Steven M. Bay, Kenneth D. Jenkins,
and Philip S. Oshida

A NEW BIOASSAY BASED ON ECHINOCHROME PIGMENT SYNTHESIS

There is a need for a marine toxicity test that is sensitive, simple, rapid, and inexpensive. Such a test has been devised, and it can be used to detect toxicity in seawater samples having low contaminant concentrations. This report explains how the test is conducted and where it might be used.

A simple, rapid, and quantitative embryo-larval bioassay that uses changes in echinochrome pigment synthesis as an indicator of seawater toxicity has been developed to allow increased use of sensitive sea urchin bioassays where time, resources, and technical expertise may be limited. Several 48h embryo-larval tests were conducted with lowered salinity, increased concentrations of copper, municipal wastewaters, harbor seawater and offshore seawater as the potential toxicants.

Embryo-larval bioassays have proven to be very sensitive indicators of seawater contamination. Such embryo-larval tests with sea urchins (Kobayashi, 1972, 1974, 1976, 1977) and oysters (Cardwell *et al* 1977a, 1977b, 1977c and Woelke, 1972) have detected the areal extent of industrial-related toxicity in the sea and determined the decreases in size of affected areas as discharges were reduced. Echinoderm eggs, embryos, and larvae have also been used to measure the toxicity of marine waters (Oshida *et al*. 1980), wastewater discharges (Oshida *et al*. 1981), heavy metals (Kobayashi and Fujinaga, 1976), chlorinated hydrocarbons (Oshida, 1977), and petroleum hydrocarbons (Davis *et al*. 1981). In these studies the effects of the toxicants were determined by measuring decreased survival and development of the embryo after 48 to 96 hours.

The echinoderm egg undergoes a rapid and complex sequence of development during the first 96 hours after fertilization. The egg divides repeatedly to form a hollow ball of cells called a blastula; a part of the blastula's wall then invaginates to form the gut (gastrulation). The cells of the gastrula continue to divide and specialize, forming the skeleton and internal organs as the embryo progresses through the prism (or pyramid) and pluteus stages. Evaluation of toxic responses usually involves the microscopic examination and classification of these eggs and larvae as "normal" or "abnormal" according to often complex and somewhat subjective morphological criteria. The time and expertise required to examine many such samples is often prohibitive.

Echinochrome pigments are found almost exclusively in sea urchins and sand dollars (Boolootian, 1966). These pigments are easily extracted and measured. Echinochromes are usually re-

stricted to the soft tissues and are often the dominant quinone pigment during embryonic and larval development (Boolootian, 1966). The function of echinochrome is not fully understood, but evidence suggests that it plays a role in the regulation of lipid peroxidation (Koltsova et al., 1981). De novo synthesis of echinochrome occurs during embryonic development in many sea urchins and initiation of synthesis usually coincides with the events of gastrulation (Asashima, 1971). In Strongylocentrotus purpuratus, echinochrome production occurs just prior to gastrulation and proceeds at a linear rate for at least 20 hours (Chaffee and Mazia, 1963). Changes in the normal sequence or rate of sea urchin embryo development affects echinochrome synthesis (Asashima, 1971; Chaffee and Mazia, 1963). This linearity of echinochrome synthesis and the sensitivity of this pigment production to alterations in rates of embryonic development suggested that accumulation of echinochrome might represent an effective indicator of normal embryonic development. As echinochrome is easily extracted and absorbs light in the visible range (wavelength maximum = 475 nm) it could provide a simple and objective colorimetric measure of embryonic development and a direct new approach to larval bioassays.

Correlations between measurements of embryo response based on pigment synthesis and morphological change were made. Information from eight similar salinity experiments conducted during a six month period is presented for analysis of variations in embryo sensitivity and echinochrome production. Also included are data that show the response of the embryos to a toxicant (copper chloride), to seawater sampled from both within a harbor and from offshore, and to municipal wastewater.

METHODS

Exposure to Toxicants and Morphological Analysis

Gametes of the purple sea urchin, Strongylocentrotus purpuratus, used in all experiments, were collected, exposed to toxicants, and cultured according to the methods of Oshida et al. (1981). Each bioassay sample contained the same number of eggs (approximately 30,000) in 900 ml of seawater. Precautions were taken to ensure that initial egg densities in each breaker were very similar.

Eggs in the control solutions matured, gastrulated, and developed to the prism stage during the 48 hours following fertilization. At 48 hours, embryo subsamples were examined for morphological development and echinochrome production. The embryos in each sample were examined microscopically for two parameters to evaluate morphological development. One parameter used to describe developmental stage was the number of embryos which had undergone gastrulation (G), a critical process during development. This number also represented those embryos which were producing echinochrome. The percentage of these gastrulated embryos that developed to the prism stage at 48 hours (P) was the second parameter used. This percentage also indicated the relative amount of echinochrome produced by the embryos. The value of each parameter (G or P) was independent of the other; therefore, a sample with a low number of gastrulas could have had a high percentage of those gastrulated embryos at the prism stage.

