

## Observation of microbial mats in radioactive hot springs

Kazue TAZAKI

*Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan*

*E-mail: Kazue Tazaki: kazuet@kenroku.kanazawa-u.ac.jp*

*Phone: +81-76-264-6512*

*Fax: +81-76-264-6545*

**Abstract :** Microbial mats can accumulate heavy metals and radio nuclides through precipitation and complexation on and within the cell surface containing hydroxyl and carboxyl groups. Processes involving bacterial oxidation-reduction will alter the mobility of the heavy metal pollutants in water. The microbial immobilization of radio nuclides and other metals was compared for the high temperature and strongly acidic conditions of Tamagawa Hot Springs in Akita Prefecture and the low temperature and neutral pH conditions of Misasa Hot Springs in Tottori Prefecture, Japan. Both hot springs produced microbial mats indicating the Ra and Rn radioactivity was higher than that of the surrounding water. Here, bacterial biomineralization in the radioactive hot springs was described by nanometer-resolution observations using electron microscopic techniques. The analytical results showed the bacterial immobilization of radio nuclides and other metals. The reddish-brown microbial mats at Tamagawa Hot Springs had radioactivity of 5700 cps and were composed of  $\text{Ca}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{HSO}_4^-$  and  $\text{SO}_4^{2-}$ , which had formed sulfur-bearing compounds of barite ( $\text{BaSO}_4$ ), elemental sulfur and As-S elements. Bacterial cells were entirely encrusted with spherical grains having diameters of 100–200 nm, suggesting that heavy metals were transported from strongly acidic hot spring waters (pH of 1.2) to sediments. On the other hand, the green microbial mats at Misasa Hot Springs were characterized by a neutral pH of 7.2 and a temperature of 42°C. The radioactive microbial mats (11.9  $\mu\text{Sv/h}$ ) were mainly composed of cyanobacteria of *Oscillatoria* spp. and *Phormidium* spp., which are associated with minerals of ferrihydrite, Mn oxides and calcite. The  $^{226}\text{Ra}$  content of the microbial mats was  $6.9 \times 10^4 \text{ Bq/kg}$ , which was higher than that of the hot spring water ( $1.4 \times 10^{-1} \text{ Bq/kg}$ ). In addition, in reddish-brown microbial mats, coccus and bacillus type bacteria associated with extracellular polymers around the cell. The results suggest that the microorganisms in the microbial mats grow and decay with a metabolic reaction, and then accumulate heavy metals. It is possible that the capability of radioactive immobilization can be used to counteract the disastrous effects of radionuclide-polluted water and sediments.

**Keywords:** Microbial immobilization, radionuclide, cyanobacteria, hot springs, Ra, sulfur-bearing compounds, capsule

## Introduction

Microbial mats can accumulate heavy metals and radio nuclides through precipitation and complexation on and within the cell surface containing hydroxyl and carboxyl groups. Processes involving bacterial oxidation-reduction will alter the mobility of the heavy metal pollutants in water. The interaction between water, microorganisms, and radioactive materials on the Earth's surface is of great importance because large amounts of radioactive material could be released by microbial activity (Macfarlane and Miller 2007; Stroes-Gascoyne 2007). U-, Ra-, and Rn- biotic interactions could play important roles in radionuclide cycling, involving microorganisms in soils, ground water, and organic matter (Beveridge et al. 1983; Haas et al. 1998; Suzuki et al. 2003; Tazaki et al. 2003). Filamentous fungi, yeasts, algae, and bacteria have been evaluated for U biosorption (Lovley 1995). Rn converted from Ra is a radioactive gaseous element that mainly emits  $\alpha$ -rays. Although Rn inhalation has been thought to be hazardous in general, springs containing Rn have been reported to have therapeutic effects on senile brain disorders and hypertension. Another known effect of Rn springs is the promotion of the effects of tissue perfusion agents such as adrenaline in plasma (Yamaoka et al. 2004).

Tamagawa and Misasa Hot Springs in Japan are well known for producing Ra, Rn, and radioactive minerals such as hokutolite (plumbian barite). However, only a few studies have been conducted on Ra- and Rn-bearing microbial mats with nanometer-resolution observations in the context of environmental remediation. Hokutolite has a chemical composition characterized as (Ba, Pb)SO<sub>4</sub> and has high radioactivity owing to the presence of Ra isotopes and their daughters. Recently, studies on the interaction by which living microorganisms bind metals have been carried out at Tamagawa Hot Springs (Tazaki and Watanabe, 2004). It is probable that the presence of Th in hokutolite is due to the co-precipitation with (Ba, Pb)SO<sub>4</sub> from the hot spring water. The Ra–Rn hot spring environments, which support a wide diversity of microbial life, are poorly understood.

