

# Uptake of Trace Metals and Rare Earth Elements from Hornblende by a Soil Bacterium

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*Analysis of trace elements released from hornblende between pH 6.5 and 7.5 in the presence of *Arthrobacter* sp. shows that Fe, Ni, V, Mn, and, to a lesser extent, Co are preferentially released into solution relative to bacteria-free experiments. This enhanced release into solution could be due to contributions from the slightly lowered pH, the presence of low molecular weight organic acids (LMWOAs), or the presence of a catechol siderophore in experiments with bacteria. The best explanation for enhanced metal release is siderophore complexation at the mineral surface followed by release to solution. However, the relative rates of metal release to solution in these experiments do not strictly follow the trend predicted by the relative ordering of metal hydrolysis, which might be predicted for siderophore-promoted dissolution. For some of these metals, release to solution is fast initially in biotic experiments, but concentrations in solution reach a steady state value or decrease with time as the bacteria cell numbers increase exponentially. Lack of enhanced release to solution for some metals and decreases in release rate with time for others may be explained by uptake into bacteria. Many of the metals predicted to strongly complex with siderophore (including Al, Ti, Fe, Cu) are heavily taken up into cellular material. The relative ordering of organic ligand–element complexation may therefore partially explain the relative ordering of uptake of trace metals and rare earth elements into cell material. Fractionation of heavy rare earth elements taken up into cellular material is also very strong, and increases from Ho to Lu. Strong fractionation in uptake of some elements by bacteria may create biological signatures either in the mineral substrate or in any mineral precipitates associated with the cellular material.*

**Keywords** hornblende, rare earth elements, siderophores, soil bacteria, trace elements

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Roughly one-third of the ~100 elements in the periodic table are known to be bioessential. Of these elements, the macronutrients C, O, N, H, P, Ca, Mg, K, S, Na, and Cl constitute the bulk of the cells (Mason and Moore 1982). Of the remaining trace element micronutrients, many are metals found specifically in proteins. In fact, of all known proteins, one-third to one-half are metalloproteins where the metals participate as catalysts, are important structurally, or activate certain processes (Butler 1998). For example, Wackett, Orme-Johnson, and Walsh (1989), Ferris, Shotyk, and Fyfe (1989), and Sprott (1989) point out that V is known to be used in at least one nitrogenase; Mn is used in many enzymes including superoxide dismutase in *E. coli*; Fe is used in ~70 proteins; Co is used in B<sub>12</sub>-containing enzymes and at least two other enzymes; Ni is used in urease, several hydrogenases, CO dehydrogenase, and in at least one reductase; Cu is used in >30 proteins (many of which are oxidases and oxygenases); Zn is used in ~80 proteins; and Mo is used in nitrogenase, some oxidases, and reductases, and some formate and CO dehydrogenases. In addition, W has been identified in at least one enzyme of one species. Uses for other metals may await discovery.

Although uptake of metals by microbes has been documented by many workers (e.g., Beveridge and Fyfe 1985), relatively little is known about the uptake mechanisms of the metal micronutrients for environmentally important microbes. Crustal abundance of these elements varies from Fe (5%) > Mn (950 ppm) > Zn (~70 ppm) ≈ Ni (75 ppm) > Cu (55 ppm) > Co (25 ppm) > Mo (1.5 ppm) (average continental crust, Mason and Moore 1982), and many of these metals are insoluble under conditions of bacteria growth. Beveridge (1989a) argued that use of some of the more rare metals by microbes may have evolved previous to the Proterozoic when hydrothermal venting and leaching through mafic and ultramafic rocks was more vigorous—under such conditions, Mg, Cr, Co, Mn, Ni, Cu, precious metals, and Fe may have been more highly concentrated in environmental solutions, and therefore perhaps more bioavailable.

Despite the low concentrations of metals in most environments, Fe is the only bioessential element thought to require a specific organic ligand for transport (Neilands 1989; Butler 1998). These Fe(III)-specific organic ligands (siderophores) are secreted by many organisms for sequestration and uptake of Fe under neutral, aerobic conditions. The most common groups of siderophores are the hydroxamates and the catecholates (Winkelmann 1991).

In previous papers (Liermann, Kalinowski, Brantley, and Ferry 2000; Kalinowski, Liermann, Brantley, Barnes, and Pantano 2000), we showed that siderophores released by soil microbes accelerate the release of Fe from hornblende, a common silicate, in buffered and unbuffered media. The mechanism of Fe leaching was inferred to result from siderophore complexation on the hornblende surface followed by metal release. Hornblende was chosen for these studies because it often contains a high concentration of minor or trace elements needed by soil microorganisms (e.g., Fe, Mn, Cu, Zn, V, Mo, Ni, and Co). Of these elements, Fe is of particular interest because of its low bioavailability in aerobic environments at neutral pH.

While the exact chemistry of the catecholate siderophore produced by the *Arthrobacter* sp. used in our earlier experiments has not been identified, it has been shown that the tendency of metal ions to complex with ligands such as catechol with negatively charged oxygen atoms correlates with the tendency of the metals to hydrolyze (e.g., Morel and Hering 1993; Hernlem, Vane, and Sayles 1996). The best example of this relative ordering is the observation that the tendency for divalent metals to complex with organic ligands increases according to the Irving-Williams series:  $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ . Therefore, even without knowledge of the exact siderophore structure, trends in the siderophore-metal complexation constants can be predicted from knowledge of the first hydrolysis constants of the metals. Such trends, along with our previous observations, would suggest that soil microbes should also accelerate the release of other trace metals from silicates to solution.

Of particular note are the rare earth elements (REE) whose similar physicochemical properties suggest predictable fractionation in natural systems. Over the past three decades, REE geochemistry has become a powerful tool in both igneous and aqueous geochemistry. However, in spite of their wide use, little is known about the *biogeochemistry* of the REE. Considering the ubiquity of organisms and biofilms in natural environments this is a major shortcoming. The few studies that looked into the effects of microbes and/or biofilms on REE behavior focussed, for example, on the microbial mediation of Ce(III) oxidation (Moffett 1990), on the characteristics of REE complexation with organic ligands on marine particle surfaces (Byrne and Li 1995), and on REE uptake by the marine macroalgae *Ulva lactuca* L. (Stanley and Byrne 1990).

One of the features that make the REE so useful in studies of altered igneous rocks, clastic sediments, and soils is their immobility compared to other (trace) elements. However, it appears that this immobility is primarily due to the effectiveness with which the REE, once they are mobilized during fluid-rock interaction, are immobilized again due to incorporation into secondary minerals or sorption onto particle surfaces (Bau, Usui, Pracejus, Mita, Kanai, Irber, and Dulski 1998). The overall mobility of the REE during fluid-rock interaction, however, can be expected to increase dramatically when ligands are available that form strong (polydentate) REE solution-complexes and thereby significantly increase the REE transport capacity of the fluid. Because organic compounds such as polycarboxylic and aminocarboxylic acids are known to form very stable complexes with the REE, REE mobility during fluid-rock interaction may be drastically increased by organic acids and siderophores produced by microbes, perhaps changing REE distribution patterns.

However, because of the enormous affinity of siderophores for Fe(III), in many cases where the siderophore concentration in solution, [L], is lower than the Fe(III) concentration, [Fe(III)], the ligand is completely bound to Fe, and little to no other metal-siderophore complexes are formed (Morel and Hering 1993). The extreme specificity of many siderophores for Fe(III) in solution might imply therefore that siderophore-promoted metal leaching from solids would be very Fe(III)-specific. Indeed, Liermann, Kalinowski, et al. (2000a) and Kalinowski et al. (2000) both observed that neither Si nor Al release rates from hornblende were enhanced in the presence of siderophore-producing arthrobacter even though the Fe release was enhanced markedly. However, Duhme, Hider, Naldrett, and Pau (1998) suggested that some microbes produce multiple siderophores (e.g., *Azotobacter vinelandii*, *Pseudomonas aeruginosa*) with different metal-binding capacities, and that, under some conditions, some of these siderophores may be important in binding metals other than Fe(III). These "extra" siderophores may therefore be involved in solubilization of metals other than Fe from soil minerals.

Here, our earlier work is extended by measuring release rates of essential and nonessential trace elements. If the specificity of siderophores for Fe(III) holds also for surface complexation, then release of trace elements other than Fe would not be expected to be enhanced in the presence of bacteria. Furthermore, in our previous work, no attempt was made to measure bacterial uptake of Fe or other metals. In this paper, we summarize measurements of both trace metal release to solution and uptake into bacteria during dissolution of hornblende.

