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Toxicity of Cadmium-Copper-Nickel-Zinc Mixtures to Larval Purple Sea Urchins (*Strongylocentrotus purpuratus*)

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Wastewater and ambient water samples typically contain complex mixtures of trace organic and metal constituents, but the interactive effects of these mixtures are poorly understood. Trace metals are often present at concentrations below individual toxic thresholds. Metals may act additively in complex mixtures, resulting in situations where the combined effect of multiple metals results in sample toxicity (Masnado et al. 1995). However, chemical combinations may also act antagonistically or synergistically (Kraak et al. 1994), complicating the identification of specific constituents responsible for observed biological effects.

Water chemistry monitoring programs usually consider only individual metals and generally ignore mixtures (Enserink et al. 1991; Masnado et al. 1995). Because ambient samples are rarely contaminated with a single metal, it is necessary to better understand how the bioavailable fractions of metals interact with the organism to determine the causes of observed toxicity. Metals interact with organisms in different ways, particularly at the cellular level. Competition for binding sites within cell membranes, with metallo-enzymes, metallothioneins, or target molecules with metal-specific sensitivities can create antagonistic and synergistic interactions that deviate from straight addition (Sharma et al. 1999).

Previous studies conducted to assess the interactive effects of metals on a variety of species have demonstrated that similar combinations of metals can produce varying results (Negilski et al. 1981; Kraak et al. 1994; Masnado et al 1995). Recent research has addressed metal toxicity in fish by examining the biotic ligand model (Di Toro et al. 2001), but few studies have examined the effects of metal mixtures with invertebrates, particularly larval marine invertebrates. Because marine invertebrate larval development toxicity tests are used extensively for effluent monitoring and for screening environmental samples (APHA 1998), it is important to understand how larval test organisms respond to metal mixtures. We investigated the toxicity of all possible combinations of four trace metals (copper, zinc, cadmium, and nickel) to sea urchin embryos to determine whether they acted synergistically, additively or antagonistically. This study is intended to facilitate the interpretation of chemical analyses and toxicity identification evaluation (TIE) results so that mixtures observed in environmental or wastewater samples could be better related to observed biological effects.

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MATERIALS AND METHODS

Copper and zinc (essential metals) and nickel and cadmium (non-essential metals) were chosen for the study because they are commonly present in effluent and ambient samples. The bioavailability of these metals in solution can be altered through binding with particulate matter, as well as organic ligands and colloids. To minimize the variation in test solution metal bioavailability, a single batch of 0.45 μm-filtered seawater (34‰) was used in all tests. This water was collected at the initiation of the study and stored in the dark at 4°C in high-density polyethylene carboys. It was assumed that the amount of organic material available for metal complexation in the stored seawater remained constant throughout the study.

Each combination experiment was replicated three times. Metal stock solutions were prepared from $CdCl_2$, $CuCl_2$, $NiCl_2$, and $ZnSO_4$ at the following concentrations: Cd – 100,000 μg/L, Cu – 10,000 μg/L, Ni – 1,000,000 μg/L, and Zn – 10,000 μg/L. These single stock solutions were stored in high-density polyethylene volumetric flasks and used in all experiments. Stock solutions were analyzed as described below to determine percent deviation from nominal concentrations. Based on the chemical confirmation of stock solutions and experimental dilutions, metal results are presented as nominal concentrations of total metal.

Each binary mixture experiment was composed of three tests: two single metal tests and a combination test. The toxic unit approach used was similar to that of Kraak et al. (1994). The single metal tests included six concentrations prepared so that the expected EC50 (concentration causing abnormal development to 50% of the larvae) was evenly bracketed. These test concentrations were determined in previous laboratory experiments. The six concentrations for the combination test were prepared by combining one half of each of the single metal concentrations. EC50s were calculated using the Trimmed Spearman-Karber method, or by linear interpolation if there was an incomplete range of responses, using Toxcalc 5.0 software (Tidepool Scientific Software, McKinleyville, CA, USA). Using the single metal EC50s, toxic units (TUs) were calculated for each single metal concentration (TU = concentration divided by EC50). We determined the TUs contributed by each metal in the binary test concentrations by dividing these TU values in half (0.5 TUx + 0.5 TUy). Once the EC50 was determined for the mixture, the individual metal concentrations present at the EC50 were calculated using linear equations. If toxicity was additive, the sum of individual metal TUs would be 1 at the mixture concentration causing a 50% effect. Mixture TUs significantly less than one indicated that lower than expected concentrations of the individual metals caused a 50% toxic effect in the mixture, thus suggesting synergy. Mixture TUs significantly greater than one suggested antagonistic interactions.