In this study, more-detailed studies on the radioactive microbial mats at Tamagawa and Misasa Hot Springs in Japan using electron microscopic methods are discussed. Emphasis is given to the radiochemistry of the microbial mats through the determination of  $\gamma$ -rays. The elemental composition and distribution of radioactive microbial mats are also examined using scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray fluorescence (XRF) spectroscopy, X-ray powder diffraction (XRD) analysis, and Fourier transform infrared (FT-IR) absorbance spectroscopy, and are used to elucidate nanometer-scale relationships among microorganisms and bio-precipitated materials at the two hot springs. Ra and Rn as well as other metals found with minerals on indigenous bacterial cell walls are described.

## Samples and Methods

Measurements of water pH, oxidation-reduction potential (ORP), electrical conductivity (EC), water temperature, and dissolved oxygen (DO) content were carried out

in the field. The hot spring water measurements were taken using portable inspection meters (Horiba and Toa Co.) in the months of December for 2002, 2003, 2004, and 2006. Radioactivity was measured using a  $\beta$ -ray Geiger–Muller survey meter and a Geiger counter ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -rays) (Aloka Company TGS-136), and the radio activities of air, spring water, microbial mats and rocks were compared.

In this study, reddish-brown microbial mats at Tamagawa Hot Springs and both green and reddish-brown microbial mats at Misasa Hot Springs were collected for detailed observation by electron microscopy.

#### *XRD analysis*

The mineralogical characteristics of microbial mats collected from Tamagawa and Misasa Hot Springs were analyzed using XRD (Rigaku Rinto 1200) with a Cu-K $\alpha$  generator at 40 kV and 30 mA for identification of minerals and the crystalline state of the products during biomineralization.

#### *Optical and fluorescence microscopy*

The microbial mats were observed with an optical microscopy to recognize living microorganisms and minerals. Wet samples were placed on a glass slide and stained with 4',6-diamidino-2-phenylindole (DAPI, 50  $\mu$ g/ml). DAPI-stained DNA in bacteria fluoresced a blue color that could be seen under ultraviolet rays (wavelength of  $\sim$ 365 nm). In contrast, minerals with DAPI staining fluoresced yellow.

#### *Energy dispersive X-ray fluorescence (ED-XRF) spectroscopy*

Chemical compositions of the microbial mats collected from Tamagawa Hot Springs and Misasa Hot Springs were determined by ED-XRF analysis using a JEOL JSX-3201 (Rh-K $\alpha$ ) instrument, which was operated at an accelerating voltage of 30 kV under vacuum conditions.

#### *FT-IR absorbance spectroscopy*

The microbial mats at Misasa Hot Springs were examined by FT-IR absorbance spectroscopy (Jasco FT-IR-610, MICRO-20) to evaluate the chemical bonding of organics and inorganics. A small quantity of microbial mats was suspended in distilled water. A drop of the suspension was mounted on a fluorite disk (CaF<sub>2</sub>, 0.5 mm thick) for FT-IR analysis. After air-drying, the cells were selected under the IR microscope showing organic chemical bonding.

#### *Scanning electron microscopy-energy dispersive X-ray (SEM–EDX) spectroscopy*

The microbial mats were fixed with glutaraldehyde solution (2.5% final concentration) for 1 hour. After a sample was washed and substituted with *t*-butyl alcohol, it was frozen in liquid nitrogen and dried using a freeze-drying apparatus. The freeze-dried sample was mounted onto copper stubs with double-sided adhesive carbon tape for SEM

(JEOL JSM-5200 LV) at an accelerating voltage of 15 kV. The scanning electron microscope was equipped with an EDX spectrometer (Philips EDAX PV 9800) and the chemical composition of the microbial mats was analyzed. Bacteria (*Oscillatoria* spp. and *Phormidium* spp.) were identified using Bergey's Manual of Determinative Bacteriology (Ninth Edition).

#### *Electron microscopic techniques*

Wet samples of microbial mats mounted on a micro-grid were observed with a transmission electron microscope (JEOL TEM- 2000-EX). After air-drying, the samples were observed without coating at an accelerating voltage of 160 kV in the bright-field mode. Energy-filtering transmission electron microscopy (EF-TEM) and scanning transmission electron microscopy-energy dispersive X-ray (STEM-EDX) spectroscopy were used for nm scale observations. Complimentary techniques of EF-TEM (JEOL JEM-2010 FEF) using a field emission gun, an in-column energy filter, an EDX spectrometer (JEOL JED-2300), and the scanning transmission mode of an advanced scanning imaging device were carried out for semi-quantitative analyses of the microbial mats at an accelerating voltage of 200 kV. STEM-EDX was also used for the point analysis of chemical compositions of nanometer-order.

