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Toxicity Evaluation of Complex Metal Mixtures Using Reduced Metal Concentrations: Application to Iron Oxidation by *Acidithiobacillus ferrooxidans*

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In this study, we investigated the inhibition effects of single and mixed heavy metal ions (Zn^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}) on iron oxidation by *Acidithiobacillus ferrooxidans*. Effects of metals on the iron oxidation activity of *A. ferrooxidans* are categorized into four types of patterns according to its oxidation behavior. The results indicated that the inhibition effects of the metals on the iron oxidation activity were noncompetitive inhibitions. We proposed a reduced inhibition model, along with the reduced inhibition constant (α_i), which was derived from the inhibition constant (K_i) of individual metals and represented the tolerance of a given inhibitor relative to that of a reference inhibitor. This model was used to evaluate the toxicity effect (inhibition effect) of metals on the iron oxidation activity of *A. ferrooxidans*. The model revealed that the iron oxidation behavior of the metals, regardless of metal systems (single, binary, ternary, or quaternary), is closely matched to that of any reference inhibitor at the same reduced inhibition concentration, $[I]_{reduced}$, which defines the ratio of the inhibitor concentration to the reduced inhibition constant. The model demonstrated that single metal systems and mixed metal systems with the same reduced inhibitor concentrations have similar toxic effects on microbial activity.

Keywords: *Acidithiobacillus ferrooxidans*, metal toxicity, reduced inhibition constant, reduced metal concentration, reduced inhibition model

As natural resources are progressively being depleted, development of new technologies in recovering precious metals from low-grade ores is becoming important. Bioleaching is one of the most recently developed processes and is currently being commercialized in several countries [1, 5, 12, 28–30]. It utilizes the biological activity of bacteria or fungi to dissolve and extract metals from an insoluble solid. The process therefore is an environment-friendly technology with lower cost for operation and energy than conventional technologies such as chemical leaching [5].

One of the representative microorganisms used in the bioleaching process is *Acidithiobacillus ferrooxidans* [1, 18, 20, 27, 30]. *A. ferrooxidans* is an autotrophic bacterium that can oxidize the reductive compounds of iron and sulfur. Metal leaching by *A. ferrooxidans* proceeds *via* either direct or indirect oxidation by Fe^{3+} produced by oxidation of Fe^{2+}. Bioleaching efficiency is determined by various factors including nutrients, O_2, CO_2, pH, temperature, substrates, heavy metal concentrations, surfactants, and microbial activity [5, 17]. Heavy metals in high concentration have a clear negative impact on bacterial biomass, various enzyme activities, and C and N mineralization [8, 11, 21, 33]. Therefore, promising microorganisms for bacterial leaching applications are mine-dwelling microorganisms, which show a high tolerance against metal toxicity owint to high concentrations of metals in leachate obtained during bioleaching of ores.

The tolerance of *A. ferrooxidans* to metal ions is well documented in previous studies [8–11, 13–15, 21, 22, 30, 33]. For example, the tolerance of *A. ferrooxidans* to individual metal ions is known to be 10–800 mM of Cu^{2+}, 160–1,000 mM of Ni^{2+}, 80–750 mM of Cd^{2+}, and 400–1,000 mM of Zn^{2+} [7–10, 14, 21, 23, 29]. However, as bacterial leaching is usually applied to solutions containing a mixture of metal ions rather than a single metal ion, knowledge on the effect of other metals on the iron oxidation activity of *A. ferrooxidans* is crucial to control the bacterial leaching process.

Few studies have addressed this issue. The effect of metal concentration on iron oxidation activity in complex metal mixtures is more difficult to evaluate than in a single metal system. The contribution of each component in a mixture of metal ions to the toxicity value is not proportional to the weight fraction or relative numbers of ions in the mixture. This necessitates development of a new model that can

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interpret the relationship between metal mixtures and toxicity value in a more efficient and accurate way.

In the present study, therefore, we investigated the inhibition effects of single and mixed heavy metal ions such as Zn^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} on the rate of iron oxidation of *A. ferrooxidans*. On the basis of our experimental results, we also proposed a reduced inhibition model, which can allow conversion of the different toxicity of each component to that of the reference metal.

MATERIALS AND METHODS

Strain and Medium

A. ferrooxidans (ATCC 19859) cultivated in 9 K medium was used throughout this study. The 9 K medium contained the following; 3.0 g/l of $(NH_4)_2SO_4$, 0.1 g/l of KCl, 0.5 g/l of K_2HPO_4, 0.5 g/l of $MgSO_4 \cdot 7H_2O$, 0.01 g/l of $Ca(NO_3)_2$, and 45.0 g/l of $FeSO_4 \cdot 7H_2O$ (equivalent to 9 g/l of ferrous iron). The pH of the medium was adjusted to 2.0 with sulfuric acid. *A. ferrooxidans* was innoculated to the 9K medium and incubated at 30°C for 3 days. The culture broth was centrifuged at 3,500 rpm for 5 min to remove jarosite particles. The supernatant was then collected and further centrifuged at 8,000 rpm for 20 min to harvest the cells. The harvested cells were resuspended in the mineral salt medium and used as an innoculum. The composition of the mineral salt medium was identical to that of the 9 K medium except for $FeSO_4 \cdot 7H_2O$.

Effect of Metal Ion Concentration on Iron Oxidation Rate

All the experiments were carried out in 250-ml flasks. The flasks were charged with 100 ml of 9 K medium, and single or mixed metal ions in the form of sulfate salts were added to the 9 K medium. Heavy metal ions added to the flasks were Cu^{2+} ($CuSO_4 \cdot 5H_2O$), Zn^{2+} ($ZnSO_4 \cdot 7H_2O$), Ni^{2+} ($NiSO_4 \cdot 6H_2O$), and Cd^{2+} ($CdSO_4 \cdot 7H_2O$). Detailed descriptions of the metal ion combinations used are listed in Table 1. Response surface methodology (RSM) was used to set up all the experimental conditions listed in Table 1. The RSM enabled assessment in effects of multiple parameters, either alone or in combination, on response [6, 35]. The main idea of RSM is to use a set of designed experiments to obtain an optimal response. Based on the culture conditions of a single metal, the mixed metal compositions in Table 1 were designed to assess the effect of metal toxicity on the activity of *A. ferrooxidans* in multiple metal systems.

The inoculum of *A. ferrooxidans* was inoculated into a 250-ml flask containing 9 K medium; its initial optical density was 0.2 at 600 nm. The inoculum with high cell concentration was used to minimize the effect of cell growth on the iron oxidation during the experiments. The flasks were incubated at 30°C and 200 rpm. The culture broth from each flask was sampled every 6 or 12 h, followed by centrifuging at 12,000 rpm for 5 min to remove jarosite particles and bacterial cells, which might interfere with absorbance measurements by a UV/Vis spectrophotometer. Ferrous iron, ferric iron, and total iron concentrations of the culture broth sample were determined by using the method described in Ryu *et al.* [29]. All the experiments were performed in duplicate.

