GROWTH INHIBITION OF *CELLULOMONAS FLAVIGENA* INDUCED BY COPPER AND ZINC: DETERMINATION OF TOXICITY THRESHOLDS AND THE COMBINED EFFECTS OF COPPER AND ZINC ON THE GROWTH OF A CELLULOLYTIC-FERMENTING BACTERIA[1]

A. Ruhs[2], L. Figueroa[2] and T. R. Wildeman[2]

Extended Abstract: The effective use of anaerobic passive treatment systems (APTS), such as sulfate-reducing bioreactors, to treat acid mine drainage will help to mitigate water contamination from mines located in remote areas as well as cut current treatment costs. One drawback to APTS has been an observed decline in long-term performance. Several environmental factors, such as initial metals concentration and temperature, may contribute to observed declines in sulfate reduction carried out by the microorganisms. APTS contain a complex microbial ecosystem, and metal toxicity could be indirectly affecting sulfate-reduction by inhibiting other important microbes. Previous research has found that organisms capable of degrading cellulose (cellulolytic-fermenters) are dominant within a sulfate reducing bioreactor (Pruden et al., 2005), and their ability to produce viable substrates for the sulfate-reducing bacteria is the rate-limiting step in sulfate reduction (Logan, 2003). This investigation examines the individual effect of zinc and copper, and then the combination of both metals, on a pure culture of cellulolytic fermenters, specifically *Cellulomonas flavigena* (ATCC 482).

C. flavigena exhibited 50% growth inhibition between a copper concentration of 0.00188 mM and 0.0038 mM. Further investigation of growth inhibition within this range of copper concentrations is currently underway. Glucose consumption was also much less in the copper containing bottles, as expected due to the low biomass observed in these bottles. The pH remained relatively constant throughout the experiments, staying within an optimal microbial growth range between pH 6 and 7. Possible changes in solution phase metals concentration and organic acid production throughout the experiments were monitored, and these results will be available during the ICARD conference. Concentrations of Zn(II) at 0, 0.125, 0.25 and 0.5 mM were also examined to determine the effects on cell growth for *C. flavigena*. The results for zinc toxicity and a binary metal mixture will be presented at the 2006 ICARD conference.

Additional Key Words: Anaerobic Passive Treatment System, sulfate reducing bioreactor

---

[1] Poster paper presented at the 7th International Conference on Acid Rock Drainage (ICARD), March 26-30, 2006, St. Louis MO. R.I. Barnhisel (ed.) Published by the American Society of Mining and Reclamation (ASMR), 3134 Montavesta Road, Lexington, KY 40502

[2]Alison Ruhs, Graduate Research Assistant, Environmental Science and Engineering, Colorado School of Mines, Golden, CO 80401, Linda Figueroa, Associate Professor, ESE, CSM, Golden, CO 80401, Tom Wildeman, Professor, Chemistry and Geochemistry, CSM, Golden, CO 80401.

https://doi.org/10.21000/JASMR06021768

The toxicity effects of Cu(II) and Zn(II) were determined based on increased lag time for cell growth, inhibited total cell protein, and lower specific growth rates that were measured over 240 hours, or ten days. All of the experiments were performed in quadruplicate, and graphical analysis was based on averages of the data collected. Cell protein was measured to establish growth and total cell biomass, and was determined with a Coomassie Protein Assay Reagent Kit, using a procedure developed by Sani et al. (2001). The Cu^{+2} concentrations of 0, 0.015 and 0.05 mM were initially used based on Cu^{+2} concentrations found to inhibit *D. desulfuricans*, a sulfate-reducing bacterium, (Sani et al., 2001). This gave us a starting place for establishing toxicity thresholds for *C. flavigena*. Sani et al. (2001) found that *D. desulfuricans* displayed 50% growth inhibition at a copper concentration of 0.016 mM. This was not the case for *C. flavigena*, as can be seen in Fig. 1. *C. flavigena* exhibited much greater than 50% inhibition of growth at 0.015 mM Cu concentration.

