SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 29, No. 12, pp. 2669–2675, 2010

Printed in the USA
DOI: 10.1002/etc.338

EFFECTS OF FERROUS SULFATE, INOCULUM HISTORY, AND ANIONIC FORM ON LEAD, ZINC, AND COPPER TOXICITY TO *ACIDITHIOBACILLUS CALDUS* STRAIN BC13

JOHN E. ASTON,† BRENT M. PEYTON,† BRADY D. LEE,‡ and WILLIAM A. APEL*‡
†Department of Chemical and Biological Engineering, Montana State University, Bozeman, Montana, USA
‡Biological Systems Department, Idaho National Laboratory, Idaho Falls, Idaho, USA

(*Submitted* 23 *March* 2010; *Returned for Revision* 20 *April* 2010; *Accepted* 19 *July* 2010)

Abstract—The current study reports the single and combined toxicities of Pb, Zn, and Cu to *Acidithiobacillus caldus* strain BC13. The observed half-maximal inhibitory concentrations (IC50), ± 95% confidence intervals, for Pb, Zn, and Cu were 0.9 ± 0.1 mM, 39 ± 0.5 mM, and 120 ± 8 mM, respectively. The observed minimum inhibitory concentrations (MIC) for Pb, Zn, and Cu were 7.5 mM, 75 mM, and 250 mM, respectively. When metals were presented in binary mixtures, the toxicities were less than additive. For example, when 50% of the Pb MIC and 50% of the Cu MIC were presented together, the specific growth rate was inhibited by only 59 ± 3%, rather than 100%. In addition, the presence of ferrous iron in the growth media decreased Pb and Zn toxicity to *A. caldus* strain BC13. The importance of inoculum history was evaluated by pre-adapting cultures through subsequent transfers in the presence of Pb, Zn, and Cu at their respective IC50s. After pre-adaptation, cultures had specific growth rates 39 ± 11, 32 ± 7, and 28 ± 12% higher in the presence of Pb, Zn, and Cu IC50s, respectively, compared with cultures that had not been pre-adapted. In addition, when cells exposed to the MICs of Pb, Zn, and Cu were harvested, washed, and re-inoculated into fresh, metal-free medium, they grew, showing that the cells remained viable with little residual toxicity. Finally, metal chlorides showed more toxicity than metal sulfates, and studies using sodium chloride or a mixture of metal sulfates and sodium chloride suggested that this was attributable to an additive combination of the metal and chloride toxicities. Environ. Toxicol. Chem. 2010;29:2669–2675. © 2010 SETAC

Keywords—Acidithiobacillus Toxicity Lead Zinc Copper

Acidithiobacillus caldus is a gram-negative bacterium that oxidizes sulfur and reduced sulfur compounds for energy, and that can fix carbon dioxide as a sole carbon source [1,2]. *Acidithiobacillus caldus* grows from pH 1 to 4, with optimal growth between pH 2 and 3, and from 32 to 50°C, with optimal growth at 45°C [1]. These traits make *A. caldus* well suited for growth in many biomining systems [3–6], where recent studies suggest that it may play a significant role in metal mobilization. McGuire et al. [7] reported that microbial communities containing *A. caldus* were observed to leach more Fe from pyrite, arsenopyrite, and marcasite than communities without *A. caldus* [7]. Dopson and Lindstrom [8] reported that twice as much Fe was leached from arsenopyrite when an Fe oxidizer, *Sulfobacillus thermosulfidooxidans*, was cocultured with *A. caldus*, as compared with when *S. thermosulfidooxidans* was cultured alone [8]. In addition, *A. caldus* was observed to enhance Cu recovery by oxidizing S formed during the biomining of chalcopyrite [9]. These studies suggest an important role for *A. caldus* in commercial biomining; however, few direct studies of metal interactions and toxicities to *A. caldus* have been published.

The toxicity of metals to microorganisms has been well documented, and several general reviews have been written covering this subject [10–15]. Specific to the work presented here, multiple studies have reported that the related microorganisms, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, have relatively high tolerance to Zn and Cu when presented individually [16–21] or combined [18,22]. However, toxicity studies with *A. caldus* have been limited to the metalloid As [23–25]. Recent work by Watkin et al. [26] compared Fe, Cu, Zn, Ni, and Co tolerances of several new isolates with those of several known strains, including *A. caldus* strain KU, but in-depth inhibition studies were not done [26].

The purpose of the current study was to test the single and combined toxicities of various metals to *A. caldus* strain BC13, including the effects of metal mixtures, inoculum history, metal concentration and speciation, and the use of different metal anionic forms to determine which conditions should be considered in future work with *A. caldus* strain BC13 and possibly other microorganisms. Specifically, the current study is a comprehensive report on the effects of Pb, Zn, and Cu on the growth of *A. caldus* strain BC13, including effects of single versus combined metal toxicity, effects of high ferrous iron concentrations on Pb, Zn, and Cu toxicity, effects of prior exposure to Pb, Zn, and Cu, and a comparison of metal sulfate and metal chloride toxicity. Zinc and Cu were chosen because of their commercial relevance and ubiquity in biomining and acid-mine environments. Lead was chosen as a control test because *A. caldus* has not been identified in environments containing high levels of galena. By identifying the effects of various environmental conditions on the efficacy of these metals toward *A. caldus* strain BC13, this report significantly increases the current understanding of this microorganism and how the conditions of biomining and acid-mine drainage environments may affect its metal tolerance.

