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Proceedings of the

Twelfth

PACIFIC NORTHWEST INDUSTRIAL WASTE CONFERENCE

Published by

UNIVERSITY OF WASHINGTON

COLLEGE OF ENGINEERING

Department of Civil Enginering

in cooperation with

THE INDUSTRIES AND UNIVERSITIES OF THE PACIFIC NORTHWEST

November 1965

BAY MUSSEL EMBRYO BIOASSAY

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During the past 10 years the embryos and larvae of oysters and some species of clams have been employed to quickly assess (48 hours or less) the biological effects of various substances in marine water samples. (Walne 1958, Woelkie 1960, Davis 1961, K. Okubo and T. Okubo 1962, Hidu 1965, and others). The best materials have included some insecticides, herbicides, antibiotics, detergents, a variety of other compounds, and a few industrial effluents. The common Bay mussel, Mytilus edulis L. possesses certain unique features which suggest that its embryos and larvae also might be employed advantageously in routine laboratory bioassays made in saltwater samples--much in the same ways that guppy fish and the crustaceans, Daphnia ("water-fleas") are used as test organisms at some freshwater stations. At our Yaquina Bay laboratory, mussel-embryo bioassays have been conducted with a variety of substances and some industrial effluents. Most of these have been made in cooperation with Mr. Russell Blosser, regional engineer for the National Council for Stream Improvement who is stationed at the University. Some of these tests were designed for exploring biological changes in stabilization of pulpmill effluents as measured by effects on bivalve larvae. Currently, Professor Fred Burgess and his associates of the Oregon State University Engineering Experiment Station have joined in an expanded investigation involving the degradation of pulpmill wastes in marine waters. This study also will include the biological evaluation of the efficacies of a variety of waste treatments by utilizing mussel and other bivalves, fishes and other representative marine species as test organisms.

Favorable factors in support of the Bay mussel for use as a universal bioassay marine invertebrate are:

1. Its wide geographic distribution in the intertidal zones of estuaries, bays, and on some ocean shore areas provides a common test organism available at many marine stations located particularly in the northern hemisphere. It occurs in the north Pacific, from the Arctic Ocean into Baja, California and as far south as Japan on the Asiatic Coast; on the eastern coast of North America from Greenland

to Cape Hatteras, North Carolina; and in Europe, from the White Sea to the Mediterranean and northern Africa. It is also reported from both coasts of lower South America; and from Australia and New Zealand. (Soot-Ryen 1955).

2. Spawning can be readily stimulated by chemical means; and gametes (eggs and sperm) are available, at least in our area, at all times of the year.

3. The embryology of the mussel is well known and documented (Field 1922) as are the abnormal embryos and larvae (Rattenburg and Berg 1954).

4. The embryos are inimically affected by a wide variety of chemical compounds and perhaps by differences in ecological factors occurring naturally in saltwater and experimentally in saltwater.

Our use of the Bay mussel embryos for testing purposes resulted from a chance laboratory happening. In 1958, while observing the reactions of several kinds of bivalves (oysters, clams, and mussels) in a strong saltwater concentration of Kraft mill effluent (KME), it was noticed that several of the mussels discharged eggs and sperm (Breese, Millemann and Dimick 1963) while the oysters and clams were not stimulated to spawn. Then, later, it was discovered that gravid ("sexually ripe") adults were present in the bay at all seasons of the year. Thus, mussel embryos were readily obtainable for bioassay purposes at any time when needed. Subsequently, it was found that KCL (2 gr/liter) in saltwater solutions also caused vigorous spawning of gametes which resulted in viable embryos and normal larvae.

METHOD

A mussel embryo bioassay essentially consists of exposing the eggs and sperm and the ensuing embryos to different concentrations of particular substances in seawater for 48 hours. At the end of this time, the numbers of normal (shelled-straight hinged) larvae and abnormal (nonshelled or imperfectly shelled) larvae are counted in each of the test solutions. In some bioassays, the fertilized eggs are counted and then related to numbers of surviving larvae at the termination of the tests.

