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Optical and X-Ray Imaging Analysis of Chemical Elements Associated with Microbial Communities

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Abstract—We analyze the distribution of chemical elements in microbial communities from various hydrothermal ecosystems (the Kurile–Kamchatka volcanic belt, the Baikal rift zone) and various laboratory cultures by means of microXFA-SR. We describe various techniques for combining optical and microXFA-SR X-ray cell images. The combination of images allows us to demonstrate the accumulation of chemical elements in cells of bacteria, cyanobacteria, and diatom and green algae.

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INTRODUCTION

Places where hydrothermal waters issue onto the Earth's surface are characterized by intense growth of microbial communities. The basic factors of the microorganisms' habitat in these systems are temperature, extreme pH values, anaerobiosis, and inflows of volcanogenic substances. Recent studies of some basic thermal springs in the Baikal rift zone allow us to study features of the microelement distribution in such microbial communities [1, 2]. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray fluorescence analysis using synchrotron radiation (Siberian Center for Synchrotron and Terahertz radiation, Institute of Nuclear Physics, Novosibirsk) helped to determine the exact distribution of elements Ge, Mo, W, Li, Cs, Rb, Sr, Ba, Mn, etc. We established that microbial mates play a pivotal role in the accumulation of various chemical elements that are distributed differently between the organic and mineral substances of microbial mates. The distributions of K, Mn, Ni, Cu, Zn, and Fe are thus normal; Ca, Rb, and Sr are almost completely bound with the mineral part; and Ga, Ge, and Br are accumulated by the mates' organic material.

No less important and interesting are recent investigations of the accumulation and distribution of elements in the microbial communities of the thermal springs of the Kurile–Kamchatka volcanic belt [3]. The authors obtained the first data on the elemental content in microbial films of the thermal sources of Geyser Valley and the Uson caldera. Alkali and alkaline earth elements (Li, Rb, Cs, Sr) were shown to predominate in the microelemental composition. The basic contents of anionogene elements (Mo, Sb, P, Ge) were determined. The especially high As contents in both the microorganism habitat and the material of microbial mates are of special interest in light of recent reports concerning the microorganisms' ability to exist without phosphorous by replacing it with arsenic in biomolecules, including DNA [4]. Hg contents of more than 300 ppm [3] were also found in the microbial community of the Uson caldera. The stability of microorganisms in combination with the collection of extreme factors (temperature, pH, high concentrations of heavy metals) testifies to their high biotechnological potential. The study and classification of such valuable genetic material would undoubtedly be of benefit to various areas of nano- and biotechnology, ecology and medicine.

Experiments to study microbial communities and individual microorganisms are now being conducted by numerous synchrotron radiation centers in Russia and abroad [5, 6]. These works are state-of-the-art, highly relevant, and interesting from a scientific point of view, since the microorganisms are being studied as bases for new biotechnologies in the food industry, bioenergy, etc.

The aim of this work was to study the distributions of chemical elements in microbial communities, both mineral and organic (bacteria, cyanobacteria, and diatom and green algae), by means of microXFA-SR, and to compare them to data obtained by optical methods.

EXPERIMENTAL

Our investigations of the elemental composition of microbial communities were conducted at the Institute of Nuclear Physics, Siberian Branch, Russian Academy of Sciences, in association with the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences. Samples of microbial communities were studied by means of optical spectroscopy to

obtain optical images of biological objects (cards); the optical cards were subsequently used in microelemental analysis performed on a scanning electron microscope (SEM) equipped with an energy dispersion detector (Hitachi S-3400V, Oxford Instruments, INCAx-act) at the Siberian Center for Synchrotron and Teraherz Radiation, Siberian Branch, Russian Academy of Sciences. Using the SEM in INP enabled us to determine the content of light elements ($Z < 15$). Experiments to study the distribution of chemical elements in microorgasnisms were performed on ANKA-FLUO timeline [7–9]. Using the focusing monochromatic SI beam in the micro RFA mode enabled us to raise the relative threshold of finding heavy elements ($Z > 15$) by at least two orders of magnitude relative to SEM, and obtain two-dimensional cards of the distribution of chemical elements in living objects with micron resolution. The optical and microRFA-SI X-ray images were then combined.

