USING ENZYME BIOASSAYS AS A RAPID SCREEN FOR METAL TOXICITY[1]

E.P. Blumenstein[2], J.F. Ranville[3], L.M. Choate[4], and P.E. Ross[5]

Abstract: Mine tailings piles and abandoned mine soils are often contaminated by a suite of toxic metals, which were released in the mining process. Traditionally, toxicity of such areas has been determined by numerous chemical methods including the Toxicity Characteristic Leachate Procedure (TCLP) and traditional toxicity tests using organisms such as the cladoceran *Ceriodaphnia dubia*. Such tests can be expensive and time-consuming. Enzymatic bioassays may provide an easier, less costly, and more time-effective toxicity screening procedure for mine tailings and abandoned mine soil leachates.
This study evaluated the commercially available MetPLATE™ enzymatic toxicity assay test kit. The MetPLATE™ assay uses a modified strain of *Escherichia coli* bacteria as the test organism. Toxicity is defined by the activity of β-galactosidase enzyme which is monitored colorometrically with a 96-well spectrophotometer. The study used water samples collected from North Fork Clear Creek, a mining influenced water (MIW) located in Colorado. A great benefit to using the MetPLATE™ assay over the TCLP is that it shows actual toxicity of a sample by taking into account the bioavailability of the toxicants rather than simply measuring the metal concentration present. Benefits of the MetPLATE™ assay over the use of *C. dubia* include greatly reduced time for the testing process (~2 hours), a more continuous variable due to a greater number of organisms present in each sample (100,000+), and the elimination of need to maintain a culture of organisms at all times.

Additional Key Words: enzyme bioassay, metal contamination, mine tailings, contaminated soils, toxicity testing, MetPLATE™

---

[1]Paper was presented at the 2005 National Meeting of the American Society of Mining and Reclamation, Breckenridge CO, June 19-23 2005. Published by ASMR, 3134 Montavesta Rd. Lexington, KY 40502.

[2]Eric P. Blumenstein, Graduate student, Department of Environmental Science and Engineering, Colorado School of Mines, Golden, CO 80401, [3]James F. Ranville, Associate Professor, Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO 80401, [4]LaDonna M. Choate, United States Geological Survey, [5]Philippe E. Ross, Professor, Department of Environmental Science and Engineering

Proceedings America Society of Mining and Reclamation, 2005 pp 98-107
DOI: 10.21000/JASMR05010098
https://doi.org/10.21000/JASMR05010098

Introduction

An important aspect of mining influenced water (MIW) is in determining which mine tailings piles and abandoned mine soils within the watershed will generate toxic effects to the aquatic organisms. Traditional hazard evaluation for mine tailings piles and abandoned mine soils has been done using the Toxicity Characteristic Leachate Procedure (TCLP) or by exposing organisms, such as the cladoceran *Ceriodaphnia dubia*, to various concentrations of leachates from the soils. Such tests can be expensive, time-consuming, and may not take important variables such as bioavailability into account. Enzymatic bioassays may provide an easier, less costly, and more time-effective toxicity screening procedure for mine tailings and abandoned mine soil leachates.

Several enzyme and microbial assays have been proposed for assessing the toxicity of environmental samples (Bitton and Dutka, 1986; Bitton and Koopman, 1992; Wells et al., 1998; Bitton and Morel, 1998). Most of the proposed toxicity tests measure the general toxicity of a sample which can arise from organic or inorganic toxicants. The MetPLATE™ enzymatic toxicity assay test kit focuses on the specific determination of heavy metal toxicity (Bitton et al., 1994). Because the toxic components of MIW are most often heavy metals, the MetPLATE™ test kit is an appealing enzymatic bioassay for our application. As such, this study compares the results of the MetPLATE™ enzymatic toxicity assay test kit to those from the more traditional *C. dubia* toxicity tests mentioned above.

The MetPLATE™ assay uses a modified strain of the *Escherichia coli* bacteria as the test organism. When the *E. coli* bacteria are not stressed, they produce the enzyme β-galactosidase, which cleaves a chromogenic substrate. Conversely, when the *E. coli* bacteria are stressed, they cleave lesser amounts of substrate or no substrate at all. The inhibition of the enzyme can be measured colorometrically with a 96-well spectrophotometer. Using the MetPLATE™ assay has benefits over both the TCLP and *C. dubia* testing. A great benefit to using the MetPLATE™ assay over the TCLP is that it accurately portrays the actual toxicity of a sample by taking into account the bioavailability of the toxicants rather than simply measuring the metal concentration present. Benefits of the MetPLATE™ assay over the use of *C. dubia* include greatly reduced time for the testing process (~2 hours), a more continuous response due to a greater number of organisms present in each sample (100,000+), and the elimination of need to maintain a culture of organisms.

