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Developmental Toxicity of Copper, Chromium, and Aluminum Using the Shrimp Embryo Teratogenesis Assay: *Palaemonid* with Artificial Seawater

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Palaemonetes pugio, or daggerblade grass shrimp, has been the focus of several recent investigations because it is an excellent candidate for determining developmental toxicants in the estuarine environment. Grass shrimp embryos, which are incubated externally on the female, have been exposed in the laboratory to a variety of environmental contaminants. Fisher and Foss (1993) describe a relatively sensitive 12-d toxicity test using the grass shrimp embryos in natural filtered seawater. Rayburn et al. (1996) developed and compared the sensitivity of a 12-d and 4-d assay using the embryos of the grass shrimp. Rayburn and Fisher (1997) compared the 12-d and 4-d assay using embryos of the grass shrimp with three carrier solvents. These papers showed that the shrimp assay was consistent and repeatable.

Previous researchers have used filtered natural seawater for their assays. However, artificial seawater may be preferable to natural seawater because natural seawater can vary in composition and has the potential to be contaminated. Fisher and Foss (1993) demonstrated, in one experiment, that artificial seawater may be used, however they chose to use filtered natural seawater in most of their assays. In addition to experimental consistency, artificial seawater may be used to maintain adults that produce satisfactorily developing embryos in a constant environment. Therefore, researchers, without immediate access to natural seawater, can perform this estuarine toxicity assay.

Metals in the estuarine environment are a constant problem due to the role of human activity since the industrial age (Goyer, 1996). Demonstrated here is the toxic response of grass shrimp larvae to three metals. Copper was selected because Rayburn and Fisher (1999) provided data on copper toxicity in natural seawater. This provides baseline information for determining if shrimp respond differently in artificial sea salts or those kept in natural filtered seawater. Two metals not previously tested with the shrimp assay, chromium VI and aluminum were also selected. Chromium VI has been shown to cause developmental anomalies in rats and many river systems receive this pollutant (Kanojia et al. 1998; Vijayram and Geraldine 1996). Aluminum is ubiquitous in the environment and, recently, acid rain has been increasing the amount of aluminum in biological systems (Groyer 1996).

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One objective was to demonstrate that grass shrimp could be maintained in the laboratory using artificial sea salts and produce viable embryos for toxicological study. Subsequently, it was important to determine whether their embryos responded similarly to those tested in natural filtered seawater. Specifically, the objective was to assess the toxicity of copper, chromium VI and aluminum using this shrimp assay using grass shrimp kept in artificial seawater.

MATERIALS AND METHODS

Chromium (VI) was obtained (Fisher Scientific Inc Atlanta Georgia) as potassium dichromate in 1000 ug/ml stock solution. copper and aluminum were obtained (Buck Scientific Inc, East Norwalk, CT) in puro graphic stock solutions of 997 ug/ml and 998 ug/ml, respectively. The diluent was 20-ppt artificial seawater (Coralife Scientific Grade Marine Salt, Energy Savers Unlimited, Carson CA). Experimental pH was measured prior to embryo exposure and adjusted to a range between 7.6 to 8.1.

Grass shrimp were collected using dip or seine nets from relatively uncontaminated estuaries near Pensacola Beach, FL and Dauphin Island, AL for this study. They were placed in a cooler with aeration and transported in estuary water. At Jacksonville State University (JSU), the shrimp were acclimated to laboratory temperature (~24°C) and 20-ppt salinity. Each shrimp was identified as *P. pugio* or non-*P. pugio* then placed into separate 10-gallon aquaria with sponge filters in 20-ppt artificial seawater. Only *P. pugio* species collected between October 1998 and August 1999 were used for this study. Aquaria were kept on a 16-hr light:8-hr dark cycle. The aquaria were cleaned once every 2-3 weeks with 20 percent of the water being renewed. Temperature was maintained between 22 $\pm$5 °C. The grass shrimp were fed Tetrafin goldfish flakes daily and supplemented with brine shrimp 1- 2 times a week. *P. pugio* spontaneously breed in laboratory continuously throughout the year. To determine if embryos would grow into adults, while raised in artificial sea salts, a clutch of larvae (approximately 100) were placed into a 5-gallon aquarium that was filtered, aerated and allowed to develop.

Gravid females were selected and observed for embryos in the tissue cap stage of development. Embryos were gently removed with forceps, separated, and placed into 24 well cell-culture plate, one embryo per well. Two mls of 20-ppt seawater or its corresponding test solution was added to each well. Each assay was run for 12-d, from the tissue cap stage (3-days old @ 27°C) to two days post hatch (15 days old @ 27°C). Embryos were incubated in the dark, at 27 °C, and rotated at 60 rpms. Three experiments were performed with one control and at least four increasing concentrations for each metal which gave an increasing response of toxicity for each of the concentrations and were able to be used for calculations of LC50 values. The N for each test solution was 24.

