AN ABSTRACT OF THE THESIS OF

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Title THE USE OF THE EMBRYONIC STAGES OF THE BAY MUSSEL, MYTILUS EDULIS LINNAEUS, AS A BIOASSAY TOOL WITH SPECIAL REFERENCE TO SODIUM PENTACHLOROPHENATE

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(Major professor)

A series of experiments was conducted at Oregon State University's Yaquina Bay Fisheries Laboratory during 1960 and 1961 to determine the suitability of the mussel embryo test for bioassay work in marine waters. Specific objectives of this study included the determination of the seasonal availability of gametes, sensitivity of the embryos to dilute concentrations of the experimental toxicant sodium pentachlorophenate (NaPCP), reproducibility of bioassay results, and general laboratory techniques necessary for this type of test.

Adult mussels were stimulated to spawn by immersion in a 6-8 percent (by volume) mixture of Kraft Mill Effluent in sea water. This spawning method proved to be a rapid and reliable means of obtaining viable gametes during all seasons of the year. From the information obtained in this study, plus additional spawning data to provide more complete annual coverage, it appears that gametes are readily available from January through August (30-80 percent spawning)

and available to a lesser extent from September through December (15-25 percent spawning success).

The normal development of mussel embryos includes the formation of a fully-shelled veliger stage at 40-48 hours ($20^{\circ} \pm 2\ C^{\circ}$). The presence of an environmental stress (toxicant) causes a percentage of the embryos to develop in an abnormal manner, i.e. do not develop shells. A mussel bioassay consists of exposing developing embryos to various concentrations of a toxicant in seawater, allowing the embryos to develop for 48 hours at $20^{\circ} \pm 2^{\circ}$ C., and determining the percentages of fully-shelled larvae in each concentration. Twenty-four bioassays were conducted using NaPCP as the experimental toxicant. In 19 of these tests, the percentages of normal larvae were based on the number of larvae surviving at the end of an experiment. In the remaining five bioassays, counts were made of the numbers of fertilized eggs originally present in each container to obtain information on the effect of mortality on bioassay results.

Experiments conducted to determine the effect of salinity on mussel embryo development indicated that the optimum salinity was in the range of 24-28 p.p.t. (salinities from 16-32 p.p.t. were tested). In another series of experiments both salinity and NaPCP concentration were varied. In these tests embryos reared in sea water with a salinity of 28 p.p.t. were less susceptible to the effects of NaPCP than at either 20 or 24 p.p.t.

In the 24 bioassays the NaPCP did not appear to affect shelling at 0.2 mg/l and completely prevented shelling at 0.6 mg/l. The average EC_{50} for all of the experiments was approximately 0.40 mg/l

with a range of 0.28 to 0.57 mg/l. Although the percentages of embryos surviving decreased with increasing concentrations of the toxicant, EC_{50} values computed using the numbers of larvae at 48 hours did not differ significantly from values based on the original numbers of fertilized eggs.

Measurements of shell lengths of 48-hour embryos indicated that differences in shell lengths may be a means of detecting inimical effects not shown by percentages of normal larvae alone.

The mussel embryo test, as described in this report, is a rapid and sensitive bioassay method. Additional tests may be required to relate mussel toxicity data to that of other species of interest.

THE USE OF THE EMBRYONIC STAGES OF THE BAY MUSSEL, *MYTILUS EDULIS* LINNAEUS, AS A BIOASSAY TOOL WITH SPECIAL REFERENCE TO SODIUM PENTACHLOROPHENATE

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INTRODUCTION

The possible use of the embryonic stages of the bay mussel, Mytilus edulis Linnaeus, for bioassaying toxic materials in marine waters is the subject of this report. The original impetus for this study was a result of the discovery that Kraft Mill Effluent (KME) stimulated spawning in mussels during all seasons of the year. In 1957, W. P. Breese, while testing the toxic effects of KME on several species of bivalves at the Yaquina Bay Fisheries Laboratory (now the Oregon State Marine Science Center), noted that the effluent readily stimulated the mussels to spawn. With the discovery of an effective method of obtaining gametes, the possibility of employing mussel larvae for bioassay work was suggested.

A mussel embryo bioassay essentially consists of exposing developing embryos to different concentrations of some toxicant, in this case sodium pentachlorophenate, for a period of 48 hours. At the end of this time numbers of normal (shelled) and abnormal (nonshelled) larvae are counted. During 1957 and 1958, Richard Toner, then a graduate student at Oregon State University, explored the feasibility of using the embryonic stages of the bay mussel to bioassay spent sulfite liquor (SSL) (1961). The results obtained by Toner appeared promising in that there was almost always a decrease in numbers of normal larvae with

increasing concentrations of the liquor, but certain problems were encountered which were not adequately solved. Most of these problems were minor difficulties in technique; however the important consideration of rearing high percentages (80-90 percent) of normal larvae in the absence of toxicant was never completely resolved. In the majority of Toner's experiments the numbers of normal larvae in the control dishes (those dishes which contained sea water only) were less than 50 percent of the original numbers of fertilized eggs. This difficulty indicated that perhaps certain environmental factors, e.g., water temperature, type of container, salinity of the rearing water, etc., were not optimum for the bioassays, and that more experimentation along these lines could increase the reliability of the tests.

During 1960 and 1961 studies were conducted at the Yaquina Bay Fisheries Laboratory using sodium pentachlorophenate (NaPCP) as the experimental toxicant. The main objectives of these experiments were to improve techniques and to standardize environmental testing conditions. Most of the actual experimental work was conducted during the summer months with occasional experiments at other times to confirm the availability of mussel gametes during all seasons of the year. The research performed during 1960 was mainly exploratory and by 1961 techniques had been considerably improved, resulting in the data which forms the basis of this report.

The use of organisms to evaluate the effects of various chemicals and wastes on the marine environment is widespread, especially in relation to the shellfish industry. Davis (1961) reported on the use of embryonic and larval stages of two east coast bivalves, the quahog,

Venus mercenaria Linnaeus, and the American oyster, Crassostrea virginica (Gmelin), to study the toxicity of several pesticides. At the Yaquina Bay Fisheries Laboratory, the Threespine stickleback, Gasterosteus aculeatus Linnaeus, has been used to estimate weekly changes in the toxicity of pulping process effluent from a Kraft pulp mill. Mature Native Pacific coast oysters, Ostrea lurida Carpenter, and Pacific oysters, Crassostrea gigas (Thunberg) have been used for several years in long-term (six months to three years) bioassays of pulp mill effluents by Woelke (1965) and by the Yaquina Bay Fisheries Laboratory. Okubo and Okubo (1962) described a bioassay method utilizing the embryonic stages of M. edulis, C. gigas, and two sea urchins. These organisms were selected because of overlapping spawning seasons which insured availability of gametes from one organism during all seasons of the year. The development of eggs fertilized in various concentrations of several chemicals was compared to the development in plain sea water (control) to estimate the effect of the experimental toxicant.

In general, the use of the above animals entailed certain limitations for bioassay work, principally that of obtaining test organisms. Spawning clams and oysters often required several days (compared to one to two hours with the KME method), and was not always productive throughout the year (see Loosanoff, 1954, for a description of the methods used to spawn these bivalves). Although Okubo and Okubo (1962) employed bay mussel embryos, they obtained gametes by temperature stimulation, i.e., gradual increases in water temperature over a period of several days with sharp rise in temperature on the day gametes were

required. Marine fish, especially those of a size small enough to be used in bioassays, are normally available only seasonally and are often difficult to collect. The long-term bioassays were conducted for several months to achieve measurable results. It is hoped that the use of the mussel embryo test, which employs a rapid method of obtaining gametes and requires only 48 hours, might prove to be an effective means of estimating the effects of industrial, municipal, and domestic effluents in marine waters.

The research for this report was conducted under the auspices of the Department of Fisheries and Wildlife, Oregon State University. R. E. Dimick, Professor of Fisheries, and W. P. Breese, Assistant Fishery Biologist, supervised the study. All results presented in this paper were obtained from experiments conducted at the Yaquina Bay Fisheries Laboratory.

MATERIALS

Experimental Animals

The bay mussel occurs along the Pacific coast of North America from Alaska to Baja California and on the Atlantic coast from Greenland to North Carolina. On the European side of the Atlantic, bay mussels are found from the White Sea and into the Mediterranean Sea and northern Africa. This species is also found along the coast of Asia from the Bering Sea to the Sea of Ocholok and the islands of Japan. Soot-Ryan (1959) includes both coasts of South America in the distribution by describing the localities where many of the sub-species of M. edulis are found. This wide geographic distribution includes most of the localities where marine problems involving pollution by industrial wastes might be expected to occur. Thus the bay mussel has the distribution required to become a truly cosmopolitan test organism.

The normal development of the bay mussel has been described by Field (1922) and by Rattenbury and Berg (1954), although the latter authors were principally concerned with abnormal development. During the summer of 1960 the normal development of the embryo from fertilization through shelling was observed extensively. The formation of the stage of particular interest in this study, the fully-shelled stage, normally occurred at 40-48 hours (at $20^{\circ} \pm 2^{\circ}$ C.) from the time of fertilization, although in two instances completely shelled larvae were observed at 24 hours. When the fertilized eggs were subjected to

conditions of environmental stress (presence of toxicant, low salinity, etc.) many of the embryos failed to develop the typical straight-hinged shell. Figure 1 illustrates the appearance of the "D-shape" larvae at 48 hours. These larvae possess well developed powers of locomotion (by means of cilia or the velar lobe) and transparent valves capable of completely enclosing the digestive and locomotor organs. Figure 2 is a photomicrograph of 48 hour larvae exposed to 0.4 mg/l of NaPCP containing approximately 50 percent normal larvae. The small, dark larvae, which resembled advanced trochophore larvae and were mobile, were considered abnormal. These observations formed the basis for the criterion for normality described in the following section.

Experimental Toxicant

The sodium pentachlorophenate (NaPCP) used in this study was a commercially available technical grade form containing approximately 97 percent active ingredient. Preliminary investigations into the suitability of NaPCP indicated that it was highly soluble in water, had no apparent oxygen demand at the concentrations tested, and no significant effect on the pH of the sea water in the tests.

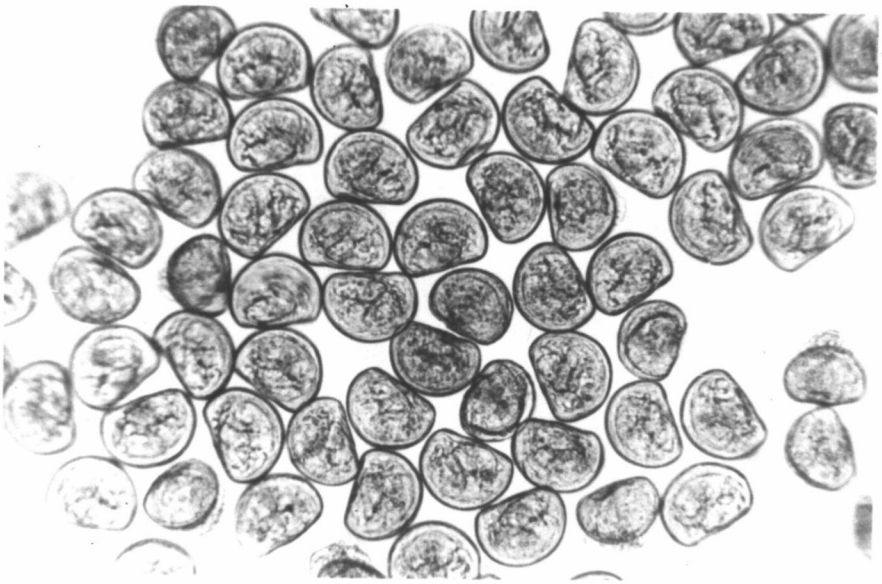

Figure 1. Normal mussel larvae, "D-shape", at 48 hours. 100x.