MATERIALS AND METHODS

Microorganism and growth conditions

Acidithiobacillus caldus strain BC13 (ATCC 51757), henceforth referred to as BC13, was grown in a basal salts medium [1]. The medium was autoclaved for 15 minutes at 121°C and

* To whom correspondence may be addressed (William.Apel@inl.gov).
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

22 psi, and the pH was then adjusted to 2.5, using 6 normal sulfuric acid. A filtered (0.2 μm) metal sulfate solution of Pb, Zn, or Cu was added from a stock solution. The concentrations of metal in the stock solutions were adjusted to ensure that an equal volume could be added to each flask. A filter-sterilized (0.2 μm) solution of potassium tetrathionate was then added to a concentration of 5 mM, as an electron donor, and ambient carbon dioxide provided the sole carbon source. Cells preserved at 4°C in nanopure water (17.4 MΩ), with the pH adjusted to 3.0 using 6 normal sulfuric acid, provided the initial inoculum. Aliquots that provided initial cell densities of approximately 5×10^7 cells/ml were used. Cells were cultured in 125-ml Erlenmeyer flasks (75 ml medium volume), fitted with foam stoppers, and shaken at 150 rpm in a temperature-controlled incubator at 45°C.

Experimental design and statistics

Initial growth inhibition studies were carried out at relatively large concentration intervals to roughly estimate the minimum inhibitory concentrations (MICs) of Pb, Zn, and Cu. After this was done, more detailed experiments were carried out to quantify the inhibitory effects of each metal in the relevant concentration range. In experiments that determined the effects of ferrous iron on metal toxicity, ferrous iron was added to concentrations up to environmentally relevant conditions. Each experiment was repeated in triplicate under identical conditions, so that average values and 95% confidence intervals could be calculated for specific growth rates at each metal concentration and condition tested. To determine the half-maximal inhibitory concentrations (IC50s) and corresponding 95% confidence intervals, linear regressions were calculated using the LINEST function in Microsoft Excel®. Using this method, data points from each set of a triplicate across a range of concentrations contributed to determining the reported IC50s. No statistical confidence was assigned to the no-observable-effect concentration, lowest-observable-effect concentration, and MIC, because these values were determined graphically from average values.

Determining metal toxicity

Cell concentrations were measured by using direct cell counts at 12-h intervals with a Petroff-Hauser counting chamber (Hausser Scientific) and a phase-contrast microscope (Zeiss). To perform cell counts, an aliquot of growth medium was added to the counting chamber to volume (5×10^{-5} mm^3/grid). Cells were then counted across grids from the middle and all sides of the counting grid to account for any possible spatial variations. In more cell-dense samples, each cell in individual 0.05×0.05 mm grids was counted until a minimum of 400 cells were counted. In less cell-dense samples, each cell in a minimum of 20 small (0.05×0.05 mm) grids was counted. The observed specific growth rates were calculated from the resultant growth curves and used to quantify inhibition.

To determine combined metal toxicity, binary mixtures of Pb and Zn, Pb and Cu, and Zn and Cu were prepared. Concentrations were proportional to their respective MICs and, assuming additive toxicities, mixed to produce a total metal concentration proportional to an effective MIC. For example, to produce a mixture containing Pb and Zn equivalent to 50% of an effective MIC, the final growth medium would contain: $0.5 \cdot \left(\frac{\text{MIC}_{\text{Pb}}}{2} + \frac{\text{MIC}_{\text{Zn}}}{2}\right)$. Linear regressions were used to calculate expected toxicities between tested data points using the LINEST function in Microsoft Excel. From these regressions, estimated contributions toward the total effective toxicity from each metal were calculated.

Similar experiments were conducted to determine whether ferrous iron affected the toxicity of Pb, Zn, or Cu to BC13. Each metal was added to a concentration equal to its previously calculated IC50, and ferrous iron sulfate was added to concentrations of 0, 25, 50, 75, or 100 mM. The concentration of ferrous iron in each stock was adjusted so that an equal volume was added to each flask. Lead-, Zn-, and Cu-free controls were performed to determine whether ferrous iron alone affected BC13 in the absence of Pb, Zn, or Cu.

Determining effects of previous metal exposure

Cells were prepared as described earlier and inoculated into growth medium containing Pb, Zn, or Cu concentrations equal to the previously calculated IC50. During the late-log growth phase, cells were harvested and washed as previously described, then inoculated into fresh medium containing the same metal concentration. This process was repeated three times to allow cells to adapt to Pb, Zn, or Cu. During the fourth growth cycle, cell concentrations were measured using direct counts as described previously, and the specific growth rates were calculated.

Determining metal chloride toxicity

Cells were prepared as described previously, but metal chlorides were used instead of metal sulfates. Chloride salts of Pb, Zn, or Cu were introduced at initial concentrations equal to the previously calculated IC50s of the respective metal sulfate counterparts. To determine whether chloride ions contributed directly to cell inhibition, sodium chloride was added to metal-free growth media at concentrations of 0, 50, 100, and 200 mM. In control experiments, lead, zinc, or copper sulfates were added to concentrations proportional to their previously calculated IC50s, and sodium chloride also was added to concentrations of 0, 50, 100, or 200 mM. In these experiments, cell concentrations were measured as described previously, and specific growth rates were calculated for comparison.

Modeling metal complexation and precipitation

Visual MINTEQ (version 2.53) software was used to predict complexation and precipitation of media components using activities from the Debye-Huckel equation and the default MINTEQA2 thermodynamic database. The temperature was set to 45°C, and the proton concentration was calculated from the pH, which was set at 2.50. The saturation index (defined as the log of the ion activity divided by the solubility product) was used to predict metal precipitation. Compounds with a positive saturation index were set to infinite saturation, to allow for their precipitation. Each experimental medium condition tested was modeled in this manner. The statistical software MINITAB was then used to construct matrix plots and perform primary component analyses to determine relations between metal complexation and the observed specific growth rates at various metal concentrations.

