A STUDY OF ZINC METAL TOXICITY ON THE CELLULOLYTIC BACTERIA IN ANAEROBIC PASSIVE TREATMENT SYSTEMS[1]

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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 draw back to these systems has been the inhibition of sulfate reduction with high concentrations of metals. APTS contain a complex microbial ecosystem, and metal toxicity could be indirectly affecting sulfate-reduction by inhibiting other important microbes. If microbes such as the cellulolytic - fermenting bacteria are inhibited from producing viable substrate for the sulfate-reducing bacteria, then the rate of sulfate reduction over time in APTS will ultimately decline.

We examined the toxic effect of zinc, a common metal found in acid mine drainage, on a pure culture of *Cellulomonas flavigena*, a cellulolytic - fermenting bacteria. Serum bottles containing *C. flavigena*, at two protein concentrations of 250 and 500 mg/L, were exposed to initial zinc concentrations of 0, 20, and 40 mg/L and monitored over a 9 hour period. The extent of inhibition on *C. flavigena* activity correlated best (r^2=0.93) with the mass ratio of zinc uptake to cell protein. Final zinc concentrations ranged from 0.9 to 2.2 mg/L. Zinc uptake was operationally defined as the total zinc removed from solution and includes sorption and internalization. Initial and final dissolved zinc concentration did not correlate well with extent of inhibition. In the presence of higher biomass the relative rate of glucose utilization was 20 to 50% higher in the presence of zinc than at lower biomass concentration. The concurrent internalization of metals with sorption and precipitation processes can produce inhibition in the presence of low metal concentration. Thus low effluent metal concentration may not be indicative of the extent of inhibition experienced by the microbes. The inhibitory effect of metals on cellulolytic - fermenting bacteria is an important aspect to consider when establishing the limitations of sulfate reducing biozones.

Additional Key Words: water treatment, sulfate reducing bioreactor

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Introduction

Surface mining and deep mining activities have been found to enhance a phenomenon known as acid mine drainage (AMD), which is caused by the oxidation of sulfide minerals, and typically leads to acidic conditions with high levels of dissolved metals and sulfate. AMD is detrimental to aquatic life and expensive to treat. There are currently thousands of abandoned mines throughout the Western United States, 51,700 of these abandoned mine sites are within EPA Region 8, and many of these mines are in remote locations (Western Governor's Association 1998). Benner et al. (1997) estimated that 5,000 to 10,000 miles of streams in the western United States are impacted by AMD. Typical treatment for AMD would include the addition of alkaline chemicals, such as lime, in an attempt to buffer the acidic water and precipitate out dissolved metals. Such traditional site remediation techniques can be very expensive and are not feasible for remote and abandoned mining-related sites (USEPA, 1995). Due to a need for a cost effective, long-term, and low maintenance remediation technique, passive treatment systems, such as permeable reactive barriers (PRB's) and man-made wetlands, have become increasingly important for the remediation of remote mining locations.

Anaerobic passive treatment systems (APTS) rely on sulfate-reducing bacteria (SRB) for remediation of AMD. The SRB reduce the sulfate ion to sulfide, which leads to an increase in pH and precipitates the dissolved metals as insoluble sulfide metals (Barton and Tomei, 1995; Utgikar et al., 2000). Unfortunately the presence of heavy metals can be harmful to the biological process of the SRB by deactivating enzymes, denaturing proteins, and competing with essential cations (Mazidji et al., 1992; Mosey and Hughes, 1975). Several research studies have been conducted in an attempt to quantify the toxic impact of metal ions on SRB. Utgikar et al. (2003) looked at the direct toxicity effects of zinc and copper ions on acetate-utilizing culture of SRB and found the ultimate toxic concentrations of zinc and copper to be 20 mg/L and 12 mg/L, respectively. Heavy metals have been reported to reach toxic concentrations for SRB over a wide range, spanning from a few mg/L to as much as 100 mg/L (Booth and Mercer, 1963; Hao et al., 1994; Loka Bharathi et al., 1990; Poulsen et al., 1997; Saleh et al., 1964; Temple and Le Roux, 1964; Utgikar et al., 2001).