## Methods

Powdered hornblende (Gore Mountain, NY) with a grain size of 250–429  $\mu\text{m}$  was ultrasonically cleaned several times in acetone until supernatant was transparent. Trace element composition of the hornblende was analyzed by digestion and chemical analysis using inductively coupled plasma-mass spectrometry (Finnigan MAT-Element double focusing sector field ICP-MS). Details are summarized in Tables 1a, 1b. Analyses of major elements on

**TABLE 1a** Trace element content of hornblende and suspended material

	Hornblende <sup>1</sup> (ppm)	% RSD <sup>2</sup>	Bacteria without hornblende <sup>2</sup> ( $\mu\text{g/L}$ )						Bacteria with hornblende <sup>2</sup> ( $\mu\text{g/L}$ )						
			2-1-1	2-1-2	2-2-1	2-2-2	4-1-1	4-1-2	4-2-1	4-2-2	4-3-1	4-3-2			
			14.2	13.4	5.1	7.6	21.4	205	179	174	169	190			
Al	Conc. % <sup>4</sup>	<3	14.2	13.4	5.1	7.6	21.4	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.02
Ba	Conc. %	<2	0.4	0.4	0.4	0.4	6.5	6.3	6.3	12.4	12.4	12.5	2.5	2.5	2.8
Co	Conc. %	<10	0.004	0.007	0.017	0.007	0.31	0.35	0.35	0.27	0.27	0.27	0.34	0.34	0.36
Cr	Conc. %	<9	0.14	0.13	0.15	0.14	0.97	0.93	0.93	1.16	1.16	1.24	0.69	0.69	0.63
Cu	Conc. %	<5	1.11	1.02	0.71	0.87	11.1	12.0	12.0	13.9	14.4	14.4	12.7	12.7	12.4
Fe	Conc. %	<8	3.67	3.62	4.24	3.96	2.97	3.22	3.22	3.71	3.85	3.85	3.40	3.40	3.33
Li	Conc. %	<11	0.00	0.01	0.01	0.01	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mn	Conc. %	<5	0.72	0.72	0.71	0.66	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Mo	Conc. %	<9	0.07	0.06	0.06	0.06	3.63	3.61	3.61	6.44	6.44	6.69	4.69	4.69	4.91
Ni	Conc. %	<20	0.10	0.19	0.07	0.11	0.58	0.56	0.56	0.75	0.75	0.76	0.65	0.65	0.64
Rb	Conc. %	<4	0.01	0.01	0.01	0.01	4.15	3.98	3.98	5.34	5.34	5.44	4.63	4.63	4.54
Sb	Conc. %	<17	0.004	0.004	0.004	0.004	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00
Si	Conc. %	<7	12.3	12.4	20.9	37.8	0.12	0.12	0.12	0.12	0.09	0.09	0.08	0.08	0.11
							0.34	0.33	0.33	0.32	0.26	0.26	0.22	0.22	0.30
							0.012	0.013	0.013	0.014	0.015	0.015	0.011	0.011	0.009
							0.052	0.057	0.057	0.062	0.067	0.067	0.047	0.047	0.039
							79.7	75.0	75.0	61.8	70.7	70.7	59.9	59.9	80.3
							0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002

Sr	Conc.	130	<2	0.13	0.15	0.12	0.14	0.40	0.41	0.41	0.41	0.40	0.41	0.33	0.36
	%							0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Ti	Conc.	4700 <sup>3</sup>	<16	0.13	0.12	0.12	0.19	9.60	9.65	9.65	9.65	5.15	7.89	8.86	
	%							0.01	0.01	0.01	0.01	0.01	0.01	0.01	
U	Conc.	0.06	<10	0.003	0.003	0.005	0.004	0.75	0.70	0.70	0.85	0.89	0.65	0.61	
	%							48.3	44.9	44.9	55.0	56.9	41.8	39.0	
V	Conc.	88	<14	0.02	0.02	0.02	0.02	0.20	0.20	0.20	0.13	0.14	0.19	0.22	
	%							0.01	0.01	0.01	0.01	0.01	0.01	0.02	
Zn	Conc.	120	<2	3.29	2.56	2.36	2.05	5.47	7.20	7.20	9.21	9.07	8.73	7.00	
	%							0.30	0.40	0.40	0.51	0.50	0.48	0.38	

<sup>1</sup> Approximately 0.25 g samples were heated by microwave in 1 ml H<sub>2</sub>O, 5 ml conc. HNO<sub>3</sub>, 1 ml conc. HCl, and 3 ml conc. HF in CEM HP 500+ vessels for three cycles of 100% power (2 min), 75% power (5 min), and 55% power (5 min). Vessels were manually rotated between heating. After heating, solutions were evaporated to near dryness, redissolved in 10 ml solution (10% HNO<sub>3</sub> + 10% HCl) with gentle heating. Digested solutions were diluted to 100 ml and stored in HDPE container.

<sup>2</sup> Bacteria pellet was dissolved after drying in 0.6 mL of conc., HNO<sub>3</sub> at 60°C, diluted to 10 mL and analyzed. Multiple analyses of individual samples of digested bacteria yielded % RSD values as noted. Where no concentration data are listed, % RSDs were extremely high (>30%). Concentrations of metals in cellular material are reported as µg element/L of culture solution sampled at 28 days. Two experiments were run without (2-1-1, 2-2) and three with hornblende (4-1, 4-2, 4-3). Each of these runs was analyzed in replicate (e.g., 2-1-1, 2-1-2), yielding four or six analyses total as shown. No corrections have been completed on these data (see text or compare to corrected data in Figures 3-5). Omitted data indicate concentrations below detection.

<sup>3</sup> Analysis of major element concentration from XRAL, Inc. by X-ray fluorescence, averaged for two samples.

<sup>4</sup> Percentage of metal in the hornblende extracted into suspended cellular material.

**TABLE 1b** Rare earth element content of hornblende and suspended material

	Hornblende <sup>1</sup> (ppm)	% RSD	Bacteria without hornblende <sup>2</sup> ( $\mu\text{g/L}$ )						Bacteria with hornblende <sup>2</sup> ( $\mu\text{g/L}$ )													
			2-1-1		2-1-2		2-2-1		2-2-2		4-1-1		4-1-2		4-2-1		4-2-2		4-3-1		4-3-2	
La	Conc. % <sup>4</sup>	3.38	<3	0.009	0.004	0.005	0.003	0.031	0.032	0.037	0.035	0.027	0.073									
Ce	Conc. %	11.1	<6	0.013	0.005	0.006	0.005	0.08	0.08	0.15	0.15	0.06	0.15									
Pr	Conc. %	1.65	<6	0.002	0.001	0.001	0.001	0.009	0.008	0.007	0.007	0.008	0.018									
Nd	Conc. %	7.23	<7	0.005	0.002	0.003	0.002	0.037	0.038	0.033	0.032	0.034	0.062									
Sm	Conc. %	1.93	<6	0.0002	0.0002			0.010	0.010	0.009	0.009	0.008	0.009									
Eu	Conc. %	0.68	<7					0.03	0.03	0.03	0.03	0.03	0.03									
Gd	Conc. %	1.40	<7					0.004	0.004	0.003	0.003	0.003	0.003									
Tb	Conc. %	0.24	<6					0.03	0.03	0.03	0.03	0.03	0.03									
Dy	Conc. %	1.30	<5					0.009	0.009	0.007	0.007	0.008	0.010									
Ho	Conc. %	0.23	<7					0.04	0.04	0.03	0.03	0.04	0.05									
								0.002	0.002	0.001	0.001	0.001	0.002									
								0.04	0.04	0.03	0.03	0.03	0.04									
								0.009	0.009	0.008	0.008	0.008	0.009									
								0.05	0.05	0.04	0.04	0.04	0.04									
								0.002	0.002	0.002	0.002	0.002	0.002									
								0.06	0.06	0.06	0.06	0.05	0.06									

Er	Conc. %	0.61	<6	0.0001	0.0001	0.009	0.009	0.010	0.010	0.008	0.008
Tm	Conc. %	0.08	<6			0.002	0.002	0.003	0.003	0.002	0.002
Yb	Conc. %	0.61	<8	0.0001	0.0001	0.019	0.019	0.023	0.022	0.016	0.016
Lu	Conc. %	0.09	<6			0.20	0.20	0.24	0.24	0.16	0.17
						0.003	0.003	0.004	0.004	0.002	0.003
						0.21	0.21	0.27	0.28	0.16	0.18

<sup>1</sup>Approximately 0.25 g samples were heated by microwave in 1 ml H<sub>2</sub>O, 5 ml conc. HNO<sub>3</sub>, 1 ml conc. HCl, and 3 ml conc. HF in CEM HP 500+ vessels for three cycles of 100% power (2 min), 75% power (5 min), and 55% power (5 min). Vessels were manually rotated between heating. After heating, solutions were evaporated to near dryness, redissolved in 10 ml solution (10% HNO<sub>3</sub> + 10% HCl) with gentle heating. Digested solutions were diluted to 100 ml and stored in HDPE container.

<sup>2</sup>Bacteria pellet was dissolved after drying in 0.6 mL of conc. HNO<sub>3</sub> at 60°C, diluted to 10 mL, and analyzed. Multiple analysis of individual samples of digested bacteria yielded % RSD values as noted. Where no concentration data are listed, % RSDs were extremely high (> 30%). Concentration of metals in cellular material are reported as µg element/L of culture solution sampled at 28 days. Two experiments were run without (2-1, 2-2) and three with hornblende (4-1, 4-2, 4-3). Each of these runs was analyzed in replicate (e.g., 2-1-1, 2-1-2), yielding four or six analyses total as shown. No corrections have been completed on these data (see text or compare to corrected data in Figures 3-5). Omitted data indicate concentrations below detection.

<sup>3</sup>Analysis of major element concentration from XRAL, Inc. by X-ray fluorescence, averaged for two samples.

<sup>4</sup>Percentage of metal in the hornblende extracted into suspended cellular material.

separate samples by X-ray fluorescence (XRF) by commercial laboratory (XRAL Activation Services Inc.) are also reported.

A bacterial isolate obtained from hornblende-containing soil from Gore Mountain New York was used in this work (Kalinowski et al. 2000). The isolate was chosen because it exhibited robust growth in Fe-deficient medium in the presence of hornblende. Scanning electron microscopy of the isolate showed a rod-coccus growth cycle typical for arthrobacter (Keddie, Collins, and Jones 1989) and partial sequencing and analysis of the 16S rRNA gene revealed the isolate to be closely related to *Arthrobacter oxydans* (similarity rank of about 0.594). Following Kalinowski et al. (2000), this bacterium is therefore referred to as an arthrobacter.

