Marine Pollution Bioassay by Sea Urchin Eggs, An Attempt to Enhance Accuracy, II

By

Naomasa Kobayashi

Biological Laboratory, Doshisha University, Kyoto 602, Japan

With Tables 1–6

The author proposed in 1971 the use of sea urchin eggs and embryos as indicatory materials in marine pollution bioassay and actually this was applied to the survey of the sea water pollution in the Inland Sea of Japan (Kobayashi *et al.*, 1972). The method was then improved to enhance the sensitivity by using aged eggs (Kobayashi, 1974). Later, it was found that the sensitivity to chemicals varies from fertilization to metamorphosis and the effects on the formation of pluteus were more pronounced than in any other earlier stages (Kobayashi, 1977). It seems that the sperm activity is the most sensitive to chemicals, and that the fertilization and gastrulation are more sensitive than the first cleavage, blastulation and pluteus formation (Kobayashi, 1980).

This paper is to show the results of experiments to see the enhancement in sensitivity to chemicals of the fertilized eggs obtained after the pre-treatment of both sperms and eggs, in respective developmental stages from fertilization to pluteus. First, the experiments were made with the chemicals: Cu sulphate, Zn chloride, alkyl benzyle sulphate (ABS) and ammonia chloride and then with the test water collected from around Hatakejima Island. Comparisons were made among the results of experiments with the fertilized eggs derived from normal and differently pre-treated sperms or eggs in various test water checking their sensitivity in respective developmental stages from fertilization to pluteus. Lastly, a new manual and revised ranking are proposed to apply the results of mentioned experiments actually to the bioassay.

Materials and Methods

Eggs and sperms of *Anthocidaris crassispina* (A. Agassiz) (in May, July and September) or *Hemicentrotus pulcherrimus* (A. Agassiz) (in January) were left intact for some time in each test water before they were used for insemination, then the rates of fertilization, first cleavage, gastrulation, pluteus formation and some anomalies

Publ. Seto Mar. Biol. Lab., 30(4/6), 213–226, 1985. (Article 9)

in the development were checked. Eggs were obtained by the current KCl-method, being washed several times with fresh sea water, and were used as soon as possible, within 1 hour at the latest. Sperms were obtained from testes within 1 hour after these were taken out of the test. The sperm density for insemination was standardized at about 1 dry sperm:100,000 sea water in volume. If necessary, the preliminary check of eggs was made to see if the fertilization membrane was elevated in 3 minutes after insemination in over 91% of eggs and if the well synchronized first cleavage occurred in over 91% of them in the control laboratory water.

Eggs (pre-treated 3 or 6 h in the test water before fertilization) were inseminated with sperms (pre-treated 5 min in the test water before fertilization) and left in respective test water samples. Rates of successful fertilization, first cleavage, gastrulation and pluteus formation and of some abnormalities in development were checked at 26°C or 19°C.

Series of concentrations of the chemicals to be tested were prepared by successive dilution of the original solution (chemicals —$1:10^4$–10^3 sea water by volume $=100-1{,}000$ ppm).

Firstly, the percent of eggs with elevated fertilization membrane to the total eggs observed was read. The first cleavage occurred in most cases about 90 minutes after the insemination at 19°C (warmed) or 50–60 minutes after the insemination at 26–28°C. Then, the rate and state of the first cleavage, namely proportions of undividing cells, normal two cells and multi-cells caused by polyspermy were checked at respectively adequate time. One or two hundred eggs were fixed with 5% formaldehyde at a time for this examination. Next, the state of swimming embryos exclusive of those deposited on the bottom, namely proportions of permanent blastulae, normal gastrulae and abnormal exogastrulae were checked about 20 hours at 19°C or 14–18 hours at 26–28°C after the insemination. One or two hundred embryos were fixed at a time for this check. Lastly, the state of swimming larvae exclusive of those deposited on the bottom, namely proportions of abnormal plutei and normal plutei were checked about 36 hours at 19°C or 24–30 hours at 26–28°C after the insemination. In normal plutei at this time, the body contour has clearly changed already from that in previous stages, the digestive tract is completed and spicules are fully developed. In abnormal plutei, however, the formation of these structures is retarded markedly. In every examination, one or two hundred eggs, embryos or larvae were checked and the examination was repeated 3 times on different batches in respective water samples. Comparisons were made on the phases from fertilization to pluteus between the developments in the test water of the fertilized eggs respectively resulting from normal and differently pre-treated sperms or eggs.