Passive treatment systems however are comprised of a complex microbial consortium, the SRB being only one of many different genera of bacteria present in a sulfate reducing biozone. The survival of SRB is closely related to the health of the microbial community in which they live. Master's thesis research done by Miranda Virginia Logan, from Colorado School of Mines, looked at the possibility "that sulfate reduction in passive treatment systems was limited by one or more upstream microbial activities that function as rate-limiting steps in generating substrate for sulfate-reducing bacteria" (Logan, 2003). Logan's results indicated that the hydrolysis of cellulose was the rate-limiting step for the support of sulfate reduction. Because SRB are unable to breakdown cellulose directly for their energy needs they must rely on cellulolytic - fermenting bacteria to provide the necessary substrates, such as lactate and butyrate. Considering the fact that PRB's and constructed wetlands (CW) are comprised mainly of organic material in the form of cellulose, one can see the need to look at the environmental constraints necessary to maintain a healthy community of cellulolytic - fermenting bacteria, who are responsible for the hydrolysis of cellulose into cellobiose and glucose and subsequent fermentation to lactate and butyrate.

Unpublished data from Logan's Master's work showed that nickel inhibited overall sulfate reduction in column experiments, but when lactate (a known substrate for many sulfate reducers)

was added to bioassays of the column material the observed extent of sulfate reduction from inhibited and uninhibited columns were the same. This suggests that the "upstream" microbial populations were affected by the nickel and were unable to produce the substrates needed by the sulfate reducing population. To date, very little work has been done to determine the toxicity affects of metal ions on the bacteria that hydrolyze cellulose. Without resolving the inhibitory effects of metals on bacteria such as the cellulolytic - fermenters, we could fail to completely understand the applicability of APTS. Here, we looked at the toxic effects of zinc ions on the cellulolytic - fermenting bacteria, specifically *Cellulomonas flavigena*, in an attempt to answer two hypotheses: 1) The extent of *C. flavigena's* inhibition by zinc will be dependent on the final dissolved zinc concentration, and 2) increased biomass will help reduce the toxic effects of zinc on *C. flavigena*. One approach many toxicity studies use is the determination of the LC_{50}, or the solution concentration of a toxic substance that will render a loss of activity or death for 50% of the population. This is difficult to quantify as bacteria are able to absorb and/or internalize many metals, and this can occur concurrently with other reactions in the system, such as precipitation. The rate and extent of metal sorption to cell surfaces or internalization are not well understood, and few studies have been done to determine the rate of these reactions and its importance relative to the rate and extent of chemical precipitation. A study conducted by Hu et al., (2003) looked at the impact of sorption and internalization for Cu, Zn, Ni, and Cd on nitrification inhibition. They found that inhibition by these metals was related to their intracellular fraction and the slow kinetics for internalization indicated that metal inhibition can easily be under predicted (Hu et al., 2003). The fact that LC_{50} could under-predict metal inhibition, and because little is known about the kinetics of sorption and internalization relative to metal precipitation, we may find that microbes within an APTS are actually being greatly inhibited even with the precipitation of heavy metals as metal sulfides.

Activity inhibition in this study was determined by comparing the removal of glucose in control bottles, containing no zinc, with those containing either 20 or 40 mg/L of zinc. *C. flavigena* is a facultative anaerobe, that can survive with or without the presence of oxygen, and is able to hydrolyze cellulose extracellularly to produce glucose which it then uptakes for its energy needs. We chose to use glucose, rather than cellulose, because glucose is the substrate directly utilized by the microbes for energy and growth. The microbes also breakdown glucose rapidly, which saved time, and more importantly, allowed us to focus on the changes in the rate of substrate utilization rather than growth. Shorter experiments minimized the possibility of significant changes in cell numbers and focused on changes in substrate utilization rather than the toxicity effects on biomass production. This approach is different from similar toxicity studies conducted with SRB (Poulson et al., 1997; Sani et al., 2001; Utigikar et al., 2001; Utigikar et al., 2003) which looked at the effects of certain metal concentrations on cell growth.

By determining the toxic effects of common AMD heavy metals, such as zinc, on cellulolytic - fermenting bacteria present in sulfate-reducing biozones, we may be able to predict in advance where anaerobic wetlands or sulfate reducing bioreactors will be successful. Performing toxicity tests looking at the effects of other heavy metals, in a similar manner as this research, could help establish parameters for treatment systems and mitigate some unnecessary costs associated with installing APTS in locations where long-term benefits cannot be achieved. By recognizing realistic limitations of APTS we can begin to focus on ways to supplement or compliment APTS and improve AMD treatment technologies.