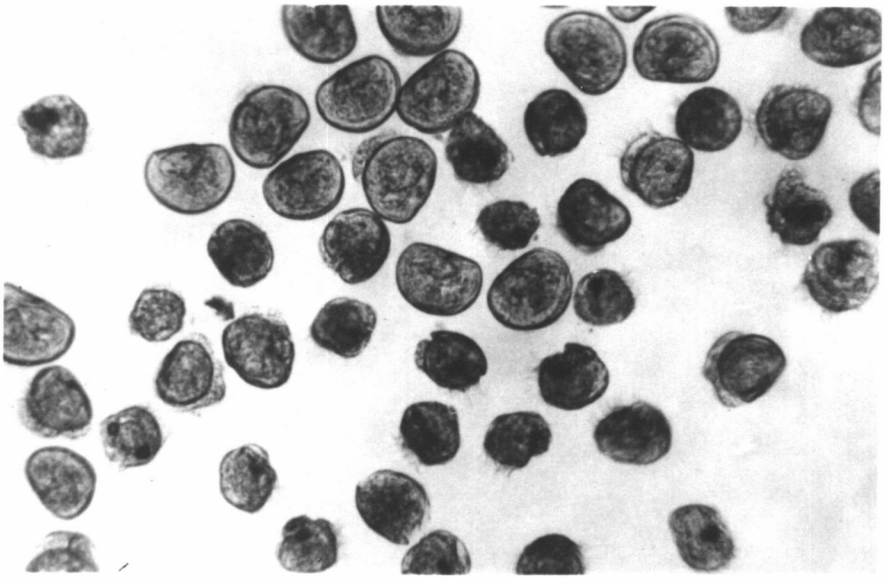

Figure 2. Normal and abnormal (unshelled) mussel larvae at 48 hours. 100x.

METHODS

The procedures employed during this investigation will be explained more fully in the following paragraphs but can be briefly outlined at this point. Eggs and sperm were obtained from mussels and fertilized in sea water (salinity of 25 p.p.t.) containing varying concentrations of sodium pentachlorophenate (NaPCP). The embryos were then allowed to develop for 48 hours at a constant temperature of 20° ± 2°C. At the end of this period, the larvae were killed and, with the aid of a binocular microscope, the numbers of normal (D-shaped) and abnormal (non-shelled or partially shelled) larvae determined. Inasmuch as this study was mainly exploratory, many procedures were tried and discarded before improved techniques were evolved and utilized. The two procedures developed, referred to in this report as procedures "A" and "B", essentially differed only in the way in which the percentages of normal larvae were calculated. In procedure A, the number of fertilized eggs in each dish was known only approximately, whereas in procedure B a count was made of the number of eggs placed in each container at the beginning of the experiment. At the end of the 48-hour period, numbers of normal and abnormal larvae in each container were counted and the percentage of normal larvae computed according to the following formulae:

1. Procedure A

$$P_A = \frac{\text{Number of normal larvae per container}}{\text{Number of normal + number of abnormal}} \times 100$$

2. Procedure B

$$P_B = \frac{\text{Number of normal larvae per container}}{\text{Number of fertilized eggs originally present}} \times 100$$

The results of bioassays using these two procedures will be the same if no mortality occurs during the 48 hour test period.

Mussel Spawning

Subsequent to the completion of this study, Breese, Milleman and Dimick (1965) published a rather complete description of the Kraft effluent method of inducing spawning in mussels, along with comparisons of some other methods employed by various investigators, therefore the procedures will only be summarized in this report. Adult mussels were normally collected the day before an experiment from pilings and floating docks located in Yaquina Bay. After removing extraneous organisms and materials, such as mud and barnacles, mussels were placed in a basket and stored at room temperature until the following morning. Approximately one to one and one-half hours before the projected beginning of an experiment, 24 to 48 mussels were placed in individual stacking dishes and covered with sea water containing six to eight percent (by volume) Kraft Mill Effluent (KME). Males normally commenced spawning at the end of the first half hour and could easily be detected by the streams of sperm coming from the mantle cavity. Usually after another 20 to 30 minutes, ripe females started to discharge eggs, normally in the form of short or long rod-like packets, although occasionally as single eggs. Egg color varied from female to female with

some yellow, some pink or orange and still others were white. As soon as spawning was noted, the mussel was thoroughly rinsed with fresh sea water (containing no KME) and placed in a dish containing salt water. The mussels were then allowed to spawn until sufficient numbers of gametes were available to conduct an experiment. Figures 3 and 4 show sperm and eggs from mussels that were allowed to spawn completely. The cloudy water in the dish in Figure 3 was caused by countless millions of sperm swimming in the water.

General Laboratory Techniques

Many of the techniques employed in this investigation are routinely used in bioassay studies with other organism and need only be mentioned at this time. Freedom from chemical contamination was of extreme importance, especially with respect to the glassware used. In order to avoid the possible inimical effects of soap and detergent residues on the developing embryos, dishes used as test containers were cleaned by flushing with cold running water from nontoxic pipes, scrubbing with a synthetic sponge, re-rinsing and allowing the dishes to air dry. Whenever graduated cylinders, pipettes, volumetric flasks, etc., were used, care was taken to insure that each container was used for only one solution, such as the toxicant, or sperm, eggs, etc.

Water used for rearing the larvae was collected from Yaquina Bay on the day of an experiment and transported to the laboratory in five gallon glass jars. Salinity was then determined to the nearest 0.1 p.p.t. with the use of hydrometers and then diluted to 25 p.p.t. with

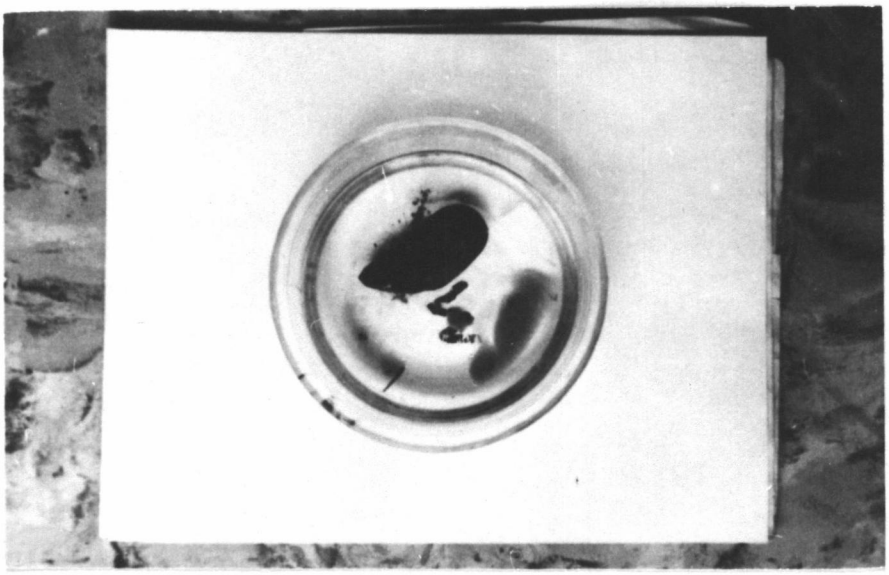

Figure 3. Female mussel allowed spawn completely. Approximately 1/3x.

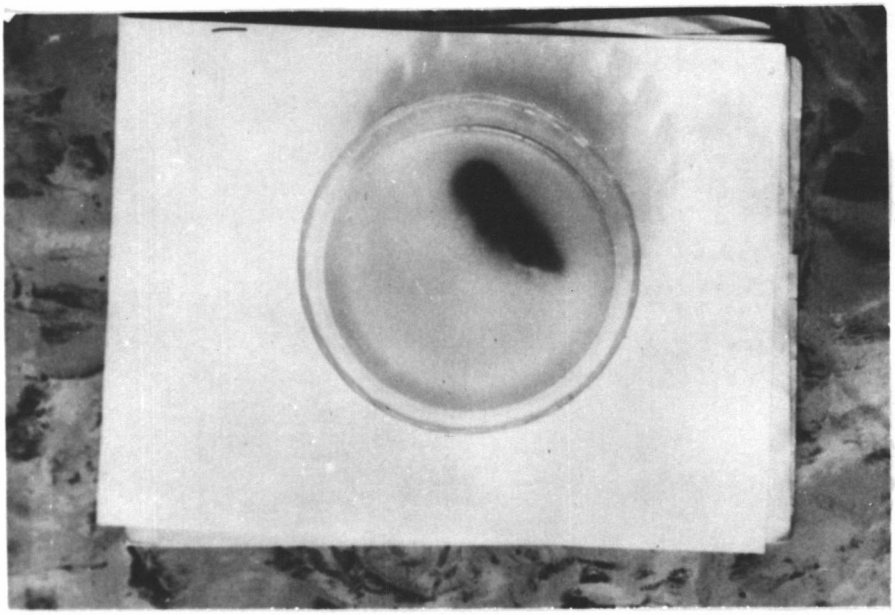

Figure 4. Male mussel allowed to spawn completely. Approximately 1/3x.

springwater from a metal-free system located at the laboratory. After dilution suspended solids, detritus, plankton, etc., were removed by passing the water through a continuous-flow centrifuge.

Experimental concentrations of the toxicant were prepared from a 0.25gm/l stock solution which was made fresh at the beginning of each summer and used until the following fall. Actual preparation of this solution consisted of weighing 0.250 (± 0.001 grams) grams of technical grade NaPCP on a semi-micro electric balance and completely dissolving the compound in one liter of glass-distilled water. Results of bioassays conducted throughout the study indicated that the stock solution did not noticeably decrease in toxicity during the three-month period. After dilution to experimental concentrations, determinations of hydrogen ion concentration and dissolved oxygen concentration were made with a laboratory pH meter and the Winkler method, respectively. These quantities were then redetermined at the completion of an experiment.

Experimental Procedures

Environmental Conditions. Before beginning actual bioassay studies, preliminary work was conducted to determine the optimum environmental conditions conducive to the normal development of the bay mussel. Because of the limited facilities available at the Yaquina laboratory in 1960 and 1961, it was not deemed feasible to investigate the temperature requirements of the developing embryos, however the

temperature selected (20°C.) did not appear to have an adverse effect during the testing period.

Approximate salinity tolerance of the embryos was determined by allowing the fertilized eggs to develop in water of various salinities from 16 to 32 p.p.t. with no toxicant present. Additional tests were conducted with water of different salinities, but in these tests the embryos were exposed to varying concentrations of NaPCP.

Although pyrex dishes were used throughout this study, tests were conducted to explore the possibility of utilizing plastic containers of various types. In these experiments larvae were exposed to different concentrations of NaPCP in both types of containers of the same general shape and volume (pyrex and plastic petri dishes).

These preliminary studies were conducted utilizing the techniques outlined below for Procedure A and the results computed using formula 1 (P_A).

Procedure A. After mussel spawning had progressed to the extent that an adequate number of gametes were available, several thousand eggs from a single female were collected and placed in a beaker containing sea water. Although eggs from one female were used for each separate experiment, occasionally eggs from two or three females were tested on the same day to determine the viability of eggs from different females. Eggs were collected from any of the spawning females, although preference was usually given to those eggs from females ejecting single eggs, primarily because a homogenous suspension could be more easily obtained with eggs of this type. The number of eggs per

milliliter was then estimated by counting the numbers of eggs in five, one milliliter aliquot samples. Using the mean of these five samples as the concentration of eggs per milliliter, the volume of sea water in the beaker was then adjusted to provide an approximate final concentration of 1000 eggs per milliliter.

The bioassays were conducted using one pint pyrex dishes as test containers. The final volume of liquid in all the bioassays considered in this report was 200 milliliters, although volumes of 100 and 300 milliliters were occasionally employed in preliminary work. The experimental concentrations of NaPCP were prepared in duplicate by the addition of pre-determined amount of the stock solution, 10 milliliters of egg suspension, one-half milliliter of sperm suspension and enough sea water required to make a final volume of 200 milliliters. Duplicate concentrations were prepared individually. Concentrations ranged from 0.0 mg/l (control) to 1.0 mg/l, generally in 0.1 mg/l or 0.2 mg/l increments.

Fertilization was normally completed within an hour after the mussels had commenced spawning. At the end of one and one-half hours eggs were examined under a binocular microscope for the formation of polar bodies, thus indicating that fertilization was successful. If it appeared that complete fertilization had occurred, which was almost always the case, the dishes were transferred to a constant temperature room, $20^{\circ} \pm 2^{\circ}$ C. In those few instances where complete fertilization did not occur, the cell suspensions were discarded, the dishes washed and rinsed, and the experiment was attempted with gametes from different parents.

