Experimental Assessment of Microbial Effects on Chemical Interactions Between Seafloor Massive Sulfides and Seawater at 4 °C

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Abstract
To assess the effects of microbes on the exchange of Cu, Zn, and P between seafloor massive sulfide (SMS) deposits and seawater, we monitored the variation of the concentrations of Cu, Zn, and P in the artificial seawater of reaction systems that did or did not also include slabs and microbes originating from an SMS sample at 4 °C for 71 days. Dissolution of Cu and Zn from the slabs was observed when microbes were present or absent. Zinc from the slabs dissolved 1.4–2.3 fold more rapidly when microbes were present. In the presence of slabs and microbes, the rate of removal of P from the artificial seawater was the sum of the individual removal rates associated with the slabs and microbes. Six bacterial phylotypes including Halomonas and Marinobacter were present at the end of the experiment as shown by PCR-based analysis targeting 16S rRNA genes. These bacteria probably contribute to the release of Zn from the SMS slab and removal of P from the artificial seawater. Our results provide further insights into the role(s) of microbes on the geochemical interactions between SMS deposits and seawater.

Keywords
Bacteria • Batch experiment • Elemental cycle • Microbial dissolution • Seafloor massive sulfides
9.1 Introduction

Seafloor massive sulfide (SMS) deposits occur at and around the plate boundaries, e.g., mid-ocean ridges, back-arc basins and volcanic arcs (Hannington et al. 2011). These SMS deposits consist of relatively insoluble sulfide minerals, e.g., pyrite (FeS2), chalcopyrite (CuFeS2), sphalerite (ZnS), and galena (PbS), and also contain trace elements such as Au, Ag, Co and in some cases, Ni (Herzig and Hannington 1995). The hydrothermally active and inactive vents of chimney- and mound-shaped SMS deposits are microbe-rich environments, with microbial densities of up to 10^{10} cells (g sulfide)^{-1} (Kato et al. 2010; Schrenk et al. 2003; Suzuki et al. 2004; Takai and Horikoshi 1999), which are densities comparable with those in soils and animal bowels (Whitman et al. 1998). Dissolved H2, H2S, CH4 and Fe^{2+} supplied from hydrothermal fluids in the active sulfide vents serve as energy sources for the microbial communities. Conversely, microbial communities in inactive sulfide vents apparently use metal sulfides as energy sources (Edwards et al. 2003a). An in situ examination of SMS ores with time suggested that sulfide minerals on the seafloor are highly weathered by microbes (Edwards et al. 2003b). However, a quantitative assessment of the elements released from or adsorbed by SMS deposits mediated by microbes had not been done prior to this report.

Considering the high levels of heavy metals in the SMS deposits and the ubiquity of these deposits on the seafloor (Hannington et al. 2011; Herzig and Hannington 1995), an experimental study of the change with time of the SMS components via microbial activity is important to understand how such events impact the oceanic biogeochemical cycles. The concentrations of certain heavy metals, e.g., Fe, Zn, Mn, Cu and Ni, which are essential for oceanic microbial viability, are extremely small (<0.1 μM) in the ocean (Morel and Price 2003; Sohrin and Bruland 2011). The exchange of these heavy metals between seawater and SMS deposits is potentially needed for the maintenance of important oceanic microbial ecosystems.

Here, we report the effects of microbes on the exchanges of elements between SMS samples and seawater by simulating the environmental conditions of deep seafloor (i.e., low temperature, weakly alkaline pH and a small amount of organic carbon). The goals of this study were (i) to measure the kinetics of the dissolution of certain metals and P from a recovered SMS sample and (ii) to assess if and how microbial communities affect the kinetics.

9.2 Materials and Methods

9.2.1 Sample Collection

A portion of massive sulfide ore (sample ID, D903-R1) was collected from a hydrothermal vent field, called the Archaean site (12°56.35′N, 143°38.0′E; depth, 3,076 m), in the Southern Mariana Trough during the YK05-09 cruise (July to August 2005) by the manned submersible Shinkai 6500 (JAMSTEC, Japan) of the R/V Yokosuka (JAMSTEC, Japan). A bathymetric map of the vent field is shown in a previous report (Kato et al. 2010) and also in Seama et al. (Chap. 17). The SMS sample (Suppl. 9.1a) was washed with filter-sterilized seawater and crushed into fist-sized, sub-surface, non-oxidized samples, using an autoclave-sterilized hammer and chisel in a clean box on board. Some of the samples were stored at −80 °C in DNA/RNA-free plastic tubes for DNA extraction and the others were stored at 4 °C for inoculation and mineralogical studies.