Results

Effects of some chemicals.

The effects of various chemicals upon the developmental stages from

Table 1. Effects of Cu, Zn, ABS and Ammonia on egg development in *Anthocidaris crassispina*.
Tests: July 1981; water temperature: 26°C.
normal eggs+normal sperms

Chemicals	Concs. ppm	Time after insemination: 3 min fertiliz. membrane formation %	1 h 1-cell state %	1 h 2-cell stage %	1 h multi-cell polyspermy %	15 h permanent blastula %	15 h normal gastrula %	15 h exo-gastrula %	24 h abnormal pluteus %	24 h normal pluteus %	Ultimate state
Control		98 (>90)	3	97 (>90)	0	2	98 (>90)	0	2	98 (>95)	normal
	0.5	36	65	25	10	96	4	0			permanent blastula
	0.2	71	32	48	20	23	77	0	100	0	retardation
$CuSO_4 \cdot 5H_2O$	0.1	83	16	53	31	17	83	0	67	33	retardation
	0.05	95	7	93	0	5	95	0	18	82	retardation
	0.02	98	3	97	0	2	98	0	2	98	normal
	0.5	45	59	41	0	34	49	17			permanent blastula
	0.2	74	27	60	13	17	51	32	100	0	retardation
$ZnCl_2$	0.1	83	16	82	2	6	83	11	69	31	retardation
	0.05	96	6	94	0	3	97	0	16	84	retardation
	0.02	97	5	95	0	1	99	0	2	98	normal
	10	48	54	46	0						cytolysis
	5	77	25	75	0	36	64	0	100	0	retardation
ABS	2	81	21	79	0	18	82	0	48	52	retardation
	1	94	8	92	0	7	93	0	17	83	retardation
	0.5	97	4	96	0	2	98	0	3	97	normal
	10	48	55	45	0						cytolysis
	20	72	30	65	5	79	21	0			permanent blastula
NH_4Cl	5	84	14	82	4	19	81	0	83	17	retardation
	2	93	9	91	0	7	93	0	20	80	retardation
	1	97	3	97	0	3	97	0	1	99	normal

Table 2. Effects of Cu, Zn, ABS and Ammonia on egg development in *Anthocidaris crassispina*.
Tests: July 1981; water temperature: 26°C.
pre-treated eggs (3 h)+normal sperms

Chemicals	Concs. ppm	Time after insemination: 3 min fertiliz. membrane formation %	1 h 1-cell state %	1 h 2-cell stage %	1 h multi-cell polyspermy %	15 h permanent blastula %	15 h normal gastrula %	15 h exo-gastrula %	24 h abnormal pluteus %	24 h normal pluteus %	Ultimate state
Control		99 (>90)	1	99 (>90)	0	2	98 (>90)	0	1	99 (<95)	normal
	0.5	24	76	10	14	98	2	0			permanent blastula
	0.2	66	35	39	26	27	73	0	100	0	retardation
$CuSO_4 \cdot 5H_2O$	0.1	83	17	48	35	16	84	0	76	24	retardation
	0.05	94	9	91	0	5	95	0	17	83	retardation
	0.02	98	3	97	0	1	99	0	3	97	normal
	0.5	49	55	45	0	36	33	31			exogastrula
	0.2	76	29	56	15	16	41	43	100	0	exogastrula
$ZnCl_2$	0.1	91	17	78	5	7	84	9	71	29	retardation
	0.05	95	4	96	0	4	96	0	18	82	retardation
	0.02	98	3	97	0	2	98	0	3	97	normal
	10	46	57	43	0						cytolysis
	5	75	31	69	0	35	65	0	100	0	retardation
ABS	2	83	19	81	0	16	84	0	54	46	retardation
	1	95	9	91	0	9	91	0	19	81	retardation
	0.5	94	4	96	0	3	97	0	2	98	normal
	20	44	59	41	0						cytolysis
	10	73	31	63	6	83	17	0			permanent blastula
NH_4Cl	5	89	18	82	0	19	81	0	81	19	retardation
	2	94	7	93	0	9	91	0	18	82	retardation
	1	96	6	94	0	4	96	0	1	99	normal