### **Results**

The study areas of Tamagawa Hot Springs in Akita Prefecture and Misasa Hot Springs in Tottori Prefecture are on the island of Honshu in Japan (Fig. 1).

#### *Tamagawa Hot Springs*

Tamagawa Hot Springs is a potentially useful area for the study of radioactivity in hydrothermal environments, and the site has the highest production of hot spring water (9,000 L/min) in Japan. The characteristics of the hot spring water were strong acidity with a pH of 1.2, high temperature of 99.5°C, ORP of 140 mV, EC of 29 mS/cm, a DO concentration of 0.8 mg/L, and high  $\beta$ -ray counts. The  $\beta$ -ray counts of reddish-orange microbial mats (5700 cps) were 2–100 times those of the hot spring water (50–90 cps), sediments (1850–2420 cps), hokutolite (1800–3200 cps) and air (80–100 cpm) on 22 October 2003.

The aqueous chemistry of hot spring water was mainly HCl associated with high concentrations of  $\text{Ca}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{HSO}_4^-$ , and  $\text{SO}_4^{2-}$  (Tazaki and Watanabe 2004). Using the Geiger–Muller counter, the reddish-brown microbial mats were found to have radioactivity of 5000 cpm whereas the background in the air had radioactivity of 100 cpm (Fig. 2 A, B). The  $\beta$ -ray counts of the microbial mats were 50–100 times those of the hot spring water and the air. The area used to produce the radioactive mineral hokutolite.

Small cubic hokutolite, 10  $\mu\text{m}$  in diameter, was found in the reddish-brown microbial mats under SEM–EDX observation. The hokutolite was rich in S and Ba and had a small amount of Pb (as indicated in Fig. 2 C). The XRD analysis of the microbial mats identified



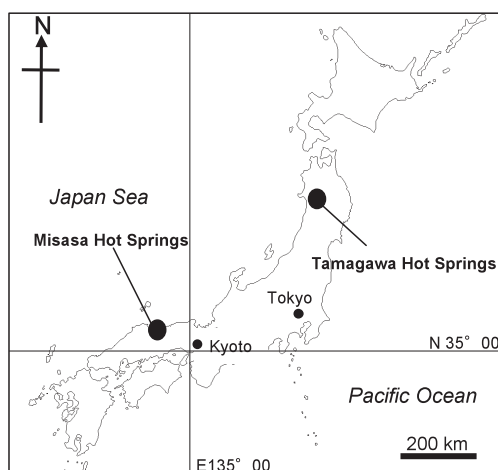


Fig. 1. Locality map of sampling places at Tamagawa and Misasa Hot Springs in Japan.

