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Effects of sulfide on sulfate reducing bacteria in response to Cu(II), Hg(II) and Cr(VI) toxicity

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Sulfate reducing bacteria (SRB) is identified as the primary organisms responsible for the treatment of heavy metal wastewater. However, most heavy metals can inhibit the growth of SRB during heavy metal treatment processes. Sulfide is a metabolic product of SRB and it can precipitate or reduce heavy metals. This study focused on the effects of sulfide on SRB resistance to Cu(II), Hg(I) and Cr(VI) toxicity. First, we considered the existence style of various heavy metals with and without sulfide addition by sequential extraction experiments. Second, the particle size distribution was evaluated and the cell structure during the metabolism of a SRB culture, containing different heavy metals, was analyzed by particle size distribution and TEM analyses. Third, the evolution of sulfate under the influence of different concentrations of heavy metals with and without sulfide addition was investigated to evaluate SRB activity. The results indicated that sulfide played an important role in alleviating and even eliminating the toxicity of Cu(II), Hg(II) and Cr(VI). We also discuss the mechanism of sulfide on SRB resistance to Cu(II), Hg(I) and Cr(VI) toxicity.

sulfide, heavy metal toxicity, sulfate reducing bacteria, copper, chromium, mercury

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With the development of metallurgy-related industries like metal finishing and electroplating, massive amounts of acid wastewater are generated and released into the environment [1]. These waste streams commonly contain high levels of sulfate and dissolved metals. The discharge of this heavy metal contaminated wastewater into the environment can be devastating to aquatic and terrestrial ecosystems [2]. Conventionally, the most widely used method to treat these effluents is chemical neutralization with hydroxide followed by the precipitation of metals. However, this method has serious limitations due to the production of large amounts of unstable metal hydroxides leading to high disposal and dewatering costs for the produced sludge [3]. The biological treatment of acidic and metal-containing wastewater is an attractive alternative. The main advantage of this process over chemical neutralization is that less sludge is generated and insoluble compounds such as metal oxides or sulfides are produced that are easily separated and recycled [4–6]. SRB have been identified as the primary bacteria for use in the biological treatment of heavy metal containing wastewater.

While SRB are capable of various metal transformations, metals can also inhibit their growth [7–9]. Sulfide which is produced biologically during sulfate reduction by SRB could precipitate [10,11] or reduce [12,13] heavy metal cation. This decreases or even eliminates the toxicity of the heavy metals toward SRB. To our knowledge, few systematic investigations on the effects of sulfide on SRB resistance to heavy metal ion toxicity and of biologically produced precipitates on SRB metabolism have been conducted. We, therefore, studied the toxic metal ions Cu(II), Hg(II) and Cr(VI), which are usually found in waste streams. The mechanism of sulfides in the presence of SRB in response to Cu(II), Hg(II) and Cr(VI) toxicity and the

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biologically produced precipitates produced during SRB metabolism were investigated.

1 Materials and methods

1.1 Source of the inocula

SRB from the *Desulfovibrio* family were used in this study. They were obtained from an expanded granular sludge bed (EGSB) reactor that had treated effluent with high sulfate and ethanol concentrations for two years. Ten percent (V/V) of the inoculum was successively transferred to oxygen-free sterilized vessels containing the modified Postgate medium [14] and this is summarized in Tables 1 and 2. The vessels were filled with deionized water immediately to provide anaerobic conditions. The SRB cultures were incubated at 35°C. The inoculation procedure was repeated 3 times. Each cultivation process lasted 7 d to ensure sufficient SRB growth. After the enrichment cultivation, the concentration of SRB in the inoculum was found to be around 2.0×10^7 cfu mL^{-1}.

1.2 Analytical methods

The sulfate concentration was measured using a DX-500 ion chromatography (Dionex, USA). The eluent used was NaOH (30 mmol L^{-1}) and the flow rate was 1.5 mL min^{-1}. An IonPac AC11 column and an ED50 electrochemical detector were also used. The working current of the suppressor was 100 mA.