Reduced Inhibition Model

One of the major problems encountered in commercial bacterial ore leaching is the very low growth rate of *A. ferrooxidans*. This consequently makes insufficient cell populations and difficult cell growth determination [4]. Therefore, the majority of previous studies have not considered cell growth in evaluating the iron oxidation activity of *A. ferrooxidans* [9–11, 13, 21, 23, 29]. To address the problem in cell growth measurement, we developed a simple kinetic model of iron oxidation that is independent of cell growth; this model was then used to evaluate the effect of metal toxicity on the activity of *A. ferrooxidans*.

Various inhibition models such as competitive, noncompetitive, and uncompetitive models can be used to explain the effect of an inhibitor on microbial activity. These models cannot be applied to mixed inhibitors, however, and only provide comparative information on the inhibition intensity of microbial activity resulting from each individual inhibitor.

The Michaelis-Menten equation was employed to perform a kinetic analysis of iron oxidation by *A. ferrooxidans*. An apparent maximum

Table 1. Experimental conditions for various metal systems.

System	Metal ions added	Metal concentrations (mM)
Single metal systems	Cu^{2+}	0, 47, 94, 142, 160, 189
	Zn^{2+}	0, 230, 460, 690, 920
	Ni^{2+}	0, 51, 102, 153, 204, 850, 1,020
	Cd^{2+}	0, 27, 53, 80, 107, 440, 660
Binary and ternary metal systems	$Cu^{2+}/Zn^{2+}/Ni^{2+}$	0/0/0, 94/460/102, 0/460/102, 189/460/102, 94/460/0, 94/460/204, 94/0/102, 94/920/102, 47/230/51, 47/690/51, 47/230/153, 47/690/153, 142/230/51, 142/690/51, 142/230/153, 142/690/153
	$Cu^{2+}/Zn^{2+}/Cd^{2+}$	0/0/0, 94/460/53, 0/460/53, 189/460/53, 94/460/0, 94/460/12, 94/0/53, 94/920/53, 47/230/27, 47/690/27, 47/230/80, 47/690/80, 142/230/27, 142/690/27, 142/230/80, 142/690/80
	$Cu^{2+}/Ni^{2+}/Cd^{2+}$	0/0/0, 94/102/53, 0/102/53, 189/102/53, 94/0/53, 94/204/53, 94/102/0, 94/102/12, 47/51/27, 47/51/80, 47/153/27, 47/153/80, 142/51/27, 142/51/80, 142/153/27, 142/153/80
	$Zn^{2+}/Ni^{2+}/Cd^{2+}$	0/0/0, 460/102/53, 460/0/53, 460/204/53, 460/102/0, 460/102/12, 0/102/53, 920/102/53, 230/51/27, 690/51/27, 230/51/80, 690/51/80, 230/153/27, 690/153/27, 230/153/80, 690/153/80
Quaternary metal systems	$Cu^{2+}/Zn^{2+}/Ni^{2+}/Cd^{2+}$	0/0/0/0, 16/152/17/9, 31/306/34/18, 47/460/51/27, 63/612/68/36, 79/765/85/44

iron oxidation rate ($V_{m,app}$) and Michaelis constant (K_m) were obtained to determine the effect of individual metal toxicity on iron oxidation activity. The inhibition on the iron oxidation of *A. ferrooxidans* due to the presence of a single metal inhibitor takes the form of noncompetitive inhibition where $V_{m,app}$ is decreased with increase in the inhibitor's concentration.

The noncompetitive inhibition model is expressed as follows:

$$V=\frac{V_m}{\left(1+\frac{[I]}{K_I}\right)\left(1+\frac{K_m}{[S]}\right)}=\frac{V_{m,app}}{\left(1+\frac{K_m}{[S]}\right)} \quad (1)$$

where, $V_{m,app}=\frac{V_m}{\left(1+\frac{[I]}{K_I}\right)}$

where K_I is an inhibition constant, and [S] and [I] are the concentrations of substrate (Fe^{2+}) and inhibitor, respectively. As can be seen from Eq. (1), when the rate of the apparent reaction ($V_{m,app}$) is one-half of the maximum value, the inhibitor concentration is equal to the inhibition constant. So, K_I means the inhibitor concentration or the toxicity showing one-half of the maximum reaction rate (or microbial activity). Since the inhibitor is generally present as a mixture in the microbial reaction solution, an advanced inhibition model, which can be applied to mixed as well as single inhibitors, is needed. Therefore, we now propose a new model (the reduced inhibition model) allowing the conversion of the effects of various inhibitors on microbial activity to that of the reference inhibitor. Central to the proposed model is the reduced inhibition constant, defined as the ratio of the inhibition constant (or inhibitor concentration) of each metal species to that of a reference inhibitor metal species:

$$\alpha_i=\frac{(K_I)_i}{(K_I)_{ref}}=\frac{[I]_i}{[I]_{ref}} \quad (2)$$

If Cu^{2+} is the reference compound, the reduced inhibition constant is as follows:

$$\alpha_i=\frac{(K_I)_i}{(K_I)_{Cu}}=\frac{[I]_i}{[I]_{Cu}} \quad (3)$$

This means that the reduced inhibition constant represents the tolerance of any other inhibitor relative to that of the reference inhibitor. The reduced inhibitor concentration is the conversion of an inhibitor concentration to a reference inhibitor concentration *via* the reduced inhibition constant, and is expressed mathematically as

$$[I]_{reduced}=\frac{[I]_i}{\alpha_i} \quad (4)$$

The basic idea behind the reduced inhibition constant is that all the inhibitors behave alike at the same reduced concentration.

The new model enables universal and simple evaluation of inhibition effects in complex mixed systems. The reduced inhibition model is obtained with modification of Eq. (1) by the reduced inhibition constant that is derived from the inhibition constant (K_I) and inhibitor concentration [I]:

$$V=\frac{V_m}{\left(1+\frac{[I]_i}{(K_I)_i}\right)\left(1+\frac{K_m}{[S]}\right)}=\frac{V_m}{\left(1+\frac{[I]_i}{\alpha_i(K_I)_{ref}}\right)\left(1+\frac{K_m}{[S]}\right)} \quad (5)$$

$$=\frac{V_m}{\left(1+\frac{[I]_{reduced}}{(K_I)_{ref}}\right)\left(1+\frac{K_m}{[S]}\right)}=\frac{V_{m,app}}{\left(1+\frac{K_m}{[S]}\right)}$$