Table 3. Effects of Cu, Zn, ABS and Ammonia on egg development in *Anthocidaris crassispina*.
Tests: July 1981; water temperature: 26°C.
normal eggs+pre-treated sperms (5 min)

Chemicals	Concs. ppm	Time after insemination: 3 min fertiliz. membrane formation %	1 h 1-cell state %	1 h 2-cell stage %	1 h multi-cell polyspermy %	15 h permanent blastula %	15 h normal gastrula %	15 h exo-gastrula %	24 h abnormal pluteus %	24 h normal pluteus %	Ultimate state
Control		96 (>90)	5	95 (>90)	0	3	97 (>90)	0	2	98 (>95)	normal
	0.2	18	84	16	0	82	18	0			permanent blastula
	0.1	66	36	49	15	24	76	0	100	0	retardation
$CuSO_4 \cdot 5H_2O$	0.05	85	14	77	9	16	84	0	81	19	retardation
	0.02	94	7	93	0	4	96	0	18	82	retardation
	0.01	96	6	94	0	2	98	0	3	97	normal
	0.2	54	57	43	0	64	4	32			exogastrula
	0.1	71	32	63	5	18	41	41	100	0	exogastrula
$ZnCl_2$	0.05	86	18	82	0	6	78	16	83	17	retardation
	0.02	91	11	89	0	4	96	0	24	76	retardation
	0.01	97	4	96	0	2	98	0	1	99	normal
	5	48	56	44	0						cytolysis
	2	74	32	68	0	29	71	0	100	0	retardation
ABS	1	85	17	83	0	17	83	0	69	31	retardation
	0.5	95	6	94	0	5	95	0	19	81	retardation
	0.2	94	8	92	0	3	97	0	2	98	normal
	10	51	52	41	7						cytolysis
	5	69	32	60	8	81	19	0			permanent blastula
NH_4Cl	2	86	17	79	4	16	84	0	92	8	retardation
	1	91	8	89	3	4	96	0	18	82	retardation
	0.5	95	6	94	0	2	98	0	4	96	normal

Table 4. Effects of Cu, Zn, ABS and Ammonia on egg development in *Anthocidaris crassispina*.
Tests: July 1981; water temperature: 26°C.
pre-treated eggs (3 h)+pre-treated sperms (5 min)

Chemicals	Concs. ppm	Time after insemination: 3 min fertiliz. membrane formation %	1 h 1-cell state %	1 h 2-cell stage %	1 h multi-cell polyspermy %	15 h permanent blastula %	15 h normal gastrula %	15 h exo-gastrula %	24 h abnormal pluteus %	24 h normal pluteus %	Ultimate state
Control		98 (>90)	3	97 (>90)	0	2	98 (>90)	0	2	98 (>95)	normal
$CuSO_4 \cdot 5H_2O$	0.2	17	84	16	0						cytolysis
	0.1	52	58	31	11	83	17	0			permanent blastula
	0.05	84	28	67	5	24	76	0	94	9	retardation
	0.02	92	11	89	0	11	89	0	83	17	retardation
	0.01	97	4	96	0	4	96	0	16	84	retardation
	0.005	96	5	95	0	2	98	0	3	97	normal
$ZnCl_2$	0.2	49	56	44	0	74	16	10			permanent blastula
	0.1	66	35	60	5	64	15	21			permanent blastula
	0.05	76	28	72	0	21	48	31	100	0	retardation
	0.02	94	8	92	0	2	93	5	81	19	retardation
	0.01	96	5	95	0	3	97	0	16	84	retardation
	0.005	97	4	96	0	1	99	0	1	99	normal
ABS	5	38	64	36	0						cytolysis
	2	61	44	56	0	34	66	0	100	0	retardation
	1	89	17	83	0	16	84	0	84	16	retardation
	0.5	92	9	91	0	11	89	0	65	35	retardation
	0.2	96	5	95	0	4	96	0	18	82	retardation
	0.1	98	4	96	0	1	99	0	2	98	normal
NH_4Cl	10	34	74	15	11						cytolysis
	5	69	34	51	15	100	0	0			permanent blastula
	2	89	14	86	0	68	32	0	100	0	retardation
	1	96	6	91	3	16	84	0	80	20	retardation
	0.5	94	7	93	0	4	96	0	19	81	retardation
	0.2	97	4	96	0	2	98	0	5	95	normal

pre-fertilization to pluteus formation were compared one another and the results are given in Tables 1 to 4. As seen in these, the effects upon the stages from fertilization to pluteus formation seemingly conform to those reported already in the previous papers (1971, 1974 and 1977), but some exception. The chemicals are arranged in the Tables in the order of their inhibitory effects.

