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Effects of Copper, Cadmium, and Zinc on the Hatching Success of Brine Shrimp (*Artemia franciscana*)

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Abstract. Previous studies indicate that the hatching success of brine shrimp (*Artemia franciscana*) cysts is surprisingly sensitive to ambient metal concentrations. These studies estimated median effective concentrations (EC_{50}s) of 7, 5, and 28 μg l^{-1} for Cd, Cu, and Zn, suggesting that the hatching end point for *A. franciscana* is the most sensitive tested to date for Cd and Zn in saline environments and comparable in sensitivity with the most sensitive tested to date for Cu. Furthermore, these data suggest that brine shrimp are at significant risk from Cu and Zn in Great Salt Lake (GSL), UT, where ambient concentrations as high as 10 and 14 μg l^{-1}, respectively, have been measured. Given that brine shrimp appear to be successfully reproducing in GSL, we hypothesized that these toxicity values were either biased low as a result of an artifact of the test method used or that site-specific water-quality conditions in the lake had decreased metal bioavailability such that brine shrimp could successfully reproduce. To test these hypotheses, we initiated a step-wise series of experiments. First we investigated the effects of pretreatment of brine shrimp cysts with antibiotics on brine shrimp sensitivity to metals because previous investigators as part of their test methods have used antibiotics. Next we considered the effect of ionic composition of the artificial test media on sensitivity. Finally, we evaluated the effects of the site-specific water quality of the GSL on metal bioavailability and toxicity. Results indicate that pretreatment of cysts with antibiotics had no effect on sensitivity. However, we were unable to repeat the previous values for Cd and Zn, obtaining EC_{50}s of 11,859 and 289 μg l^{-1} for Cd and Zn, respectively. For Cu, however, we estimated an EC_{50} of 12 μg l^{-1}, so we conducted further testing on the artificial media, adjusting the media composition to better reflect the Ca^{2+} and HCO_3^- concentration of normal seawater. This increased the EC_{50} to 28 μg l^{-1}. Finally we evaluated the toxicity of Cu in GSL water and obtained an EC_{50} of 68 μg l^{-1}, suggesting that the increased dissolved organic carbon in GSL has a significant protective effect. Overall, the results of this study suggest that brine shrimp hatching success is not particularly sensitive to Cd and Zn, but it is sensitive to Cu. However, site-specific water-quality conditions ensure that brine shrimp cyst hatching success is not significantly affected by any of these metals at the normal background concentrations that occur in GSL (<15 μg l^{-1}).

Generally, brine shrimp (*Artemia* spp.) have not been considered particularly sensitive to metals. Numerous researchers have demonstrated that relative to other aquatic organisms, brine shrimp range from moderately sensitive to insensitive to a wide range of metals. Most of these studies have considered the short-term (acute) toxicity of metals to either nauplii or adult life–stage shrimp (Brown and Ahsanullah 1971; Saliba and Krzyz 1976; Browne 1980; Jayasekara *et al.* 1986; Liu and Chen 1987), although several studies have also exposed brine shrimp to metals for an entire life cycle (Gebhardt 1976; Brix *et al.* 2003a; Brix *et al.* 2004b).

The exception to these data are experiments evaluating the effects of metals on the hatching success of brine shrimp (Bagshaw *et al.* 1986; Rafiee *et al.* 1986; MacRae and Pandey 1991). These studies included a series of experiments using a standardized method to estimate the median effective concentrations (EC_{50}s) of 7, 5, and 28 μg l^{-1} for Cd, Cu, and Zn, respectively, for brine shrimp hatching success. These data suggest that this particular end point (hatching success) makes brine shrimp one of the most sensitive species tested to date in a saline environment. In fact, when compared with toxicity data for marine species, these EC_{50}s represent the lowest toxicity values measured for Cd and Zn and are comparable with the lowest values measured for Cu (United States Environmental Protection Agency [USEPA] 1987, 2001, 2003).

The Great Salt Lake (GSL), UT, is one of the primary habitats for *A. franciscana*. Ambient monitoring of metal concentrations in GSL indicates Cu concentrations ranging from 3.7 to 10.2 μg l^{-1} and Zn concentrations ranging from 8.0 to 14.1 μg l^{-1} in the oxic portion of the water column (Tayler *et al.* 1977; Domagalski *et al.* 1990). Cd is always <2 μg l^{-1}. The overlap between effect levels on hatching success observed by Bagshaw *et al.* (1986) and ambient Cu and Zn concentrations in the lake suggests brine shrimp may be at substantial risk from exposure to these two metals.