Particle size was measured using a laser diffraction sensor (Mastersizer 2000, from Malvern) and determined using a sphere of the same volume.

Copper, chromium and mercury concentrations were measured by inductively coupled plasma spectroscopy (ICP) on a Perkin Elmer Plasma 2000 ICP-OEM (USA).

Table 1 Chemical composition of the modified Postgate medium

Composition	Concentration (g L^{-1})	Composition	Concentration (g L^{-1})
KH_2PO_4	0.5	NH_4Cl	1.0
$CaCl_2$	0.06	$MgSO_4\cdot7H_2O$	1.5
Na_2SO_4	1.5	Yeast extract	1.0
Ethanol	3 mL L^{-1}	Trace element solution[a)]	1 mL L^{-1}

a) see Table 2.

Table 2 Chemical composition of the trace element solution in the modified Postgate medium

Composition	Concentration (mg L^{-1})	Composition	Concentration (mg L^{-1})
$FeCl_2\cdot4H_2O$	1500	$MnCl_2\cdot4H_2O$	100
$ZnCl_2$	70	H_3BO_3	62
$Na_2MoO_4\cdot2H_2O$	36	$NiCl_2\cdot6H_2O$	24
$CuCl_2\cdot2H_2O$	17	EDTA	500
HCl(37%)	7 mL L^{-1}		

The concentration of Cr(VI) was measured using a spectrophotometer with diphenylcarbazide as the chromogenic reagent. The concentration of Cr(III) was calculated as the difference between the Cr and Cr(VI) concentrations.

Transmission electron microscopy (TEM): Samples were placed in a fixative which consists of 2.0% glutaraldehyde in a sodium cacodylate buffer (0.1 mol L^{-1}, pH 7.2). Cells were postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in a resin. Sections were collected on copper grids and contrast-stained with uranyl acetate and lead citrate. The samples were viewed using a transmission electron microscope (JEM-1400) at a magnification of 15000.

Sequential extraction procedure: Samples were filtered through a filter with a pore size of 0.22 μm. The filtrate was collected to measure the soluble heavy metal concentrations. A 5-step extraction method [15] was used to determine the distribution of the heavy metals in the filter cakes. This included: (1) the determination of an exchangeable fraction with $MgCl_2$; (2) a carbonate fraction with NaAC; (3) an Fe-Mn oxide fraction with $NH_2OH\cdot HCl$; (4) an organic fraction with $NH_4AC/HNO_3/H_2O_2$ and (5) a residual fraction with HNO_3/HF/HClO/HCl.

1.3 Experimental design

To determine the influence of sulfide on the distribution of Cu(II), the PSD of the particles and the structure of the SRB from the SRB metabolism culture, experiments were carried out in two 150 mL vials containing 100 mL of modified Postgate media. $CuCl_2$ was added to each vial to give a copper concentration of 50 mg L^{-1} and Na_2S was added to one vial to give a sulfide concentration of 100 mg L^{-1}. After that, 10 mL of the inoculum was inoculated in each vial and the pH of each vial was adjusted to 7.0±0.2 using 0.01 mol L^{-1} HCl or 0.01 mol L^{-1} NaOH. The vials were then filled immediately with deionized water and incubated at 35°C for 5 d. The samples in the vial were collected at the beginning and end of the metabolism of the SRB in each vial to evaluate the distribution of copper, the PSD of the particles and the structure of the SRB in the culture. The experimental design for Hg(II) and Cr(VI) was the same as that for Cu(II). $HgSO_4$ and $KCrO_4$ were added as sources of Hg(II) and Cr(VI) in these experiments, respectively.

