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Toxicity of Zinc, Cadmium and Copper to the Shrimp *Callianassa australiensis*. II. Effects of Paired and Triad Combinations of Metals*

D. S. Negilski**, M. Ahsanullah*** and M. C. Mobley

Marine Science Laboratories, Ministry for Conservation; Queenscliff, Victoria 3225, Australia

Abstract

The acute toxicity of zinc, cadmium and copper to *Callianassa australiensis* (Dana) was assessed in paired and triad combinations in 14 d toxicity tests. Predicted mortalities following exposure to various mixtures of metals were evaluated according to two models proposed by Plackett and Hewlett (1967) for the non-interaction of toxicants: (a) independent dissimilar action and (b) simple similar action. However, based on the rejection of these two models and analyses of the experimental results for each metal alone, it was concluded that the metals in all paired mixtures acted in an interactive manner. For the triad mixture, mortalities of shrimp were predictable by the independent dissimilar action model. Toxicities of mixtures of metals were also assessed using the toxic-unit concept. However, mortalities in all mixtures were overestimated by this method. Some problems concerning the prediction of mortalities in mixtures of toxicants are discussed.

Introduction

Although marine animals may be exposed in the field to many different combinations of toxicants and environmental conditions, most acute toxicity experiments have considered only the individual effects of these variables. Relatively little is known about the combined effects of mixtures or of those combinations which may be dangerous.

In addition, most information on the effects of mixtures of toxicants on aquatic organisms has been based on the toxic-unit concept (e.g. Herbert and Van Dyke, 1964; Sprague and Ramsay, 1965; Brown, 1968; Brown and Dalton, 1970; Calamari and Marchetti, 1973; Eaton, 1973; NAS/NAE, 1973; Nimmo and Bahner, 1976). This assumes that all poisons contribute in a similar way to the overall toxicity of a mixture, although, *a priori*, it is illogical to expect poisons of different toxicological properties and having different concentration-response curves to contribute in this manner (Brown, 1968).

However, there are several other schemes that have been proposed for the classification and description of the effects of mixtures of toxicants. Bliss (1939) suggested that mixtures may act according to: (a) independent joint action (where each toxicant acts on a different site); (b) similar joint action (where each toxicant acts on the same site); or (c) synergistic action. Gaddum (1948) classified the action of toxicants according to effects which were more-than-additive, additive, less-than-additive or antagonistic. Plackett and Hewlett (1967) developed the classification of non-interactive (independent dissimilar or simple similar) and interactive (complex similar or dependent dissimilar) models.

The objective of this study was to assess the acutely lethal effects of zinc, cadmium and copper in paired and triad mixtures on the initial assumption that they acted according to the model for independent dissimilar action (Plackett and Hewlett, 1967; Finney, 1971). Where this assumption was not satisfied, simple similar, dependent dissimilar and complex similar models were considered.

Materials and Methods

Procedures for field collections, acclimation of *Callianassa australiensis* (Dana), preparation of metal stock solutions, experimental procedures and general data analyses are given in the preceding paper (Ahsanullah *et al.*, 1981a).

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** Present address: Lilly Research Laboratories, P. O. Box 708, Greenfield, Indiana 46140, USA

*** Correspondence and reprint requests should be addressed to M. A. at his present address: Australian Atomic Energy Commission, Research Establishment, Private Mail Bag, Sutherland, New South Wales 2232, Australia

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Test solutions for pairs of metals were prepared in 10 litre Perspex tanks by diluting the appropriate quantity of metal stock solution with sea water to 8 litres, adding the proper quantity of the second metal stock solution, and further diluting the mixture to 10 litres. When a mixture of three metals was tested, the appropriate dilutions were to 5, 8 and 10 litres. The test solutions were then slowly transferred to each experimental tank containing the test shrimp. In each experiment, one control and several concentrations were used (Table 1). The test concentrations in each tank were measured daily, immediately before renewal of test solutions.

From comparisons of the 14 d dose-mortality curves for each metal tested singly, experiments with paired and triad combinations were designed according to Plackett and Hewlett (1967) and Finney (1971) to test the independent dissimilar action model. The expected mortalities for the mixture were calculated according to the formula:

$$P = 1-(1-P_1).(1-P_2),$$

or

$$P = 1-(1-P_1).(1-P_2).(1-P_3),$$

where

P = percentage of individuals predicted to respond to the mixture of metals; P_1, P_2, P_3 = percentage of individuals responding to each metal, based on data from the individual dose-response curves and measured chemical concentrations from mixture test solutions.