9.2.2 Experimental Medium

The artificial seawater (ASW) was a modification of that described in the previous report (Jannasch et al. 1996) and contained 20.0 g NaCl; 3.0 g MgCl2·6H2O; 6.0 g MgSO4·7H2O; 1.0 g (NH4)2SO4; 0.2 g NaHCO3; 0.3 g CaCl2·2H2O; 0.5 g KCl; 0.015 g KH2PO4, 1 mL of vitamin solution (DSMZ medium 141; http://www.dsmz.de) and 1 mL of marine trace element solution (DSMZ medium 511), in 1 L of distilled water. The pH value of the ASW was 7.3 before autoclaving and 8.1 afterwards. To simulate the low concentrations of organic compounds found in deep seawater, Bacto yeast extract (BD Difco, NJ, USA) was added from an autoclave-sterilized stock solution to render the concentration to be 10 mg (corresponding to 3.3 mg of carbon) L^{-1} in the final medium as described previously (Jannasch et al. 1996).

9.2.3 Batch Experiments

A sample of the interior of the SMS was cut into small slabs (10 × 10 × 1 mm; Suppl. 9.1b) using a low speed diamond saw (Struers Minitor, Westlake, OH, USA). Each slab weighed 0.35 ± 0.05 g. The relative surface area of each slab was 5.72 ± 0.01 m^2 g^{-1}, as determined by the BET-N2 method (Seishin Enterprise Co., Ltd., Tokyo, Japan). The slabs were cleaned and sterilized by soaking them in ethanol
and acetone for 2 h each according to Edwards et al. (2000) and then dried by flushing with N₂ gas at room temperature.

Sterile 250-mL glass culture flasks, each containing 100 mL of ASW and capped with sponge plugs, were used to contain the incubations. Each flask contained one of the following systems: ASW with slabs and an inoculum; ASW with only slabs; ASW with only an inoculum; only ASW. The contents of all systems were prepared in duplicate, except for the system of only ASW. The inoculum was prepared from a slurry of the mostly oxidized surface of the SMS sample. The cell density of the slurry was 3.62 ± 0.65 × 10⁷ cells mL⁻¹ (corresponding to 2.22 ± 0.38 × 10⁶ cells (g sulfide)⁻¹) determined by fluorescence microscopy (Kato et al. 2009a). The systems containing slabs and an inoculum are denoted as MC1 and MC2; those with only slabs as C1 and C2; those with only an inoculum as M1 and M2; and the negative control, without slabs and an inoculum as N (i.e., blank test), respectively. MC1, MC2, C1 and C2 each contained 10 slabs. One milliliter of the slurry was added into each of MC1, MC2, M1 and M2. These reaction systems were incubated in a cold room at 4 °C.

An aliquot of 3 mL (1 mL for direct cell counting and 2 mL for chemical analyses) was removed from each culture with a sterile disposable syringe at 4, 42, 140, 332, 782, and 1698 h after the start of the experiment. Before sampling, each medium was gently withdrawn and returned by action 1698 h after the start of the experiment. Before sampling, each medium was gently withdrawn and returned by action 1698 h after the start of the experiment.

To determine the chemical composition of the retrieved SMS sample, 0.1 g of the slabs was pulverized and decomposed in HNO₃-HF-HClO₄ solution at 90 °C. The solution was evaporated at 90 °C. The residue was dried at 140 °C and then dissolved in 1.25 mL HCl-HNO₃ solution at 90 °C. The concentration of each element was determined by multi-channel inductively coupled plasma-optical emission spectrometry (ICP-OES) (SPS5500; SII NanoTechnology, Chiba, Japan). The chemical composition of the sample was 42.9 wt% Fe (weight per total weight); 694 ppm Zn; 594 ppm Al; 114 ppm Co; 106 ppm Cu; 97.4 ppm Mg; 72.9 ppm Ba; 46.0 ppm Ca; 32.4 ppm Cd; 30.0 ppm Ni; 23 ppm Na; 9.3 ppm V; 6.90 ppm Pb; 6.80 ppm Sr; 4.0 ppm Mn. We assumed that the remaining solid, which could not be quantified, was sulfur. The uncertainties for the ICP-OES analysis were within ±5 % for Al, Ba, Ca, Cd, Cu, Fe, Mg, Rb, Sr and Zn, and approximately ±10 % for Co, Mn, Na, Ni and V. The detection limit for the aforementioned elements was <10 ppb. Ikehata et al. (Chap. 22) characterized the mineral content of a bulk sample of this SMS of D903-R1, and showed that it was primarily composed of cryptocrystalline pyrite and marcasite with lesser amounts of sphalerite, chalcopyrite, and barite.