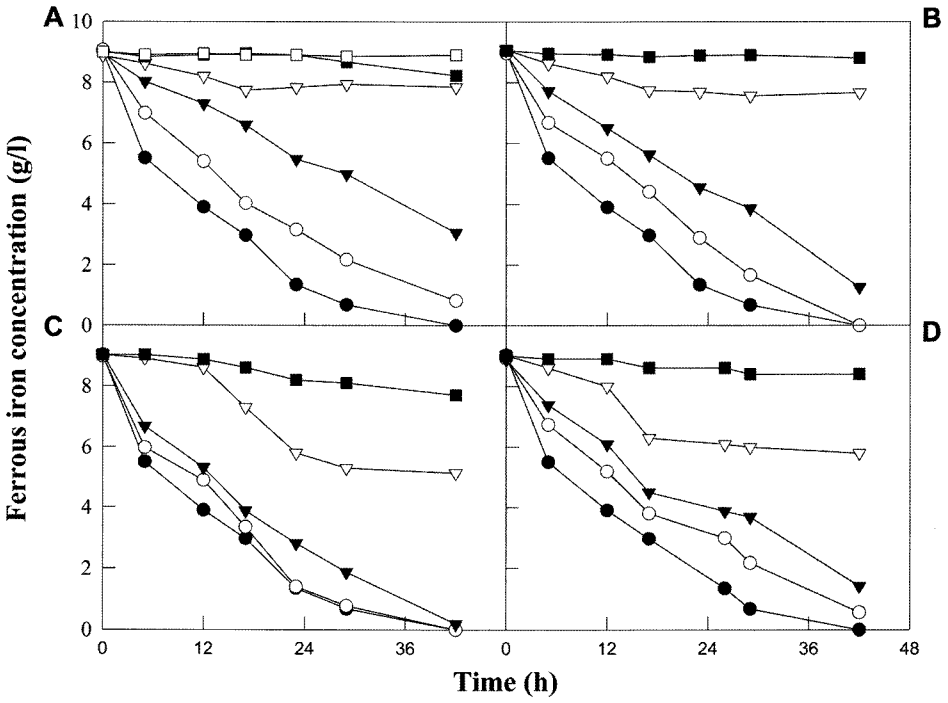

Fig. 1. Time profiles of Fe^{2+} concentration in 9 K medium in the presence of a single metal.
A. Cu^{2+} (mM): ●, 0; ○, 47; ▼, 94; ▽, 142; ■, 160; □, 189. **B.** Zn^{2+} (mM): ●, 0 ; ○, 230; ▼, 460; ▽, 690; ■, 920. **C.** Ni^{2+} (mM): ●, 0; ○, 102; ▼, 204; ▽, 850; ■, 1,020. **D.** Cd^{2+} (mM): ●, 0; ○, 53; ▼, 107; ▽, 440; ■, 660.

For a single inhibitor:

$$V_{m,app}=\frac{V_m}{\left(1+\frac{[I]_i}{\alpha_i(K_I)_{ref}}\right)}=\frac{V_m}{\left(1+\frac{[I]_{reduced}}{(K_I)_{ref}}\right)} \quad (6)$$

For multiple inhibitors:

$$V_{m,app}=\frac{V_m}{\left(1+\sum_{i=1}^{n}\frac{[I]_i}{\alpha_i(K_I)_{ref}}\right)}=\frac{V_m}{\left(1+\sum_{i=1}^{n}\frac{[I]_{reduced}}{(K_I)_{ref}}\right)} \quad (7)$$

where a_i is the reduced inhibition constant of inhibitor i, $(K_I)_{ref}$ is the inhibition constant of the reference inhibitor, and $(K_I)_i$ is the inhibition constant of inhibitor i.

RESULTS

Effect of Heavy Metals on Iron Oxidation by *A. ferrooxidans*

We evaluated the effects of metals (single, binary, ternary, and quaternary metal mixtures) on the Fe^{2+} oxidation activity of *A. ferrooxidans*. Fig. 1 shows the profiles of iron oxidation by *A. ferrooxidans* for each single metal system. The iron oxidation activity of *A. ferrooxidans* in the presence of 0–189 mM Cu^{2+} decreased with increasing Cu^{2+} concentration and was completely inhibited at or above 160 mM Cu^{2+} (Fig. 1A). The strain was able to oxidize iron over a very broad concentration range of Zn^{2+} (0–920 mM), but was completely inhibited above 920 mM Zn^{2+} (Fig. 1B). Oxidation in the presence of Ni^{2+} (0–1,020 mM) or Cd^{2+} (0–660 mM) similarly decreased with increasing their concentrations (Figs. 1C and 1D) and was completely inhibited above 1,020 mM and 660 mM, respectively.

Fig. 2 shows the typical iron oxidation by *A. ferrooxidans* in 9 K medium in the presence of a ternary metal mixture. Inhibition increased with increasing total metal concentration, but the effect was more substantial with increasing Cu^{2+} concentration. Among the ternary metal mixtures used in this study, the Ni/Cd/Zn mixture (Fig. 2B) was the only system without Cu^{2+}. The inhibition effect of metal on iron oxidation by *A. ferrooxidans* was not significant for the Ni/Cd/Zn mixture, whereas the three Cu^{2+}-containing ternary mixtures (Figs. 2A, 2C, and 2D) showed considerable inhibition effects with increasing Cu^{2+} concentration from 47 to 142 mM. This

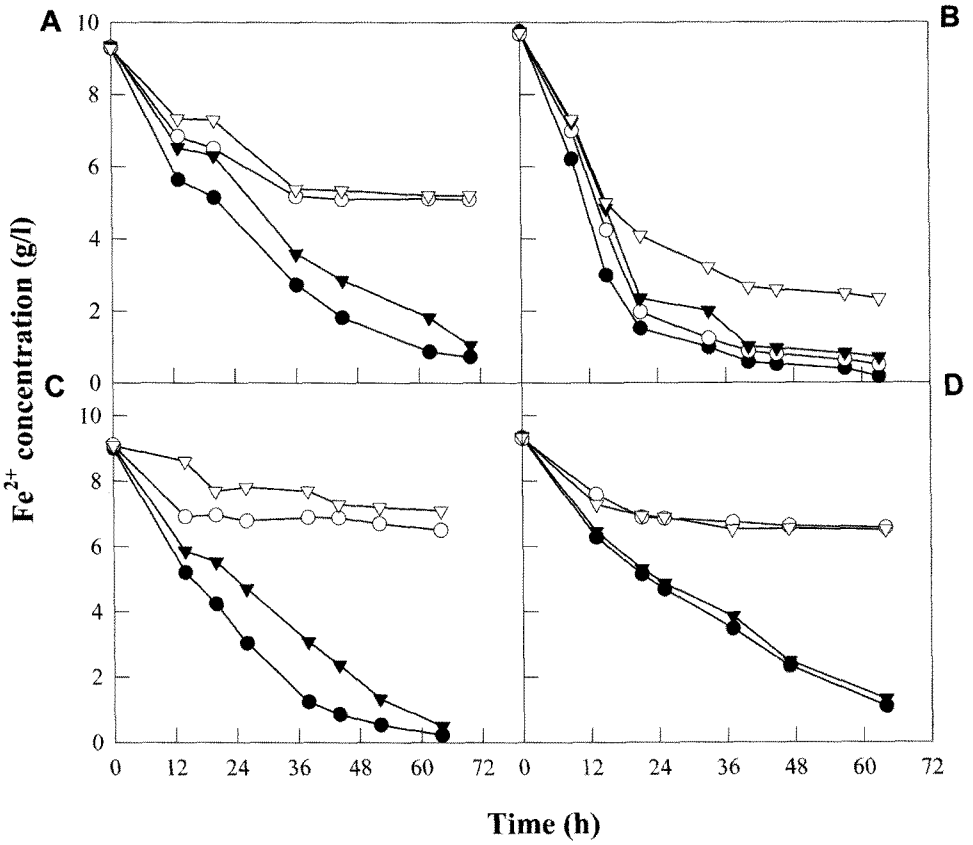

Fig. 2. Time profiles of Fe^{2+} concentration in 9 K medium in the presence of a ternary metal mixture.
A. $Cu^{2+}/Zn^{2+}/Ni^{2+}$ (mM): ●, 47/230/51; ○, 142/230/51; ▼, 47/230/153; ▽, 142/230/153. **B.** $Zn^{2+}/Ni^{2+}/Cd^{2+}$ (mM): ●, 230/51/27; ○, 230/51/80; ▼, 230/153/27; ▽, 230/153/80. **C.** $Cu^{2+}/Zn^{2+}/Cd^{2+}$ (mM): ●, 47/230/27; ○, 142/230/27; ▼, 47/230/80; ▽, 142/230/80. **D.** $Cu^{2+}/Cd^{2+}/Ni^{2+}$ (mM): ●, 47/27/51; ○, 142/27/51; ▼, 47/80/51; ▽, 142/80/51.