Experiments with and without bacteria and with and without hornblende (four conditions in all) were completed in 1000 ml borosilicate glass flasks covered with Al foil at ambient temperature. Glassware was pretreated as follows: rinsed with Alconox detergent, rinsed with deionized H<sub>2</sub>O, soaked in Nochromix (GODAX Laboratories, Inc.) acid bath ( $\geq 15$  min), rinsed, soaked in 1:10 HNO<sub>3</sub> bath ( $\geq 24$  h), rinsed, soaked in deionized H<sub>2</sub>O ( $\geq 12$  h). Polypropylene sample bottles were pretreated similarly, without the Nochromix.

Flasks were filled with 500 ml sterilized MM9 medium (Schwyn and Neilands 1987) prepared from ultrapure chemicals and deionized H<sub>2</sub>O. To remove trace elements, 5 g of Chelex resin were mixed into every 100 ml of casamino acid solution made for this medium, mixed for 1 h, and then removed by filtration. All experiments were run from the same stock MM9 solution. Preautoclaved hornblende powder (7.00 g) was added to some flasks.

Some flasks were inoculated with 100  $\mu$ l of a 1-week-old (late log to stationary phase) culture of *Arthrobacter* sp. grown in 5 ml MM9 medium in a glass test tube. Then, 10 ml were sampled from each flask periodically. Samples were filtered (using a 0.2  $\mu$ m cellulose acetate filter), and aliquots were measured for pH. Remaining aliquots were frozen for analysis by ICP-MS. No attempt was made to analyze the oxidation state of released elements (for example, both Fe(II) and Fe(III) were analyzed and reported as [Fe]). Aliquots of the inoculum culture and several 100  $\mu$ l samples were also serially diluted and plated for enumeration of bacteria.

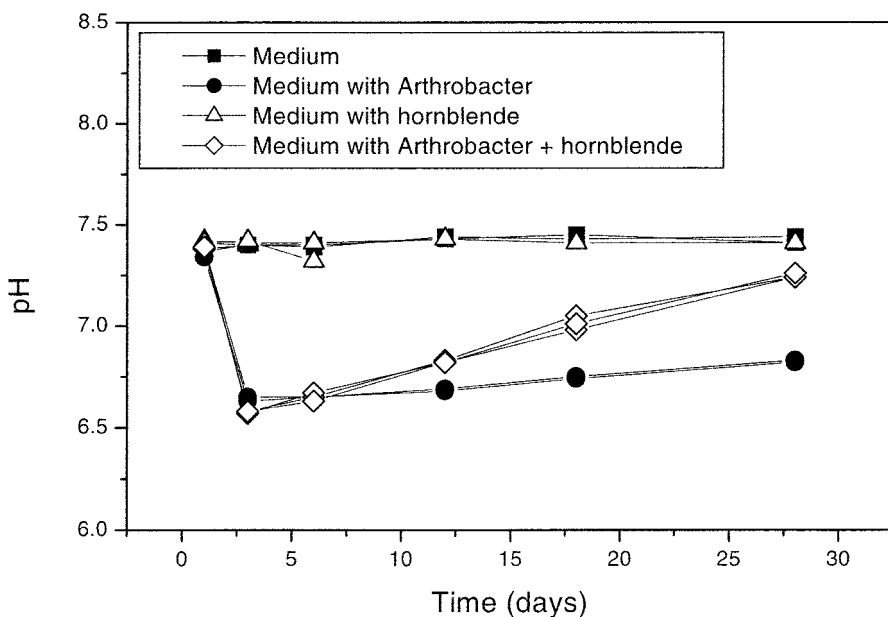
Upon experiment completion (28 d), two 100-ml portions of bacteria-containing cultures were centrifuged to pellet cells. Before centrifugation, these 100-ml samples contained floating bacteria but little to no hornblende, as hornblende settled to the bottom of the flask before sampling. Then, 50-ml aliquots of these supernatants were also filtered for ICP-MS analysis. Cell pellets were dried at 60°C and weighed. The hornblende powders were rinsed 4 times with deionized H<sub>2</sub>O to further separate cells from powder, ultrasonicated 20 min in 0.2 mg/ml of lysozyme in deionized H<sub>2</sub>O, rinsed 3 times with deionized H<sub>2</sub>O to separate remaining cells from the powder, ultrasonicated 15 min in acetone, and ultrasonicated for 10 min with Optima-grade acetone.

In order to stain for extracellular polysaccharides, samples of *Arthrobacter* sp. cells grown in the presence of hornblende were heat-fixed onto glass microscope slides, stained for 1 min w/ a 1:9 dilution of Alcian Blue (prepared 1% w/v in 95% ethanol): deionized water and counterstained for 5–10 sec with carbofuchsin. The stained samples were air-dried and viewed under phase contrast microscopy.

## Results

Measurements on plates showed similar cell counts through day 18 for flasks with and without hornblende; however, by day 28, significantly larger cell numbers had grown in flasks with hornblende present (Table 2). The average dry mass of cells of cultures containing hornblende was about 4 times that from cultures grown without hornblende after 28 days incubation.



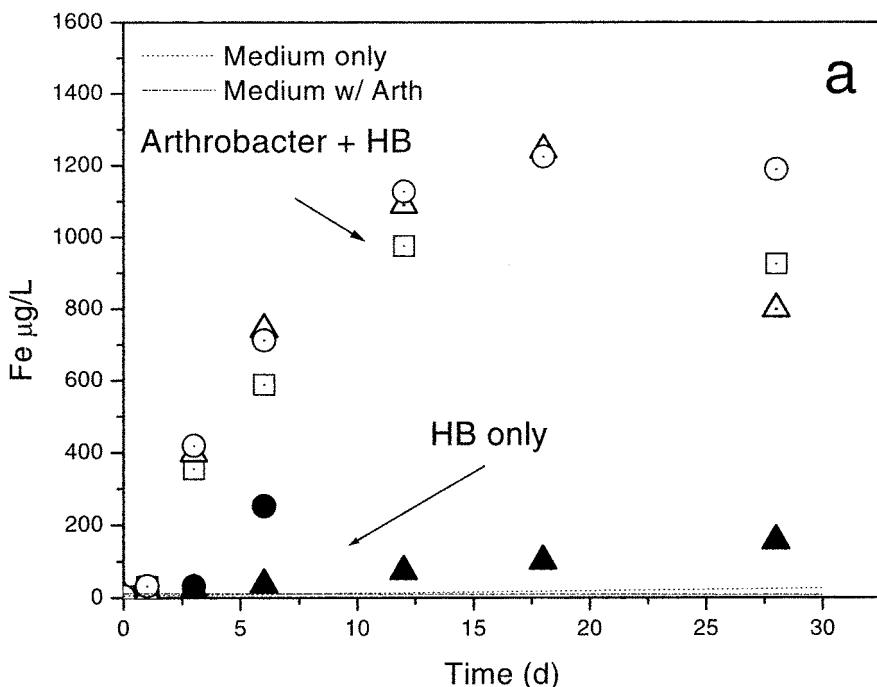


**FIGURE 1** Changes in pH measured in flask experiments as a function of time for the four experimental conditions listed.

The pH ( $=7.40 \pm 0.02$  at day 1) of medium only or medium + hornblende experiments changed insignificantly ( $\sim 0.2$  pH units) over 8 d (Figure 1). In contrast, experiments with medium + bacteria dropped by  $\sim 0.7$  pH units within 3 days, after which time the pH increased slightly (to pH 6.83). Similarly, experiments with medium + bacteria + hornblende dropped in pH (by  $\sim 0.8$  pH units) over 3 d but then increased to values almost coincident with the bacteria-free experiments (pH at day 28 = 7.25).

ICP-MS analysis of dissolved Fe, Mn, V, and Ni documented significantly or slightly enhanced rates of release of metal with time for experiments inoculated with *Arthrobacter* sp. (Figure 2). Rate of release of Co may also have been slightly enhanced (Figure 2, Table 1a). For these metals, the dissolved concentrations are also significantly higher than in medium or medium + bacteria control experiments. Of these 5 elements, however, only Fe, Ni, and V show higher concentrations at 28 d for experiments with bacteria as compared to experiments without (Table 1a). Mn and Co showed lower dissolved concentrations at 28 days in experiments with bacteria than without. Rates of release of other elements not reported in Table 1a either showed no consistent increase with time, were not reproducible, or were not significantly greater than values for experiments with medium or medium + bacteria only.

Several elements found in hornblende, Zn, Sr, and Ni also showed little to no enrichment in cellular material when grown with hornblende. In contrast, elemental analyses of U, Yb, Rb, Sc, Li, Cu, Cr, Ba, V, Co, Mn, Ti, Fe, and Al all showed significant enrichment in the bacteria pellets grown with hornblende powder as compared to those grown without powder present (Table 1a, Figure 3). However, tiny black spots that might be hornblende were observed in the bacteria cells grown with hornblende before digestion. To correct for this slight contamination, concentration of elements in cellular material grown with hornblende plotted in Figure 3 were corrected assuming that much of the Si in the cell material was due to hornblende powder (hornblende analysis summarized in Table 1a). Analyses were corrected so that Si concentration of cellular material grown with and without hornblende were the same.



