FRI-UW-8207
June 1982

SEA URCHIN SPERM BIOASSAY OF COPPER ASSOCIATED WITH NATURAL SEAWATER ORGANIC CONCENTRATES

By

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This work was supported by
The College of Ocean and Fishery Sciences,
University of Washington
and was conducted in conjunction with
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Submitted:

June 11, 1982

Approved:

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TABLE OF CONTENTS

LIST OF TABLES

1.0 INTRODUCTION

Literature on toxicity of metals to animals clearly shows that the degree of metal toxicity depends on many factors including 1) animal species and life stage, 2) mode and duration of exposure, 3) physical factors (i.e., temperature, salinity or alkalinity, pH, etc.), and 4) chemical form of the metal and its physical/chemical interaction with other environmental components (i.e., particulates, dissolved organics and inorganics, etc.).

One factor which has received little attention in aquatic bioassay studies is the interaction of metals and naturally occurring organic substances and the degree which organics might combine with metals to mitigate either acute or chronic toxicity. A study by Lewis et al. (1972) showed that particulate (clays and diatoms) and soluble substances (ascorbic acid, unchlorinated primary sewage, and extracts of humic acid and soils) reduced the toxicity of copper to the calanoid copepod Euchaeta japonica. Recent works by Crecelius et al. (1982) and Zamuda and Sunda (1982) have also shown that sediment or nitrilotriacetic acid (NTA)-complexed copper is less bioavailable to marine invertebrates for bioaccumulation than ionic copper.

Sperm cells and egg fertilization responses (especially of sea urchins) have long been favorite tools of developmental biologists and cytochemists. The effects of copper on sperm activation, viability, longevity, motility, and egg fertilizing capability have been investigated by various workers. Lillie (1921) and Heslinga (1976) found that immediate egg fertilization by Arbacia and Echinometra sperm was reduced in as little as 190 and 180 µg/l copper, respectively. Young and Nelson (1974) noted reduced sperm

motility for Arbacia in 159 µg/l copper. Most recently Stober et al. (in prep.) found that copper was toxic to sea urchin sperm in 60-min exposures at concentrations of 59, 25, 23, and 2 µg/l (EC50's) depending on species tested.

Work by Tyler (1953) has shown that metal chealating agents such as Ethylenediaminetetraacetic acid (EDTA), Diethyldithiocarbamate (DEDTC) and 8-Hydroxyquinoline (Oxine) are effective in maintaining sperm viability/motility in otherwise toxic concentrations of metals. This information gradually led to speculation by Barron et al. (1948) as to the mode of metal interaction with sperm cells. It has long been known that levels of various metals commonly present in sea water aid in activation of sperm when shed into sea water, but that excess metal concentrations kill or inactivate sperm. Barron hypothesized that sperm cells contain two types of sulfhydryl (-SH) groups: "soluble" and "fixed," and that low levels of metals aid activation by combining with the soluble -SH groups thereby possibly inactivating a negative feedback inhibiting sperm respiration. "Excess" metal ions, however, would combine with the fixed -SH groups which are important components of cellular enzyme systems, thereby interfering with overall cellular metabolism.

The sensitivity of sperm cells to "free" or "ionic" metals in conjunction with the rapidity and high degree of experimental design variability (i.e., number of replicates, concentrations, etc.) possible with sperm assays should provide a quick and sensitive tool for determining the relative toxicities of natural organics-bound metals in sea water. The experiments detailed in this report were conducted to assess the

potential of using a sperm bioassay procedure (Dinnel et al. 1982, Stober et al. in prep.) to define gradations of toxicity of copper associated with concentrates of naturally occurring marine organic substances in sea water.

2.0 MATERIALS AND METHODS

Green sea urchins (Strongylocentrotus droebachiensis) were collected in January 1982 from Tongue Point on the Strait of Juan de Fuca and maintained in flowing sea water (temperature 9.0 ± 1°C, salinity 28 ± 1 °/oo, pH 8.0 ± 0.2) at the West Point laboratory on Puget Sound.

Three test samples contained organics of three different molecular weight ranges (concentrated from natural sea water by cellulose acetate polymer membranes) in an essentially freshwater matrix. Two other samples were artificial sea water, one of which was the supernatent from a bacterial suspension. Each sample was measured for specific gravity, salinity (direct conversion from the specific gravity measurements), pH, and five metals (Table 1). Activated carbon-filtered natural sea water (salinity = 27-29 °/oo, pH = 7.8-8.0) served as control water.

Sperm bioassays were conducted by spawning a male and female urchin separately by injection of 1 ml 0.5 M potassium chloride (KCl) through the peristomal membrane into the coelomic cavity. The sperm were mixed in about 100 ml of natural sea water (30 °/oo salinity) after 30 min and subsampled for counts in a hemacytometer. The sperm were diluted to a density of 4×10^7 sperm/ml 60 min after initiation of spawning. Sperm (0.1 ml = 4×10^6) were added to each test tube containing 0.025 - 10 ml

Table 1. Selected physical-chemical parameters in the five test samples.