Approximately one to two hours before the termination of an experiment or bioassay, a few drops of neutral red stain in distilled water were added to each dish. This stain aided in counting the larvae and assisted in the differentiation of the normal and abnormal larvae because the abnormal larvae, called anomalies, appeared considerably darker than the normal, shelled larvae. At 48 hours from the time of fertilization, the larvae were killed with a solution known as "AFA", a killing and preservative agent containing isopropyl alcohol, formalin and glacial acetic acid. This solution was used for the following reasons: five or six drops killed all the larvae within a few minutes; shelled larvae died with valves closed, which aided in identification; and the utilization of the agent in rearing dishes did not appear to have any residual effects causing harm to the developing embryos.

The large numbers of larvae originally present in each test dish, and the comparatively large volume of water, necessitated the use of aliquot-sample type of counting technique. The contents of each test dish were poured into a 500 milliliter Erlenmeyer flask, the flask then stoppered and the contents agitated for one minute. A five milliliter automatic pipette was immediately inserted and a sample withdrawn from the center of the container. The sample was then discharged into a petri dish and the dish placed on a grid etched in plexiglass. The petri dish and grid were next placed on the stage of binocular microscope and the larvae counted at a magnification of 45X. The development of the shell was used at the test criteria, a shelled larvae with

a straight hinge was considered normal and an unshelled, or partially shelled larvae, abnormal (see Figures 1 and 2). The data were then recorded as percent normal as computed by formula 1 (P_A). In many instances the lengths of 20 to 40 shelled larvae from each concentration were measured by means of the micrometer eyepiece.

In reporting the data, an additional quantity was determined, the EC_{50}, or the estimated concentration which had the designated effect (prevented normal shelling) on 50 percent of the organisms. The EC_{50} was derived by plotting the dosage-response curve on semi-logarithmic graph paper for each bioassay with the concentration of the toxicant on the arithmetic scale and the percent normal larvae on the logarithmic scale. A straight line was then drawn between the two successive concentrations on either side of the 50 percent line. The concentration at the point of intercept with the 50 percent line was considered the approximate EC_{50} value.

In order to evaluate the reliability of the sampling method used in this study, occasionally several samples were withdrawn from the same test dish and the variation among the samples determined. In all instances the samples were returned to the dish before withdrawing the next sample.

Procedure B. This procedure differed from A in only one basic aspect, that it required an accurate determination of the numbers of eggs present in each test container at the beginning of an experiment. Because of the difficulty encountered in counting the eggs in the one

pint pyrex dish, due mainly to the sloping sides and rounded bottoms of the dishes, another type of test container was needed. Several types were examined, with the 20 x 100 millimeter pyrex petri dishes being found suitable.

Approximately 5000 eggs were fertilized in one pint pyrex dishes containing the experimental concentrations. After one and one-half hours each test dish was stirred with a glass rod and twenty-five milliliters of egg suspension were transferred to a correspondingly marked petri dish. After the transfer, the numbers of fertilized eggs (by this time all fertilized eggs were normally in the second polar body stage or undergoing the first cell division) were counted and the dishes moved to the constant temperature room. Any unfertilized eggs were noted.

Termination of the bioassay at 48 hours was accomplished as in Procedure A: the larvae were stained with neutral red and killed with "AFA". The normal and abnormal larvae in each dish were differentiated and counted by placing the petri dish on the plexiglass grid and counting at 45X under the binocular microscope. The percentages of normal larvae were calculated using both formulae 1 and 2 (P_A and P_B).

EXPERIMENTAL RESULTS

During this study emphasis was directed to evaluating the effect of sodium pentachlorophenate on the normal embryonic development of the bay mussel; however, a portion of the experimental effort was allotted to the investigation of other environmental and procedural factors which might have influenced the reliability and/or usefulness of mussel embryos in marine water bioassays. The data obtained during the investigation of these latter factors (including mussel spawning, effect of salinity, evaluation of sampling techniques and the effect of the toxicant on larval size) are included in this section, along with the results of the NaPCP bioassays.

Mussel Spawning

Mussel spawning was attempted on 102 different occasions during the summers of 1960 and 1961 and only on three of these attempts either a male or female failed to spawn, thus preventing bioassay work on these particular days. Appendix Table A is a compilation of the mussel spawning data obtained during this study with some results obtained by W. P. Breese. The spawning data of Breese, from the same general period of time as this study and by the same spawning methods, are included to present a more comprehensive annual spawning pattern. Figure 5 is a graphic representation of the average monthly spawning percentages calculated from the data in Table A. Average monthly spawning was

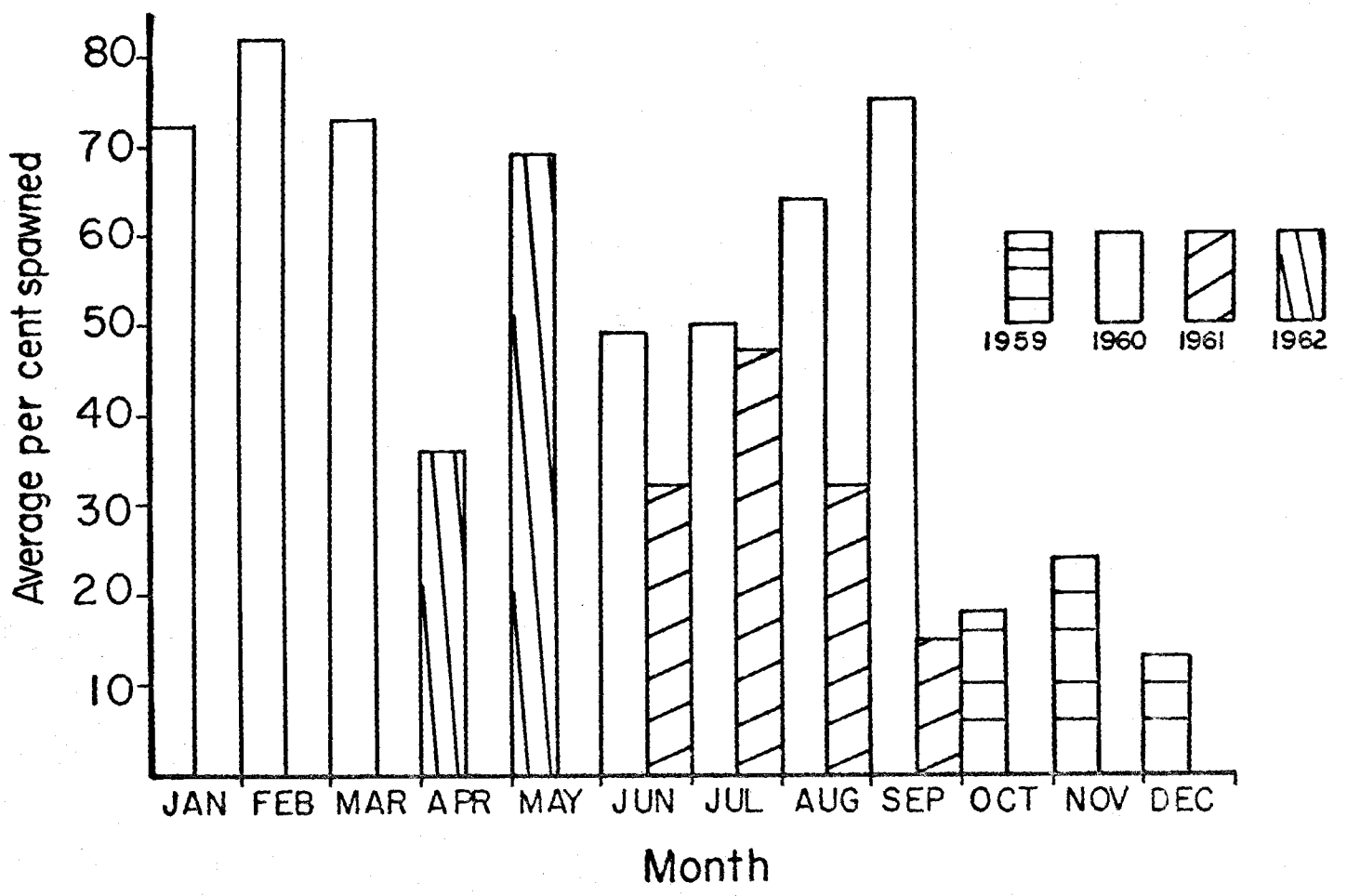

Figure 5. Average monthly percentages of mussel spawning after stimulation by Kraft Mill Effluent. All available data.

highest in February 1960 (82 percent) and lowest in December 1959 (13 percent). From the information in Figure 5 it appears that gametes were readily available during the months of January through August (lowest average spawning during this period was approximately 30 percent) and were available to a lesser extent from September through December (from 15 to 25 percent spawning). A comparison of the spawning from the two summers indicates that there may be considerable variation from year to year. During the months of August and September spawning was significantly lower in 1961 as compared with 1960 (August - 64 percent in 1960 vs 32 percent in 1961 and September - 75 percent vs 15 percent). There was little difference in spawning in June and July between the two years (June - 49 percent in 1960 vs 32 percent in 1961 and July - 50 percent vs 47 percent).

The overall sex ratio of the mussels that spawned was 0.77 (females to males) indicating that males were more readily stimulated to spawn.

Effect of Salinity on Normal Development

During the summer of 1960 experimentation was conducted to determine the approximate optimum salinity requirements of the developing embryos. The results of the first series of experiments, those in which salinity was the only variable tested, are summarized in Table 1. The highest percentages of fully shelled larvae were obtained at a salinity of 28 p.p.t. (from 90 to 96 percent fully shelled at the

end of 48 hours); although in three of the four experiments, approximately 90 percent of the larvae in 24 p.p.t. water were normal. In the experiment conducted on August 24, 1961, only 47 percent of larvae at 24 p.p.t. were normal, perhaps due to low egg viability (the percentages of normal larvae at the other salinities tested were also lower on the 24th than on any other day). The highest salinity tested (32 p.p.t.) appeared to be too high for maximum development in that only 70-80 percent of the larvae were normal at the end of the test period. The percentages of larvae developing normally at 20 p.p.t. varied widely (from 7.7 percent to 70 percent) indicating that this salinity may be marginal for the normal development of mussel larvae. No fully shelled larvae were observed at the end of 48 hours in water with a salinity of 16 p.p.t.

Table 1. Effect of salinity on percentage of normal development of mussel larvae. Percentages are averages of duplicate samples.

	Salinity (p.p.t.)				
Date	16	20	24	28	32
8-24-60	0	8	47	90	72
8-25-60	0	52	93	95	78
8-28-60		25	90	96	
9-1-60		70	90	95	

Percentages computed using the following equation:

$$P_A = \frac{\text{Number of normal larvae at 48 hours}}{\text{Number of normal larvae + number of abnormal larvae}} \times 100$$

The results of another series of salinity experiments, in which both salinity and concentration of NaPCP were varied, are presented in Table 2. These results further substantiated the findings of the above experiments, i.e., a salinity of 28 p.p.t. is near the optimum for the normal development of mussel larvae. In both of the experiments conducted at 28 p.p.t., 95 percent of the control larvae were normal and 75 to 80 percent of the larvae at 0.4 mg/l were fully shelled at the end of 48 hours. At a salinity of 24 p.p.t. controls also had high percentages of normal larvae (approximately 90 percent normal), but the percentages of shelled larvae decreased to approximately 30 percent at 0.4 mg/l. At the lowest salinity tested, 20 p.p.t., there were few normal larvae in controls (25 and 50 percent) and no normal larvae at 0.3 or 0.4 mg/l of NaPCP.

In Figure 6 the dose-effect curves obtained using the water adjusted to the three different salinities on August 25, 1960 are illustrated. As the salinity increases, the curves are displaced upward along the abscissa (percent normal larvae) indicating that the toxicity of the NaPCP decreased. This point can be further emphasized by an examination of the EC_{50}'s of the three curves. At a salinity of 20 p.p.t., the percentages of normal larvae in the various concentrations of NaPCP were too low to accurately determine an EC_{50}. The EC_{50} for a salinity of 24 p.p.t. was 0.32 and at 28 p.p.t. the percentages of normal larvae were never low enough (less than 50 percent) to establish a median effective concentration. It is probable that the developing embryos are more resistant to the effects of the toxicant at or near the optimum salinity value.

Table 2. Effect of various salinities and concentrations of NaPCP on normal development of mussel larvae. Percentages are averages of duplicate samples.