To analyze the chemical elements in microbial communities, field work was done to select samples of microbial communities growing under the conditions of high contents of such elements as As, Hg, Fe, Ge, and S. The geography of the samples included the Baikal rift zone and the Kurile–Kamchatka volcanic belt, since hot springs of volcanic fluids containing increased surface concentrations of various elements are found under conditions of increased volcanic activity. Samples of microbial communities from the Zavarzin hot springs (U-1), the Thermofil hot springs (U-5), the Stromatit hot springs (Kurile–Kamchatka volcanic belt), and the Garga hot springs (Barguzin Valley) were used in the early stage.

The samples were collected along the river beds of thermal brooks in sterile containers. Samples of water, ground precipitation, and microbial mats were taken on the investigated sites. Some of the samples were preserved with either 4% formalin or 96% ethanol. Some of the samples were kept alive.

In addition to the natural samples, three strains in this experiment were isolated from the investigated zones for which the accumulation of various elements from the environment had been demonstrated using an electron microscope equipped with an energy dispersion detector. We used this approach to preliminarily estimate the elemental content in our natural samples. Diatom algae with characteristic sizes of 10–20 μm were found to accumulate Br, P, Cl, S, Mn, and Fe with concentrations of 0.1–0.5 wt %, bacteria and cyanobacteria 1–2 μm in size accumulate Cl, P, S, Cr, and Fe at levels of 0.1–0.5 wt %. The minimum limit of the electronic microscope detection of chemical elements was 0.1–0.05 wt %, which fell sharply for chemical elements with $Z > 15$. The concentration of chemical elements in microorganisms under this limit could not be determined using an electronic microscope. Using the monochromatic SI beam in the microXFA-SR mode on the ANKA-FLUO timeline (Germany, Karlsruhe) allowed us to lower the minimum detection limit to 10^{-3}–10^{-5} wt %.

Obtaining Optical Images

Bacterium morphotypes and the composition and number of cells in control and hybridized samples were studied using the optical and luminescent Karl Zeiss microscopes (Axio Imager M1 and Axioskop 2 Plus, Germany) at the Interinstitute Center for Collective Use in the Microscopic Analysis of Biological Objects (Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk).

To visualize microorganisms in the samples, we obtained images using objectives with powers of ×100 or ×63. This could not be accomplished without immersion, so we selected an organic transparent optical film to replace the glass covers. Bacteria cannot be analyzed using a microXFA-SR glass cover since it must be removed, which in turn disturbs the objects of investigation and prevents us from creating an exact optical card of the microorganisms' disposition. We used an organic milar film 5 μm thick with Zn microadmixtures. The images were treated with the MetaSystems ISIS and AxioVision Rel.4.8 programs.

Conducting FISH

Fluorescent in situ hybridization (FISH) was used for the visual separation of mineral and organic parts and for better study of the microorganisms' cells. Hybridization was performed under the conditions described in the standard protocols [11]. Probes marked with fluorescent CY-5 and FAM labels were synthesized by the MEDIGEN company (Novosibirsk). The reaction was conducted with simultaneous use of all of the probes. To control hybridization, we used DAPI dye bound to samples' organic parts [12].

Obtaining Optical Images on Various Organic Supports (e.g., Organic Glass, Milar, Capton)

Since we cannot use standard slides as supports in performing X-ray fluorescent analyses of chemical elements in microorganisms, we selected various supports (e.g., organic glass, quartz, sapphire, milar, capton) and studied their properties. The investigated supports had various physicochemical and optical properties (e.g., thickness, strength, optical transparency, autofluorescence) and contained inclusions of different chemical elements (e.g., milar contained Zn, while sapphire and quartz contained Al and Si, respectively). Our studies demonstrated that most supports have autofluorescence that makes it difficult (and in some cases) impossible to obtain high-quality and informative optical images of biological objects in reflected light. The minimum limit of chemical element detection was proportional to the ratio of a characteristic elemental peak to the background. The background depended in turn directly on the support's thickness (kompton, elastic, back scattering). For the maximum possible elemental detection limit, we found that the thickness of the support was comparable to the sizes of microorganisms themselves. A

