Quantification of Toxic and Inhibitory Impact of Copper and Zinc on Mixed Cultures of Sulfate-Reducing Bacteria

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Abstract: The adverse effects of copper and zinc on an acetate-utilizing mixed cultures of sulfate-reducing bacteria (SRB) at concentrations below the toxic concentration (minimum metal concentration at which no sulfate reduction is observed) are reported in this paper. Mathematical models were developed to incorporate the toxic and inhibitory effects (defined as the reduction in bacterial population upon exposure to the metal and the decrease in the metabolic rate of sulfate reduction by the SRB, respectively) into the sulfate-reduction biokinetics. The characteristic toxicity and inhibition constants were obtained from the measurements of bacterial populations and dissolved metal concentrations in serum bottle studies conducted at 35°C and pH 6.6. Both copper and zinc had toxic and inhibitory effects on SRB. The toxicity constants for copper and zinc were 10.6 and 2.9 mM^{-1}, respectively, indicating that exposure to copper resulted in a higher mortality of SRB than did exposure to zinc. The values of the inhibition constants were found to be 17.9 ± 2.5 and 25.2 ± 1.0 mM^{-1} for copper and zinc, respectively. This implies that dissolved zinc was slightly more inhibitory to SRB than copper. The models presented in the paper can be used to predict the response of a sulfate-reduction bioreactor to heavy metals during acid mine drainage treatment. **Biotechnol Bioeng* **82**: 306–312, 2003.
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INTRODUCTION

Heavy metals impact bacterial communities adversely by deactivating enzymes, denaturing proteins, and competing with essential cations (Mazidji et al., 1992; Mosey and Hughes, 1975). The impact of dissolved metals is dependent upon a number of factors. Binding to environmental constituents, interactions with ions, biosorption, and biotransformations tend to mitigate the toxic effects of metals (Babich and Stotzky, 1981a,b, 1985; Chen et al., 2000; Gadd and Griffiths, 1978).

Table I presents a literature summary of the characteristic toxic concentration (concentration causing a cessation of sulfate reduction activity) of various heavy metals to pure strains and mixed cultures of sulfate-reducing bacteria (SRB), which can be used for the remediation of acid mine drainage (AMD) (Dvorak et al., 1992; Utgikar et al., 2000). The metals should be removed from the AMD stream prior to the biotreatment to prevent their adverse impact on the SRB (Utgikar et al., 2002).

A SRB culture exposed to a heavy metal at a concentration below the characteristic toxic concentration for that metal will continue its sulfate reduction activity, although at a lower rate, ultimately reducing the metal concentration to zero by precipitating it as sulfide. While the adverse effects (of metals) are known and described qualitatively, they remain to be discussed in quantitative terms from a kinetic viewpoint. The objective of this study was to develop mathematical rate expressions that incorporate the adverse effects into the biokinetics of sulfate reduction. A theoretical discussion of the adverse effects is presented along with the technique to modify the biokinetic rate expression to account for these effects. The characteristic parameters describing the adverse effects of zinc and copper on an acetate-utilizing mixed culture of SRB were determined experimentally. The choice of these two metals was dictated by their abundance in the acid mine water from the Berkeley Pit, Butte, Montana (Davis and Ashenberg, 1989).

Theory

Equations (1) and (2) represent the microbial sulfate reduction reaction (utilizing acetate) and the metal sulfide precipitation reaction, respectively (Brierly and Brierly, 1997).

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Table I. Heavy metal toxicity to SRB.

Metal	SRB strain	Toxic concentration, mg/L	Reference
Cu	*Desulfovibrio* strains	20–50	Booth and Mercer (1963)
	Desulfovibrio strains	3	Temple and Le Roux (1964)
	Desulfovibrio strains	2–20	Saleh et al. (1964)
	Mixed culture	4–20	Hao et al. (1994)
	Mixed culture	12	Utgikar et al. (2001)
Zn	Mixed culture	25–40	Hao et al. (1994)
	Mixed culture	20	Utgikar et al. (2001)
	Desulfovibrio desulfuricans	13	Poulson et al. (1997)
Pb	Mixed culture	75–80	Hao et al. (1994)
	Strain L-60[a]	125	Loka Bharathi et al. (1990)
Cd	Mixed culture	>4–20	Hao et al. (1994)
	Strain L-60[a]	54	Loka Bharathi et al. (1990)
Ni	Mixed culture	10–20	Hao et al. (1994)
	Desulfovibrio desulfuricans	10	Poulson et al. (1997)
Cr	Mixed culture	60	Hao et al. (1994)
Hg	Strain L-60[a]	74	Loka Bharathi et al. (1990)
Mixture (Cr, Ni, Cu, Cd, Zn, Pb)	Mixed culture	20	Hao et al. (1994)