RESULTS

Single metal toxicity

Figure 1a shows the effect of Pb concentrations on the specific growth rate of BC13. Similarly, the effects of Zn (Fig. 1b) and Cu (Fig. 1c) also are shown. Lead was the most toxic of the three metals tested, with an IC50 of 0.94 ± 0.13 mM, and an MIC of 7.5 mM. An IC50 and an MIC of

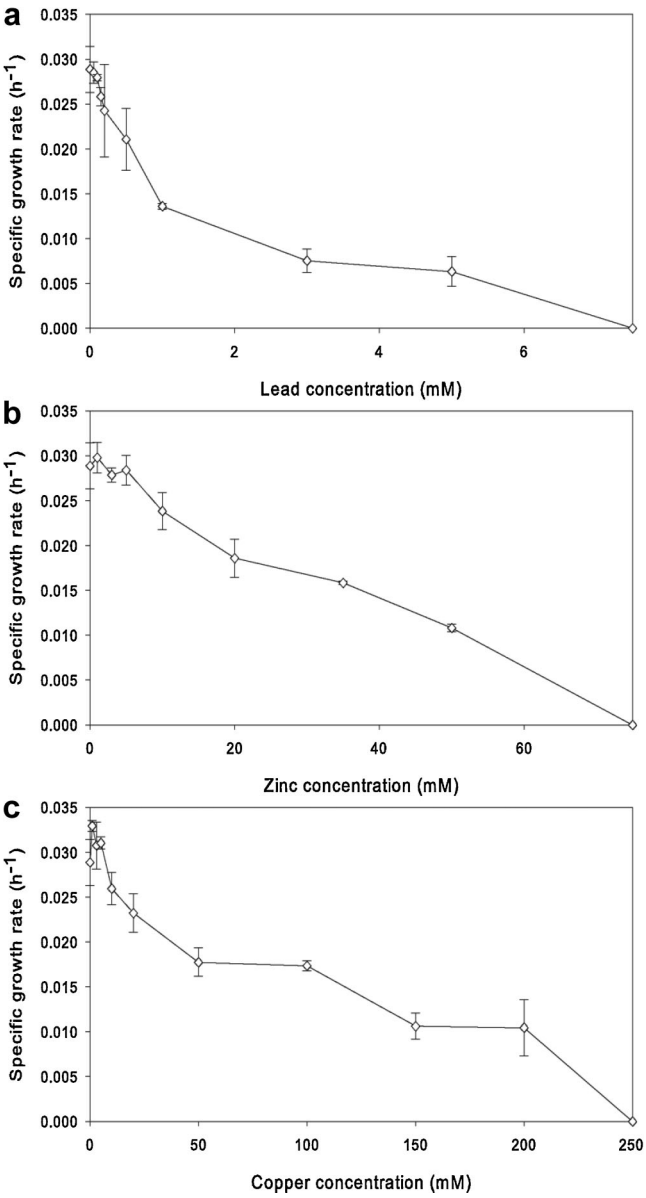

Fig. 1. Effect of (**a**) lead, (**b**) zinc, and (**c**) copper sulfate on the specific growth rate of *Acidithiobacillus caldus* strain BC13. Error bars represent 95% confidence intervals.

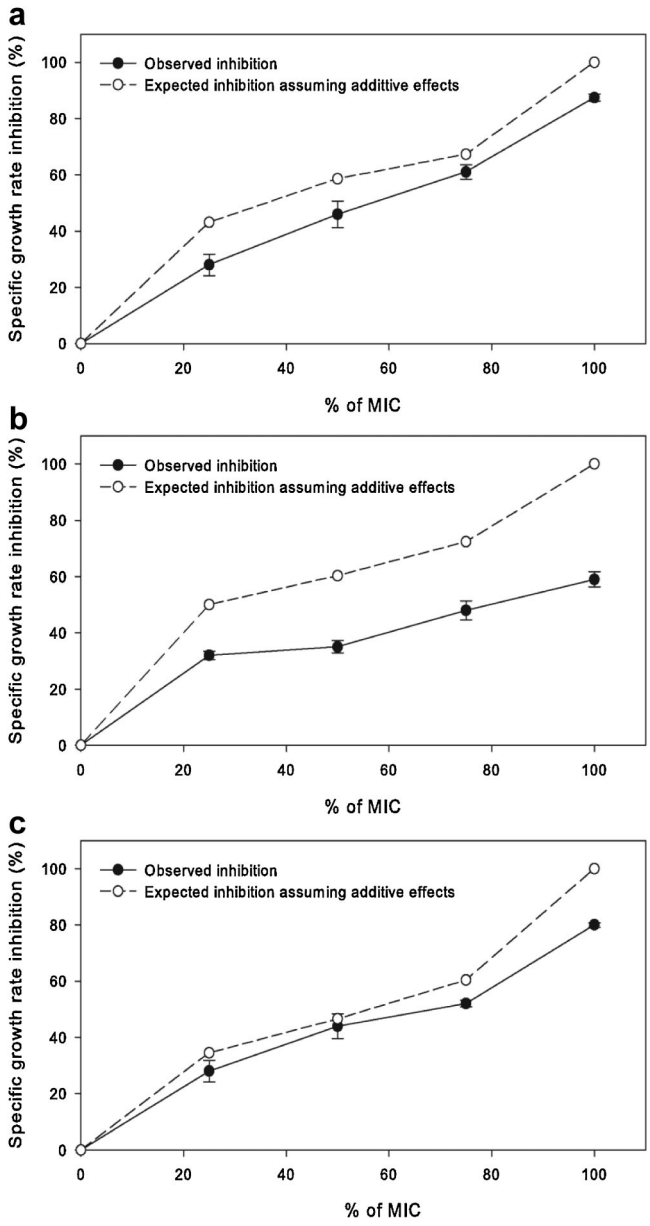

Fig. 2. Observed inhibition compared with predicted inhibition, assuming additive toxicity, of binary mixtures of lead and zinc (**a**), lead and copper (**b**), and zinc and copper (**c**) to strain *Acidithiobacillus caldus* BC13. The *x* axis represents the percentage of the minimum inhibitory concentration (MIC) calculated, assuming additive effects. Error bars represent 95% confidence intervals. The MIC concentrations for lead, zinc, and copper were 7.5, 75, and 250 mM, respectively.