The MetPLATE™ assay was evaluated by comparing results with traditional toxicity tests. This study explored the potential use of MetPLATE™ enzymatic toxicity assays in determining the toxicity of metals in mining impacted soils (MIS). Toxicity evaluation of soils will rely on using MetPLATE™ on a water leachate procedure that is still under development. In the initial phase of the study, reported in this paper, we examined metal toxicity for a surface water sample collected from the North Fork Clear Creek and laboratory prepared solutions. This initial stage of the study addresses the following questions:

- Is the MetPLATE™ assay able to give accurate and reproducible results for lab samples and field samples?
- Can a correlation be formed between the MetPLATE™ assay and traditional aquatic toxicity tests?

Sampling and Analytical Methods

Sampling Methods

Sampling occurred on the 26th of May 2004 along the North Fork and Main Stem of Clear Creek just west of Golden, CO. The sampling sites of interest in this paper are NCC-SW-31, NCC-SW-28, NCC-SW-27, NCC-SW-16, NCC-SW-12, NCC-SW-6, and NCC-SW-3. Location of the sampling sites for the North Fork and Main Fork of Clear Creek are displayed in Fig. 1 below.

Water samples were collected with 500 mL and 5-gallon plastic containers after being washed twice with the river water at each site. Nitrile gloves were worn when handling all sample bottles and taking all samples. Upon returning to the Colorado School of Mines (CSM), the water samples were refrigerated until use and used no later than 36 hours after collection. All water samples, dilutions, and metal spikes were handled and prepared at the U.S. EPA Region VIII Laboratory and a USGS Central Region laboratory, explicitly following the U.S. EPA's protocols (U.S. EPA, 2002). Cu and Zn were added using single metal analytical standards dissolved in nitric acid.

Analytical Methods

Procedures used for the enzymatic assay, *C. dubia* tests, and elemental analyses are shown individually below.

MetPLATE™ Toxicity Assay. The MetPLATE™ kit (University of Florida, Gainesville, Florida) includes freeze-dried *E. coli* ("Bacterial Reagent"), moderately hard water ("Diluent"), phosphate buffered enzyme substrate ("Buffer"), and one 96-well microplate. The freeze-dried bacterial reagent is rehydrated into 5.0 milliliters (ml) of diluent and is mixed thoroughly by vortexing until a uniform suspension is obtained. A volume of 0.1 ml of bacterial reagent is then added to 0.9 ml of solution or a dilution thereof. The mixture is then vortexed and incubated at 35˚C for 60 minutes. At the end of the 60-minute exposure period a 0.2 ml aliquot of the suspension is dispensed in a well of the assay microplate. Then, 0.1 ml of the substrate is added to each well. Upon mixing in each of the wells, the microplate is incubated an additional 60 to 90 minutes at 35˚C for color development. The intensity of the resulting purple color gives an indication of enzyme (β-galactosidase) activity and is inversely proportional to the toxicity of the sample. Absorbance is measured at 575 nanometers (nm) using a 96-well microplate reader (PowerwaveX 340). All toxicity tests and samples are run in triplicate (Bitton et al., 1994). An example of a typical MetPLATE™ assay after incubation that is measured in the 96-well spectrophotometer can be seen in Fig. 2.