Chi-squared values were calculated between expected and observed mortalities according to Finney (1971), to test for significant differences. The tolerances of individuals were considered to be uncorrelated (Finney, 1971).

The expected mortalities in the simple similar action model were found from the individual dose-response curves, by combining the mixture concentrations into the equivalent of a dose of one toxicant, i.e.,

$$z = z_1 + \rho z_2,$$

where z_1 and z_2 are doses of the two toxicants and ρ is the corresponding relative potency ratio (Finney, 1971).

In all mixtures, if mortalities were observed in the controls, then results were corrected according to Abbott's formula (Finney, 1971), although the mortalities were never greater than 10%.

Results

Zinc-Cadmium Mixtures

Observed mortalities of *Callianassa australiensis* exposed for 14 d to mixtures of zinc and cadmium were significantly different from expected mortalities based on independent dissimilar action (Table 1). The observed values were lower than those expected from this model, except at the three lowest concentrations, and were similar to those expected for Zn alone. At concentrations of 1.22 Zn + 0.51 Cd and 1.18 Zn + 0.53 Cd (mg l^{-1}), the observed values were also similar to those expected for Cd alone. At the lowest mixture concentration the observed mortality was much higher than was expected for either metal acting by itself. In general, it appears that observed mortalities for the mixture were similar to those for zinc alone and much higher than those expected for cadmium.

Toxic units for the mixture ranged from 1.42 to 2.11, even though observed mortalities in 7 of the 9 concentrations were 50% or less. This overestimate of toxicity by the toxic unit concept is in agreement with the estimates given by the simple similar model (Table 1).

Zinc-Copper Mixtures

Chi-squared values (Table 1) revealed a significant difference between expected and observed mortalities based on the dissimilar independent hypothesis. Observed mortalities for each mixture were always less than expected. At the two highest test concentrations (Table 1), mortalities observed in the mixture were similar to those expected for either metal alone. As concentrations decreased, observed mortalities were more similar to those for copper alone, except at a concentration of 0.11 mg Cu l^{-1}, where they were twice as high as would have occurred for copper alone.

The strength of each mixture was also calculated in terms of toxic units. Values ranged between 1.0 and 2.4 and appeared to overestimate the toxicity of mixtures.

Cadmium-Copper Mixtures

Observed mortalities were significantly different from mortalities expected according to independent dissimilar action (Table 1), being higher than expected for 7 out of 9 concentrations tested.The concentration ratio between cadmium and copper increased markedly as mixture concentrations decreased, and this may have caused a change in the toxic interaction between the metals.

Toxic units ranged between 0.76 and 1.73 and appeared to overestimate the toxicities of mixtures at concentrations lower than 0.37 mg Cd l^{-1} + 0.078 mg Cu l^{-1} (Table 1). At concentrations higher than these, toxic units ranged between 1.03 and 1.73; we are not aware of a way to evaluate expected mortalities in mixtures with values greater than 1.0 toxic unit, for comparison with observed mortalities.

Zinc-Cadmium-Copper Mixtures

Observed and predicted mortalities based on independent dissimilar action were not significantly different

Table 1. *Callianassa australiensis.* Concentrations of paired and triad mixtures of Zn, Cd and Cu with expected percent mortalities for each metal alone, expected percent mortalities for mixtures according to both independent dissimilar action and simple similar action, and observed mortalities and toxic units for each mixture concentration. These tests lasted 14 d. Dose-mortality curves for Zn and Cu, and Cd and Cu are not parallel, so simple similar action model not considered

