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Influence of sulfur-oxidizing bacteria on the budget of sulfate in Yugama crater lake, Kusatsu-Shirane volcano, Japan

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Abstract. Sulfur-oxidizing bacteria, *Thiobacillus thiooxidans*, were found in a highly acidic ($pH = 1\sim1.5$) crater lake, Yugama, seasonally flowing streams and soil in the catchment area of the crater. *Thiobacillus ferrooxidans* was also found in some of the streams but not in the lake itself. The lake water contains aqueous carbon dioxide, hydrogen sulfide, polythionates and elemental sulfur in suspension which are the substrates for the growth of the sulfur-oxidizing bacteria as no organic compounds except for the microorganisms themselves were detected. The bacteria isolated from the Yugama water preferred polythionates in the following order: $S_4O_6^{2-} > S_5O_6^{2-} > S_6O_6^{2-}$. On the other hand, H_2S was more rapidly consumed by the bacteria than polythionates which were followed by elemental sulfur. In the case of test-tube incubation, the optimum pH of the solution for growth of the bacteria was between 1.0 and 1.5, and for cultivation in growth medium plates between 2.5 and 3.5. The bacteria hardly proliferated at pH 0.5 or below. In accordance with these characteristics of the bacteria, numbers of the bacteria in the surface Yugama crater lake water were at minimum (<a few cells/mL) in February and at maximum (10^6 cell/mL) in August. The bacterial activity changed in accordance with the surface lake water temperature, but not necessarily with the variations in H_2S and polythionates concentrations of the lake water. Based on the variation in sulfur isotope ratios of sulfate and experimentally determined rate of oxidation of H_2S in the lake water, the sulfate production rate by the bacteria in the catchment area and the lake were estimated to 9.5 and 8.4 g/m^2/day, respectively, during the period from 1988 to 1990 when the volcanic activity at Yugama was at minimum. Also stream, hydrothermal, H_2S-oxidated SO_4^{2-} inputs and outputs by seepage and precipitation have been calculated as 4.1, 32, 0.56, 36, and 1.2 ton/day, respectively.

Introduction

Some of crater lakes in the world contain quite acidic waters with pH values frequently less than 2.0 and temperatures are sometimes as high as the boiling point of water. Such environments seem to be too harsh for the existence of biomass. Many earlier studies, however, have demonstrated that in neutral or alkaline hot springs some kinds of bacteria can live and even grow in boiling water or superheated water under pressure (Bott & Brock 1969; Jørgensen et al. 1992). Also in warm (<50 °C) and acidic (pH as low as 2.5) crater lakes microbial activity has been observed (Ljunggren 1960; Ivanov & Karavaiko 1966; Satake & Saijo 1974; Schwartz & Schwartz 1979). However, Brock and Darland (1970) suggested that high acidity may add an additional environmental stress on microbial growth as high temperature does. In fact, bacterial growth has not been observed in crater lake waters with pH less than one, e.g. in Yugama crater lake, Japan (pH 0.9 in 1968, Satake & Saijo 1974) and Ruapehu Crater Lake, New Zealand (0.8 in 1991, Takano et al. 1994a).

The authors have investigated volcanic eruptions which occurred at highly acidic crater lakes from an aqueous sulfur speciation point of view (Takano & Watanuki 1990; Takano et al. 1994a). Volcanic gases are continuously injected into crater lakes from subaqueous fumaroles, whereupon sulfur-containing gases such as hydrogen sulfide and sulfur dioxide react with each other, producing elemental sulfur, polythionates and other sulfur compounds in intermediate oxidation states. Until now, the contribution of sulfur bacteria in the crater lake and its vicinity to the aqueous sulfur budget of the crater lake system has remained unclear. In this paper we have studied how much impact any sulfur bacteria would have on the budget of sulfate in the Yugama crater lake, if they were in fact present there.

Yugama crater lake setting

Yugama crater lake is one of the craters on the top of Kusatsu-Shirane volcano located 150 km northwest of Tokyo (Figure 1a). This andesitic stratovolcano has been active since the first recorded explosion in 1882 (Tsuya 1933), containing a quite acidic (pH 1–1.8) lake, Yugama. The lake is about 270 m in diameter, with a surface area of 6.7×10^4 m^2, volume of 7.2×10^5 m^3 and average depth of 15 m (Ohba et al. 1994), and has no surface overflow (Figure 1c). The most active subaqueous fumarolic vent is located at the deepest point (24 m) where a molten sulfur pool is formed depending on the activity of the fumaroles and discharges numerous sulfur spherules up to the lake surface (Takano et al. 1994b). The lake water always contains a suspension of fine particles of sulfur, mainly formed through a reaction between aqueous sulfur dioxide and hydrogen sulfide in the lake water. The temperature of the lake has always been higher than the atmospheric temperature, because of a continuous heat input from the several vents on the lake floor. The catchment area of the Yugama crater lake, 4.3×10^5 m^2 (Ohba et al. 1994), is shown in Figure 1b. There are several small inflows within the rim of the crater, the largest of which cuts through the north wall. These only flow surface water in a rainy season or during intensive rainfall.

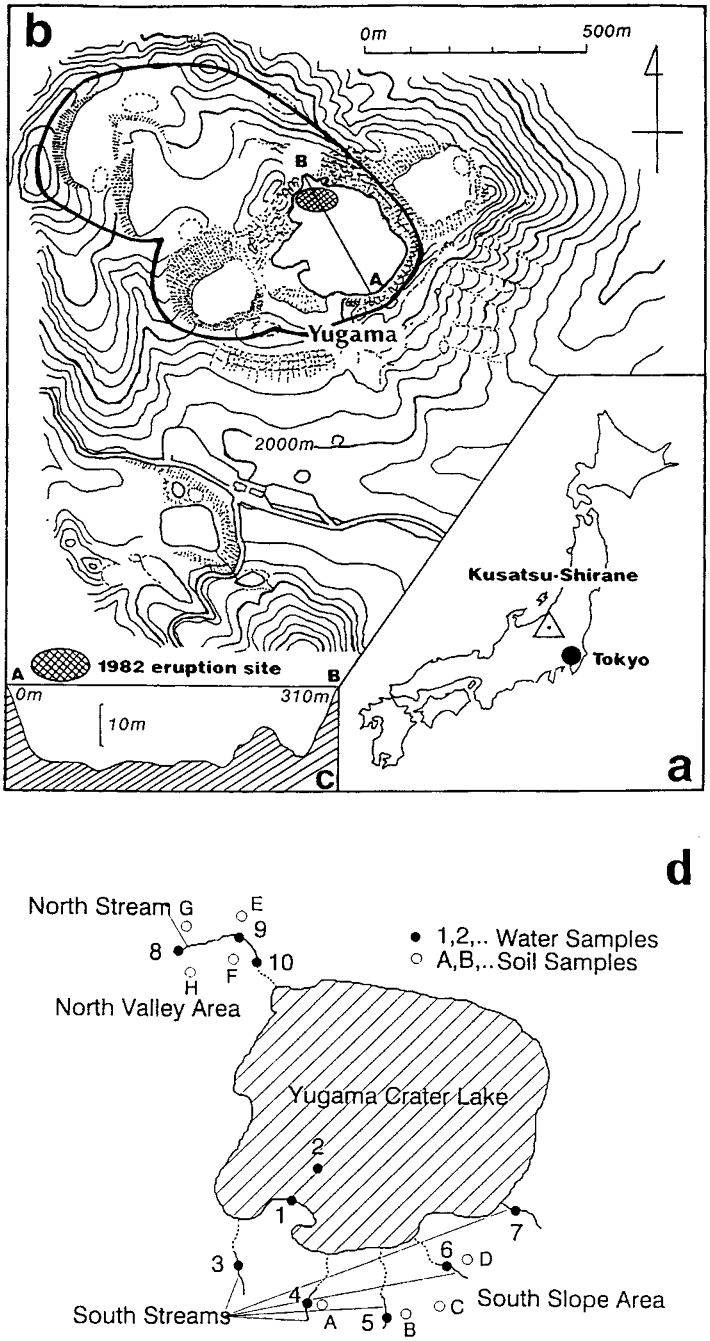

Figure 1. The location of Kusatsu-Shirane volcano (**a**), topographical map of the summital area of the volcano (**b**) and the cross section of the lake along the line A–B (**c**). A bold circle in **b** represents the catchment area of the Yugama crater lake. Filled and open circles with the numerical numbers and alphabets (**d**) are the points of sampling waters and soils, respectively.