Extraction of Echinochrome

Embryos contained in 800 ml of each bioassay sample were extracted for echinochrome (Bay et al. in press). Embryos were removed from the sample with a Nitex screen (mesh size 44 μm) and transferred to 15 ml polyethylene culture tubes containing seawater. After the sea urchin larvae were packed by centrifugation (20 min. at 1,000 x g) the seawater above the pellet was removed by aspiration. The pellet was washed with 10 ml of 95 percent ethanol to remove in-

terfering substances (e.g. chlorophyll) and centrifuged at 1,000 x g for 15 minutes. Following removal of the supernatant, 3 ml of acidified ethanol (1 part 25 percent HCl and 3 parts 95 percent ethanol) was added to each tube. Extraction of the echinochrome into the acidified ethanol was rapid once the pellet was disrupted with a stirring rod.

Extracts were centrifuged to remove embryo debris and the echinochrome content (E) of each sample was determined by measuring the absorbance of the orange-colored supernatant at 475 nm with a Turner spectrophotometer. The concentration of echinochrome in each sample was proportional to the absorbance. A test solution was identified as toxic if the amount of echinochrome measured (absorbance) was less than that produced by embryos in the control seawater. In most cases, extracts were stored at 0°C for up to 72 hours before absorbance was measured. Controls indicated that storage at this temperature caused less than five percent loss of pigment absorbance.

Particulates in wastewater contribute significant amounts of interfering color to echinochrome extracts at some effluent concentrations. This interference was corrected by subtracting the absorbance of a blank from that of each test sample in the wastewater experiment. A blank containing only diluted wastewater was made up for each effluent concentration of the start of the bioassay and run through the pigment extraction procedure with the embryo samples at the end of the experiment.

Toxic Solutions and Water Samples

Solutions of varying salinity were mixed by adding deionized water to natural seawater to produce seawater salinities of 33 (control) 28, 26, 25 and 21 parts per thousand (ppt). Samples with nominal copper concentrations of 0.001 to 0.040 mg/1 were produced by adding appropriate amounts of $CuCl_2$ to seawater.

Bioassays were also conducted using seawater from 11 Los Angeles Harbor stations (one meter beneath the surface), from 4 offshore stations (3 depths/station), and Orange County Sanitation District's final effluent. The Los Angeles Harbor seawater samples were collected from the stations indicated on Figure 1 and the offshore samples from those stations shown on Figure 2. The samples were held overnight at 11°C prior to conducting the bioassay in duplicate.

The final effluent from the Orange County Sanitation District is discharged 7.25 km offshore in the Pacific Ocean. The final effluent sample tested was collected at the treatment plant prior to discharge at sea. A 24 h composite sample was collected by treatment plant personnel in an acid-washed, kilned, glass bottle. The sample was brought to the laboratory and held overnight at 11°C prior to conducting the bioassay. For use in the bioassay, the effluent was diluted to different concentrations with filtered (3 μm) natural seawater. The dilutions used were between 152:1 and 10:1 (seawater:effluent). The effluent dilutions were tested in triplicate.

Statistical Analysis

Echinochrome production in each solution was examined for correlations with morphological development. Each datum was expressed as a percentage of the control value to compensate for variable control response in each experiment (Tattersfield and Morris, 1924). Seawater salinities causing 50 percent response levels (EC 50) were estimated for the echinochrome and morphological data using the Trimmed Spearman-Karber method (Hamilton, *et al.* 1977). Relationships between the morphological development parameters and pigment concentration were examined using correlation and partial correlation analysis (Nie, *et al.* 1975). As changes
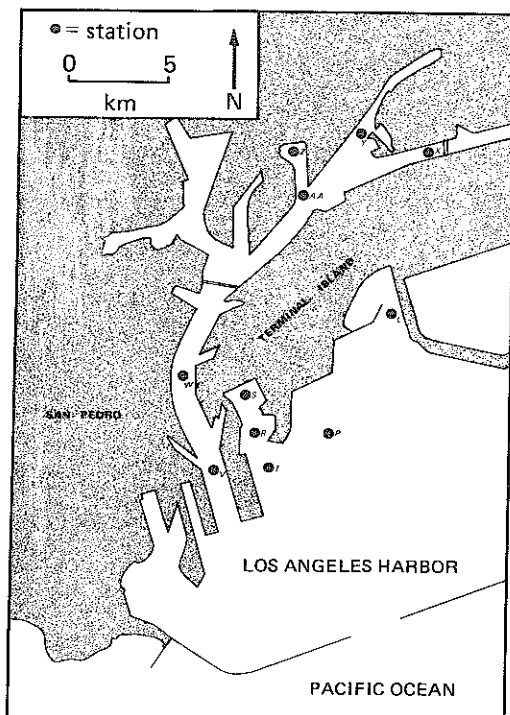
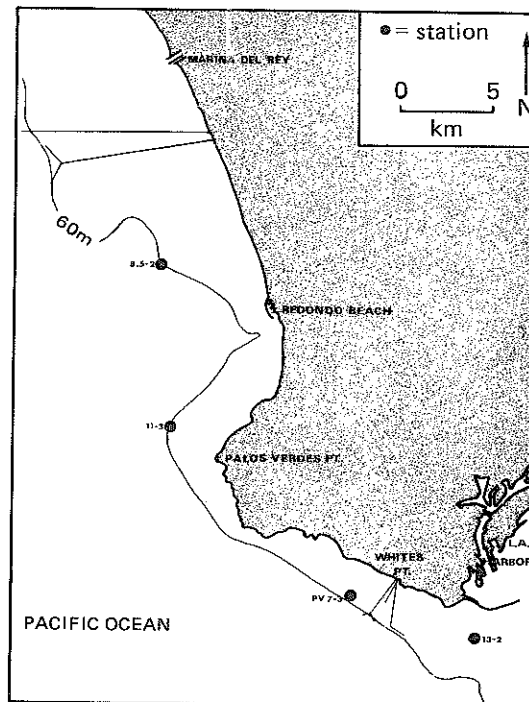Figure 1. Location of surface water sampling stations in Los Angeles Harbor.