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However, several uncertainties exist regarding the methods used by Bagshaw *et al.* (1986) in their toxicity tests. First, to decrease the potential for bacterial attack on emerging nauplii, the Bagshaw *et al.* methodology includes soaking brine shrimp cysts overnight at 4°C in test media containing 50 U ml^{-1} penicillin, 50 mg l^{-1} streptomycin, and 0.2% (w/v) sodium tetraborate. Second, the test media used in the Bagshaw *et al.* methodology has an ionic composition that differs substantially from natural seawater and from GSL water. Specifically, Ca^{2+} and HCO_3^- concentrations in the test media are 1.3 and 0.5 mM compared with 10 and 2.4 mM in natural seawater (Garrison 1998). The organic carbon content of the Bagshaw *et al.* media is very low (<0.04 mM) compared with natural waters.

To evaluate whether one or both of these factors (antibiotic pretreatment, test media ionic composition) contributed to the apparent high sensitivity of brine shrimp to metals during cyst hatching, we conducted experiments to evaluate the effects of antibiotic pretreatment and test media ionic composition on the sensitivity of brine shrimp to metals during cyst hatching.

Table 1. Ionic composition of dilution waters

Parameter	Bagshaw *et al.* media	Synthetic seawater	GSL
Ca^{2+}	1.4	10.3	1.6
Mg^{2+}	48.1	49.2	40.9
Na^+	423	485	492
K^+	7.2	8.3	15.6
Cl^-	478	410	413
SO_4^{2-}	25.5	21.7	19.1
CO_3^{2-}	0.023	0.214	0.089
HCO_3^-	0.470	2.171	0.905
PH	7.8	8.1	8.1
DOC	<0.04	<0.04	0.63

[a] All units in mM except pH, which is in standard units.

Methods and Materials

Experimental Design

Our general experimental design was to first evaluate the effects of antibiotic pretreatment by conducting metal toxicity tests with brine shrimp cysts that had and had not been pretreated with antibiotics. Testing was conducted with Cd, Cu, and Zn in these experiments. This was followed by a series of experiments (using Cu only) in which test media with different ionic compositions were used to evaluate the importance of ionic composition on Cu bioavailability and subsequent toxicity. This testing culminated in an experiment using water collected from GSL to evaluate the potential effects of other naturally occurring constituents (*e.g.*, dissolved organic carbon [DOC]) on Cu bioavailability and toxicity.

Test Organisms and Chemicals

Brine shrimp cysts were purchased from Argent Chemical Laboratories (Redmond, WA), which certified them to be *A. franciscana* collected from GSL. Cysts were stored in the dark at 4°C until used for testing. All test substances and antibiotics were obtained from Sigma Chemical (St. Louis, MO). Reagent-grade cadmium chloride, copper chloride, and zinc sulfate were used to conduct all studies. Penicillin G sodium salt, streptomycin sulfate salt, and reagent grade sodium tetraborate were used in experiments assessing the effects of antibiotics on metal sensitivity.

Toxicity Test Methods

Initial experiments that assess the effects of antibiotics on brine shrimp hatching success during metal exposure were conducted according to the methods of Bagshaw *et al.* (1986). Briefly, brine shrimp (*A. franciscana*) cysts were soaked overnight in test media at 4°C. Two treatments of cysts were soaked, one in test media only and one that also included a mixture of antibiotics at the concentrations specified in the Bagshaw *et al*. study, *i.e.*, 50 units ml^{-1} penicillin, 50 mg ml^{-1} streptomycin, and 0.2% w/v of sodium tetraborate. After soaking overnight, toxicity tests were initiated with treated and untreated cysts for each of the three metals evaluated (Cd, Cu, and Zn).

Toxicity tests consisted of a dilution water control and six to eight treatments, each tested in quadruplicate. Dilution water for these studies was the test media described by Bagshaw *et al.* (1986) (Table 1). Test chambers were 50-ml polypropylene test tubes containing 40 ml test solution. Ten cysts were introduced to each test chamber and placed in a temperature-controlled water bath (25°C). After 48 hours of exposure, hatching success in each replicate was enumerated and samples for total recoverable and dissolved metal concentrations collected.