The lake was once drained through a tunnel constructed at the south crater wall for sulfur mining (Tsuya 1933). The sulfur-poor sediments for mining were discarded on the west bank of the crater floor. In winter the lake surface is frozen, but not completely in recent years, except for the period of quiescent volcanic activity during 1988–1990. The atmospheric temperature in winter frequently falls down to -15 °C. No plants were found on the inside crater slope except at the north wall where small patches of grasses are sporadically seen.

Experimental

Sampling

The Yugama crater lake waters were sampled at the two points: Station 1 was at the lake shore (Figure 1d) for taking surface lake water; Station 2 was in the lake, about 30 m off Station 1, 8 m deep and 1.5 m above the lake floor, where the lake water was periodically pumped up to the Station 1 through Tygon® tubing (8 mm i.d. × 35 m long), using a batter-driven diaphram pump. Waters from the small streams were taken at five points in the south wall area within the crater (Figure 1d, Stations 3∼7). Also waters were sampled at three points along the north stream (Figure 1d, Stations 8∼10).

Soil samples were collected at eight points (Figure 1d, A∼H). All of them were taken from an uppermost layer of the soil. Except for Station C, each soil sample was collected at a site less than 1 m from each stream.

Chemical analysis

Hydrogen sulfide in water was determined *in situ* by the gas detector tube method (Togano & Ochiai 1987). Hydrogen sulfide and SO_2 evolved from a sample solution by adding 20% sulfuric acid were sucked into a graduated gas-detecting tube (2.5∼60 ppm H_2S in air: Gastec 4LL; 0.2∼5 ppm in air: Gastec 5Lb) in which the packed material changed color from white to brown due to the formation of PbS. The detection limit for H_2S goes down to 0.1 ppm H_2S_{aq} and 1 ppm SO_{2aq}. Elemental sulfur ($\geq$1 ppm S) was extracted from samples with spectroscopic-grade cyclohexane (Wako Chemicals) and determined by UV spectrometry at 267.5 nm (Ozawa 1966). Thiosulfate ($\geq$1 ppm) was detected by the method proposed by Ohsawa (1992). Polythionates ($\geq$0.2 ppm) were determined by microbore high performance liquid chromatography with an ODS column (Spherisorb ODS2) and a UV detector at 220 nm (Takano & Watanuki 1988). The mobile phase was 0.1 M potassium dihydrogen phosphate containing 25% acetonitrile and

0.1% tetra-n-butylammonium hydroxide and the pH of the solution adjusted to 3.5 with concentrated ($>85\%$) phosphoric acid. Sulfate (≥ 2 ppm) was determined by ion chromatography with a polystylene column (Shodex IC I-524A) and a conductometer (Shodex IC CD-2). The mobile phase solution was 3 mM phthalic acid, the pH of which was adjusted to 4.0 by adding 1% tris(hydroxymethyl)aminomethane. Iron (Fe^{2+}, $Fe^{3+} > 0.2$ ppm) was determined colorimetrically using 1,10-phenanthroline monohydrate. Hydrochloric acid was added to the water samples at the time of sampling for the determination of ferrous iron.

Bacterial strains

Thiobacillus thiooxidans JCM3867 and *Thiobacillus ferrooxidans* JCM3863 were obtained from Japan Collection of Microorganisms in the Institute of Physical and Chemical Research (RIKEN), and used in experiments of bacterial digestions of sulfur and iron compounds.

Media for growth

The compositions of media for sulfur-oxidizing bacteria are basically similar to those used by Tuovinen & Kelly (1973). The salt medium, Fe(II) solution, energy source solution except potassium tetrathionate and solidification solutions, were sterilized by autoclaving at 121 °C for 20 min, separately, and the potassium tetrathionate solution was sterilized by using a membrane filter (Millipore® HV, 0.22 μm). After sterilization, the components were immediately mixed and 15 mL of 1 L mixture was poured into a petri dish to be solidified at room temperature. The solidificant (Gelrite® and $CaCl_2 \cdot 2H_2O$) was omitted from the components for the liquid media.

Counting and isolation of bacteria in water and soil samples

Viable cell counting and isolation of bacteria in water and soil samples were performed as described:

Viable cell count in water samples: Each 0.1 mL of the solutions prepared from the water samples by a $1 \sim 10^4$ stepwise dilution were spread by using a L-formed glass stick on a separate medium. These plates were incubated in the atmosphere at 25 °C for 2 weeks, and formed colonies were counted. Numbers of viable bacterial cells in the original water samples (cell number/mL) were calculated based on their counts.

Viable cell count in soil samples: Numbers of viable bacterial cells were determined by counting colonies formed on the plate media spread 0.1 mL of the solutions prepared by shaking 1:5 (w/v) mixture of the soils and deionized water for 20 min and then filtering the solutions through Toyo Rohsi® No. 2 filter. The number of bacteria in the solutions obtained from the soil samples were calculated by counting the formed colonies as in the water samples.

Total cell count in liquid cultures: Numbers of total bacterial cells in the liquid culture was determined by the microscopic counting method using a bacterial counting chamber (depth: 0.02 mm, Erma Tokyo).

Isolation of bacteria from the Yugama lake and the area around the lake: Each 0.1 mL of the water samples was spread on the medium plate as the viable cell counts, and after incubation at 25 °C for two weeks, some of the colonies formed on the plate were separately picked up and inoculated into different test tubes with fresh liquid medium to incubate and obtain a pure culture. The pure cultures which showed stable growth after repeating transfer into a fresh medium and incubation, have been maintained in our laboratory as isolated strains.

Digestion of sulfur compounds by bacteria

Bacterial digestions of various sulfur compounds were observed for the following media:

1) Yugama and stream waters; each of sodium hydrosulfide, potassium polythionates and elemental sulfur or these mixtures were added to 10 mL of the Yugama crater lake water or the stream waters. The solutions were incubated in test tubes or hypodermic syringes (in case of H_2S) at various temperatures. Control solutions were made by filtering the sample solutions through 0.22 μm Millipore® membrane filters.
2) Soil-extracted solutions; the mixtures of soil and distilled water (1:2 w/v) were shaken and filtered through Toyo Roshi® No. 2 filters. One mL of this solution was added to 10 mL of the culture medium (KH_2PO_4, 0.12 g; $(NH_4)_2SO_4$, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; deionized water, 1000 mL) containing 0.15 g of fine grained pyrite powder or elemental sulfur as energy sources.

The effect of pH of the lake water on the activity of sulfur-oxidizing bacteria was examined by adjusting the pH of the solution to a desired value with aqueous ammonia or sulfuric acid and incubating it at 25 °C for more than a week.

Results

Chemical compositions of Yugama, stream waters, and soil extracts

The chemical composition of the Yugama crater lake varies from time to time depending upon the volcanic activity at the crater. The following is that on June 22 1985, about two years after the most recent phreatic eruptions in 1982∼83: Temperature 19.7 °C, pH 1.54, NH_4^+ 0.034 (0.0019), Na^+ 49.5 (2.15), K^+ 221 (0.53), Ca^{2+} 440 (11), Mg^{2+} 67 (2.8), Fe^{2+} 204 (3.65), Al^{3+} 219 (8.12), Cl^- 1820 (51.3), SO_4^{2-} 2450 (25.5), PO_4^{3-} 6.5 (0.068), $S_4O_6^{2-}$ 140 (0.63), $S_5O_6^{2-}$ 180 (0.70), $S_6O_6^{2-}$ 45 (0.16) (mg/L and mM in parentheses). Furthermore, dissolved O_2, H_2S and suspended elemental sulfur are 1∼9, 5∼15 and about 10 mg/L, respectively. Iron, sulfate, pH and numbers of sulfur- and iron-oxidizing bacteria in small stream waters inside the crater are listed in Table 1. Table 2 also lists concentrations of sulfate, total iron and numbers of sulfur- and iron-oxidizing bacteria in the soil extracts together with sulfur contents of the soils.

Identification of sulfur bacteria in Yugama crater lake environments

Table 3 lists the characteristics of the sulfur bacteria which were detected *in situ* in the Yugama water by a differential phase microscope. The table also lists type strains of *T. thiooxidans* JCM3867 and *T. ferrooxidans* JCM3863 for comparison with the bacteria in the lake water. The bacterium isolated from the lake water is a rod-like ($0.3 \times 1.0\ \mu$m) organism with a single flagellum and is negative in Gram staining. The optimum pH and temperature for growth are 2.5∼3.5 and 20∼25 °C, respectively. It needs free oxygen and uses elemental sulfur, thiosulfate, polythionates and less actively ferrous ions, but never consumes organic substances. Based on these results the bacteria from the Yugama crater lake water are temporarily identified as *T. thiooxidans* with infrequent cohabitation of *T. ferrooxidans*, although they are to be identified through a molecular approach.