Mixture concentrations (mg l^{-1})			% mortality expected for each metal alone			% mortality expected by independent dissimilar action	Observed mortality (%)	% mortality expected by simple similar action	Combined toxic units
Zn +	**Cd**		**Zn**	**Cd**					
1.22	0.51		63.4	58.3		85.1	60	100	2.10
1.18	0.53		56.5	64.4		84.5	70	100	2.11
1.14	0.40		48.1	20.8		58.9	50	99.8	1.81
1.15	0.39		50.2	17.9		59.1	50	88.8	1.80
1.08	0.35		35.2	9.3		41.2	40	99.4	1.65
1.04	0.36		27.1	10.5		34.8	30	99.3	1.64
1.07	0.33		33.1	5.3		36.7	40	99.0	1.60
0.96	0.34		13.6	6.8		19.5	20	98.2	1.53
0.93	0.30		9.8	2.2		11.8	30	96.0	1.42
						$\chi^2 = 8.2$*		$\chi^2 = 4\,578.5$***	
Zn +	**Cu**		**Zn**	**Cu**					
1.25	0.250		69.7	59.3		87.7	66		2.40
1.23	0.260		65.2	60.5		86.3	60		2.44
1.24	0.140		67.9	39.1		80.5	33		1.82
1.22	0.110		63.6	33.0		75.6	66		1.64
1.18	0.110		56.1	33.0		70.6	70		1.61
1.14	0.063		48.1	18.1		57.5	21		1.32
1.15	0.053		50.2	14.5		57.4	0		1.28
1.10	0.051		40.3	13.8		48.8	20		1.22
1.11	0.027		41.6	5.3		44.7	10		1.11
1.07	0.042		33.8	10.6		40.8	10		1.15
1.00	0.025		20.5	4.6		24.2	0		1.00
						$\chi^2 = 59.3$***			
Cd +	**Cu**		**Cd**	**Cu**					
0.49	0.139		50.9	40.0		70.5	90		1.73
0.47	0.133		43.2	38.6		65.1	100		1.66
0.46	0.110		41.8	32.7		60.8	90		1.52
0.42	0.102		27.2	30.5		49.4	100		1.39
0.40	0.040		19.4	9.8		27.3	80		1.03
0.37	0.078		11.8	23.1		32.2	20		1.17
0.31	0.062		2.8	17.7		20.0	30		0.96
0.33	0.026		5.7	5.0		10.4	10		0.81
0.29	0.032		1.4	7.0		8.3	20		0.76
						$\chi^2 = 38.1$***			
Zn +	**Cd** +	**Cu**	**Zn**	**Cd**	**Cu**				
1.11	0.51	0.132	41.6	58.3	38.3	85.0	90		2.70
1.09	0.47	0.086	37.4	44.7	25.7	74.3	90		2.36
1.13	0.44	0.079	45.9	32.3	23.5	72.0	89		2.30
1.11	0.41	0.052	41.6	23.9	14.2	61.9	78		2.08
1.06	0.44	0.059	31.0	34.0	16.7	62.1	50		2.13
1.03	0.41	0.079	25.2	23.9	23.5	56.5	80		2.15
1.02	0.39	0.056	23.3	17.9	15.6	46.9	30		1.98
1.09	0.33	0.041	36.7	5.8	10.2	46.5	56		1.84
1.04	0.35	0.047	27.1	8.7	12.4	41.7	56		1.87
0.95	0.35	0.034	12.2	8.5	7.7	25.9	20		1.72
						$\chi^2 = 9.4$ (NS) (10.5)			

*Significant at 0.05 level; *** significant at 0.001 level

($P > 0.05$) over the range of concentrations tested (Table 1), although observed mortalities were higher than expected values for 7 out of the 10 concentrations. Based on the measured test concentrations, observed mortalities were always higher than those that would have been expected for each metal alone.

Toxic units calculated for each mixture ranged between 1.72 and 2.70 and appeared to overestimate the toxicities of the mixtures. On average, the toxic units were higher for the triad mixture concentrations than for any of the mixtures with pairs of metals.

Discussion

Initially, we believed that zinc, cadmium and copper acted on different sites within the shrimp and therefore tested these metals on *Callianassa australiensis* on the assumption that they would act in a dissimilar manner, the simplest model being independent action. However, the results showed that for all paired combinations of metals, there were significant differences between observed and expected mortalities; this has led us to reject the hypothesis of independent dissimilar action as an adequate predictor of the lethal effects of paired combinations of these metals for the given experimental conditions. The model was then considered for simple similar action, but probit-regression analyses showed that the individual 14 d dose-mortality curves for zinc and copper, and cadmium and copper were not parallel (Ahsanullah *et al.*, 1981a), which suggested that these pairs of metals may have different mechanisms of action (Bliss, 1939) and therefore would not act in a simple fashion (Finney, 1971). Plackett and Hewlett (1967) also considered parallel probit lines to be a necessary condition for simple action, if the question of amount administered versus the amount reaching the site of action is ignored. Although there was no evidence that 14 d dose-mortality curves for zinc and cadmium were not parallel, observed mortalities for mixtures of these metals were much higher than those predicted, if the mixture had acted according to simple similar action. As a result, we have concluded that zinc-cadmium, zinc-copper and cadmium-copper mixtures exert their toxic action in an interactive manner.