Figure 2. Offshore stations where water samples were taken from the surface, mid-depth and bottom.

in both morphological parameters often occurred in the same sample, correlation analysis was unable to determine which parameter (G or P) affected echinochrome production. To make this type of determination, partial correlation analysis was utilized. This procedure eliminated the confusing effects of interactions between G and P and allowed us to determine which of the morphological parameters had a significant effect upon echinochrome synthesis.

RESULTS

Similar patterns of response to water of various salinities were evident in both the echinochrome and morphological measurements (Figure 3). All three indices had 50 percent response levels at salinities between 25 and 26 ppt. Standard correlations between pigment absorbance and either the number gastrulated or percent at prism stage were highly significant in all cases (Table 1). Partial correlation coefficients revealed that echinochrome was always strongly related to the number of embryos reaching the gastrula stage. A significant relationship between pigment levels and the percentage of fully developed prisms was also found.

Similar patterns of response were also evident when either echinochrome concentration or morphological development were examined in embryos exposed to copper (Figure 4). Reductions in the number of gastrulated embryos with increasing copper concentration were not as great and were more variable than the responses observed in the salinity experiments. Partial correlation coefficients for these data indicated that the degree of post-gastrulation development to the prism stage had the strongest effect on pigment concentration (Table 1).

For embryos exposed to Orange County Sanitation District municipal wastewater, standard correlations again indicated a very significant correlation between the echinochrome and both the number of sea urchins that gastrulated and the percentage of these gastrulas that developed

Figure

Percentage of control — SALINITY (PPT)

Legend:
- △——△ Echinochrome ± SE
- ●——● Number gastrulated ± SE
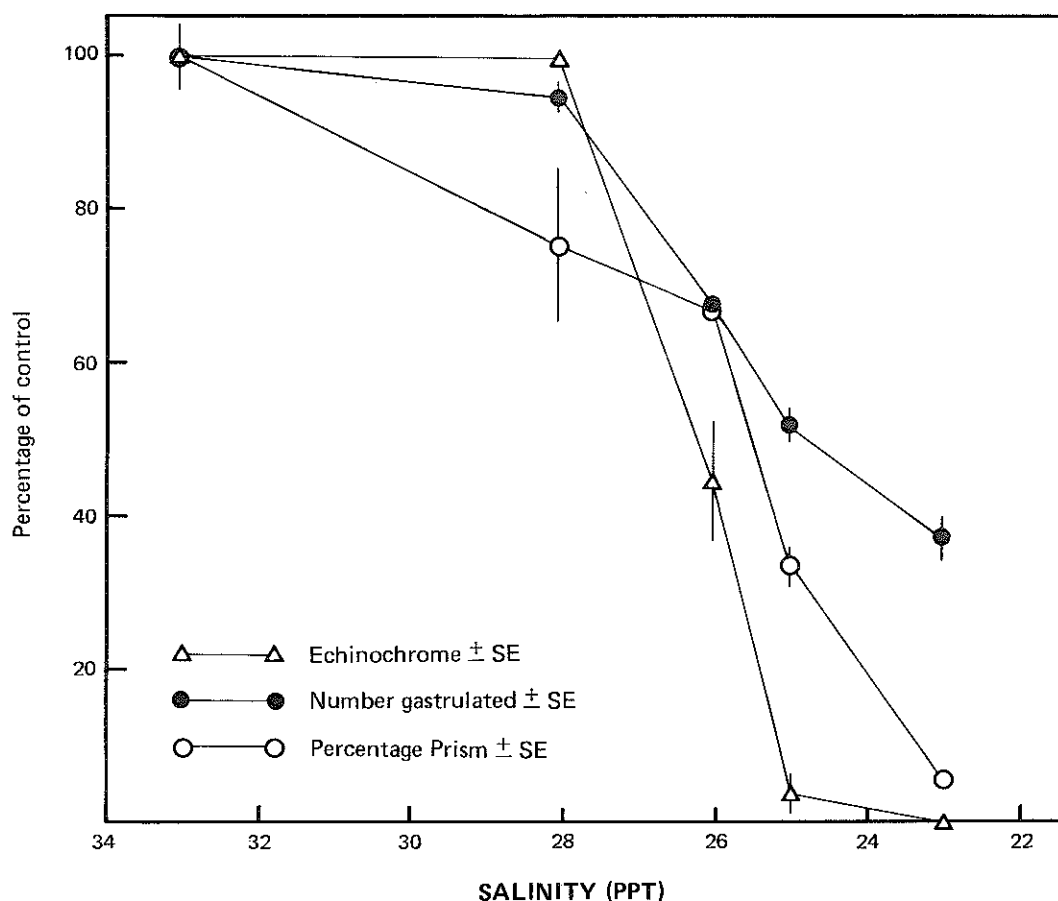- ○——○ Percentage Prism ± SE

Figure 3. 48-hour embryo development and echinochrome
production in response to lowered salinity. Data are
from experiment C (Table 1).