Parameter	Sample[1] A	B	C	D	E
60°F specific gravity	1.006	1.004	1.001	1.023	1.020
"Salinity" ($^{o}/_{oo}$)[2]	8.9	6.3	2.4	31.1	27.2
pH	7.4	7.1	6.3	8.0	7.3
Copper (µg/l)	2,760	2,300	320	<10	<10
Silver (µg/l)	6	<4	9	<4	<4
Zinc (µg/l)	43	33	19	10	13
Lead (µg/l)	<100	<100	<100	<100	<100
Cadmium (µg/l)	<200	<200	<200	<200	<200
Total organic carbon (mg/l)	33	62	19	N.M.	N.M.[3]

[1] Sample A = >300,000 molecular weight (XM 300 retentate)
B = 10,000 - 20,000 molecular weight (UM 10 retentate)
C = 1,000 - 5,000 molecular weight (UM 2 retentate)
D = artificial sea water
E = artificial sea water (bacterial suspension)

[2] Salinity equivalent to direct conversion of specific gravity readings.

[3] N.M. = Not measured.

of test sample (diluted as necessary to 10 ml with control water) or control water 90 min after initiation of spawning and incubated at 12°C for 60 min. Minor reductions in test salinities (i.e., $\leq$10%) due to dilution by the test samples should not cause a significant change in the test results. At the end of the 60-min sperm exposure period 1 ml of washed eggs (2000 eggs/ml) was added to each tube and 20 minutes were allowed for fertilization before fixing with 1 ml of concentrated formaldehyde solution. The final sperm-to-egg ratio in each tube was 2000:1. Each sample was later assessed for percentage unfertilized eggs by noting the presence or absence of the obvious fertilization membranes in a subsample of 100-200 eggs.

3.0 RESULTS AND CONCLUSIONS

A range-finding set of experiments conducted 3 March 1982 tested 0.25-10% volumes of the organic concentrates and 25-100% of the artificial seawater samples in natural sea water. The results of these tests are presented in Tables 2 and 3, respectively. Egg fertilization in the two artificial seawater samples (with and without bacteria) was essentially the same as the natural seawater controls at all dilutions (Table 3). Hence, there was no significant toxic component in either of these samples as indicated by sperm bioassay.

Organic concentrate samples A and B showed toxic responses at concentrations in the 2.5-10% (V/V) range. Sample C was non-toxic at 10% (Table 2). Samples A and B additionally showed a stimulatory response (i.e., fertilization success greater than controls) at concentrations of 0.5-1.0% and sample C showed stimulation at 2.5-10%.

The purpose of these experiments was to determine the effect of

Table 2. Percentage of unfertilized eggs from 60-min green sea urchin sperm bioassays of dilutions of natural seawater organic concentrates and equivalent nominal copper concentrations associated with each sample (see Table 1). These tests were conducted on 3 March 1982.

Percent of sample in natural sea water	Sample A[1]		Sample B[2]		Sample C[3]	
	% eggs unfertilized	[Cu] (µg/l)	% eggs unfertilized	[Cu] (µg/l)	% eggs unfertilized	[Cu] (µg/l)
10.0	100	276	100	230	14*	32
7.5	100	207	100	172	17*	24
5.0	98	138	99	115	15*	16
2.5	37	69	54	58	19*	8
1.0	10*	28	15*	23	58	3
0.5	19*	14	19*	12	52	2
0.25	44	7	39	6	59	1

Controls (5) percent unfertilized = 37, 52, 37, 56, 54.

Control mean and 95% confidence limits = 47.2 ±26.1 (21.6 - 73.3).

[1] Sample A = >300,000 molecular weight

[2] Sample B = 10,000 - 20,000 molecular weight

[3] Sample C = 1,000 - 5,000 molecular weight

* Significant (p = 0.05) stimulatory effect.

Table 3. Percentage of unfertilized eggs from 60-min green sea urchin sperm bioassays of dilutions of artificial sea water and artificial sea water with bacterial suspension.

Percent of sample in natural sea water	Artificial sea water*	Artificial sea water (with bacteria)*
100 (no dilution)	51	47
75	58	37
50	57	40
25	56	54

* None of these values indicate a significant stimulatory or toxic effect ($p = 0.05$). See Table 2 for control data and statistics.

concentrated natural seawater organics on the toxicity of copper to sperm. Our intention was to add known amounts of copper to each concentrate to determine this information. However, as summarized in Table 1, significant amounts of copper were already present in each of the organic concentrates. The concentrations of silver, zinc, lead, and cadmium were determined to be insignificant relative to sperm viability (especially when diluted by 90% or more). Therefore, based on the amounts of copper already present in these samples, we simply conducted the bioassays using dilutions of the concentrates without added copper and expressed the results as the nominal concentration of copper in each dilution as calculated from the value measured for each whole concentrate. Based on these calculations, 50% effective concentrations (EC50's) were roughly in the 2.5-5.0% dilution range for samples A and B. This range corresponds to copper concentrations of approximately 69-138 and 58-115 µg/l, respectively. Sample C was non-toxic at a calculated copper concentration of 32 µg/l (Table 2). Stimulatory responses for each sample were noted at equivalent copper concentrations of 14-28, 12-23, and 8-32 µg/l for samples A, B, and C, respectively.