Growth of T. thiooxidans in the Yugama water

To determine what sulfur compounds the bacteria use best for growth in the Yugama water, polythionates, hydrogen sulfide, and elemental sulfur and/or their mixtures were added to the lake water.

Polythionates: Potassium tetra-, penta-, and hexathionates were separately added to 10 mL of the Yugama water sampled on Sept. 6, 1990 and incubated at 30 °C. The rates of consumption of the thionates are in the order of

Table 1. Sulfate, iron concentrations of the stream waters and their bacterial populations.

A. South stream

Sampling point	Sampling date	Water temperature (°C)	pH	SO_4^{2-} (ppm)	ΣFe (ppm)	$Fe^{3+}/\Sigma Fe$ (%)	Sulfur-oxidizing bacteria (cells/mL)	Iron-oxidizing bacteria (cells/mL)
3	1990.8.4	16.4	2.01	4180	320	8	9.0×10^3	3.2×10^3
4	1990.8.4	18.9	2.20	4380	400	36	3.8×10^5	1.2×10^4
5	1990.8.4	21.7	2.37	3840	470	15	3.6×10^5	5.9×10^3
6	1990.8.4	22.2	2.08	7750	1360	18	6.5×10^5	2.6×10^4
7	1990.8.4	20.5	2.89	6220	860	29	2.3×10^5	3.0×10^3
3	1990.11.7	4.2	2.07	2260	130	70	7.9×10^3	–[1]
4	1990.11.7	4.5	2.38	2930	320	57	6.4×10^3	–
5	1990.11.7	4.1	2.51	3320	410	30	5.8×10^3	–
6	1990.11.7	4.5	2.08	5920	890	39	3.4×10^3	–
7	1990.11.7	2.6	2.01	11100	1830	25	1.0×10^2	–

B. North stream

Sampling point	Sampling date	Water temperature	pH	SO_4^{2-} (ppm)	ΣFe (ppm)	H_2S (ppm)	Sulfur-oxidizing bacterial	Iron-oxidizing bacteria
8	1990.7.14	17.8	3.38	500	12	38[2]	4.5×10^4	n.d.[4]
9	1990.7.14	17.7	2.75	800	28	–	1.0×10^4	n.d.
10	1990.7.14	19.1	2.70	870	43	–	8.5×10^3	n.d.
8	1990.11.8	13.3	3.92	810	–	22[3]	1.7×10^3	–
9	1990.11.8	5.6	2.98	940	18	–	2.5×10^4	–

[1] Not determined.
[2] Sampled and determined on Sept. 9, 1990.
[3] Sampled and determined on Aug. 4, 1990.
[4] Not detected.

$S_4O_6^{2-} > S_5O_6^{2-} > S_6O_6^{2-}$, which were all used up within three to four days. The population of the bacteria increases from the initial 4.1×10^5 to $(4.0{\sim}7.9) \times 10^6$ cells/mL (Figure 2).

The mixture of the three thionates were also added to the Yugama water (sampled on Aug. 4, 1990) containing initially 8.4×10^5 cells/mL of *T. thiooxidans* and incubated *in vitro* at 30 °C. The polythionate with a shorter sulfur chain is more immediately consumed by the bacteria, that is, in the same order as in the addition of a single thionate.

Hydrogen sulfide: Potassium tetrathionate plus sodium hydrogen sulfide or tetrathionate alone was added to 30 mL of the crater lake water sampled on Sept. 6, 1990 and incubated *in vitro* at 30 °C. The initial population of the

Table 2. Chemical and microbial characteristics of the soils sampled near the south and north streams.

Sampling point	Water content[1] (%)	pH[2]	SO_4^{2-}[2] (ppm)	ΣFe[2] (ppm)	Elemental sulfur (%)	Sulfur-oxidizing bacterial 7 bacteria (cells/g)[3]	Iron-oxidizing (cells/g)[3]
A	16.4	2.46	10300	840	2.0	6.0×10^2	n.d.[4]
B	18.9	2.98	1880	22	2.1	3.0×10^2	n.d.
C	21.7	2.50	41600	3800	11.3	4.5×10^3	n.d.
D	22.2	2.70	17000	340	3.7	1.1×10^4	n.d.
E	20.5	1.89	18500	1180	17.7	n.d.	n.d.
F	4.2	2.65	4260	1000	1.7	1.1×10^3	n.d.
G	4.5	2.39	7120	1870	1.4	1.3×10^4	n.d.
H	4.1	3.29	540	14	0.28	2.8×10^4	n.d.

[1] Water content was measured by loss on drying samples at 75 °C.
[2] These were measured for the solutions prepared by shaking a mixture (1:5 w/v) of the untreated soils and distilled water and then filtered through Toyo Roshi No. 2 filters.
[3] Numbers of the bacteria per 1 g of the soils.
[4] Not detected.

Table 3. Microbial characteristics of the strains isolated from the Yugama crater lake.

Strains	YT-1	YT-2	YT-3	YP-1	YP-2	YP-3	*T. thiooxidans* JCM3867	*T. ferrooxidans* JCM3863
Colony shape	s[1]	s	s	s	s	s	v[2]	i[3]
Color	w–y[4]	w–y	w–y	w–y	w–y	w–y	w–y	w–y
Size (mm)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	pin point
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Size (μm)	0.3 × 0.6	0.3 × 1.0	0.3 × 1.0	0.3 × 0.7	0.3 × 1.0	0.3 × 1.0	0.5 × 1.1–2.0	0.5 × 1.0
Flagella	+	−	−	+	+	+	−	S or Tuft
Spore	−	−	−	−	−	−	−	−
Gram staining	−	−	−	−	−	−	−	−
O_2 requirement	+	+	+	+	+	+	+	+
Temperature (°C)								
Growth range	15–30	15–30	15–30	15–30	15–35	15–30	10–37	
Growth optimum	20–25	25	25	20	25	25	28–30	15–20
pH								
Growth range	2.0–5.0	1.5–5.0	1.5–4.5	2.0–5.5	1.0–5.0	1.0–5.0	0.5–6.0	2.5–5.8
Growth optimum	3.0	2.5	3.0	3.5	2.5	2.5	2.0–3.5	2.5–5.8
Chemotrophic								
Elemental-S	+	+	+	+	+	+	+	+
Thiosulfate	+	+	+	+	+	+	+	+
Polythionate	+	+	+	+	+	+	+	+
Ferrous iron	±	−	−	±	−	−	−	+
Heterotrophic								
Yeast extract	−	−	−	−	−	−		
Casamino acid	−	−	−	−	−	−		
Sucrose	−	−	−	−	−	−	−	−(+)
Glucose	−	−	−	−	−	−	−	−(+)

[1] Smooth
[2] Variant
[3] Irregular
[4] Whitish yellow

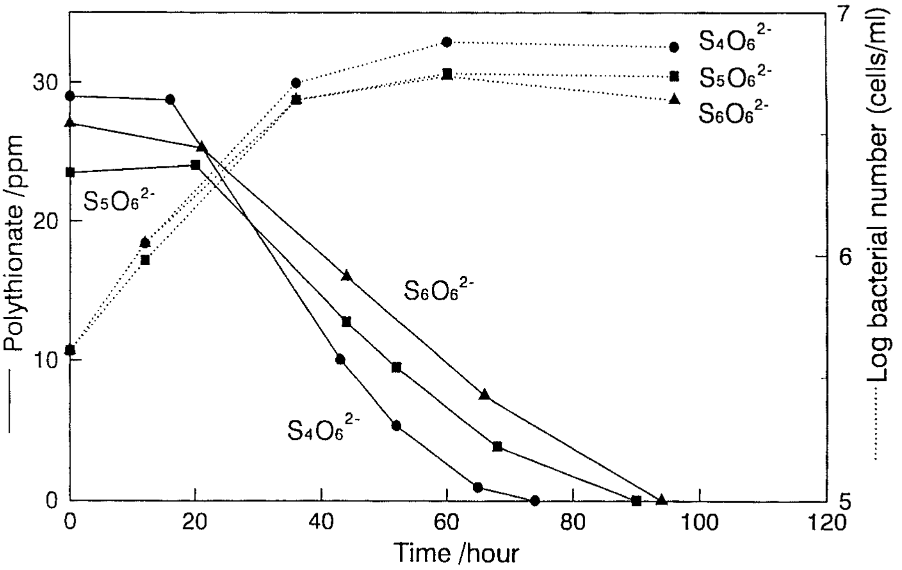

Figure 2. Three discrete bacterial digestions of potassium polythionates in the Yugama crater lake water at 30 °C. Solid and broken lines stand for the variations in polythionate concentration and population of *T. thiooxidans*, respectively.

bacteria was 4.1×10^5 cells/mL. A control solution was prepared by filtering the lake water through an 0.22 μm membrane filter. Figure 3 shows that:

a) the consumption rate of hydrogen sulfide is affected not by coexisting tetrathionate but presumably by dissolved atmospheric oxygen;

b) and the bacteria use H_2S first as an energy source, and then tetrathionate after the former was totally consumed.