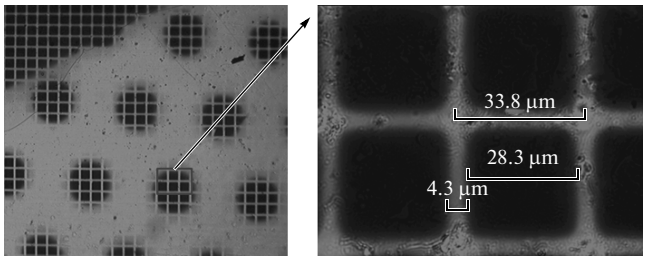

Fig. 1. Coordinate network from gold on capton. Thickness of the capton, 12.5 μm; thickness of gold, 50 μm; white areas, capton; black areas, gold; circle diameter, 125 μm.

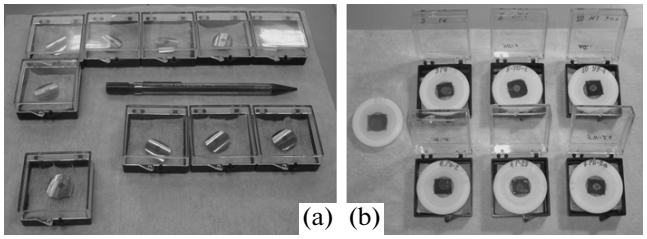

Fig. 2. (a) Capton films after coating with gold spray; (b) photographs of samples with microorganisms.

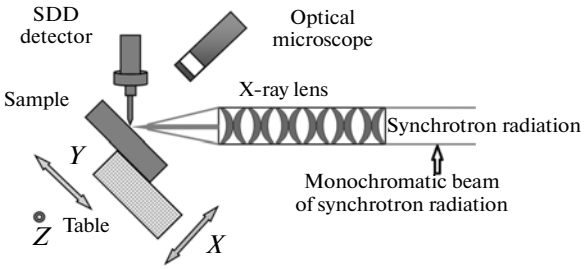

Fig. 3. Scheme of the experiment on the ANKA-FLUO beamline (Karlsruhe, Germany).

polyamide kapton film (DuPont) 12.5 μm thick was selected as our support.

Placing Coordinate Markers on Kapton

A gold coordinate net 50 nm thick was deposited onto kapton via magnetron evaporation (Fig. 1). The net was used to find microorganisms on a sample quickly. Before depositing the gold, the capton film was washed carefully and heated to 200°C; ionic etching was then performed in argon plasma. The capton films after gold deposition are shown on Fig. 2 along with photographs of samples with microorganisms.

RESULTS AND DISCUSSION

Two-dimensional cards of the distribution of chemical elements in microorganisms were obtained with micron resolution on the ANKA-FLUO station. Figure 3 shows the scheme of our experiment. An monochromatic radiation energy of 20 keV was used in the experiment. The energy resolution of the monochromator was 2×10^{-2}; the radiation source was a rotating magnet with $E_{crit} = 6$ keV. The size of the monochromatic X-ray beam was 5.7 μm horizontally and 2.8 μm vertically. Parabolic crossed component refraction lenses were used as our focusing X-ray optics [10]. The phonone beam at an energy of 20 keV was more than 10^8 photons per μm^2. The scanning area was 100 μm horizontally and 100 μm vertically, encompassing the gold markers for the superimposing of X-ray and optical images. Each vertical step was 2.5 μm and each horizontal step was 5 μm (usually 40×20 points); the scanning time at each point was 5–10 s. Each step (scanning point) contained an XFA-SR spectrum. Each card contained 800–1200 X-ray XFA-SR spectra. Figure 4 shows two XFA-SR spectra. Photon counts are plotted on the vertical axis, while the photon energy in eV is plotted on horizontal axis. The spectra measuring time is 10 s. The total loading on the detector can be estimated from these