Cu:

In the higher concentrations of Cu, the eggs or embryos developed in somewhat abnormal states. Retarded formation of the fertilization membrane occurred. Polyspermy occurred and irregular cleavage was induced at higher concentrations. Retarded development was evident in most cases, but no exogastrulae appeared in any cases.

The effects seemed more pronounced in the following order, pre-treated eggs +pre-treated sperms>normal eggs +pre-treated sperms>pre-treated eggs+normal sperms, normal eggs+normal sperms.

Zn:

Eggs and embryos developed somewhat abnormally in higher concentrations of Zn. Retarded formation of the fertilization membrane occurred. Polyspermy occurred in higher concentrations. Retardation of development was evident in most cases. Exogastrulae appeared in higher concentrations.

The effects seemed more pronounced in the following order, pre-treated eggs +pre-treated sperms>normal eggs+pre-treated sperms>pre-treated eggs+normal sperms, normal eggs+normal sperms.

ABS:

Higher concentrations of ABS reduced the development or caused the cytolysis of cells. There was no evidence of polyspermy. The development was anomalous or retarded even in lower concentrations. No exogastrulae appeared in any cases.

The effects seemed more pronounced in the following order, pre-treated eggs +pre-treated sperms>normal eggs+pre-treated sperms>pre-treated eggs+normal sperms, normal eggs+normal sperms.

Ammonia:

Quantitatively, the influence of ammonia was similar to that of ABS. Some reduction in fertilization membrane formation occurred at the highest concentration. Polyspermy occurred in higher concentrations. Retardation of the development was evident in most cases. Cytolysis occurred at the highest concentration. Exogastrulae did not appear in any cases.

The effects seemed more pronounced in the following order, pre-treated eggs +pre-treated sperms>normal eggs+pre-treated sperms>pre-treated eggs+normal sperms, normal eggs+normal sperms.

Summing up the above-mentioned results of experiments, the effects of 4 tested chemicals seemed similarly more pronounced in the following order, pre-treated eggs+pre-treated sperms>normal eggs+pre-treated sperms>pre-treated eggs+normal sperms and normal eggs+normal sperms.

Table 5a. Results of the Sept. 6, '82 experiment with eggs of *Anthocidaris crassispina*.
Wind; 0. Test water temperature; 26°C. 0 mins. old sperms. 3 hrs. old eggs. *After Ranking II 1974

Location (depth)	Fertiliz.	First cleavage (50 min.)			Gastrulation (17 hrs.)			*Degree of inhibitory effect
	membrane formation	1 cell	2 cell (normal)	multi-cell (polyspermy)	permanent blastula	gastrula (normal)	exo-gastrula	
(m)	%	%	%	%	%	%	%	
Running sea water of Laboratory	99.0	1.5	98.5	0	1.0	99.0	0	
	98.5	1.5	98.5	0	1.5	98.5	0	0
	98.0	2.0	98.0	0	1.0	99.0	0	
Water from open sea side of Hatakejima Surface	98.5	1.5	98.0	0.5	1.5	98.5	0	
	98.0	2.0	97.0	1.0	2.0	98.0	0	0
	97.5	2.5	96.0	1.5	1.5	98.5	0	
Bottom (25)	97.0	3.5	95.0	1.5	2.0	98.0	0	
	97.5	3.0	95.5	1.5	2.5	97.5	0	0
	96.0	4.0	94.0	2.0	3.0	97.0	0	
Water from land side of Hatakejima Surface	98.5	2.0	97.0	1.0	2.0	98.0	0	
	98.0	2.5	96.0	1.5	2.5	97.5	0	0
	97.0	4.0	93.5	2.5	3.0	97.0	0	
Bottom (7)	90.5	14.0	84.5	1.5	3.0	97.0	0	
	90.0	13.5	84.5	2.0	2.5	97.5	0	1
	90.5	13.5	84.0	2.5	4.0	96.0	0	
Sea water from Tsunashirazu cove Surface	97.0	3.5	95.0	1.5	3.5	96.5	0	
	96.0	4.0	93.5	2.5	4.0	96.0	0	1
	96.5	4.0	93.0	3.0	4.0	96.0	0	
Bottom (5)	86.5	14.0	83.5	2.5	13.5	86.0	0.5	
	85.5	15.0	82.0	3.0	13.0	87.0	0	3 somewhat delayed development
	85.0	15.5	81.0	3.5	15.0	85.0	0	