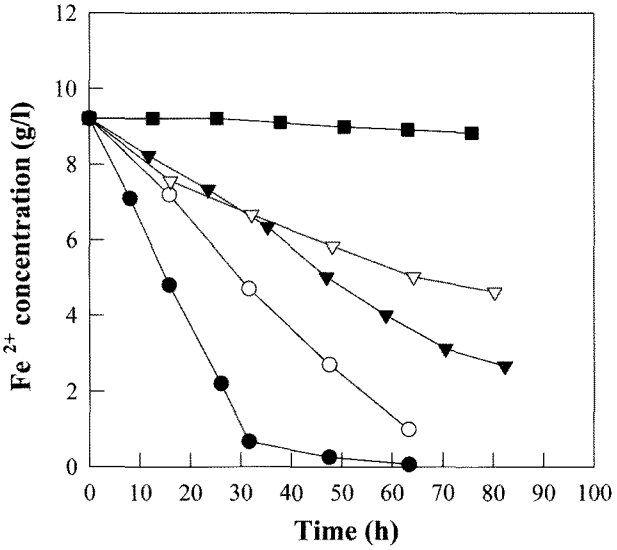

Fig. 3. Time profiles of Fe^{2+} concentration in 9 K medium in the presence of a quaternary metal mixture.
$Cu^{2+}/Zn^{2+}/Ni^{2+}/Cd^{2+}$ (mM): ●, 16/152/17/9; ○, 31/306//34/18; ▼, 47/460/51/27; ▽, 63/612/68/36; ■, 79/765/85/44.

suggested that even within a metal mixture, the inhibition of iron oxidation by the bacterium was mainly governed by the most toxic metal. The increase in the inhibition effect of metals on iron oxidation with increasing total metal concentration is shown clearly for the quaternary metal mixtures, illustrated in Fig. 3.

Effect of Metal Concentration on Iron Oxidation Rate.

Fig. 4 shows the rate of iron oxidation *vs.* total metal concentration, as calculated from the results shown in Figs. 1–3. For the single metal systems, the iron oxidation rate clearly decreased with increasing metal concentration. The order in toxicity of the metals studied was $Cu^{2+}>Cd^{2+}>Zn^{2+}>Ni^{2+}$, as shown in Fig. 4A. In binary, ternary, and quaternary metal mixtures, the iron oxidation rate decreased with increasing total metal concentration, but only for low total metal concentrations (Figs. 4B and 4C). These results suggest that the inhibition effect is dependent on the composition of metals within the mixture owing to the different toxicity of individual metal affecting on the iron oxidation activity of *A. ferrooxidans.*

Reduced Inhibition Model

An apparent maximum iron oxidation rate ($V_{m,app}$) and Michaelis constant (K_m) were used to determine the effect of individual metal toxicity on iron oxidation activity. The relationship between $V_{m,app}$ obtained from the kinetic analysis and metal concentration is shown in Fig. 5. The maximum oxidation rate, V_m, in the absence of inhibitor was 12.2 $mM \cdot h^{-1}$ and K_m was 92.5 mM. The inhibition of metal on the Fe^{2+} oxidation rate by *A. ferrooxidans* was identified as

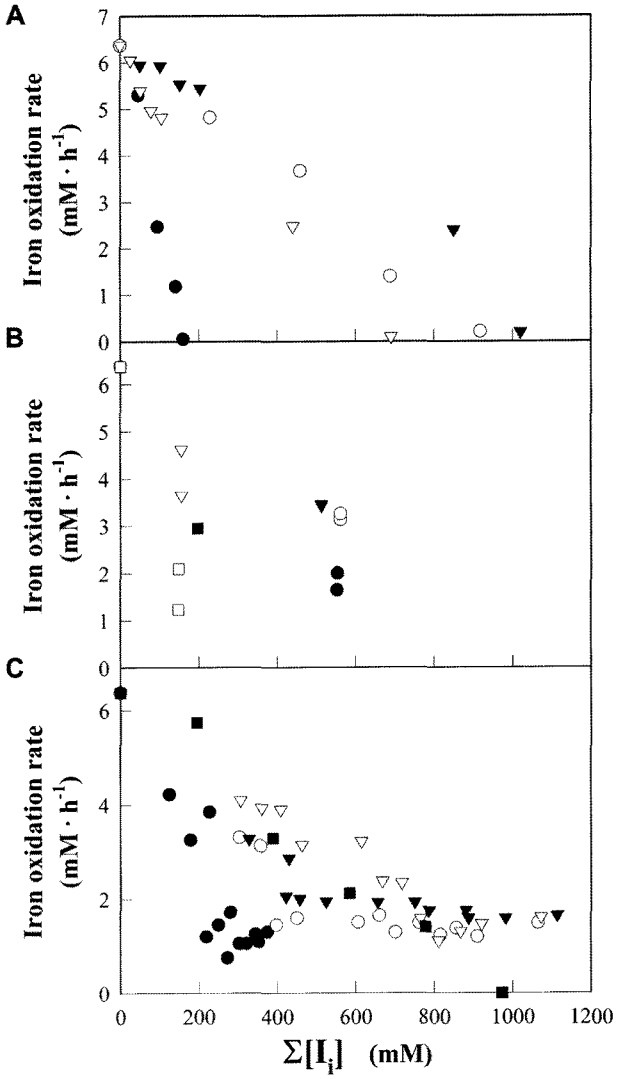

Fig. 4. Iron oxidation rates for different metal concentrations.
A. Single metal: ●, Cu^{2+}; ○, Zn^{2+}; ▼, Ni^{2+}; ▽, Cd^{2+}. **B.** Binary metals: ●, Cu^{2+}/Zn^{2+}; ○, Zn^{2+}/Ni^{2+}; ▼, Zn^{2+}/Cd^{2+}; ▽, Ni^{2+}/Cd^{2+}; ■, Cu^{2+}/Ni^{2+}; □, Cu^{2+}/Cd^{2+}. **C.** Ternary and quaternary metals: ●, $Cu^{2+}/Ni^{2+}/Cd^{2+}$; ○, $Cu^{2+}/Zn^{2+}/Cd^{2+}$; ▼, $Cu^{2+}/Zn^{2+}/Ni^{2+}$; ▽, $Zn^{2+}/Ni^{2+}/Cd^{2+}$; ■, $Cu^{2+}/Zn^{2+}/Ni^{2+}/Cd^{2+}$.

noncompetitive inhibition, where $V_{m,app}$ decreased with increase in the inhibitor's concentration.