39 ± 0.5 and 75 mM, respectively, were observed for Zn, whereas Cu was the least toxic metal tested, with an IC50 and MIC of 120 ± 8.2 and 250 mM, respectively (Table 1).

Combined metal toxicity

To determine the combined toxicity of Pb, Zn, and Cu, metals were presented in binary mixtures in ratios proportional to their individual IC50s. Binary metal mixtures containing ratios of 12.5, 25, 37.5, and 50% of each metal's respective MIC was used. Assuming additive toxicity when mixed, the effective overall metal concentrations were then 25, 50, 75, and 100% of an effective MIC. However, Figure 2 shows that the toxicities were less than additive. For example, when 25% of the Pb MIC was mixed with 25% of the Zn MIC, the observed specific growth rate was 0.016 ± 0.001/h, compared with a predicted specific growth rate of 0.012/h, calculated assuming additive toxicities (Fig. 2a). Similar results were seen when Pb and Cu, and Zn and Cu, were mixed at varying concentrations (Fig. 2b, c).

Effect of ferrous iron on metal toxicity

Ferrous iron gave significant protection to BC13 from Pb and Zn toxicity. Figure 3 shows that cultures exposed to a concentration of Pb equal to the IC50 exhibited specific growth rates of 0.014 ± 0.001, 0.032 ± 0.001, and 0.030 ± 0.001/h when ferrous iron was added to concentrations of 0, 50, and 100 mM, respectively. Similarly, the observed specific growth rates of cultures in the presence of the Zn IC50 were 0.016 ± 0.001, 0.023 ± 0.001, and 0.028 ± 0.001/h when ferrous iron was added to 0, 50, and 100 mM, respectively. However, when this experiment was performed using Cu, the effect was significantly decreased, as observed specific growth rates were 0.017 ± 0.001, 0.014 ± 0.000, and 0.019 ± 0.001/h when ferrous iron was added to concentrations of 0, 50, and 100 mM,

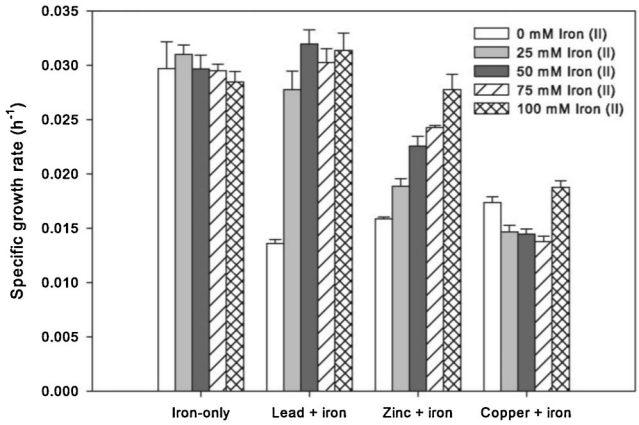

Fig. 3. The effect of ferrous iron added to concentrations of 0, 25, 50, 75, or 100 mM on lead, zinc, and copper toxicity to *Acidithiobacillus caldus* strain BC13 when added at concentrations equal to the previously calculated half-maximal inhibition concentrations (0.94 mM, 39 mM, and 120 mM for lead, zinc, and copper, respectively). Error bars represent 95% confidence intervals.

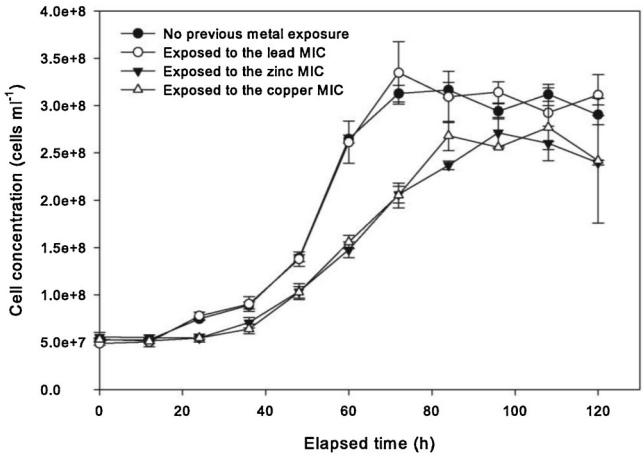

Fig. 5. Growth of *Acidithiobacillus caldus* strain BC13 in metal-free cultures after cells were harvested from cultures containing minimum inhibitory concentrations of lead, zinc, or copper, or 7.5, 75, and 250 mM, respectively. Error bars represent 95% confidence intervals.

respectively (Fig. 3). In separate control experiments, ferrous iron was added to concentrations of 0, 50, and 100 mM with no Pb, Zn, or Cu added. At these concentrations, the observed specific growth rates were 0.030 ± 0.001, 0.030 ± 0.001, and 0.028 ± 0.001/h, suggesting that ferrous iron did not significantly affect the growth of BC13 by itself (Fig. 3). Visual MINTEQ predicted that more than 96% of the Fe remained as aqueous ferrous iron at the concentrations used in the current study.

Effect of prior metal exposure on metal toxicity

Figure 4 shows that the specific growth rate increased 39 ± 11, 32 ± 7, and 28 ± 12% when cultures were pre-adapted, through subsequent transfers, to Pb, Zn, or Cu, respectively. In addition to increased specific growth rates, the lag phase of cultures pre-adapted to Pb, Zn, or Cu decreased by 12, 24, and 48 h, respectively (data not shown).