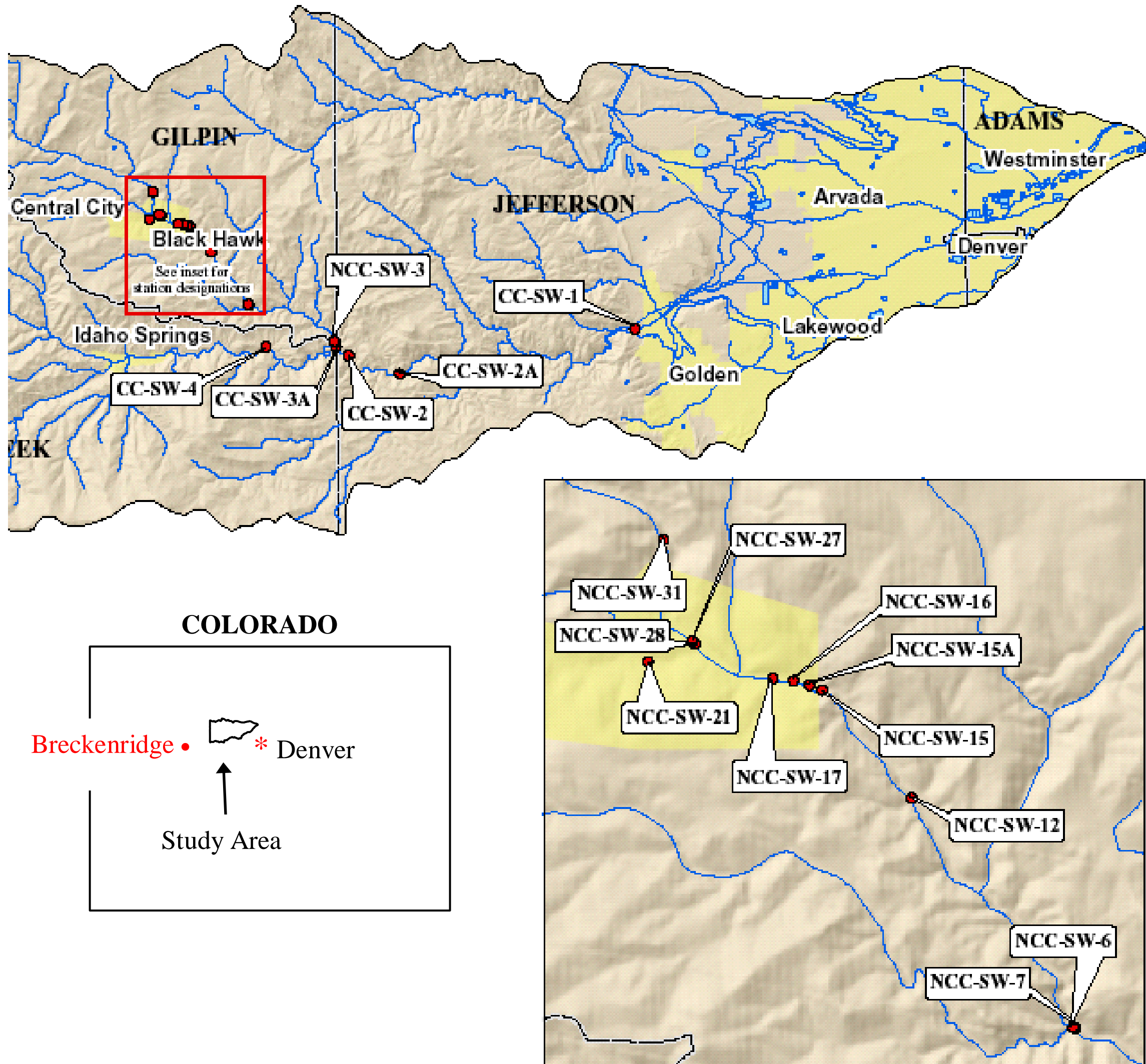

Figure 1 – Sampling Sites on Clear Creek

Ceriodaphnia dubia Toxicity Test. The procedure used for this test was the United States Environmental Protection Agency's standard operating procedure (SOP) for *Ceriodaphnia dubia* toxicity testing (U.S. EPA, 2002). The EPA SOP 2002.0 calls for a static test that is 48 hours long and the samples are held at 20˚C ± 1˚C. Each day consists of 16 hours of daylight and 8 hours of dark, to simulate a diurnal cycle. Each individual test chamber is a 30 mL plastic cup that is filled 15-20 mL with the sample liquid. The EPA uses six different dilutions of sample liquid (100%, 50%, 25%, 12.5%, 6.25%, and 0%) with four replicate chambers of each dilution and five test organisms per chamber, for a total of 20 test organisms per sample dilution. The dilution water is moderately hard reconstituted water for studies with synthetic water samples and clean river water taken upstream of the contaminant influence for studies with field-collected water samples. Each of the test organisms must be less than 24 hours old and are fed for two hours prior to transfer into the test chamber.