**FIGURE 2** Release of metals from hornblende (= HB) to solution as a function of time for experiments with and without bacteria. Symbols represent experiments with hornblende present. For experiments with hornblende and *Arthrobacter* (open symbols labeled “*Arthrobacter* + HB”), data for three replicates are shown. For experiments with hornblende but without bacteria (labelled “HB only”), only one of three replicate experiments remained uncontaminated after day 7. Levels of metal concentration in control flasks with medium only and medium with *Arthrobacter* (= Arth) are also indicated by lines, although in many cases these concentrations are below detection limits. In some cases, apparent decreases in concentration in the two controls may only indicate that concentrations were below detection. In one case, concentrations were so low (and negative) that data are not plotted. (Continued)

For each experiment with cells, two 100-ml aliquots were sampled and centrifuged to collect cell mass. The cell mass per 100-ml aliquot extracted for each experiment at 28 days often differed markedly (Table 2). Despite this difference, the observed concentration of metals suspended in solution did not differ greatly for aliquots for each sample (Table 1) when normalized on a mass per L basis. This observation was consistent with the finding that concentrations of metals in cellular material differed from aliquot to aliquot when normalized on a mass per g cellular material basis (averages shown in Figure 3).

After staining with Alcian Blue, the cells grown with hornblende appeared pinkish red in color in phase contrast microscopy, with a blue layer around the cells. In Alcian Blue staining, acidic polysaccharides are stained blue by the Alcian dye while cells are stained pinkish red by the counterstain, carbolfuchsin (Murray, Doetsch, and Robinow 1994).

Although no data for dissolved rare earth elements (REE) were collected due to lack of sufficiently large solution samples and low concentrations, further information on trace element behavior during interaction of *Arthrobacter* sp. with hornblende may be gleaned from the distribution of the rare earth elements in cellular material (Figure 4). Concentrations of the middle and heavy REE (MREE (Sm to Ho) and HREE (Ho to Lu), respectively)

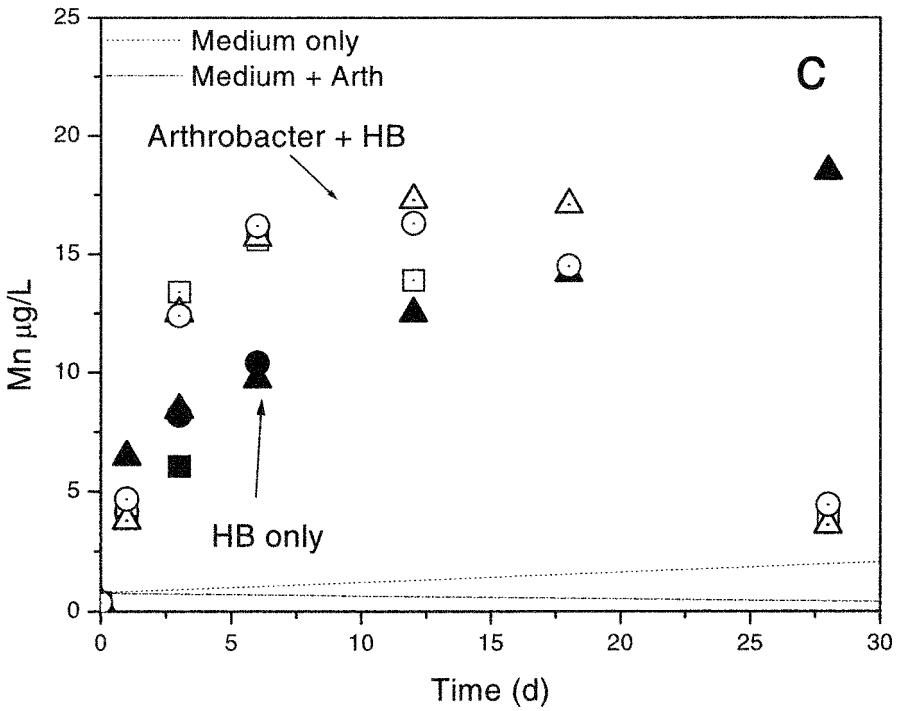
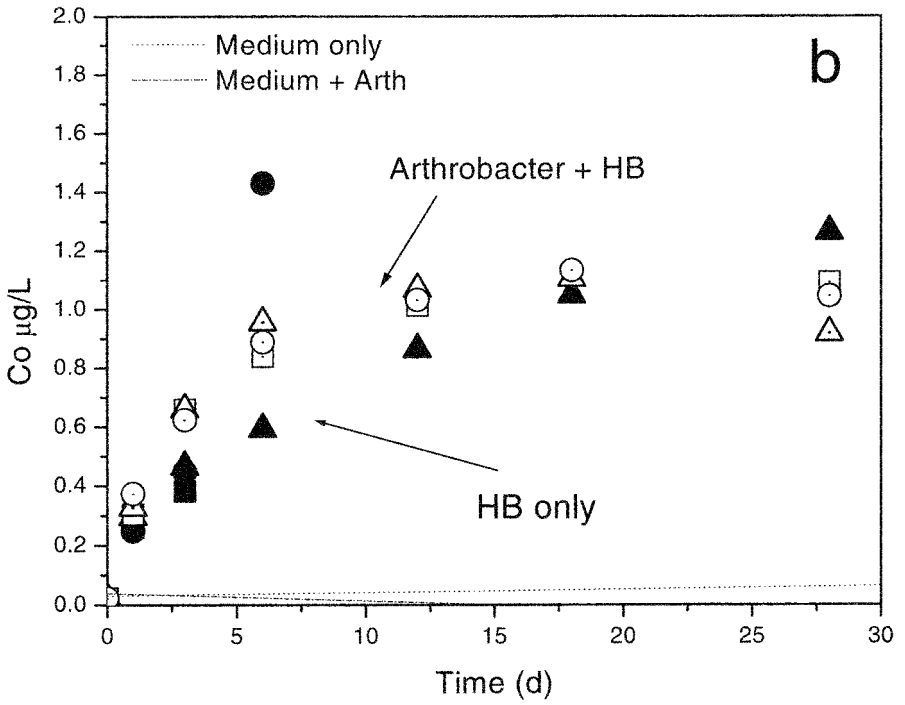


FIGURE 2 (Continued)

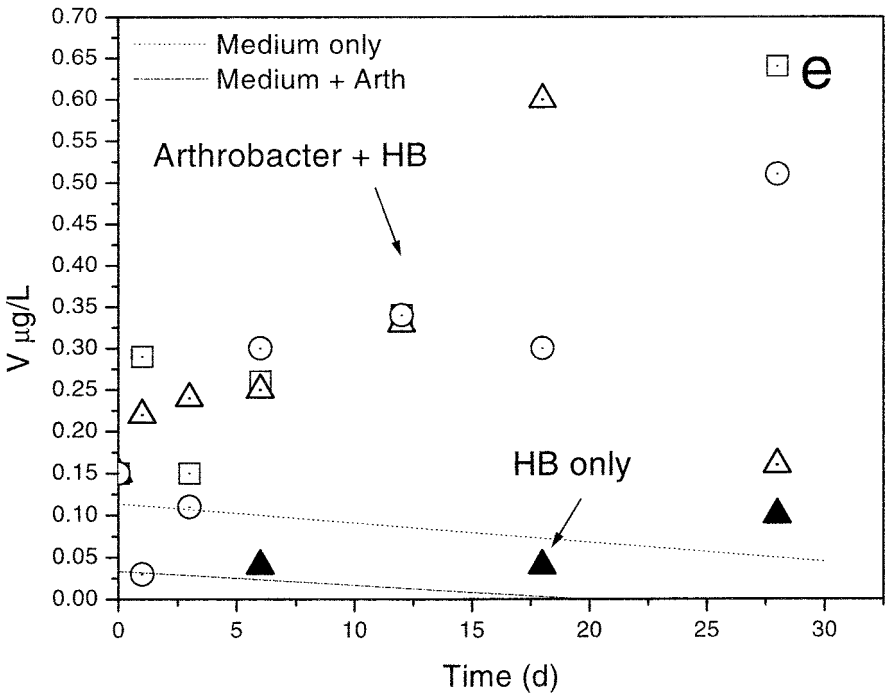
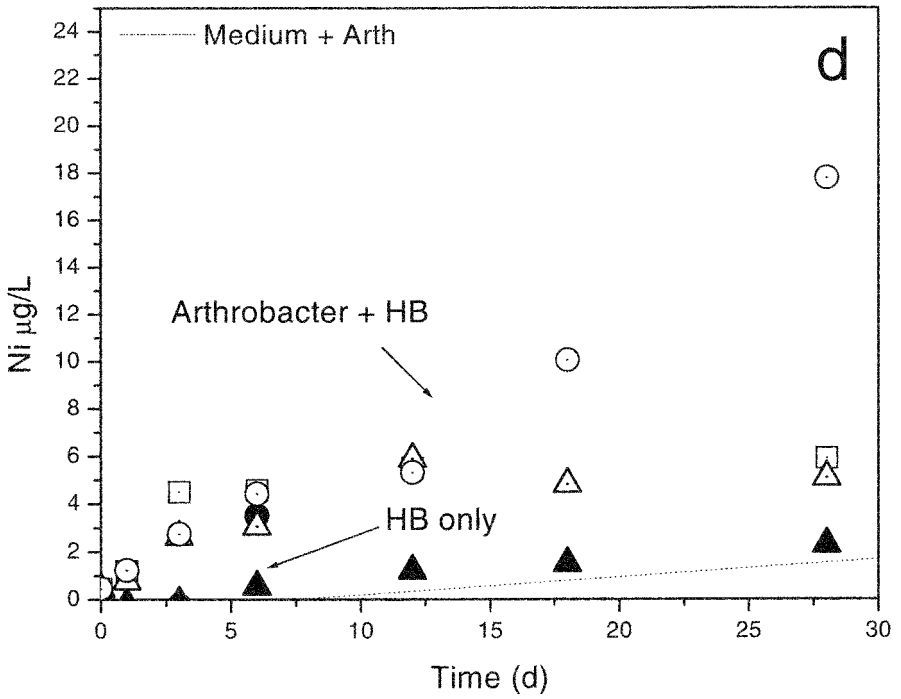
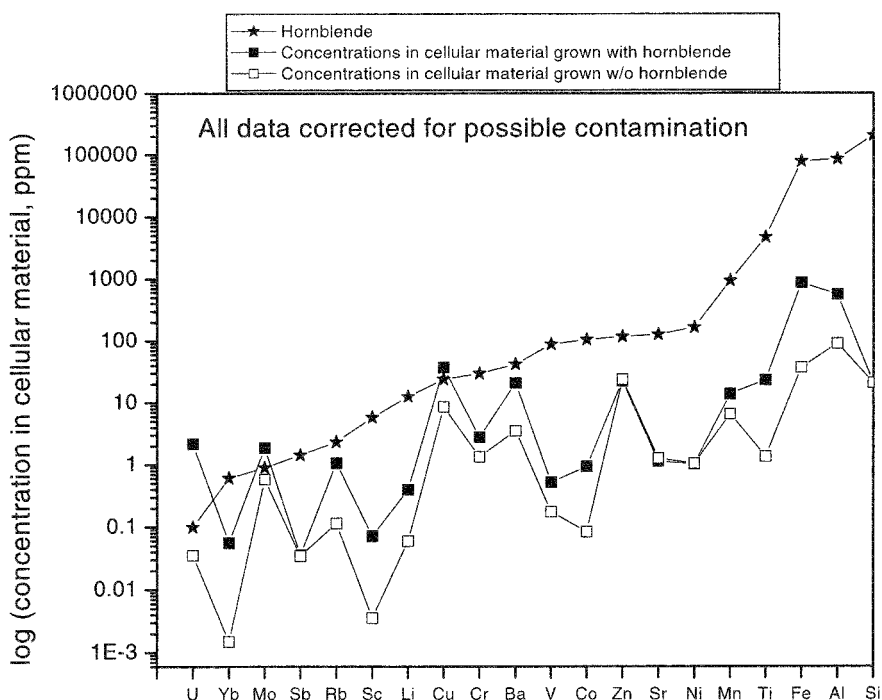