Figure 5 shows that cells collected from media containing the MIC of Pb, Zn, or Cu were able to resuscitate and grow in fresh, metal-free medium. After being exposed for 120 h to the MIC of Pb, then re-inoculated into fresh, metal-free medium, cultures grew with no residual inhibition and attained a final cell concentration of 107 ± 7% of the final cell concentration observed for cultures that had not been exposed to Pb. However, when cells were collected from medium containing the MIC of Zn or Cu after 120 h of exposure, and re-inoculated into fresh, metal-free medium, the cultures grew to final cell concentrations of only 83 ± 1 and 83 ± 23% of the final cell concentration observed in cultures with no prior exposure to Zn or Cu, respectively. The observed specific growth rates of cells exposed to MICs of Pb, Zn, and Cu for 120 h were 0.032 ± 0.003, 0.028 ± 0.002, and 0.030 ± 0.003/h, respectively, after being re-inoculated into fresh, metal-free medium. Cells that had not been pretreated by the MICs of Pb, Zn, or Cu exhibited an observed specific growth rate of 0.029 ± 0.003/h, suggesting that there were no significant residual affects on the observed specific growth rates (data not shown).

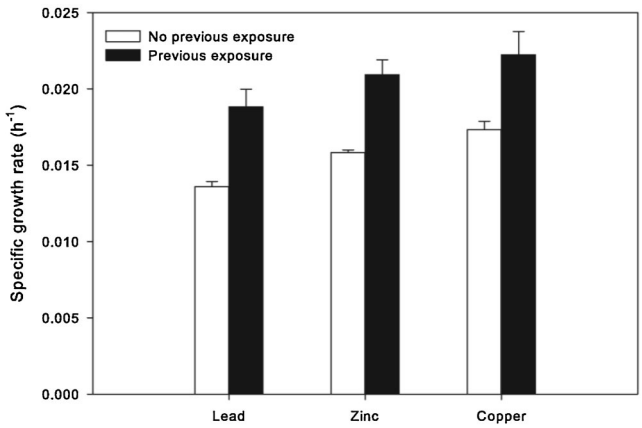

Fig. 4. Effect of prior exposure to lead, zinc, and copper on the specific growth rate of *Acidithiobacillus caldus* strain BC13. Cells were adapted through subsequent culturing and transfers in the presence of the half-maximal inhibitory concentrations of lead, zinc, or copper (0.94 mM, 39 mM, and 120 mM, respectively). Error bars represent 95% confidence intervals.

Comparison of metal chloride to metal sulfate toxicity

Figure 6a shows that when lead, zinc, and copper chlorides were added at concentrations equal to the IC50s of their respective sulfates, the observed specific growth rates were lower than those observed for the metal sulfates. For Pb, this difference was relatively minor, 0.012 ± 0.001/h versus 0.014 ± 0.000/h, respectively. However, in the case of Zn and Cu, the differences were more pronounced. The specific growth rate observed when zinc chloride was used was 0.012 ± 0.001/h, compared with 0.016 ± 0.000/h when zinc sulfate was added. Similarly, the specific growth rate observed when copper chloride was added was 0.012 ± 0.000/h, compared with 0.017 ± 0.001/h when copper sulfate was used.

The specific growth rate of BC13 also decreased when only sodium chloride was added to metal-free medium. When sodium chloride was added to concentrations of 0, 50, 100, and 200 mM, the specific growth rates were 0.029 ± 0.002, 0.027 ± 0.001, 0.023 ± 0.001, and 0.021 ± 0.001/h, respectively. In other experiments, lead, zinc, and copper sulfate were added to concentrations equal to their respective IC50s, and the sodium chloride concentration was varied. The inhibition observed in these tests suggested that the metal and chloride toxicity effects are additive (Fig. 6b).

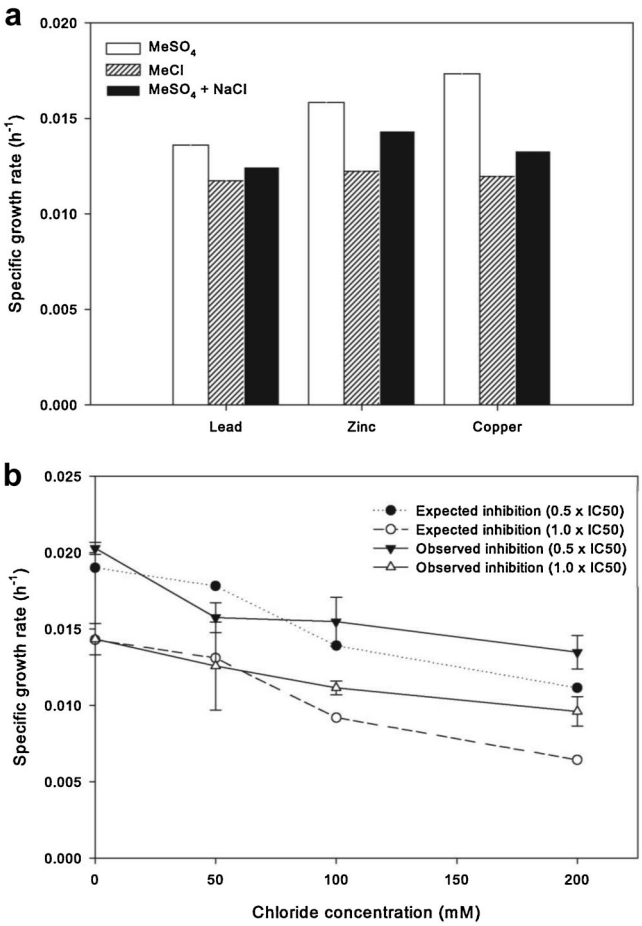

Fig. 6. (**a**) Effect of lead, zinc, and copper on the specific growth rate of *Acidithiobacillus caldus* strain BC13 when added as either metal sulfates, metal chlorides, or metal sulfates with a corresponding concentration of sodium chloride. In each case, metals were added to concentrations equal to previously calculated half-maximal inhibition concentrations (IC50s) for the corresponding metal sulfates, or 0.94 mM, 39 mM, and 120 mM, for lead, zinc, and copper, respectively. (**b**) Predicted and observed effect of chloride concentrations on the specific growth rate of *A. caldus* strain BC13 with zinc sulfate added to a concentration equal to either 50% or 100% of the previously calculated IC50 (39 mM). Similar results were observed with lead and copper (data not shown). Error bars represent 95% confidence intervals.