To determine the influence of sulfide on SRB resistance to Cu(II) toxicity, experiments were carried out in twelve 150 mL vials. These were divided into two groups (designated group A and group B). 100 mL of modified Postgate media was added to each vial. The copper concentration in group A was 0, 5, 10, 20, 30, 50 mg L^{-1} after the addition of $CuCl_2$, respectively. Ten milliliters of the inoculum was subsequently added to each vial. The pH of each vial was adjusted to 7.0±0.2 with 0.01 mol L^{-1} HCl or 0.01 mol L^{-1} NaOH. The vial was then filled immediately with deionized water and incubated at 35°C for 5 d. The experimental conditions for group B were same as that for group A except for

extra sulfide addition before inoculation. The sulfide concentration in each vial in group B was 100 mg L^{-1}. The evolution of sulfate in each vial was investigated to evaluate the activity of SRB. The experimental design for Hg(II) and Cr(VI) was the same as that for Cu(II).

2 Results and discussion

2.1 Influence of sulfide on the distribution of heavy metals

The distribution of heavy metals in the vials was analyzed by sequential extraction experiments. The results are shown in Figure 1.

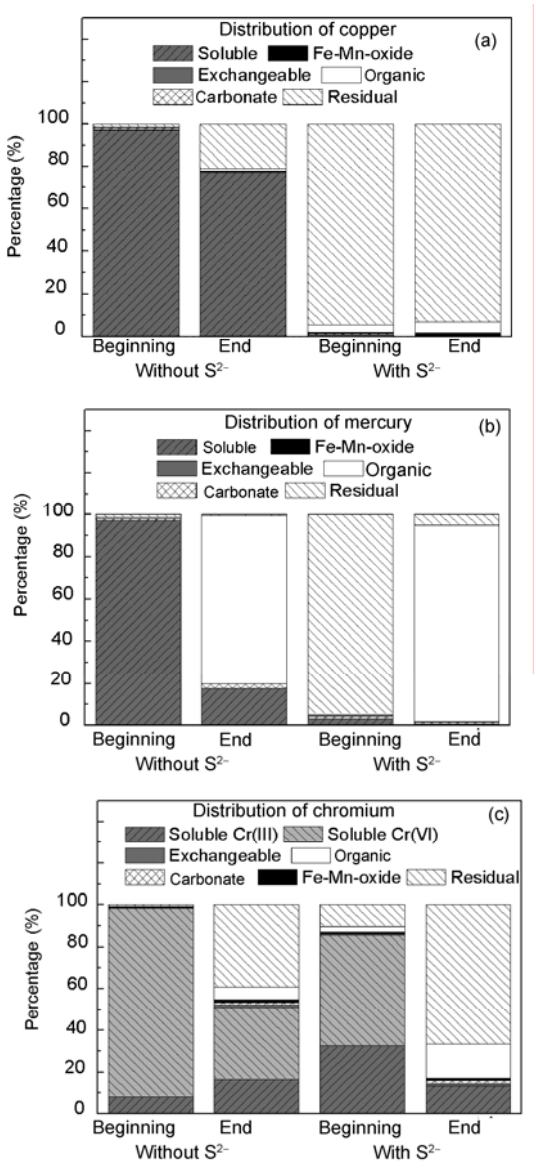

Figure 1 Distribution of heavy metals in the SRB metabolism system with and without sulfide. (a) With Cu(II) addition; (b) with Hg(II) addition; (c) with Cr(VI) addition.

As shown in Figure 1(a), without the addition of sulfide almost all the copper (97.3%) was distributed in the soluble fraction at the start of the experiment. After cultivation for 5 d, most of the copper (76.8%) was still distributed in the soluble fraction and some copper (21.2%) appeared in the residual fraction. With sulfide addition, most of the copper transferred from the soluble fraction to the residual fraction at the start of the experiment because 94.1% of the copper was distributed there. After cultivation for 5 d the distribution of copper in the residual fraction was still 93%. The remaining copper was distributed in the organic fraction (5.4%).