The K_I and α values for each metal species were calculated using the data in Fig. 5 along with Cu^{2+} as the reference metal. The K_I and α values for Cu^{2+}, Ni^{2+}, Cd^{2+}, and Zn^{2+} are

Table 2. K_I and α values for the metals.

	Metal			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Cd^{2+}
K_I (mol/l)	0.07	0.57	0.67	0.19
α	1.00[a]	8.14	9.57	2.71

[a]Reference metal.

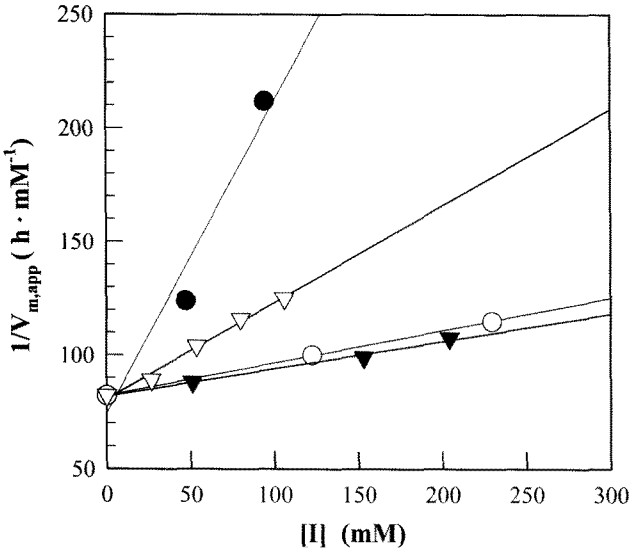

Fig. 5. Noncompetitive inhibition of iron oxidation by metals. ●, Cu^{2+}; ○, Zn^{2+}; ▼, Ni^{2+}; ▽, Cd^{2+}.

summarized in Table 2. By using the appropriate equations among (4), (5), and (6) or (7) for each system, we converted the concentration of each metal to the reduced concentration; the relationships of the two concentrations to apparent maximum iron oxidation rate ($V_{m,app}$) are illustrated in Figs. 6A and 6B. Cu^{2+} was selected as the reference inhibitor because of its high toxicity compared with the other metals. The result in Fig. 6B clearly demonstrated that with conversion of [I] to $[I]_{reduced}$, the $V_{m,app}$ value was always the same at the equivalent $[I]_{reduced}$ value regardless of metal type.

To confirm the applicability of the reduced inhibition model, we converted the concentration of each metal presented in Fig. 4 for single, binary, ternary, and quaternary metal systems to the reduced concentration; the results were replotted against iron oxidation rate, as shown in Fig. 7. After this conversion, the iron oxidation rates for all metal systems, regardless of system type, were positioned close to the reference line (solid line) at the reduced concentration ($[I]_{reduced} \leq 150$ mM). This indicated that the inhibition effect of Ni, Cd, and Zn on the iron oxidation activity of *A. ferrooxidans* is similar to that of the reference compound, Cu^{2+}, at this range of $[I]_{reduced}$.

Iron Oxidation Patterns in the Presence of Complex Metal Mixtures

Iron oxidation by *A. ferrooxidans* in single, binary, ternary, and quaternary metal systems shows diverse patterns, as previously revealed in Figs. 1–3. However, careful analyses indicated that these patterns can be categorized into four types (Fig. 8). These are as follows: Type I: the same oxidation pattern with no heavy metal system (control) and almost complete oxidation of ferrous ions in the medium by *A. ferrooxidans*; Type II: substantial iron oxidation followed by a sudden decrease in iron oxidation rate; Type III: initial iron

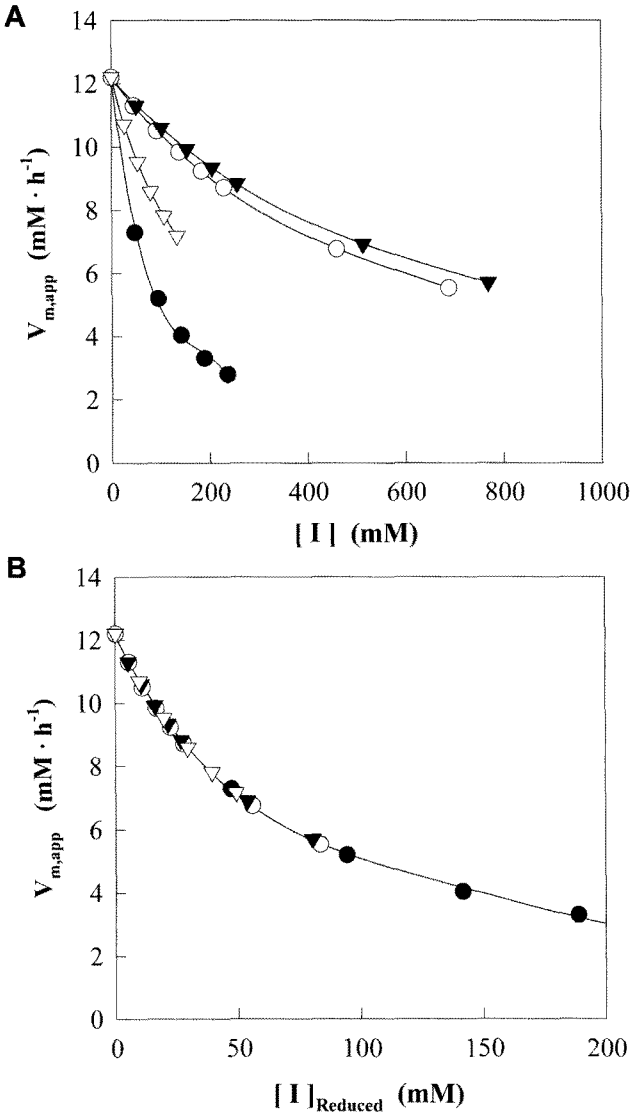

Fig. 6. Maximum iron oxidation rates for different metal concentrations (**A**) and for reduced metal concentrations where Cu^{2+} is the reference compound (**B**).
●, Cu^{2+}; ○, Zn^{2+}; ▼, Ni^{2+}; ▽, Cd^{2+}.

oxidation followed by complete impediment of oxidation owing to the inhibition effect of heavy metal; and Type IV: complete absence of iron oxidation owing to the severe inhibition effect of heavy metal.