Table 1. Correlations of echinochrome absorbence (E) with the number of gastrulated embryos (G)
or percentage at prism stage (P). Changes in echinochrome caused by each toxicant were always
strongly related to morphological changes.

Treatment	Treatment Range	Experiment date	N	Standard correlations		Partial correlations	
				E vs G (p)[a]	E vs P (p)	E vs G (p)	E vs P (p)
A. salinity	33-23 ppt	13 Aug 81	8	0.9162(0.001)	0.8966(0.001)	0.6245(0.067)	0.5057(0.123)
B. salinity	33-23 ppt	8 Oct 81	13	0.9485(0.001)	0.9654(0.001)	0.5593(0.029)	0.7316(0.003)
C. salinity	33-23 ppt	2 Dec 81	12	0.9486(0.001)	0.8802(0.001)	0.7596(0.003)	0.2233(0.255)
D. copper	.004-.04 mg Cu/1[b]	4 Aug 81	14	0.6491(0.006)	0.9142(0.001)	-0.0124(0.484)	0.8464(0.001)
E. wastewater	.6-10%[c]	21 Mar 82	22	0.8267(0.001)	0.8236(0.001)	0.5483(0.005)	0.5380(0.006)
F. Harbor seawater		8 Oct 81	21	0.1303(0.277)	0.7867(0.001)	0.5220(0.006)	0.8473(0.001)
G. Offshore seawater		8 Oct 81	25	0.7424(0.001)	-0.0092(0.482)	0.7509(0.001)	0.1687(0.205)