A second set of experiments was conducted on 22 April 1982 to establish firm EC50's for samples A and B. The results of these tests are shown in Table 4. The EC50's established by computer probit analysis (Finney 1971) with an attached FORTRAN program for 95% fiducial limits (Litchfield and Wilcoxon 1949) were 83.6 (80.9-86.4) and 87.5 (84.8-90.3) µg/l copper for samples A and B, respectively (Table 5).

Previous green sea urchin sperm bioassays of copper in activated carbon-filtered natural sea water produced EC50's between 27.5 and 58.7 µg/l

Table 4. Sixty-min green sea urchin sperm bioassay of samples A and B with narrowed concentration ranges to determine 50% effective concentrations (EC50) based on nominal copper concentrations in each sample (see Tables 1 and 2). These tests were conducted on 22 April 1982.

Percent of sample in natural sea water	Sample A[1]		Sample B[2]	
	% eggs unfertilized	[Cu] (µg/l)	% eggs unfertilized	[Cu] (µg/l)
5.5	98	151.8	—	—
5.0	96	138.0	79	115.0
4.75	97	131.0	75	109.2
4.5	94	124.2	98[3]	103.5
4.25	87	117.3	66	97.8
4.0	76	110.4	60	92.0
3.75	84	103.5	57	86.2
3.5	70	96.6	35	80.5
3.25	50	89.7	25	74.8
3.0	46	82.8	18	69.0
2.5	23	69.0	11	57.5
2.0	13	55.2	4	46.0

Controls (6) 0, 0, 2, 4, 1, 0 $\bar{x}$ = 1.2% unfertilized.

[1] Sample A = >300,000 molecular weight (XM 300 retentate)

[2] Sample B = 10,000 - 20,000 molecular weight (UM 10 retentate)

[3] This value not included in calculation of the EC50.

Table 5. Estimated and calculated EC50's and 95% fiducial limits for samples A, B, and C based on nominal concentrations of copper.

Test date	Sample	EC50 (µg/l copper)	95% fiducial limits (µg/l copper)
3 March 1982	A	69-138	—
	B	58-115	—
	C	>32	(non-toxic at 10%)
22 April 1982	A	83.6	80.9-86.4
	B	87.5	84.8-90.3

with fiducial limits ranging from 12.1 to 81.7 µg/l (Table 6) (Stober et al. in prep.)

Several tentative conclusions may be drawn from these experiments:

1) The artificial sea water (with or without bacteria) was non-toxic to sperm.

2) Organic concentrate samples A and B were toxic to sea urchin sperm. This toxicity was probably due, for the most part, to copper. Copper was equally toxic in these two samples as shown by essentially equal EC50's. Sample C was non-toxic to sperm at concentrations as great as 10% (32 µg/l copper).

3) Each organic concentrate sample showed a stimulation response (i.e., fertilization greater than the controls) at copper concentrations ranging from 8-32 µg/l.

4) Comparison of these test data with past green sea urchin sperm bioassays of copper in natural sea water suggests that copper associated with concentrated natural organics from sea water is slightly less toxic than copper in unenriched sea water. Confirmation of this must await additional carefully designed and carefully controlled experiments. Some recommendations for future experiments include:

a) Conduct side-by-side sperm assays of copper in natural sea water and sea water containing natural concentrated organics.

b) Conduct sperm assays with metals/organics in low-metal, low-organic artificial sea water.

c) Conduct sperm assays of copper in sea water enriched with appropriate "off-the-shelf" organics.

Table 6. Results of green sea urchin sperm toxicity tests of copper conducted in natural sea water (activated carbon-filtered) during 1981-82. Test conditions: 2000 sperm/egg ratio, sperm exposure time = 60 min, 27 °/oo salinity, temperature 12°C, pH 7.8-8.2.

Test date	EC50 (µg/l) copper)	95% fiducial limits
6 May 1981	50.9	49.1-52.8
8 May 1981	27.5	12.1-81.7
4 February 1982	>32.9	—
5 February 1982	58.7	51.1-67.8

d) Conduct assays of copper-organic combinations using sperm of a variety of other animals (i.e. oysters, mussels, salmon, other species of urchins).

e) Conduct additional bioassays of copper-organics using other life stages (i.e., embryos, larvae, etc.).

f) Determine toxicity of additional metals associated with a variety of organics.

4.0 ACKNOWLEDGEMENTS

This work was supported by The School of Ocean and Fishery Sciences, University of Washington. The Municipality of Metropolitan Seattle (METRO) donated space and the seawater intake system for the West Point laboratory.

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