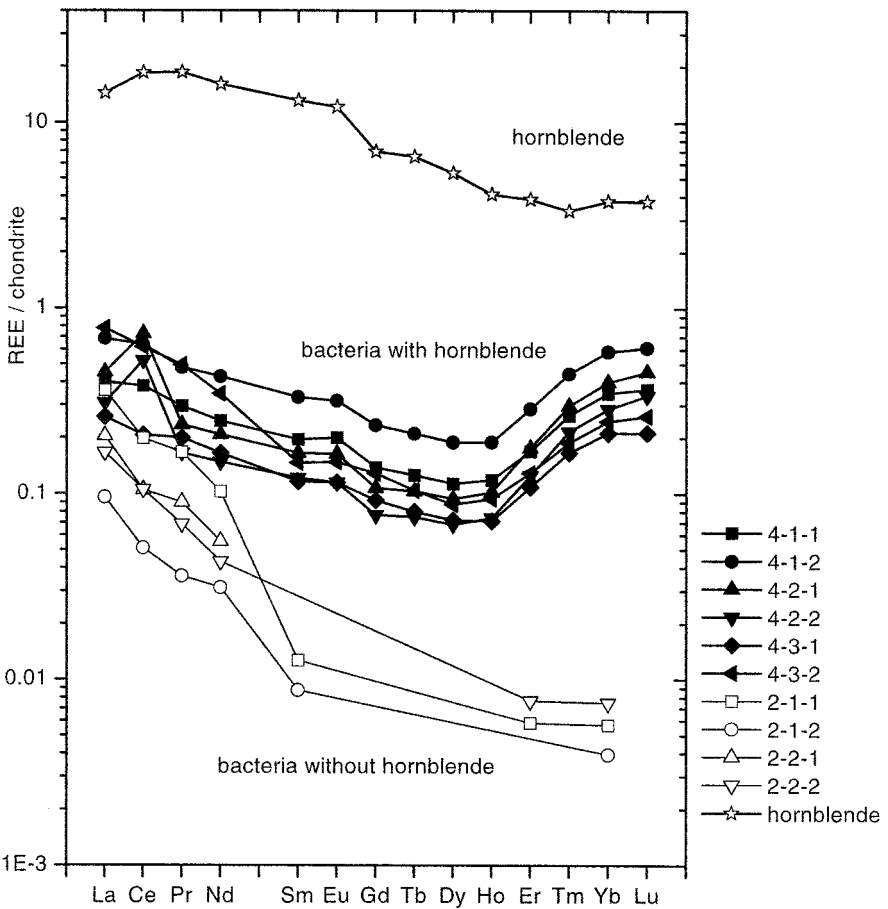
FIGURE 2 (Continued)



**FIGURE 3** Log (concentration) of metal in suspended cellular material or hornblende powder vs. element for 20 elements found in measured abundance in hornblende (see Tables 1a, 1b). Elements are listed in the order of increasing abundance in hornblende. Concentrations were measured in bacteria grown with and without hornblende present as described in Tables 1a, 1b. Concentrations in cell material grown with hornblende present was corrected by assuming some Si in cell material was due to contaminating silicate powder. All elemental concentrations were corrected using the hornblende concentration data assuming that the Si concentration in bacteria grown with and without hornblende were the same. Concentrations are given as  $\mu\text{g}$  element/g dry cell material, or  $\mu\text{g}/\text{g}$  hornblende powder. Data points for cell material represent averages for 2 (no hornblende) or 3 (with hornblende) experiments where each experiment was measured in replicate (Tables 1a, 1b).

are significantly higher in bacteria pellets grown with hornblende than in those that grew without hornblende present (Figure 4). However, due to the large spread (up to a factor of 5) of concentrations of light REE (LREE; La to Sm) in bacteria grown without hornblende, and because the concentrations of LREE sometimes reached the levels found in the bacteria pellets grown with hornblende, LREE contamination was inferred to have resulted from the reaction vessels or medium. We focus here on the behavior of the HREE from Ho to Lu.

Chondrite-normalized REE patterns  $\text{REE}_{\text{CN}}$ ; C1-chondrite from Anders and Grevesse 1989) of bacteria pellets grown with hornblende decrease from La to Ho and increase from Ho to Lu (Figure 4). As per standard practice, chondrite normalization is used to present the data in a manner whereby anomalies are easily noticed. All  $\text{REE}_{\text{CN}}$  patterns are free from anomalies with the exception of both aliquots of experiment 4-2, that show a positive Ce anomaly. Except for these Ce anomalies and more pronounced enrichment of La, Ce, Pr, and Nd in 4-3-2 (which is presumed due to contamination), all  $\text{REE}_{\text{CN}}$  patterns are very similar to each other.  $\text{REE}_{\text{CN}}$  patterns of the hornblende powder and of the bacteria pellets grown



**FIGURE 4** Chondrite-normalized (C1-chondrite from Anders and Grevesse 1989) REE patterns for: hornblende used in the experiments, pellets of bacteria grown without hornblende (experiments 2-x-x), and pellets of bacteria grown with hornblende (experiments 4-x-x). Contamination might have affected LREE distribution from La to Dy, but not that of the HREE from Dy to Lu. Note preferential bacterial removal of HREE from the hornblende (increasing pattern from Dy to Lu). Also note the positive Ce anomalies in experiment 4-2-x, suggesting (partial) oxidation of Ce(III) to Ce(IV).

without hornblende are significantly different from cells grown with hornblende present. Both lack the HREE enrichment seen in the bacteria pellets grown with hornblende (for example,  $Yb_{CN}/Ho_{CN}$  of the hornblende is 0.9 compared to  $\sim 3$  (4-1-1), for example, while showing similar LREE distribution.

Although HREE are removed from the hornblende during interaction with the bacteria, this HREE removal is considerably less effective than removal of Cu, Cu/Yb ratios of 4-1-1 and 4-1-2 ( $\sim 600$ ) were significantly higher than that of the hornblende ( $\sim 40$ ).

## Discussion

For all experiments with bacteria, growth of cells was accompanied by a decrease in pH (Figure 1). Analysis of organic acid production by this arthrobacter using ion chromatography (IC) for similar experiments was reported by Liermann, Barnes, Kalinowski, Xi-angyang, and Brantley (2000). Only the following acids were investigated: formic, acetic,

oxalic, malonic, citric, and phthalic acid. In general, the total organic carbon of samples ( $> 3000$  mg/L) was comprised of longer chain organic molecules than those identified by IC. However, the IC showed the presence of acetic and formic acid, and small amounts of oxalic and citric acids. These low molecular weight organic acids (LMWOAs) may therefore be responsible for the drop in pH in the first three days of the experiment. The rise in pH after 3 d may be caused by bacterial exudates in the bacteria without hornblende experiment, and by exudates and neutralization accompanying hornblende dissolution in the bacteria + hornblende experiment (Figure 1).