[a]Resembles *Desulfosarcina.*

$$\underset{\text{(electron donor)}}{CH_3COO^-} + \underset{\text{(electron acceptor)}}{SO_4^{2-}} \rightarrow HS^- + 2HCO_3^- \quad (1)$$

$$\underset{\text{(metal ion)}}{Me^{2+}} + \underset{\text{(biogenic sulfide)}}{HS^-} \rightarrow \underset{\text{(metal sulfide)}}{MeS\downarrow} + H^+ \quad (2)$$

The rate of sulfate reduction by SRB (reaction 1) can be described by Monod kinetics (Robinson and Tiedje, 1983):

$$-\frac{dS}{dt} = \frac{k\,S\,X}{K_S + S} \quad (3)$$

Han and Levenspiel (1988) have presented an in-depth discussion of the various mathematical expressions used to describe the inhibition kinetics in presence of an interfering species in microbial systems. Competitive inhibition of biochemical reactions is often modeled using the Haldane equation. Aiba et al. (1968) used an empirical inverse exponential model to describe the effect of product inhibition in alcohol fermentations. Similar expressions have been used to describe the adverse effects of substrates and other interfering species (Bellgardt, 1991; Edwards, 1970). The models reported in the literature almost exclusively involve either a substrate or a product involved in the biochemical reaction, and they do not describe inhibition by heavy metals.

The broad non-specific impact of metals on microbial activity (Mazidji et al., 1992) may manifest itself through two distinct modes of action: (1) causing mortality of less-tolerant species leading to a decrease in total number of viable SRB and species diversity, and (2) reducing the metabolic rate of the surviving culture. The first effect causing death of cells may be described as a toxic effect, while the second effect related to the rate of reaction may be treated as an inhibitory effect. This distinction is made in order to facilitate and simplify the quantitative analysis of the adverse effects.

Mathematically, the reduction in total number of viable SRB translates to a decrease in X (SRB concentration) in Eq. (3). A decrease in the metabolic rate, on the other hand, can be incorporated in the biokinetics by a reduction in rate constant (k). Equation (3) is therefore modified in presence of a heavy metal as

$$-\frac{dS}{dt} = \frac{k(M)\,S\,X(M)}{K_S + S} \quad (4)$$

It should be noted that the dissolved metal concentration decreases [due to precipitation (reaction 2)] as the sulfate-reduction progresses even though the metal itself does not participate in the sulfate-reduction bioreaction [Eq. (1)] either as a product or a reactant (substrate). Under these conditions, the toxic and inhibitory effects of the metal species can be conveniently incorporated into the biokinetics using the following inverse exponential relationships:

$$X(M) = X_0 \exp(-K_T\,M) \quad (5)$$

and

$$k(M) = k_0 \exp(-K_I\,M) \quad (6)$$

It can be seen that both $X(M)$ and $k(M)$ decrease with in-

creasing M, indicating that the adverse effects become larger as the metal concentration increases and reduce to the base values (X_0 and k_0) in absence of the metal (at $M = 0$).

Determination of Toxicity and Inhibition Constants

Equation (5) indicates that K_T will be the negative of the slope of the straight line ln[$X(M)$] vs. M. From Eqs. (1) and (2),

$$\frac{dM}{dt} = \frac{dS}{dt} \tag{7}$$

Combining Eqs. (4)–(7),

$$-\frac{dM}{dt} = \frac{k_0 \exp(-K_I M)\, S\, X_0 \exp(-K_T M)}{K_S + S} \tag{8}$$