Combination experiments with three and four metals were conducted in a similar manner except that single metals were added at one-third and one-fourth the concentrations used in single metal tests. To determine the statistical significance of mixture additivity, t-tests were used to compare the TU sums from three replicate mixture tests to the constant 1 (straight additivity). Each replicate value for this comparison was the sum of individual metal TUs at the EC50 concentration for the mixture, as described above.

Nominal concentrations of metals associated with toxic effects were verified by analyzing two samples per dose-response experiment. Metals were analyzed at the California Department of Fish and Game Trace Metal Analytical Facility at Moss Landing, CA, using Inductively Coupled Plasma Mass Spectrometry (ICP MS, US EPA method 1638). Based on standard reference materials, instrument accuracy ranged from 1.0 to 5.6% and precision ranged from 0.1 to 1.6%. Accuracy of nominal concentrations compared to measured concentrations ranged from 0.3 to 20.4%, and precision ranged from 0.3 to 16.0%. Based on these analytical results and documentation of quality assurance measures, the toxicity data are presented using nominal concentrations.

Toxicity tests were conducted using embryos of purple sea urchins (*Strongylocentrotus purpuratus*, US EPA 1995). Sea urchins were collected from the Monterey County coast (California, USA), and held at the Marine Pollution Studies Laboratory (MPSL) at ambient seawater temperature and salinity until testing. On the day of a test, animals were induced to spawn through osmotic shock and gametes were collected. Eggs were fertilized and embryos were introduced to test containers. Test containers were polyethylene-capped, 20-mL glass scintillation vials containing 10 mL of sample. Each test container was inoculated with approximately 250 embryos (25/ml). After a 96-hour exposure at 15°C and 34‰ salinity, larvae were fixed in 5% buffered formalin. Larvae in each container were examined under an inverted light microscope at 100x to determine the proportion of normally developed larvae. Dissolved oxygen and pH were within the acceptable range for purple urchins.

RESULTS AND DISCUSSION

The single metal dose response tests conducted in this study produced accurate EC50 values for the four metals. Individual point estimates are listed in Table 2, but are summarized in Table 1.

Binary metal combinations tested with sea urchin embryos produced all three types of interactions. Synergistic responses were noted for Cd+Cu, Cu+Ni and Ni+Zn (Table 2). In each of these cases, the toxic units for the individual metals added up to less than one at the mixture concentration eliciting a 50% embryo

Table 1. Mean 96-hour larval development EC50 values calculated from single-metal dose response tests.

	Cd	Cu	Ni	Zn
Mean EC50 (µg/L)	342.3	15.3	341.4	96.9
SD (µg/L)	137.6	2.2	84.8	12.2
N	12	15	8	15

response, and this difference from 1 TU was statistically significant ($p<0.05$). An additive response occurred with Cd+Ni, where the mixture EC50 occurred at a concentration at which individual metal toxic units added up to approximately one. Antagonistic responses occurred with Cd+Zn and Cu+Zn. The most variable response occurred in the Cd+Ni pair, with TU sums ranging from 0.848 to 1.345 in the three replicate experiments. Trinary metal combinations produced an antagonistic response for Cd+Cu+Zn, as the TU sums were significantly greater than 1 (Table 2). Cd+Cu+Ni produced an additive response and Cd+Ni+Zn produced a generally synergistic response, but because of the variability of TU sums, the mean response was not statistically different from additive. Variability of the TU sums in these experiments was caused by the variable TU contribution of cadmium. Cu+Ni+Zn produced a synergistic response. The four-metal experiment produced a mean response that was synergistic (Table 2).