Metal complexation and precipitation

Visual MINTEQ predicted the complexation and potential precipitation of Pb, Zn, and Cu over the range of concentrations and combinations used in these experiments. The primary dissolved constituents of Pb, Zn, and Cu were aqueous divalent metal cations and metal sulfates, regardless of whether the metals were added as metal sulfates or metal chlorides. Lead was predicted to remain soluble up to a concentration of 20 μM. Concentrations used in these experiments were beyond this value, and visual precipitation of lead was observed. No precipitation was predicted or observed for Zn or Cu.

Visual MINTEQ modeling results were entered into the multivariant statistical software MINITAB and compared against changes in the corresponding observed specific growth rates. Matrix plots and primary component analyses suggested that for all the metals, only changes in the total metal concentrations correlated strongly with changes in observed specific growth rates, and although speciation occurred, it did not significantly affect specific growth rates (data not shown).

Table 1. Toxicity of lead, zinc, and copper sulfates to *Acidithiobacillus caldus* strain BC13 described using the no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), half-maximal inhibitory concentration (IC50), and the minimum inhibitory concentration (MIC)[a]

	NOEC (mM)	LOEC (mM)	IC50	(mM) MIC (mM)
Lead	0.10	0.15	0.94 ± 0.13	7.5
Zinc	1.0	3.0	39 ± 0.46	75
Copper	5.0	10	120 ± 8.2	250

[a] Inhibition was quantified by changes in the specific growth rate in the presence of metals. ± Values indicate 95% confidence intervals.

DISCUSSION

Single toxicity of lead, zinc, and copper

The BC13 grew in millimolar concentrations of Pb, Zn, and Cu, with Pb being the most toxic metal tested, having an MIC of 7.5 mM. Interestingly, *A. caldus* has not been isolated from environments containing high concentrations of galena [27], and results reported here suggest that these environments may contain Pb concentrations too high for significant *A. caldus* activity (Fig. 1a). The BC13 exhibited relatively high tolerances for Zn and Cu, with MICs of 75 and 250 mM (Fig. 1b and c). This is not surprising, because many of the environments in which *A. caldus* has been identified have high concentrations of Zn and Cu [4,27–29].

Previous work with *A. caldus* strain KU reported MIC values of 65 g/L (993.9 mM) for Zn and only 1.5 g/L (23.6 mM) for Cu [26], suggesting a Zn tolerance significantly higher than that observed here, and a Cu tolerance significantly lower. This previous work did not report methods for quantifying growth, or describe the growth medium used [26], making a direct comparison difficult. These differences may be attributable to strain-to-strain variance in metal tolerance, medium composition, or possibly inoculum history. Regardless, BC13 and strain KU appear to be quite tolerant to Zn and Cu.

Comparisons with other acidithiobacilli

High resistance to Zn and Cu is not unprecedented among the acidithiobacilli. Aside from previous work with *A. caldus* strain KU [26], *Acidithiobacillus ferrooxidans* has been observed to grow on ground S in a medium containing 100 mM Cu [30]. Furthermore, strain KU can facilitate sphalerite leaching in the presence of 25 mM Zn and chalcopyrite leaching in the presence of 10 to 25 g/L Cu (158–397 mM) [18,30]. Barreira et al. [16] and Chen et al. [17] made similar observations while working with *A. thiooxidans*.

With observed MIC values of 75 mM and 250 mM for Zn and Cu, respectively, the current study suggests that BC13 has a level of tolerance to Zn and Cu similar to that of *A. ferrooxidans* and *A. thiooxidans*. However, key differences can be seen between this work and previous work with acidithiobacilli. First, the current study used a soluble substrate (tetrathionate), which prevented the formation of biofilms that may provide some protection from metals [31], and second, the current study characterized toxicity directly with respect to cell growth, rather than inhibition of leaching kinetics.

Effects of combined metals

Metals presented in binary mixtures exhibited less than additive toxicity toward BC13, suggesting an aspect of competitive inhibition (Fig. 2). In addition, the significant decrease in Pb and Zn toxicity in the presence of ferrous iron (Fig. 3) is

also quite interesting, given that many environments from which *A. caldus* has been isolated also contain high concentrations of Fe relevant to the concentrations used in the present study [4,27–29]. This suggests that BC13 may exhibit catabolic activity (leaching) in iron-rich environments with Pb, Zn, and Cu concentrations higher than the respective MIC values reported here. Previous studies also have observed less than additive toxicity in binary-metal systems. For example, Gikas [32] observed that Ni(II) and Co(II) exhibited similar individual toxicities to microbes growing in an activated sludge; however, when presented in combination, their toxicities were significantly reduced [32].

Effect of inoculum history

One aspect of cell culturing that is often overlooked in toxicity studies is inoculum history. In the current study, preadaption to Pb, Zn, or Cu increased specific growth rates of BC13 significantly when it was subsequently exposed to heavy metals (Fig. 4). Similar results have been observed by others [33–35] and may indicate higher tolerances in environments in which species have had prolonged exposure to metals.