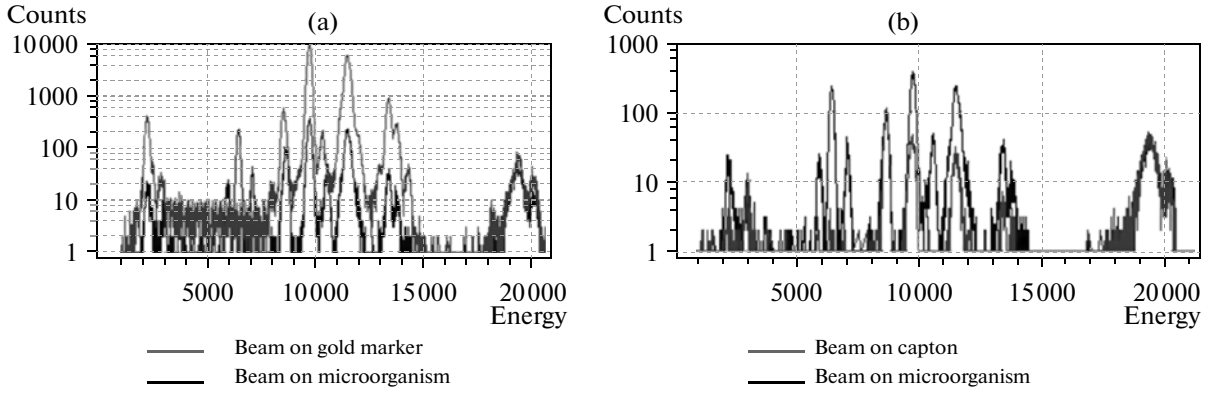

Fig. 4. (a) Roentgenofluorescent spectrum using synchrotron radiation from a gold marker and a microorganism; (b) roentgenofluorescent spectrum from a capton and a microorganism.