Table 5b. Results of the Sept. 6, '82 experiment with eggs of *Anthocidaris crassispina*.
Wind; 0 Testwater temperature; 26°C. 5 mins. old sperms. 3 hrs. old eggs. *After Improved ranking (Ranking III)

Location (depth)	Fertiliz.	First cleavage (50 min.)			Gastrulation (17 hrs.)			Pluteus (26 hrs.)		*Degree of inhibitory effect
	membrane formation	1 cell	2 cell (normal)	multi-cell (polyspermy)	permanent blastula	gastrula (normal)	exo-gastrula	abnormal pluteus	normal pluteus	
(m)	%	%	%	%	%	%	%	%	%	
Running sea water of Laboratory	98.0	2.0	97.5	0.5	1.5	98.5	0	2.5	97.5	
	99.0	1.5	98.5	0	2.0	98.0	0	1.5	98.5	0
	97.5	2.5	97.0	0.5	2.0	98.0	0	2.5	97.5	
Water from open sea side of Hatakejima Surface	98.5	1.0	98.5	0.5	2.0	98.0	0	4.0	96.0	
	98.5	1.5	97.5	1.0	2.5	97.5	0	3.5	96.5	0
	98.0	2.0	97.0	1.0	3.0	97.0	0	3.0	97.0	
Bottom (25)	96.0	2.0	96.5	1.5	4.0	96.0	0	4.5	95.5	
	97.5	2.5	96.5	1.0	4.5	95.5	0	4.0	96.0	1
	95.0	6.0	90.5	3.5	3.5	96.5	0	5.5	94.5	
Water from land side of Hatakejima Surface	98.0	3.5	96.0	0.5	3.0	96.0	1.0	5.0	95.0	
	98.5	2.0	97.5	0.5	6.0	93.5	0.5	3.5	96.5	1
	96.5	4.0	94.0	2.0	6.5	93.0	0.5	6.0	94.0	
Bottom (7)	97.0	3.0	96.0	1.0	13.5	85.0	1.5	15.5	84.5	
	96.5	5.0	93.5	1.5	15.5	83.5	1.0	14.0	86.0	3 development somewhat delayed
	95.5	5.5	91.0	3.5	17.0	83.0	0	17.0	83.0	
Sea water from Tsunashirazu cove Surface	96.5	5.5	92.0	2.5	3.0	97.0	0	4.5	95.5	
	95.0	6.0	89.5	4.5	4.5	94.5	1.0	3.5	96.5	1
	94.5	6.0	91.0	3.0	7.0	93.0	0	5.5	94.5	
Bottom (5)	87.5	14.5	83.5	2.0	15.0	85.0	0	16.0	84.0	
	86.0	15.5	81.0	3.5	15.5	84.0	0.5	16.5	83.5	3 development somewhat delayed
	85.0	16.0	80.5	3.5	17.5	82.5	0	18.0	82.0	

Effects of marine pollution.

The marine pollution bioassay was carried out using the aged eggs and sperms as in the above-mentioned experiments with some chemicals, on the water samples collected at the four stations around Hatakejima Island as in the cases reported in previous studies (Kobayashi, 1974). (See Table 5 in this report and Tables 1, 2 in "Biological Data" in this volume).

The effects of aged eggs (3, 6 hours old) and aged sperms (5 minutes old) upon the fertilization, the first cleavage, gastrulation and pluteus formation in the polluted sea water were learned by comparing one another the figures concerning these developmental processes, obtained in respective test water samples and those observed in the control water, the running sea water of the laboratory, using aged eggs (3, 6 hours old) and normal 0 minute old sperms. It was found, then, that the effects become larger with the age of germ cells on one side and naturally more pronounced with the degree of pollution of the water on the other side. Actually the effects were most remarkable in the water samples from the cove of Tsunashirazu where the water is polluted most heavily by sewage and waste products of fish rearing, etc.