After 48 hours, each test chamber is examined to determine what affect the sample water had on the organisms. The endpoint of the test is mortality and the measured and reported value is the lethal concentration at which 50% of the organisms have died (LC_{50}). The result of a test is valid if 90% of the organisms in the control (0% dilution) survive.

Figure 2 - MetPLATE™ 96-well Microplate with Colorometric Response

Elemental Analyses. The water samples were analyzed for elemental concentrations using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) at CSM. Approximately 10 ml of filtered sample, acidified with nitric acid, was required. The samples were then analyzed on a Perkin Elmer Optima 3000 ICP-AES for 31 elements including those of importance for this study: Cu and Zn. Cu and Zn were chosen because they are the two metals that are thought to cause toxicity to the biota in the Clear Creek stream system. All concentration results are given in milligrams per liter (mg/L). During the ICP-AES analysis, an internal standard of scandium is used to correct for variations in sample uptake and plasma conditions. Concentration check standards are analyzed in the beginning and after every 20 samples to monitor the stability of all analytical conditions. The relative standard deviation of the water sample results is about ±5% for concentration that are greater than 10 times the limit of detection.

Results

MetPLATE™ Assay Results

Results for the initial stage of the study are reported and displayed below. Fig. 3 shows a typical result from a MetPLATE™ assay. In this graph the different site waters are plotted along the x-axis with the flow direction being from left to right, and the absorbance is plotted along the y-axis. The spectrophotometer measures absorbance which allows for a quantitative measurement of the concentration at which 50% of the organisms are adversely affected (EC_{50}). The spectrophotometer measures a high absorbance reading when there are healthy *E. coli* in the wells, and a low absorbance reading when the *E. coli* are being negatively impacted by toxicants in the sample waters. The absorbance that lies halfway between that of the positive and negative controls is used to compute the EC_{50} for the sample.

As Fig. 3 clearly indicates, the more diluted the sample water is, the less toxic it is to the organisms. The exception to this is the major mine water input site (SW 27), which remains almost entirely toxic until the 12.5% dilution. There is a lower absorbance reading at the higher site water dilutions because the *E. coli* are not healthy and are not producing the ß-galactosidase enzyme, and therefore, not cleaving the chromogenic substrate. These were the expected results and display the relationship of absorbance versus contaminant concentration.