The pH of each system was measured with a TWIN pH meter (HORIBA, Kyoto, Japan). Concentrations of Cu, Zn and P in the ASW samples with time were measured using the ICP-OES system as described above. The detection limits were 2.25 nmol L⁻¹ for Cu, 239 nmol L⁻¹ for P, and 5.54 nmol L⁻¹ for Zn. The uncertainty for the P measurement was ±5 % and that for the Cu and Zn measurement was approximately ±10 %. We also assessed the concentrations of the other elements that had been detected in the SMS sample; however, no clear temporal trends were observed because of their low concentrations (data not shown). The concentration of each element was corrected for the decrease in the sample volume and the loss of element mass during the sampling using the following equation (Eq. 4 in Wu et al. 2007):

\[
C_{j,i} = \frac{C_{j,i}V_0 - (j-1)V_s}{V_0} + \sum_{h=1}^{j-1} Ch_{i}V_s
\]

where \(C_{j,i} \) is the measured concentration of element \(i\) in the \(j\)th sample (\(j = 1, 2, ..., 7\)), and \(C_{j,i} \) is the measured concentration. \(V_0\) is the initial volume (0.1 L), \(V_s\) is the sample volume (0.003 L), and the term \(\sum_{h=1}^{j-1} Ch_{i}V_s\) accounts for the total mass of element \(i\) extracted during the samplings.
The rate constant \( \frac{dC}{dt} \) of dissolution for each element was calculated for the 332–1,698 h period (Suppl. 9.2), a time when the pH values of the ASW were between 7.3 and 7.5 (Fig. 9.1a and Suppl. 9.3). The release or removal rate \( R \) of each element to or from the ASW, respectively, was calculated using the corresponding rate constant \( \frac{dC}{dt} \), the initial fluid volume \( V_0 \) (0.1 L), the relative surface area \( A \), the total mass of the slabs in each system \( m \), and Eq. 5 in Wu et al. (2007):

\[
R = \frac{dC}{dt} \frac{V_0}{A m}, \tag{9.2}
\]

The values for \( R \) are listed in Table 9.1.

### 9.2.5 16S rRNA Gene Clone Library Construction and Phylogenetic Analysis

The 16S rRNA gene analysis was performed as described (Kato et al. 2009a, b). Partial 16S rRNA genes in extracted genomic DNA were amplified by PCR with the prokaryote-universal primer set, Uni515F and Uni1406R (Kato et al. 2009a). The PCR products were cloned and the nucleotide sequences of randomly selected clones were determined. Nucleotide sequences were aligned using ClustalW 2.0.12 (Larkin et al. 2007). Sequences with at least 97 % similarity according to DOTUR (Schloss and Handelsman 2005) were treated as the same phylotype. Maximum-likelihood (ML) trees were constructed using PHYML (Guindon and Gascuel 2003). Bootstrap values were calculated using 100 replicates.
The initial pH values of the incubations were between 8.2 and 9.3. Results of the calculation for Zn were similar for M1 or M2 and C1 or C2 (Suppl. 9.2). The temporal changes in cell densities are shown in Fig. 9.1e, with decreases seen in the MC1, MC2, M1 and M2 systems to 10^4–10^5 cells mL^-1 at 140 h and increases up to 10^6–10^7 cells mL^-1 by 1,698 h. The cell densities in the C1, C2 and N systems could not be reliably counted by microscopy (<10^5 cells mL^-1). Cell colonies were observed on the MC1 and MC2 slabs at the end of the experiment (6.92 ± 4.55 and 5.76 ± 3.28 × 10^5 cells cm^-2, respectively; Suppl. 9.4).