This general type of bivalve bioassay using oysters and clams is in no way original on our part but has been employed by several investigators. Each has

added modifications to fit specific needs and conditions. A thorough discussion of laboratory procedures involved, particularly pertaining to the Pacific oyster and some clams, has been presented by Charles Woelke (1960) in a paper entitled "Bioassay - The Bivalve Larvae Tool."

Adult mussels used for obtaining eggs and sperm are everywhere abundant in most of our bays where adequate salinity patterns prevail throughout the life cycle. They are normally found attached to floats, piling, rocks, and other solid structures, usually in the intertidal zone.

The mussels are first cleaned of extraneous material such as barnacles, debris, etc. They are then placed in individual stacking dishes immersed in seawater containing KCL. Males normally start spawning within 30 minutes and females usually begin discharging eggs within another 20 to 30 minutes. As soon as spawning is noted, the mussel is thoroughly rinsed and placed in a separate beaker of diluent seawater. In this way, eggs and sperm from individual mussels may be kept separate and thus the parentage of resulting embryos may be identified should inherent viability of embryos and larvae become a question.

Test solutions are prepared usually from Yaquina Bay water adjusted when necessary to a salinity of 25 p.p. t. with uncontaminated spring water. Generally, the diluent test water is exposed to ultra-violet light and it is sometimes subjected to continuous-flow centrifuging. Test solutions are then prepared within effective concentration ranges, usually predetermined by a screening test. Measured saltwater volume of eggs and sperm are introduced into the glass test dishes. In this way, fertilization, if not inhibited, takes place in the test solutions. Then the dishes are transferred to constant temperature rooms and/or incubators.

Approximately an hour or two before termination of a bioassay (46 to 47 hours) a drop or two of neutral stain in distilled water is added to each test dish. This assists in the later differentiation of abnormal from normal larvae. Forty-eight hours from the time of fertilization the larvae are killed and numbers of normal and abnormal larvae are recorded for each test solution concentration. Sample sizes of 150 larvae (abnormal and normal) are made and occasionally counts are recorded for duplicate samples. Generally, replications are employed unless space in incubators is limited. Usually in report-

ing test results, percentages of normal larvae are used and relative percentages when results of several bioassays are considered as combined groups. For comparison, the Median Effective Concentration (EC 50) is estimated graphically. The EC 50 is the estimated concentration of test solution which is expected to produce 50 percent abnormal larvae (or conversely 50 percent normal larvae).

EMBRYONIC DEVELOPMENT

Embryonic development normally follows an orderly pattern of structural changes occurring at specific time periods (Field 1922). At 20° C the developmental larva schedule is approximately as follows:

1. The egg is at first somewhat oval in shape when discharged but soon changes to nearly round shape (approximately 70 microns in diameter) and rapidly starts development to the metaphase stage of development.

2. Within about 20 minutes after the sperm penetrates the egg membrane, the first polar body appears. This is followed by a second polar body in about 10 minutes.

3. Then a polar lobe becomes evident and first and second cleavages occur during the 50 to 90 minute period following insemination.

4. The blastual ("many celled") become free swimming at the 4 1/2 to 5-hour period.

5. Gastrulation takes place and a larval form (unshelled) called the trochophore appears about 20 hours after fertilization.

6. Shells (valves) begin appearing in about 40 to 43 hours and then the larvae, called veliger, which posses well-formed straight-hinges shells (D shape) occurs from 43 to 45 hours elapsed time after fertilization.

EXAMPLES OF BIOASSAY RESULTS

The results from five typical bioassays were selected to illustrate how the mussel-embryo test is being employed at our Yaquina Bay Laboratory.

Kraft Mill Effluent (KME).

Composite 7-day samples of "strong waste" (minus wash and service waters) were collected at weekly intervals from a non-bleach type of mill. Seven of

these samples were passed through a stabilization apparatus which incorporated a series of glass jars, each holding 18 liters. Diluted KME, 25 percent by volume, was pumped from a reservoir into the first jar; and from there the flow was by gravity into each successive jar. Flow rates were maintained at approximately 2-day retention in each jar. Mild aeration was provided in the reservoir before the diluted sample entered the first jar to assure complete mixing and an aerobic environment. Such a passage system, in some respects, simulated a travel route of an effluent passing down an estuary during a 10-day retention period.