Subsequent studies on the effect of water chemistry on Cu toxicity were conducted according to the same methods described previously, but cysts were not soaked overnight in the test media before initiating exposure. Three different waters were used in these experiments: (1) the test media described by Bagshaw *et al.* (1986); (2) an artificial seawater with the ionic composition of normal seawater; and (3) water collected from GSL adjusted to the osmolality of the artificial seawater (approximate 6-fold dilution) (see Table 1 for chemical composition). Adjustment of osmolality for GSL water was accomplished by addition of deionized water. Full-strength GSL water was not tested because previous experiments have demonstrated that cysts cannot hatch in water with such high osmolality (Brix *et al.* unpublished).

Analytical Chemistry

Water samples for dissolved metals were filtered through a 0.45-µm filter and then acidified immediately by addition of HNO_3 (trace-metal grade; Fisher Scientific) to a final concentration of 1%. Metal concentrations were analyzed by graphite furnace atomic absorption (Varian 220Z; Varian, Australia) after either solvent extraction (Kinrade and Van Loon 1974) or iron coprecipitation (Weisel *et al.* 1984) to eliminate salt matrix effects. Dilution waters were analyzed for Na^+, K^+, Ca^{2+}, and Mg^{2+} by atomic absorption (Varian 220FS; Varian); for Cl^- and SO_4^{2-} by anion chromatography (DIONEX DX120, CA); for pH (PHM220 meter fitted with an Accumet combination glass electrode; Radiometer, Copenhagen, Denmark); and for total carbon dioxide using a Corning 962 carbon dioxide analyzer (UK). The fraction of total carbon dioxide present as HCO_3^- and CO_3^{2-} was estimated using CO2Sys software (Lewis 1996). DOC in the dilution waters was determined by high-temperature catalytic oxidation using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan).

Statistical Analysis

Toxicity data generated in this study were statistically analyzed by probit analysis using the computer software program ToxCalc, v 5.0 (Tidepool Scientific Software, McKinleyville, CA). For each test, the 48-hour EC_{50} (and its 95% confidence limits) was determined. Modeling copper speciation in the three test waters was accomplished using a metal speciation program as described by Millero and Pierrot (1998).

Results and Discussion

Measured metal concentrations across all tests remained stable for the duration of testing and were within ±20% of nominal concentrations. All statistical analyses were performed based on the mean of the measured test concentrations (Table 1). Hatching success in the control ranged between 70% and 95% in the different tests.

Initial testing to evaluate the effects of antibiotic pretreatment on cyst hatching sensitivity indicated that antibiotics had no effect on metal sensitivity (Table 2). The 48-hour EC_{50}s for Cd were 11,859 μg l^{-1} and 10,569 μg l^{-1} for untreated and treated cysts, respectively. Similarly, the 48-hour EC_{50}s for Cu were 11.8 and 11.3 μg l^{-1} for untreated and treated cysts, respectively. For Zn, the untreated cyst 48-hour EC_{50} was 289 μg l^{-1} and the treated cyst EC_{50} was 307 μg l^{-1}. Subsequent experiments using artificial seawater and GSL water diluted to the same osmolality resulted in EC_{50}s of 28 and 68 μg l^{-1} Cu, respectively (Table 2).

Modeling of copper speciation in the three different test waters indicated a significant difference in the fraction of free Cu ion (Cu^{2+}) present in the artificial seawater and GSL water compared with the Bagshaw media (Table 3). The largest differences in speciation between the three waters were the percentages of Cu^{2+} and $Cu(CO_3)_2$ present. In the Bagshaw *et al.* (1986) media, 18.6% of the Cu was present as Cu^{2+} compared with only 3.3% and 5.1% in the artificial and GSL waters. In the latter two waters, the decrease in Cu^{2+} was accompanied by an increase in $Cu(CO_3)_2$.

We investigated whether low effect levels obtained by Bagshaw *et al.* (1986) were a result of either the antibiotic treatment of brine shrimp cysts or the ionic composition of the test media they used. Our experiments show that antibiotic treatment of the cysts does not influence their sensitivity to Cd, Cu, and Zn. For all three metals, EC_{50}s determined for treated and untreated cysts were statistically indistinguishable. However, for Cd and Zn, we estimated EC_{50}s approximately 1,500 and 10 times higher than those previously estimated by Bagshaw *et al.* (1986). Our results are much more consistent with the acute sensitivity of other organisms that have been tested in saline waters with these two metals (USEPA 1987, 2001), suggesting that brine shrimp are not at risk from ambient concentrations of Cd and Zn in GSL.