Materials and Methods

Growth of bacteria

A pure culture was used, specifically *Cellulomonas flavigena* (ATCC 482), purchased from the American Type Culture Collection (Rockville, MD). The microbes were grown in autoclaved Erlenmeyer flasks containing beef nutrient broth, base for nonfastidious microorganisms (ISO 9000 registered, 8 g/l; Difco, Sparks, Md.) (Sani et al., 2002). Inoculation of the bacteria was completed under a sterile hood and the flasks were plugged with styrofoam in order to avoid any microbial contamination while still allowing for the diffusion of oxygen. *C. flavigena* was grown at ambient temperature on a shaker table under aerobic conditions to speed up the growth process. The log growth phase for *Cellulomonas* was determined in aerobic conditions at ambient temperature, by taking multiple Optical Density readings throughout a 42 hour period, which showed that the culture was well into a log growth phase within 24 hours and through 42 hours. Based on these findings, the bacteria were allowed to grow aerobically for 36 hours before they were concentrated down by centrifuging at 10,000 g (the acceleration of gravity, $1g = 9.8\ m.s^{-2}$) for 10 minutes. The floating broth was removed and the bacteria were washed with a 1.3 mM KCl solution and resuspended. The entire process was repeated 3 times to insure that all the broth was removed, and finally the pellet was resuspended in 1.3 mM KCl and inoculated into capped, sterile, serum bottles using a disposable sterile needle and syringe.

Purging and inoculation of bacteria

One crucial aspect of the APTS, in which microbial treatment occurs, is the absence of oxygen. Therefore, it was necessary to perform all of the experiments as anaerobic batch studies. First, solutions of buffer and zinc were put into autoclaved serum bottles. Glucose was used as the food source for the bacteria, which was autoclaved and added to the bottles in order to obtain a concentration of 2 mM. The serum bottles were closed with a septum and a metal crimp. They were then purged with argon for 20 minutes, and a redox dye indicator (Resazurin) was used at a concentration of 0.5 mg/L to insure anoxic conditions are established. Argon was used because it is an inert gas. CO_2 could not be used because the formation of carbonic acid and related species would occur as the CO_2 dissolved in water, which would cause fluctuations in pH as well as interact with the zinc in solution.

Microbial activity is affected by pH, thus a buffer called PIPES was used to minimize pH changes during the experiments. This buffer was chosen for a couple of reasons, one being that it is known for successfully autoclaving (no variation of pH is noted after autoclaving, pK_a of 6.8), and secondly, this buffer is very unlikely to complex and/or form a solid with zinc. A concentration of 100 mM of PIPES was used in each serum bottle. Three solutions were made with the different concentration of zinc in PIPES, and the pH was adjusted to 7.

After concentrating the bacteria by centrifuging, calculated volumes of bacteria were added to the bottles via the septa by use of a syringe in order to obtain the appropriate final protein concentrations of 250 mg/L and 500 mg/L within each serum bottle. Each sample was duplicated in order to validate the results. The bottles were set up as indicated in table 1.

Varying the concentration of zinc helped determine the inhibitory effects of zinc on *C. flavigena*'s activity. By using two different concentrations of protein (or bacteria), the effect of biomass concentration on reducing inhibition of *C. flavigena's* activity while in the presence of zinc was also established.

Table 1 Varying concentrations of protein and zinc used in serum bottles

Protein concentration (mg/L)	Zinc concentration (mg/L)
250	0
250	20
250	40
500	0
500	20
500	40

Determination of protein concentration and optical density

A typical cell is comprised of about 50% protein, and this allows for a correlation to be developed between cell numbers and protein concentrations. Protein concentration was determined with a Coomassie Protein Assay Reagent Kit. This dye works by binding with amino acids such as arginine and lysine, which will then absorb light at 595 nm, and can be measured with a spectrophotometer. This procedure was conducted in the same manner as the procedure used by Sani et al. (2001). Another method involved the measurement of optical density at 600 nm. This method was used in order to get a rough idea of how many cells were present before performing the Coomassie assay.

Determination of changes in glucose concentration

Each serum bottle initially contained 2 mM concentration of glucose. A Colorimetric method was used for determining the concentration of glucose throughout the experiment (Dubois et al., 1956.) The phenol – sulfuric acid assay is a broad spectrum method for carbohydrates, which measures both mono- and polysaccharides. All absorbance readings were performed at a wavelength of 490 nm.

Parameters followed

In order to assess the activity of *C. flavigena*, the glucose concentration was measured every one to two hours, along with the pH, for a total of nine hours. Glucose is the energy source for *Cellulomonas*, thus the decrease of glucose concentration was directly correlated with the activity of the cells, and the rate of substrate utilization could be inferred for each serum bottle from the amount of glucose consumed during the experiment. The concentration of protein was also measured during this period, using the Coomassie Protein Assay, to determine the extent of growth or biomass production during the study. The concentration of zinc was determined at the end of the experiment with an ICP analysis (Perkin Elmer, Optima 3000). This was done in order to quantify the amount of zinc that remained in solution relative to the initial zinc concentration.