Sulfate concentration in the acid mine waters is typically in thousands of mg/L (Davis and Ashenberg, 1989), which is 2–3 orders of magnitude higher than the toxic metal concentrations (ca. 30 mM of sulfate at 3,000 mg/L vs. 0.3 mM of Zn at 20 mg/L). It can be treated as a constant during the time required to reduce the metal concentration from M to zero. Equation (8) can then be rearranged in the following form:

$$-\frac{1}{X(M)}\frac{dM}{dt} = K_{lum} \exp(-K_I M), \tag{9}$$

where K_{lum} is a lumped constant defined by

$$K_{lum} = \frac{k_0\, S}{K_S + S} \tag{10}$$

dM/dt is obtained by measuring metal concentration as a function of time. The plot of natural logarithm of the left-hand side of Eq. (9) vs. M will be a line with slope $-K_I$. However, this will require measurement of $X(M)$ at each point in time. The determination of bacterial population estimates is generally time consuming and cumbersome. The problems associated with these measurements can be minimized by utilizing only the initial rate of decrease of metal concentration. Mathematically,

$$\left(-\frac{1}{X(M)}\frac{dM}{dt}\right)\bigg|_{t=0} = K_{lum} \exp(-K_I M) \tag{11}$$

An experimental scheme in which viable SRB populations are estimated after exposure to heavy metals and the progress of sulfate reduction is monitored through the measurement of metal concentration would yield both the toxicity constant (K_T) and inhibition constant (K_I) as described above.

MATERIALS AND METHODS

SRB Stock Culture

The SRB culture used in the present study was obtained from a 20-L master reactor containing an acetate-utilizing mixed culture of SRB maintained at 35°C and a pH of 7.5 ± 0.5. The initial seed source for the master reactor was the anaerobic digester sludge obtained from a domestic sewage treatment plant in Cincinnati, Ohio. Sulfidogenic conditions were provided in the master culture reactor by maintaining an excess of sulfate over acetate (the organic substrate utilized by the mixed culture) to insure the dominance of SRB over methanogens. A drain-and-fill schedule for the master culture reactor involved a weekly replacement of 20% of the volume by fresh Postgate's C medium (Atlas, 1993) modified to contain acetate instead of lactate. An aliquot of the master culture was washed thoroughly by suspending it in distilled deionized water and centrifuging at 2,500g for 10 min in a CRU-5000 IEC Centrifuge (Fisher Scientific, Pittsburgh, PA). This procedure was repeated three times to ensure the removal of traces of dissolved sulfide prior to its use as inoculum for the toxicity/inhibition studies. Dissolved sulfide can react and render non-toxic an equivalent amount of heavy metal (Lawrence and McCarty, 1965), and it has to be removed to eliminate its confounding effect on SRB.

Nutrient Medium Design

Metal tolerance of a culture is a function of metal bioavailability (Hines and Jones, 1982) and precipitation alters the concentration of dissolved metals. The nutrient medium was designed to eliminate metal precipitation, which is a confounding factor in toxicity studies (Poulson et al., 1997). The pH of the medium was adjusted to 6.60 ± 0.05 to minimize the precipitation of metals as hydroxides. Similarly, the phosphate concentration was lowered [from the value recommended for most sulfate reducers (Atlas, 1993)] to preclude precipitation as phosphates. The pH and C/P ratios are still within the range of optimum conditions determined for some sulfate reducers (Okabe and Characklis, 1992; Reis et al., 1992). The nutrient solution composition used in the study is shown in Table II. The nutrient solution was sterilized by autoclaving at 121°C for 15 min after preparation.

Serum Bottle Studies

Metal ion (copper or zinc) containing solutions were prepared by adding stock metal sulfate (copper or zinc) solu-

Table II. Nutrient medium composition.

Component	Concentration, mg/L
Ammonium chloride (NH_4Cl)	1,000
Potassium phosphate (KH_2PO_4)	50
Sodium acetate (CH_3COONa)	6,000
Sodium sulfate (Na_2SO_4)	4,500
Yeast extract	1,000
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	300
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	60
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	60
Ferrous sulfate ($FeSO_4 \cdot 5H_2O$)	4