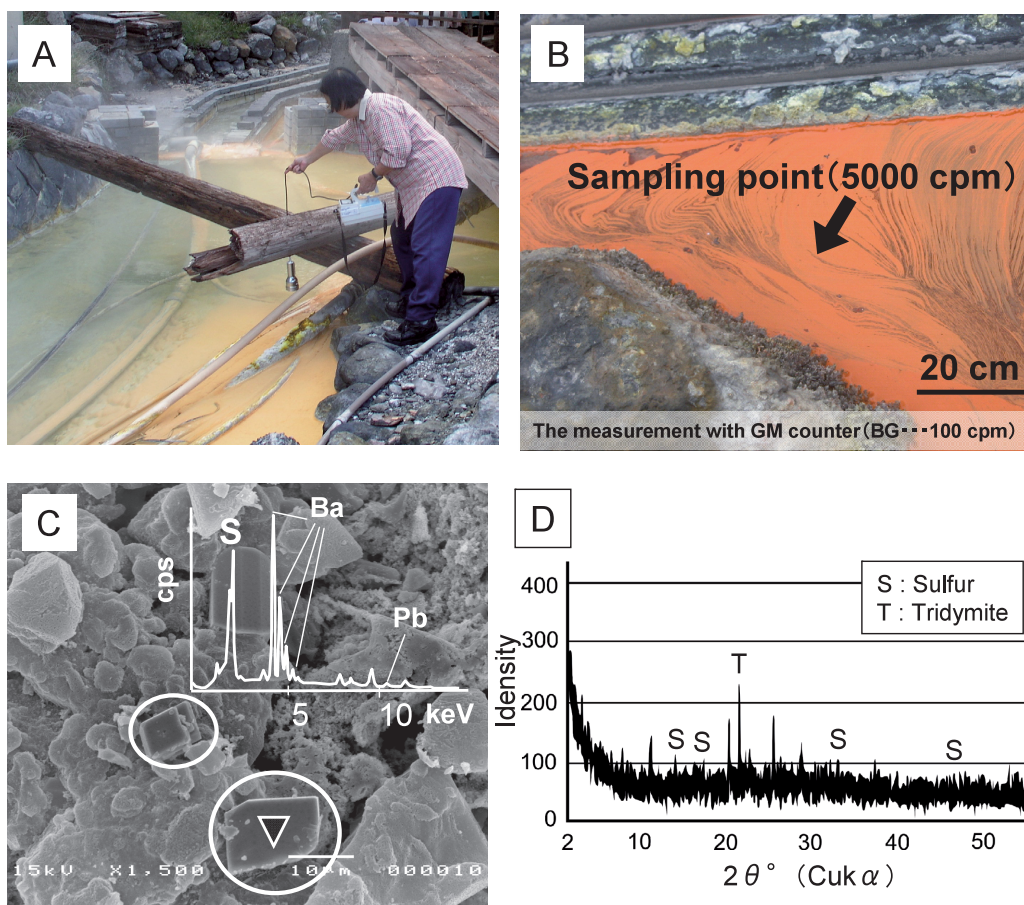


Fig. 2. Reddish brown microbial mats (A, B), the SEM-EDX micrograph of Hokutolite (C) and the XRD pattern (D) in the microbial mats collected from Tamagawa Hot Springs.

low quantities of crystalline sulfur and tridymite. Hokutolite was not identified because its small amounts and quantity were too small for this method (Fig. 2 D). In addition, the ED-XRF analysis of the microbial mats clearly indicated granular Hokutolite (Fig. 3 C), associated with As element (Figs. 3 A and B). The STEM-EDX micrograph of the bacterial cells showed a thick cell wall encrusted with Fe component associated with P, Ca, and As elements. The result suggests that the existence of these materials around them defend from radioactivity (Fig. 4).

### Misasa Hot Springs

The characteristics of the hot spring water at Misasa Hot Springs were a neutral pH of 7.2, low temperature of 42.0°C, ORP of 226 mV, EC of 1.3 mS/cm, and DO concentration of 1.5 mg/L, which were associated with radioactive microbial mats. The microbial mats accumulated elements such as  $^{238}\text{U}$  (6.4 Bq/kg),  $^{226}\text{Ra}$  ( $6.9 \times 10^4$  Bq/kg),  $^{228}\text{Ra}$  ( $8.3 \times 10^4$  Bq/kg),  $^{228}\text{Th}$  ( $8.8 \times 10^3$  Bq/kg), Fe (total of  $2.2 \times 10^5$  ppm) and Mn (total of  $6.2 \times 10^4$  ppm). The counts for the microbial mats were several hundred times those for the water (Fujisawa and Tazaki 2007).