Results

The average for duplicate batch experiment measurements were used for all figures and related results, because duplicate measurements were within less than 10% difference of one another.

pH fluctuations during experiment

The change in pH versus time is highlighted in Fig. 1 and 2. As anticipated, fluctuations in pH were small during the experiments. Thus, zinc should be the only inhibitory factor on cell activity.

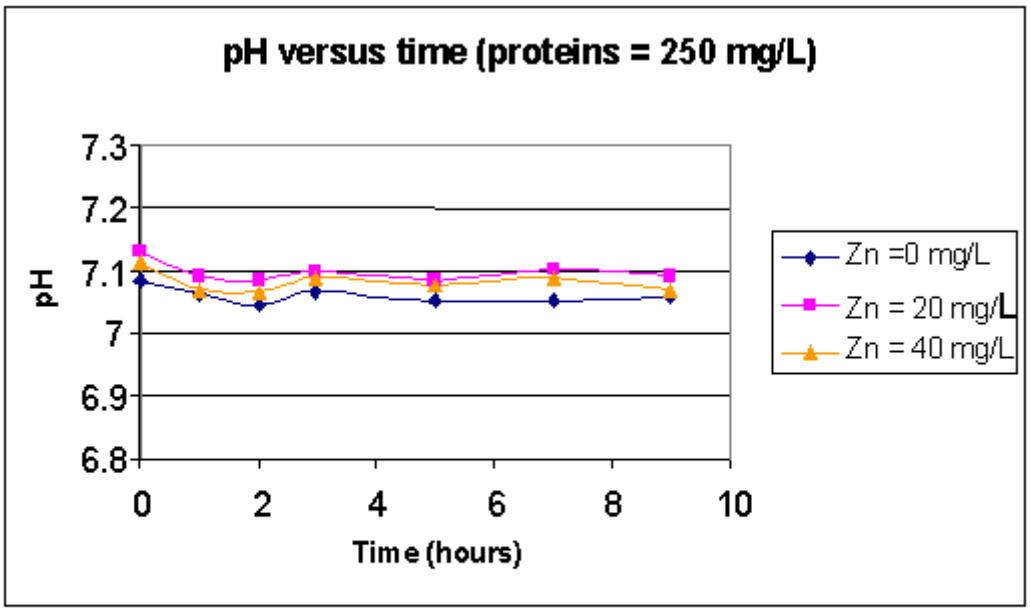

Figure 1 Fluctuations in pH during experiment for serum bottles containing 250 mg/L of protein

Variation in protein concentration throughout experiment

The concentration of protein stayed relatively constant throughout the experiments as seen in Table 2. Part of the experimental design was to minimize the change in biomass concentration over time as to focus on zinc effects on substrate utilization and not growth.

Consumption of glucose at varying zinc concentrations; an indication of cell activity inhibition

The consumption of glucose was used as an indicator of cell activity. Healthy, active bacteria will more readily breakdown glucose than bacteria that have been inhibited. Plots of glucose concentration with time are presented for initial zinc concentrations of 0, 20 and 40 mg/L and biomass concentrations of 250 and 500 mg/L as protein, Fig. 3 and Fig. 4 that follows.