16S rRNA gene clone libraries were constructed from the genomic DNA extracted from the ASW samples and SMS slabs at the end of the experiment. The Inoc library is that from microbes in the original inoculum. The MC1q, MC2q, M1q and M2q libraries are from microbes in the MC1, MC2, M1 and M2 ASW samples, respectively. The MC1cp and MC2cp libraries are from microbes in the MC1 and MC2 slabs, respectively. The total numbers of the analyzed clones are 91, 45, 46, 47, 48, 20 and 20 for Inoc, MC1q, MC1cp, MC2cp, MC2q, M1q and M2q, respectively. 16S rRNA genes were not detected by PCR from the ASW samples and SMS slabs from the C1 and C2 systems or from the ASW of the N system.

The clones in the Inoc library were affiliated with the following taxonomic groups (Fig. 9.2 and Suppl. 9.5): Actinobacteria (5.4 % of the total number of clones), Bacteroidetes (2.2 %), Chloroflexi (1.1 %), Planctomycetes (6.5 %), Alphaproteobacteria (16.3 %), Deltaproteobacteria (2.2 %), Gammaproteobacteria (63.0 %), Zetaproteobacteria (1.1 %), Verrucomicrobia (1.1 %) and Euryarchaeota (1.1 %). Many of the phylotypes in the Inoc library are similar to uncultured environmental clones recovered from the sulfide chimneys of the inactive vents (Kato et al. 2010), from the oceanic basaltic rocks (Santelli et al. 2008) and marine sediments but are distantly related to known species with <95 % of 16S rRNA gene similarity. Phylotypes (the
Fig. 9.2 Phylogenetic trees for Proteobacteria. The ML trees for (a) Gammaproteobacteria and (b) Deltaproteobacteria, Alphaproteobacteria and Zetaproteobacteria were inferred using 753 and 752 homologous positions in the alignment dataset, respectively. Mariprofundus ferrooxydans (EF493243) or Halomonas aquamarina (AJ306888) were used as the out groups for each tree (data not shown). Bootstrap values (>50%) based on 100 replicates are shown at the branch points. The scale bar represents 0.1 nucleotide substitutions per sequence position. The clone numbers are shown in bold type. The numbers that follow in brackets indicate the number of detected clones.
representative clones: inoc38 and 51) related to Mariprofundus ferrooxydans (95 % similarity) of Zetaproteobacteria and Ferrimicrobium acidiphilum (89 % similarity) of Actinobacteria, which are iron-oxidizing bacteria, were also detected in the Inoc library. In contrast to the great diversity of the microbial community in the original inoculum, only six phylotypes were detected at the end of the experiment (Table 9.2). These phylotypes were completely different from those in the Inoc library (Fig. 9.2 and Suppl. 9.5), probably because the original phylotypes had been out-competed during the incubation periods.

### 9.3.3 Microbial Effects on Chemical Interaction on Sulfide Deposits

The bacterial species, e.g., Halomonas and Marinobacter, found at the end of the experiment should be minor constituents of the microbial community of the in situ SMS deposits because they were not detected in the original inoculum. The species present at the end of the experiment were probably selected by the experimental conditions. These species may have influenced the release of Zn and removal of P. Remarkably, these species have been detected in SMS samples in various deep-sea fields (Edwards et al. 2003b; Kaye et al. 2010; Rogers et al. 2003), suggesting that they may play a role in the release of Zn and removal of P in situ.

Our results suggest that microbial action accelerated Zn dissolution from the SMS slabs. Iron-oxidizing bacteria convert Fe$^{2+}$ to Fe$^{3+}$, such that Fe$^{3+}$ can then react with sphalerite (ZnS) as oxidants to release Zn$^{2+}$ (Fowler and Crundwell 1999) as follows:

$$4\text{Fe}^{2+} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Fe}^{3+} + 2\text{H}_2\text{O} \quad (9.3)$$

$$\text{ZnS} + 2\text{Fe}^{3+} \rightarrow \text{Zn}^{2+} + 2\text{Fe}^{2+} + \text{S} \quad (9.4)$$

In fact, the SMS samples contain sphalerite (Ikehata et al. Chap. 22). The genus Marinobacter includes an iron-oxidizing bacterium that has been isolated from an SMS sample (Edwards et al. 2003c). In addition, local acidification on the sulfide slabs by microbes might also have accelerated the dissolution of Zn in our systems, similar to that of silicate dissolution by Arthrobacter sp. (Liermann et al. 2000). The genus Halomonas includes acid-producing bacteria (Sanchez-Porro et al. 2010). Our 16S rRNA gene analysis indicates that the Marinobacter sp. and Halomonas sp. were attached to the slabs. Thus, the activity of these species likely contributed to the acceleration of the Zn dissolution from the sulfide slabs. In contrast to the dissolution of Zn, we did not observe a significantly accelerated Cu dissolution from the slabs.