The objective of this study was to investigate metal interactions so that biological effects observed in environmental or wastewater samples containing metal mixtures can be better interpreted. The results demonstrate few consistent trends, and indicate that additive, synergistic and antagonistic responses with larval sea urchins are all possible with different combinations of metals. The six binary combinations produced all three possible outcomes, as did the four trinary combinations, while a mixture of all four metals produced synergistic toxicity. Although there is not much agreement in the literature about metal mixture toxicity (Kraak et al. 1994; Sharma et al. 1999), Enserink et al. (1991) suggested that mixtures involving large numbers of metals are additive, but mixtures involving two or three metals are unpredictable. This point is demonstrated in the literature (Negilski et al. 1981; Enserink et al. 1991; Kraak et al. 1994; Masnado et al. 1995); however, the four-metal combinations in this study did not produce additive responses for sea urchins.

The toxicity of metals to sea urchin larvae can be affected by metal speciation and bioavailability, and by cellular mechanisms. Metals in solution can bind to particulate matter, organic ligands, and colloids. Depending on the source of dilution water, differences in binding capacity can alter metal speciation, and affect toxicity (Paulson and Gendron 2001). It is well established that metals toxicity can be reduced through the formation of less bioavailable complexes. This study attempted to minimize the variability associated with binding by

Table 2. Sea urchin metal mixture results. Toxic Unit contributions at the 96-hour larval development mixture EC50 and individual EC50s listed for each test.

Metal Mixture	Test 1 TU	EC50	Test 2 TU	EC50	Test 3 TU	EC50	TU Mean	Result
Cd	0.406	290.1	0.364	462.5	0.422	179.0	0.397	
Cu	0.234	16.0	0.347	15.4	0.180	13.6	0.254	
TU Sum	0.640		0.711		0.602		**0.651**	Synergistic
Cd	0.689	237.4	0.441	389.9	0.467	380.8	0.532	
Ni	0.656	244.9	0.407	408.0	0.503	348.7	0.522	
TU Sum	1.345		0.848		0.971		**1.055**	Additive
Cd	0.850	290.1	0.678	462.5	1.004	179.0	0.844	
Zn	1.177	86.0	1.314	100.9	0.813	90.7	1.101	
TU Sum	2.027		1.992		1.817		**1.945**	Antagonistic
Cu	0.200	14.4	0.139	16.1	0.159	14.7	0.166	
Ni	0.381	244.9	0.176	408.0	0.212	348.7	0.256	
TU Sum	0.581		0.315		0.371		**0.422**	Synergistic
Cu	0.529	15.8	0.595	13.0	0.365	19.9	0.496	
Zn	0.891	98.0	0.783	99.4	0.881	81.9	0.852	
TU Sum	1.420		1.378		1.246		**1.348**	Antagonistic
Ni	0.300	244.9	0.227	408.0	0.195	348.7	0.241	
Zn	0.271	111.6	0.358	102.8	0.292	93.4	0.307	
TU Sum	0.571		0.585		0.487		**0.548**	Synergistic
Cd	0.417	197.9	0.171	679.0	0.407	227.9	0.332	
Cu	0.133	20.2	0.270	14.5	0.222	13.3	0.208	
Ni	0.200	412.6	0.295	419.7	0.333	267.1	0.276	
TU Sum	0.750		0.736		0.962		**0.816**	Additive
Cd	0.507	462.5	1.091	179.0	0.765	339.5	0.788	
Cu	0.475	15.4	0.452	13.6	0.626	13.1	0.518	
Zn	0.729	100.9	0.692	90.7	0.870	95.6	0.764	
TU Sum	1.711		2.235		2.261		**2.069**	Antagonistic
Cd	0.472	197.9	0.114	679.0	0.261	227.9	0.282	
Ni	0.215	412.6	0.189	419.7	0.220	267.1	0.208	
Zn	0.279	108.5	0.227	119.1	0.192	97.8	0.233	
TU Sum	0.966		0.530		0.673		**0.723**	Additive
Cu	0.119	20.2	0.169	14.5	0.137	13.3	0.142	
Ni	0.179	412.6	0.19	419.7	0.213	267.1	0.194	
Zn	0.219	108.5	0.211	119.1	0.183	97.8	0.204	
TU Sum	0.517		0.570		0.533		**0.540**	Synergistic
Cd	0.116	237.4	0.091	389.9	0.205	380.8	0.137	
Cu	0.095	14.4	0.066	16.1	0.103	14.7	0.088	
Ni	0.127	244.9	0.082	408.0	0.194	348.7	0.134	
Zn	0.158	111.6	0.107	102.8	0.137	93.4	0.134	
TU Sum	0.496		0.346		0.639		**0.494**	Synergistic

utilizing a single batch of filtered seawater for all exposures. It was also assumed that most strong binding sites were already saturated at concentrations where metal toxicity begins to affect biological organisms in natural waters (Paulson and Gendron 2001). Although the bioavailability of a metal may change when it is mixed with other dissolved metals, it was assumed that a single batch of water spiked with toxic concentrations of the test metals would produce similar bioavailable fractions among tests. Attempts were made to limit variability, but it should be noted that toxicological data from one type of water should not be extrapolated to a different type without considering differences in speciation (Luoma 1995).