[a] p = significance of correlation coefficient
[b] nominal Cu concentrations
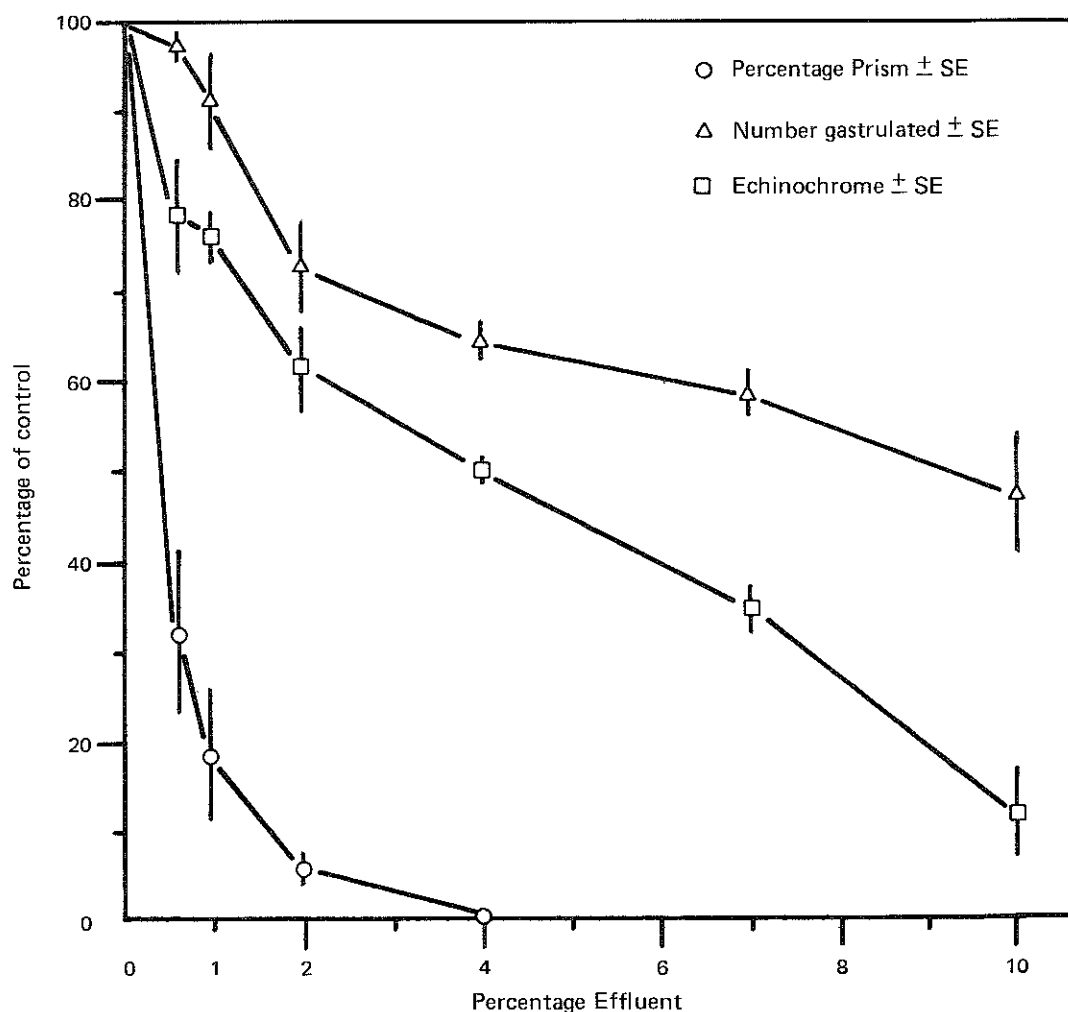[c] percent effluent
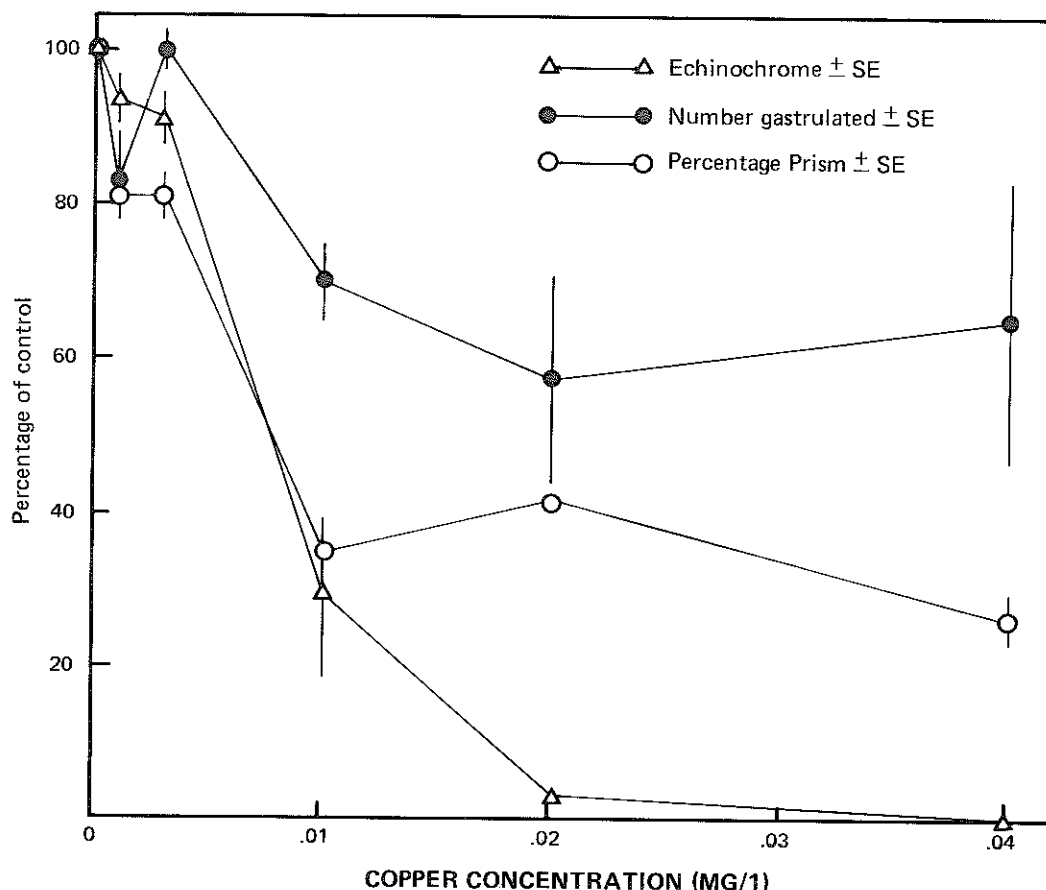
Figure 4. 48-hour embryo development and echinochrome production in response to copper concentration.

to the prism stage. Partial correlations also showed that enchinochrome pigment production was related to both of the aforementioned morphological parameters (Table 1). The results from the wastewater experiment (Figure 5) show how the amount of echinochrome produced appears to give an integrated measurement of the effects of toxicants on both gastrulation and development to prism.

Echinochrome and morphological measurements showed similar patterns of response to Los Angeles Harbor seawater. Large changes in echinochrome were observed at stations X, Y, Z, and AA (Figure 6). Corresponding reductions in the percentage of gastrulated embryos reaching the prism stage were usually found at these stations, while changes in the number of gastrulated embryos were not as frequent. The standard correlation between pigment absorbance and percent at prism stage was highly significant, while that between pigment absorbance and number gastrulated was not as great (Table 1). Partial correlation coefficients revealed that echinochrome was strongly related to the percentage of urchins that reached the prism stage when exposed to Los Angeles Harbor waters (Table 1); a significant relationship between echinochrome and number gastrulated was also found.

Reductions in echinochrome content were also observed in embryos exposed to seawater taken from offshore sites (Figure 7). Reduced pigment levels in these samples were highly correlated with reductions in the number of gastrulated embryos (Table 1). The percentage of embryos

Figure 5. 48-hour embryo development and echinochrome production in response to Orange County final sewage effluent toxicity.

reaching the prism stage was not reduced in any of the samples. Partial correlation analysis of these data showed that echinochrome levels were strongly influenced by changes in the number gastrulated and not related to the percentage at the prism stage.