	Salinity					
	20 p.p.t.		24 p.p.t.		28 p.p.t.	
NaPCP (mg/l)	8-25-60	8-28-60	8-25-60	8-28-60	8-25-60	8-28-60
0.0	52	25	93	90	96	96
0.2	24	26	78	80	83	94
0.3	0	0	61	72	78	86
0.4	0	0	32	30	74	82

Percentages computed using the following equation:

$$P_A = \frac{\text{Number of normal larvae at 48 hours}}{\text{Number of normal larvae + number of abnormal larvae}}$$

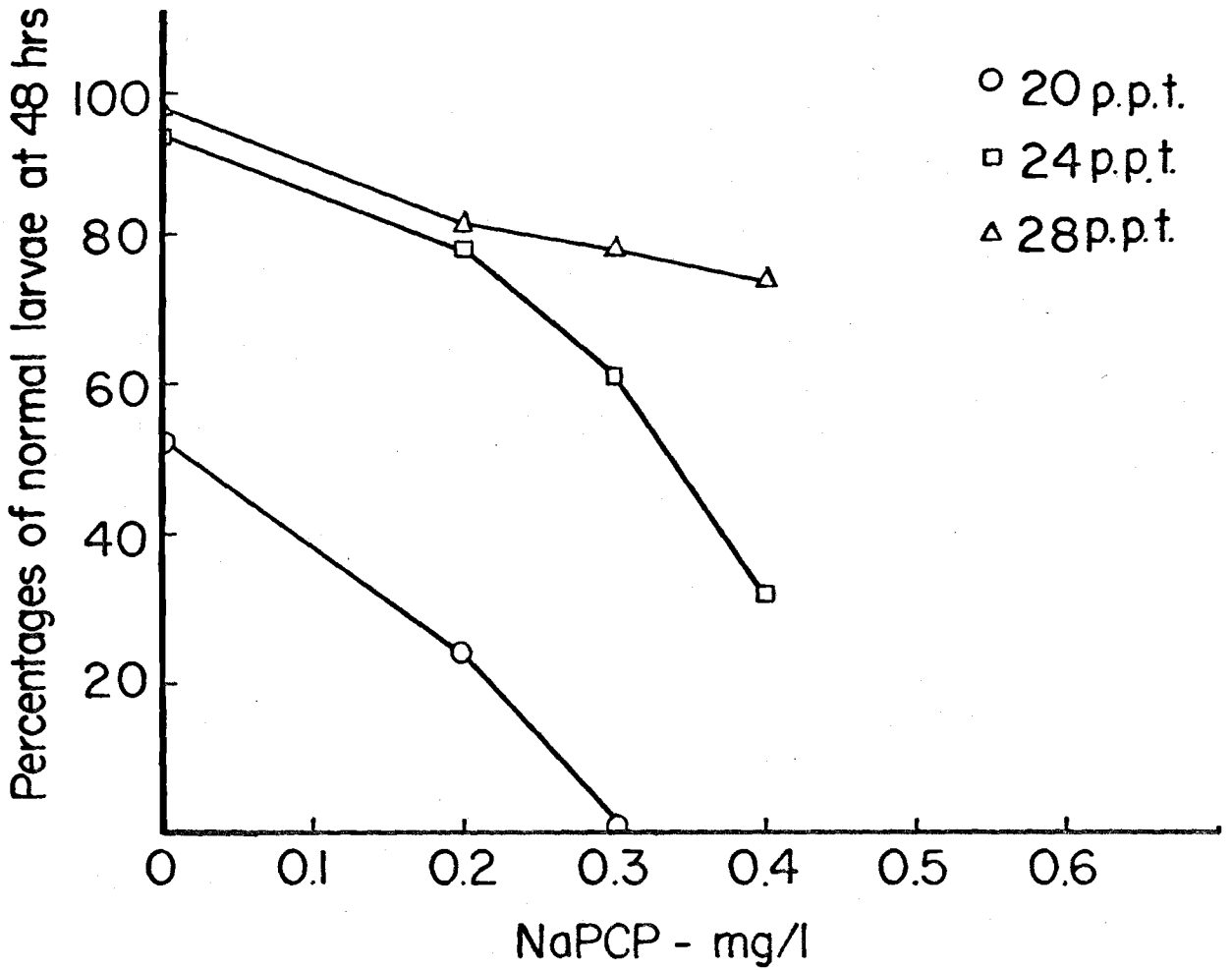

Figure 6. Effect of salinity and NaPCP on 48 hour normal development of mussel embryos.

Effect of NaPCP on Hydrogen Ion and Dissolved Oxygen Concentrations

During some preliminary experiments, hydrogen-ion and dissolved oxygen concentrations were determined at the beginning and end of the bioassays. These determinations were made on replicate concentrations of the toxicant, containing developing embryos, although no counts were made of the larvae. Tables 3 and 4 contain the results of two sets of these analyses. From the data in Table 3 it is apparent that NaPCP does not change the pH of sea water at the concentrations tested. In both of these experiments there was little or no difference in pH values between controls and 0.6 mg/l, either at the beginning or the end of the test period.

Table 3. Effect of NaPCP on hydrogen ion concentration (pH).

Date	NaPCP (mg/l)	pH 0.0	0.2	0.4	0.6
8-25-60	Start	8.03	8.02	8.02	8.02
	Finish	8.0	7.98	7.98	8.0
8-28-60	Start	7.87	7.87	7.87	7.87
	Finish	7.85	7.86	7.85	7.87

In Table 4 the differences in dissolved oxygen values between controls and 0.6 mg/l vary from -0.18 to +0.01 (considering both beginning and end values) indicating that the NaPCP had no appreciable

oxygen demand at the concentrations tested. The reduction in dissolved oxygen concentrations (approximately 0.5 ppm in all cases measured) during the bioassay is due to the change in water temperature from about 15°C. to 20°C.

Table 4. Effect of NaPCP on dissolved oxygen concentrations.

Date	NaPCP (mg/l)	Dissolved oxygen (p.p.m.) 0.0	0.2	0.4	0.6
8-25-60	Start	6.68	6.58	6.49	6.50
	Finish	6.12	6.10	6.07	6.10
8-28-60	Start	6.72	6.67	6.61	6.73
	Finish	6.11	6.11	6.10	6.08

Plastic versus Glass Containers

In August 1961 four experiments were conducted to determine the possible influence of the type of container material on the toxicity of NaPCP. The test containers used in this series of bioassays were molded plastic and pyrex glass petri dishes. Eggs and sperm were added to duplicate dishes of each type containing concentrations of NaPCP from 0 to 1.0 mg/l in 0.2 mg/l steps. The results of these experiments are contained in Table 5. A comparison of the EC_{50}'s obtained in each type of container for individual experiments indicates that there was a reduction of toxicity in the plastic dishes. In three of

Table 5. Comparison of plastic vs glass containers. Percentages are averages of duplicate samples.

Concentration NaPCP (mg/l)	Date 8-5-61		8-7-61		8-8-61		8-11-61	
	Plastic	Glass	Plastic	Glass	Plastic	Glass	Plastic	Glass
0.0	99	98	93	99	90	97	97	98
0.2	98	98	93	98	94	98	90	98
0.4	98	90	83	92	91	40	87	94
0.6	63	6	26	25	0	0	80	60
0.8	10	0	0	0	0	0	57	0
1.0	0	0	0	0	0	0	5	0

Percentages calculated using the following equation:

$$P_A = \frac{\text{Number of normal larvae at 48 hours}}{\text{Number of normal larvae + number of abnormal larvae}}$$

the four pairs of bioassays, the EC_{50} was higher in the plastic containers (0.65 vs 0.50; 0.48 vs 0.36 and 0.82 vs 0.63 for plastic and glass, respectively), and in the fourth there was little difference. Another indication of the loss of toxicity in the plastic containers was the presence of normal larvae in concentrations of 0.8 (two instances) and 1.0 mg/l (one instance) of NaPCP. As will be pointed out in another section, shelled larvae were not noted in these concentrations when using glass containers.

Evaluation of Sampling Technique

The sampling method used in procedure A (mixing sample thoroughly and withdrawing a five milliliter sample) was tested by removing and counting a series of ten, five milliliter samples from a control dish (volume of 200 milliliters). The results of this test are presented in Table 6. The sample sizes varied from 375 to 456 larvae with an average of 419 larvae per five milliliter sample. The percentage of normal larvae for the ten samples had a range of 3.5 percent (77.9 to 81.4 percent normal larvae) with an average value of 79.4 percent. Based on the range of values noted in this experiment, differences of less than seven percent ($\pm$ 3.5 percent) are of doubtful significance.

Table 6. Variation among a number of successive five milliliter samples from a single test container.

Sample number	Sample size	Normal	Abnormal	Percent normal larvae
1	449	351	98	78.2
2	403	320	83	79.4
3	417	334	83	80.1
4	401	313	88	78.1
5	456	362	94	79.4
6	375	300	75	80.0
7	433	346	87	79.9
8	447	364	83	81.4
9	407	317	90	77.9
10	406	323	83	79.6

Procedure A Bioassay Results

During the summers of 1960 and 1961 several bioassays were conducted employing the procedures outlined for Procedure A. These procedures consisted of exposing approximately equal numbers of fertilized mussel eggs to several concentrations of NaPCP for 48 hours. At the end of this period the percentages of normal larvae were determined by counting aliquot samples of the surviving larvae. These bioassays were conducted with the objectives of standardizing techniques and evaluating

the reproducibility of mussel embryo test results over a three month period. The results of 19 bioassays conducted in 1961 have been selected for presentation because of the high percentages of normal larvae in control cultures (more than 90 percent normal in most instances) and the more or less standardized laboratory conditions under which the experiments were conducted.

Although the 19 separate experiments will be considered, pertinent features of the bioassays in general can be illustrated by examining, in some detail, the results of two typical tests conducted in June, 1961, Table 7. The results of these two experiments were similar in several respects. In both bioassays the percentages of normal larvae decreased with increasing concentrations of NaPCP, from approximately 90 percent in controls to no straight-hinged larvae at a concentration of 0.6 mg/l. At 0.2 mg/l the percentages of normal larvae were about 90 and 95 percent of the control values in experiments one and two respectively. The percentages of normal larvae at the next highest concentration tested, 0.3 mg/l, were lower than controls by about 20 percent in both experiments. At 0.4 mg/l the approximate EC_{50} values were noted with approximately 50 percent of the larvae developing normal shells. Only an average of 14 and 4 percent of the larvae developed the typical straight-hinged shell in the presence of 0.5 mg/l of the toxicant.

The dose-effect curves of these two bioassays (averages of duplicate dishes) are plotted in Figure 7. The close agreement between the shape of the two curves further illustrates the similarity of

Table 7. Results of two typical bioassays showing variation between duplicates of same concentrations of NaPCP.