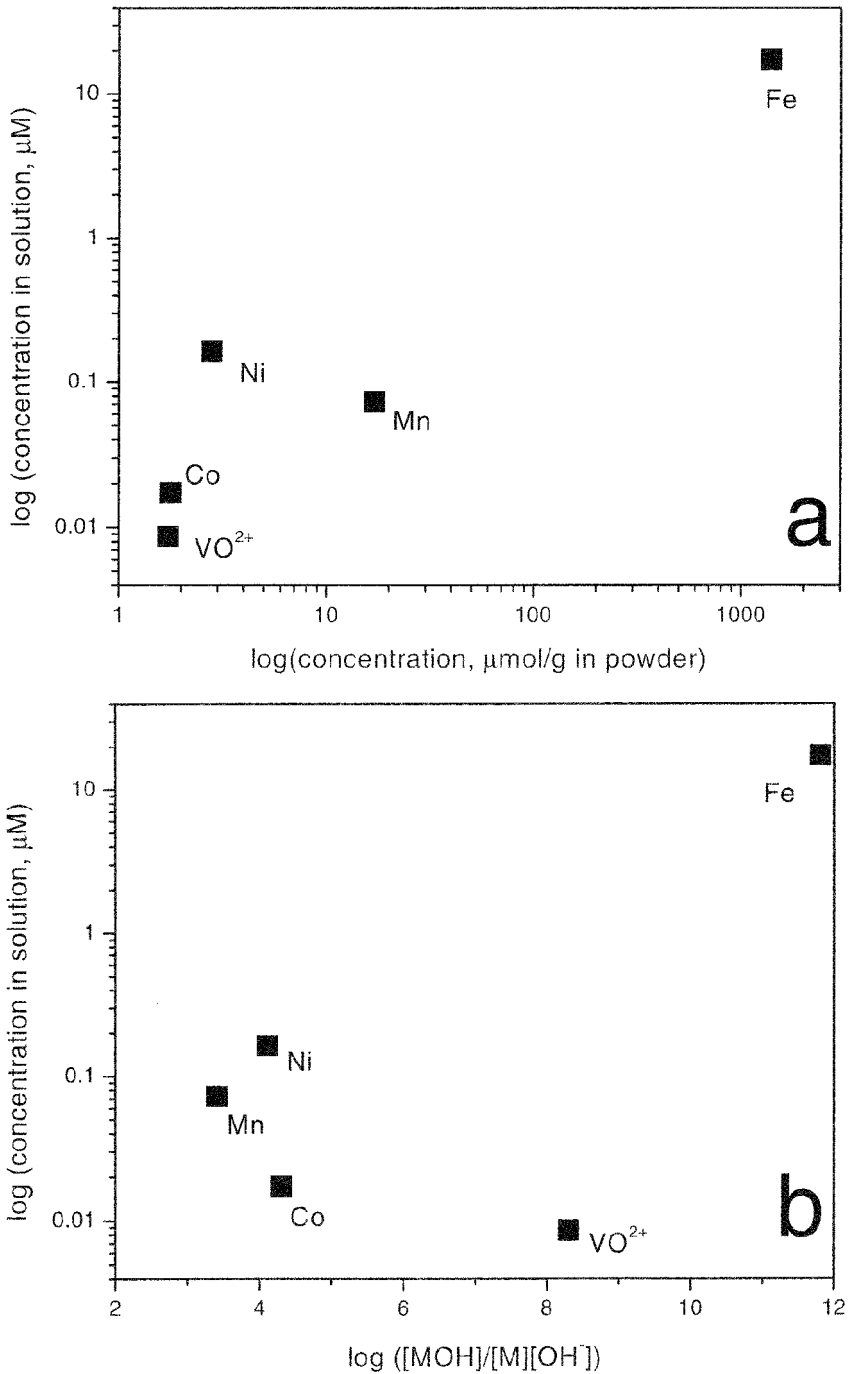
Siderophore complexation with Fe(III) will also yield protons to solution, so some of the early decrease in pH could be related to complexation reactions of the siderophore. Specifically, the *Arthrobacter* sp. isolate used here has been shown to produce a catecholate siderophore using a chemical assay (Kalinowski et al. 2000). Typical absorption maxima for catechol were also detected for the siderophore with high performance liquid chromatography using UV detection.

### **Release to Solution**

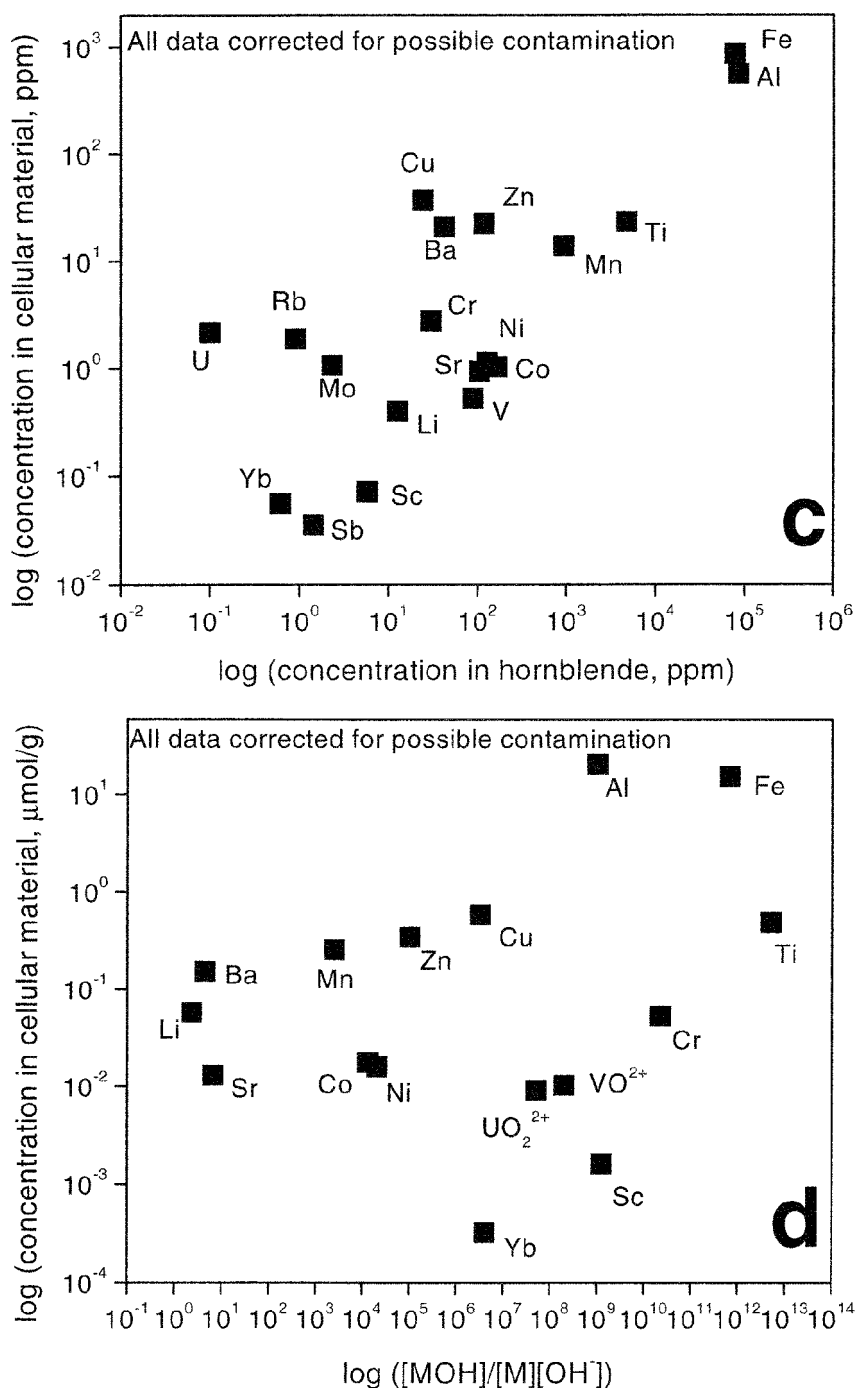
Enhanced release rates of Fe, Mn, V, Ni, and (possibly) Co in the presence of bacteria in the first half of the experiments could be related to the slightly lower pH, the chelating capacity of LMWOAs, or the presence of the catecholate in the experiments with bacteria. However, the release rates of most silicates are not pH-dependent between pH 6 and 7.5, where these experiments were run (White and Brantley 1995), so little if any enhancement in release rate would be expected. Likewise, if LMWOAs were enhancing metal release rates we would expect an enhanced release of Al into solution, which is not observed (Kalinowski et al. 2000). The best explanation for enhanced metal release is siderophore complexation at the mineral surface and release of metal-siderophore complexes to solution.

The enhanced release rates of Mn, V, Ni, and (possibly) Co from hornblende in the presence of bacteria early in the experiment suggest that the extreme specificity for Fe(III) observed for siderophores in solution does not preclude the formation of surface complexes with other metals. Kalinowski et al. (2000) observed no free catecholate present in experiments with arthrobacter and hornblende: free catecholate was documented only in hornblende-free experiments with arthrobacter. This observation indicates that the concentration of free siderophore ligand, [L], is very small in the flask experiments when hornblende is present. If the equilibrium constant is very high for association in solution, and if  $[\text{Fe(III)}] \geq [\text{L}]$ , we would expect little complexation with metals in solution other than Fe(III) at equilibrium. However, the rate of metal hydrolysis and release of metal-complex to solution from the hornblende surface could be faster than the rate of siderophore-metal exchange at surface sites, suggesting that no such equilibrium is attained at the mineral surface. Surface complexes would thus form with all metals at the silicate interface and many metals could be preferentially released into solution.