As shown in Figure 1(b), without the appearance of sulfide mercury was mainly distributed in the soluble fraction at the start of the experiment as 97.2% of the mercury was in the soluble fraction. After cultivation for 5 d the distribution of mercury changed significantly. Most of the mercury (79.8%) was distributed in the organic fraction while the remaining mercury (17.7%) mainly distributed in the soluble fraction. With sulfide addition, almost all the mercury (94.7%) appeared in the residual fraction at the start of the experiment. After cultivation for 5 d, the distribution of mercury was similar to that obtained without sulfide addition. 93.2% of the mercury was present in the organic fraction. The remaining mercury was mainly found in the residual fraction. Only 0.2% of the mercury was found in the soluble fraction.

As shown in Figure 1(c), in the absence of sulfide most of the chromium was present as soluble ions and 90.3% of the chromium was present as soluble Cr(VI) while 8.2% of the chromium was present as soluble Cr(III). After cultivation for 5 d, 39.2% of the chromium transferred to the residual fraction and the remaining chromium was still distributed in the soluble fractions because 34.4% of the chromium was present as soluble Cr(VI) and 8.2% of the chromium was present as soluble Cr(III). Upon sulfide addition a larger amount of soluble Cr(VI) transformed into soluble Cr(III) at the start of the experiment because 32.5% of the chromium was present as soluble Cr(III) and 53.3% of the chromium was present as soluble Cr(VI). The remaining chromium was found in the residual fraction, which contained 10.6% chromium. After cultivation for 5 days more chromium was distributed in the residual fraction, which contained 66.6% chromium. The remaining chromium was mainly distributed in the organic fraction and the soluble fraction with contents of 16.2% and 13.3% chromium, respectively. All the chromium that distributed in the soluble fraction was Cr(III) and no soluble Cr(VI) was present.

2.2 Particle size distribution analysis

The PSD of the particles in the vials obtained after sulfide addition are shown in Figure 2.

As shown in Figure 2(a), only one peak is present in the PSD profile for the sample without heavy metals and for the

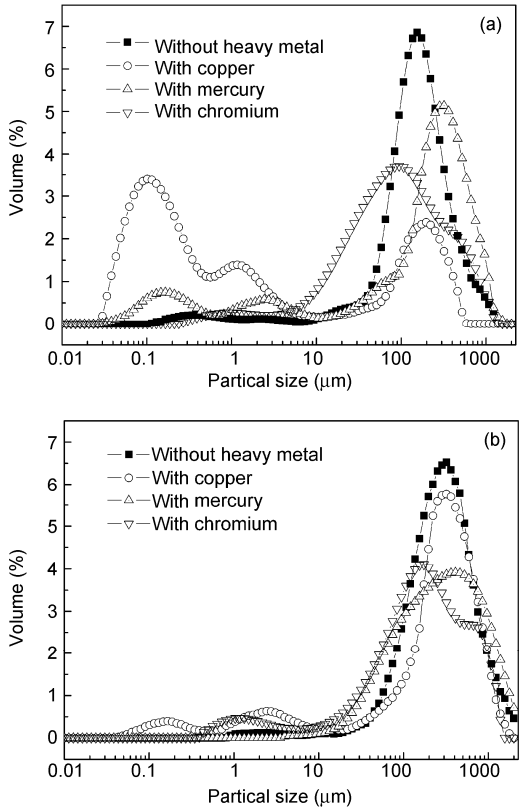

Figure 2 PSD of the particles from the SRB metabolism culture with different heavy metals in the presence of sulfide. (a) At the start of the experiment; (b) at the end of the experiment.

sample containing chromium with their main peaks being centered at 100 and 80 μm, respectively. However, the profile of the latter was lower and wider. The PSDs of the samples that contained copper or mercury were more heterogeneous. For the sample containing copper, the three main PSD peaks were centered at 0.1, 1 and 150 μm, respectively. For the sample containing mercury the main PSD peak was centered at 300 μm and two smaller peaks were present at around 0.2 and 2 μm. After the SRB were metabolized for 5 d the distribution of particles in the other vials changed significantly except for the vial without heavy metals. The PSD profile for the vial without heavy metals still had only one peak, which was centered at 250 μm. For the sample containing copper, the height of the peaks around 0.1 and 1 μm decreased while the peak that was around 150 μm moved to 300 μm and it increased in height. For the sample containing mercury, the main PSD peak was centered at 400 μm. The old peaks around 0.2 and 2 μm disappeared and a new small peak centered at 1 μm appeared. For the sample containing chromium, two main peaks appeared at around 150 and 700 μm in the PSD profile.