Mortality was observed daily and malformations determined on day 10 of development (day 7 of the assay). The embryo is clearly visible and mortality was assessed by embryo being opaque and degrading from day 0 to 3 of the assay, after day 3 mortality was determined by lack of a heart beat. Rayburn et al. (1996) indicated that day 10 of development was the best time to ascertain embryonic abnormalities. The 12-d LC50 was obtained using the Litchfield-Wilcoxon Probit analysis (Tallarida and Murray 1980).

RESULTS AND DISCUSSION

Sixty adult female and 53 male *P. pugio* organisms were collected from varying locations along the coast of Alabama and Florida, at three different times between 1998 and 1999 (Table 1). The first collection indicates a ratio of 1 to 2.04 of *P. pugio* to non-*pugio* organisms, and a female to male ratio of 1.78 to 1. The second collection indicates a ratio of 1.48 to 1 of *P. pugio* to non-*pugio* with a female to male ratio of 1 to 2.88. The third collection indicates a ratio of 18.33 to 1 of *P. pugio* to non-*pugio* with a female to male ratio of 1.69 to 1. The sites had different ratios of *P. pugio* to non-*pugio* (*P. vulgaris, P. intermedius*) due to location and time of year. This indicates the importance of identifying species and sex of collection to ensure embryos are from the correct species and an acceptable sex ratio because all sites had non-*pugio* present and varying female to male ratios.

All shrimp taken back to Jacksonville State University did well in the artificial seawater mixture and produced embryos during a 6-12 month period. Thus, artificial seawater is sufficient to maintain adults and produce viable embryos. Adult populations steadily decreased over time and required stocking.

Table 1. Total number of adult grass shrimp collected by site/time, sex and species.

Date | Location	Sex	P. pugio (#)	Non-pugio (#)
10/23/98 | Pensacola FL, Big Sabine Point	Male	27	57
	Female	16	31
3/28/99 | Dauphin Island AL, Airport estuary	Male	6	10
	Female	31	15
8/23/99 | Pensacola FL, I-10 Bridge	Male	20	2
	Female	13	0
	Juvenile	77	4

Mostly juveniles were captured from the August sampling (Table 1). These juveniles were young-of-the-year from clutches in springtime. The juveniles in artificial sea salts began producing clutches of embryos approximately 2 to 4 months after acclimating to laboratory conditions.

The average control mortalities for the 12-d shrimp embryo test for Cu, Cr, and Al were 8/72, 6/72, and 6/72 respectively, for a total mortality of 9.7% (21/216). None of the control embryos exhibited developmental delay or anomalies. Results

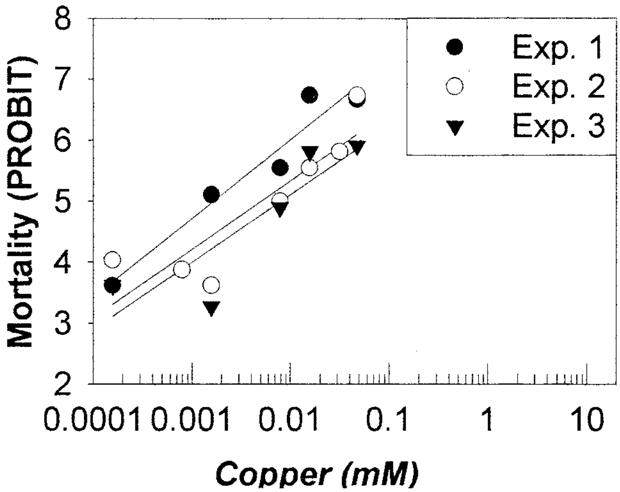

Figure 1. The concentration-response mortality curves generated from results of copper exposures. Graph represents three independent tests using the 12-d assay. Response is given in probit values; a probit of 5 = 50% mortality.

indicate that adults kept in artificial sea salts produced embryos of acceptable quality and quantity for toxicological assessment, i.e. that develop through hatching with an acceptable mortality rate. Embryos grown entirely in artificial sea salts developed into juveniles by 2 months had fully developed and reproduced clutches of their own at four months. The life span and ability to produce viable clutches of embryos of laboratory reared grass shrimp is at least 19 months. Thus, experimental data show that under controlled conditions in the laboratory, *P. pugio* are able to produce viable offspring.

Table 2. Three individual experimental LC50 (m*M*) and 95% CI values for copper.