Elemental sulfur: To 10 mL of the crater lake water sampled on Sept. 6, 1990, fine powder of precipitated elemental sulfur with or without potassium tetrathionate was added and the mixture was incubated *in vitro* at 30 °C. As shown in Figure 4, no noticeable difference in the consuming rate of tetrathionate was detected for both solutions. The population of the bacteria increased from 4.1×10^5 to 3.2×10^6 cells/mL. The organism showed no sign of using elemental sulfur within the three-day incubation.

Growth of T. thiooxidans *and T. ferrooxidans in inflow streams*

Bacteria in streams in the south crater wall area: Table 1A shows that the water from the small streams are strongly acidic (pH 2~2.9) and contain a great amount of sulfate ions mostly exceeding those of the Yugama water. This is also the case for the total iron concentration of the stream water. No

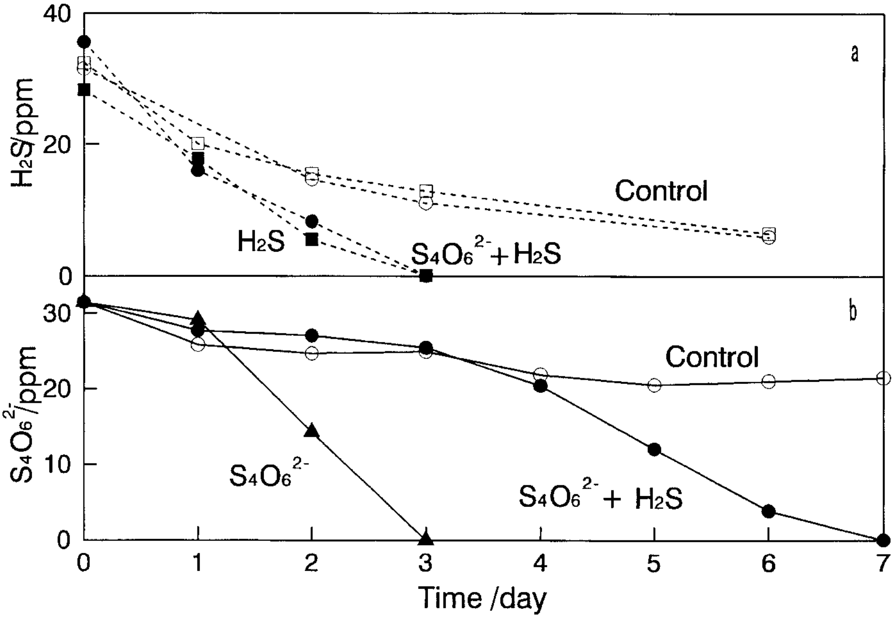

Figure 3. Bacterial digestions of hydrogen sulfide (**a**) and tetrathionate (*b*) in the Yugama crater water at 30 °C. The control solutions (open circle and square) were prepared by filtering the crater water through an 0.22 μm membrane filter and dissolving each of the following reagents: sodium hydrosulfide (solid square), potassium tetrathionate (solid triangle) or sodium hydrosulfide plus potassium tetrathionate (solid circle).

detectable amount of reduced sulfur compounds were found in the stream water. Both *T. thiooxidans* and *T. ferrooxidans* were detected for all stream waters in this area. The population of *T. thiooxidans* was always greater than that of *T. ferrooxidans* (three to almost 100 times of the latter).

The populations of *T. thiooxidans* in these streams are as much as that of the Yugama crater lake water (ca. 10^5 cells/mL in summer) but decreased to ca. 10^3 cells/mL in winter. This is also the case for the Yugama water. Non-filtered and filtered (Control) south stream waters (St. 3 and St. 5) to which ferrous ions were added were incubated *in vitro* at 25 °C. Within a week almost all ferrous ions had been oxidized to ferric whereas both control solutions changes little in ferric ion concentrations (Figure 5).

Bacteria in a stream in the north crater wall area: The water from the stream is characterized by a higher pH value, much lower sulfate and iron concentrations than those from the south streams (less than 25% and 15% of the latter, respectively). The population of the bacteria in the water are one order less than those in the south streams. Incubation of the bacteria at 35 °C using

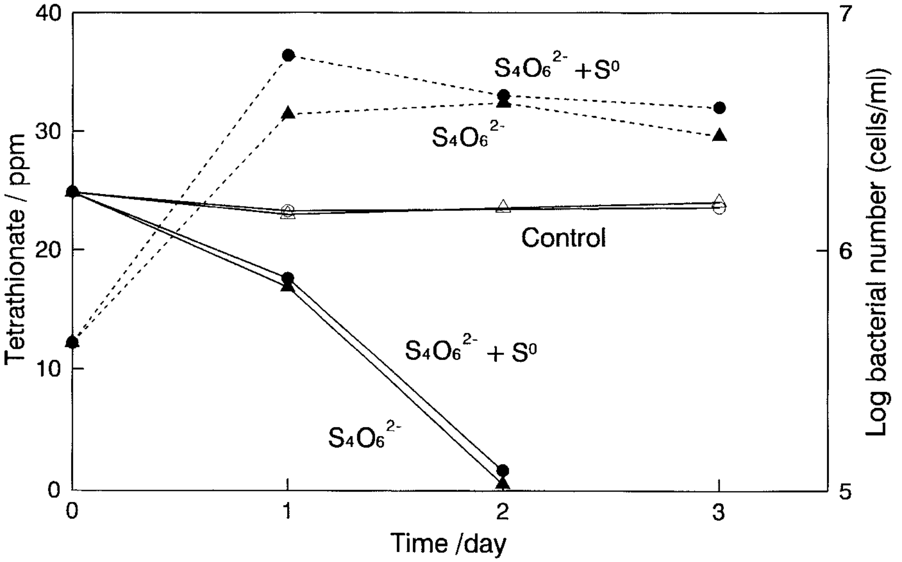

Figure 4. Bacterial digestions (solid lines) of tetrathionate in the Yugama water mixed with elemental sulfur (circle) or without it (triangle) at 30 °C. The growths of the bacteria are shown by broken lines. Solid and open symbols stand for the unfiltered and filtered solutions (control), respectively.

the stream water showed an increase in the cell number from 10^4 to ca. 10^6 cells/mL in consumption of hydrogen sulfide added to the water. No *T. ferrooxidans* but *T. thiooxidans* was detected in the water into which hydrogen sulfide gas was injected from a part of the stream floor and elemental sulfur was observed to deposit. Through a microscope a lot of the bacteria were observed around particles of the elemental sulfur.

To examine whether the bacteria can oxidize elemental sulfur, the stream water was added to the culture medium with colloidal elemental sulfur as the sole energy source. After incubation at 30 °C for a week, much sulfate ions were released into the solution (control: ca. 100 ppm; St. 8: 3700 ppm; St. 9: 3900 ppm), while the control solution (a filtered stream water) showed only a small increase in SO_4^{2-} (Figure 6). The floor of the stream assumed a dark-brown color. A microscopic inspection of the dark-brown precipitates on the floor indicates that this is *Pinnularia braunii* var. *amphicephala,* one of the acidphilic diatoms.

