Toxicity of Metal Mixtures to *Daphnia magna* – Comparison of Laboratory and Field Data

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Abstract In aquatic systems impacted by mine wastes, organisms are usually exposed to metal mixtures instead of individual metals. For example, legacy acid mine drainage continues to pollute the North Fork of Clear Creek (NFCC) in central Colorado, USA. In November 2012, we collected water samples from NFCC and deployed *Daphnia magna* as in-stream biomonitoring tools. Survival and whole-body concentrations of key electrolytes in *D. magna* decreased at sites that, as a result of mine drainage inputs, had metal concentrations elevated above the upstream reference sites. In parallel laboratory toxicity tests in which *D. magna* were exposed to binary and ternary metal mixtures, approximately additive or less-than-additive toxicity occurred.

Keywords North Fork Clear Creek, acid mine drainage, aquatic geochemistry, cadmium, copper, iron, zinc

Introduction

In the mid-to-late 1800s, mining and mineral processing operations extracted valuable metals from the Colorado Mineral Belt. These processes led to unexpected environmental consequences as a result of acid mine drainage (AMD) generation. Currently an AMD-contaminated section of the North Fork of Clear Creek (NFCC) in central Colorado cannot sustain an aquatic vertebrate population and has a depauperate population of aquatic macroinvertebrates. Therefore, the United States Environmental Protection Agency (USEPA) has placed a high level of urgency for remediation of the stream. As a consequence of the AMD discharge into NFCC, pH and alkalinity are lowered, but water hardness and concentrations of dissolved metals (*e.g.* Cd, Cu, Ni, Zn) and suspended sediments are increased in comparison to upstream waters. The combined effects of these water quality parameters and the dissolved organic carbon(DOC) concentration control the aquatic toxicity of individual metals (Meyer *et al.* 2007). However, the consequences of organism exposure to metal mixtures are less well-understood. Laboratory toxicity testing of binary and ternary metal mixtures in standard media can provide insights into potentially non-additive effects. Field studies, while less controlled than laboratory toxicity tests, offer the opportunity to examine effects under realistic conditions and compare to results of laboratory tests. Our study goal was to gain a fuller understanding of the impact of metal mixtures on aquatic life in NFCC using both approaches. Thus, we conducted *D. magna* toxicity tests in the laboratory coordinated with *in situ* deployment of *D. magna* and concurrent water sampling to see if toxicity in a field setting could be predicted from laboratory toxicity tests.

Methods

Water Sampling

Six sampling sites represent changes in water chemistry as NFCC flows southeast to join Clear Creek. Two AMD point sources (Gregory Incline [GI] and National Tunnel [NT]) enter the stream in the town of

Blackhawk (Fig. 1). A relatively pristine reference site (Site 1) that has low metal concentrations is ≈1 km upstream from GI. This upstream site contains fish and numerous species of benthic macroinvertebrates that are characteristic of uncontaminated Rocky Mountain streams. Site 2 is immediately downstream of the two point sources, and the water chemistry and stream health are severely affected by the AMD inputs. Sites 3 and 4 are situated immediately above and below the city of Blackhawk's wastewater treatment plant (WWTP), ≈9 km downstream of Blackhawk. Site 4 is the last NFCC site before the confluence with the main stem of Clear Creek. Sites 5 and 6 on the main stem of Clear Creek are located upstream and downstream of the confluence with NFCC.

Grab samples of stream water, for analysis of major water quality parameters and metals, were collected using polypropylene containers on November 7, 15, and 17, 2012. Samples were filtered for dissolved metals in the field, and unfiltered samples were collected for total metals and total organic carbon (TOC)analyses. The TOC samples were collected in pre-combusted (500 °C) amber glass bottles.

In situ Biological Monitors

D. magna (a freshwater cladoceran commonly employed in standardized aquatic toxicity tests) were deployed at the six field sampling sites on November 15, 2012, for a 48-h instream exposure in polypropylene test chambers that allowed free flow of water through the chamber. Surviving Daphnia were rinsed sequentially with Milli-Q water and 10 mL of 0.1 mM EDTA for 2 min to remove particles and weakly surface–bound metals, oven dried (60 °C, 48h), weighed to the nearest 0.1 mg, extracted with 1 mL of trace-metal-grade nitric acid at 20 °C for 24h, and diluted to 10 mL with Milli-Q water before analysis for elemental content.