tions to the nutrient medium. The pH of the spiked solutions was adjusted to 6.60 ± 0.05. The nutrient medium without copper or zinc was designated as the no-metal control (referred hereafter simply as the control). The control media were spiked with ferrous ion that served as surrogate ion for measurement of metabolic activity. It should be noted that the term "no-metal" means absence of either copper or zinc. The control and metal-containing media were then dispensed into 125-mL butyl rubber stopper-aluminum crimp seal Pyrex serum bottles. The bottles were autoclaved and seeded with mixed SRB culture in an anaerobic chamber (Model 855-AC, Plas-Labs, Lansing, MI) after cooling. The inoculated bottles were incubated at 35°C in a New Brunswick rotary shaker. Samples were withdrawn from the serum bottles within 30 min after inoculation for the estimation of bacterial populations using a most-probable number technique. The serum bottles were also sampled for the analysis of dissolved metal concentrations as a function of time. Samples were withdrawn by piercing the stopper with a B-D disposable needle (#305194) attached to a B-D disposable syringe (#309603) through a 0.2-μm Fisherbrand nylon syringe filter (#09-719-C, Fisher Scientific Company, Pittsburgh, PA).

Analytical Methods

The dissolved metal concentrations were determined by an inductively coupled argon plasma emission spectroscopy [Method 3120 (APHA, 1998)] on a Perkin-Elmer Optima 3300 DV instrument. The pH of the solutions was measured by an Accumet (AR-25) pH/mV/ISE meter (Fisher Scientific, Pittsburgh, PA).

A Most Probable Number (MPN) Technique for Enumeration of SRB

A most probable number (MPN) technique, similar to those reported in literature (Jain, 1995; Vester and Ingvorsen, 1998) was used in this study for the estimation of viable SRB population. Postgate B medium was modified by replacing sodium lactate by 3 g/L of sodium acetate and the pH adjusted to 7.0 ± 0.2 to maintain the similarity between the constituents of the media used in toxicity studies and MPN tests. Difco anaerobic agar (1.5 g/L) was also added to the medium to obtain semisolid medium; 9 mL of the medium was dispensed into anaerobic aluminum crimp seal, butyl-rubber stoppered Hungate tubes each (#2048, Bellco Glass, Vineland, NJ) and autoclaved at 121°C for 15 min. Six to 8 serial 10-fold dilutions of SRB samples were prepared in deaerated, distilled deionized water inside the anaerobic chamber and 1 mL of the appropriate dilution was inoculated into the semisolid modified Postgate B medium. Five MPN tubes were set up at each dilution, incubated at 35°C in a controlled temperature room and observed for the blackening (ferrous sulfide precipitate) for the detection of SRB activity. MPNs were calculated using the most probable number calculator program developed by Klee (1993).

RESULTS AND DISCUSSION

The concentration profiles of zinc and copper at various concentrations are shown in Figures 1 and 2, respectively. The control data (no copper or zinc) are shown in Figure 1 along with the data for zinc. Ferrous ion served as the surrogate ion in control samples as mentioned earlier. The toxicity and inhibition constants were calculated using the procedure explained in the theory section above. The initial SRB populations were plotted as a function of initial metal concentration as shown in Figure 3. The toxicity constants (K_T) were calculated from the slope of the line [Eq. (5)] and were found to be 10.6 mM^{-1} for copper and 2.9 mM^{-1} for zinc.

Figure 4 shows the graphical representation of Eq. (11) used to calculate the inhibition constants (K_1). The initial rates of change of metal concentrations (dM/dt at $t = 0$) were obtained from the metal concentration measurements shown in Figures 1 and 2. The zero metal concentration data point was obtained from the control data. The values of the inhibition constants were 18.3 mM^{-1} for copper and 25.5 mM^{-1} for zinc. The coefficients of correlation (r^2) for lines in Figure 4 were 0.97 for copper and 0.92 for zinc. The data used in calculating toxicity constants did not fit the linear model as well (Fig. 3) with the correlation coefficients (r^2) being 0.76 for copper and only 0.50 for zinc. These lower numbers are due to primarily the inherent uncertainty of measurements of the population estimates using the MPN method. The 95% confidence interval limits typically vary by an order of magnitude for any MPN result (APHA, 1998). The analysis of the kinetics presented in the paper requires estimation of viable SRB population, possible only through the use of MPN methods, and the values of the characteristic constants are affected by the accuracy limitations of the MPN method. The molecular biology techniques, although more accurate, are unable to distinguish between viable and nonviable SRB (Jain, 1995). The results indicate that SRB population numbers were much less sensitive to zinc than copper, possibly due to different mecha-