Analyses of metal concentrations in whole shrimp and tissues (Ahsanullah *et al.*, 1981b) suggest that zinc and cadmium may exert their toxic effects in mixtures as in the complex similar model, since each metal appears to enhance the accumulation of the other. Further, as was mentioned above, their 14 d dose-mortality curves are parallel, which may also indicate that the metals are acting mainly on a similar site within *Callianassa australiensis*.

In the case of zinc-copper and cadmium-copper mixtures, copper appears to interact with both zinc and cadmium when causing death in shrimp, as is clear from an examination of metal-accumulation data. However, there is no clear evidence for determining an interactive model that best predicts the effects of these pairs of metals. If it is assumed that these pairs of metals mainly exert their toxic action on different sites (as deduced by the differences in slopes between 14 d dose-response curves and information from the literature), then dependent dissimilar action best describes the acutely lethal effects of both of these mixtures; thus, mixtures of cadmium and copper are more toxic than was expected and mixtures of zinc and copper are less toxic (Table 1) for the independent model.

In contrast to the results for mixtures of pairs of metals, the observed mortalities in the Zn-Cd-Cu mixture were not significantly different ($P > 0.05$) from predicted mortalities based on independent dissimilar action. However, considering the results from the experiments with pairs of metals, we cannot support the acceptance of this model for the triad mixture without further experimentation to provide more evidence on which to base the hypothesis test.

In some studies, the effects of mixtures of zinc and cadmium, zinc and copper, and zinc, cadmium and copper have been reported as "synergistic" or "more than additive" (Sprague and Ramsay, 1965; Hutchinson and Czyrska, 1972; Wissmar, 1972; Eisler and LaRoche, 1972; Eaton, 1973; Eisler and Gardner, 1973; D'Agostino and Finney, 1974). In others, combinations of zinc and copper (Lloyd, 1961; Brown and Dalton, 1970) and zinc and cadmium (Thorp and Lake, 1974) have been reported as "strictly additive", according to the toxic-unit concept (Sprague, 1970). Nimmo and Bahner (1976) reported "no dramatic toxic interaction of the combination of methoxychlor and cadmium, or of the combination of methoxychlor-cadmium-PCB to *Penaeus duorarum*." Further, they suggested that "the toxicities of the combinations when compared to each toxicant tested singly, were independent and additive . . . although synergism might exist in the environment."

It appears that much of the literature reported for effects of mixtures of toxicants on both marine and freshwater biota have been based on the toxic-unit concept alone. This method does not have a sound theoretical framework (Brown, 1968), even though there are empirical examples supporting the concept (Sprague, 1970). It is merely a guide to the levels of toxicants in mixtures that need investigation. This concept appears to be merely a redefinition of the simple similar action model but, unlike that model, it has been used to characterize the toxicity of mixtures of pollutants without regard for the dose-mortality curves of the individual constituents, in particular the shapes of the slopes. There appears to be a need for new models that take interaction into account when attempting to explain such results.

In the present study, when the toxicities of mixtures of metals were assessed according to the toxic-unit concept, the expected mortalities of shrimp in all paired and triad mixtures were overestimated (Table 1). Even when concentrations of mixture constituents were as high as one-third of the 14 d LC_{50} values, mortalities were negligible. These results compare favourably with those of Lloyd and Orr (1969), who reported that the toxicity of a mixture failed to add up when concentrations of constituents were less than one-fifth of the lethal threshold concentration.

Possible explanations for some of these differences in results reported by various investigators may be the different rates of absorption of the metals for different concentrations and environmental test conditions, differences in the physiological condition of test organisms, and differences in models hypothesized and interpretation of results. From the results, it is obvious that no clear trends have emerged that would permit a simple generalization. Indeed, several factors influence and complicate prediction of the effects of mixtures of toxicants.