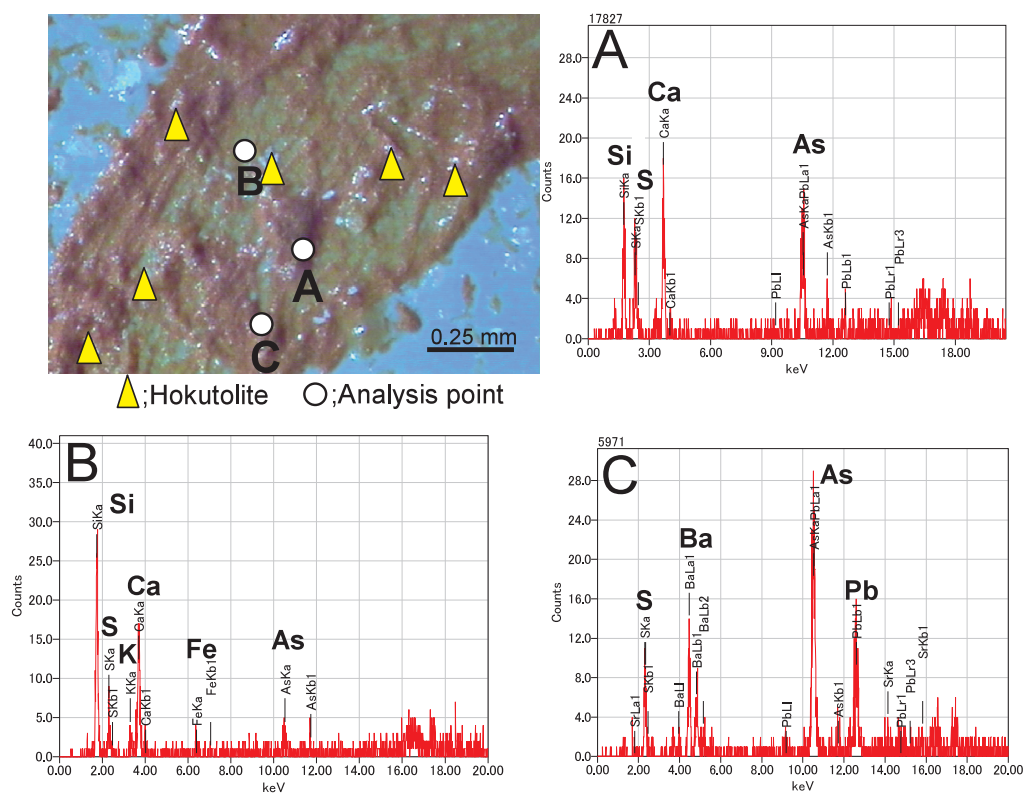


Fig. 3. ED-XRF analyses of the microbial mats at Tamagawa Hot Springs, showing Hokutolite small grains (arrows), and analytical points, showing the presence of As element and Hokutolite (C).

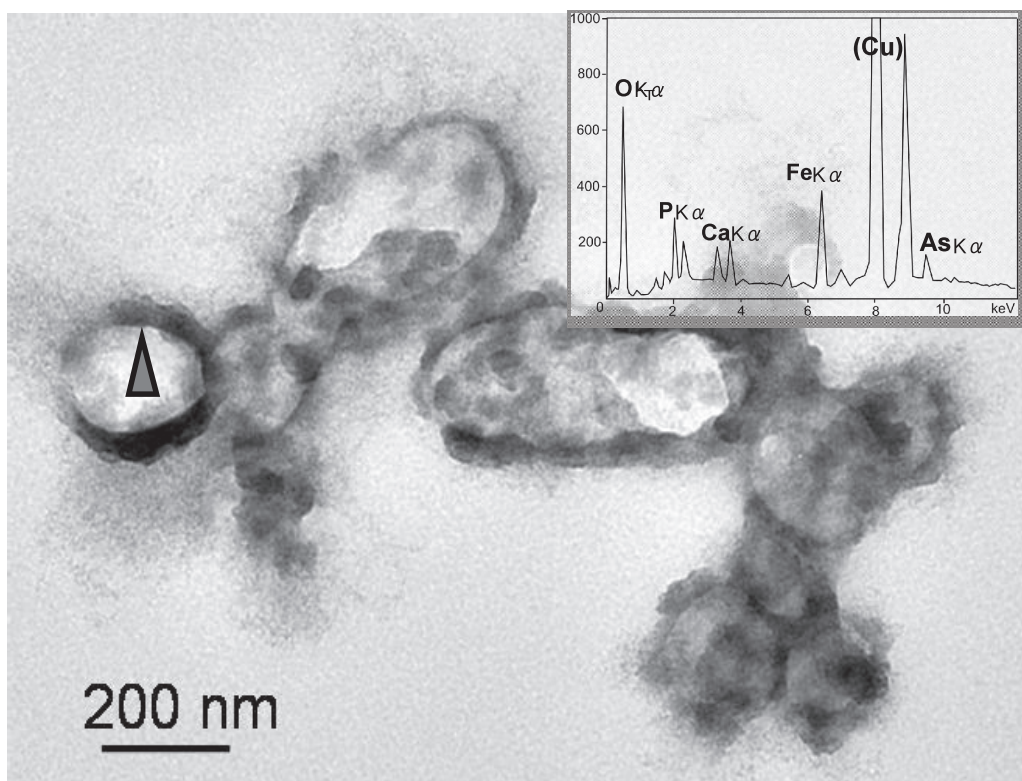


Fig. 4. STEM-EDX micrograph of unstained thin-section of Fe-rich coatings around the cells at Tamagawa Hot Springs. The area contains small amount of P, Ca, and As (Cu peak is due to supporting grid)(an arrow).

The Misasa Hot Springs water had aqueous chemistry of Fe (0.10 ppm), Mn (0.12 ppm), and  $^{226}\text{Ra}$  (0.14 Bq/kg), whereas the microbial mats had quite high concentrations of Fe ( $2.2 \times 10^5$  ppm), Mn ( $6.2 \times 10^4$  ppm), and  $^{226}\text{Ra}$  ( $6.9 \times 10^4$  Bq/kg), indicating high radioactivity conditions. Using the Geiger counter, the radio activities of the background (0.18  $\mu\text{Sv/h}$ ), surface of granite (0.30  $\mu\text{Sv/h}$ ), hot spring water (0.17  $\mu\text{Sv/h}$ ), and microbial mats (11.9  $\mu\text{Sv/h}$ ) were measured in the field (Fig. 5). The green and reddish-brown parts of the microbial mats were collected from the spring source and areas of water overflow for this study (Fig. 5, top). The ED-XRF chemical analysis showed the microbial mats contained high concentrations of Mn and Fe components associated with Si, S, and Ca and As (Fig. 5, middle left). The mineralogical composition determined from the XRD analysis of the microbial mats was mainly ferrihydrite and calcite (Fig. 5, middle right). The FT-IR analysis indicated abundant organic materials, showing chemical bonds of C–H, C=O, and CNH in the organics, suggests that microbial mats can accumulate heavy metals and radio nuclides through precipitation and complexation on and within the cell surface containing hydroxyl and carboxyl groups (Fig. 5, bottom).