The age limit for the maintenance of the normal developmental conditions, such as the usual higher rates of fertilization and the first cleavage over 91%, was found again to be 6 hours in *Hemicentrotus* and 3 hours in *Anthocidaris*.

Considerations and Proposal of a New Manual for Bioassay

There have been many researches dealing with the comparative effects of chemicals or polluted water on the fertilization and further development in normally inseminated eggs (normal eggs+normal sperms) of sea urchins (i.e., Kobayashi 1971 etc.). The effects of various chemicals or polluted water were found more pronounced by using the aged eggs (pre-treated eggs+normal sperms), in other words the aged eggs were found more sensitive to the pollutants (Kobayashi, 1974).

Further, it was found, in comparing the effects of chemicals on various developmental stages of sea urchins, that the sperm test (normal eggs+pre-treated sperms) was the most sensitive than any other processes in reducing the fertilization and further development (Kobayashi, 1980). Similar findings have been reported already by Renzoni (1974) and Hagström & Lönning (1977) for oil-dispersant etc. Stober, Dinnel & Crumley (1979) and Dinnel, Stober & Dijulio (1981) reported that the relative sensitivity of sperms was higher than that of eggs in sea urchins. In the latter work, the sea urchin sperm bioassay for sewage etc. was carried out using declining fertilization success as an indication of reduction in sperm viability.

In the present experiments, the sensitivity to the 4 chemicals seemed more pronounced in the order, pre-treated eggs+pre-treated sperms>normal eggs+pre-treated sperms>pre-treated eggs+normal sperms, normal eggs+normal sperms. However, Crawford and Gates (1981) reported that the effect of drilling fluid on fertilization of sand dollar eggs was in the order, treated sperm+treated eggs, normal

sperm+treated eggs>treated sperm+normal eggs>normal sperm+normal eggs.

It has already been reported by many researchers (Wilson & Armstrong, 1951–1961, Hagström & Lönning, 1973, Pagano, Esposito, Giordano & Hagström, 1978, Wilson, 1981, Castagna, Sinatra, Scalia & Capodicasa, 1981, and Pagano, Esposito & Giordano, 1982, etc.) that chemicals or polluted water affect pluteus formation in various sea urchins. And actually the effects of some pollutants and polluted water upon the developmental stages were more pronounced in pluteus formation that in any of other stages prior to it (Kobayashi, 1977). As seen in those experiments, in marine pollution bioassay using sea urchin eggs, it is necessary to check the later developmental stages as far as possible, at least as late as pluteus formation.

Therefore, the significance of the results obtained by a short-time bioassay of checking only the earlier developmental stages from fertilization to gastrulation should be estimated properly on the background of more strict results that will be obtained by checking the earlier through later stages, at least from pre-fertilization to pluteus formation. In other words, the most sensitive method for marine pollution bioassay using the sea urchin development seems to be found in checking from fertilization with pre-treated eggs+pre-treated sperms to pluteus formation in the test water.

Thus, an improvement of the method of grading the pollution degree proposed by Kobayashi (1974, the Ranking II) was attempted on more exact results of experiments checking the developmental stages from the fertilization with aged eggs and aged sperms to pluteus formation of sea urchins. And here the new Ranking III is proposed in Table 6. In this, the check of the abnormal and normal formation of pluteus was adopted with a weight of the same percentage as that of gastrulation in the Ranking II. In general, the present Ranking III is more exact than the Ranking II. So that the Ranking II will not be used hereafter by the present author. The Ranking I by Kobayashi (1972) to check only the stages from the fertilization with 0 hour old eggs and 0 minute old sperms to the gastrulation may be suitable to treat a large number of heavily polluted water samples in a limited time. But, when only a smaller number of less polluted water samples are checked and higher sensitivity is requested, the Ranking III will be much better applied. The advantage of this ranking is especially noted when the pollution is caused by organic matters. For instance, the bottom water sample from the land side of Hatakejima Island (Table 1) was judged as grade 1 in the Ranking I but as grade 3 in the Ranking III. Therefore, for the bioassay of the marine pollution caused by heavy industries or chemical factories the Ranking I may be applied better in general than the Ranking III. The sea water pollution around Hatakejima Island is not yet so heavy and is caused mainly by organic substances. Therefore, the Ranking III may be more suitable to check the pollution in this area than the Ranking I. Probably actual choice may depend mainly upon the number of water samples. Then, it seems necessary to propose here a new manual of bioassay for the Ranking III, that follows next procedures:

Table 6. An improved ranking (the Ranking III) of the sea water pollution by using aged eggs and aged sperms of sea urchins.

