial necrosis and other damage in oysters chronically poisoned with mixtures of DDT, toxaphene, and parathion. The major histopathology in oysters exposed for 24 weeks to the polychlorinated biphenyl Aroclor (not a pesticide) was atrophy of the diverticular epithelium (Lowe et al., 1972). Pauley and Sparks (1965; 1966) studied inflammatory reactions and histological changes in oysters injected with turpentine (also not a pesticide) and reported necrosis and sloughing of intestinal and kidney epithelia. Hassanein et al. (1968) reported extensive tissue necrosis in a larval lepidopteran exposed for 24 hours to Sevin.

Vacuolization of epithelial cells, dissolution of their components, and sloughing of sensitive epithelial surfaces in my clams shows that the toxic action of Sevin is at least in part superficial. The siphon, gills, suprabranchial gland, and mantle are all directly exposed to water and therefore to Sevin. This demonstration that Sevin causes severe, superficial, histological damage suggests that damage to the nervous system through cholinesterase inhibition may not be the only or even the primary cause of death of exposed clams.

The pathology primarily responsible for death of the clams probably was gill necrosis. Loss of the lateral cilia or cessation of their movement stops circulation of water through the gill filaments, and the disjunction of the filament cells disrupts the flow of oxygenated blood, thus causing anoxia. Death of clams exposed to

Sevin in the field may be attributed to the same cause. In the previous field study I reported that clam mortalities occurred within 24 hours after application of Sevin to mud flats. Levels of Sevin on and in the mud were maximal and exceeded water solubility for part of this time. In the present study, siphons were lost by clams after only 10 hours of exposure, and gill necrosis was severe among clams exposed to 30 mg/liter of Sevin for 24 hours. Although not a lethal factor in my laboratory tests, loss of the frontal cilia would allow accumulation of sediment on the gills of clams in the field, thereby contributing to the anoxia. In addition to the stress created by loss of cilia-bearing cells from the gills, the other histopathological changes found in laboratory-exposed clams could also affect long-term survival of clams exposed in the field. Necrosis and loss of epithelial surfaces on the siphon and mantle could provide portals of entry for disease agents. Loss of the inhalant siphon would impair food gathering and interfere with respiration by loss of contact with the mud flat surface. Damage to the suprabranchial gland could reduce feeding activity and cleaning of the gills and palps if mucus production were diminished.

Embryological Study

The most sensitive stage of early development of M. edulis larvae exposed to Sevin occurs in the first hour or so following

fertilization. The calculated 1-hour EC_{50} value was 5.3 mg/liter for exposure at the appearance of the first polar body. Stewart et al. (1967) who also used mussel larvae whose parents were collected in Yaquina Bay, Oregon, reported a 48 hour EC_{50} value of 2.3 mg/liter for Sevin. It is significant that exposure of larvae for 48 times the period used in my tests resulted in an EC_{50} value only 2.3 times lower than my value cited above. This small difference supports the view that sensitivity to Sevin is highest shortly after fertilization, because a 1 hour exposure did not require appreciably more Sevin to do the same damage observed in a 48 hour exposure.

1-naphthol has been reported to be more toxic than Sevin to molluscan embryos (Stewart et al, 1967; Butler et al., 1968). There was no significant difference in toxicity of these two compounds to the two early stages of M. edulis against which both were tested; this suggests that embryos are probably most sensitive to 1-naphthol after the first polar body stage.

One of the most pronounced effects of Sevin on M. edulis embryos was reduction in the rate of development or complete cessation of cleavages. Retardation of development at later stages of M. edulis larvae exposed to linear dodecylbenzenesulphonate (LAS) was reported by Granmo (1972). The rate of development from fertilization to the trochophore stage was normal. However, by 96 hours, when all controls were veligers, larvae exposed to 0.3 ppm

or more of LAS were still trochophores. Granmo also found that LAS concentrations above 1 ppm entirely prevented fertilization, and 0.3 ppm decreased it by 50% from controls. Exposures to Sevin concentrations as high as 60 mg/liter did not prevent ferilization in my tests.

My comments concerning the biochemical systems that may be affected by Sevin can be only speculative ones. The greater sensitivity of eggs following fertilization may be partially due to increased permeability, a typical consequence of fertilization (Monroy, 1965), that would allow a greater influx of Sevin and 1-naphthol into the egg. Once Sevin is in or on a newly fertilized egg, there are many enzyme-mediated processes that Sevin might inhibit. Generally, Sevin is classed as a cholinesterase inhibitor (Casida, 1963) and what effects it may have on other enzymatic systems is not clear (Grosch and Hoffman, 1973). After fertilization, the biochemical activity in the egg and early embryo is greatly increased. The DNA content of embryos rises significantly and DNA-dependent RNA synthesis begins as early as the first cleavage (Gross, 1968). Interference with RNA synthesis, and consequently with protein synthesis, can alter and eventually stop development. Grosch (1973) reported significant effects on reproduction of Artemia salina exposed to breakdown products of several pesticides, including three of Sevin. He stated that reductions in numbers and viability of the progeny of exposed

parents were attributable to cytogenic destructive action of naphthalene and carbamate type compounds on the stem cell components of the gonads and on cells of the cleaving embryo. Grosch (1973) and Grosch and Hoffman (1973) also noted that naphthalene compounds related to Sevin and its derivatives are known spindle poisons affecting the mitotic apparatus and, therefore, cleavages. Certainly the reduction in cleavages or failure of cleavages to occur in M. edulis embryos exposed to Sevin and 1-naphthol could be due to destruction of enzyme systems responsible for spindle formation. The damage of spindles could also explain unalignment of cleavages observed in my tests.

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