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Malformations and Inhibitory Tendencies Induced to *Brachydanio rerio* (Hamilton-Buchanan) Eggs and Larvae due to Exposures in Low Concentrations of Lead and Copper Ions

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Many tests on metallic toxicity measure the effect of one metal or mixtures using toxic units. While these procedures have generally yielded good results, many aspects of pollution problems are left unanswered. Many effluent discharges of industrial wastes contain combinations of metals especially the so-called heavy ones. How these combined toxicants affect the eggs and larvae of aquatic animals can only be a matter of speculation. Furthermore, the use of classical LC_{50} and MATC (maximum acceptable toxicant concentration) methods seem inadequate in toxicological studies when the test animals are highly motile. Bioaccumulation and biomagnification of metals by aquatic organisms pose some problems in setting up and determining what may be tolerable concentrations to these animals. Previously accumulated metals will not only modify new toxic threshold responses if the same metals are encountered again but will offer some degrees of acquired resistance to new thresholds.

To minimize these shortcomings, an orthogonal factorial design experiment was used in this study. It is a well known fact that the effect of one factor and simultaneous combinations of factors can be studied effectively using factorial design (FISHER 1942). The eggs and larvae of fish were used in this study as indicator organisms for testing the single effect of lead and copper ions and their combinations. For the bioassay, Brachydanio rerio eggs were chosen since it was found that they can detect in distilled water copper concentrations of 3-4 ug/L. Besides, the eggs are transparent. The adult fish are highly prolific all the year round if well cared for and, above all, they have been extensively used in laboratory work.

In this paper it will be shown that the presence of lead ions in distilled water induce epithelioma and that copper ions cause spirality of the nervous system. But lead ions antagonised the effect due to copper by suppressing abnormality and inhibitory tendency due to copper.

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MATERIALS AND METHODS

Brachydanio rerio eggs spawned naturally and at nigh blastula stage (HISAOKA AND BATTLE 1958) were used in the bioassay. The choice of stage reduced the chances of egg mortality common at earlier stages and it also afforded better screening from unfertilized eggs. A series of test containers capable of housing 10 L of toxicant solution each and some conical flasks of 250 mL by volume were provided for incubating the eggs. Lead and copper nitrate salts, reagent grade, supplied the metallic ions.

Concentrations of 0, 36, and 72 ppb of lead and copper were singly and in combinations prepared in 10 L of distilled water, making 9 combinations in all. The concentrations of lead and copper were analysed one day after preparation and 5 days after the termination of the experiment with atomic absorption spectrophotometer to check both the accuracy of the prepared concentrations and the effect of adsorption and evaporation. The concentrations were constant. Each conical flask housed 90 eggs and the flask was immersed into 10 L of test container. The experiment was replicated six times. Pre-trial experiment with 18 ppb of lead concentration did not yield any observable effect on the eggs so the concentrations of both lead and copper were raised to 36 and 72 ppb for the sake of orthogonality (SNEDECOR AND COCHRAN 1967). The alkalinity of distilled water used in making the solutions was 1.6 ppm by weight of calcium carbonate, and all solutions were prepared a day before use and were thermostatically heated at 26 $\pm$ 0.5 ^{0}C. Artificial aeration was provided and petri dishes of 5 cm in diameter containing plexiglass slides with centered bores of 1 mm each were used for counting the eggs in an Leitz Ortholux microscope. By dropping water from the pipette, the eggs rolled over allowing their developmental stages to be determined. Daily renewal of solutions in the flasks from the main toxicant containers reduced accumulation of metabolites and the sticking of the eggs to the bottom of the flasks.

The criteria used in assessing toxicological effect of the ions were the malformations and inhibitory tendencies to hatching. The inhibited pro-larval percentages were calculated from the original 90 eggs. The pro-larvae that were inhibited turned whitish with time, and once about 4% of the pro-larvae turned white it was certain that inhibition has started. The pro-larvae in the 0 x 0 solution that still failed to hatch when well over 80% had hatched were regarded as being naturally inhibited. For the mathematical interpretation analysis of variance was first carried out on the

inhibition degrees and the significance of each effect determined. A regression analysis of the type Y in function of X_1 and X_2 was then made (X_1 was concentrations in copper and X_2 was concentrations in lead). Y was inhibition to hatching in percentage. By substitution of the independent variables X_1 and X_2 optimum inhibition was obtained.