An inspection of table 1 shows the composite results of seven separate bioassays made at seven-say intervals and expressed in mean relative percentages or normal larvae. The seven control groups ranged from 85 to 99 percent normal larvae with a mean of 95 percent. The median Effective Concentration (EC 50) values in percent by volume of the KME are the amounts estimated to produce 50 percent abnormal larvae and these estimates are recorded in the right hand column.

Note that in each series (original samples and each of the five retention groups) the percentages of normal larvae decreased with increases in KME concentrations; and that increases in normal larvae also occur in each KME concentration, except for two minor occurrences, with increases in retention time. It is also evident by inspection, and by increases in EC 50 values that the greatest improvement occurred in the first two days of stabilization. It was during the first two days stabilization that approximately 90 percent of the BOD was removed.

Although the two-day increments in normal larvae after the first two days stabilization were small (EC 50 value increased from 5.7% KME at end of second day stabilization to 8.2% by end of the 10-day period), nevertheless, a significant improvement in normal larvae production was evident throughout the 10-day stabilization period totaling approximately 81.6 percent decrease in toxicity as evidenced by EC 50 values of 1.5 and 8.2% KME.

In another series of bioassays, significant improvements in "strong-waste" samples of KME were noted after 50 percent solutions were held in saltwater for twenty-four hours, and further marked reduction in toxicity was indicated when

TABLE 1

KME Stabilization Results from 10 Day Retention Samples
Mean relative percentages of normal larvae based on seven bioassays

KME % by volume	Percent normal larvae									Estimated EC 50 % KME
	0,5	1	2	3	5	7	9	11	14	
Original samples	94.7	72.2	35.6	10.3	0					1.5
2 day retention		94.3	90.5	82.5	65.5	37.2	4.0			5.7
4 day retention			98.3	84.5	77.6	57.0	29.4	8.3		7.1
6 day retention				88.8	76.1	58.2	32.3	13.1		7.2
8 day retention				90.4	74.3	61.1	45.6	31.2	7.4	8.1
10 day retention					79.8	67.1	42.0	26.5	13.8	8.2

duplicate saltwater mixtures were aerated vigorously for 24 hours. Table 2 presents the estimated EC 50 obtained from four mussel-embryo tests. Mean toxicity decrease in the nonaerated mixture was approximately 37.1 percent and about 76.3 percent in the aerated mixture.

A series of four exploratory bioassays was conducted with the chemical compounds representing the volatile components known to occur in spent sulfite liquor (SSL). These included acetic and formic acids, methanol, ethanol, acetone, furfural, p-cymene, formaldehyde (as sodium formaldehyde bisulfide), and acetaldehyde (as acetaldehyde sodium) in mixed proportions as to occurrence in SSL adjusted to 10 percent solids. Table 3 gives the mean relative percentage of normal larvae produced in the approximately combined volatiles calculated to occur in SSL concentrations ranging from 250 to 4000 p.p.m. The percentages or normal larvae in control solutions ranged from 93.4 to 99.7 percent with a mean of 95.6 percent normal. The data indicates that no harm occurred to mussel embryos in the presence of prepared mixtures of volatiles thought to be approximately equivalent to 500 ppm SSL. Serious reduction in normal larvae occurred in mixtures equivalent to 1500 ppm SSL and in the greater concentrations of 2000, 3000, and 4000 ppm and a slight decrease was indicated (91.4 relative % normal larvae) to have occurred in 1000 ppm equivalent volatile mixtures.

A research project on the effects of the newer insecticides on representative estuarial organisms, particularly those compounds which might be employed in the control of oyster and clam pests has recently begun at Yaquina Bay under the direction of Dr. R. E. Mellemann. It is expected that mussel-embryo tests will be routinely employed in monitoring of toxic concentrations into continuous-flow aquaria containing a variety of organisms. Table 4 presents the mean results of the insecticidal carbonate, Sevin, in percentages of normal mussel larvae produced in concentrations ranging from 0.8 to 3.2 mgs/liters of 80 percent wettable powder. The indications were that 0.8 mg/liter concentrations did not inimically effect mussel embryos but did so in the 1.6 and 3.2 mg mixtures.