Exp	LC50 m*M*	95% CI
1	0.008	0.0015 - 0.0437
2	0.002	0.0008 - 0.0034
3	0.005	0.0015 - 0.0166

Copper has a mean 12-d LC50 of 0.005 m*M* (averaged from values in Table 2) with a standard deviation (STD) of 0.003. This gives, for the grass shrimp tests for copper, a coefficient of variation (CV) of 60%. The Probit graph (Fig 1) illustrates that copper yields generally a linear response and has a repeatable concentration response curve. Copper caused malformations similar to those reported in Rayburn and Fisher (1999). Malformations were commonly observed in concentrations above the LC50 (0.005 m*M*). Mortality is within an order of magnitude as compared to reports by Rayburn and Fisher (1999) and Burton et al. (1990) which had an LC50 of between 0.014 and 0.005 m*M*.

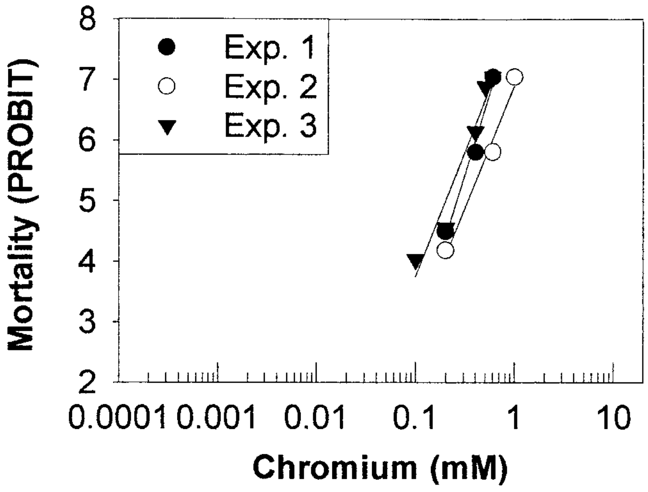

Figure 2. The concentration-response mortality curves generated from results of chromium exposures. Graph represents three independent tests using the 12-d assay. Response is given in probit values; a probit of 5 = 50% mortality.

Marino-Balsa et al. (2000) reported LC50 values for Cu of 0.0519 m*M* for larval stages of *Palaemon serratus*. Hutchinson et al. (1994) showed an LC50 for Cu of >0.0034 m*M* for larva marine fish. This indicates that the grass shrimp embryo assay is relatively sensitive to Cu. This indicates that the grass shrimp kept under these conditions produced embryos that performed comparatively well as compared to other assays.

Table 3. Three individual experimental LC50 (m*M*) and 95% CI values for chromium.

Exp	LC50 m*M*	95% CI
1	0.280	0.240 - 0.330
2	0.330	0.240 - 0.500
3	0.200	0.150 - 0.270

The mean 12-day LC50 for chromium is 0.270 m*M* (averaged from values in Table 3) with a STD of 0.0655. This gives a CV of 24%. The Probit graph (Figure 2) is similar to copper in that a straight line is produced and there is a repeatable concentration response. Overall, in mortality, chromium provides the most repeatable results as compared between the three metals. No severe abnormalities were observed with chromium. Marino-Balsa et al. (2000) reported LC50 values for Cr of 0.231 m*M* for larval stages of *Palaemon serratus*. Lee et al. (2000) reported reduced hatching success with Cr III. Hutchinson et al. (1994) showed an LC50 value of 0.61 m*M* for Cr for larval marine fish. This also shows grass shrimp embryo assay is as or more sensitive than other assays. However,

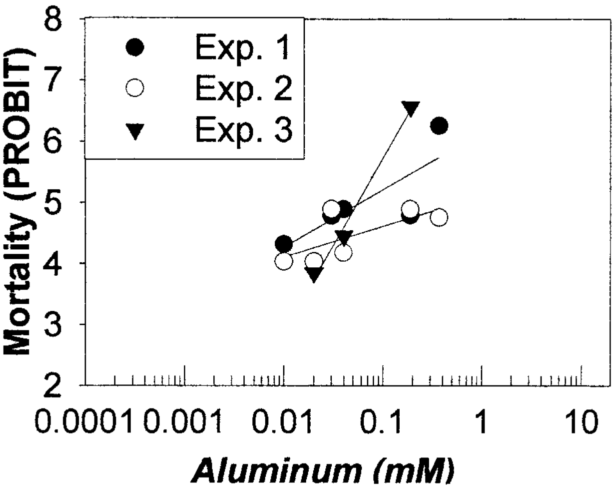

Figure 3. The concentration-response mortality curves generated from results of aluminum exposures. Graph represents three independent tests using the 12-d assay. Response is given in probit values; a probit of 5 = 50% mortality.

according to the articles mentioned above, copper was more toxic than chromium VI which was in agreement with the shrimp embryo assay.