Number	Date	Concentration	Dish Number	Number of larvae			Percent Normal
				Normal	Abnormal	Total	
1	6-26-61	0.0	1	124	10	134	92.5
			2	106	6	112	94.6
		0.2	1	108	20	128	84.3
			2	114	24	138	82.6
		0.3	1	84	37	121	69.4
			2	74	25	99	74.7
		0.4	1	38	36	74	51.4
			2	38	30	68	55.9
		0.5	1	5	22	27	18.5
			2	9	73	82	10.9
		0.6	1	0			0
			2	0			0
2	6-28-61	0.0	1	269	25	294	91.5
			2	281	29	310	90.6
		0.2	1	281	26	307	91.5
			2	232	57	289	80.3
		0.3	1	252	114	366	68.9
			2	221	110	331	66.8
		0.4	1	170	178	348	48.9
			2	105	144	249	42.2
		0.5	1	9	176	185	4.9
			2	7	183	190	3.7
		0.6	1	0			0
			2	0			0

1. Computed using the following equation:

$$P_A = \frac{\text{Number of normal larvae at 48 hours}}{\text{Number of normal + number of abnormal larvae at 48 hours}}$$

2. Number of larvae obtained in a 5 ml sample.

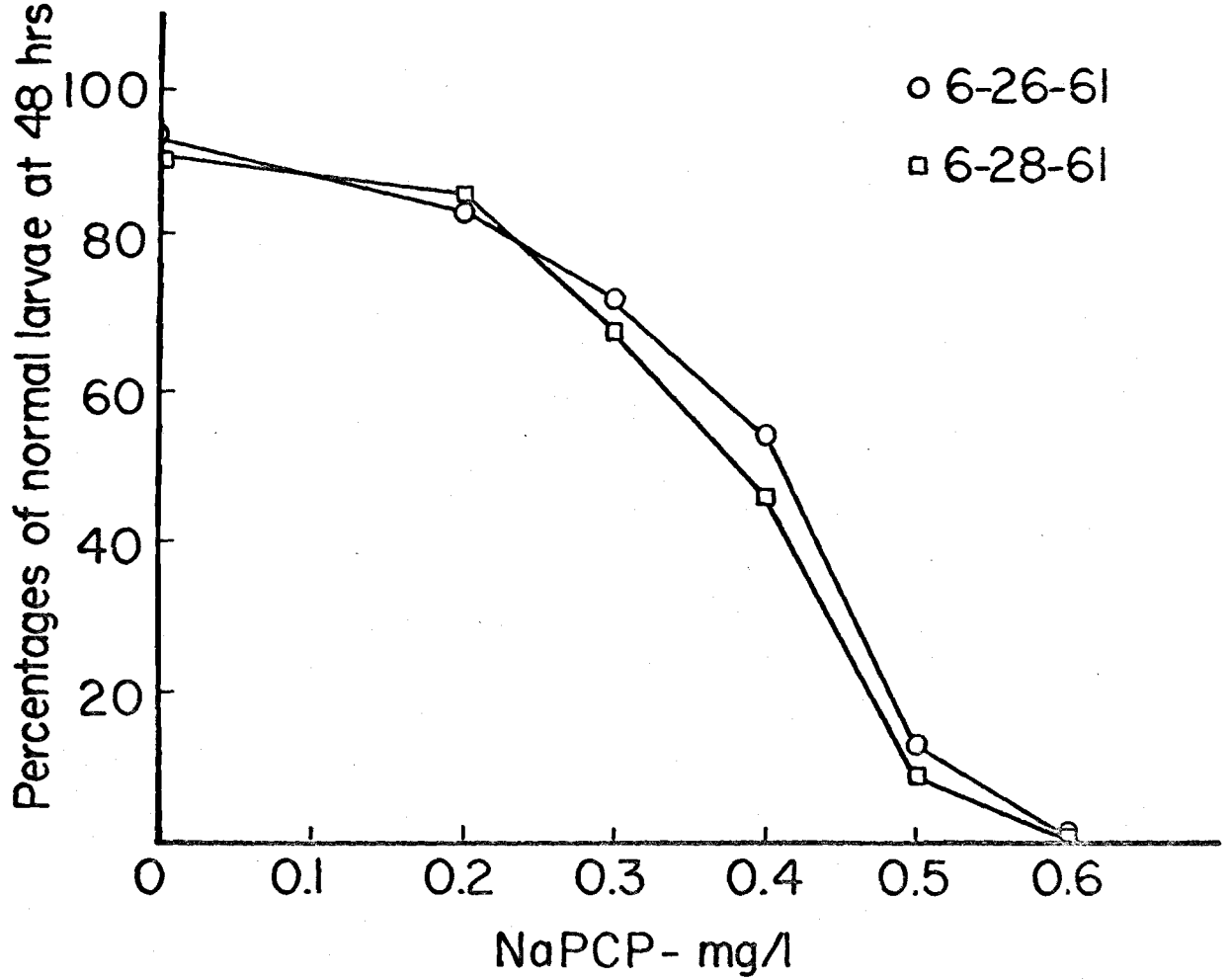

Figure 7. Results of two bioassays showing effect of NaPCP on normal development of mussel embryos.

results of two bioassays conducted on different days and with gametes from different parents. The sigmoid shape of the curves is typical of curves plotted from bioassay data obtained in this study, and is also typical of dose-effect curves in general. At both ends of the curves changes in concentration resulted in little or no change in percentages of normal larvae and there is a central section where small increases in concentration greatly influenced the ability of the larvae to develop normally.

A comparison of the results of individual dishes within each concentration also illustrates the agreement between the two tests. There were only two instances (experiment 1, 0.5 mg/l and experiment 2, 0.2 mg/l) where the differences in percentages of normal were greater than seven percent. As was pointed out previously, the method used to sample the larvae at the termination of an experiment is probably accurate to only $\pm$ 3.5 percent, therefore recorded differences between duplicates of less than seven percent cannot be considered significant. In the other duplicates the differences were on the order of one to five percent. Only one instance (experiment 1, 0.5 mg/l) was noted where there was a relatively large difference between duplicate dishes (27 _vs_ 82 larvae) in total numbers of larvae at 48 hours. The general agreement in sample sizes taken from duplicate concentration indicates that the desired objective of placing approximately equal numbers of fertilized eggs in each dish was realized.

In both bioassays there were decreases in total numbers of larvae in the samples as the concentration increased (approximately

55 and 40 percent fewer at 0.5 mg/l as compared to controls in bioassays 1 and 2 respectively). In the following section, Procedure B bioassay results, the possible effects of this differential larval mortality at various concentrations of NaPCP on percentages of normal larvae will be presented.

The results of the 19 bioassays are presented in Appendix B. The percentages of normal larvae, averages of duplicates within each concentration, have been calculated based on the observed data (actual) and relative to controls as 100 percent normal. Relative percentages are included to help determine if those experiments in which controls are less than 90 percent normal can provide useful information.

In general, the data for the 19 bioassays closely resembled the results of the two experiments discussed previously. In all instances there were lower percentages of normal larvae in the highest concentration when compared to control larvae. In Figure 8, average relative percentages for each concentration tested, along with the range of values, have been plotted. The curve follows the general shape of the curves for experiments 1 and 2 quite closely. At 0.2 mg/l the range of values was small (81 to 99 percent normal) and the average value of 94.5 percent normal suggests that this toxicant caused little effect. The upper concentration limit at which some larvae reached the straight-hinged stage at 48 hours was 0.6 mg/l. This level was tested 14 times and on three occasions normal larvae (possessing a straight-hinged shell, although these larvae did not attain the size of control larvae) were present at the end of the test period. In these three bioassays percentages of normal larvae were variable (6, 25, and 45

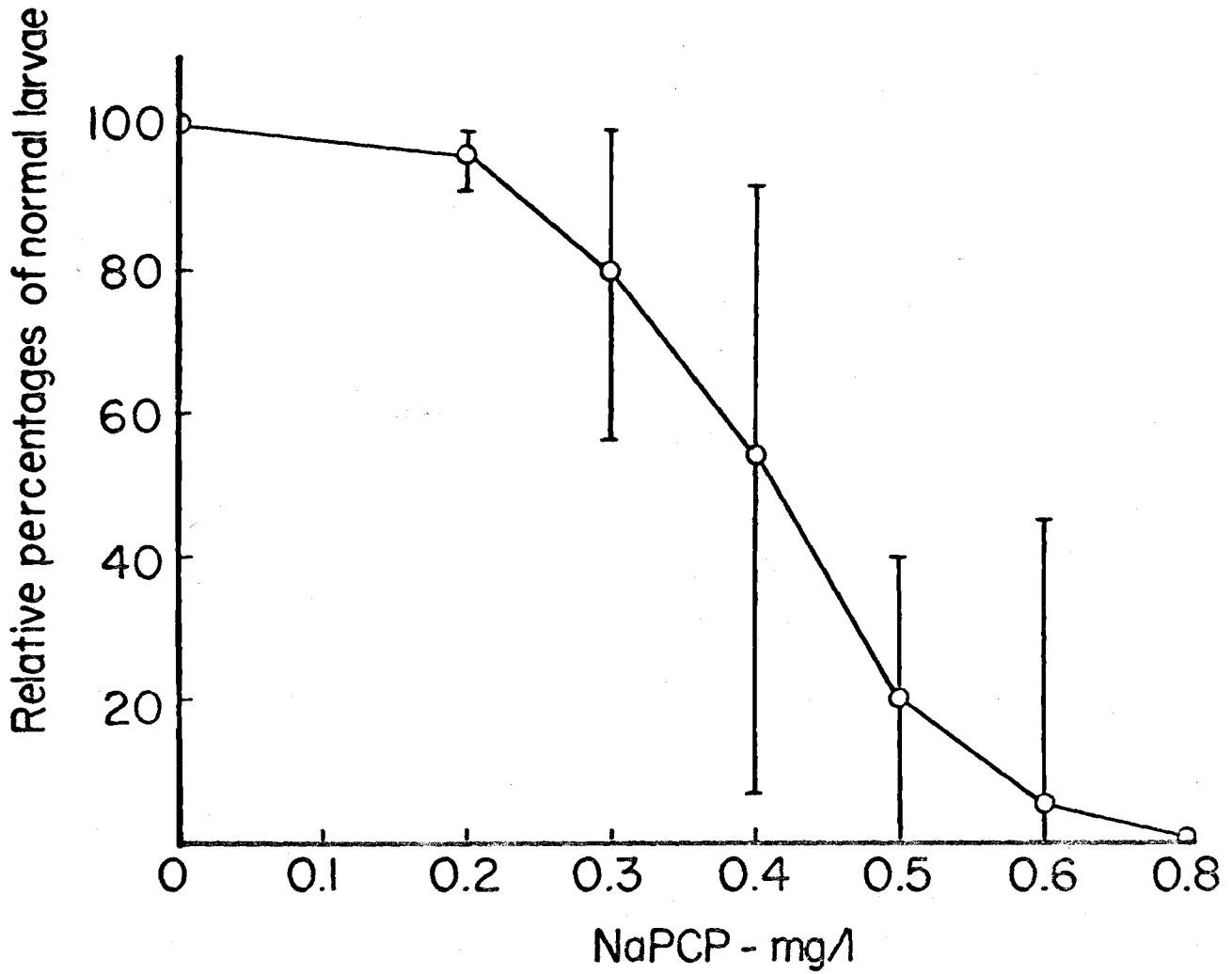

Figure 8. Effect of NaPCP on normal development of mussel embryos. Results of 19 Procedure A bioassays.

percent) and the total numbers of larvae in the samples were considerably less than in controls (approximately 51, 49 and 96 percent fewer) suggesting that this concentration was near the limit of embryonic tolerance. From the foregoing data it appears that the effective range, the interval between the concentration which produced no effect and the concentration which caused a complete lack of shelling, was approximately between 0.2 mg/l and 0.6 mg/l under the testing conditions of these experiments.

At 0.5 mg/l, tested a total of ten times, the percentages of normal larvae varied between 0 and 40 percent with an average of approximately 20 percent. The average estimated mortality for larvae exposed to this concentration of NaPCP was 57 percent with a range of 38 to 92 percent.

The widest variation in the dose-effect relationship studied was noted at 0.4 mg/l. This concentration was tested 19 times and the results ranged from almost no effect (94 percent normal) to almost complete harm (6 percent normal) with an average of approximately 53 percent. In general the comparison of total numbers of larvae at 0.4 mg/l with controls indicated that mortality was less than noted at 0.5 mg/l (range of no mortality to 90 percent with an average of approximately 28 percent).

At a concentration of 0.3 mg/l the relative percentages varied from 54 to 99 percent with an average of 79 percent. As the graph in Figure 8 illustrates, the variation was much less than noted at 0.4 mg/l

with controls indicated that mortality was less than noted at 0.5 mg/l (range of no mortality to 90 percent with an average of approximately 28 percent).

At a concentration of 0.3 mg/l the relative percentages varied from 54 to 99 percent with an average of 79 percent. As the graph in Figure 8 illustrates, the variation was much less than noted at 0.4 mg/l and about the same as for 0.5 and 0.6 mg/l. On an average there were approximately 25 percent fewer larvae in this concentration than in controls.

The percentages of larvae in controls were almost uniformly above 90 percent and often above 95 percent normal. In only two instances did the percentages drop below the 90 percent level, experiments 3 (77.8 percent) and 4 (88.2 percent). Although little difficulty was encountered in rearing larvae to the D-shaped stage during the summer of 1961, there was a period of one week in August 1960 when larvae did not develop normally in controls. Spawning was as usual during this period and fertilization appeared to be successful. Swimming larvae were usually noted, but development seemed to stop before the trochophore stage. This problem was not encountered at any other time during the study. Since development proceeded normally both immediately prior to and after this period, some factor in the bay water may have been responsible.

The EC_{50} values listed in Table 8 deserve special consideration since it is in this form that mussel embryo test results will most

Table 8. EC_{50} values of 19 NaPCP bioassays using Procedure A.