Bacteria in soil

To clarify the origin of sulfate and iron in the streams, the soils around the streams were extracted with deionized water. Table 2 shows that almost all

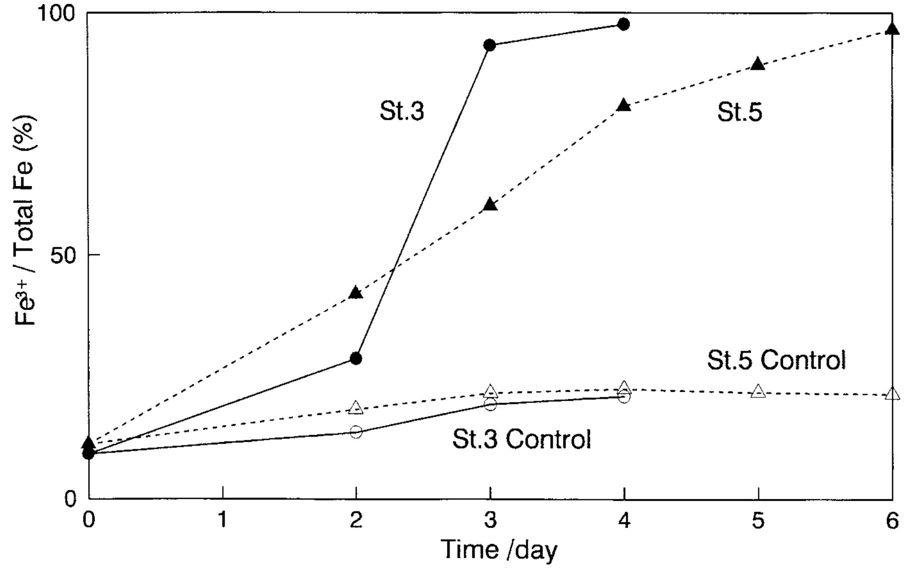

Figure 5. Bacterial oxidation of ferrous iron in the South Stream waters. St. 3 and St. 5 represent the waters sampled at Station 3 and Station 5, respectively. The initial numbers of the bacteria for the stations are 3.2×10^3 and 5.9×10^3 cells/mL, respectively and were incubated in a test tube at 25 °C. Controls were made by filtering the waters through an 0.22 μm membrane filter.

the extracted solutions were quite acidic (pH = 1.8∼3.3) and contain high concentrations of SO_4^{2-} (540∼41600 ppm) and iron (14∼3800 ppm). Dried soils contain elemental sulfur of 0.28∼17.7% (ave. 5%). No iron-oxidizing bacteria were found in the soil-extracted solution, while the bacteria oxidizing tetrathionate (3×10^2∼2.8×10^4 cells/g) were also detected from all soils except that from Station E (Figure 7a).

Apart from elemental sulfur, pyrite in the soil is also a probable energy source both for sulfur-oxidizing and iron-oxidizing bacteria. The soil-extracted solutions were added to the growth media with fine-grained pyrite as the sole energy source. For comparison bacteria in the Yugama crater lake water and the stream waters in the south area were incubated using the growth media at 25 °C for 75 days. Figure 7b shows that only solutions containing the stream waters exhibited a remarkable increase in aqueous sulfate and iron.

Factors controlling activity of bacteria in Yugama crater region

The influence of temperature and pH on bacterial activity in the crater lake was examined. Tetra-, penta- and hexathionates were separately added as the sole energy source to the lake water containing the sulfur-oxidizing bacteria

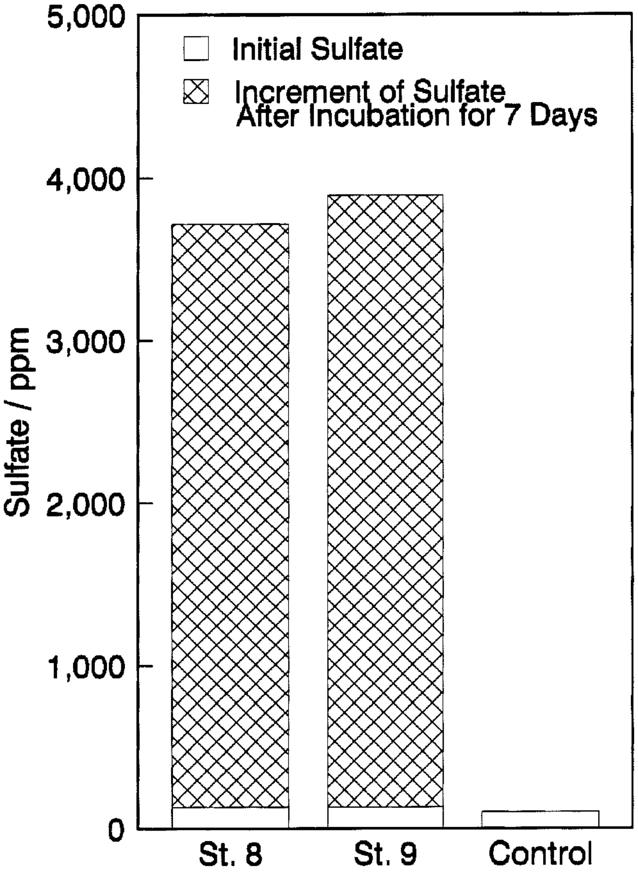

Figure 6. Production of sulfate from elemental sulfur by the bacteria in the North Stream waters sampled at Station 8 and Station 9. Open and hatched bars represent initial sulfate and increment of sulfate produced by bacteria, respectively. The bacteria were incubated at 30 °C in a test tube for 7 days. Experimental conditions are described in **Experimental**.

which were incubated *in vitro* at 2, 15 and 25 °C. At 2 °C no tetrathionate was used even after a month. The bacteria incubated at 15 °C took about twice as much time as those at 25 °C to completely consume the thionate. When the bacteria were incubated at 35 °C, they took only a half of the time for the incubation at 25 °C to completely consume tetrathionate. Thus the rate of polythionate consumption by the bacteria becomes about twice at an every 10 °C temperature increase.

At pH = 0.5 no change in the concentration of tetrathionate was observed even after a month. Tetrathionate, however, was totally consumed by the bacteria in a higher pH (1, 1.5, 2 and 2.5) solution. The detectable consumption started earliest at pH = 1.5 and last at pH = 1.0.

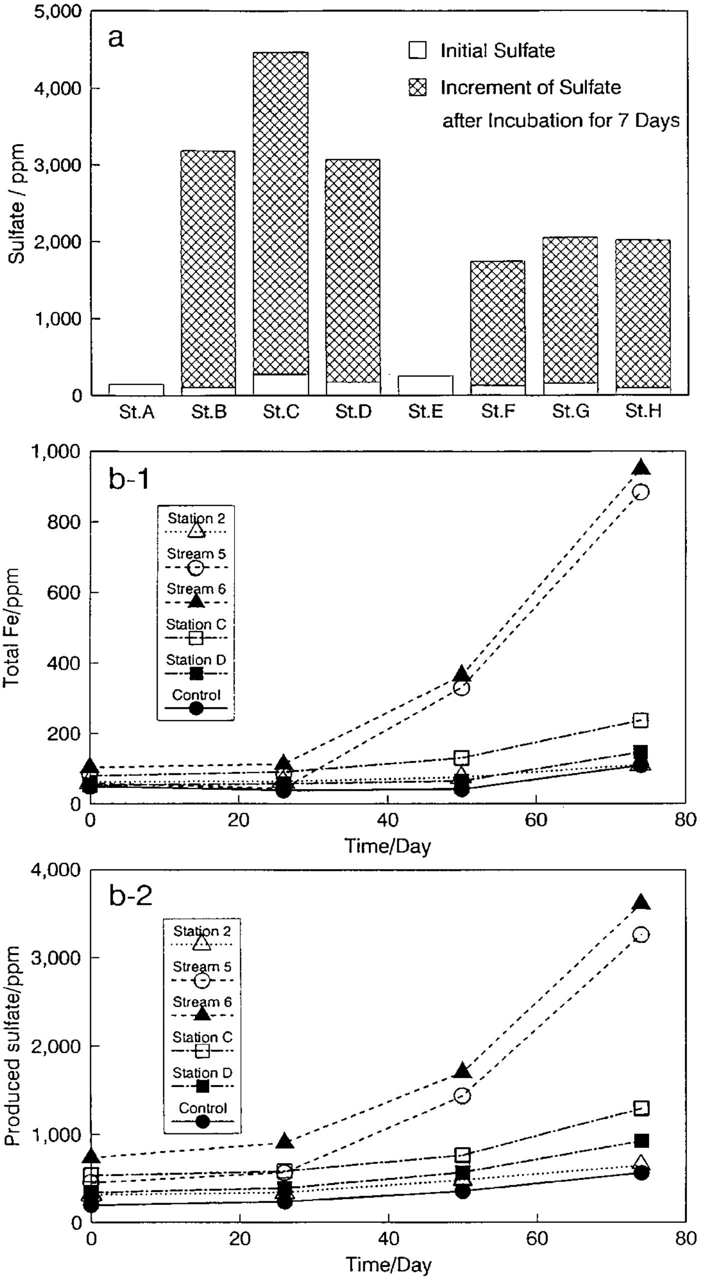

Figure 7. **a**. Production of sulfate from elemental sulfur at 30 °C in the salt media (10 mL) to which 1.0 mL of each soil extract was added (see Experimental). St. A~St. H stand for the location of the soil samples. **b**. Bacterial digestion of pyrite at 25 °C. Total iron (**b-1**) and sulfate (**b-2**) released through incubation of the culture media mixed with the lake water (Station 2), South Stream waters (Station 5; Station 6), the soil extracts (Station C; Station D) and Control solutions (culture media with no sample solutions).