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Literature Cited

Ahsanullah, M., D. S. Negilski and M. C. Mobley: Toxicitiy of zinc, cadmium and copper to the shrimp *Callianassa australiensis*. I. Effects of individual metals. Mar. Biol. *64*, 299–304 (1981a)

Ahsanullah, M., D. S. Negilski and M. C. Mobley: Toxicity of *liensis*. III. Accumulation of metals. Mar. Biol. *64*, 311–316 (1981b)

Bliss, C. I.: The toxicity of poisons applied jointly. Ann. appl. Biol. *26*, 585–615 (1939)

Brown, V. M.: The calculation of the acute toxicity of mixtures of poisons to rainbow trout. Wat. Res. *2*, 723–733 (1968)

Brown, V. M. and R. A. Dalton: The acute lethal toxicity to rainbow trout of mixtures of copper, phenol, zinc and nickel. J. Fish Biol. *2*, 211–217 (1970)

Calamari, D. and R. Marchetti: The toxicitiy of mixtures of metals and surfactants to rainbow trout. Wat. Res. *7*, 1453–1464 (1973)

D'Agostino, A. and C. Finney: The effect of copper and cadmium on the development of *Tigriopus japonicus*. *In*: Pollution and physiology of marine organisms, pp 445–463. Ed. by F. J. Vernberg and W. B. Vernberg. New York: Academic Press 1974

Eaton, J. B.: Chronic toxicity of a copper, cadmium and zinc mixture to the fathead minnow. Wat. Res. *7*, 1723–1736 (1973)

Eisler, R. and G. R. Gardner: Acute toxicology to an estuarine teleost of mixtures of cadmium, copper and zinc salts. J. Fish Biol. *5*, 131–142 (1973)

Eisler, R. and G. LaRoche: Elemental composition of the estuarine teleost *Fundulus heteroclitus*. J. exp. mar. Biol. Ecol. *9*, 29–42 (1972)

Finney, D. J.: Probit analysis, 333 pp. London: Cambridge University Press 1971

Gaddum, J. H.: Pharmacology, 3rd ed. 504 pp. London: Oxford University Press 1948

Herbert, D. W. M. and J. M. Van Dyke: The toxicity to fish of mixtures of poisons. II. Copper-ammonia and zinc-phenol mixtures. Ann. appl. Biol. *53*, 415–421 (1964)

Hutchinson, T. C. and H. Czyrska: Cadmium and zinc toxicity and synergism to floating aquatic plants. Wat. Pollut. Res. Can. *7*, 59–65 (1972)

Lloyd, R.: Effect of dissolved oxygen concentration on toxicity of several poisons to rainbow trout. J. exp. Biol. *38*, 447–455 (1961)

Lloyd, R. and L. D. Orr: The diuretic response by rainbow trout to sublethal concentrations of ammonia. Wat. Res. *3*, 335–344 (1969)

NAS/NAE.: National Academy of Sciences–National Academy of Engineering: Water quality criteria 1972, 594 pp. Washington D.C.: U.S. Government Printing Office 1973

Nimmo, D. R. and L. H. Bahner: Metals, pesticides and PCB's: toxicity to shrimp singly and in combination. *In*: Estuarine processes, Vol. 1. pp 523–532. Ed. by M. Wiley. New York, San Francisco & London: Academic Press Inc.: 1976

Plackett, R. L. and P. S. Hewlett: A comparison of two approaches to the construction of models for quantal response to mixtures of drugs. Biometrics *23*, 27–44 (1967)

Sprague, J. B.: Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. Wat. Res. *4*, 3–32 (1970)

Sprague, J. B. and B. A. Ramsay: Lethal levels of mixed copper-zinc solutions for juvenile salmon. J. Fish. Res. Bd Can. *22*, 425–432 (1965)

Thorp, V. J. and P. S. Lake: Toxicity bioassays of cadmium on selected freshwater invertebrates and the interaction of cadmium and zinc on the freshwater shrimp, *Paratya tasmaniensis* Riek. Aust. J. mar. Freshwat. Res. *25*, 97–104 (1974)

Wissmar, R. C.: Some effects of marine drainage on primary production in Coeur d'Alene River and Lake, 61 pp. Ph.D. thesis, University of Idaho 1972

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