Additional factors that influence the toxicity of metals can include the interactions of non-essential metals with essential metals, the formation of metal-protein complexes, and the organism age and stage of development (Viarengo 1985). Previous studies have generally reported the effects of metal combinations on adult organisms. Recent research has emphasized metal impacts on adult freshwater fish using the biotic ligand model. The cellular membrane processes at work in the urchin embryo might be similar to those of a fish gill, but it must be noted that larval organisms in this study began their exposure at the one-cell stage, therefore, metal interactions took place on a cellular level, rather than through organ systems such as the gill. Therefore, the biotic ligand model may not be applicable to describe metal toxicity to developing sea urchin embryos, particularly when metal mixtures are considered.

Although there have been numerous studies concerning the biochemistry of the purple sea urchin embryo, few specific processes have described the mechanisms of metal toxicity in the early developmental stages of urchins. Within a generalized cell the essential metals copper and zinc exist in homeostatic balance. Copper acts as a catalyst for many enzyme systems, and zinc is essential for the proper structure and function of cell membranes (Viarengo 1985). Of the non-essential metals, cadmium can disrupt the ionic balance and alter the permeability characteristics of cell membranes (Viarengo 1985), and nickel can cause mutagenicity in cells (Fletcher et al. 1994).

Radenac et al. (2001) report that larval urchins accumulated metals, and in the case of Cu and Zn, actively took up the metal when reared in concentrations below lethal limits. Concentrations of metal in the tissues were related to the frequency of larval abnormalities. Excess amounts of metals entering a cell can cause increased metallothionein production and changes in lysosomal activity (Langston 1990). These processes help control the homeostasis of existing cellular metals and help in detoxifying excess metals.

Increased amounts of essential metals inhibit natural cell functions and can be toxic through displacement of other metals at binding sites (George 1990). As metal concentrations increase, metal substitution reactions take place and the inhibition of enzyme activity and the destabilization of the structural components of cellular molecules occur. Interactions among metals with similar properties

might cause various reactions through competition for binding sites at membrane transporters, metallo-enzymes, metallothioneins, or target molecules with specific sensitivities (Sharma et al. 1999). When zinc was associated with copper, cadmium, or both metals in these experiments, an antagonistic response occurred. This might have been because these three metal ions have similar physical and chemical properties, with the toxicity being more related to binding site competition than specific cellular processes (George 1990).

The results of water quality monitoring are often interpreted on the basis of single compounds, and chemical interactions are often overlooked or poorly understood. Concentrations of metals that do not cause adverse effects when tested individually can cause toxicity when tested at the same concentrations in mixtures. Enserink et al. (1991) tested mixtures in which individual metal concentrations were at the maximum levels for Dutch water quality criteria, and found the mixtures to be highly toxic to *Daphnia magna*. Similarly, Masnado et al. (1995) tested samples containing combinations of metals at permitted concentrations and found them to be toxic to *C. dubia*.

Results of metal mixture tests with a range of species demonstrate that interactive effects are unpredictable and sometimes contradictory (Sharma et al 1999). However, knowledge of metal interactions for specific species is still helpful for monitoring and assessment studies because it facilitates interpretation of chemistry data from ambient and effluent samples. Although recent studies have investigated predictive models for metal toxicity (Newman et al. 1998), predicting the toxicity results of mixtures have not been as successful. The results of this study could not be predicted based on existing knowledge of chemical interactions and cellular metabolism. Information from the present study can be applied to toxicity identification evaluations (TIEs) and effluent chemical measurements to estimate whether various combinations of metals would contribute to observed toxicity in effluent or environmental samples. This study adds to our understanding of the toxicity of metal mixtures, but is limited by its use of total metal concentrations. Future work should examine bioavailable fractions and seek to quantify metals in their cationic forms

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