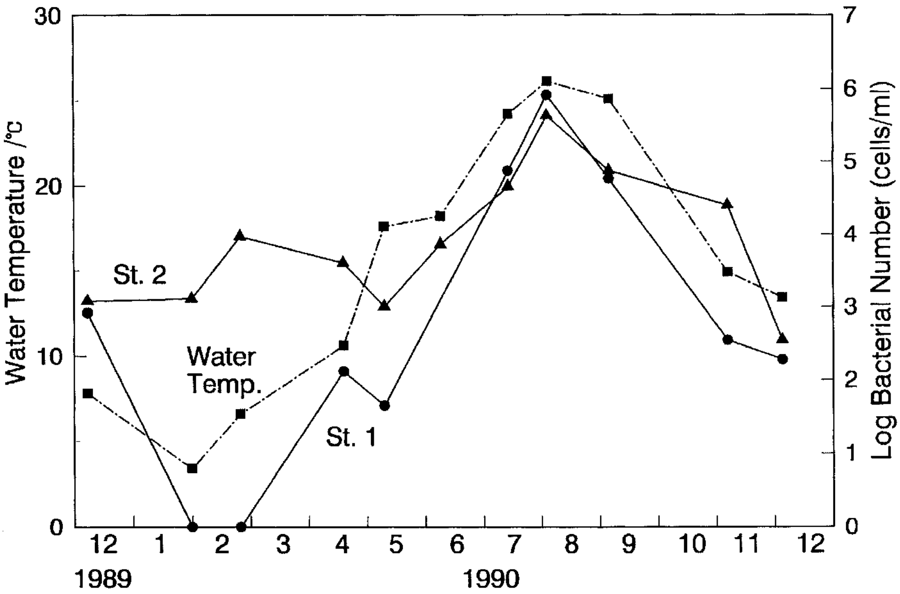

Figure 8. Seasonal variations in the bacterial population at Stations 1 and 2 and temperature of the surface lake water at Station 1.

Seasonal variation in bacterial activity at Yugama crater lake

The population of bacteria in the lake water is closely correlated with the lake temperature, especially that of the surface lake water (St. 1) than that of the water sampled at the depth of 8 m from the surface (St. 2) (Figure 8). No bacteria were detected in the surface water in January and February, but $10^3 \sim 10^4$ cells/mL of the bacteria were found at St. 2 during the same period. The maximum population (ca. 10^6 cells/mL) was observed in August and the minimum in February at both stations.

Discussion

Sulfur compounds and nutrient substances in Yugama water

The lake water dissolves or suspends reduced sulfur compounds such as H_2S, SO_2, $S_xO_6^{2-}$ and S° whose concentrations vary depending on the activity of the subaqueous fumaroles (Takano & Watanuki 1990). Polythionates are formed through the reactions between hydrogen sulfide and sulfur dioxide in the water (Takano 1987) and one of such reactions is:

$$H_2S + 3SO_2 = S_4O_6^{2-} + 2H^+ \qquad (1)$$

Polythionates ($S_xO_6^{2-}$: x = 4∼15) were detected in the Yugama water (Takano 1990). Another reduced sulfur compound which is likely to exist in the water is thiosulfate formed through the following reactions:

$$S_xO_6^{2-} + HSO_3^- \rightleftarrows S_{x-1}O_6^{2-} + S_2O_3^{2-} + H^+ \tag{2}$$

or

$$S^\circ(\text{liquid}) + HSO_3^- \rightarrow S_2O_3^{2-} + H^+ \tag{3}$$

However, $S_2O_3^{2-}$ was not detected, probably due to low (<1 ppm) HSO_3^- in the water (Ohsawa 1992), although Webster (1989) detected more than 10 ppm of $S_2O_3^{2-}$ in Ruapehu Crater Lake, New Zealand where much more SO_2 was often detected (ca. 10 ppm, Takano et al. 1994a).

Substantial nutrients for the bacteria, phosphorus in PO_4^{3-} and nitrogen in NH_4^+, are 2.1 and 0.026 mg/L, respectively. Phosphorus is 100 times as concentrated, and nitrogen is almost the same level as those in average Japanese river waters (Kobayashi 1960). Heavy metals such as zinc, copper and manganese some of which are indispensable or hazardous for fauna are 0.11, 0.03 and 1.0 mg/L, respectively (S.M. Fazlullin, pers. comm.). Those heavy metal concentrations of the Yugama water are less than those of the Maly Semiachik crater lake water in which *T. thiooxidans* was detected (B. Takano, unpublished data). Thus from the nutrient and energy source point of view, Yugama crater lake during 1988∼90 was an appropriate environment for the growth of *T. thiooxidans*.

Distribution of sulfur-oxidizing bacteria in the Yugama crater lake environment

The Yugama crater lake environment is divided into three categories from the bacterial habitation point of view: Yugama crater lake, inflows and soil on the gentle slope of the inner crater wall.

Yugama crater lake: The pH of the water used to be 1∼1.2 before the phreatic eruptions in 1982∼83. During the eruption much rock and mud were thrown into the lake basin from the vents at the north fringe of the crater lake (Figure 1b). This event caused a gradual increase in pH up to 1.8 in 1983, and since then the value has slowly been decreasing to 1.2 in 1995. The pH range Yugama has experienced for these 10 years since 1982 has been appropriate for *T. thiooxidans* to proliferate (Table 3). The isolated strains from six points (Figure 1d) at Yugama can not grow in the media whose pH is adjusted to 0.5, although the type strain can grow even at this pH (Table 3).

There are several strongly acidic crater lakes in the world, such as Ijen in Indonesia (pH 0.2 in 1993; Delmelle & Bernard 1994). Poás in Costa Rica (pH

<0 in 1987∼1990; Rowe et al. 1992a) and Ruapehu in New Zealand (pH 0.8; Takano et al. 1994a). In these three lake waters no bacterial activity has been observed. The crater lake water at Volcano Maly Semiachik in Kamchatka showed the pH value of 0.95∼1.0 at 9 °C in 1992 when only small numbers of *T. thiooxidans* (48 cells/mL) were detected near the subaqueous fumarolic vent. On the other hand, even at lower water temperature (8 °C), 10^2∼10^4 cells/mL of the bacteria were detected in the whole area of the lake in 1993 when the pH of the water increased to 1.1∼1.2. These observations strongly indicate that the lower limit in pH for *T. thiooxidans* to grow is 1.0 in crater lake environments.

The optimum temperature for growth of *T. thiooxidans* is 30∼35 °C (Table 3). The water temperature of Yugama (0∼25 °C) has always been higher than atmospheric temperature (−15∼20 °C). At the end of the last century the temperature of Yugama was almost boiling (98 °C, Ohashi 1919). Since then no such high water temperature was recorded except for the time of 1982∼83 eruptions when a high surface temperature (56 °C) was observed down to the depth of 1.3 m for only three days (Shimoya 1985). Thus except in these periods of abnormally high water temperature and low temperature in winter (0∼10 °C), *T. thiooxidans* grows in the lake. A trace activity of *T. ferrooxidans* observed for the water may be due to an inflow of the bacteria from the streams to the lake. They can not grow in the lake water because the pH is less than 2 (Table 3).

Among the reduced sulfur compounds in the water, hydrogen sulfide is most preferentially consumed by the bacteria, polythionates the second, and elemental sulfur the last (Figures 3, 4). Of the three polythionates, tetrathionate is most preferred, then pentathionate and finally hexathionate. This order in bacterial consumption of the sulfur compounds is not parallel with oxidation states of sulfur of the compounds, but seems to be related to the molecular sizes of these compounds. According to Meyer (1976), the smallest molecular diameters of H_2S, $S_4O_6^{2-}$ and cyclic S_8 are 1.8, 1.9 and 3.5 Å, respectively. The smallest aqueous H_2S molecule may most easily pass through microbial cell walls. Polythionates have almost the same diameters, but they are chain-like molecules which are more than 5 Å long. Cyclic S_8 molecules are crown-like, usually hydrophobic, and much more chemically inactive than the others, although Steudel et al. (1987) observed that the surface of elemental sulfur occurring in polythionate formation adsorbs long-chain polythionate molecules. This may result in a stable dispersion of hydrophobic sulfur molecules in the lake water. The bacteria in this case may use the long-chain polythionates in preference to the elemental sulfur. From the molecular-size point of view, thiosulfate and sulfite ions may be less preferred by the bacteria than hydrogen sulfide but more than polythionates. Matsumoto & Watanuki (1992) pointed out that the lake is quite poor in organic material except a trace amount of microorganisms themselves. Therefore no heterotrophic organisms may live or grow in this environment, even though the activity of uncultured chemotrophic microorganism(s) could not be ruled out from the organic geochemical point of view.