To test whether the final concentration of element in solution at 28 d was controlled by availability in the hornblende powder, the average concentration of dissolved element is plotted vs. concentration in the powder (Figure 5a). The correlation between total dissolved metal concentration and concentration in powder documents that availability of metal influenced release to solution, as expected. In contrast, dissolved concentrations of metals do not show as strong a trend as a function of the first hydrolysis constant of the metal at zero ionic strength (Figure 5b). The importance of solubilization of metals by chelates with oxygen-donating ligand groups may be more important for some metals than others (e.g., Figure 5b). Assuming that siderophore complexation accelerates release of metals from the hornblende surface, another effect probably influences the relative metal concentrations in solution.



**FIGURE 5** (a) Log (average dissolved concentration) for five elements measured at 28 days vs. concentration in hornblende powder for experiments with hornblende + bacteria. (b) Log (average dissolved concentration) vs. first hydrolysis constant of the same metals shown in (a). Note that other elements analyzed are not shown because they either showed no consistent increase with time, were not reproducible, or were not significantly greater than values for experiments with medium or medium + bacteria only. (*Continued*)



**FIGURE 5** (Continued) (c) log (average concentration) of metal in suspended cell material ( $\mu\text{g}$  element/g dry cell material) plotted vs. log (concentration,  $\mu\text{g}$  element/g powder) in hornblende. (d) log (average concentration) in cellular material ( $\mu\text{mol}$  element/g dry cell material) plotted vs. log of first hydrolysis constant of metals. Hydrolysis constant data derived from NIST (1998) for ionic strengths = 0. Concentrations in cellular material were corrected as described in text for contamination by hornblende powder.

### Bacterial Uptake

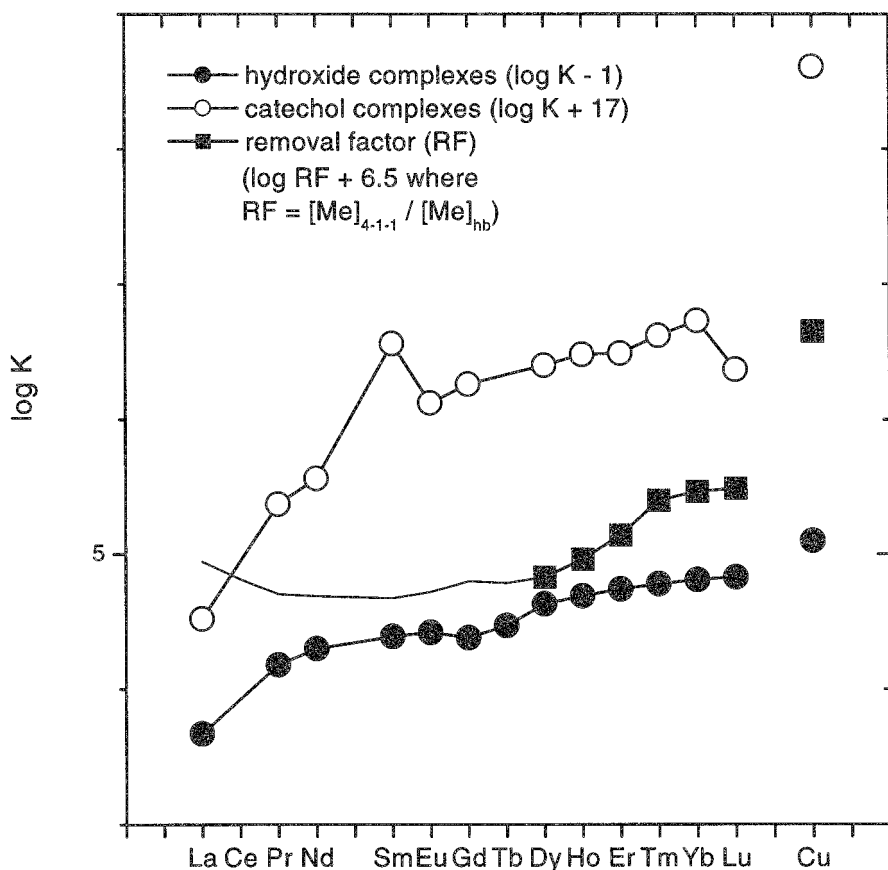
Concentrations of metals in solution could reflect the competing effects of release from the mineral surface due to siderophore complexation, and bacterial uptake. A clear increase in metal concentration in cellular material is observed with increasing metal content of the hornblende powder (Figure 5c). However, metal concentration in cells also generally increases with the first hydrolysis constant of the metal (Figure 5d). For example, the Irving-Williams series could explain some of the trends in the metal data. Specifically, although Mn (934 ppm in hornblende powder), Co (105 ppm), Ni (165 ppm), and Zn (117 ppm) are more concentrated in the powder than Cu (23.6 ppm), significantly more Cu is taken up into the bacterial cells, as predicted by the series ( $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$ ) (Figure 5d). Several authors have similarly argued that Cu can strongly bond to the Fe transport system in other organisms (e.g., Hudson and Morel 1990). Both the metal concentration in the powder (availability) and the tendency toward complexation with the siderophore (extractability) contribute to the metal uptake into the cell.

Several metals are taken up by bacteria in extreme proportion to their concentration in the hornblende powder. In particular, Cu, Mo, and U were all taken up such that more than 2% of total metal in the hornblende was extracted (Tables 1a, 1b, Figure 3). This observation is consistent with extremely high ligand-metal complexation constants, and/or dissolution of small impurity phases containing these elements. The presence of impurity phases was unavoidable given the large mass of hornblende used in these experiments. Liermann et al. (2000b) have reported the presence of pervasive chlorite, and occasional biotite, plagioclase, and garnet in this hornblende. Very small accessory phases of unidentified composition may also be present.

The most striking feature documented by the REE data in Figure 4 is the preferential removal of HREE from the hornblende. Removal of Lu, for example, is about five times as effective as removal of Dy. This relationship agrees well with the idea that relative trace element mobilization from hornblende by *Arthrobacter* sp. may be *qualitatively* described by the relationship between the elements' first hydrolysis constants. The first hydrolysis constants (ionic strength = 0) of the REE increase with increasing mass number from 5.5 for  $\log K^{\text{La}}$  to 6.6 for  $K^{\text{Yb}}$  (NIST, 1998). Note, however, that REE fractionation related to formation of organic REE complexes is complex and the approach using metal hydrolysis just indicates a general trend. Organic REE complexes with monocarboxylic acids, for example, show rather low stabilities and only minor differences along the REE series, whereas REE complexes with dicarboxylic, polycarboxylic, and aminocarboxylic acids show higher stabilities and more pronounced differences between the LREE and the HREE (e.g., Byrne and Li 1995). If siderophore-REE complexation is occurring, as inferred, we would predict that the relative increase in  $\log K$  from the LREE to the HREE would be extreme, which agrees well with the observed strong fractionation of HREE.

As an example of this stronger predicted fractionation for ligands with stronger association constants, the relative extraction of Cu and Yb can be compared. The  $\log K$  for hydroxide-metal complexation is close to about 6 for both Cu and Yb suggesting that Cu-Yb fractionation due to metal hydrolysis in inorganic systems should be minor (Figure 5d). In contrast, catechol complexes of Cu are significantly more stable than those of Yb:  $\log K_{\text{catechol}} = -8.39$  for Cu and  $\log K = -10.27$  for Yb (NIST 1998). The latter suggests major Cu-Yb fractionation and preferential Cu mobilization in situations where Cu and Yb behavior is affected by organic (catechol) complexation compared to inorganic systems. The extreme fractionation manifested in Figure 5c is consistent with complexation by an extremely strong ligand.

The relative trend in metal content of bacteria at 28 d (on a mol/g basis) increased in the order, Cd (present at concentrations so low that data are not reported) < Ni < Mn < Zn < Cu < Al ~ Fe (Figure 5d). This trend shows some similarity to trends for trace metal



**FIGURE 6** Stability of hydroxide and catechol complexes (data from NIST, 1998) and removal factor; (note that  $RF^{La}$  to  $RF^{Tb}$  may be affected by contamination) for REE and Cu. Removal factors are normalized to that of Lu. Although catechol and hydroxide complexation may both cause Dy-Yb fractionation, only catechol complexation also fractionates Cu and Yb, consistent with mobilization of elements by siderophore complexation.

concentrations reported for marine plankton, although such concentrations are extremely variable (Brumsack 1986). For example, trends reported for metal content of marine phytoplankton as summarized by Stumm and Morgan (1996) include:  $Cd < Ni \approx Cu \approx Mn < Zn < Fe < Al$  (Martin et al. 1976);  $Cu \approx Ni < Cd < Zn < Mn < Al \approx Fe$  (Martin and Knauer 1973);  $Cd \approx Cu < Ni < Zn < Mn < Al < Fe$  (Collier and Edmond 1983). Brumsack (1986) suggests on the basis of a critical assessment of these and other data that  $Cd < Ni < Cu \ll Zn$  for marine plankton. For all of these measurements, separation of inorganic particulates from cells may be a problem. Similar to our conclusions here, for marine plankton, metal concentrations are thought to be a function of both the extent to which an element is involved in biological cycling and the concentration in the seawater (Brumsack 1986).