Inhibitory degree	Stage / Grade	Fertiliz. membrane formation	First cleavage: 1 cell	First cleavage: 2 cell (normal)	First cleavage: multi-cells* (polyspermy)
Violent inhibition	5	0– 50%	100–50%	0– 50%	15–100%
Strong inhibition	4	51– 60	49–40	51– 60	12– 14
Moderate inhibition	3	61– 70	39–30	61– 70	9– 11
Weak inhibition	2	71– 80	29–20	71– 80	6– 8
Slight inhibition	1	81– 90	19–10	81– 90	3– 5
Non-inhibition	0	91–100	9– 0	91–100	0– 2

Inhibitory degree	Gastrulation: permanent blastula	Gastrulation: gastrula (normal)	Gastrulation: exogastrula*	Pluteus formation: abnormal	Pluteus formation: normal	Remarks**
Violent inhibition	100–25%	0– 75%	15–100%	100–25%	0– 75%	development stopped in early stages
Strong inhibition	24–20	76– 80	12– 14	24–20	76– 80	development delayed or deformed
Moderate inhibition	19–15	81– 85	9– 11	19–15	81– 85	development somewhat delayed and deformed
Weak inhibition	14–10	86– 90	6– 8	14–10	86– 90	
Slight inhibition	9– 5	91– 95	3– 5	9– 5	91– 95	
Non-inhibition	4– 0	96–100	0– 2	4– 0	96–100	

*: Rather infrequent. **: Notes when such features were seen on over 50% of the checked embryos. Hours to insemination are 3 hours in summer (water temperature 26–28°C) for *Anthocidaris* eggs, 9 hours in autumn (water temperature 13–16°C) for *Pseudocentrotus* eggs and 6 hours in winter (water temperature 17–19°C, warmed) for *Hemicentrotus* eggs (after Kobayashi, 1984).

1. Unfertilized eggs are kept in a glass bowl filled with respective test water for some hours before they are inseminated. Hours to insemination are 3 hours in summer in *Anthocidaris* (at water temperature 26–28°C), 9 hours in autumn in *Pseudocentrotus depressus* (A. Agassiz) (at water temperature 13–16°C) and 6 hours in winter in *Hemicentrotus* (at water temperature 17–19°C). The water temperature should be maintained stably in autumn and winter, when the temperature is variable.

2. It is desirable that a preliminary check is done to see if the fertilization membrane is elevated in 3 minutes after insemination on over 91% of eggs and the first cleavage occur synchronously over 91% of them.

3. Sperms are left in a glass bowl filled with respective test water at the density of about 1 dry sperm:100 test water in volume for 5 minutes before they are used for insemination.

4. When this assay is applied to see the inhibitory effects of different sea

sediment samples, the following procedures may be a way. The water is removed from the sample as far as possible and then the sample is homogenized by stirring. One part in volume of the sediments is mixed with 9 parts of control (normal) sea water; the mixture is shaken for 5 minutes, then kept still for 6 hours. The supernatant water is assayed as in the case of polluted water.

5. In grading, take the lowest figure for normal features but the highest for abnormal ones, of course exceptional figures should be excluded. The grade of the pollution is represented by the highest grade throughout the whole indicatory features checked, as this will decide the survival rate (Kobayashi, 1971).

Summary

To improve the previous methods of marine pollution bioassay presented by the author (1971, 1974 and 1977), especially to enhance the sensitivity, some experiments were made with the developmental stages of the sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*, from pre-fertilization to pluteus formation. The sperms and eggs were left in the test water for some time before they were used for insemination. Rates of fertilization, first cleavage, gastrulation, pluteus stages and some anomalies of development in the test water were then checked. Various abnormalities, i.e. the reatrded formation or absence of the fertilization membrane, polyspermy, irregular cleavage and development, cytolysis etc., occurred most sensitively in the series of experiments with the pre-treated sperm+pre-treated eggs; especially markedly the irregularity was seen at the stage of pluteus. This result seems to be applicable to improve the bioassay of marine pollution. For instance, the effects of heavy metals (Cu and Zn) and other chemicals (ABS and Ammonia) upon the pre-treated sperms and eggs are significant. Results of a short-term marine pollution bioassay checking only the earlier developmental stages from fertilization to gastrulation should be assessed against the background of the more strict results that will be obtained by checking the earlier through later stages, at least from the pre-fertilization to pluteus formation.