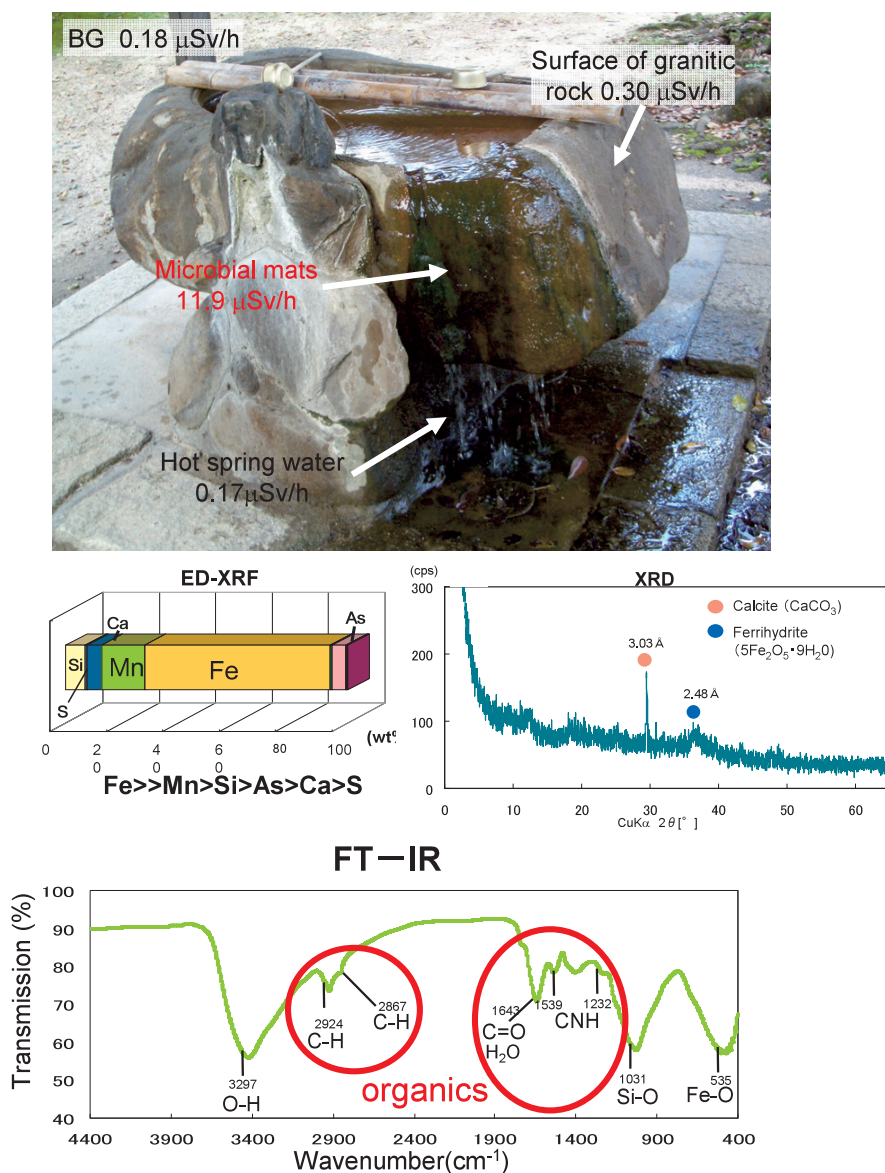


Fig. 5. Sampling points at Misasa Hot Springs in Tottori Prefecture in Japan, showing higher radioactivity of the green and reddish brown microbial mats (11.9  $\mu\text{Sv/h}$ ; an arrow in centre) than that of hot spring water (0.17  $\mu\text{Sv/h}$ ; an arrow in bottom), granite (0.30  $\mu\text{Sv/h}$ ), and back ground in the air (0.18  $\mu\text{Sv/h}$ ; BG). Energy dispersive X-ray fluorescence analysis (ED-XRF) of the microbial mats showed Mn and Fe- rich compositions (center left). X-ray powder diffraction analysis (XRD) of the microbial mats shows mainly ferrihydrite and calcite minerals (center right). Fourier transform-infrared absorbance spectroscopy (FT-TR) of the microbial mats showed rich in organics (circles)(bottom).