Indications that numbers of normal mussel larvae may vary greatly with salinity differences within the range of 20 to 28 p.p.t. in prepared saltwater solutions of sodium pentachlophenate (NaPCP) were suggested from results of two

TABLE 2

Summary of KME - Mussel Larvae EC 50 Valves
in 24 hour KME saltwater samples

Sample dates	Estimated EC 50 -- KME % volume 7/1	7/14	7/21	7/2	Mean	Toxicity percent decrease
Original sample	1.7	1.7	2.4	1.2	1.7	--
KME-Saltwater mixture after 24 hours	2.6	2.8	3.5	2.1	2.7	37.1
KME - Saltwater mixture aerated 24 hours	6.0	8.0	8.4	6.3	7.4	76.3

TABLE 3

Combined SSL Volatile Effect on Mussel Larvae
Based on known compounds in SSL at 10% solids; and 4 bioassays

	Equivalent volatiles in SSL p.p.m. 250	500	1000	1500	2000	3000	4000
Relative percentages normal larvae	98.3	99	95.8	91.4	73.2	45.6	16.8

TABLE 4

Effects of Sevin (80% wettable powder) on Mussel Larvae
Mean percentage of normal larvae averages of 4 replications
(Bioassay by Eugene Stewart)

Sevin mg/liter	Percent normal larvae	
	Actual	Relative
0.0	90	100
0.8	90	100
1.6	81	90
3.2	26	29
EB 50	2.4 mg/1	2.5 mg/1

TABLE 5

Salinity and NAPCP Effect on Mussel Larvae
Percentages Normal Larvae
averages of duplicate samples
(Bioassays by Randall Brown)

NaPCP mg/liter	Salinity					
	20 0/00		24 0/00		28 0/00	
	A	B	A	B	A	B
0.0	52.1	24.5	92.7	89.6	95.7	96.3
0.2	23.9	26.4	78.1	80.0	82.5	93.5
0.3	0.0	0.0	60.9	71.9	78.3	86.4
0.4	0.0	0.0	32.0	29.8	74.2	81.5

eparate bioassays conducted by Randall Brown, a graduate student in fisheries. ble 5 presents his results from the two separate bioassays (A and B) in per- ntages of normal larvae produced in prepared NaPCP solutions (0.2 to 0.4 mg/liter) justed to salinities of 20, 24, and 28 p.p.t. From an inspection of his data, is evident that a greater number of normal larvae occurred in the two 28 p.p.t. linity groups (A and B) than in corresponding solutions of 24 and 20 p.p.t. linity. Also, it was evident that lowest production occurred in the 20 p.p.t. linity control and NaPCP concentrations and that no normal larvae were obtained n 0.3 and 0.4 mg/l solutions. Of course, similar NaPCP-Salinity bioassays must e made for testing other possible factors which might have influenced the ndicated results that normal mussel larvae increase with increases in salinity ncentrations within the range of 20 to 28 p.p.t.; and also that the toxicity f NaPCP (0.2 to 0.4 mg/l) to mussel embryos decrease with salinity increases n the range of 20 to 28 p.p.t.

Several modes of determining water quality conditions in marine waters have been tried, including chemical, physical and biological. Preliminary experimental work with Bay mussel embryos seems to indicate it is a reliable biological test organism. Much work is yet to be done in perfecting the bioassay but results from preliminary tests, as I have just attempted to illustrate, should point out the potential value of this organism in industrial waste treatment studies and other similar water quality problems of estuaries and bays.

Our experiences in rearing a variety of bivalve embryos and larvae suggest that ecological factors such as temperatures and salinity optimums may vary significantly for different species. Therefore, we have under study, the effects of temperatures on the embryos of mussels and other bivalves as basis for improved understanding of bioassay results.

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