The types of iron oxidation patterns of *A. ferrooxidans* for the four heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}) in single to quaternary metal systems are summarized in Table 3. Type I patterns were common in the systems with low total metal concentration and low concentration of Cu^{2+} ($\leq$100 mM), regardless of the metal system. Type II patterns were evident in binary and ternary systems without Cu^{2+}, and in quaternary systems at reduced metal concentrations of 79 mM and 113 mM. Type III patterns appeared in a total of 39 systems where the concentrations of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+} were

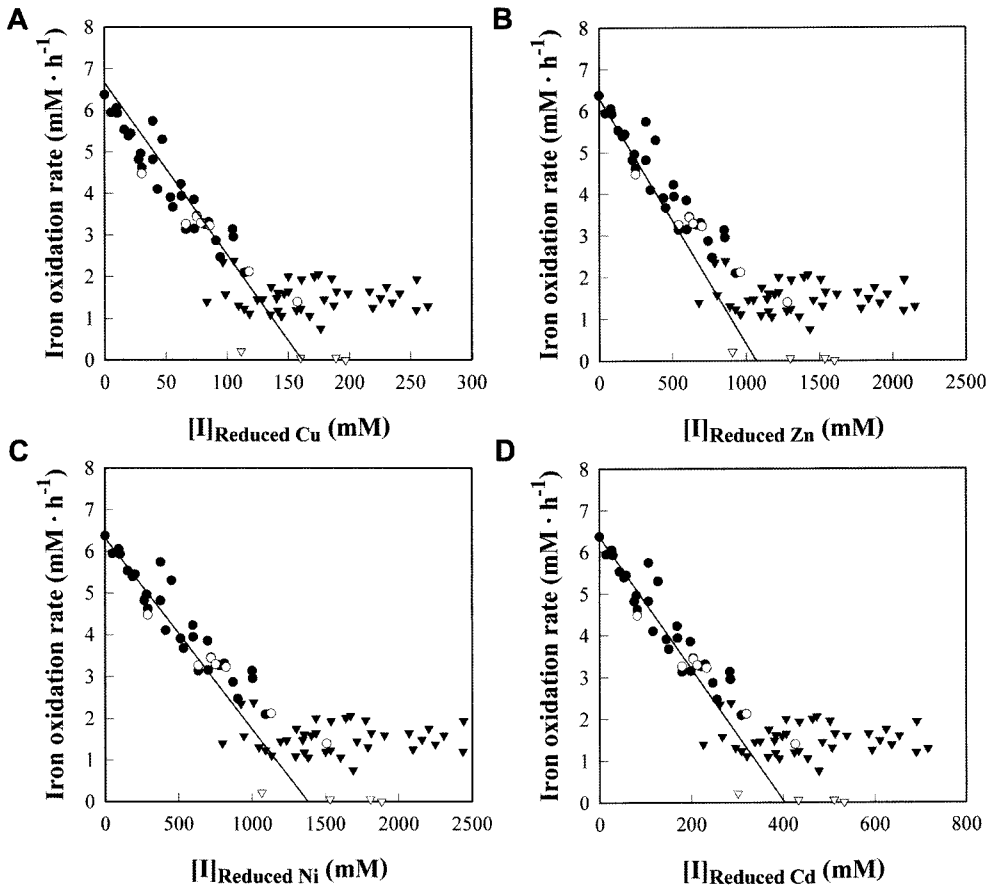

Fig. 7. Iron oxidation rates versus reduced metal concentration.
—, Reference metal (Cu^{2+}); ●, single system; ○, binary system; ▼, ternary system; ▽, quaternary system.

142, 690, 850, and 440 mM, respectively, or the reduced concentrations of the metal mixture were above 100 mM. Type IV patterns were shown in five single metal systems (160 or 189 mM Cu^{2+}, 920 mM Zn, 1,020 mM Ni^{2+}, and 660 mM Cd^{2+}) and a quaternary system with the reduced metal concentration of 197 mM. These results clearly demonstrated that the pattern of iron oxidation shifted from Type I to Type IV with increasing total metal concentration or increasing concentration of highly toxic heavy metals such as Cu^{2+}.

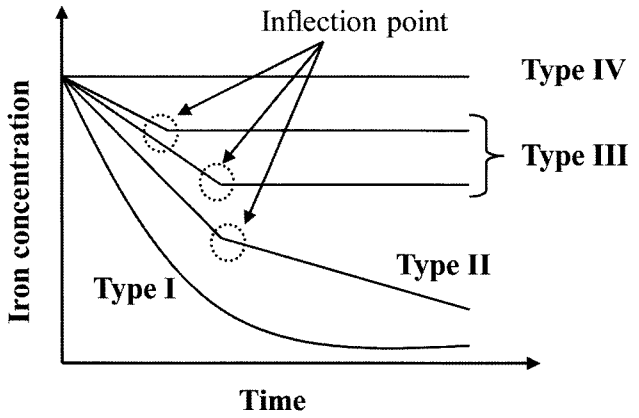

Fig. 8. Patterns of Fe^{2+} oxidation by *A. ferrooxidans* in the presence of inhibitor metal compounds.

Discussion

The metal toxicity order in this study was Cu^{2+}>Cd^{2+}>Zn^{2+}>Ni^{2+} and the maximum tolerance concentration (MTC) values for each metal were 142 mM Cu^{2+}, 690 mM Zn^{2+}, 850 mM Ni^{2+}, and 440 mM Cd^{2+}. Nevertheless, a wide range of MTC and the order of metal toxicity can be found in the literatures [7–10, 13, 21, 23]. For example, Cabrera *et al.* [8] found that the toxicity order for *A. ferrooxidans* was Cd^{2+} (89 mM)>Cu^{2+} (160 mM)>Zn^{2+} (459 mM)>Ni^{2+} (511 mM). This discrepancy may be due to the difference of strains and/or experimental conditions employed.

On the contrary, the study in the toxicity of multiple inhibitors on the activity of *A. ferrooxidans* is rather scarce in the literatures [9]. When a complex mixture of metals is present in *A. ferrooxidans* medium, it is difficult to separate the inhibition effect of an individual metal on the iron

Table 3. Types of iron oxidation rate patterns for different toxic metal systems.[a]