2.3 TEM analysis of SRB

TEM images of the SRB from the vials containing sulfide are shown in Figure 3. Compared with the TEM image of the bacteria in the vial without heavy metals (Figure 3(a)), a black layer was observed on the outside of the cell wall in the vial containing copper (Figure 3(b)). The cell in the vial containing mercury (Figure 3(c)) was no different to that in Figure 3(a). In Figure 3(d), many large particles were present on the cell from the vial containing chromium. The deposited particles had a loose structure. The composition of the black layer and selected particles in Figure 3(b) as well as the particles in Figure 3(d) were analyzed by energy-dispersive X-ray (EDX). The results are listed in Table 3. The black layer shown in Figure 3(b) was mainly composed of S(45.87%), Cu(44.14%), P(6.43%) and C(1.35%). The ratio of S to Cu was 1.04, which is consistent with the formation of covellite (CuS)(K_{sp}=6.3×10^{-36}). CuS was clearly predominant in the black layer. The particle that was analyzed in Figure 3D was mainly composed of C(51.84%), O(24.87%), Cr(13.93%) and P(7.09%). This particle was mainly composed of organic substances because of the high carbon content, and some chromium was deposited because the chromium was mainly distributed in the residual fraction (Figure 1(c)).

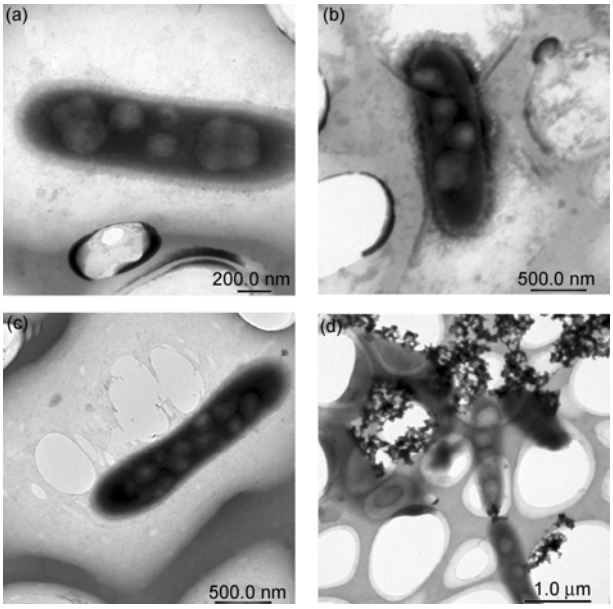

Figure 3 TEM images of SRB cells showing the appearance of sulfide. (a) Without heavy metal addition; (b) with Cu(II) addition; (c) with Hg(II) addition; (d) with Cr(VI) addition.

Table 3 Elemental composition of the particles in Figure 3(b) and (d)

Element	S	Cu	P	C	O	Si
Precipitates with Cu(II) addition (%)[a)]	45.87	44.14	6.43	1.35	1.12	1.09
Element	C	O	Cr	P	Ca	Na
Precipitates with Cr(VI) addition (%)[a)]	51.84	24.87	13.93	7.09	2.02	0.25

a) Atomic percentage.

2.4 Influence of sulfide on SRB heavy metal toxicity

As noted in section 1.3, the evolution of sulfate in each vial for different heavy metal concentrations with and without sulfide are shown in Figure 4.