Laboratory Toxicity Tests

D. magna were cultured in moderately hard reconstituted (MHR) water (USEPA 2002; nominal pH of 7.4-7.8, alkalinity of 57–64 mg/L as $CaCO_3$, and hardness of 80–100 mg/L as $CaCO_3$) and were tested in MHR water to which DOC was added as Suwannee River fulvic acid at 3 mg DOC/L. Standard 48-h lethality tests using <24-h-old neonates were conducted for all single-metal (Cd, Cu, Ni, and Zn) and metal-mixture tests (USEPA 2002). Negative controls and four replicate test groups consisting of five Daphnia each were employed for each tested concentration. Temperature, pH, alkalinity, and concentrations of Cd, Cu, Ni, Zn, and major inorganic anions and cations were monitored during the tests; and hardness was calculated from the measured Ca and Mg concentrations. Because the concentration-response curves for individual metals can vary from test to test, we usually conducted a Cd-only, Cu-only, Ni-only,as well as Zn-only test concurrent with the binary-mixture tests. Additionally, we conducted numerous toxicity tests with each binary metal pair to determine within-day and
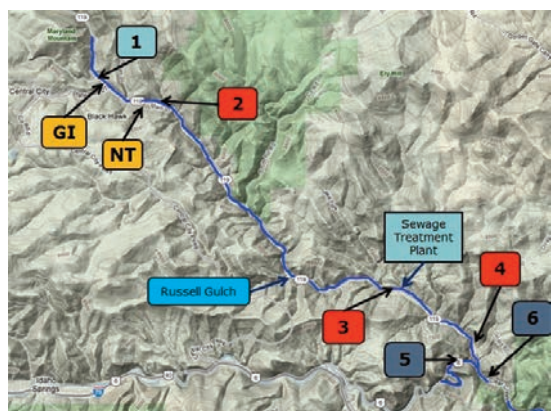

Fig. 1 *Map of the sampling sites on the North Fork and mainstem of Clear Creek in central Colorado*

among-week variability in results, to evaluate whether any apparently less-than-additive, additive, or more-than-additive toxicity result was due to variability alone.

Chemical Analyses

During the field study, we measured pH (VWR SP80PC meter/VWR 14002–860 combination gel electrode), alkalinity (HACH Model AL-DT Alkalinity Test Kit), and ferrous iron (HACH DR/890 Colorimeter/1-10 phenanthrolene AccuVac ampules) in the field. Later in the laboratory, unfiltered and filtered (0.45 µm) water samples from field sites were acidified with trace-metal-grade nitric acid before being analyzed for major elements and metals using inductively coupled plasma–optical emission spectrometry (ICP-OES; Perkin Elmer Optima 5300 DV). Water hardness was calculated from the measured Ca and Mg concentrations. Unfiltered water was also analyzed for total organic carbon (TOC) concentration by persulfate oxidation/UV irradiation (Sievers531 °C TOC analyzer). Acid-digested *D. magna (*see above) were analyzed for elemental content using ICP-OES.

During laboratory toxicity tests, the same general analyticalprocedures (APHA 2012) and instruments were used. However, pH was determined using a ThermoScientific Orion2 Star Benchtop Meter/ThermoScientific Orion 815600 Ross Combination pH/conductivity probe.

Results
Laboratory Toxicity Tests
In Cu-Zn binary mixture tests, the toxicity always appeared to be either slightly more-than-additive (*i.e.* the observed mortality was slightly greater than the predicted mortality) or additive (*i.e.* the observed mortality equaled the predicted mortality) when based on dissolved-metal concentrations, whether Cu was varied while Zn was held constant (Fig. 2a), or vice versa (results not shown). Similarly, the toxicity of Cu-Ni mixtures always appeared to be more-than-additive or additive (results not

shown). However, the toxicity of these Cu-Zn mixtures was less-than-additive when based on the concentrations of Cu and Zn bound to the biotic ligands, when predicted using a multi-metal biotic ligand model (results not shown). These results indicate that the dominant determinant of toxicity was Cu-Zn or Cu-Ni competitive binding to DOC, thus leaving more Cu^{2+}, Ni^{2+}, or Zn^{2+} available to bind to the biotic ligand than would have been available at the same dissolved-metal concentration in a Cu-only, Ni-only, or Zn-only test. Furthermore, these results demonstrate that conclusions about the additivity of metal-mixture toxicity can differ considerably when the frame of reference changes from dissolved metal to biotic-ligand-bound metal.