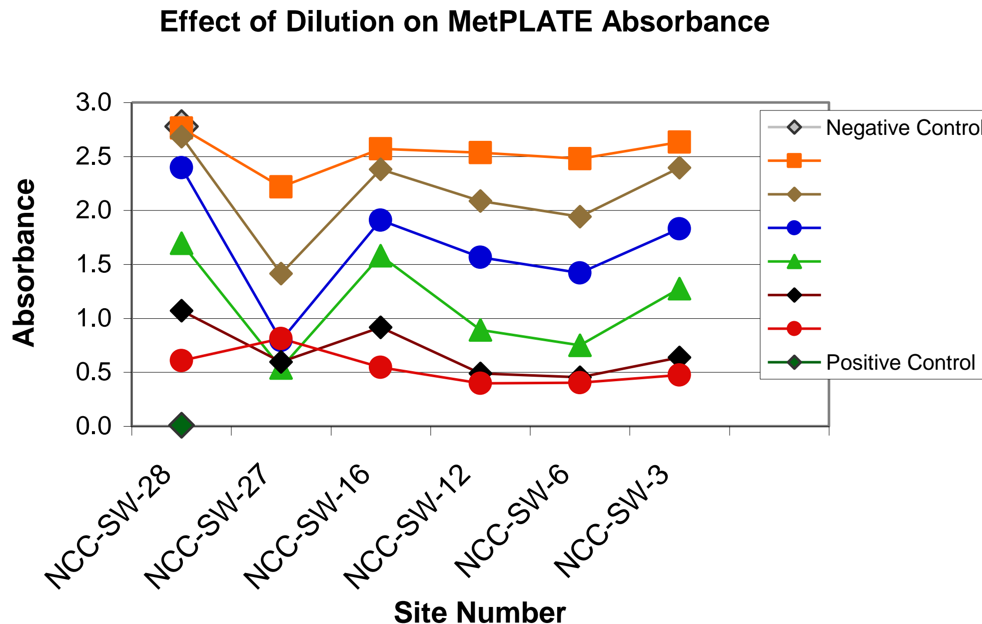

Figure 3 - MetPLATE™ Dilutions for Site Sample Waters

Relative absorbance versus the concentration of Cu and Zn for a MetPLATE™ enzyme assay using a dilution of the sample is shown in Fig. 4 below. The dilution level of 12.5% sample water was chosen because it most nearly represents the EC_{50} for the MetPLATE™ assay at the site, as shown in Fig. 3. The MetPLATE™ assay provides a good correlation between absorbance and the log of the metal concentration. The closed symbols represent the absorbance of the MetPLATE™ assay with regard to Cu concentration, while the open symbols represent the absorbance of the MetPLATE™ assay with regard to Zn concentration. The absorption vs. Cu relationship resulted in an R^2 value of 0.842 while the Zn vs. absorption relationship provides a slightly higher R^2 value of 0.936. Both metals showed a dose response relationship with increasing concentration. However, the fact that both metals were present in the sample makes it difficult to identify the metal responsible for toxicity.

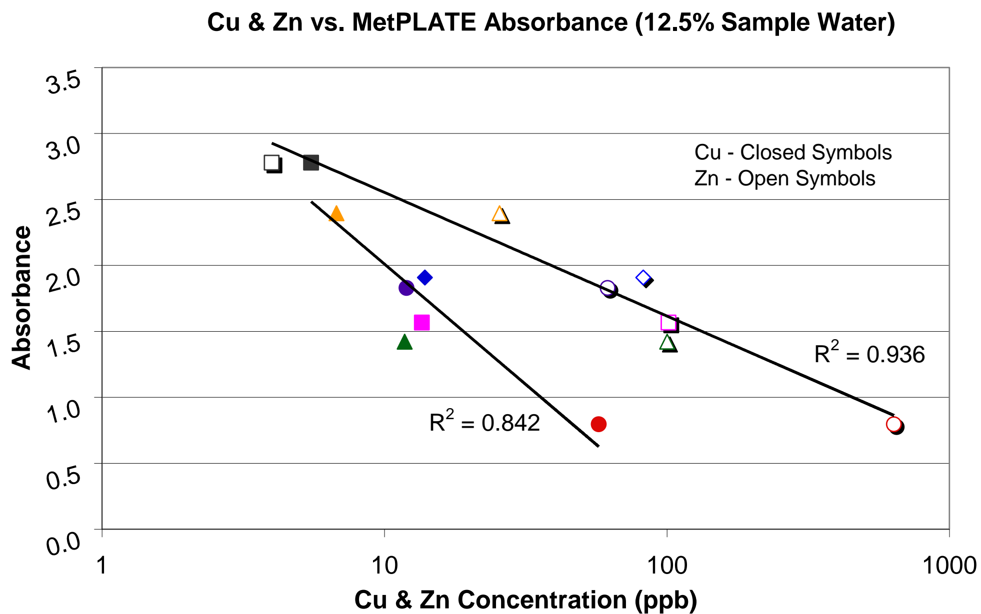

Figure 4 - Correlation Between Absorbance & Metal Concentration

After it was determined that the MetPLATE™ assay produced a dose response for Cu and Zn, a comparison was made between the MetPLATE™ assay and traditional *C. dubia* toxicity tests to determine if there was a significant correlation between the two. Experimental results of these comparative field sample studies are presented below.

MetPLATE™ and *C. dubia* Field Sample Comparisons

Before conducting MetPLATE™ and *C. dubia* field sample comparison experiments, a literature review was performed to determine if a prior such study had been done. The only study that used MetPLATE™ and *C. dubia*, under the same experimental conditions

as those mentioned above, was that conducted by Nelson and Roline (1998). MetPLATE™ EC_{50}s and *C. dubia* LC_{50}s for Cu and Zn from the Nelson and Roline (1998) study, and the results from the MetPLATE™ and *C. dubia* field sample comparison of this study, are shown in Table 1.

Table 1. MetPLATE™ & *C. dubia* Comparison Values

	MetPLATE™ EC_{50}		***C. dubia* LC_{50}**	
	Nelson and Roline*	This Study	Nelson and Roline*	This Study
Copper (ppb)	113±41	128	18±3	13
Zinc (ppb)	128±65	114	128±23	60

* (Nelson and Roline, 1998)

Figs. 5 and 6 display the comparison of the MetPLATE™ assay to the *C. dubia* test from this study graphically, for Cu and Zn, respectively.