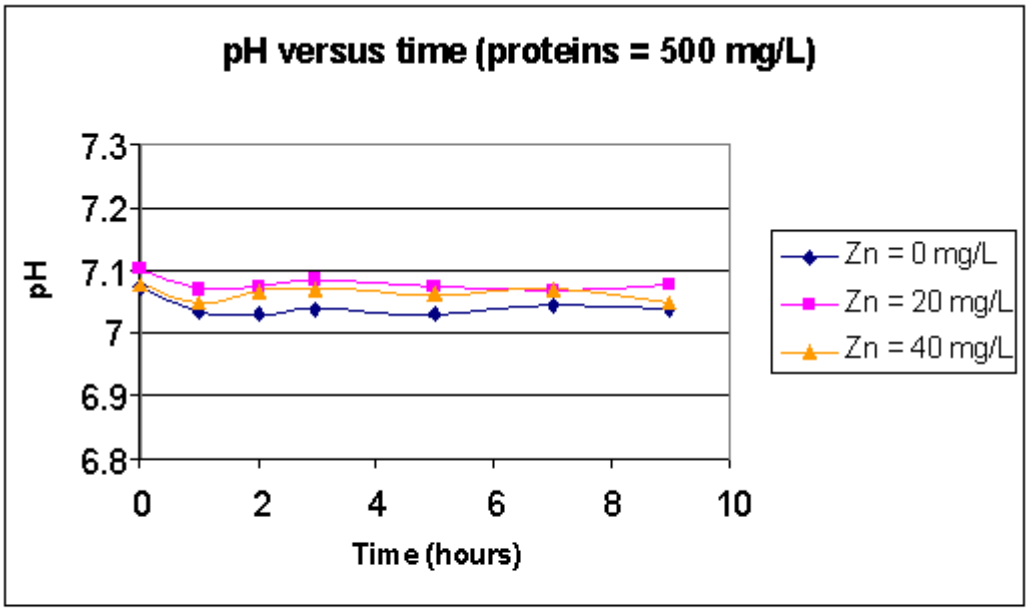

Figure 2 Fluctuations in pH during experiment for serum bottles containing 500 mg/L of protein

Table 2 Protein concentration measured at the beginning of the experiment and at the end.

Theoretical concentration of protein (mg/L)	Concentration of zinc (mg/L)	Protein concentration at the beginning of the experiment (mg/L)	Protein concentration at the end of the experiment (mg/L)
250	0	215	212.5
250	20	235	235
250	40	225	225
500	0	490	470
500	20	490	470
500	40	485	495

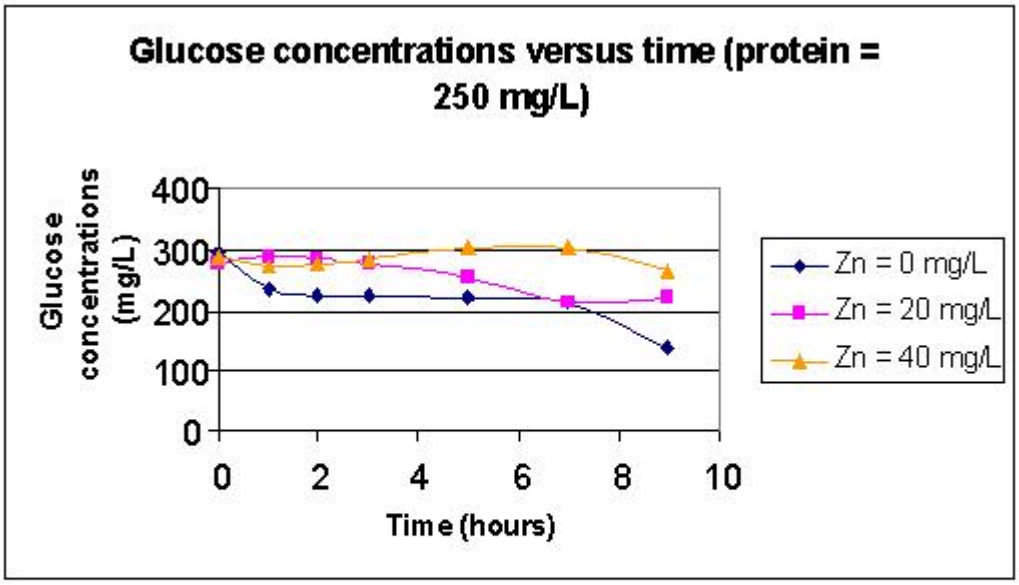

Figure 3 Fluctuations in glucose concentration for serum bottles containing 250 mg/L of protein with varying zinc concentration.