In contrast, in Cd-Cu, Cd-Ni, and Cd-Zn binary mixture tests, the toxicity appeared to be either less-than-additive (*i.e.* the observed mortality was less than the predicted mortality) or additive (*i.e.* the observed mortality equaled the predicted mortality) when based on dissolved metal concentrations (Figs. 2b, 2c, and 2d). Most notably, Ni and Zn concentrations less than 1/10 of the Ni-only or Zn-only median lethal concentration (LC50) protected against partial to 100 % Cd lethality (Figs. 2c and 2d); and Cd concentrations approximately 10x the Cd-only LC50 had to be added to the Cd-Ni and Cd-Zn mixtures before mortality began increasing above the background mortality caused by the constant Ni or Zn concentration (results not shown), again demonstrating a large protective effect of Ni and Zn against Cd toxicity. These results demonstrate a protective effect of higher-concentration metals (Cu, Ni, or Zn) against the toxicity of a more lethal metal that is present at much lower concentrations. These results also indicate that the dominant determinant of toxicity was Cd-Cu, Cd-Ni, or Cd-Zn competitive binding to the biotic ligand, whereby the relatively higher concentration of Cu^{2+}, Ni^{2+}, or Zn^{2+} allowed less Cd^{2+} to bind to the biotic ligand than would have been bound at the same dissolved-Cd concentration in a Cd-only test.
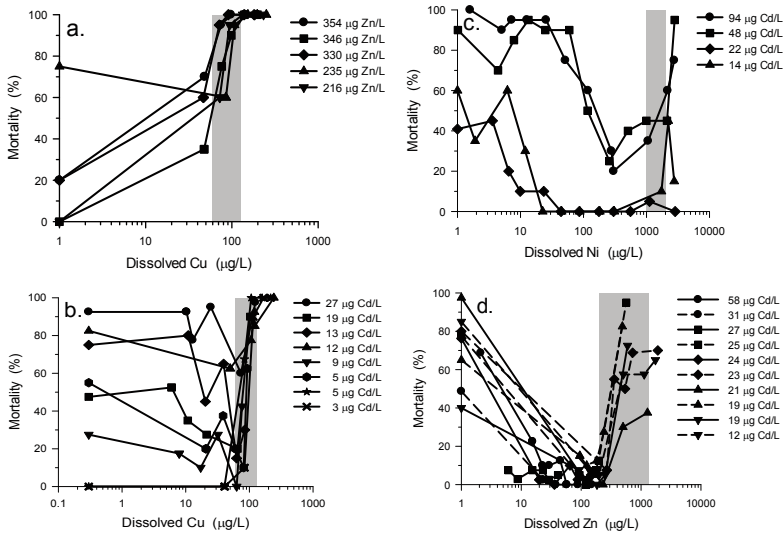
Fig. 2 Laboratorytoxicity of binary mixtures to D. magna. (a) Constant Zn with variable Cu. (b) Constant Cd with variable Cu. (c) Constant Cd with variable Ni (d) Constant Cd with variable Zn. The vertical gray bars indicates the range of concentrations at which the variable metal caused 50 % mortality in single metal tests.

An analogous protective effect against Cd toxicity also occurred in ternary Cd-Cu-Zn mixtures, without any apparent more-than-additive interactions between Cd and Zn (results not shown).

November 2012 Field Study

The pH and alkalinity of NFCC decreased and the water hardness increased at Site 2 after the two AMD point sources entered the stream, and the pH and alkalinity continued to decrease as the water flowed downstream to Site 3 (Table 1). These continued decreases of pH and alkalinity could have been due to slow Fe^{2+} oxidation, manifested as the continuing high concentration of dissolved Fe throughout the ≈ 9-km reach between Sites 2 and 3. Influx of the WWTP effluent considerably increased the pH, and alkalinity in NFCC at Site 4, but the hardness and TOC concentration increased

only slightly. Temperatures ranged from -0.5 to 3 °C at the beginning of the *D. magna* deployment.