Patterns	Metal system	Metal concentrations (mM)
Type I	Single	Cu^{2+}: 0, 47, 94 Zn^{2+}: 0, 230 (28), 460 (56) Ni^{2+}: 0, 51 (5), 102 (11), 153 (16), 204 (21) Cd^{2+}: 0, 27 (10), 53 (20), 80 (30), 107 (40)
	Binary	Cu^{2+}/Ni^{2+}: 94/102 (105), Cu^{2+}/Cd^{2+}: 94/53 (114), Zn^{2+}/Ni^{2+}:460/102 (66), Zn^{2+}/Cd^{2+}: 460/53 (75), Ni^{2+}/Cd^{2+}:102/53 (30),
	Ternary	$Cu^{2+}/Zn^{2+}/Ni^{2+}$: 0/0/0, 47/230/51 (80), 47/230/153 (91) $Cu^{2+}/Zn^{2+}/Cd^{2+}$: 0/0/0, 47/230/27 (85), 47/230/80 (104) $Cu^{2+}/Ni^{2+}/Cd^{2+}$: 0/0/0, 47/51/27 (62), 47/51/80 (82), 47/153/27 (73), 47/153/80 (93) $Zn^{2+}/Ni^{2+}/Cd^{2+}$: 0/0/0, 230153/27 (54), 230/51/80 (63), 230/51/27 (43), 230/153/80 (73), 460/102/12 (71)
	Quaternary	$Cu^{2+}/Zn^{2+}/Ni^{2+}/Cd^{2+}$: 0/0/0/0, 16/152/17/9 (40)
Type II	Binary	Zn^{2+}/Ni^{2+}: 460/102 (67) Ni^{2+}/Cd^{2+}: 102/53 (30), Cd^{2+}/Zn^{2+}: 53/460 (75)
	Ternary	$Zn^{2+}/Ni^{2+}/Cd^{2+}$: 460/102/53 (86)
	Quaternary	$Cu^{2+}/Zn^{2+}/Ni^{2+}/Cd^{2+}$: 31/306//34/18 (79), 47460//51/27 (113)
Type III	Single	Cu^{2+}: 142, Zn^{2+}: 690 (84), Ni^{2+}: 850 (89), Cd^{2+}: 440 (162)
	Binary	Cu^{2+}/Zn^{2+}: 94/460 (150)
	Ternary	$Cu^{2+}/Zn^{2+}/Ni^{2+}$: 94/460/102 (160), 189/460/102 (255), 94/460/204 (171), 94/920/102 (216), 47/690/51 (136), 47/690/153 (147), 142/230/51 (175), 142/69/51 (231), 142/230/153 (186), 142/690/153 (242) $Cu^{2+}//Zn^{2+}/Cd^{2+}$: 94/460/53 (169), 189/460/53 (264), 94/460/12 (154), 94/920/53 (225), 47/690/27 (141), 47/80/690 (160), 142/27/230 (180), 142/27/690 (236), 142/230/80 (199), 142/690 /80 (255) $Cu^{2+}/Ni^{2+}/Cd^{2+}$: 94/102/53 (124), 189/102/53 (219), 94/204/53 (135), 94/102/107 (144), 142/51/27 (157), 142/51/80 (177), 142/153/27 (168), 142/153/80 (188) $Zn^{2+}/Ni^{2+}/Cd^{2+}$: 160/204/53 (97), 920/102/53 (142), 690/51/27 (99), 690/51/80 (119), 690/153/27 (110), 690/153/80 (129)
Type IV	Single	Cu^{2+}: 160, 189, Zn^{2+}: 920 (112), Ni^{2+}: 1,020 (160), Cd^{2+}: 660 (243)
	Quaternary	$Cu^{2+}/Ni^{2+}/Cd^{2+}/Zn^{2+}$: 79/85/44/765 (197)

[a]Numbers in parentheses indicate the total metal concentrations where each metal concentration is converted as the reduced metal concentration using Cu^{2+} as the reference metal.

oxidation of the bacterium. The toxicity of metals for unadapted and adapted strains of *A. ferrooxidans* in the presence of binary and ternary combinations of metal ions such as Cu^{2+}, Fe^{3+}, and Zn^{2+} was estimated in terms of toxicity index (TI) by Das *et al.* [9]. They only reported the toxicity behavior of the adapted cells in the presence of the above metal ions in comparison with that of wild unadapted cells under similar conditions. The TI used was defined as the ratio of the time required for completion of ferrous ion oxidation by the strain with dissolved metal ion to that required by the wild unadapted strain without any dissolved metal ion.

Several studies had been conducted to investigate the combined effect of heavy metals on organisms [2, 19, 24–26, 34]. In the ecotoxicological studies, the combined effects of multiple chemicals are generally analyzed using the toxic unit (TU) approach, which tested the response addition model for the toxicant mixture [19, 24–26, 34]. Concentration in the mixtures was expressed by TU with fractions of their median effective concentration (EC_{50}=1 TU).

$$TU_i = \frac{C_i}{(EC_{50})_i}$$

The sum of TU in the mixtures can be expressed by the following equation:

$$\sum TU_i = \sum_{i=1}^{n} \frac{C_i}{(EC_{50})_i}$$

and TU was calculated after exposure for a certain period of time.

These models had been developed basically for individual toxicants, and the effect of multiple toxicants interactions can be predicted or interpreted from the sum of the toxic units of the individual compounds [25]. The TU approach appeared to be a good model in estimation of the combined effect of metals, but has certain limitations. Only the sum of TU in the mixture represents whether or not the toxicity behavior is an antagonistic, additive, or a synergistic effect [2, 8, 25, 26]. With the TU approach therefore, it is difficult to predict

the inhibition behaviors of cell growth or activities with varying concentrations of the toxicant in the mixture.

In this study, the reduced inhibition constant derived from the inhibition constant (K_I), which was obtained from kinetic analysis of cell growth and activity, has been introduced in lieu of the TU approach. As shown in Figs. 6–7, this study clearly reveals that the reduced inhibition constant from our proposed reduced inhibition model enables the conversion of complex inhibitor concentrations into concentrations of the reference compound (*e.g.*, Cu^{2+}). Such a conversion allows the systematical separation of the toxicity effects of individual inhibitors within a complex mixed system, thus providing an efficient evaluation. In other words, in mixed metal systems containing many types of metals, the effects of these mixed inhibitors on cell activities can be normalized to the effect of the reference inhibitor *via* the reduced inhibition concentration derived from the reduced inhibition constant.

Furthermore, the oxidation patterns are categorized into four types, as shown in Fig. 8. This is one of the most useful approaches in evaluation of the toxicity (or inhibition behavior) in a single as well as in mixed toxicants. The reduced inhibition model and TU approach can be applicable to Type I and II patterns, but they are insignificant in evaluation of the toxicity for Type III and IV patterns. Most of Type III is the mixture rather than the single system and is the metal system having either high concentration of a highly toxic metal such as Cu^{2+} or a number of metal ions (Table 3). In Type III, the antagonistic effect is observed even at high reduced metal concentrations. This effect is probably due to disparities in the binding opportunities of the inhibitor (or heavy metal) to active sites of enzymes; this is in turn related to the iron oxidation activities of *A. ferrooxidans* and cell activity. For instance, the binding opportunity of a highly toxic metal such as Cu^{2+} to active sites of enzymes is expected to be less in the systems mixed with other toxic metals than in single metal systems.