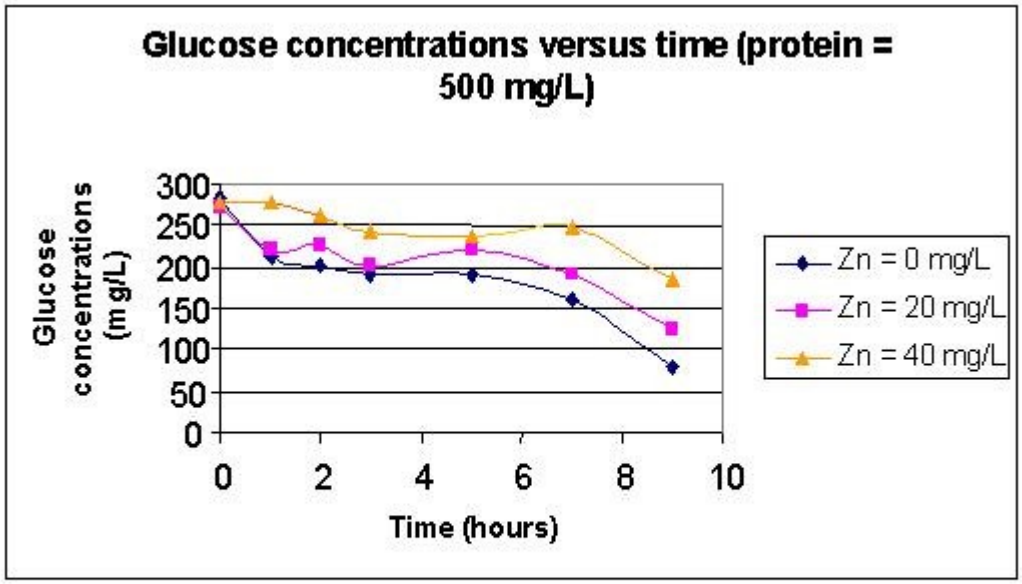

Figure 4 Fluctuations in glucose concentration for serum bottles containing 250 mg/L of protein with varying zinc concentration.

The comparisons of glucose consumption between serum bottles containing different biomass concentrations are shown as plots of glucose concentration with time in Figures 5, 6, and 7 and as percent of control rate in Table 3 below. Glucose was removed to a greater extent when biomass concentration was higher

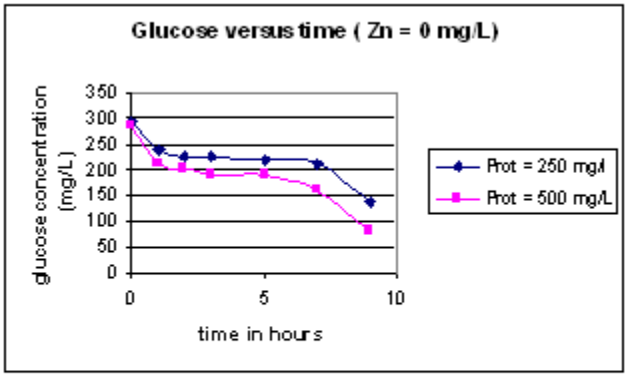

Figure 5 Comparison of the fluctuations in glucose concentration for the control serum bottles containing 250 mg/L verses 500 mg/L of protein.

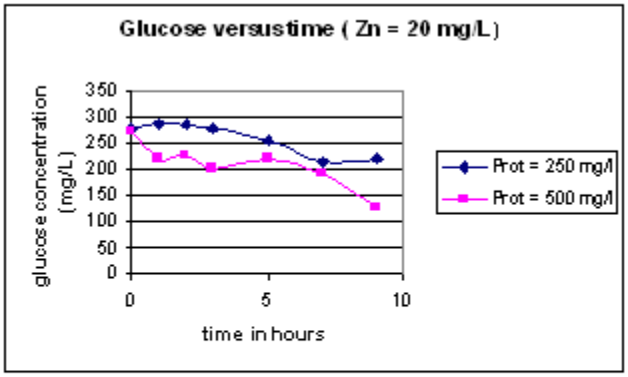

Figure 6 Comparison of the fluctuations in glucose concentration for serum bottles containing 250 mg/L verses 500 mg/L of protein at a zinc concentration of 20 mg/L initially.

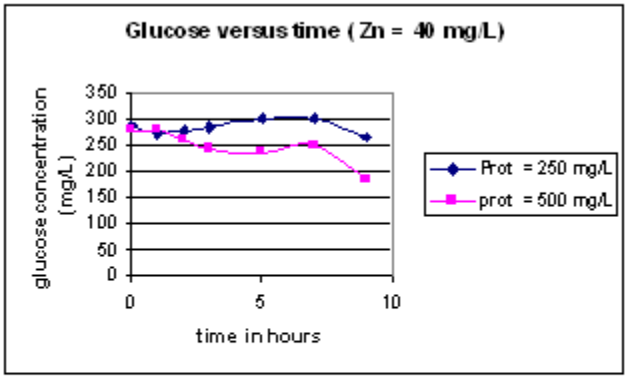

Figure 7 Comparison of the fluctuations in glucose concentration for serum bottles containing 250 mg/L verses 500 mg/L of protein at a zinc concentration of 40 mg/L initially.

Table 3 Effect of initial zinc concentration on average rate of glucose utilization

Initial Zn, mg/L	% of control	
	0.25 g/L protein	0.5 g/L protein
0	100	100
20	41	78
40	19	45

ICP measurements:

ICP results show that zinc in solution drops dramatically by the end of the experiment. See Table 4 below for results.