In contrast, for Cu we estimated an EC_{50} of 12 μg l^{-1}, only twice that previously observed by MacRae and Pandey (1991) and still within the range of concentrations reported for GSL. We hypothesized that this low toxicity value might be influenced by the low Ca^{2+} and HCO_3^- concentrations in the Bagshaw *et al.* (1986) media. This media had only 1.3 mM Ca^{2+} and 0.5 mM HCO_3^- compared with concentrations in natural seawater of 10 and 2.3 mM, respectively (Garrison 1998). Although this discrepancy exists when compared with natural seawater, the ionic composition of GSL, when diluted to the same osmolality as the Bagshaw *et al.* media, has a comparable Ca^{2+} concentration (1.6 mM) and has HCO_3^- concentration only two times higher (1.0 mM).

Results of copper speciation modeling indicate that substantially less Cu^{2+} (generally considered the toxic form of Cu) was present in the artificial seawater and GSL waters than in the Bagshaw *et al.* (1986) media. This shift in speciation appears to be more driven by the difference in pH in the Bagshaw media (7.9) compared with the other two media (both 8.1) rather than difference in the total carbon dioxide concentrations in the media. This increase in pH results in significantly more CO_3 in the latter two media, promoting formation of $Cu(CO_3)_2$ (Table 3).

Although differences in inorganic Cu speciation explain the lower toxicity observed in the artificial seawater compared with the Bagshaw *et al.* (1986) media, they do not explain the difference in toxicity observed between the artificial seawater (12 μg l^{-1}) and GSL water (68 μg l^{-1}). We propose that the difference in toxicity between these two waters is a result of the relatively high DOC concentrations in GSL, which will complex with Cu and decrease bioavailability. GSL water, diluted to the same osmolality as the Bagshaw media, has 0.63 mM DOC compared with <0.04 mM in the Bagshaw media and artificial seawater. Predicting the effect of this DOC on Cu bioavailability is not readily accomplished in GSL water. Complexation models for high ionic strength waters are not well developed, and the composition of GSL DOC, which is largely derived from autochthonous sources, is not well characterized. However, several studies have demonstrated that DOC decreases Cu bioavailability in seawater (Lorenzo *et al.* 2002; Arnold 2004). It is therefore very likely that a significant percentage of the inorganic species predicated are actually associated with a DOC–Cu complex (Table 3).

In conclusion, we were unable to repeat the previous observations of Bagshaw *et al.* (1986) with respect to the hypersensitivity of brine shrimp hatching when exposed to Cd and Zn, and our results are much more consistent with the extant literature in terms of the general toxicity of these metals in saline waters. Our experiments did however corroborate previous observation by Bagshaw *et al.* regarding the high sensitivity of brine shrimp hatching to Cu when exposed in the artificial media used by these investigators. This high sensitivity is not the result of antibiotic pretreatment of the cysts. As would be ex-

Table 2. Measured EC_{50}s in brine shrimp toxicity tests

Test	EC_{50} (μg l^{-1})	95% CI (μg l^{-1})
Cadmium - no antibiotics	11,859	10,267–13,217
Cadmium - antibiotics	10,569	8,666–12,327
Zinc - no antibiotics	289	240–357
Zinc - antibiotics	307	241–375
Copper - no antibiotics	11.8	9.8–14.1
Copper - antibiotics	11.3	4.8–22.7
Copper - artificial seawater	28.2	10.9–51.9
Copper - Great Salt Lake	68.3	UC

CI = Confidence interval.
UC = Unable to calculate.

Table 3. Estimated Cu speciation in three test waters

	% Total		
Cu Species	Bagshaw *et al.* Media	Artificial Seawater	GSL
Cu^{2+}	18.63	3.32	≤ 5.10[a]
CuOH	0.00	0.00	0.00
$Cu(OH)_2$	0.00	0.00	0.00
$CuHCO_3$	0.08	0.06	≤ 0.04
$CuCO_3$	70.59	67.51	≤ 73.46
$Cu(CO_3)_2$	5.20	28.29	≤ 20.25
$CuSO_4$	5.50	0.81	≤ 1.16

[a]Included for the GSL speciation estimates because all inorganic species are likely significantly overestimated given the DOC present in this water.

pected, observed Cu toxicity is strongly influenced by Cu speciation, and Cu is approximately 2.5- and 6-fold less toxic when brine shrimp cysts are exposed in artificial seawater and GSL water compared with the media used by Bagshaw *et al.* Based on test results in GSL water and reported Cu concentrations in GSL, we conclude that brine shrimp are not currently at risk from Cu in GSL.

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