Another aspect of inoculum history examined here was the effect of exposure to the MICs of Pb, Zn, and Cu. Cells harvested from medium containing the MIC of Pb showed no residual effects, whereas cells harvested from media containing the MIC of Zn or Cu showed some residual effect but still grew quite well (Fig. 5), suggesting that Pb, Zn, and Cu may simply slow cell growth, perhaps through increased energy requirements. However, when cells were collected from MIC exposures to Zn or Cu, they did not grow as well (Fig. 5). This may be because of residual metal strongly bound to the cells, as previous work has shown that BC13 has larger sorption capacities for Zn and Cu than for Pb [36]. The less than additive toxicities of Pb, Zn, and Cu, and the ability of BC13 to be resuscitated from exposure to heavy-metal MICs, may suggest that cells are viable and metabolically active in environments containing Pb, Zn, or Cu concentrations significantly higher than the MICs observed here.

Metal chloride versus metal sulfate toxicity

Figure 6a shows the increased toxicity of metal chlorides over metal sulfates, and Figure 6b suggests that this is attributable to additive toxicity, because additional experiments showed that chloride itself was inhibitory to BC13. This may explain why lead chloride toxicity was not significantly different from lead sulfate, because the corresponding chloride concentration was only 1.9 mM, which was not observed to be toxic when sodium chloride was added in the absence of Pb, Zn, or Cu (data not shown). Conversely, chloride concentrations associated with zinc and copper chlorides (78 and 240 mM) were toxic even in the absence of metals. This observation is supported by past work that reported chloride inhibition toward the acidithiobacilli [37], and although the chloride concentrations necessary to achieve this effect are not necessarily relevant to natural environments containing *A. caldus* [28,29], these results do emphasize the importance of metal salts chosen for inhibition studies.

CONCLUSIONS

To our knowledge, this is the first comprehensive report on Pb, Zn, and Cu toxicity to *A. caldus* and the first study reporting the toxicity of Pb to any member of the acidithiobacilli. The order of toxicity observed here was $Cu < Zn < Pb$, and the relatively high tolerances observed to Zn and Cu were comparable to those observed in other acidithiobacilli [16–18,30]. Additional studies using binary-metal mixtures and high ferrous iron concentrations were carried out to better relate the single-metal toxicity observations to in situ realities. Interestingly, these studies suggested that binary-metal mixtures, and the presence of ferrous iron, significantly decreased the toxicity of Pb and Zn to BC13. In addition, inoculum history was an important factor in metal tolerance, because cells that were allowed to adapt to Pb, Zn, and Cu through subsequent culturing showed significantly increased tolerance to these metals. Combined, these results suggest that BC13 may grow and be metabolically active in environments containing Pb, Zn, or Cu concentrations higher than the MICs observed here when these metals were presented individually.

Finally, a comparison of metal sulfate versus metal chloride toxicity suggested that metal sulfates were much less toxic to BC13, because chloride ions exhibited an inhibitory effect of their own that was approximately additive with those of Pb, Zn, or Cu.

Acidophilic chemolithoautotrophs play important roles in biomining and acid-mine drainage systems because of their tolerance and mobilization of metals [38–40]. The current study significantly improves the understanding of one such microorganism, *A. caldus* BC13, and may lead the way for future research of specific toxicity mechanisms and metal-regulated protein expression.

Acknowledgement—This work was supported by the Idaho National Laboratory Directed Research and Development program under Department of Energy Idaho Operations Office Contract DE-AC07-05ID14517, the National Science Foundation Montana Experimental Program to Stimulate Competitive Research (NSF), and the NSF Integrated Graduate Education Research Training, program (grant DGE-0654336). The authors also thank the Department of Chemical and Biological Engineering and Center for Biofilm Engineering at Montana State University for laboratory access and support.

REFERENCES

1. Hallberg KB, Lindstrom EB. 1994. Characterization of *Thiobacillus caldus* sp. Nov., a moderately thermophilic acidophile. *Microbiology* 140:3451–3456.
2. Dopson M, Lindstrom EB, Hallberg KB. 2002. ATP generation during reduced inorganic sulfur compound oxidation by *Acidithiobacillus caldus* is exclusively due to electron transport phosphorylation. *Extremophiles* 6:123–129.
3. Burton NP, Norris PR. 2000. Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis. *Extremophiles* 4:315–320.
4. Druschel GK, Baker BJ, Gihiring TM, Banfield JF. 2004. Acid mine drainage biogeochemistry at Iron Mountain, California. *Geochem T* 5:12–32.
5. Goebel BM, Stackebrandt E. 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl Environ Microbiol* 60:1614–1621.
6. Okibe N, Gericke M, Hallberg KB, Johnson DB. 2003. Enumeration and characterization of acidophilic microorganisms isolated for a pilot plant stirred-tank bioleaching operation. *Appl Environ Microbiol* 69:1936–1943.
7. McGuire MM, Edwards KJ, Banfield JF, Hamers RJ. 2001. Kinetics, surface chemistry, and structural evolution of microbially mediated sulfide mineral dissolution. *Geochem Geophy Geosyst* 65:1243–1258.
8. Dopson M, Lindstrom EB. 1999. Potential role of *Thiobacillus caldus* in arsenopyrite bioleaching. *Appl Environ Microbiol* 65:36–40.
9. Zhou QG, Bo F, Bo ZH, Xi L, Jian G, Fei LF, Hau CH. 2007. Isolation of a strain of *Acidithiobacillus caldus* and its role in bioleaching of chalcopyrite. *World J Microb Biotechnol* 23:1217–1225.
10. Gadd GM, Griffiths AT. 1978. Microorganisms and heavy metal toxicity. *Microbial Ecol* 4:303–317.
11. Nies DH. 1999. Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51:730–750.