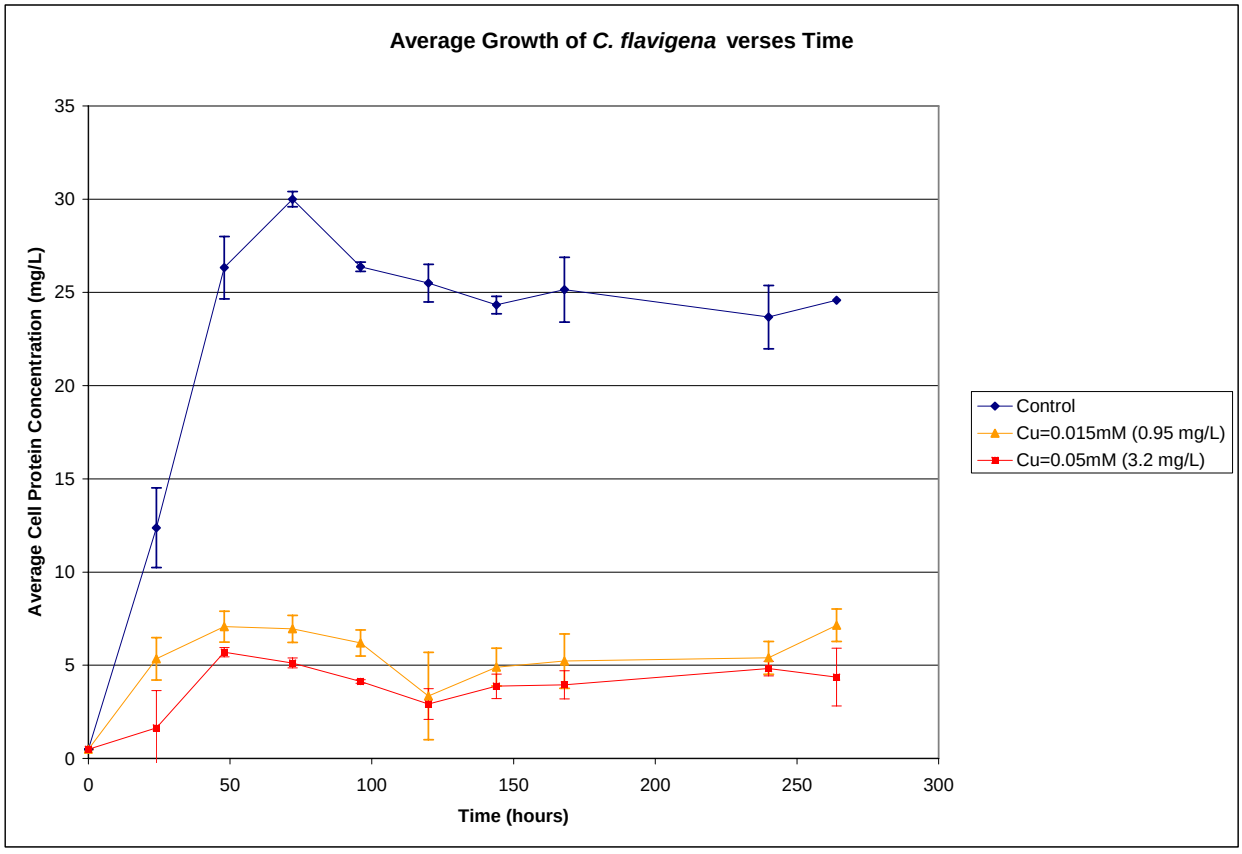

Figure 1. Average changes in cell protein concentration throughout 10 day experiment. Observation of growth kinetics for *C. flavigena* in the presence of dissolved Cu^{+2}.