Number	Date	EC_{50} (mg/l NaPCP)[2] Actual[1]	Relative
1	6-26-61	0.41	0.42
2	6-28-61	0.38	0.40
3	7-5-61	0.26	0.41
4	7-12-61	0.39	0.43
5	8-3-61	0.42	0.42
6	8-7-61	0.49	0.49
7	8-8-61	0.35	0.36
8	8-9-61	0.47	0.47
9	8-11-61	0.52	0.52
10	8-15-61[3]	0.28	0.28
11	8-15-61	0.30	0.31
12	8-15-61	0.34	0.34
13	8-16-61[3]	0.55	0.57
14	8-16-61	0.35	0.36
15	8-19-61	0.47	0.47
16	8-22-61[3]	0.33	0.33
17	8-22-61	0.46	0.46
18	8-23-61	0.42	0.43
19	9-1-61	0.31	0.32
	Mean	0.39	0.41
	Variance	0.1349	0.1178

1 As computed from observed percentages of normal larvae
2 Relative to control as 100 percent normal
3 Eggs from more than one female used on the same day

likely be tabulated in future work. These values were calculated using both actual percentages of normal larvae and percentages relative to controls as 100 percent normal.

The actual EC_{50} values of the 19 bioassays conducted during the period June 26 through September 1, 1961 ranged from a low of 0.26 to a high of 0.55 mg/l NaPCP (average of 0.39 mg/l) with a variance of 0.14. The range of relative EC_{50} values was slightly greater (low - 0.28, high - 0.57 with an average of 0.41 mg/l) although the variance was reduced to 0.12.

Considerable day to day variation was noted in the EC_{50} values, but there did not appear to be any observable trend over the nine week period. The data from the three days on which the eggs from more than one female were used in the same test yielded conflicting results. On August 15 (bioassays 10, 11, and 12) the actual EC_{50} values were in good agreement (0.28, 0.30, and 0.34) indicating that the fertilized eggs from different females were similar with respect to their tolerance of NaPCP. This conclusion is also substantiated by the data for these experiments in Appendix B showing that the actual percentages of normal larvae at various concentrations were similar in the three tests. On the following day another experiment of this type was conducted, although only two females were used. At 48 hours there was a wide difference in the observed EC_{50} values (0.55 *vs* 0.35 mg/l). Similar results were obtained on August 22 when the median tolerance limits of larvae from two females were 0.33 and 0.46 mg/l.

There were two experiments in which the controls were less than 90 percent normal (experiment 3, 77.8 and experiment 4, 88.2 percent). The actual EC_{50} value for experiment 3 was 0.26 mg/l, the lowest of the 19 EC_{50}'s, however the relative EC_{50} was 0.41, or equal to the mean of the 19 values. In experiment 4, there was little difference between the results of the two methods used to calculate the EC_{50} values (0.39 actual and 0.43 relative). Although there was some indication that experiments with less than 90 percent of the control larvae normal may provide useful data, more experimentation along these lines is required.

Procedure B Bioassay Results

Five Procedure B bioassays were conducted during the month of August, 1961. In these tests, fertilized eggs were placed in 25 milliliters of the experimental concentrations of NaPCP and then counted. At the end of 48 hours all surviving larvae were counted and the percentages of normal larvae calculated using the formulae for P_A and P_B (see page 8). Although the volume of liquid in these bioassays was only 25 milliliters, as compared to 200 milliliters in the Procedure A experiments, the number of eggs per milliliter was approximately 40 to 50 in both instances. The results of these experiments are tabulated in Table 9.

The percentages of normal larvae (P_A) are in general agreement with those values described in the previous section. Control larvae

Table 9. Results of five mussel embryo tests using Procedure B bioassay techniques.

Bioassay Number	Date	Concentration NaPCP (mg/l)	Number of Eggs	Number of Larvae			Percent Survival	Percent Normal (P_A)	Percent Normal (P_B)
				Normal	Abnormal	Total			
1	8-21-61	0.0	1,216	1,020	23	1,043	85.8	97.8	83.9
		0.2	1,497	1,297	56	1,335	89.2	95.8	85.4
		0.3	1,283	982	82	1,064	82.9	92.3	76.5
		0.4	1,322	442	568	1,010	76.4	43.8	33.4
		0.5	1,248	153	916	1,069	85.6	14.3	12.3
		0.6	1,096	0	713	713	65.1	0.0	0.0
						EC_{50}		0.39	0.36
2	8-22-61	0.0	1,547	1,503	51	1,554	100	96.7	96.7
		0.2	1,249	1,101	131	1,232	98.6	89.4	88.2
		0.3	1,488	1,282	160	1,442	96.9	88.9	86.2
		0.4	1,197	607	541	1,158	96.7	52.4	50.7
		0.5	1,315	277	931	1,108	84.3	25.0	21.1
		0.6	1,219	0	625	625	61.3	0.0	0.0
						EC_{50}		0.41	0.40
3	8-23-61	0.0	600	497	22	519	86.5	95.8	82.8
		0.2	556	478	54	532	95.7	89.8	85.5
		0.3	607	400	69	469	77.3	85.3	65.9
		0.4	642	153	298	451	70.2	33.9	23.9
		0.5	571	0	207	207	36.3	0.0	0.0
		0.6	483	0	0	0	0.0	0.0	0.0
						EC_{50}		0.37	0.34

Table 9, continued. Results of five mussel embryo tests using Procedure B bioassay techniques.

Bioassay Number	Date	Concentration NaPCP (mg/l)	Number of Eggs	Number of Larvae			Percent Survival	Percent Normal (P_A)	Percent Normal (P_B)
				Normal	Abnormal	Total			
4	8-29-61*	0.0	871	836	7	843	96.8	99.2	90.4
		0.1	1,092	1,002	11	1,013	92.9	98.9	90.8
		0.2	877	811	19	830	94.6	97.7	90.7
		0.3	919	780	75	855	93.0	91.2	91.5
		0.4	1,004	809	127	936	93.2	86.4	80.5
		0.5	947	362	477	839	88.6	43.2	38.2
		0.6	904	251	434	685	75.8	36.6	27.8
						EC_{50}		0.47	0.44
5	8-29-61*	0.0	854	818	3	821	96.0	99.6	95.7
		0.1	989	957	12	969	98.1	98.8	96.7
		0.2	767	700	19	719	93.7	97.4	91.3
		0.3	815	695	84	779	95.6	89.2	85.3
		0.4	909	653	165	818	89.9	79.8	71.8
		0.5	814	322	406	728	89.4	44.2	39.6
		0.6	846	219	476	795	93.9	27.6	25.9
						EC_{50}		0.48	0.47

* Eggs from more than one female used on same day

averaged 97.8 percent normal (from 95.8 to 99.6 percent) and 0.1 mg/l (tested twice) 95.5 percent. At 0.2 mg/l, little or no effect was noted with an average of 92.3 percent normal larvae (from 89.4 to 98.8 percent). The average value of 92.3 (85.3 to 97.7) percent shelled larvae at 0.3 mg/l was considerably higher than the average value of 79 percent observed in the 19 Procedure A bioassays. The results of the next three concentrations, 0.4 - 0.6 mg/l, varied considerable from bioassay to bioassay. At 0.4 mg/l the percentages of normal larvae ranged from 33.9 to 86.4 with an average of 59.3 percent. The range at 0.5 mg/l was from 0.0 (number 3) to 44.2 percent, with an average of 25.3 percent, slightly higher than the average of 20 percent noted when using the larger volume of liquid. At the highest concentration tested, 0.6 mg/l, there were no normal larvae observed in the first three bioassays and 36.6 and 27.6 percent in bioassays 4 and 5 respectively. These last two bioassays, set up on the same day using eggs from separate females in each test, provided unusually high percentages of normal larvae at higher NaPCP concentrations. It is possible that the eggs from these females were unusually tolerant of the NaPCP, or that some error was made in preparing the dilutions.

An examination of the 48 hour survival data indicates that, in general, there was a decrease in survival with increasing concentrations of NaPCP. The average percentage of surviving larvae decreased from a high of approximately 93-95 percent in controls, 0.1, and 0.2 mg/l to a low of 59 percent at 0.6 mg/l. The larvae in experiment 3 experienced greater mortality than in any of the other bioassays, with

no larvae surviving at 0.6 mg/l, and only 36 percent survival at 0.5 mg/l. In the other four tests, survival never fell below 61 percent at 0.6 and 84 percent at 0.5 mg/l.

Since the calculations using Procedure B employ the numbers of fertilized eggs as the denominator of the equation, and there was normally, some mortality, percentages of normal larvae calculated by this method were lower than when the formula P_A is used. Again there was always a decrease from controls to the lowest concentration tested, although in two of the experiments (4 and 5) there was no detectable difference between controls and the first three concentrations. More important than the actual calculated percentages is a comparison of the EC_{50} values obtained by the two methods. Using P_A percentages the EC_{50} values ranged from 0.37 to 0.48 mg/l with an average of 0.42 mg/l. The P_B values ranged from 0.34 to 0.47 mg/l with an average of 0.40 mg/l. These values are in good agreement with the average EC_{50} of 0.39 mg/l (actual percentages) obtained in the 19 Procedure A bioassays. These results indicate that the additional, and time-consuming step of counting the numbers of fertilized eggs present at the beginning of an experiment is probably not necessary.

Effect of NaPCP on Shell Length

While counting the larvae, it was often noted that control larvae appeared larger than those larvae reared in the presence of sodium pentachlorophenate. To substantiate this observation, samples

of shelled larvae from eight bioassays were measured. In one instance 20 larvae from four concentrations were measured, and in the other seven tests samples of 20-40 larvae from controls and the next one or two levels were measured. The results of these measurements are found in Table 10.

In all of the bioassays from which larvae were measured, control larvae were always larger than those measured from the highest concentration, and the larvae normally decreased in size with increases in NaPCP concentration. Detailed statistical analysis of the data was not performed, but a rough indication of significance can be obtained from the standard error of the mean. Two averages were considered significantly different if the averages plus their respective standard errors did not overlap. Applying this criterion, all the differences were significant, with the exception of the difference between controls and 0.1 mg/l in bioassays 7 and 8.

In the first experiment there was a decrease in average size from 100.1 microns in controls to 93.5 microns in 0.2 mg/l (standard errors of 0.79 and 0.80 respectively). The decrease in percentage of normal larvae was not significant (less than 1.0 percent decrease) indicating that shell length may be a more sensitive measurement of the effect of a toxicant than percentage shelled. The difference between percentages of normal larvae in controls and 0.4 mg/l was approximately 60 percent, and the average difference in shell length was about 20 microns, both highly significant differences.

Table 10. Effect of sodium pentachlorophenate on length of shelled larvae at 48 hours.

Number	Date	Concentration (mg/l)	Sample Size	Average Size (microns)	Standard Error	Percent[1] Normal
1	8-8-61	0.0	20	100.1	0.79	98.5
		0.2	20	93.5	0.80	97.7
		0.4	20	79.8	1.44	39.1
2	8-11-61	0.0	20	104.0	0.50	99.2
		0.2	20	100.8	0.57	98.3
		0.4	20	90.8	1.35	91.9
		0.6	20	81.1	0.97	24.8
3	8-15-61[2]	0.0	30	95.7	0.41	98.0
		0.2	30	92.2	0.39	92.3
4	8-15-61	0.0	30	99.5	0.48	98.5
		0.2	30	94.4	0.53	96.9
5	8-15-61	0.0	30	99.1	0.51	99.1
		0.2	30	94.1	0.42	98.6
6	8-23-61	0.0	40	98.6	0.34	94.5
		0.2	40	93.0	0.44	88.5
7	8-29-61[3]	0.0	40	100.3	0.39	99.2
		0.1	40	101.8	0.36	98.9
		0.2	40	97.3	0.35	97.7
8	8-29-61[3]	0.0	40	96.9	0.58	99.6
		0.1	40	94.5	0.77	98.8
		0.2	40	92.4	0.73	97.4

1 Computed using the following equation:

$$P_A = \frac{\text{Number of normal larvae at 48 hours}}{\text{Number of normal + number of abnormal larvae at 48 hours}}$$

2 Eggs from more than one female used on the same day

3 Larvae from experiments using Procedure B bioassay techniques

In the next experiment, conducted on August 11, there was significant difference in larval size between controls and 0.2 mg/l (approximately a four micron decrease in average size) whereas the percentages of normal larvae were practically identical (99.2 and 98.3 percent). Between 0.2 and 0.4 mg/l, and also between 0.4 and 0.6 mg/l, there were relatively large differences in percentages of normal larvae and corresponding differences in larval size.