The SEM–EDX analyses showed abundant cyanobacteria of *Oscillatoria* spp. and *Phormidium* spp. composed of Si, P, S, Ca and Fe other than C, whereas the mineral fragments of ferrihydrite (Fig. 6 A). The filmy materials were composed of Si, Ca, Mn, Fe and organic thin films (Fig. 6 B). TEM showed the bacterial cells encrusted with fibrous Mn minerals on cell walls (Fig. 6 C). Thin-section samples of Mn-rich microbial mats revealed a cell structure having thick capsules around the cell. Most of the bacteria were encrusted with filamentous Mn minerals. Fine extracellular polymers extending away from the cell were covered with Mn minerals, and a detailed cross section of cell structures of a cytoplasm membrane, peptidoglycan layer, outer membrane, plasma membrane, and thick capsule could be observed (Fig. 6 C). The capsule of the slime layer with Fe and Mn contained organics in the forms of protein molecules, enzymatic formations and phospholipids, as shown in the FT-IR analysis (Fig. 5, bottom).

## Discussion

The bacterial biomineralization of sulfur, tridymite, and Hokutolite at Tamagawa Hot Springs under strong acidic and high temperature conditions, and ferrihydrite, calcite, and Mn minerals at Misasa Hot Springs under neutral pH and low temperature conditions are described in this study. At both hot springs, Ra–Rn concentrations were quite high, but the bacterial cells with capsules had different structures. The results provide a basis for the design of an in situ bioremediation process using indigenous bacteria. The microbial immobilization of radionuclide and other metals in the two Ra–Rn hot springs play important roles in biomineralization, bioremediation, and the ecosystem.

The microbial mats in the hot springs had higher radioactivity than the surrounding water did as shown in Figs. 2 and 5. Most of the radionuclide was present in cell walls as shown in Figs. 4 and 6 C. The result was a gram-positive cell envelope that highly interacted with dissolved metal ions, was extremely robust, and was resistant to harsh environmental conditions (Beveridge and Fyfe 1985). Usually, gram-positive cell walls are exemplified by those of *Bacillus subtilis* and are linear polymers constructed of peptidoglycan, rich in carboxylate groups and covalently link together as they assemble around the cell (Beveridge et al. 1983). The U-loaded *B. subtilis* cell is entirely coated with extracellular precipitates, having the EDX spectrum of U (Suzuki et al. 2003). Microorganisms use a wide variety of minerals for their skeletons, including silica, apatite, Fe, Mn, several polymorphs of carbonate, uranium phosphate, and in particular, aragonite and calcite (Beveridge et al. 1983; Banfield and Nealson 1997; Tazaki 2000, 2005, 2006). TEM-EDX analysis has confirmed that most U removed from solution with kaolinite clay is associated with *B. subtilis* (Ohnuki et al. 2007). For living bio-films to consist of a variable distribution of cells and cellular aggregates, their extracellular polymers and void spaces or water channels are continuously grown, as shown by TEM in this study and reported by Costerton et al. (1995).

Bacteria in nature often exist as sessile communities called microbial films. The