In order to establish the Cu concentration that would cause 50% inhibition of total cell protein growth for *C. flavigena* an additional 5 Cu concentrations were examined, at concentrations of 0.00188 mM, 0.0038 mM, 0.0056 mM, 0.0075 mM, and 0.01 mM, respectively. The graphical interpretation of this data can be viewed in Fig. 2. Based on these findings, 50% inhibition appears to happen between a Cu concentration of 0.00188 mM and 0.0038 mM. Further investigation of growth inhibition within this range of Cu concentrations is

currently underway and will be presented during the 2006 ICARD conference.

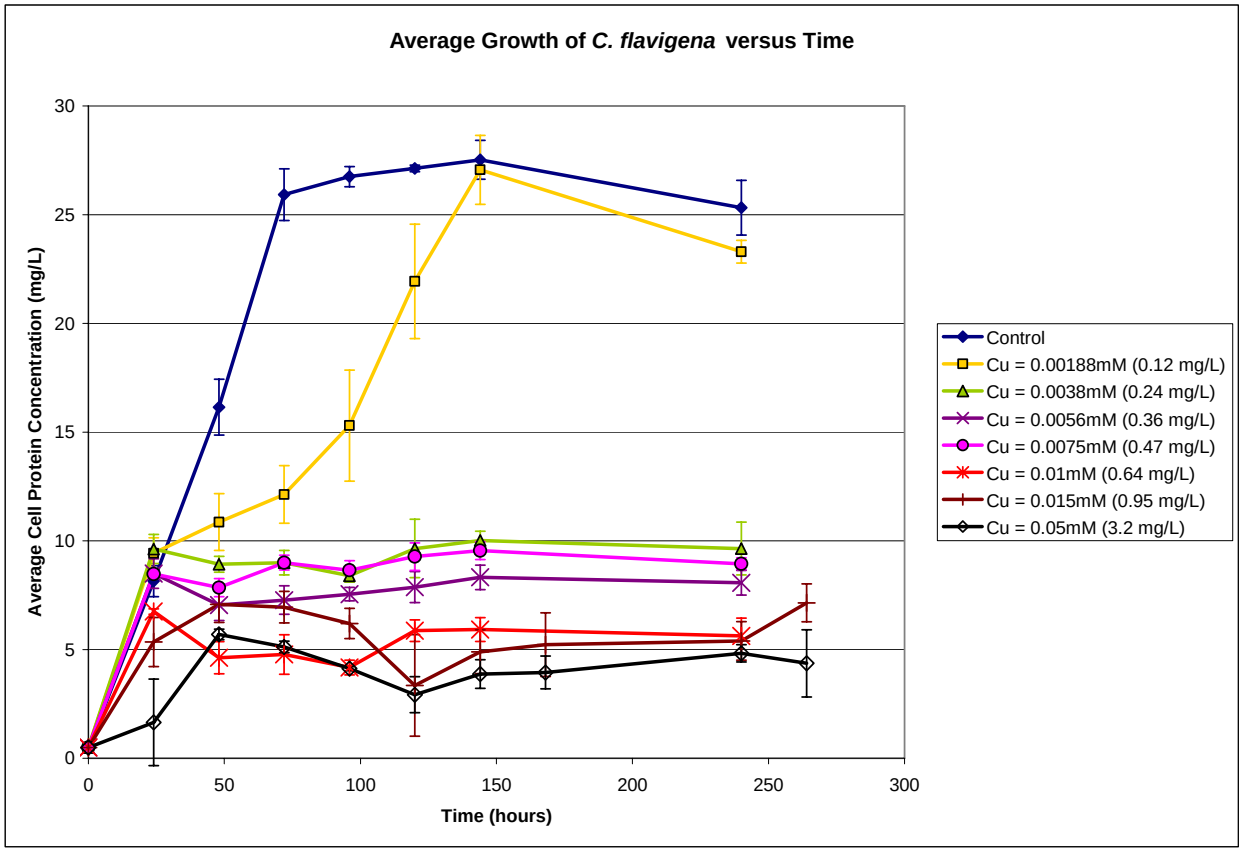

Figure 2. Average changes in cell protein concentration throughout 10 day experiment. Observation of growth kinetics for *C. flavigena* in the presence of dissolved Zn^{+2}.