In order to accept successfully the improved procedure mentioned above, a new ranking of the sea water pollution (Ranking III) is proposed as seen in Table 6. The Ranking III is more exact than the Ranking I; the former is available to see the effects of pollution more precisely especially in the cases of organic pollution.

Acknowledgments

The author wishes to express his hearty thanks to Professor Eiji Harada and the staff of the Seto Marine Biological Laboratory for facilities given to him in carrying out the research at the laboratory, particularly to Professor Emeritus Takasi Tokioka of the laboratory for advice, criticism and also for his kindness in reading the manuscript.

References

Castagna, A., F. Sinatra, M. Scalia & V. Capodicasa. 1981. Observations of the effect of Zinc on the gametes and various developmental phases of *Arbacia lixula*. Mar. Bio., 64: 285–289.

Crawford, R.B., & J.D. Gates. 1981. Effects of a drilling fluid on the development of a teleost and an echinoderm. Bull. Environm. Contam. Toxicol. 26: 207–212.

Dinnel, P.A., Q.J. Stober & D.H. Dijulio. 1981. Sea urchin sperm bioassay for sewage and chlorinated sea water and its relation to fish bioassays. Mar. Environ. Res. 5: 29–39.

Hagstrom, B.E., & S. Lönning. 1973. The sea urchin eggs as a testing object in toxicology. Acta Pharmacol. Toxicol. 32, Suppl., 1: 1–49.

———, & ———. 1977. The effects of Esso Corexit 9527 on the fertilizing capacity of spermatozoa. Mar. Poll. Bull., 8: 136–138.

Kobayashi, N. 1971. Fertilized sea urchin eggs as an indicatory materials for marine pollution bioassay, preliminary experiment. Publ. Seto Mar. Biol. Lab., 18: 379–406.

———, H. Nogami, & K. Doi. 1972. Marine pollution bioassay by using sea urchin eggs in the Inland Sea of Japan (Seto-Naikai). Publ. Seto Mar. Biol. Lab., 19: 359–381.

———. 1974. Marine pollution bioassay by sea urchin eggs, an attempt to enhance accuracy. Publ. Seto Mar. Biol. Lab., 21: 377–391.

———. 1977. Preliminary experiments with sea urchin pluteus and metamorphosis in marine pollution bioassay. Publ. Seto Mar. Biol. Lab., 24: 9–21.

———. 1980. Comparative sensitivity of various developmental stages of sea urchins to some chemicals. Mar. Biol., 58: 163–171.

———. 1984. Marine ecotoxicological testing with echinoderms. *In*: Ecotoxicological Testing for the Marine Environment, vol. 1, pp. 341–405. G. Persoone, E. Jaspers & C. Claus, Eds. State Univ. Ghent and Inst. Mar. Sci. Res., Bredene.

Pagano, G., A. Esposito, G.G. Giordano & B.E. Hagström. 1978. Embryotoxic and teratogenic effects of stylene derivatives on sea urchin development. Scand. J. Work Environm. & Health 4, Suppl., 2: 136–141.

———, ——— & ———. 1982. Fertilization and larval development in sea urchins following exposure of gametes and embryos to cadmium. Arch. Environm. Contam. Toxicol., 11: 47–55.

Renzoni, A. 1974. Influence of toxicants on marine invertebrate larvae. Thalassia Jugosl., 10: 197–211.

Stober, Q.J., P.A. Dinnel & S.C. Crumley. 1979. Development of the echinoderm sperm bioassay for testing toxic substances. Fish. Res. Inst., Univ. Wash.,: 7922.

Wilson, D.P., & F.A.J. Armstrong. 1961. Biological differences between sea waters: Experiments in 1960. J. Mar. Biol. Assoc. U.K., 41: 663–681.

———. 1981. An experimental research for phytoplanktonic algae producing external metabolites which condition natural sea waters. J. Mar. Biol. Assoc. U.K., 61: 585–607.