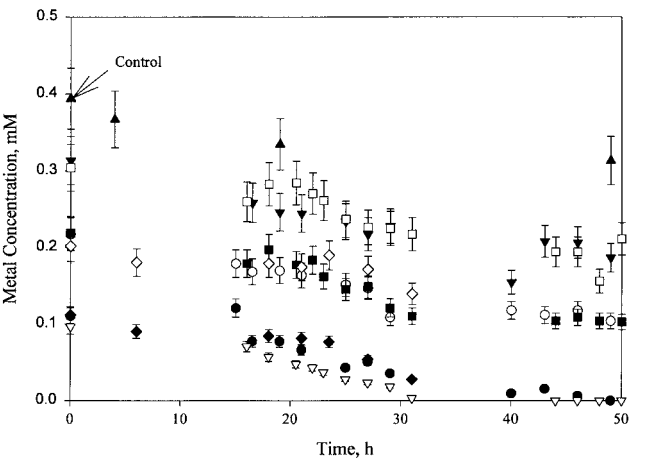

Figure 1. Metal concentration as a function of time. Initial concentration (mM): ●, 0.10; ○, 0.21; ▼, 0.32; ▽, 0.095; ■, 0.22; □, 0.32; ◆, 0.11; ◇, 0.21; ▲, control.

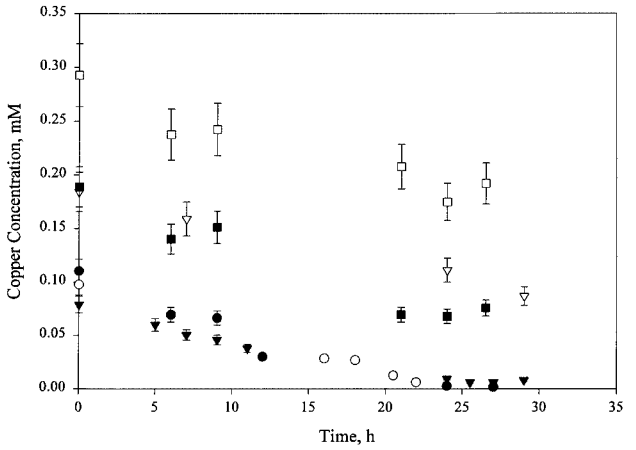

Figure 2. Copper concentration as a function of time. Initial concentration (m*M*): ●, 0.11; ○, 0.11; ▼, 0.097; ▽, 0.19; ■, 0.195; □, 0.295.

nisms of cell deactivation and death by the two metals. Zinc, unlike copper and other transition metals, has no redox-function in microbial systems, which could lead to differences in their interactions with the bacterial culture (Beveridge et al., 1997; Hughes and Poole, 1989). Further investigations into the exact mechanisms are needed for obtaining an in-depth understanding of this phenomenon.

The values of the toxicity constants indicate that copper is more toxic than zinc; that is, exposure to copper results in a greater mortality among the SRB. This finding is consistent with the reported literature values of toxic concentrations, which are lower for copper than zinc (as seen from Table I). Further, using these values of toxicity and inhibition constants, the metal concentrations that will essentially stop sulfate reduction (decrease sulfate reduction rate by a factor of 1,000) are calculated to be ca. 16 mg/L from Eq. (8). These values show good agreement with those listed in Table I.

It can also be seen that zinc has a slightly higher inhibition constant than copper. The implication of this finding is that the metabolic rate of the surviving SRB population is affected to a greater extent by the presence of zinc than copper. Consequently, the sulfide generation rate and metal

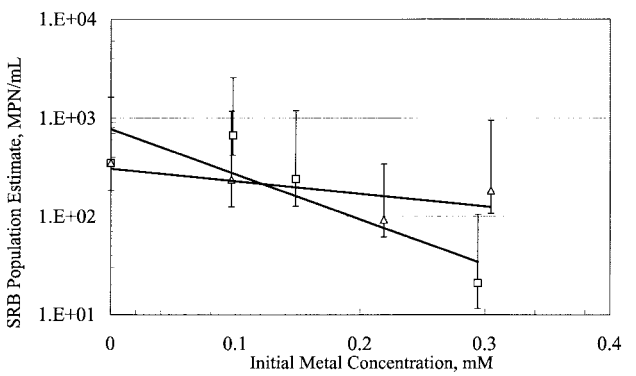

Figure 3. Toxicity of zinc and copper to SRB: □, copper; △, zinc; —, model curve.