As shown in Figure 4(a), when the initial concentration of Cu(II) was higher than 20 mg L^{-1}, the sulfate concentration barely changed during the experiment, which indicated that SRB activity was completely inhibited. When the initial concentration of Cu(II) was lower than 20 mg L^{-1} the sulfate concentration gradually decreased with time and the sulfate reduction rate decreased with an increase in the initial Cu(II) concentration. A lag phase for sulfate reduction was observed in each vial. Higher initial concentrations of Cu(II) led to a longer lag phase. This observation agreed with the results of Olive et al. [9], who reported that SRB activity was completely inhibited at Cu(II) concentrations more than 20 mg L^{-1}. With sulfide addition, the evolution of sulfate (Figure 4(b)) was vastly different to that shown in Figure 4(a). As shown in Figure 4(b), sulfate reduction was not affected when the initial Cu(II) concentration was lower than 10 mg L^{-1}. At an initial Cu(II) concentration higher than 10 mg L^{-1}, the sulfate reduction rate should decrease with an increase in the initial Cu(II) concentration. No obvious lag phase was observed for the vials shown in Figure 4(b).

As shown in Figure 4(c), when the vial contained Hg(II) a lag phase that lasted for 45 h was observed for each vial and the sulfate reduction rate was almost the same for the different Hg(II) concentrations. A similar situation was observed in Figure 4(d). Upon sulfide addition, the sulfate concentration profiles in the vials containing different Hg(II) concentrations barely changed. This observation is in agreement with the results of Chang et al. [16] who reported that Hg(II) increased the lag phase of sulfate reduction but did not affect the SRB growth rate. Compared with Figure 4(c) the lag phase time for each vial in Figure 4(d) decreased from 45 to 25 h and the sulfate reduction rate in each vial also increased.