For all experiments, the variability of replicate echinochrome values was usually similar to, or less than, that of the morphological data.

Variation in echinochrome production by control embryos was observed in experiments conducted over a six month period. Standard echinochrome extracts ranged from 0.079 to 0.175 absorbance units between experiments (Table 2). Though pigment content was variable, response to a standard salinity gradient (EC 50) was similar between experiments. During this six month interval, EC 50 values for salinity exposures ranged from 25 to 28 ppt for echinochrome and from 24 to 30 ppt for the number gastrulated (Table 2). Seawater concentrations causing 50 percent response in echinochrome concentration or number of embryos reaching gastrula stage were similar within experiments.
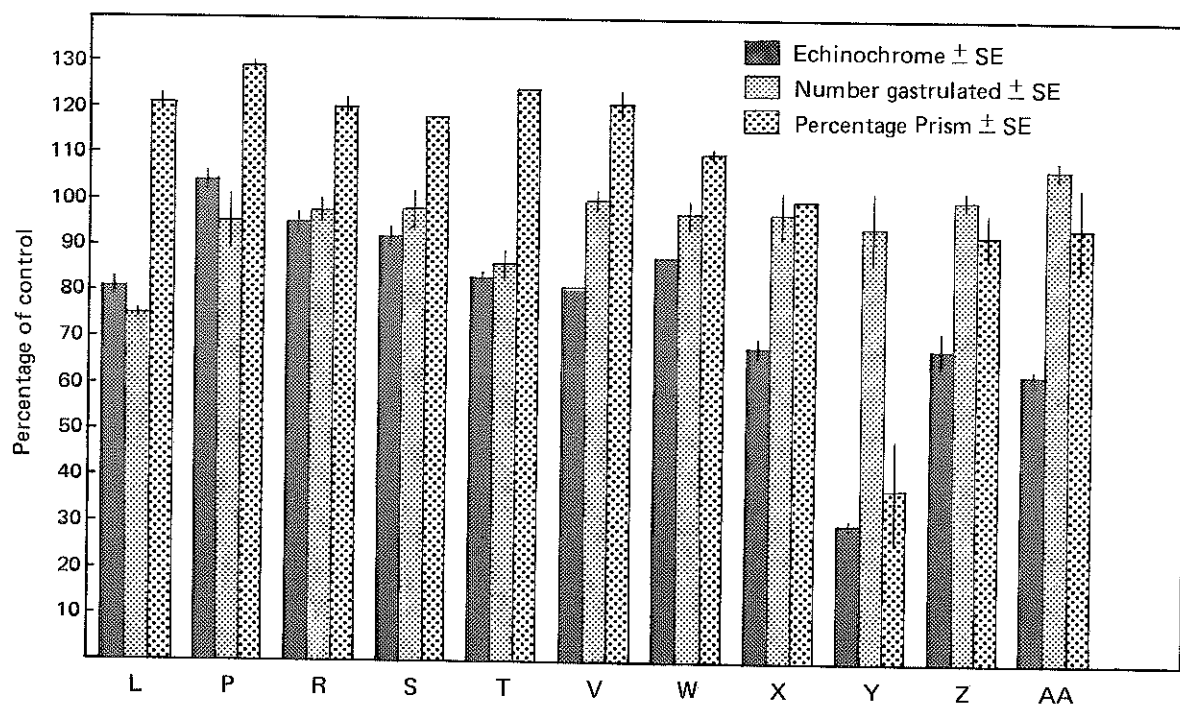
211