RESULTS

Developmental abnormalities

The presence of added copper ions in distilled water elicited remarkable malformation in the nervous system from the rhombencephalic region to the anterior margin of the dorsal fin. The region appeared helical. This helical formation (Figure 3) was specific in both the nature and shape it took. It was not determined whether this spirality occurred primarily as the effect of copper ions on the nervous system or due to secondary action by interferance with the notochord. The presence of lead ions in distilled water resulted to poor resorption of yolk in the fry (fig. 5, fig. 6), erosion of tail and fin (fig. 6),spinal curvatures (figs. 5 and 6), and out-growths from the fry which appeared to be epitheliomas (fig. 4).
In copper x lead ions mixtures, the abnormality induced by copper was suppressed by lead ions but that induced by lead (epithelioma) was not suppressed. Similarly, lead ions antagonised the inhibitory power of copper ions to hatching of Zebra fish. Higher percentage hatchings were obtained in copper/lead ions mixtures than at any copper concentration. Figure 1 showed normal egg at high blastula stage (HISAOKA AND BATTLE 1958) and figure 2 showed normal larva.

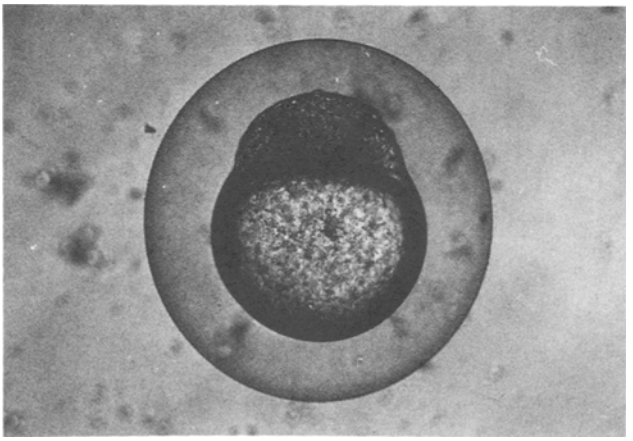

Fig. 1. Normal Egg at High Blastula stage (HISAOKA AND BATTLE 1958). x 40.

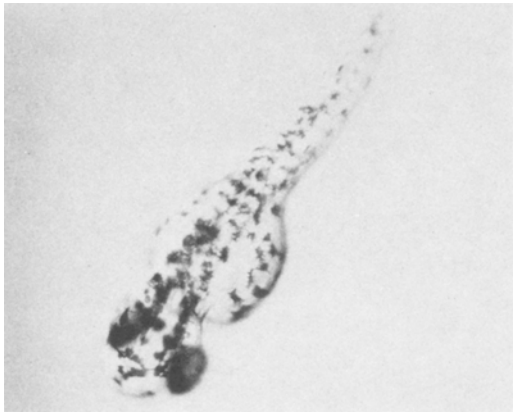

Fig. 2.Normal Larva. X 40

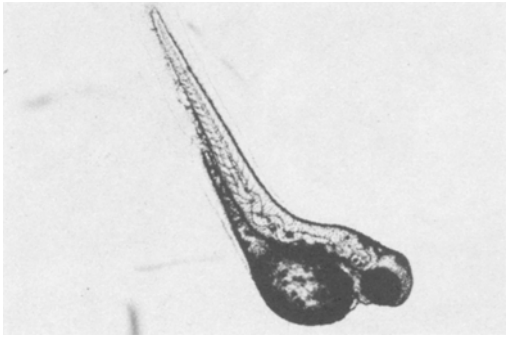

Fig. 3. Copper -Induced Spirality of the Nervous System. X 25.

Fig. 4. Epithelioma due to Lead ions. X 40.

Fig.5. Poor Resorption of Yolk due to Lead ions. X 40.

Fig. 6. Poor Resorption of Yolk and Erosion of Tail and Fin. X 40.

Hatching

Both lead and copper ions affected successful hatching which was assessed by the ability of the prolarvae to break out of the egg shells partially or fully and swim about. All the hatchings that occurred atthe time of changing the solutions were regarded as successful. Hatching occurred at the intervals of 3-5 days during the incubation period. In the 0 x 0 combination over 80% mean hatch occurred for the 6 replicates. But copper ions of 36 ppb reduced hatching to the mean of 2.4%, while copper concentration of 72 ppb gave 2.9%. Acting alone, lead concentration of 36 ppb gave the mean of 19.8%, and 27% for the concentration of 72 ppb.

A mixture of lead concentration 36 ppb x copper

concentration of 36 ppb gave mean hatch of 2.6%, lead concentration of 72 ppb x copper concentration of 72 ppb gave 14.1%. Copper 36 ppb x lead concentration of 72 ppb yielded 30.4%. But 36 ppb of lead x 72 ppb of copper gave only 11.1%. High concentration of lead ions in lead/copper mixtures antagonised copper, thereby more hatchings occurred as the toxicity of copper was checked. The presence of copper ions has both bacteriostatic and fungistic effects on the eggs as no fungal growth occurred on the eggs.

Inhibition of hatching

Lead and copper ions inhibited hatching success in Brachydanio rerio.