The pH, glucose consumption, metals concentration, and organic acid production were all monitored throughout the experiments. The pH remained relatively constant, staying within an optimal microbial growth range between pH 6 and 7, and can be seen in Fig. 3.

As can be seen in Fig. 3, the control bottles, containing no Cu, and the bottles containing a Cu concentration of 0.00188 mM, showed the most significant drop in pH. This would be expected since these bottles exhibited significantly better cell growth, which would correspond to higher organic acid production. All the bottles remained within an optimum pH range, and therefore it can be concluded that pH had no significant effect on cell growth during these experiments.

Glucose consumption was also observed during the experiment using a colorimetric method developed by Dubois et al. (1956). In Fig. 4 is shown the decrease of glucose within the bottles over time. As expected, during high growth rates the glucose drops dramatically, and the bottles containing very little biomass consumed a smaller amount of total glucose.

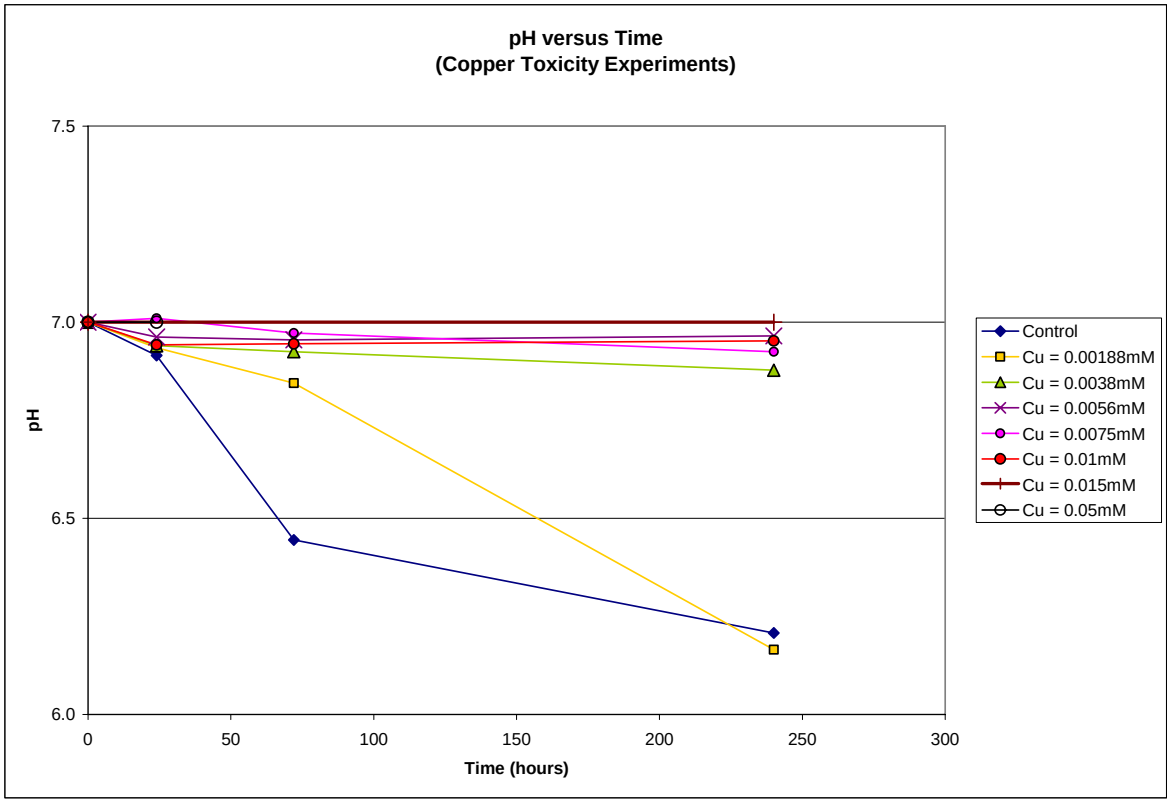

Figure 3. Fluctuations in pH observed during experiments.