Copper Conc. vs. MetPLATE Abs. and C. dubia Survival

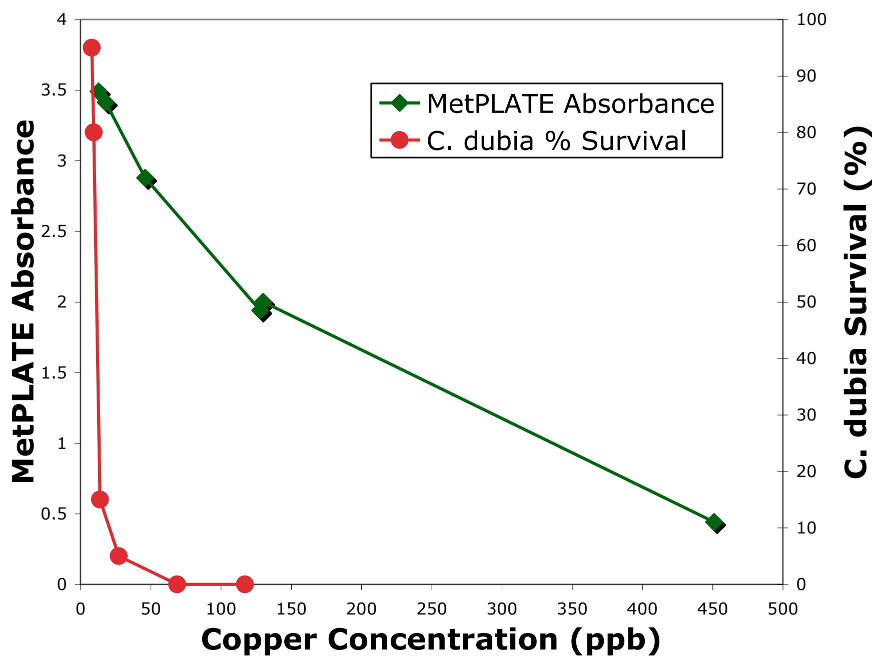

Figure 5 – Copper Concentration vs. MetPLATE™ Absorbance & *C. dubia* Survival

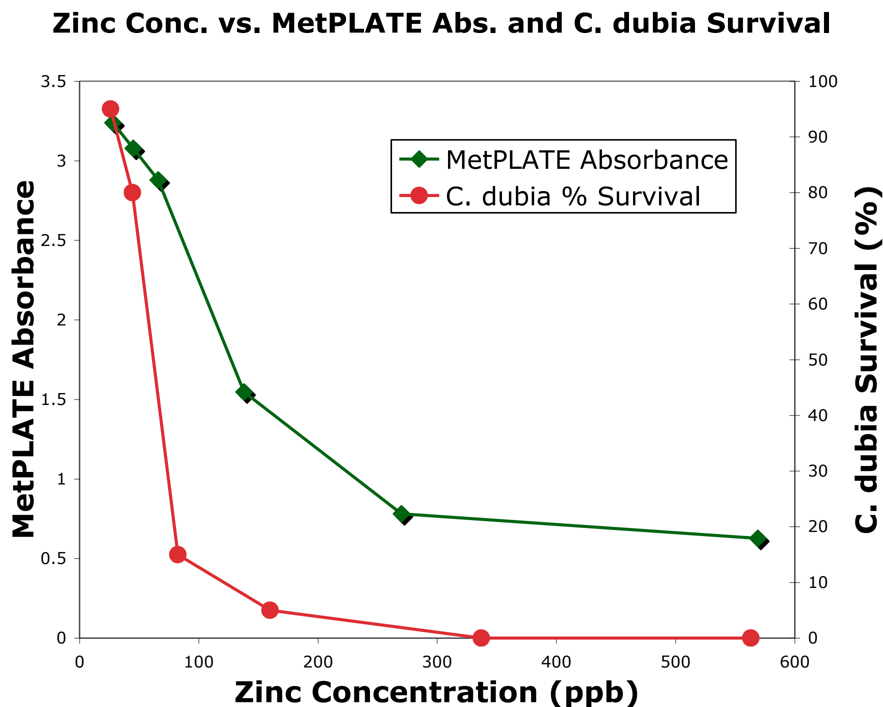

Figure 6 – Zinc Concentration vs. MetPLATE™ Absorbance & *C. dubia* Survival

Figs. 5 and 6 show that the MetPLATE™ assay does show a good correlation with the *C. dubia* test results, but the traditional *C. dubia* toxicity tests are more sensitive than are the MetPLATE™ assays. Nelson and Roline's (1998) experiments show that the EC_{50}'s for Cu are an order of magnitude different in the MetPLATE™ assay and the *C. dubia* toxicity test, while the EC_{50}'s for Zn are not statistically different between the two. These numbers are reported in Table 1 above.