Table 4 Final zinc concentration (mg/L)

	Final [Zn] (initially 20 mg/L)	Final [Zn] (initially 40 mg/L)
250 mg/L Biomass	0.87	2.15
	0.81	2.09
500 mg/L Biomass	0.87	1.71
	0.99	1.26

Discussion

Results of this experiment suggest that zinc had a negative effect upon *C. flavigena's* activity over a nine hour period, and that the removal of glucose was faster when biomass concentration was higher. The difference of inhibition between the two biomass concentrations was an important consideration in the experimental design. This allowed for a greater understanding of how the amount of initial bacteria present may reduce some of the inhibitory effects of zinc. The idea is that in an APTS containing a healthy mixture of bacteria, perhaps having a large number of bacteria, may help reduce inhibitory effects of metal ions such as zinc, which would allow for some flexibility in the concentration of zinc flowing through the APTS. The results of this study also might explain the failure of an APTS that has been stressed during the winter so that the SRB have stopped. If this occurs high concentrations of heavy metals could inhibit the fermenting bacteria. Then when spring comes, and concentrations of heavy metals in the water are still high, the fermenters are inhibited from activity; the SRB are starved and thus inactive; and eventually, the bacterial consortium is rendered useless for removal of contaminants. Such a scenario could explain the failure of the Burleigh Tunnel pilot-scale APTS (Farmer et al., 1995).

Another significant result is the reduction in solution phase zinc concentration by more than 95% over the nine hour time course; suggesting that either internalization or sorption is occurring. These processes would be happening concurrently with metal precipitation within an APTS. As for the ability of biomass to reduce zinc toxicity, a phenomenon known as biosorption seems to be a reasonable explanation for why *C. flavigena's* activity was less inhibited at higher biomass concentrations. *C. flavigena* is a gram-positive bacterium, and when grown in neutral pH conditions, this bacteria possesses a net negative charge, with groups such as COO^-, PO_3^-, and O^-, along the cell wall. Fig. 8 highlights the ultimate result of this surface charge.

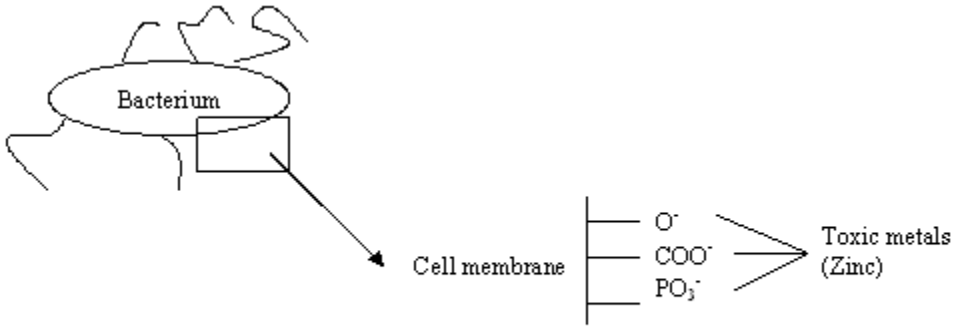

Figure 8

These groups can bind metals:

$$2R\text{-}COO^- + M^{z+} \qquad (R\text{-}COO)_2M$$

Thus, the zinc sorbs onto the surface of the bacteria by complexing with these groups. This biosorption may protect the cell by sequestering zinc on the outside of the cell, where it cannot cause damage. At high enough concentrations, the surface becomes saturated with zinc and loses its protective capacity. When comparing the glucose consumption of the serum bottles containing 250 mg/L of bacterial protein with 500 mg/L of protein, our data suggests that

inhibition from zinc is decreased with the higher biomass concentration. Figure 9 presents a summary of the all the inhibition data with rates relative to the control and the mass of zinc uptake normalized to mass of cell protein.