12. Nies DH. 2000. Heavy metal-resistant bacteria as extremophiles: Molecular physiology and biotechnological use of Ralstonia sp CH34. *J Bacteriol* 182:1390–1398.
13. Nies DH. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* 23:313–339.
14. Silver S. 1996. Bacterial resistance to toxic ions: A review. *Gene* 179:9–19.
15. Wood JM, Wang HK. 1983. Microbial resistance to heavy metals. *Environ Sci Technol* 17:582–590.
16. Barreira RPR, Villar LD, Garcia O. 2005. Tolerance to copper and zinc of *Acidithiobacillus thiooxidans* isolated from sewage sludge. *World J Microb Biotechnol* 21:89–91.
17. Chen BY, Chen YW, Wu DJ, Cheng YC. 2003. Metal toxicity assessment upon indigenous *Thiobacillus thiooxidans* BC1. *Environ Eng Sci* 20:375–385.
18. Das A, Modak JM, Natarajan KA. 1997. Studies on multi-metal ion tolerance of *Thiobacillus ferrooxidans*. *Miner Eng* 10:743–749.
19. Leduc LG, Ferroni GD, Trevors JT. 1997. Resistance to heavy metals in different strains of *Thiobacillus ferrooxidans*. *World J Microb Biotechnol* 13:453–455.
20. Natarajan KA, Sudeesha K, Rao GR. 1994. Stability of copper tolerance in *Thiobacillus ferrooxidans*. *A Van Leeuw J Microbiol* 66:303–306.
21. Tuovinen OH. 1974. Studies on the growth of *Thiobacillus ferrooxidans*. II. Toxicity of uranium to growing cultures and tolerance conferred by mutation, other metal cations and EDTA. *Arch Microbiol* 95:153.
22. Hong-mei L, Jia-jun K. 2001. Influence of Ni^{2+} and Mg^{2+} on the growth and activity of Cu^{2+}-adapted *Thiobacillus ferrooxidans*. *Hydrometallurgy* 61:151–156.
23. Dopson M, Lindstrom EB, Hallberg KB. 2001. Chromosomally encoded arsenical resistance of the moderately thermophilic acidophile *Acidithiobacillus caldus*. *Extremophiles* 5:247–255.
24. Kotze AA, Tuffin IM, Deane SM, Rawlings DE. 2006. Cloning and characterization of the chromosomal arsenic resistance genes from *Acidithiobacillus caldus* and enhanced arsenic resistance on conjugal transfer of *ars* genes located on transposon Tn*AtsArs*. *Microbiology* 152:3551–3560.
25. Tuffin M, Hector SB, Deane SM, Rawlings DE. 2006. Resistance determinants of a highly arsenic-resistant strain of *Leptospirillum feriphilum* isolated from a commercial biooxidation tank. *Appl Environ Microb* 72:2247–2253.
26. Watkin ELJ, Keeling SE, Perrot FA, Shiers DW, Palmer ML, Watling HR. 2009. Metals tolerance in moderately thermophilic isolates from a spent copper sulfide heap, closely related to *Acidithiobacillus caldus*, *Acidimicrobium ferrooxidans* and *Sulfobacillus thermosulfidooxidans*. *J Indust Microbiol Biotechnol* 36:461–465.
27. Rawlings DE. 2002. Heavy metal mining using microbes. *Annu Rev Microbiol* 56:65–91.
28. Banks D, Younger PL, Arnesen RT, Iversen ER, Banks SB. 1997. Mine-water chemistry: The good, the bad and the ugly. *Environ Geol* 32:157–174.
29. Benner SG, Blowes DW, Gould WD, Herbert RB, Ptacek CJ. 1999. Geochemistry of a permeable reactive barrier for metals and acid mine drainage. *Environ Sci Technol* 33:2793–2799.
30. Alvarez S, Jerez C. 2004. Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*. *Appl Environ Microb* 70:5177–5182.
31. Xu KD, McFeters GA, Stewart PS. 2000. Biofilm resistance to antimicrobial agents. *Microbiology* 146:547–549.
32. Gikas P. 2007. Kinetic response of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)): an isobolographic approach. *J Hazard Mater* 143:246–256.
33. Oorts KK. 2006. Discrepancy of the microbial response to elevated copper between freshly spiked and long-term contaminated soils. *Environ Toxicol Chem* 25:845–853.
34. Oorts KK. 2007. Leaching and aging decrease nickel toxicity to soil microbial processes in soils freshly spiked with nickel chloride. *Environ Toxicol Chem* 26:1130–1138.
35. Maderova L, Dawson JJC, Paton GI. 2009. Cu and Ni mobility and bioavailability in sequentially conditioned soils. *Water Air Soil Pollut* 210:63–73.
36. Aston JE, Apel WA, Lee BD, Peyton BM. Effects of cell condition, pH, and temperature on lead, zinc, and copper sorption to Acidithiobacillus caldus. *J Hazard Mater*, DOI: 10.1016/j.jhazmat.2010.07.110.
37. Kawabe Y, Chihiro I, Tadashi C. 2000. Relaxation of chloride inhibition on the biochemical activity of *Thiobacillus ferrooxidans* by Diatomaceous Earths. *J Min Mat Proc Inst Japan* 116:198–202.
38. Dopson M, Baker-Austin C, Koppineedi PR, Bond PL. 2003. Growth in sulfidic mineral environments: Metal resistance mechanisms in acidophilic microorganisms. *Microbiology* 149:1959–1970.
39. Gadd GM. 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Curr Opin Biotechnol* 11:271–279.
40. Veglio F, Beolchini F. 1997. Removal of metals by biosorption: A review. *Hydrometallurgy* 44:301–316.