### *Mechanism of Bacterial Uptake*

Beveridge and Fyfe (1985) summarized investigations of metal uptake by living cells grown in metal salt solutions, and emphasized that the anionic cell walls of bacteria have a great

ability to fix metals and to cause nucleation and growth of minerals. In particular, gram-positive bacteria such as the arthrobacter studied here have a great ability to sequester metals in their cell wall. In this sequestration, Beveridge and Fyfe (1985) argued that the first step is complexation at anionic surface sites, and the second step is precipitation of metal phases in the cell wall. Urrutia, Kemper, Doyle, and Beveridge (1992) documented that the extent of metal uptake by the cell wall is larger for dead cells than for live cells, due to competition for sites by protons for living cells.

Besides uptake into cell walls, metal uptake can also occur into the coating of macromolecules surrounding the cell wall (glycocalyx, Maier, Pepper, and Gerba 1999). This glycocalyx can be an aggregation or rigid layer of carbohydrates or proteins that is easy (slime layer) or difficult (capsule) to wash off (Beveridge 1989a, 1989b; Maier et al. 1999). Roane and Kellogg (1996) have suggested that glycocalyx provides protection to the cell against metal toxicity by complexing metals. In the cells imaged here with Alcian Blue, stained material surrounding the cells documented acidic extracellular polysaccharides.

Metals can also be taken into the cell through the cell membrane. Several workers have documented that most trace elements display a low solubility in lipids, limiting the rate of transport across bacterial membranes. Therefore, Fe-siderophore complexes are thought to be transported across the membrane by specific membrane proteins in an ATP-requiring process (Morel and Hering 1993). Fe release from the siderophore may occur at the membrane or within the cytoplasm (Guerinot 1994). In some cases (for example, marine organisms), siderophores may not be secreted but may be permanently docked in the plasma membrane (Hudson and Morel 1990), facilitating direct uptake.

A sequence for Fe uptake into the envelope (= glycocalyx + cell wall + cell membrane) and cytoplasm of the arthrobacter can be modeled:



where  $M \equiv$  represents a metal site on the surface of the hornblende,  $L - M \equiv$  represents a siderophore surface complex on the hornblende,  $LM\text{-envelope}$  represents the metal complexation site in the envelope. The significantly enhanced rate of growth observed for *Arthrobacter* sp. grown in the presence of hornblende is consistent with Fe-limitation of bacteria growth and  $LM\text{-cytoplasm}$  denotes metal within the cytoplasm. Assuming Fe-limitation, then the transport across the membrane of Fe must be relatively fast, while the rate of release of  $M$  from hornblende to solution presumably limits the growth rate.

Because metals are released from the hornblende into solution and subsequently taken up by bacteria (Tables 1a, 1b, 3), metal concentrations in solution might be expected to increase, decrease, or remain constant with time, depending upon the relative rates of these reactions (Figure 2). Specifically, if the rate of release of  $LM$  to solution from the surface of the hornblende can be expressed,

$$\frac{\partial [LM_{(aq)}]}{\partial t} = k_1 [L - M \equiv], \quad (2)$$

where  $k_1$  is the rate constant for release of metal  $-L$  complex into solution and  $[L - M \equiv]$  is the concentration of siderophore surface complexes, and if the rate of uptake into the envelope can be expressed,

$$\frac{-\partial [LM_{(aq)}]}{\partial t} = k_2 [LM_{aq}], \quad (3)$$

**TABLE 3** Trace elements in solution<sup>1</sup>

Detection Limits <sup>2</sup> (ppb)	Medium without hornblende (ppb)				Medium with hornblende (ppb)			
	Without bacteria	% RSD <sup>3</sup>	With bacteria	% RSD <sup>3</sup>	Without bacteria	% RSD <sup>3</sup>	With bacteria	% RSD <sup>3</sup>
Co	0.047 ± 0.004	37	bd <sup>2</sup>	—	1.3	3	1.02 ± 0.09	7
Fe	20 ± 10	3	bd	—	160	3	1000 ± 200	3
Mn	1.4 ± 0.5	12	bd	—	18.5	12	4.0 ± 0.4	4
Ni	1.6 ± 1.4	19	bd	—	2.29	19	9.6 ± 7.1	12
V	0.15	bd	bd	—	bd	—	0.44 ± 0.25	47

<sup>1</sup> Average ( $\pm 1\sigma$ ) for all experiments at 28 days. Number of replicates (N): for experiments w/o hornblende (N = 2); for experiments w/ hornblende w/o bacteria (N = 1, other flasks became contaminated after day 7), for experiments w/ hornblende w/ bacteria (N = 3).

<sup>2</sup> Detection limits based upon reproducibility of element concentration in medium blanks (bd = below detection). Detection limit =  $3\sigma$ , where  $\sigma$  was the standard deviation from multiple analyses of medium alone.

<sup>3</sup> %RSD represents the percent root square deviation on multiple analyses of any one sample.

where  $k_2$  is the rate of uptake of  $LM_{(aq)}$  into the envelope, then, at steady state, the following should obtain:

$$[LM_{(aq)}] = \frac{k_1}{k_2}[L - M\equiv], \quad (4)$$

Figure 2 may document steady state or pseudo steady state for several metals (e.g. Fe, Co). If we can assume that  $[L - M\equiv] \propto$  concentration of metal in hornblende, higher dissolved Fe compared to Co (Figure 2) is consistent with higher concentrations of Fe-siderophore surface complexes, as expected for this Fe-rich but Co-poor mineral. For Mn, the rate of uptake into envelope must outpace the rate of release from the hornblende surface as the cell mass grows exponentially, causing a decrease in [Mn] between 18 and 28 days (Figure 2c). The lack of a similar decrease in [Mn] between 18 and 28 days for the abiotic experiments is consistent with fast growth of bacteria in biotic experiments. Several of the other metals similarly show some evidence for less metal dissolved in solution after 18 days in the presence of bacteria, when the cell mass was observed to increase significantly.

Significant uptake of metal-siderophore complex (Table 1a) probably explains why little enhancement in release of Al to solution was observed (Kalinowski et al. 2000). In contrast, both Liermann, Kalinowski, et al. (2000a) and Kalinowski et al. (2000) observed that Al release from hornblende in bacteria-free experiments was enhanced in the presence of a commercially available siderophore (desferrioxamine B) as compared to siderophore-free experiments. In those experiments where no bacteria were present, Al release may have been observable because no bacteria were taking up the Al-siderophore complex. Our inability to measure significant dissolved Al in solution as compared to Al release from hornblende alone may also have been due to the relatively high background level of Al in the starting medium.

The observed reproducibility of dissolved metal concentrations in aliquots of supernatant from each flask experiment after centrifugation at 28 days (Table 1), despite different dry weights of cell material per aliquot (Table 2), implies that the metal concentration on a dry weight basis ( $\mu\text{g metal/g dry cell material}$ ) was not reproducible even though the concentration of suspended metal in solution ( $\mu\text{g metal/L}$ ) was reproducible. This observation is consistent with the suspended material containing at least two components, one of which is metal-rich and is present at reproducible concentrations in suspension, and one of which is metal-poor and is present in variable concentrations. Suspended material was shown with Alcian Blue staining to contain both cells and glycocalyx. If suspended material included either variable concentrations of glycocalyx or cells, then after drying, the mass of material per 100 ml aliquot would differ. At least in runs 4-2 and 4-3, cell density was reproducible (Table 2). However, Liermann et al. (2000b) previously reported significant production of glycocalyx by this *Arthrobacter* sp., and many researchers have concluded that metals can be adsorbed in glycocalyx (e.g., Beveridge 1989a, 1989b; Roane and Kellogg 1996). Our observations are therefore inconclusive as to whether the metal is present predominantly in the glycocalyx or in cells.

## Conclusions

Analysis of trace elements released from hornblende in the presence of *Arthrobacter* sp. shows that Fe, Ni, V, Mn, and, to a lesser extent, Co are preferentially released into solution relative to bacteria-free experiments during the first  $\sim 10$  days. This enhanced release could be due to contributions from the slightly lowered pH, the chelating capability of LMWOA, or the presence of a catecholate siderophore in experiments with bacteria. However, the release rates of most silicates are not pH-dependent between pH 6 and 7, where

these experiments were run (White and Brantley 1995), and little to no enhancement in metal release rates would be predicted for the slight drops in pH observed. Likewise, if LMWOAs were enhancing metal release rates by chelation, an enhanced release of Al into solution would be expected, which was not observed (Kalinowski et al. 2000). The best explanation for enhanced metal release is siderophore complexation at the mineral surface. Such a conclusion would be consistent with a model whereby, even for conditions wherein  $[L]$  is low, surface complexes form with many metals. In contrast, in solution, when  $[L] \ll [Fe(III)]$ , only Fe(III)-siderophore complexes are typically expected to form.

The metal release to solution does not strictly follow the trend predicted by the relative ordering of equilibrium with respect to metal hydrolysis. The relative concentrations of metals in the original hornblende powder and the relative ordering of organic ligand-metal complexation both contribute to the observed metal and REE uptake into bacteria cells. For some metals (e.g., Al), uptake into cell material is high even though very little enhancement of release rate of dissolved metal is observed during dissolution. Levels of dissolved metal in solution in these experiments represent a balance between rate of release from the hornblende and rate of uptake by organisms, probably into glycolyx material. Fractionation of heavy rare earth elements taken up into cellular material is also very strong, and increases from Ho to Lu. Strong fractionation in uptake of some elements by bacteria may create biological signatures either in the mineral substrate or in any mineral precipitates associated with the cellular material.

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