The in-stream contributions of Cd, Cu, Fe, and Zn from the AMD point sources are manifested as large increases in dissolved and total concentrations of those metals at Site 2 (Table 1). Trends in dissolved and particulate Cu reflect several in-stream processes, most notably pH-dependent sorption to suspended particulate iron oxides. At Site 2, Cu is largely particulate where the pH is circumneutral (6.8) and suspended iron concentration is high (15 mg/L; calculated by difference between total and dissolved Fe concentrations in Table 1). At Site 3, the particulate Cu concentration is low, consistent with the lower pH (5.7) and lower suspended particulate iron concentration (1 mg/L). Despite the higher pH (8.0) at Site 4, the particulate Cu concentration is low, likely

Site	pH	Alkalinity (mg/L as CaCO₃)	Hardness (mg/L as CaCO₃)	TOC (mg/L)	Cd (µg/L) Total	Cd (µg/L) Diss.	Cu (µg/L) Total	Cu (µg/L) Diss.	Zn (µg/L) Total	Zn (µg/L) Diss.	Fe(µg/L) Total	Fe(µg/L) Diss.
1	8.3	25	43.4	1.59	<0.1	<0.1	2.6	3.2	9	27	152	15
2	6.8	12	247.3	1.54	4.7	3.5	70.2	3.2	1,787	1,625	38,373	23,437
3	5.7	3	258.0	1.41	5.8	5.4	94.0	90.8	2,004	1,963	3,580	2,520
4	8.0	53	268.5	1.75	1.9	1.7	21.8	14.4	740	680	629	30
5	8.2	45	101.1	2.28	<0.1	<0.1	10.7	5.6	219	180	168	15
6	8.2	44	112.6	1.50	<0.1	<0.1	11.8	6.1	267	201	290	20

[a] Data are averaged from the three sampling days (November 7, 11, and 15, 2012).

Table 1 Water chemistry during 48-h field deployment of D. magna in the North Fork and main stem of Clear Creek in Central Colorado in November 2012.[a]

due to the very low suspended particulate Fe concentration (<1 mg/L), which limits the amount of available sorption sites. In contrast, the large majority of zinc in the water is dissolved. Although the significant downstream decreases in the total and dissolved Cu concentrations might be explained by the concurrent loss of suspended particulate iron, the decreases of the total and dissolved Zn concentrations must be due to direct loss to the streambed. Decreases in metal concentrations due to dilution can be ignored based on the small decrease in sulfate concentration, which is a conservative tracer in this aerobic stream system in which sulfate is not reduced to sulfides (data not shown).

Because increasing alkalinity, hardness, and TOC concentrations tend to decrease metal bioavailability and thus toxicity(as does increasing pH above approximately 6.5-7; Meyer *et al.* 2007), the major longitudinal changes in water chemistry in NFCC can have major effects on the toxicity of Cd, Cu, and Zn to aquatic organisms. Those effects were manifested in the decreased survival of *D. magna* at Sites 2 and 3, where pH and alkalinity decreased considerably even though hardness increased (Table 2). Those toxicity results are consistent with the generalization that pH and alkalinity have more of a toxicity-modifying effect on cladocerans than does water hardness (*e.g.* Meyer *et al.* 2007), but the concurrent increases of the Cd, Cu, Fe, and Zn concentrations complicate that interpretation. The major decreases of whole-body Na and K concentrations and the smaller-percentage de-

creases of whole-body Ca and Mg concentrations are also consistent with expectation, because elevated concentrations of cationic metals can disrupt iono regulation (*e.g.* Na for Cu exposure, and Ca for Zn exposure) and thus can be lethal to aquatic organisms(Meyer *et al.* 2007).