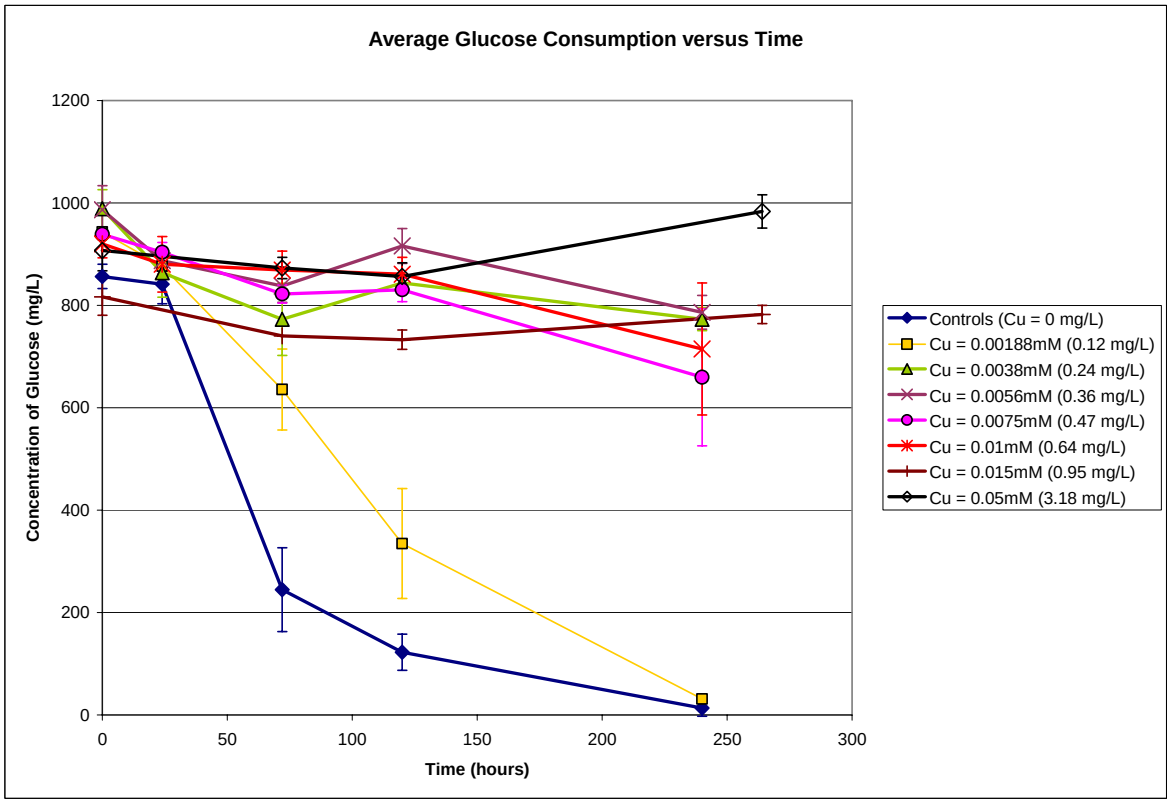

Figure 4. Average changes in glucose within experimental bottles over time.

The concentration of Cu was verified through ICP analysis (Perkin Elmer, Optima 3000). Samples were also taken throughout the experiment to determine if the dissolved Cu concentration remained constant. We wanted to establish whether or not sorption onto the cell wall occurred, causing a significant drop in bio-available copper. The ICP results from these experiments are still being analyzed and will be presented at the ICARD conference. Results regarding the production of organic acids, such as lactate and acetate, during the experiments are also being analyzed and will be available at the conference.