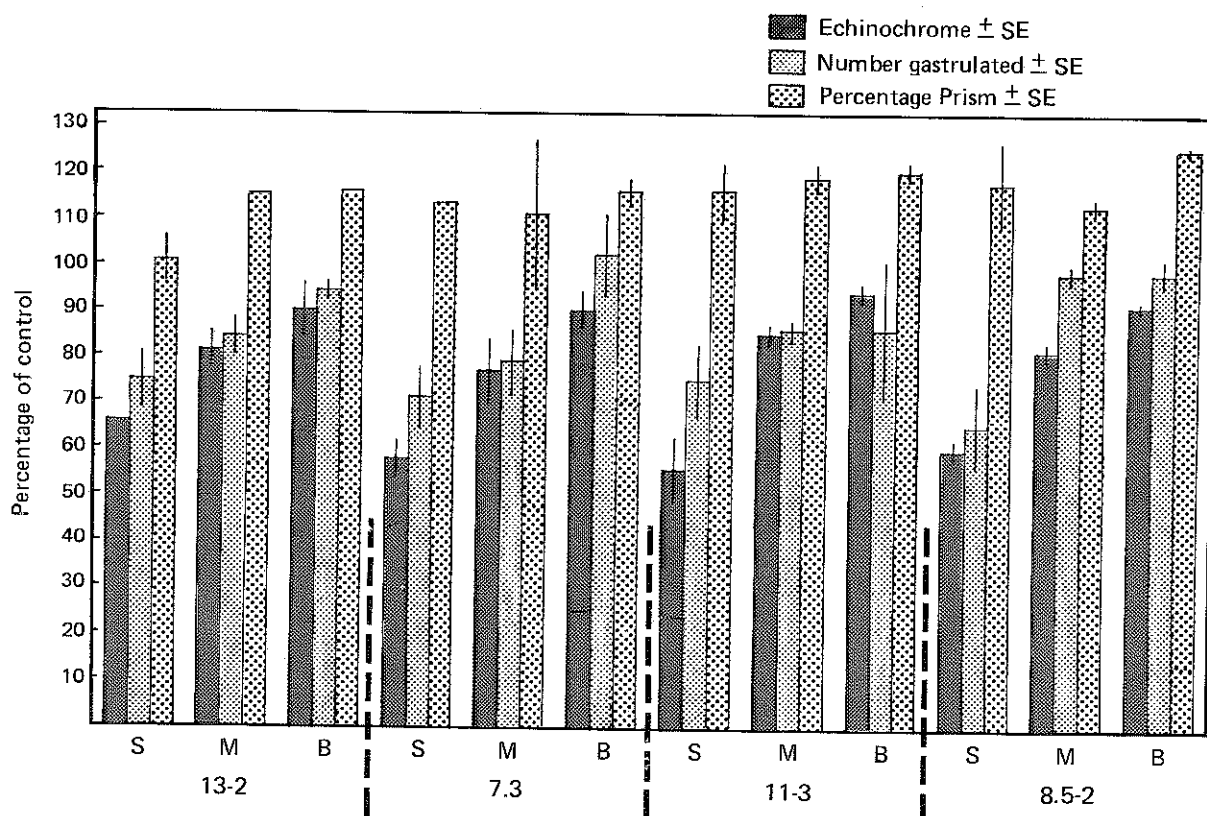Figure 6. The relationship between echinochrome production and embryo morphology in harbor water samples.

Figure 7. The relationship between echinochrome production and embryo morphology in offshore water samples.

| Experiment Date | Response to Control Water | | | Salinity Response (EC50)[d] | |
	Echinochrome mean ± SE (absorbence units, 475 nm)	number gastrulated[b] mean ± SE	Percentage prism mean ± SE	By Echinochrome (ppt)	By Number gastrulated (ppt)
28 Jul 81	0.090 ± 0.005	207 ± 22	ND[c]	25.7	27.5
4 Aug 81	0.175 ± 0.004	160 ± 6	ND	26.9	—[e]
12 Aug 81	0.123 ± 0.002	254 ± 15	84.7 ± 0.2	28.0	26.8
13 Aug 81	0.088 ± 0.008	144 ± 2	59.1 ± 9.7	26.8	27.0
16 Sep 81	0.151 ± 0.008	242 ± 4	68.9 ± 2.0	26.3	26.0
8 Oct 81	0.096 ± 0.004	217 ± 24	63.5 ± 2.4	25.7	26.3
4 Nov 81	0.110 ± 0.004	208 ± 28	61.5 ± 5.4	26.9	25.9
2 Dec 81	0.079 ± 0.002	253 ± 11	79.2 ± 9.6	26.2	24.9

[a] N = 4 for all data.

[b] Variation in number gastrulated represents differences in the number of eggs used in each experiment and not toxicity.

[c] Sample not examined for this parameter.

[d] Trimmed Spearman-Karber Method (Hamilton et al., 1977), a = 16.

[e] Unable to calculate EC50.

DISCUSSION

Change in echinochrome content is a reliable and sensitive indicator of sea urchin embryo response to the toxic effects of copper, reduced salinity, and wastewater. The data on Table 1 show that the echinochrome levels were strongly correlated with each of the following: the number of gastrulated embryos (G) and the percentage of gastrulas that reached prism stage (P). As either G or P decreased, due to experimental conditions, the echinochrome levels also decreased.

Partial correlation analysis of the data showed that echinochrome production was affected both by severe toxic responses, which prevented embryos from gastrulating (G), and by more subtle effects resulting in the retardation of the rate of post-gastrulation development (P). Differences between experiments in the significance of the partial correlation of E with G or P do not reflect changes in the sensitivity of the test, but rather variations in morphological response observed with different types of toxic samples. For example, in experiment C (Table 1), the partial correlation coefficients were 0.7596 (p = 0.003) and 0.2233 (p = 0.255) for E vs. G and E vs. P, respectively. This indicated that most of the decrease in echinochrome was due to the reduction in numbers of gastrulas. The number of embryos reaching gastrula at the lower salinities was reduced to such an extent that changes in the percentage of these gastrulas developing to prism apparently did not significantly affect echinochrome production.

Partial correlation analysis of the wastewater experiment (Table 1, Experiment E) revealed that decreased echinochrome at higher concentrations of effluent was related to reductions in both the number of embryos that gastrulated and the percentage that reached prism.

In addition to solutions created in the laboratory, the procedure can also detect toxic responses in samples of seawater collected from open coastal and harbor locations. Pigment reductions in these samples were also highly correlated with morphological changes, although the nature of the embryo response (and thus, the correlation) varied with location (Figures 6 and 7). Changes in the percentage of embryos at the prism stage were most often observed within the harbor and showed the highest correlation with echinochrome (Table 1).