Stream water: Small streams within the Yugama crater wall are usually seen to have running water only after rainfall or in rainy seasons. The waters in these streams are strongly acidic mainly due to sulfuric acid, but contain only traces of chloride (a few tens ppm of Cl^-), compared with Yugama ($Cl^- > 1000$ ppm). This may indicate that the lake sediments which were accumulated in the south and south-west area within the crater during sulfur-mining and frequent phreatic explosions have been washed out with rain and/or snow-melt, resulting in less chloride ions in the stream water. Iron in these waters is much more concentrated than in Yugama.

The origins of the high sulfate and iron concentrations of these stream waters can be explained by microbial activity. *T. thiooxidans* and *T. ferrooxidans* were detected in these waters (Table 1). Both organisms are known to coexist in sulfur-rich environments (Wakao et al. 1982) and to consume elemental sulfur and sulfide as energy sources. The soils around Yugama are rich in reduced sulfur compounds such as elemental sulfur and iron sulfide which came from the lake in the frequent explosions (10 times since 1882, Tsuya 1933) and sedimentary-sulfur mining during the period from 1880s to 1930s. Thus the bacteria utilize these sulfur compounds to produce sulfuric acid,

$$2S^\circ + 3O_2 + 2H_2O \xrightarrow[T.ferrooxidans]{T.thiooxidans} 2H_2SO_4 \tag{4}$$

$$2FeS_2 + 7.5O_2 + H_2O \xrightarrow{T.ferrooxidans} Fe_2(SO_4)_3 + H_2SO_4 \tag{5}$$

together with some inorganic reactions such as (Garrels & Thompson 1960; Wiesma & Rimstidt 1984; McKibben & Barnes 1986);

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2S^\circ. \tag{6}$$

Sulfur in pyrite cannot be directly oxidized to sulfate by *T. thiooxidans* without the cooperation by *T. ferrooxidans* (Bosecker 1984). According to Konno et al. (1991) and Sasaki (1994), *T. ferrooxidans* preferentially extracts iron from pyrite, resulting in surface excess sulfur which is oxidized to sulfate by *T. thiooxidans*. The ferrous and ferric ions thus produced from pyrite in the stream sediments may come into the stream water together with sulfuric acid produced in the soils. The isotope ratios of sulfate-sulfur (δ^{34}S) in the stream waters, however, are $-0.5\sim+2.0$‰ which are similar to those ($-5\sim-2$‰) of the suspended elementary sulfur in the lake water (Sakai 1957), but much different from that of the lake sulfate ($+20.1$ and $+22.4$‰ before 1980 and $+14.3$‰ in 1990 (Ohsawa et al. 1993; see the section below). Because sedimentary sulfides normally have a δ^{34}S value somewhere between -30 and -10‰ (Hoefs 1973) although some sulfides show rather heavy δ-values ($-16.6\sim+3.0$‰; Kaplan et al. 1963), the sulfate in the stream water should mainly come from the elementary sulfur derived from the lake floor to the soil and the source of iron therein may be altered volcanic rocks.

In the north stream water no *T. ferrooxidans* was detected. Table 1 indicates that the water dissolves only a few tens ppm of Fe but relatively higher H_2S, compared with the iron (from a few hundreds up to 2000 ppm Fe) and H_2S (null) in the south stream waters. High H_2S and probably CO_2 gases supplied from the vents on the stream floor and less Fe may be the reason for no *T. ferrooxidans* but as many *T. thiooxidans* as in south stream water. The watershed area of the north stream is an old shallow crater whose floor level is about 80 m higher than the Yugama lake surface. This area is not thickly covered with the lake sediment thrown out during explosions from Yugama or the tailings of sulfur mining. Thus the soil in this area is poor in elemental sulfur.

Soils: The inner crater slope of Yugama is covered with hydrothermally-altered projectiles of two-pyroxene andesite (Tsuya 1933) and grey clayey soils. These soils are well exposed near around the streams. Contents of elemental sulfur in the soils range from 0.3 to 18% (av. 5.0%). The bacteria separated from the soils consume elemental sulfur but hardly use pyrite for growth in the culture media (Figure 7). This fact indicates that *T. thiooxidans* but no *T. ferrooxidans* are active in the soil environments. Why is *T. ferrooxidans* inactive in the soils? The soil-extracted solutions were strongly acidic (pH $1.9\sim3.3$, av. 2.6; Table 2), indicating that pore water of the soils is much more acidic. Because *T. thiooxidans* is inactive at the pH-value less than one (Brock & Darland 1970), the pH of the pore water may be slightly over one which is still too acidic for *T. ferrooxidans* to grow. This strong acidity also explains the high concentrations of iron in the stream waters (Table 2).

Production of ^{34}S-poor sulfate and mass balance

Stable isotope balances have long been used either by themselves or in conjunction with solute balances to estimate surface and ground-water inflow or outflow from a lake as well as evaporation from the lake surface (cf. Pearson & Coplen 1978). As described before *T. thiooxidans* in the Yugama environment oxidizes elemental sulfur in the soil and hydrogen sulfide in the stream and lake water. The processes have been making the sulfate in the lake ^{34}S-poor (Ohsawa et al. 1993). This can, however, be observable only when the influx of heavy sulfate from the subaqueous vents is weak, compared with the input of the isotopically-light sulfate. The period from June 1988 to June 1990 is a time of relatively stable lake level, constant chemical composition, regular rain precipitation and water temperature (cf. Ohba et al. 1994). Therefore the Yugama crater lake during this period is an appropriate system to which a one box model can be applied for evaluating the production rate of ^{34}S-poor sulfate in the Yugama catchment area. According to Pearson & Coplen (1978), the rate of change in sulfur isotope ratio for a lake can be written:

$$\Delta(V\delta_L)/\Delta t = R_C I_C \delta_C + R_T I_T \delta_T - R_O I_O \delta_O - R_M V \delta_M + R_B V \delta_B \quad (7)$$

where *V* is the total lake volume and δ_X is the isotopic concentration of sulfate sulfur in the reservoir or a yearly flux *I of X* (=*L, C, T, O, M* and *B* which represent lake, total *c*old surface-, ground-water inflow, *t*hermal water inflow from the lake floor, *o*utflow by seepage, precipitation with *m*inerals and *b*acterial oxidation of H_2S in the lake in the unit of ton/y, respectively). R_X is an annual relative concentration factor of SO_4^{2-} for each flux I_X when R_L is unity/y.

When the total water volume of the lake *V* can be assumed constant,

$$I_C + I_T + I_R = I_E + I_O, \quad (8)$$

where I_R and I_E are influxes of rain and evaporation on and from the lake surface in ton/y, respectively. As the surface area of the lake is one fifth of the catchment area of Yugama, we can assume,

$$I_R = 1/5 I_C \quad (9)$$

Evaporation of water from the lake surface is estimated as:

$$I_E = 365 \times 24 \times 60 \times 66000 \times 10^{-6} \times 3.72 \times 10^{4-} t^3 \quad (10)$$

where *t* is the average lake water temperature (12 °C) (Sukawa 1960). This value is almost equal to:

$$I_E = 0.031V \quad (11)$$

The lake level observations from 1988 to 1993 by Ohba et al. (1994) indicate that the level can be regarded as being constant within the fluctuation of at most 1 m. Therefore

$$C_T I_T = C_O I_O \quad (12)$$

On the other hand, annual change in SO_4^{2-} concentration (S) of the lake water (ΔS_L) is expressed by S_X(X = C, T, O, B, M in g/L/y) as:

$$\Delta S_L = (S_C I_C + S_T I_T - S_O I_O)/V + S_B - S_M \tag{13}$$

The δ^{34}S value of the lake sulfate (δ_L) before 1980 was almost constant at the level of +20.2‰ and then has been decreasing to: +17.2, +15.4 and +14.3‰ on June 22, 1988, May 27, 1989 and June 6, 1990, respectively; the δ^{34}S value of the stream sulfate (δ_C) is +0.87‰ (Ohsawa et al. 1993). The rate of decrease in the δ^{34}S ($\Delta\delta_L/\Delta t$) has seemingly become constant during the period from 1988 to 1990 and is estimated to be −1.5‰/y. The δ_B is set to be −4.0‰ (Sakai 1957). The one-box model allows us to assume that R_L = $R_O \cong 1$ y^{-1}, $\delta_T \cong +20.2$‰ and $\delta_O = \delta_M = +14.3$‰. Then eq. 7 can be rewritten for the lake situation in 1990 as:

$$-1.5 = 0.87 \times R_C I_C/V + 20.2 \times R_T I_T/V - \\ 4.0 \times R_B - 14.3 \times (R_M + I_O/V) \tag{14}$$