As shown in Figure 4(e), the sulfate reduction was

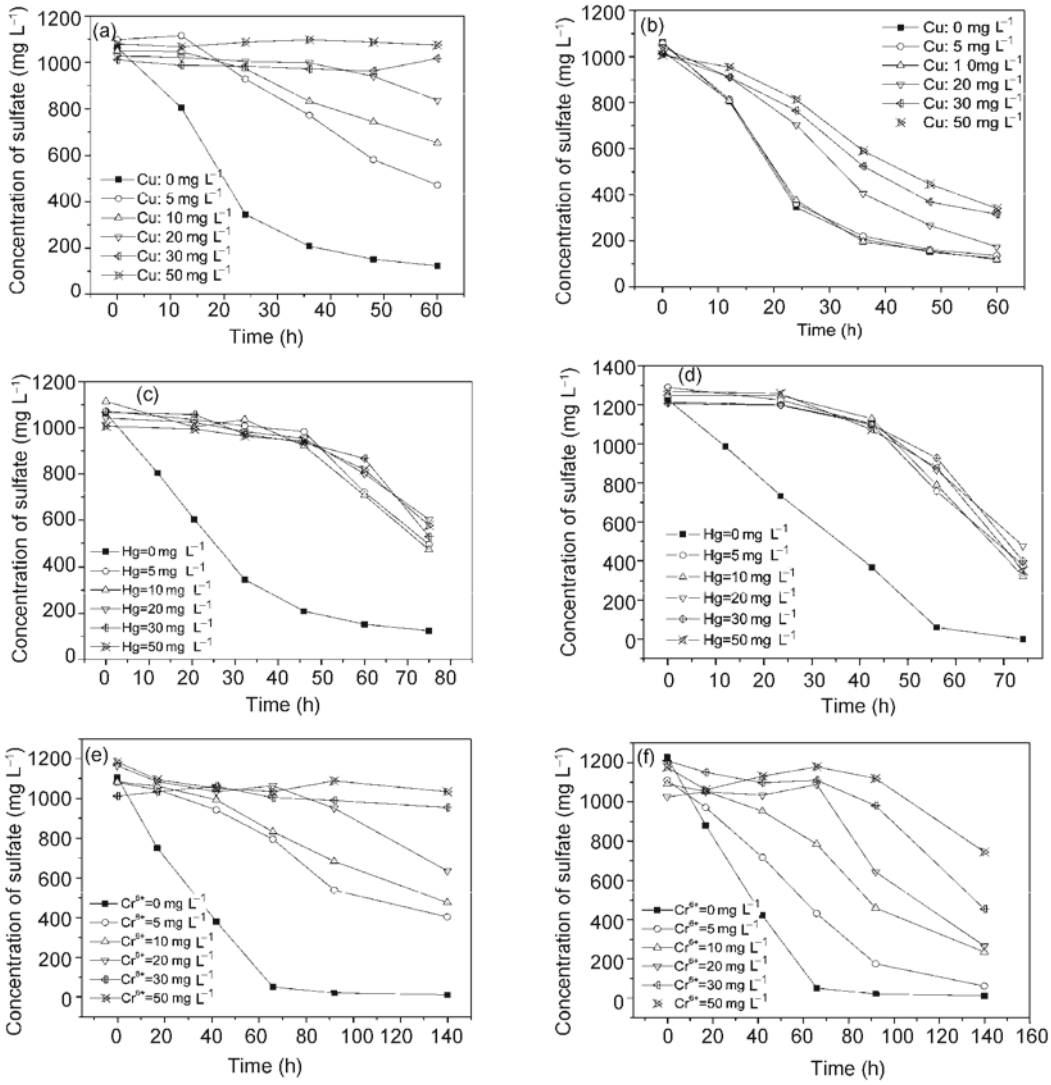

Figure 4 Evolution of sulfate for different heavy metal concentrations with and without the appearance of sulfide. (a) Cu(II) without sulfide; (b) Cu(II) with sulfide; (c) Hg(II) without sulfide; (d) Hg(II) with sulfide; (e) Cr(VI) without sulfide; (f) Cr(VI) with sulfide.

inhibited by chromium. A higher initial Cr(VI) concentration led to a lower sulfate reduction rate. An obvious lag phase was obtained for initial Cr(VI) concentrations higher than 5 mg L^{-1} and the lag phase time increased with the initial Cr(VI) concentration. As shown in Figure 4(f), with sulfide addition, the evolution of sulfate showed a similar trend to that shown in Figure 4(e). The sulfate reduction rate also decreased with an increase in the initial Cr(VI) concentration. Compared with that in Figure 4(e), the sulfate reduction rate showed an obvious increase and the lag phase in each vial showed a corresponding decrease.

According to the results discussed above, the SRB toxicity of copper is largely weakened by sulfide. As shown in Figure 1(a), Cu(II) was present in the culture in the absence of sulfide and it precipitated quickly upon sulfide addition. According to Table 3 the precipitate is CuS, which is nontoxic toward SRB. However, as shown in Figure 2(a) the particle size distribution of the precipitated CuS ranged from 0.03 to 4 μm, which is smaller than SRB. Previous studies reported that biologically produced CuS easily combines with cells [17] or agglomerate onto each other [1] because of the hydrogen bonds that surface hydroxyls form by hydration in aqueous solutions. Therefore, the produced CuS easily deposited onto SRB resulting in a black layer enveloping SRB cells (Figure 3(b)). As shown in Figure 2(b), after the SRB were metabolized for 5 d the peaks centered at 0.1 and 1 μm in the PSD profiles largely decreased, which means the amount of small particles in the SRB metabolism culture decreased. The biologically produced CuS combined with cells or agglomerated onto each other. At a low copper concentration not enough CuS was generated to completely envelope the cells and its effect on SRB activity was negligible resulting in a copper concentration lower than 10 mg L^{-1} while the toxicity of Cu(II) was completely eliminated (Figure 1(b)). When the copper concentration increased more CuS was generated and deposited on the SRB cell. After the cell was completely enveloped by covellite the metabolism of SRB was affected because of the increase in mass transfer resistance. At a copper concentration higher than 20 mg L^{-1} the sulfate reduction rate decreased with an increase in the initial copper concentration (Figure 1(b)). This indicated that the sulfide could eliminate the toxicity of Cu(II) toward SRB because of the generated covellite as the generated CuS deposited on the surface of the SRB cell. At high CuS concentrations the metabolism of SRB was affected because of the increase in mass transfer resistance.