TABLE 1

Effect of addition of heavy metal ions at various concentrations on Brachydanio rerio inhibition percent (3 x 3 factorial design with 6 replicates).

	$Pb^{2+}(X_2)$		
	0 ppb	36 ppb	72 ppb
0 ppb	12.22 20.00 14.44 12.22 8.89 13.33	82.22 76.67 52.22 78.89 61.11 37.78	44.44 67.78 72.22 56.67 53.33 55.55
$Cu^{2+}(X_1)$ 36 ppb	90.00 86.67 72.22 74.44 87.78 71.11	67.78 75.56 44.44 75.56 37.78 68.89	32.22 36.67 35.56 74.44 52.22 22.22
72 ppb	66.67 48.89 57.78 31.11 64.44 50.00	13.33 20.00 62.22 66.67 74.44 34.44	47.78 41.11 42.22 51.11 46.67 34.44

In the 0 x 0 combination hatching failed to occur by 13.52% while the highest tested copper concentration (bottom left) gave 53.15% and highest lead concentration (top right) yielded 58.33% (Table 1). On purely additive basis highest tested copper + the highest tested lead concentration gave 53.15% + 58.33% =111.48%. In fact highest combinations of copper and lead ions inhibition was 43.89%. Thus a marked antagonism is

evident.

Analysis of variance showed that an increase of lead ions from 0 to 72 ppb has no significant linear or quadratic effect on inhibition to hatching of Brachydanio rerio (Table 2). An increase of the concentration in copper ions from 0 to 72 ppb has no significant linear effect but has a definite significant quadratic effect on inhibition to hatching.

TABLE 2

Complete analysis of table 1 (inhibition percent, L = linear, Q = quadratic).

MAIN EFFECTS	Df	Sum of Squares	F ratio
Cuppric (Cu^{2+})			
Cu L	1	30.858	0.14
Cu Q	1	2678.144	12.12^{x}
Lead (Pb^{2+})			
Pb L	1	6.717	0.03
Pb Q	1	896.314	4.06
Two factor interactions			
Cu L x Pb L	1	4386.429	19.85^{x}
Cu L x Pb Q	1	2076.901	9.40^{x}
Cu Q x Pb L	1	6255.584	28.30^{x}
Cu Q x Pb Q	1	410.357	1.85
Remainder (Error)	45	9946.466	221.03

x = Highly significant at p 0.01.
Df = degrees of freedom.

A linear increase of copper concentration at any lead level has a quadratic effect on inhibition. All the interactions Cu L x Pb L, Cu L x Pb Q, and Cu Q x Pb L (minus Cu Q x Pb Q which is usually not important in analysis) are highly significant at p 0.01. The equation representing the effect is $56.73 + 0.93X_1 - 0.43X_2 - 4.98X_1^2 - 2.88X_2^2 - 13.52X_1X_2 + 9.32X_1^2X_2 + 5.37X_1X_2^2 - 1.38X_1^2X_2^2$. A large proportion of the total sum of squares, after the fitting of the mean, is ascribable to the regression equation. This confirms that an adequate fit has been made (DAVIES 1967).

It was not intended to optimize the inhibitory condition since the inhibited eggs have little chances of survival, but rather to see how combined metallic toxicants affected the eggs and fragile fry. Copper ions have both bacteriostatic and fungistic properties, egg mortality was low but copper concentration of 72 ppb affected some larvae that hatched out. They turned colourless after a day in the solution. Higher percent inhibition occurred in both copper and lead ion concentrations of 36 ppb than at either higher copper or lead concentrations. The prolarvae in these concentrations of 36 ppb of copper and 36 ppb of lead ions were observed to be less active in movement in their egg shells than those in higher concentrations. This might partly explain why more hatchings occurred in higher lead and copper ions

as hatching ability depends on many factors like hatching enzymes, temperatures, hatching media and, above all, the mechanical actions of the pro-larvae.

DISCUSSION

The ever increasing discharge of heavy metals into aquatic environments makes the use of factorial design, an important tool for multivariable ecosystem research. The simultaneous interactions of the metals with aquatic biota may not give accurate ideas on the effect of combined metallic toxicants if we use the conventional methods of assessments. However, further suggestions on the methods for predicting toxicity of mixtures of two or more pollutants on the basis of chemical measurements are made by SPRAGUE (1970), in a review paper. As of now, the literature contains very little information about the effect of mixtures of chemicals on aquatic biota, especially the eggs and fragile larvae. In the present study lead and copper ions were specific in inducing malformations to the pro-larvae and larvae of Zebra fish. Lead ions induced epithelioma, poor resorption of yolk and tail erosions, while copper induced spirality of the nervous system. Lead ions antagonised inhibitory tendency due to copper and suppressed abnormality due to copper ions.

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