P was removed from the ASW when microbes and/or slabs were present. Microbes use P as a nutrient. An increase in cell numbers with time was observed in the systems to which the inoculum had been added (Fig. 9.1e). Other reports have suggested that P is removed from seawater by adsorption onto the solid surfaces of sediments and rocks (Berner 1973; Wheat et al. 1996), which is consistent with our observation that P is removed from the systems containing only slabs (Fig. 9.1e). Notably, P was removed from the ASW more rapidly when only slabs were present, than when only microbes were present (Fig. 9.1e); however, the rate of P removal from seawater on actual environments by SMS deposits and by microbes in situ should fluctuate according to the in situ physicochemical conditions and the degree of microbial activity.

### 9.3.4 Conclusion and Perspective

In the present study, we conducted batch experiments with and without the SMS samples and microbes at 4 °C and monitored certain chemical and microbial changes in the

| Sample ID | MC1cp | MC1lq | MC2cp | MC2lq | M1lq | M2lq |
|-----------|-------|-------|-------|-------|------|------|
| **Bacteroidetes** |       |       |       |       |      |      |
| Sphingobacteria |       |       |       |       |      |      |
| Algoriphagus | 1 (2.2) | 3 (6.5) | 5 (10.6) | 9 (18.8) | 1 (5.0) | 1 (5.0) |
| **Proteobacteria** |       |       |       |       |      |      |
| Alphaproteobacteria |       |       |       |       |      |      |
| Thalassospira | 2 (4.3) | 3 (6.3) |       |       |      |      |
| **Gammaproteobacteria** |       |       |       |       |      |      |
| Halomonas | 40 (88.9) | 36 (78.3) | 34 (72.3) | 34 (70.8) | 18 (90.0) | 17 (85.0) |
| Marinobacter | 2 (4.4) | 2 (4.3) | 3 (6.4) | 1 (2.1) |       |      |
| Methylphaga | 1 (2.2) | 2 (4.3) | 4 (8.5) |       | 2 (10.0) |      |
| Pseudomonas | 1 (2.2) | 1 (2.2) | 1 (2.1) | 1 (2.1) | 1 (5.0) |      |
| **Total** | 45 (100) | 46 (100) | 47 (100) | 48 (100) | 20 (100) | 20 (100) |

Numbers in parentheses are the percentage of the clones in each taxon
reaction systems for 71 days. The dissolution rate of Zn from the SMS slabs was faster when microbes were present than in the absence of microbes, suggesting that the microbes accelerated Zn dissolution. Removal of P from the ASW was observed when microbes were present or absent and slabs were present. Our results should lead to a better understanding of the roles played by SMS deposits with microbes in the oceanic biogeochemical cycles.

Between 1 and 90 million-totons of the SMS deposits are estimated to be present in each hydrothermal field (Herzig and Hannington 1995). Given that the total amount of SMS deposits on the seafloor and their relative surface areas are approximately 100 million-totons and 6 m^2 g^-1, respectively, the flux of P adsorbed onto SMS deposits can be calculated as 4 × 10^8 mol year^-1 with the use of the averaged R value from Table 9.1 for the MC systems. This value is approximately one-hundredth of the input of P yearly into the oceans from rivers (3 × 10^10 mol year^-1) (Elderfield and Schultz 1996), which suggests that adsorption of P by SMS deposits with microbes helps control the levels of oceanic P. Similar calculations suggest that the yearly release of Zn from the SMS deposits (approx. 2 × 10^8 mol year^-1) should be much smaller than that from rivers (approx. 10^10 mol year^-1) (Elderfield and Schultz 1996). Our estimates are rough approximations because the total amount of SMS deposits are probably underestimated and the necessary in situ microbial and physicochemical information has not been adequately delineated. Additional investigations are needed to more accurately calculate the fluxes of elements associated with SMS deposits.

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