Figs. 5 and 6 and Table 1 display that the Zn EC_{50} for *C. dubia* in the field sample is a factor of two lower than what was expected from the Nelson and Roline (1998) experiments. The reason for this is that the field samples contained both Cu and Zn, while the Nelson (1998) tests were run with a single metal. The fact that the Cu EC_{50} for *C. dubia* remained the same in the field sample experiments as Nelson and Roline's (1998) experiments, while the Zn EC_{50} for *C. dubia* decreased by a factor of two, indicates that Cu is the driving force of *C. dubia* toxicity in the Clear Creek stream system.

These results suggest that a method using both bioassays could be developed to identify which metal, Cu or Zn is responsible for toxicity in MIWs that contain both metals. If the Zn *C. dubia* LC_{50} is roughly equivalent to the Zn MetPLATE™ EC_{50}, then Zn is the driving force for toxicity in the MIW. However, if the Cu *C. dubia* LC_{50} is an order of magnitude lower than the MetPLATE™ EC_{50} and the Zn *C. dubia* LC_{50} is significantly lower than the Zn MetPLATE™ EC_{50}, then Cu is most likely causing the toxicity.

Conclusions

As the above figures indicate, preliminary results indicate that it will be possible to form a correlation between traditional aquatic toxicity testing and enzyme bioassay testing. Calculating the EC_{50}'s for the enzyme tests show that there is a correlation between the MetPLATE™ assay and the *C. dubia* toxicity tests for Cu and Zn. The Cu MetPLATE™ EC_{50} is an order of magnitude higher than the Cu *C. dubia* EC_{50}, while the Zn MetPLATE™ EC_{50} is not statistically different than the Zn *C. dubia* EC_{50}. This approach using the differential responses between organisms for Cu and Zn could prove to be a very useful tool for toxicity assessment. These experiments also show that Cu, not Zn, is the driving force of *C. dubia* toxicity in the Clear Creek stream system.

Acknowledgements

Funding for this research includes an U.S. EPA Star Grant for the Center for the Study of Metals in the Environment (Grant # R-82950001; Sub-award 522120) and U.S. EPA Rocky Mountain Regional Hazardous Substances Center (Grant # R-82951501-0). The Edna Bailey Sussman Fellowship and the USGS Mendenhall Postdoctoral Fellowship provided additional funding. Sandra Spence and VelRey Lozano of the U.S. EPA Region VIII Laboratory, and many members of the CSM metals and toxicology research group provided research assistance.

Literature Cited

Bitton, G. and B. J. Dutka. 1986. Toxicity Testing Using Microorganisms. Vol. 1. Boca Raton, FL: CRC Press.

Bitton, G., K. Jung, B. Koopman. 1994. Evaluation of a Microplate Assay Specific for Heavy Metal Toxicity. p. 25-28. *In*: Arch. Environ. Contam. Toxicol. 27.

Bitton, G. and B. Koopman. 1992. Bacterial and Enzymatic Bioassays for Toxicity Testing in the Environment. p. 1-22. *In*: Rev. Environ. Contam. Toxicol. 125.

Bitton, G. and J. L. Morel. 1998. Enzyme Assays for the Detection of Heavy Metal Toxicity. *In*: Wells, P. G., K. Lee, and C. Blaise. 1998. Microscale Aquatic Toxicology: Advances, Techniques and Practice. Boca Raton, FL: CRC Press.

Nelson, S. M. and R. A. Roline. 1998. Evaluation of the Sensitivity of Rapid Toxicity Tests Relative to Daphnid Acute Lethality Tests. p. 292-299. *In*: Bull. Environ. Contam. Toxicol. 60.

U.S. EPA. 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Fresh Water and Marine Organisms. Fifth Edition. October 2002. EPA-821-R-02-012

Wells, P. G., K. Lee, and C. Blaise. 1998. Microscale Aquatic Toxicology: Advances, Techniques and Practice. Boca Raton, FL: CRC Press.