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References

1. Agate, A. D. 1996. Recent advances in microbial mining. *World J. Microbiol. Biotechnol.* **12:** 487–495.
2. An, Y. J., Y. K. Kim, T. I. Kwon, and S. W. Jeong. 2004. Combined effect of copper, cadmium, and lead upon *Cucumis sativus* growth and bioaccumulation. *Sci. Total Environ.* **326:** 85–93.
3. Baillet, F., J. P. Magnin, and A. Cheruy. 1997. Cadmium tolerance and uptake by a *Thiobacillus ferrooxidans* biomass. *Environ. Technol.* **18:** 631–638.
4. Blake II, R. C., G. T. Howard, and S. McGinness. 1994. Enhanced yields of iron-oxidizing bacteria by *in-situ* electrochemical reduction of soluble iron in the growth medium. *Appl. Environ. Microbiol.* **60:** 2704–2710.
5. Bosecker, K. 1997. Bioleaching: Metal solubilization by microorganisms. *FEMS Microbiol. Rev.* **20:** 591–604.
6. Box, G. E. P. and K. B. Wilson. 1951. On the experimental attainment of optimum conditions (with discussion). *J. Royal Statistical Soc. Series B* **13:** 1–45.
7. Brahmaprakash, G. P., P. Devasia, K. S. Jagadish, K. A. Natarajan, and G. R. Rao. 1988. Development of *Thiobacillus ferrooxidans* ATCC 19859 strains tolerant to copper and zinc. *Bull. Mater. Sci.* **10:** 461–465.
8. Cabrera, G., J. M. Gomez, and D. Cantero. 2005. Influence of heavy metals on growth and ferrous sulphate oxidation by *Acidithiobacillus ferrooxidans* in pure and mixed cultures. *Proc. Biochem.* **40:** 2683–2687.
9. Das, A., J. M. Modak, and K. A. Natarajan. 1997. Studies on multi-metal ion tolerance of *Thiobacillus ferrooxidans*. *Minerals Eng.* **10:** 743–749.
10. Dave, S. R., K. A. Ayappan, and J. V. Bhat. 1979. Biooxidation studies with *Thiobacillus ferrooxidans* in the presence of copper and zinc. *Trans IMM C* **88:** C234–C237.
11. De, G. C., D. J. Oliver, and B. M. Pesic. 1997. Effect of heavy metals on the ferrous iron oxidizing ability of *Thiobacillus ferrooxidans*. *Hydrometallurgy* **44:** 53–63.
12. Demergasso, C. S., P. A. Galleguillos P., L. V. Escudero G., V. J. Zepeda A., D. Castillo, and E. O. Casamayor. 2005. Molecular characterization of microbial populations in a low-grade copper ore bioleaching test heap. *Hydrometallurgy* **80:** 241–253.
13. Dispirito, A. A. and O. H. Tuovinen. 1982. Kinetics of uranous ion and ferrous iron oxidation by *Thiobacillus ferrooxidans* and their elimination by subculturing in media containing $CuSO_4$. *J. Gen. Microbiol.* **129:** 2969–2972.
14. Harahuc, L., H. M. Lizama, and I. Suzuki. 2000. Selective inhibition of the oxidation of ferrous iron or sulfur in *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **66:** 1031–1037.
15. Huber, H. and K. O. Stetter. 1990. *Thiobacillus cuprinus* sp. nov., a novel facultative organotrophic metal-mobilizing bacterium. *Appl. Environ. Microbiol.* **56:** 315–322.
16. Imai, K., T. Sugio, T. Tsuchida, and T. Tano. 1975. Effect of heavy metal ions on the growth and iron-oxidizing activity of *Thiobacillus ferrooxidans*. *Agric. Biol. Chem.* **39:** 1349–1354.
17. Kawabe, Y., C. Inoue, K. Suto, and T. Chida. 2003. Inhibitory effect of high concentration of ferric ions on the activity of *Acidithiobacillus ferrooxidans*. *J. Biosci. Bioeng.* **96:** 375–379.
18. Keeling, S. E., M.-L. Palmer, F. C. Caracatsanis, J. A. Johnson, and H. R. Watling. 2005. Leaching of chalcopyrite and sphalerite using bacteria enriched from a spent chalcocite heap. *Minerals Eng.* **18:** 1289–1296.
19. Khalil, M. A., H. M. Abdel-Lateif, B. M. Bayoumi, and N. M. van Straalen. 1996. Analysis of separate and combined effect of heavy metals on the growth of *Aporrectodea caliginosa* (Oligochaeta; Annelida), using the toxic unit approach. *Appl. Soil Ecol.* **4:** 213–219.
20. Krebs, W., C. Brombaacher, P. P. Bosshard, R. Bachofen, and H. Brandl. 1997. Microbial recovery of metals from soils. *FEMS Microbiol. Rev.* **20:** 605–617.

21. Ledue, L. G. and G. D. Ferroni. 1994. The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **14:** 103–120.
22. Magnin, J., F. Bailet, A. Boyer, R. Zlatev, M. Luca, A. Cheruy, and P. Ozil. 1998. Augmentation, par régénération électrochimique du substrat, de la productiond'nue biomasse (*Thiobacillus ferrooxidans* DMS 583) pour nn procédé biologique de récupération de métanx. *Can. J. Chem. Eng.* **76:** 978–984.
23. Norris, P. R., L. Parrott, and R. M. Marsh. 1986. Moderately thermophilic mineral-oxidizing bacteria. *Biotechnol. Bieng. Symp.* **16:** 253–262.
24. Pape-Lindstrom, P. A. and M. J. Lydy. 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. *Environ. Toxicol. Chem.* **16:** 2415–2420.
25. Playle, R. C. 2004. Using multiple metal gill binding models and the toxic unit concept to help multiple-metal toxicity results. *Aquat. Toxicol.* **67:** 359–370.
26. Posthuma, L., R. Baerselman, R. P. M. Van Veen, and E. M. Dirven-Van Breemen. 1997. Single and joint toxic effect of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. *Ecotoxicol. Environ. Saf.* **38:** 108–121.
27. Rawlings, D. E. 1998. Industrial practice and the biology of leaching of metals from ores. *J. Ind. Microbiol. Biotechnol.* **20:** 268–274.
28. Rehm, H. J. and G. Reed (eds.). 1988. *Biotechnology*, Vol. 6, p. 367. Verlag Chemie.
29. Ryu, H. W., K. S. Cho, Y. K. Chang, S. D. Kim, and T. Mori. 1995. Refinement of low-grade clay by microbial removal of sulfur and iron compounds using *Thiobacillus ferrooxidans*. *J. Ferment. Bioeng.* **80:** 46–52.
30. Ryu, H. W., Y. K. Chang, and S. D. Kim. 1993. Microbial coal desulfurization in an airlift bioreactor by sulfur-oxidizing bacterium *Thiobacillus ferrooxidans*. *Fuel Proc. Technol.* **36:** 267–275.
31. Sampson, M. I. and C. V. Phillips. 2001. Influence of base metals on the oxidizing ability of acidophilic bacteria during the oxidation of ferrous sulfate and mineral sulfide concentrates, using mesophiles and moderate thermophilies. *Minerals Eng.* **14:** 317–340.
32. Silver, M. and A. E. Torma. 1974. Oxidation of metal sulfides by *T. ferrooxidans* grown on different substrates. *Can. J. Microbiol.* **20:** 141–147.
33. Tuovinen, O. H., S. I. Niemela, and H. G. Gyllenberg. 1971. Tolerance of *Thiobacillus ferrooxidans* to some metals. *Antonie van Leeuwenhoek* **37:** 489–496.
34. Van der Geest, H. G., G. D. Greve, M.-E. Boivin, M. H. S. Kraak, and C. A. M. van Gestel. 2000. Mixture toxicity of copper and diazinon to larvae of the mayfly (*Ephoron virgo*) judging additivity at different effect levels. *Environ. Toxicol. Chem.* **19:** 2900–2905.
35. Wei, W., Z. Zheng, Y. Liu, and X. Xhu. 1998. Optimizing the culture conditions for higher Lnulinase production by *Kluyveromyces* sp. Y-85 and scaling-up fermentation. *J. Ferment. Bioeng.* **86:** 395–399.