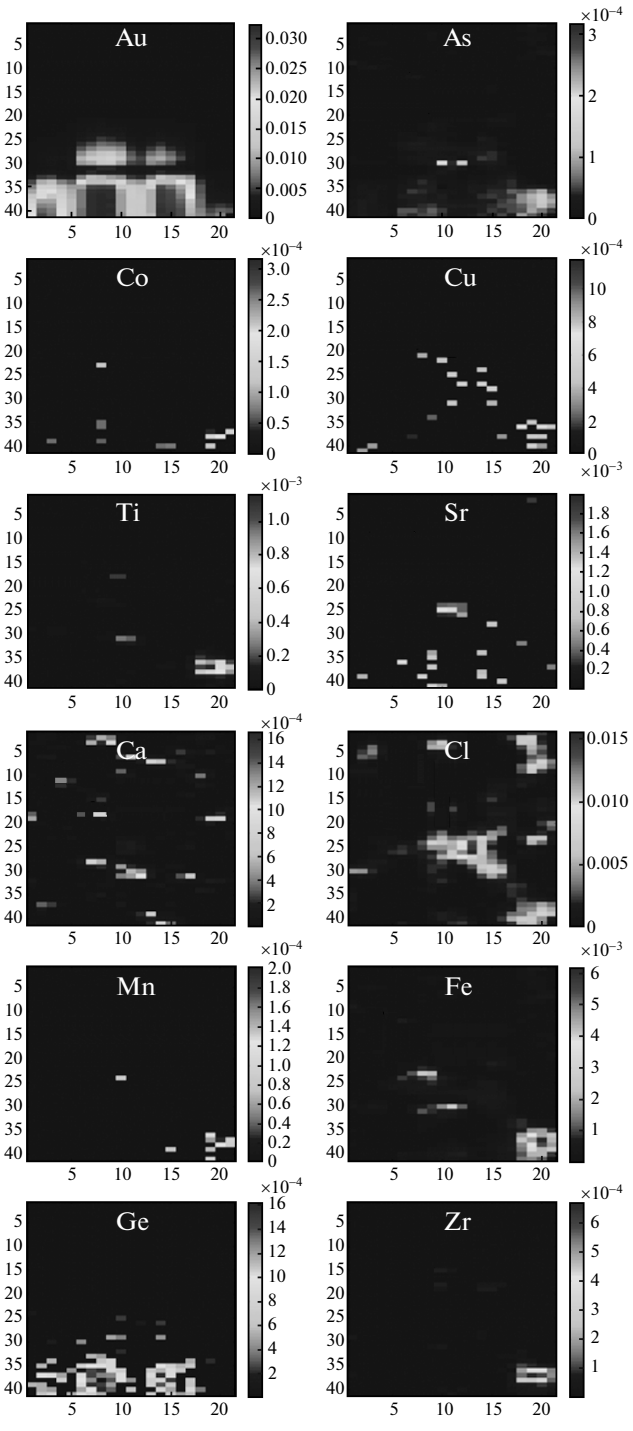

Fig. 5. Distributions of the chemical elements of one X-ray card with microorganisms (card size, 100 × 100 μm).

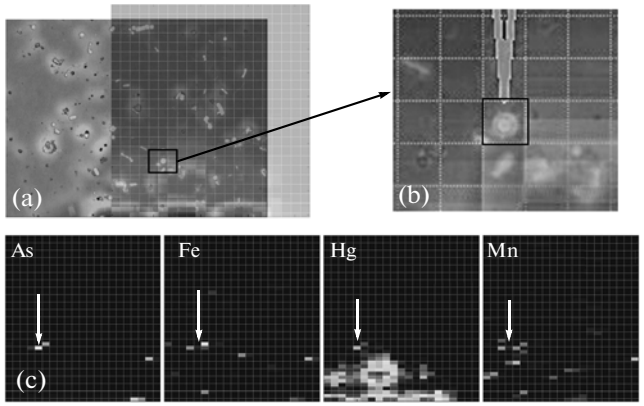

Fig. 6. (a) Combination of optical and X-ray cards (size of the card, 100 × 100 μm). (b) Magnification of combined cards; the arrow indicates cyanobacterium (the square around the cyanobacterium is 5 × 5 μm in size). (c) Distribution of chemical elements; the arrow shows the accumulation of As, Mn, Fe, and Hg coinciding with the location of a cyanobacterium cell (size of the card, 100 × 100 μm).

spectra. More than 30 cards (optical images) of our biological samples were studied.

The XFA-SR X-ray spectra were treated with the AXIL, PyMCA, and MatLab programs. The concentration of elements was calculated from its basic parameters using the PyMca program. Figure 5 shows the distributions of the chemical elements of our card with microorganisms. The steps are plotted on the vertical and horizontal axes. The vertical scale on the right is the concentration of elements in percent (1 = 100%). The optical and X-ray images were superimposed using the gold marks. The thickness of each net line was on the order of 4 μm, and stood out in sharp contrast on the X-ray element card. The maximum error obtained through the superimposing of two cards (optical and X-ray) was no more than half the width of a net line, i.e., comparable to or even less than one scanning step. The elemental composition of microorganisms was determined from the combined images. Figure 6 shows a combination of optical and X-ray cards; the arrow indicates the accumulation of As, Mn, Fe, and Hg that is coincident with the position of the cyanobacterium cells (evidently Phormidium sp) in the Thermofil U-5 hot springs.

CONCLUSIONS

A method for obtaining images of microbiological objects with specified coordinates in optical and X-ray regions was developed. Various methods for determining the elemental compositions of microorganisms through microXFA-SR were elaborated. Two-dimensional cards of the distribution of chemical elements in microorganisms were obtained with micron resolution. Optical and X-ray images (cards) were combined. Combined images were obtained for natural microbial communities of the Kurile–Kamchatka volcanic belt (the Termofil and Zavarzin hot springs), the Baikal rift zone (the Garginskii hot springs) and some laboratory cultures in the microorganism collection at the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences. The combined images were analyzed. We found that microor-

ganisms accumulate As, Hg, Fe, Mn, Cu, Ca, Co, Sr, and Ge. Our method allowed us to conduct the elemental analysis of different natural samples (laboratory cultures), to estimate the content of certain elements in a sample, to trace the regularities of the accumulation and bioaccumulation of elements by microorganism communities, and to establish likely connections between the elements and our biological samples.

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