On August 15, three bioassays were conducted using eggs from a separate female in each test. Samples of 30 larvae were measured from controls and 0.2 mg/l. In the first experiment, the percentages of normal larvae decreased by about six percent and the average length by about 3.5 microns, both considered to be actual differences. In numbers two and three, differences in percentages (1.6 and 0.5 percent) did not indicate that the NaPCP affected the larvae but in both cases there was a significant decrease in larval size of about five microns.

The larvae from the bioassay conducted on August 23 demonstrated an effect of the toxicant at 0.2 mg/l (as compared to controls) in both percentages of normal larvae and larval size.

The two Procedure B bioassays conducted on August 29 further illustrate the use of larval size to identify effects not shown by percentages of normal larval alone. In both of these tests there was little or no difference with respect to percentages of normal larvae among controls, 0.1, and 0.2 mg/l. There was also almost no difference with respect to size between controls and 0.1 mg/l. However, in

the two experiments, the larvae exposed to 0.2 mg/l were approximately four microns smaller than controls.

DISCUSSION

The data obtained in this study indicate that the mussel embryo test possesses many features necessary for use as a standard bioassay method. These features include: availability of the organism, simplicity of experimental technique, reproducibility of bioassay results, and sensitivity to dilute concentrations of toxicant. The following paragraphs will briefly discuss the results of this study in view of the criteria listed above.

Availability of the Organism

Because of the widespread distribution of this species, the question of availability is essentially reduced to that of obtaining viable gametes when required. The Kraft Mill Effluent method used to induce spawning in the bay mussel was almost always successful. Of the 102 spawning attempts during this study, there were only three occasions when experimentation was prevented due to a lack of gametes. These failures might have been avoided by using larger numbers of adult mussels in the spawning attempts.

In addition to being reliable, the KME method was simple to perform and rapid. The procedure consisted of collecting the mussels, removing mud and debris from the valves, and immersion in the liquor. Gametes were normally available within one-half to one and one-half hours after immersion.

If the bay mussel is to be used on a fairly large scale as a bioassay organism, there is a possibility that repeated collecting trips may result in a scarcity of adult mussels in the area near the laboratory. In this study adult mussels were discarded after each spawning attempt and, by the end of the second summer, a noticeable reduction in the mussel population of Yaquina Bay had occurred in collection areas. In future work, spawned and unspawned mussels could be placed in separate live-boxes thus assuring a constant supply of mature individuals.

Simplicity of Experimental Technique

The mussel embryo test is easy to conduct, requires a minimum of equipment and space, and is fairly rapid. In most instances, fertilization was completed within one and one-half hours from the time the mussels were immersed in the sea water-KME mixture. One pint pyrex dishes (approximately five inches in diameter and two and one-half inches deep) were used but smaller dishes could be used. The most time-consuming step of the procedure was counting the larvae at the end of the test period. Five to ten minutes were required to sample and count the larvae from each test container.

Distinguishing normal from abnormal larvae in the intermediate concentrations often proved difficult. Samples from these concentrations normally contained a complete series of stages of shell development, from fully shelled to completely unshelled larvae. Some of this

difficulty can be eliminated by defining a normal larvae as one possessing a shell in any stage of development. With this definition, a semi-skilled worker will not be required to estimate the degree of shell development and will also make counts by different individuals more comparable.

Reproducibility of Results

In the 24 bioassays considered in this study (19 Procedure A and 5 Procedure B) there was always a decrease in percentages of normal larvae from controls to the highest concentration tested and in most instances there was a decrease from one concentration to the next. The actual EC_{50} values for the 19 Procedure A bioassays ranged from 0.26 to 0.55 mg/l with an average of 0.39 and a variance of 0.13 mg/l. This variation does not appear to be unduly great, especially when the narrow interval between no effect and complete harm is considered (approximately 0.2 to 0.6 mg/l). With an interval this narrow, changes in the quality of the diluent water or inaccuracies in preparing the experimental concentrations could have an effect out of proportion to their magnitude.

Two significant factors affecting the reproducibility of the bioassay results were actual biological differences in the mussel embryos and changes in the quality of the bay water. Little can be done to reduce the effect of the first of these variables, but it might be worthwhile to attempt to standardize water quality. In future work

with mussel embryos, some effort should be expended for the development of a standard sea water for use in the bioassay. Although this water would not be suitable for bioassays measuring the effect of a pollutant in the aquatic environment (because it will be chemically different from any natural water), it should prove advantageous for use in tests to estimate temporal changes in toxicity or to obtain relative toxicities of different compounds.

Sensitivity of Embryos

Little information is available on the toxicity of sodium pentachlorophenate to aquatic organisms, therefore it is difficult to compare the sensitivity of mussel embryos with that of other commonly used test organisms. Crandall and Goodnight (1959), using the freshwater fathead minnow, Pimephales promelas, found an estimated 24-hour TL_m (median tolerance limit) of approximately 0.32 to 0.35 mg/l. Chapman (1965) working with potassium, instead of sodium, pentachlorophenate in fresh water, determined the 48-hour TL_m for a cichlid fish, Cichlosoma bimaculatum, to be approximately 0.37 mg/l (standing water bioassay) also Weber (1965) found the tolerance limit of NaPCP to the guppy (Lebistes reticulatus), Tubifex, and Daphnia to be about 0.5 mg/l. It is doubtful if the toxicity data in these references are directly comparable with the results of bioassays conducted in this study (fresh water vs salt water, mortality vs physiological effect, different experimental techniques, etc.) but the reported values are in

close agreement with an average 48-hour EC_{50} value of 0.39 mg/l found for the mussel embryos.

Several factors were found to affect the toxicity of NaPCP to fish. Crandall and Goodnight (1959) noted that lowering the pH or raising the temperature increased the toxicity of NaPCP. Mean survival time for fish exposed to one mg/l decreased from more than 24 hours at a pH of 8.9-9.0 to about 28 minutes at 5.9-6.0. The effect of temperature was less marked but mean survival time decreased from 260.4 ± 10.2 minutes at 10° C. to 46 ± 2.1 minutes at 26° C. Weber (1965) reported that the time for lethal action could be lengthened by increasing the hardness of the water and shortened by increasing the dissolved oxygen content or the temperature. In the tests with mussel embryos, NaPCP was less toxic to the developing embryos at a salinity of 28 p.p.t., than at 20 or 24 p.p.t. Although these factors, with the exception of salinity, were not varied experimentally in this study, their effect on the toxicity should be noted. Some of the variation in test results may have been due to relatively minor fluctuations in temperature, salinity dissolved oxygen content or pH.

Another aspect of sensitivity involves the response of the test organism to changes in concentration of the toxicant. As was mentioned previously, the effective range of the NaPCP, with respect to shell development in the mussel embryo, was between 0.2 and 0.6 mg/l. Each increase in concentration normally caused a decrease in the percentage of normal larvae (as compared to next lowest concentration) at 48 hours. Preliminary data on the effect of NaPCP on shell length

indicated that this measurement may be able to distinguish differences in concentration not shown by percentages of normal larvae alone.

Evaluation of Mussell Embryo Test

It appears that the difficulty experienced by Toner (1961) of rearing high percentages of normal larvae in control cultures has been resolved by using sea water with a salinity of 25 p.p.t. (as compared to 20 p.p.t. used by Toner) and more standardized laboratory techniques. With low percentages of normal larvae in controls, there is some doubt as to the cause of abnormal development in those cultures exposed to the toxicant. The American Public Health Association, et al, (1965) in a section devoted to fish toxicity bioassay procedures, suggest that at least 90 percent of the test organisms be alive and unaffected at the termination of an experiment. The entire bioassay probably should be done again if a lower percentage survives. Of the 24 bioassays conducted in this study, only two had less than 90 percent shelled larvae in controls.

Conversations with other workers in the field of marine pollution indicated that many were not completely satisfied with the use of the mussel embryo bioassay. Some thought that the data would not be directly applicable to the determination of the effects of pollution on economically important species. Because oysters are the most commercially important molluscs in the Pacific Northwest, it was suggested that oyster embryos might be more properly used as bioassay organisms.

Woelke (1960), working with Pacific oyster embryos and spent sulfite liquor, found that increasing concentrations of the toxicant caused the same effect noted with mussel embryos, i.e., reduced the percentages of embryos that developed normal shells at 48 hours. The use of oyster embryos suffers from the disadvantages that mature oysters must be conditioned for some time prior to spawning and that during certain periods of the year, oysters cannot be induced to spawn.

To establish the relationship between mussel toxicity data and that obtained with the use of other species of interest, comparative bioassays could be conducted using a variety of toxicants. Okubo and Okubo (1962) reported on the effects of several chemicals on the development of bay mussel, Pacific oyster, and two species of sea urchin embryos. These authors found that in nearly all instances mussel and oyster embryos were similar in sensitivity and both were slightly less sensitive than developing sea urchin embryos. In these experiments gametes were obtained without the use of artificial spawning methods (although mussels were spawned by temperature stimulation); therefore the different test organisms were selected so that the overlapping of spawning seasons would provide some gametes during all seasons. Stewart, Milleman, and Breese (1967) recently reported on the toxicity of Sevin, a pesticide, and its hydrolytic product to 10 species of marine animals, including crustaceans, molluscs, and fishes. The results of these experiments also indicated that bay mussel and Pacific oyster embryos were similar in sensitivity.

More studies of this type are necessary if mussel embryo data are to be used to predict the effect of a pollutant on the marine environment. For studies of marine pollution the mussel embryo test might be effectively used as part of a standard series of bioassays which also includes a crustacean and a fish species.

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APPENDICES

APPENDIX A

MONTHLY MUSSEL SPAWNING IN RESPONSE TO STIMULATION BY KRAFT MILL EFFLUENT AT YAQUINA BAY FISHERIES LABORATORY

Date	Number				Percent Spawning	Sex Ratio (Female:Male)
	Attempted	Females	Males	Total		
10-1-59	28	2	6	8	28.6	0.33
10-2-59	28	1	1	2	7.2	1.00
10-7-59	28	2	1	3	10.7	2.00
10-8-59	28	1	2	3	10.7	0.50
10-18-59	20	2	1	3	15.0	2.00
10-29-59	28	5	5	10	35.8	1.00
10-30-59	28	2	2	4	14.3	1.00
11-5-59	24	2	2	4	16.7	1.00
11-12-59	20	2	6	8	40.0	0.33
11-13-59	20	2	4	6	30.0	0.50
11-29-59	24	2	1	3	12.5	2.00
12-5-59	24	2	2	4	16.7	1.00
1-25-60	19	7	2	9	47.5	3.50
1-26-60	20	10	4	14	70.0	2.50
1-27-60	20	4	8	12	60.0	0.50
1-29-60	20	7	9	16	80.0	0.78
2-2-60	20	4	4	8	40.0	1.00
2-4-60	29	9	10	19	65.6	0.90
2-11-60	30	5	0	5	16.7	
2-12-60	30	13	4	17	56.7	3.26
2-24-60	30	10	7	17	56.7	1.42
2-25-60	20	12	6	18	90.0	2.00
2-27-60	30	12	6	18	60.0	2.00
3-1-60	30	10	9	19	63.3	1.11
6-17-60	43	13	10	23	53.5	1.30
6-20-60	20	2	5	7	35.0	0.40
6-22-60	30	5	6	11	36.7	0.83
6-23-60	30	7	8	15	50.0	0.88
6-27-60	30	7	15	22	73.3	0.47
6-28-60	30	5	7	12	40.0	0.71

APPENDIX A (Continued)