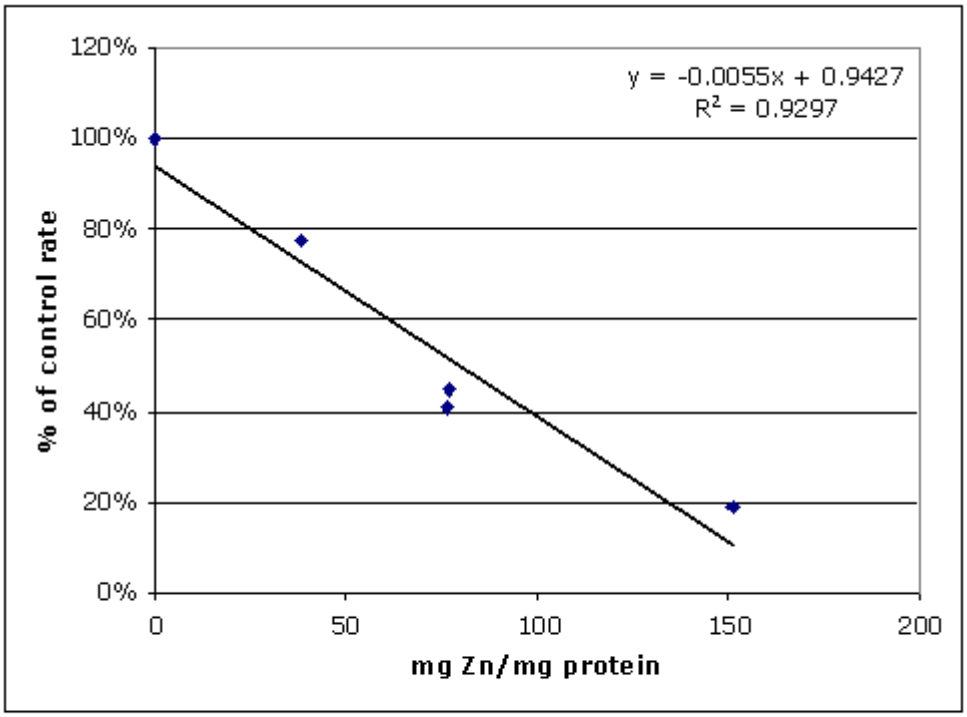

Figure 9 Average rate of glucose consumption relative to uninhibited controls versus the average mass of zinc uptake per unit mass of cell protein.

Cellular sorption and internalization are complicating factors of a solution phase LC_{50} approach. Absorption and/or internalization of metals is happening concurrently with other reactions in the system, such as precipitation, and very little work has been done to determine the kinetics of sorption and internalization relative to the kinetics of metal precipitation. Inhibition of microbes within an APTS maybe under predicted even when effluent metal concentrations are low. The ICP results show a significant drop in solution phase zinc, suggesting that the zinc is being sorbed or internalized by the microbes. If the above scenario tends to be the case, then the problem it creates could possibly be combated by significantly increasing the initial biomass concentrations.

Similar toxicity experiments with zinc and other metals have been conducted looking at SRB (Poulson et al., 1997; Sani et al., 2001; Utigikar et al., 2001; Utigikar et al., 2003). These studies primarily observed the effects these metals had on microbial growth, rather than inhibition on the rate of substrate utilization. Although there have been no published research looking at toxicity effects of heavy metals on *C. flavigena*, and the majority of published toxicity studies focused on cell growth inhibition rather than short-term activity, the results of the above mentioned articles are worth comparing with our findings. All of these studies found inhibition of growth to one degree or another based on the concentration of heavy metals present, but as stated earlier, the

metal concentration ranges reported to cause toxic effects varied greatly between studies (Booth and Mercer, 1963; Hao et al., 1994; Loka Bharathi et al., 1990; Poulsen et al., 1997; Saleh et al., 1964; Temple and Le Roux, 1964; Utgikar et al., 2001). It seems logical that this experiment suggests that *C. flavigena* is being inhibited by heavy metals since SRB have exhibited the same effect. The next logical step is to determine the maximum possible dose for common AMD metals on all critical microbes within an APTS, these microbes primarily being the sulfate-reducers and the cellulolytic-fermenting bacteria.

The results presented in this paper are part of an on-going study to understand the role of cellulolytic fermenters in maintaining a healthy sulfate reducing community. Further tests will be conducted in at least triplicate in order to determine statistical significant differences between the control bottle and the zinc containing bottles. These tests are currently underway and the data collected from these experiments will be discussed during the presentation given in June 2005 at the National Meeting of the American Society of Mining and Reclamation. It would also be useful to perform these toxicity studies at several more different zinc concentrations, ranging from 30 to 200 mg/L of zinc, to obtain a greater understanding of a probable toxicity threshold for zinc concentration, while concurrently examining the effects of varying biomass concentration in further detail to help in our understanding of *C. flavigena's* biosorption of zinc. The next set of experiments conducted will take a closer look at metal sorption and internalization to determine metal partitioning coefficients and internalization kinetics, which will be compared to precipitation reactions that are happening concurrently within an APTS. Finally we will look at toxicity effects on growth, and use cellulose instead of glucose, to gain a greater understanding of metal toxicity effects on these organisms. Toxicity tests will ultimately need to be conducted on a mixed culture, to better model a sulfate reducing biozone, in order to establish a more accurate zinc toxicity threshold for APTS.

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