Because SRB were identified as the primary organisms responsible for monomethylmercury (MeHg) production during the biotic transformation of inorganic mercury and Hg(II) through energy-dependent uptake systems [16,18,19], the toxic resistance of SRB to Hg(II) was higher than that of other bacteria. At an initial Hg(II) concentration lower than 50 mg L^{-1} sulfate reduction was not completely inhibited (Figure 4(c)). As shown in Figure 4(c) and (d), the toxicity of Hg(II) on SRB did not increase with an increase in the Hg(II) concentration independent of sulfide addition to the SRB metabolism culture. This could be caused by the high atomic weight of Hg (200.6) and the high toxic resistance of SRB to Hg. When sulfide was present, HgS ($K_{sp}=1.6\times10^{-52}$) [20] was generated, resulting in most of the mercury distributing into the residual fraction and in low solubility mercury being found (Figure 1(b)). Additionally, the toxicity of Hg(II) toward SRB was alleviated correlating to a decrease in the lag phase of sulfate reduction as well as an increase in the sulfate reduction rate (Figure 4(d)). The mercury content was also distributed in the organic fraction (Figure 1(b)). However, precipitating Hg(II) as HgS did not inhibit the generation of MeHg, because mercury was mainly distributed in the organic fraction after the SRB had metabolized for 5 d. Compared with Hg(II), the toxicity of MeHg toward the bacteria was higher. Therefore, sulfide alleviated the toxicity of Hg(II) toward SRB by distributing Hg(II) but it did not prevent the methylation of mercury and thus could not alleviate the harm mercury causes the bacterium and the environment. It is necessary to separate the generated HgS from SRB and to avoid the methylation of generated HgS by SRB.

Cr(VI) is a strong oxidable metal ion especially under acid conditions [21]. It can be reduced to Cr(III) by sulfide and shows low toxicity toward SRB. The generated Cr(III) can subsequently precipitate as $Cr(OH)_3$ after reacting with the OH^- generated by the oxidation of organic substances. $Cr(OH)_3$ is easily absorbed by other organic molecules because of its surface hydroxyl groups [22] and this was confirmed by the results. The particles shown in Figure 3(d) are mainly composed of organic substances and deposited chromium. The larger particle size causes them not to completely envelop the cells compared to those shown in Figure 3(b). The generated particles in the vial containing chromium showed no toxicity toward SRB. With the appearance of sulfide, the Cr(VI) reduction rate increased resulting in more Cr(III) and subsequently $Cr(OH)_3$. Therefore, more chromium precipitated in the residual fraction with sulfide addition after SRB had metabolized for 5 d (Figure 1(c)). The toxicity of Cr(VI) toward SRB was weakened by the decrease in soluble Cr(VI). However, as shown in Figure 4(f) the appearance of sulfide did not eliminate the toxicity of Cr(VI). SRB metabolism was inhibited by the production of Cr(III) as a result of Cr(VI) reduction by the sulfide. Therefore, the Cr(VI) reduction rate determined the toxicity of Cr(VI) toward SRB.

3 Conclusions

This study shows the effects of sulfide on SRB in response to Cu(II), Hg(II) and Cr(VI) toxicity. In the presence of sulfide, Cu(II) precipitated as CuS, which removed Cu(II) toxicity. However, the generated CuS depositing on the surface

of SRB cells caused the inhibition of SRB metabolism because of an increase in the mass transfer resistance. Hg(II) precipitated as HgS by a sulfide interaction and decreased the toxicity of Hg(II) toward SRB. Precipitating Hg(II) as HgS did not inhibit the generation of MeHg and, therefore, the sulfide did not eliminate the harm Hg(II) on bacteria and the environment. The toxicity of Cr(VI) was weakened and not eliminated by sulfide interaction, because of an increase in the Cr(VI) reduction rate in the presence of sulfide. The rate of Cr(VI) reduction determined the toxicity of Cr(VI) toward SRB.

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