Concentrations of Zn^{+2} at 0, 0.125, 0.25 and 0.5 mM were also examined to determine the effects on cell growth for *C. flavigena*. A Zn^{+2} concentration of 0.5 mM was chosen because of previous toxicity research we had conducted that examined inhibition of glucose utilization by *C. flavigena* in the presence of Zn^{+2} at 0.5 mM. This preliminary research was conducted over a 9 hour period with cell biomass concentration held constant at 250 mg/L cell protein. The results from this prior research show significant inhibition of substrate utilization by *C. flavigena* at Zn^{+2} concentrations of 0.5 mM based on the decline in rate of glucose consumption by *C. flavigena*, which can be seen in Fig. 5 below:

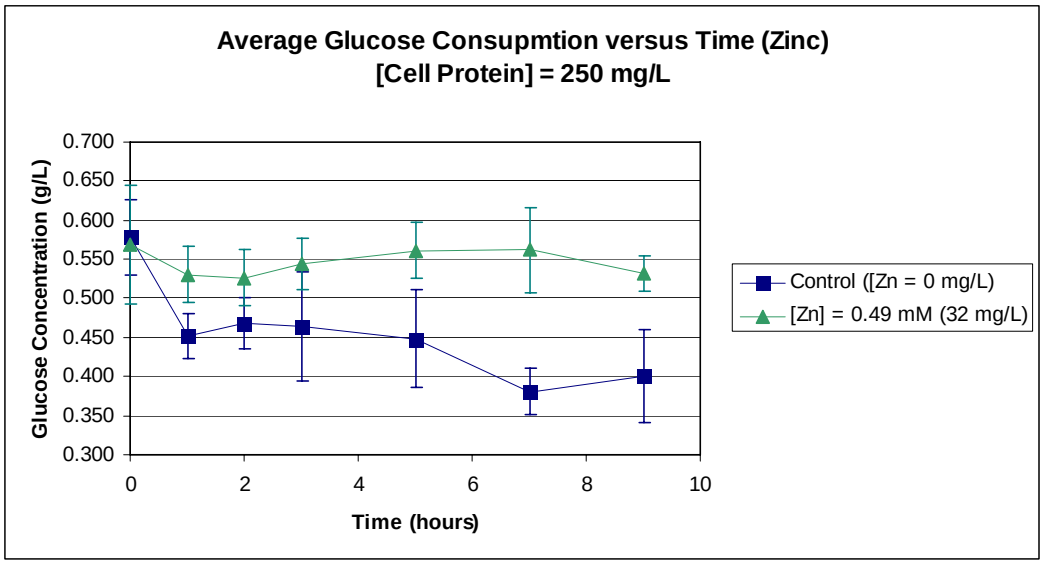

Figure 5. Average changes in glucose concentration during 9 hour experiment.

The serum bottles containing no Zn consumed glucose at a faster rate than the serum bottles containing Zn^{+2}. Data collection for this study is ongoing and additional results for the poster are expected by January 2006.

Literature Cited

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. "Colorimetric method for determination of sugars and related substances". *Anal. Chem. 28*:350.

Logan, M.V. 2003. "Microbial activity and the rate-limiting step in degradation of cellulose-based organic material to support sulfate reduction in anaerobic columns treating synthetic mine drainage". M.S. Thesis, T5797, Colorado School of Mines, Golden, CO.

Pruden, A., H. Hong, L.Y. Inman, M.V. Logan, C. Sans, K.F. Reardon, and L.A. Figueroa. 2005. "Microbial characterization of sulfate-reducing columns remediating acid mine drainage". *In Press, ASMR Conference.*

Sani, R.K., B. Peyton, W. Smith, W. Apel, and J. Petersen. 2001. "Copper-Induced Inhibition of Growth of *Desulfovibrio desulfuricans* G20: Assessment of its Toxicity and Correlation with Those of Zinc and Lead.". *Appl. And Environ. Microbiol. 67*:4765-4772.

Sani, R.K., G. Geesey, and B.M. Peyton. 2001. "Assessment of lead toxicity in *Desulfovibrio desulfuricans* G20: influence of components of lactate C medium". *Adv. Environ. Research. 5*: 269-276.