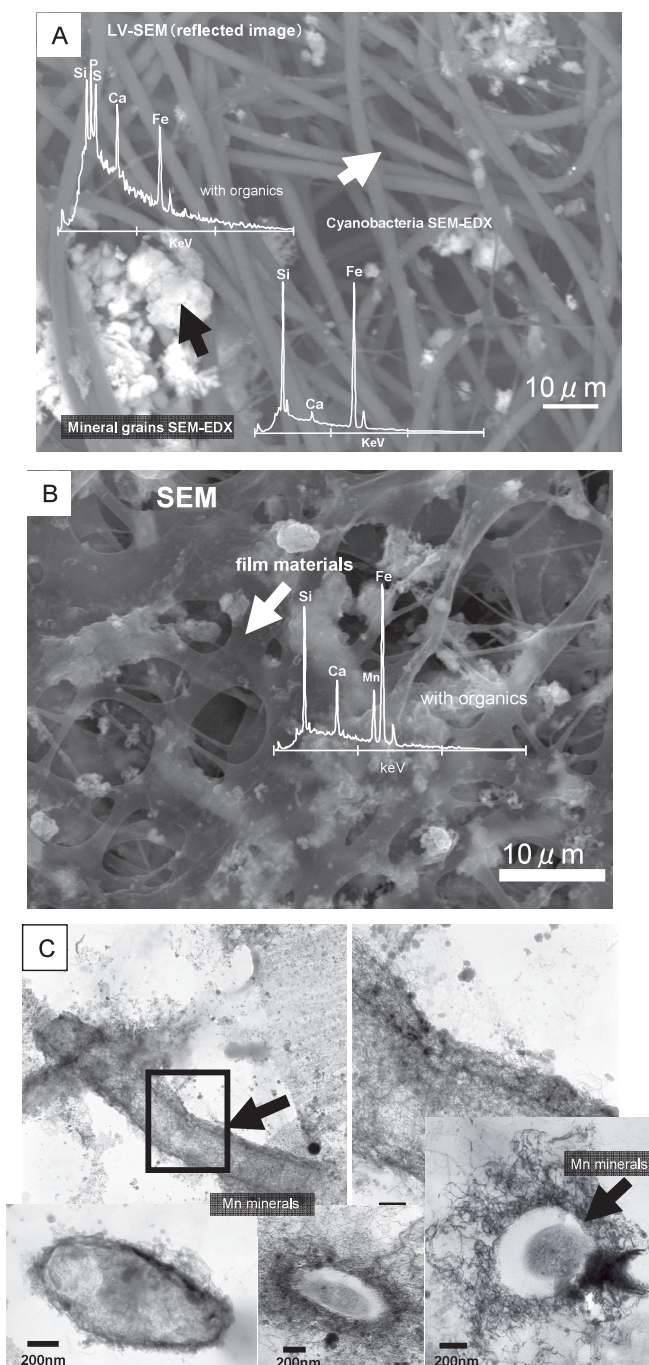


Fig. 6. Scanning electron micrographs of reflected image show cyanobacteria attaching to detrial silicate and carbonate fragments (A), thin film materials with organics contain large amount of Si, Ca, Mn and Fe (B), and unstained thin-section of Mn-rich coatings collected from microbial mats. The living bacteria are encrusted with filamentous Mn minerals. Fine extracellular polymers extending away from the cell are covered with Mn minerals (C; arrows).

communities of *Pseudomonas aeruginosa* in the microbial films develop structures that are morphologically and physiologically differentiated from those of free-living bacteria (Davies et al. 1998). A repeatable pattern of cell death and lysis occurs in bio-films of *Pseudomonas aeruginosa* during the normal course of development (Webb et al. 2003).

Effective biological remediation and biological treatments of radioactive materials from localized sources have also received much attention. There has been increased interest in the microbial uptake of radioactive materials as one of the primary ways by which Ra and Rn are eliminated from hot spring hydrothermal sites. Active resistance to the radioactive materials is a widely distributed trait among bacteria under different conditions (Beveridge and Fyfe 1985; Yoshida 1994; Costerton et al. 1995; Abdelouas et al. 1998; Davies et al. 1998; Suzuki et al. 2003; Tazaki and Watanabe 2004). Bacterial cells can accumulate heavy metals through ion exchange, precipitation, and complexation on and within a cell surface containing hydroxyl, carboxyl, and phosphate groups. Processes involving bacterial oxidation-reduction will occur after heavy-metal pollutants become mobile in soil (Yong et al. 2006). The bacterial surface might enhance the heavy metal adsorption of Mn, Fe, As, Ba, and Pb, which are associated with radioactive elements (Ra and Rn), at a pH of 1 or 7 under high temperature (98°C) or low temperature (42°C) conditions at Tamagawa and Misasa Hot Springs, respectively.

## Conclusion

Microbial immobilizations of radionuclides at Tamagawa Hot Springs in Akita Prefecture and Misasa Hot Springs in Tottori, Japan, were studied. The hot springs are a potentially useful area for the study of high radioactivity in environments rich in Ra–Rn. The water of Tamagawa Hot Springs had a high temperature of 98°C and was strongly acidic with a pH of 1.2, whereas the water of Misasa Hot Springs had a temperature of 42°C and neutral pH of 7.2. Both waters had high EC values owing to the reduced microbial mats having high radioactivity; the microbial mats were 100 times more radioactive than the surrounding groundwater. Barite and elemental sulfur were found to be associated with abundant bacteria in the reddish-brown microbial mats at Tamagawa Hot Springs. The bacterial cells were entirely encrusted with spherical grains with diameters of 100–200 nm. The heavy metals have been transported from the strongly acidic hot spring water to sediments. On the other hand, the green microbial mats at Misasa Hot Springs contained a high concentration of  $^{226}\text{Ra}$  ( $6.9 \times 10^4$  Bq/kg), which was present in living cyanobacteria (*Oscillatoria* spp. and *Phormidium* spp.) with ferrihydrite, Mn oxides and calcite. This suggests that  $^{226}\text{Ra}$  is adsorbed to the capsular surfaces of cyanobacteria and coccus and bacillus bacteria in microbial mats, thus producing capsules and slime layers of extracellular polymers around cells that protect against radionuclides and other metals in hot springs. It is possible that the capability that specific bacteria for immobilizing heavy metals can be used to counteract the disastrous effects of radionuclide pollution.



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