Table 4. Three individual experimental LC50 (m*M*) and 95% CI values for aluminum.

Exp	LC50 m*M*	95% CI
1	0.030	0.005 - 0.240
2	0.550	0.191 - 1.600
3	0.050	0.010 - 0.350

Mean LC50 for aluminum is 0.210 m*M* (averaged from values in Table 4) with a STD of 0.294 for aluminum. This gives a CV of 140%. The Probit graph (Figure 3) illustrates that aluminum generates a positive slope with a repeatable concentration response curve, but it is more variable than the previous metals tested. However, if the high LC50 is removed from the data set then mean LC50 is 0.04 m*M* with a STD of 0.014 and a CV of 35%. Although no severe abnormalities were observed, embryos exposed to higher concentrations were lethargic and failed to respond well to stimuli. Clark and Hall (1985) exposed embryos of the narrow mouthed toad (*Gastrophryne carolinensis*) to aluminum in the laboratory at pH 7.0 for 7 days following fertilization. An LC50 of 0.0019 m*M* was reported, which showed more sensitivity than the grass shrimp. The grass shrimp may have showed less sensitivity due to the slightly higher pH of seawater (~8.0) or possibly the salinity could have decreased sensitivity by decreasing uptake of Al.

These results were similar to those obtained by Rayburn and Fisher (1999), illustrating that artificial seawater is a comparable substitute for natural filtered seawater. Additional support is provided by the laboratory raised shrimp that produced viable offspring. Because natural filtered seawater may vary from area to area in exact composition, the use of artificial seawater maybe a way of increasing consistency of results from different laboratories. This also indicates that laboratories without access to natural filtered seawater can perform these assays easily.

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REFERENCES

Burton DT, Fisher DJ (1990) Acute toxicity of cadmium, copper, zinc, ammonia, 3,3'-dichlorobenzidine, 2,6-dichloro-4-nitroaniline, methylene chloride, and 2, 4, 6-trichlorophenol to juvenile grass shrimp and killifish. Bull Environ Contam Toxicol 44:776-783.

Clark KL, Hall RJ (1985) Effects of elevated hydrogen ion and aluminum concentration on the survival of amphibian embryos and larvae. Canadian J Zool 63:116-123.

Fisher WS, Foss SS (1993) A simple test for toxicity of number 2 fuel oil and oil dispersants to the embryos of grass shrimp, *Palaemonetes pugio.* Mar Pollut Bull 26:385-391

Goyer RA (1996) Toxic effects of metals In: Klaassen CD (ed) *Casarett & Doull's Toxicology the basic science of poisons* McGraw Hill, New York pp 691-736

Hutchinson TH, Williams TD, Eales GJ (1994) Toxicity of cadmium, hexavalent chromium and copper to marine fish larvae (*Cyprinodon vaiegatus*) and copepods (*Tisbebattagliai*). Mar Environ Research 38:275-290

Marino-Balsa JC, Poza E, Vazquez E, Beiras R (2000) Comparative toxicity of dissolved metals to early larval stages of Palaemon serratus, Maja squinado, and Homarus gammarus (Crustacea:Decapoda). Arch Environ Contam Toxicol 39(3):345-351

Kanojia RK, Junaid M, Murthy RC (1998) Embryo and fetotoxicity of hexavalent chromium: a long-term study. Toxicol Lett 95 165-172.

Lee R., Kim GB, Maruya KA, Steinert SA, Oshima Y (2000) DNA strand breaks (comet assay) and embryo development effects in grass shrimp (*Palaemonetes pugio*) embryos after exposure to genotoxicants. Mar Environ Res 50:553-557.

Rayburn JR, Fisher WS (1997) Developmental toxicity of three carrier solvents using embryos of the grass shrimp, *Palaemonetes pugio.* Arch Environ Contam Toxicol 33:217-221.

Rayburn JR, Fisher WS (1999) Developmental Toxicity of Copper Chloride, Methylene Chloride, and 6-aminonicotinamide to embryos of the grass shrimp *Palaemonetes pugio*. Environ Toxicol Chem 18:950-957

Rayburn JR, Glas PS, Foss SS, Fisher WS (1996) Characterization of grass shrimp (*Palaemonetes pugio)* embryo toxicity test using water soluble fraction of number 2 fuel oil. Mar Pollut Bull 32:860-866.

Tallarida RJ, Murray RB (1980) *Manual of pharmacologic calculations with Computer Programs.* Springer-Verlag, New York, NY USA.

Vijayram K, Geraldine P (1996) Regulation of essential heavy metals (Cu, Cr, and Zn)by the freshwater prawn *Macrobrachium malcolmsonii* (Milne Edwards). Bull Environ Contam Toxicol 56:335-342.