NFCC water at Sites 2 and 3 had lower alkalinity, lower TOC concentration, and higher hardness than the MHR exposure water used in the laboratory single-metal and metal-mixture toxicity tests with Cd, Cu, and Zn (pH 7.8-8, alkalinity ≈ 50-60 mg/L as $CaCO_3$, hardness ≈ 80-90 mg/L as $CaCO_3$, and DOC ≈ 3 mg/L in MHR water). At Sites 2 and 3, the Zn concentrations in NFCC were nearly double the mid-range of the median lethal concentrations (LC50 values) determined in the Zn-only laboratory toxicity tests (≈ 600 µg Zn/L); and at Site 3, the dissolved Cu concentration in NFCC was close to the mid-range LC50determined in the Cu-only laboratory toxicity tests (≈ 80 µg Cu/L). Even in the relatively high hardness water, no Daphnia survived at Sites 2 and 3 due to the high Cu and Zn concentrations, low alkalinity, and relatively low TOC concentrations. The Cd concentrations at all sites were less than the mid-range LC50determined in the Cd-only laboratory toxicity tests (≈ 8 µg Cd/L); and because Cu and Zn have moderate to large protective effects against Cd toxicity (see above), the potential contribution of Cd to the observed Daphnia mortalities at Sites 2 and 3 was probably even less. Although the hardness at Site 4 remained approximately as high as at Sites 2 and 3, the alkalinity increased

| Site | *Daphnia* 48-h survival (%) | Whole-body concentration(mg/kg dry weight tissue) | | | |
		Na	K	Ca	Mg
1	92	137	240	5,010	198
2	0	<11[a]	<17[a]	3,639[a]	148[a]
3	0	14[a]	17[a]	3,273[a]	135[a]
4	100	596	444	6,508	256
5	93	383	297	5,655	236
6	96	392	333	6,206	232

[a] Dead *Daphnia* analyzed for whole-body electrolytes at this site because no *Daphnia* survived.

Table 2 *D. magna survival and whole-body electrolyte concentrations during 48-h field deployment in the North Fork and main stem of Clear Creek in Central Colorado in November 2012.*

and the Cd, Cu, and Zn concentrations decreased. Therefore, despite the Zn concentration at Site 4 being near the LC50value determined in MHR water in the laboratory, the elevated hardness in Site 4 water (compared to MHR water) probably protected against lethality. In the main stem of Clear Creek (Sites 5 and 6), the Cd, Cu, and Zn concentrations were below the LC50 values determined for those metals in MHR water; and all the Daphnia survived, consistent with prediction for waters with those hardness and alkalinity concentrations.

Conclusions

Mine-drainage waters are complex mixtures of metals and major ions that can alter the chemistry of receiving waters in complex ways. The North Fork of Clear Creek is an example of this situation. AMD is a reactive solution; and after it enters receiving water, the speciation of metals and ions can change depending for example on temperature, pH, dissolved oxygen concentration. These reactions are kinetically limited, thus changing the concentrations of chemical constituents over time as the mixture of AMD and receiving water flows downstream. Therefore, the toxicity of the mixture can vary greatly and depends on the water chemistry at a specified point in the stream. Models of the toxicity of metal mixtures that take into account varied water chemistry (*e.g.* biotic ligand models) and interactions among the metals are needed to address these situations more accurately than current approaches that use water hardness as the only predictor of toxicity. Based on the laboratory study of metal-mixture toxicity that was conducted in conjunction with the field study, we conclude that metal-metal interactions among Cd, Cu, and Zn are generally either additive or less-than-additive based on the metal competition for binding to the biotic ligand. Failure to consider this competition can mistakenly lead to the conclusion that the mixtures are more-than-additive.

References

APHA. 2012. Standard Methods for the Examination of Water and Wastewater. 22 nded. E.W. Rice (ed.). American Public Health Association, Washington, DC.

de March B.G.E (1988) Acute toxicity of binary mixtures of five cations (Cu^{2+}, Cd^{2+}, Zn^{2+}, Mg^{2+}, and K^+) to thefreshwater amphipod Gammaruslacustris (Sars): alternative descriptive models. Can J Fish AquatSci 45:625–633

Meyer, J.S., S.J. Clearwater, T.A. Doser, M.J. Rogaczewski and J.A. Hansen. 2007. Effects of Water Chemistry onthe Bioavailability and Toxicity of Waterborne Cadmium, Copper, Nickel, Lead, and Zinc to Freshwater Organisms. SETAC Press, Pensacola, Florida, USA.

USEPA (U.S. Environmental Protection Agency). 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth edition. EPA-821-R-02-012, U.S. Environmental Protection Agency, Washington, DC, USA.

Warne MS, Hawker DW (1995) The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: the funnel hypothesis. Ecotoxicol Environ Saf 31:23–28