Morphological response by embryos exposed to offshore seawater samples was found only in the number gastrulated. As a result, a significant partial correlation was found only with the number gastrulated (Table 1).

Reductions in levels of echinochrome reflect harmful changes in sea urchin embryo development. The echinochrome procedure is not intended to examine specific responses affecting pigment levels, but rather to measure the organism's overall response to stress. By measuring reductions in echinochrome levels, one is not only monitoring a single specific response, but an integration of many aspects of embryo development which may be altered by toxicants.

The easily measured echinochrome levels show decreases in normal morphological development without microscopic examination of the larvae. This technique requires little specialized equipment, has demonstrated accuracy and sensitivity comparable to standard morphological techniques, and requires about one-fourth of the time necessary for microscopic examination. Since the procedure is more rapid and objective than microscopic examination, it may increase the use of sea urchin embryo bioassays in monitoring programs where time, resources, and technical expertise are limited.

REFERENCES

Asashima, M. 1971. Some observations on the biosynthesis of echinochrome in sea urchin embryos. J. Fac. Sci. Univ. Tokyo IV, 12, 269-77.

Bay, S. M., K. D. Jenkins, and P. S. Oshida (in press). A simple new bioassay based on echinochrome synthesis by larval sea urchins. Mar. Env. Res.

Boolootian, R. A. 1966. Physiology of Echinodermata. New York, Interscience.

Cardwell, R. D.; M. I. Carr; C. E. Woelke; and E. W. Sanborn. 1977a. Evaluation of the efficacy of sulfite pulp mill pollution abatement using oyster larvae. Aquatic Toxicology and Hazard Evaluation ASTM STP 634, 281-95.

Cardwell, R. D.; M. I. Carr; and E. W. Sanborn. 1977b. Marine receiving water quality and pulp mill effluent toxicity, near Port Angeles and Everett, Washington in 1976. Washington Department of Fisheries, Point Whitney Laboratory. Brinnon,WA

Cardwell, R. D.; C. E. Woelke; M. I. Carr; and E. W. Sanborn. 1977c. Evaluation of water quality of Puget Sound and Hood Canal in 1976. NOAA Technical Memorandum ERL MESA-21 Boulder, CO

Chaffee, R. R., and D. Mazia. 1963. Echinochrome synthesis in hybrid sea urchin embryos. Devel. Biol., 7, 502-12.

Davis, P. H.; T. W. Schultz; and R. B. Spies. 1981. Toxicity of Santa Barbara seep oil to starfish embryos II. The growth bioassay. Mar. Env. Res., 5, 287-94.

Hamilton, M. A.; R. C. Russo; and R. V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Env. Sci. Tech. 11, 714-19.

Kobayashi, N. 1972. Marine pollution bioassay by using sea urchin eggs in the Inland Sea of Japan (The Seto-Naikai). Publ. Seto Mar. Biol. Lab., 19, 359-81.

Kobayashi, N. 1974. Bioassay data for marine pollution using sea urchin eggs, 1972 and 1973. Publ. Seto Mar. Biol. Lab., 21, 411-32.

Kobayashi, N. 1976. Bioassay data for marine pollution using sea urchin eggs, 1974. Publ. Seto Mar. Biol. Lab., 22, 405-407.

Kobayashi, N. 1977. Bioassay data for marine pollution using sea urchin eggs, 1975. Publ. Seto Mar. Biol. Lab., 23, 427-33.

Kobayashi, N., and K. Fujinaga. 1976. Synergism of inhibiting actions of heavy metals upon the fertilization and development of sea urchin eggs. The Science and Engineering Review of Doshisha University, 17, 54-69.

Koltsova, E. A.; L. V. Boguslavskaya; and O. B. Maximov. 1981. On the functions of quinoid pigments in sea urchin embryos. Intl. J. Invert. Reprod., 4, 17-23.

Nie, N. H.; C. H. Hull; J. G. Jenkins; K. Steinbrenner; and D. H. Bent. 1975. SPSS: Statistical Package for the Social Sciences, New York: McGraw-Hill.

Oshida, P. S. 1977. Toxicity of chlorinated benzene to sea urchin embryos. Southern California Coastal Water Research Project. Ann. Rep. 1977. Bascom, W., ed.; El Segundo, CA.

Oshida, P. S., and T. K. Goochey. 1980. A new test for measuring seawater toxicity. Southern California Coastal Water Research Project, Bien. Rep. 1979-1980. Bascom, W., ed.; Long Beach, CA

Oshida, P. S.; T. K. Goochey; and A. J. Mearns. 1981. Effects of municipal wastewater on fertilization, survival and development of the sea urchin, *Strongylocentrotus purpuratus*. *In* Biological Monitoring of Marine Pollutants. Eds. F. J. Vernberg, A. Calabrese, F. P. Thurbert and W. B. Vernberg. New York: Academic Press.

Tattersfield, F., and H. M. Morris. 1924. An apparatus for testing the toxic values of contact insecticides under controlled conditions. Bull. Entomol. Res., 14, 223.

Woelke, C. 1972. Development of a receiving water quality bioassay criterion based on the 48-hour Pacific oyster *(Crassostrea gigas)* embryo. Washington Department of Fisheries Technical Report 9. Tacoma, WA