where $V = 7.2 \times 10^5$ m^3 (Ohba et al. 1994). R_C (=1.76 y^{-1}) is obtained from S_C (av. 3.70 g/L) and S_L (av. 2.10 g/L). R_T is estimated to be 0.783 (=2.03 × 0.81/2.1) y^{-1}, assuming that S_T and the SO_4/Cl ratio of input thermal solution are equal to the Cl concentration of the thermal solution discharged from a newly-opened vent in the 1982 eruption (C_T = 2.03 g/L) and to the lowest ratio before the eruption (=0.81, Takano & Watanuki 1990), respectively. C_O (=1.87 g/L) is the average chloride concentration of the lake during 1988∼90. S_B (=0.286 g/L) and R_B = 0.136 y^{-1} are from the experimental rate of hydrogen sulfide oxidation by *T. thiooxidans* (206 ton/y, Figure 3), taking the bacterial population (av. 3.16×10^4 cells/mL), water temperature (av. 12 °C) and the total amount of sulfate in the lake (1.51×10^3 ton) into consideration. $S_O = S_L$ and ΔS_L are assumed to be 2.10 g/L (av. lake sulfate concentration) and −0.270 g/L/y (cf. Ohba et al. 1994), respectively. Thus the total surface- and ground-water influx I_C responsible for conveying bacterially-produced ^{34}S-poor sulfate to the lake is calculated to be 4.0×10^5 m^3/y or 1.10×10^3 m^3/day. Thus the production rate of sulfate by the bacteria in the catchment area (4.3×10^5 m^2) is $I_C \times 3700$ g/day/4.3×10^5 m^2 or 9.5 g/m^2/day. Substituting the values for the corresponding terms in the preceeding equations, we obtain the following values for:

$$I_X(X = C, T, O, E, R) = 1.10 \times 10^3, 1.58 \times 10^4, 1.72 \times 10^4, \\ 6.1 \times 10, 2.2 \times 10^2 \text{ m}^3/\text{day};$$

$$S_M V = 1.2 \times 10^3 \text{ kg/day}, \quad R_M = S_M/S_L = 0.29.$$

The water fluxes I_T, and I_O, are comparable with those at Poás Volcano at its lowest volcanic activity during 1984∼86 (Rowe et al. 1992b), although they are one order of magnitude larger than those for Yugama given by Ohba et al. (1994). The other fluxes cannot be directly compared with the values by both research groups, because our definition of these fluxes are different from theirs.

The bacterial sulfate production rate of the lake is ($206 \times 10^6/365/67000 = 8.4$ g/m^2/day). This value is comparable with those for the rate of sulfuric acid formation in Yellowstone National Park (7.5∼15.1, av. 11.1 g/m^2/day; Schoen 1969). However, the value calculated by Schoen (1969) for the Oyunuma crater lake, Japan (Murozumi et al. 1966) is 75 g/m^2/day which is 8.9 times larger than that for the Yugama crater lake. Both the pH (=3) and the water temperature (42 °C) at Oyunuma are considerably higher than those at Yugama (pH 1.5, 25 °C); therefore these water conditions at Oyunuma are much more favorable for bacterial and inorganic oxidation of hydrogen sulfide than at Yugama as shown by Brock & Darland (1970).

The rate for the catchment area (9.5 g/m^2/day) is also about the same as those for the Yugama crater lake and Yellowstone National Park. The area studied by Schoen (1969) and Yugama crater lake have many subaerial or subaqueous hot springs and fumaroles and are hydrothermally more active than the Yugama catchment area where no fumarolic activity is observed. Considering the higher pH of the inlet stream waters (2.0∼2.9; Table 1) which much influences the rate of bacterial sulfate as described before, the value estimated for the Yugama catchment area seems to be reasonable as a first approximation. The sulfur movement in Yugama is schematically illustrated in Figure 9 which also shows the estimated fluxes of sulfate in the lake environment.

Conclusions

The sulfur-oxidizing bacteria *T. thiooxidans* are living in the crater lake Yugama but no *T. ferrooxidans* is there although the lake water contains much ferrous iron. *T. thiooxidans* digests sulfur compounds in the following order: hydrogen sulfide>polythionates>elemental sulfur and takes polythionates stepwise as an energy source in the order of S-chain length: $S_4O_6^{2-} > S_5O_6^{2-} > S_6O_6^{2-}$. On the other hand both *T. thiooxidans* and *T. ferrooxidans* were found in all but one small stream inside the crater. These streams appear during occasional rains and always in the rainy season, and are highly acidic with isotopically-light S sulfate. In the soil around the lake only *T. thiooxidans* was detected. Elemental sulfur in the soil is used by the sulfur-oxidizing bacteria, resulting in the production of sulfate approximately at

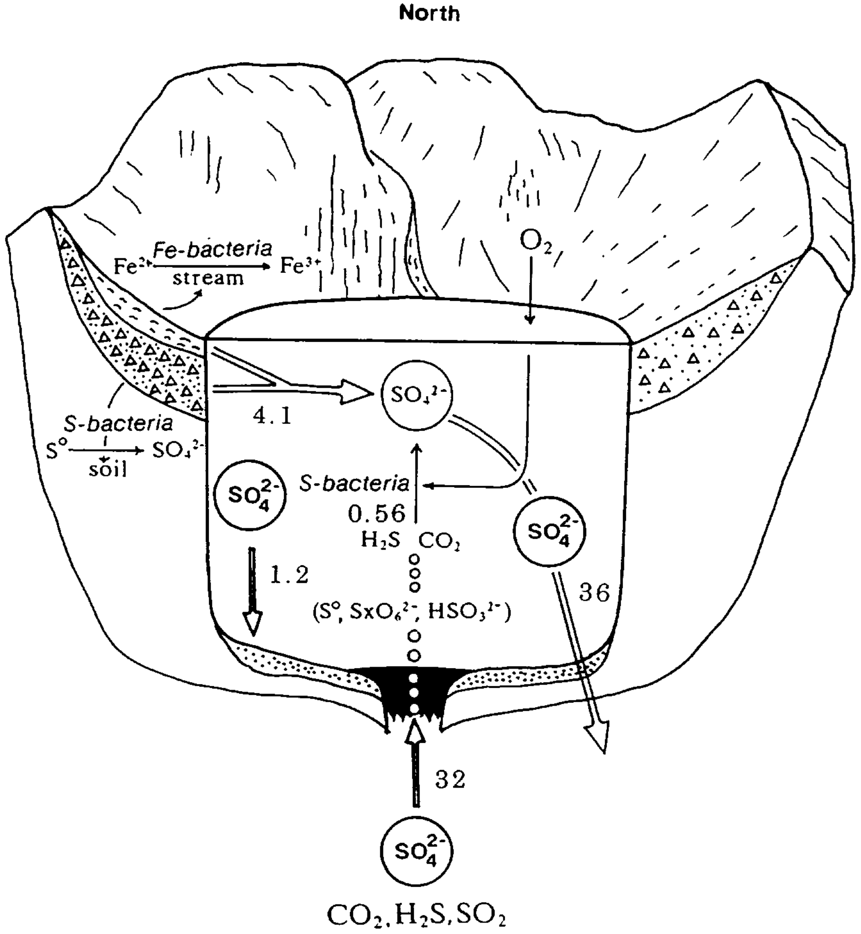

Figure 9. Schematic diagram of the sulfate budget in the Yugama crater lake environments. The sulfates encircled by thin and thick circles in the lake represent isotopically-light and heavy S sulfate, respectively. Bold numbers beside the arrows show the fluxes of sulfates (ton/day) from various origins (see the text). The black part at the lake bottom stands for liquid sulfur pools.

the rate of 9.5 g/m^2/day. This value was estimated from the rate of decrease in the δ^{34}S and concentration of sulfate in the lake water. Also hydrogen sulfide in the lake water is oxidized to sulfate by *T. thiooxidans* at the rate of approximately 8.4 g/m^2/day which was estimated from the laboratory rate of hydrogen sulfide oxidation by the bacteria incubated in the lake water. Activity of the sulfur-oxidizing bacteria in the lake is highest at pH = 1.5 and almost stops at pH < 1.0. Volcanic activity at the lake has been weakest during 1988∼1990 from the polythionate monitoring point of view, resulting in the highest pH (1.8) since 1960s. Thus the production of sulfate by the bacteria caused an observable decrease in the sulfur isotope ratio of the lake sulfate. Recently the acidity, SO_4^{2-} concentration and its δ^{34}S values of the lake water have been increasing, suggesting that the contribution of bacterial production of sulfate to the sulfate budget of the lake is becoming negligible. Because *T. thiooxidans* much prefer hydrogen sulfide to polythionates, monitoring of volcanic activity in terms of polythionates advocated by one of the present authors (B. Takano) is not significantly affected by the bacteria in the lake.

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