Date	Number				Percent Spawning	Sex Ratio (Female:Male)
	Attempted	Females	Males	Total		
7-1-60	30	5	7	12	40.0	0.71
7-3-60	36	9	8	17	47.2	1.13
7-4-60	36	8	9	17	47.2	0.89
7-8-60	44	17	12	29	65.9	1.42
7-9-60	40	10	12	22	55.0	0.83
7-13-60	49	7	6	13	26.5	1.17
7-14-60	25	2	2	4	16.0	1.00
7-17-60	43	1	5	6	13.9	0.20
7-18-60	30	15	7	22	73.3	2.14
7-25-60	24	7	14	21	87.5	0.50
7-26-60	29	9	6	15	51.7	1.50
7-28-60	29	14	8	22	75.9	1.75
7-29-60	30	11	12	23	76.6	0.92
8-1-60	31	9	13	22	71.0	0.69
8-2-60	17	8	5	13	76.5	1.60
8-4-60	20	7	3	10	50.0	2.33
8-5-60	17	4	8	12	70.5	0.50
8-7-60	16	3	7	10	62.5	0.43
8-8-60	12	3	4	7	58.0	0.75
8-9-60	20	6	4	10	50.0	1.50
8-10-60	16	3	4	7	44.0	0.75
8-13-60	20	6	8	14	70.0	0.75
8-14-60	11	2	3	5	45.4	0.66
8-15-60	20	3	13	16	80.0	0.23
8-17-60	20	6	7	13	65.0	0.86
8-22-60	28	4	9	13	45.0	0.44
8-23-60	20	5	4	9	45.0	1.25
8-25-60	15	6	7	13	86.0	0.86
8-26-60	26	10	6	16	61.5	1.66
8-27-60	20	8	7	15	75.0	1.14
8-28-60	15	8	6	14	93.3	1.33
8-30-60	20	9	3	12	60.0	3.00
9-3-60	16	4	3	7	43.7	1.33
9-6-60	20	5	11	16	80.0	0.45
9-7-60	20	7	11	18	90.0	0.64
9-12-60	25	12	8	20	80.0	1.50
9-13-60	20	7	6	13	65.0	1.17
9-16-60	20	8	8	16	80.0	1.00

APPENDIX A (Continued)

Date	Number				Percent	Sex Ratio
	Attempted	Females	Males	Total	Spawning	(Female:Male)
6-13-61	18	10	4	14	77.7	2.50
6-14-61	18	6	5	11	61.1	1.20
6-15-61	12	0	2	2	16.6	
6-16-61	18	3	2	5	27.8	1.50
6-19-61	24	2	7	9	37.5	0.29
6-20-61	36	3	7	10	27.7	0.43
6-21-61	24	3	6	9	37.5	0.50
6-21-61	12	5	3	8	66.7	1.67
6-26-61	24	1	8	9	37.5	0.13
6-26-61	12	4	7	11	91.7	0.57
6-28-61	12	0	1	1	8.3	
6-28-61	24	3	9	12	50.0	0.33
7-3-61	36	2	8	10	27.8	0.25
7-4-61	36	5	5	10	27.8	1.00
7-5-61	36	12	5	17	47.2	2.40
7-10-61	36	7	8	15	41.7	0.88
7-12-61	36	13	7	20	55.5	1.86
7-17-61	36	6	16	22	61.1	0.38
7-18-61	36	6	11	17	47.2	0.55
7-19-61	36	14	9	23	62.2	1.56
7-24-61	37	14	9	23	62.2	1.56
7-25-61	34	3	12	15	44.1	0.25
7-26-61	36	4	11	15	41.6	0.36
7-27-61	34	4	12	16	47.1	0.33
7-28-61	36	1	9	10	27.7	0.11
7-31-61	36	3	4	7	19.4	0.75
8-1-61	42	3	8	11	26.2	0.38
8-2-61	36	1	8	9	25.0	0.13
8-3-61	48	4	11	15	31.2	0.36
8-4-61	40	4	5	9	22.5	0.80
8-7-61	37	4	7	11	29.7	0.57
8-8-61	44	4	8	12	27.2	0.50
8-8-61	25	4	8	12	48.0	0.50
8-9-61	40	4	5	9	22.5	0.80
8-9-61	25	5	4	9	36.0	1.25
8-10-61	41	5	16	31	51.2	0.31
8-10-61	25	5	9	14	56.0	0.56
8-11-61	47	6	15	21	44.7	0.40
8-14-61	43	4	11	15	34.8	0.36

APPENDIX A (Continued)

Date	Number				Percent	Sex Ratio
	Attempted	Females	Males	Total	Spawning	(Female:Male)
8-15-61	48	7	10	17	35.4	0.70
8-16-61	48	4	12	16	33.3	0.33
8-17-61	41	7	13	20	47.8	0.54
8-19-61	43	4	12	16	37.2	0.33
8-21-61	48	6	15	21	43.8	0.60
8-22-61	45	4	10	14	31.1	0.40
8-23-61	35	8	5	13	37.1	1.60
8-25-61	47	4	7	11	23.4	0.57
8-28-61	36	3	5	8	22.2	0.60
8-29-61	43	3	3	6	13.9	1.00
8-30-61	44	1	5	6	13.6	0.20
8-31-61	46	2	6	8	17.3	0.35
9-1-61	43	4	1	5	11.6	4.00
9-4-61	41	9	4	13	31.7	2.25
9-5-61	40	3	2	5	12.5	1.50
9-6-61	40	0	1	1	2.5	
9-7-61	40	3	3	6	15.0	1.00
9-8-61	40	3	3	6	15.0	1.00
9-9-61	40	2	4	6	15.0	0.50
4-2-62	30	5	5	10	33.3	1.00
4-3-62	30	2	0	2	6.6	
4-4-62	30	1	0	1	3.3	
4-8-62	30	3	5	8	26.6	0.60
4-10-62	30	3	10	13	43.3	0.30
4-11-62	40	1	4	5	12.5	0.25
4-16-62	30	3	15	18	60.0	0.20
4-17-62	30	6	14	20	66.6	0.43
4-18-62	25	5	8	13	52.0	0.63
4-23-62	30	9	4	13	43.3	2.25
4-24-62	40	9	12	21	52.5	0.75
5-14-62	30	4	4	8	26.6	1.00
5-15-62	30	9	11	20	66.7	0.82
5-16-62	30	6	13	19	63.3	0.46
5-21-62	30	8	7	15	50.0	1.14
5-22-62	30	14	11	25	83.3	1.27
5-23-62	30	9	20	29	96.7	0.45
5-25-62	30	12	16	28	93.3	0.75
5-29-62	30	13	9	22	73.3	1.44

APPENDIX B

RESULTS OF 19 BIOASSAYS USING TECHNIQUES OF PROCEDURE A· PERCENTAGES ARE AVERAGES OF DUPLICATE CONCENTRATIONS

Number	Date	NaPCP Concentration (mg/l)	Number of larvae			Percent normal	
			Normal	Abnormal	Total	Actual[1]	Relative[2]
1	6-26-61	0.0	115	8	123	93.4	100.0
		0.2	111	22	133	83.5	89.4
		0.3	79	31	110	71.8	76.8
		0.4	38	33	71	53.5	57.3
		0.5	7	46	53	12.8	13.7
		0.6	0			0	0
			EC_{50} (mg/l NaPCP)			0.41	0.41
2	6-28-61	0.0	275	27	302	91.1	100.0
		0.2	267	31	298	86.1	94.5
		0.3	237	111	348	67.9	74.5
		0.4	138	161	299	46.1	50.6
		0.5	8	180	188	4.3	4.7
		0.6	0			0	0
			EC_{50}			0.38	0.40
3	7-5-61	0.0	81	23	104	77.8	100.0
		0.2	65	38	103	62.9	80.8
		0.3	56	63	119	47.3	60.8
		0.4	39	44	93	42.7	54.9
		0.5	9	48	57	15.9	20.4
			EC_{50}			0.26	0.41
4	7-12-61	0.0	153	20	173	88.2	100.0
		0.2	127	35	162	78.4	88.9
		0.3	182	43	125	65.6	74.4
		0.4	25	27	52	49.0	55.6
		0.5	0			0	0
			EC_{50}			0.39	0.43

APPENDIX B (Continued)

Number	Date	NaPCP Concentration (mg/l)	Number of larvae			Percent normal	
			Normal	Abnormal	Total	Actual[1]	Relative[2]
5	8-3-61	0.0	105	4	109	95.8	100.0
		0.2	94	10	104	90.3	94.3
		0.3	72	12	84	85.7	89.5
		0.4	36	32	68	53.7	65.1
		0.5	20	4	67	27.1	27.2
			EC_{50}			0.42	0.42
6	8-7-61	0.0	287	5	292	98.3	100.0
		0.2	348	9	357	97.5	99.2
		0.4	309	36	345	89.6	91.1
		0.6	9	135	144	6.2	6.3
		0.8	0			0	0
			EC_{50}			0.49	0.49
7	8-8-61	0.0	356	6	362	98.5	100.0
		0.2	339	8	347	97.7	99.2
		0.4	121	187	308	39.1	39.7
		0.6	0			0	0
			EC_{50}			0.35	0.36
8	8-9-61	0.0	1026	12	1038	98.8	100.0
		0.2	757	13	770	98.3	99.5
		0.3	536	20	556	96.4	97.6
		0.4	340	106	446	76.2	77.1
		0.5	34	55	89	37.9	38.4
		0.6	0			0	0
			EC_{50}			0.47	0.47

APPENDIX B (Continued)

Number	Date	NaPCP Concentration (mg/l)	Number of larvae			Percent normal	
			Normal	Abnormal	Total	Actual[1]	Relative[2]
9	8-11-61	0.0	387	3	390	99.2	100.0
		0.2	402	7	409	98.2	99.0
		0.4	311	28	338	91.9	92.6
		0.6	41	124	165	24.8	25.0
		0.8	0			0	0
			EC_{50}			0.52	0.52
10	8-15-61[3]	0.0	446	9	455	98.0	100.0
		0.2	638	53	691	92.3	94.2
		0.4	44	602	646	6.7	6.8
		0.6	0			0	0
			EC_{50}			0.28	0.28
11	8-15-61	0.0	521	8	529	98.5	100.0
		0.2	671	21	692	96.9	98.4
		0.4	84	377	451	16.3	16.5
		0.6	0			0	0
			EC_{50}			0.30	0.31
12	8-15-61	0.0	676	6	682	99.1	100.0
		0.2	678	9	687	98.6	99.5
		0.4	183	408	591	31.0	31.3
		0.6	0			0	0
			EC_{50}			0.34	0.34

[3] Eggs from more than one female used on same day

APPENDIX B (Continued)

Number	Date	NaPCP Concentration (mg/l)	Number of larvae			Percent normal	
			Normal	Abnormal	Total	Actual[1]	Relative[2]
13	8-16-61	0.0	109	10	119	91.2	100.0
		0.2	112	19	131	85.5	93.8
		0.4	99	21	120	82.4	90.3
		0.6	12	17	29	41.4	45.4
			EC_{50}			0.55	0.57
14	8-16-61	0.0	461	16	477	96.5	100.0
		0.2	449	22	471	95.3	98.7
		0.4	122	187	309	39.3	40.7
		0.6	0			0	0
			EC_{50}			0.35	0.36
15	8-19-61	0.0	514	6	520	98.8	100.0
		0.2	288	12	300	96.0	97.4
		0.3	354	6	360	98.3	99.5
		0.4	184	58	242	76.0	76.9
		0.5	16	24	40	40.0	40.5
		0.6	0			0	0
			EC_{50}			0.47	0.47
16	8-22-61[3]	0.0	1052	64	1116	94.2	100.0
		0.2	987	98	1085	90.7	96.5
		0.3	511	317	828	61.7	65.5
		0.4	108	591	699	15.5	16.5
		0.5	0			0	0
			EC_{50}			0.33	0.33

3 Eggs from more than one female used on same day

APPENDIX B (Continued)

Number	Date	NaPCP Concentration (mg/l)	Number of larvae			Percent normal	
			Normal	Abnormal	Total	Actual[1]	Relative[2]
17	8-22-61	0.0	1155	10	1165	99.1	100.0
		0.2	1082	25	1107	97.7	98.5
		0.3	983	98	1081	90.9	91.7
		0.4	833	238	1071	77.8	78.5
		0.5	219	423	652	33.6	33.9
		0.6	0			0	0
			EC_{50}			0.46	0.46
18	8-23-61	0.0	735	45	778	94.5	100.0
		0.2	569	72	641	88.8	93.9
		0.3	506	145	651	77.7	82.2
		0.4	448	305	753	59.5	63.0
		0.5	64	311	375	17.1	18.1
		0.6	0			0	0
			EC_{50}			0.42	0.43
19	9-1-61	0.0	148	10	158	93.1	100.0
		0.2	140	37	177	79.0	84.9
		0.3	79	75	154	51.4	55.2
		0.4	4	50	54	6.3	6.8
		0.5	0			0	0
			EC_{50}			0.31	0.32

[1] As computed from observed percentages of normal larvae.
[2] Relative to controls as 100 percent normal.