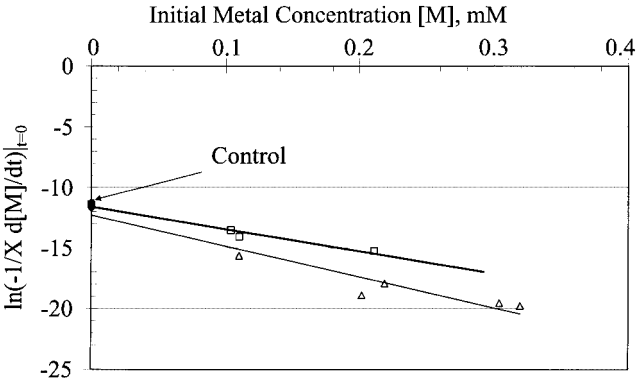

Figure 4. Inhibition of SRB by copper/zinc: □, copper; △, zinc; ●, control; —, model curve.

sulfide precipitation rate will be higher in the presence of copper than zinc, provided that the rest of the conditions—nutrient and biomass concentrations, temperature, etc.,—are the same. The time needed to reduce the metal concentration to zero will be consequently less for copper.

Acid mine drainage containing a mixture of heavy metals has a similar toxic and inhibitory impact on the metabolic processes of the sulfate-reducing bacteria. The adverse effects of individual heavy metals have been studied and reported in this study using synthetic solutions containing either zinc or copper. Additional studies are in progress for the determination of the characteristic toxicity and inhibition constants for mixtures of heavy metals and for the Berkeley Pit acid mine water.

Implications for the Operation of Sulfate-Reduction Bioreactors

The results of this study along with the data on the toxic concentrations (for example, Table I) can be used to predict the transient response of an operating sulfate-reduction bioreactor used in the biotreatment of AMD to a shock-load of heavy metal. SRB cultures are protected from the adverse effects of heavy metals by the sulfide produced by their metabolic activity (Temple and Le Roux, 1964). The SRB and consequently the bioreactor operation will be adversely affected if the shock load exceeds the protection afforded by the biogenic sulfide. The bioreactor operation will cease if the net resultant exposure of SRB to the heavy metal (after accounting for sulfide protection) is in excess of its toxic concentration. When the exposure is at a concentration less than the toxic concentration, the loss in the bacterial population can be determined from the toxicity constant values. The time needed to eliminate the metals from the bioreactor solution by precipitation as metal sulfide through the reaction with biogenic sulfide can be estimated by incorporating the inhibition constant in the sulfate reduction biokinetics.

CONCLUSIONS

A novel approach for incorporating the adverse impacts of a species not involved in the microbial reaction into the

biokinetic model is presented in this study. The toxic and inhibitory effects of copper and zinc on an acetate-utilizing mixed culture of sulfate-reducing bacteria were monitored and analyzed through the measurements of their aqueous phase concentrations. The toxic effect of the metals was manifest through the reduction in the number of viable sulfate-reducers and the inhibition of sulfate-reduction activity was correlated to the rate of decrease of the dissolved metal concentrations. Inverse exponential relationships were used in the present study to model the dependence of the two effects on the dissolved metal concentrations. The characteristic toxicity constants for zinc and copper were 2.9 and 10.6 mM^{-1}, respectively, indicating that copper was more toxic than zinc. However, the characteristic inhibition constant was slightly greater for zinc (25.2 mM^{-1}) than copper (17.9 mM^{-1}), indicating the dissolved zinc concentration inhibited the surviving SRB population to a greater extent than copper. The models developed in this study are useful in estimating the impact of heavy metals on a sulfate-reduction bioreactor. The results of this study are significant for the fundamental understanding of the toxicity and inhibition effects and the operation and control of microbial sulfate reduction process.

NOMENCLATURE

k	Rate Constant in Monod Equation (mol/(MPN/mL) · h)
K_I	Characteristic Inhibition Constant (mM^{-1})
K_{lum}	Lumped constant defined by Eq. (10) (mol/(MPN/mL) · h)
K_S	Monod Constant (mM)
K_T	Characteristic toxicity constant (mM^{-1})
M	Metal concentration (mM)
S	Substrate concentration (mM)
t	Time (h)
X	Biomass concentration (MPN/mL)

Subscripts

0	Dissolved metal concentration = 0

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