1-1.Clays and microorganisms : Biosynthesis and biomineralization

Clays and microorganisms: Biosynthesis and biomineralization

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INTRODUCTION

Various microorganisms have the ability to accumulate metallic ions from their external aquatic environments. Microorganisms play an important role in the concentration, crystallization, transportation and sedimentation of almost all elements in the Earth (Tazaki 1999; 2002). In the Earth's surface environment biofilms and microbial mats are universal, and a host of minerals is synthesized in complex biomediated processes.

Clays are also universal, and are distinguished in high water absorbent, flexible morphological structures, CEC, and capacity of heavy metal accumulation. The occurrence of clays is everywhere that is normally found in the case of microorganisms, regarding the very small or minute sizes. In laboratory experiments using microorganisms in closed systems, effects on biomineralization are distinguished. On migration the organic matter encounters fresh mineral surfaces to form clay minerals. Microorganisms under natural surface conditions primarily mediate dissolution and precipitation of clay minerals. It indicates a very close relationship between clays and microorganisms.

In the geo-, aqua-, and eco-systems, microbial mats of bacterial colonies with clays are an exciting area of study. Bacterial clay biomineralization can be found in colorful microbial mats in river, hot springs, ponds, lakes, deep sea floor, and geothermal area. The biomineral assemblages can include carbonate, silicates, Fe-Mn oxides, hydrated phosphates, sulfides, and clay minerals formed through bacterial activities. The nature of assemblage is dependent on temperature, hydrogen ion concentration (pH), oxidation, and reduction potentials (Eh), dissolved oxygen (DO), and electrical conductivity (EC) (Tazaki 1997).

In this paper the natural occurrence of bacterial bio-clays are introduced to better understand the role of bacteria in their biosynthesis and clay crystallization. For example, amorphous layer silicate, 7 and 14 Å clay mineral formation at various localities are described.

HOW BIO-CLAY MINERALS ARE OBSERVED: MATERIALS AND METHODS

Optical and electron microscope techniques are needed because both of microorganisms and clays are quite small to see it by our eye. Scanning electron microscope equipped with energy dispersive X-ray spectrometer (SEM-EDX) and transmission electron microscope (TEM) with electron diffraction techniques can be used. The bio-clay mineralization study is, of necessity, multi-disciplinary, such as geology, mineralogy, microbiology, and chemistry to know components of interpretative synthesis.

< Field work >

1.Geology and sampling

The geology of an area where bio-clays are formed is fundamental to their investigation. The geological environment affects both underground and hydro thermal waters. Since microorganisms living in microbial mats metabolize using materials from the air, water, sediments, and rocks, it is essential to record details not only of the microbial mats, but also of the surrounding geological environment, such as terrain, basement petrology, soil science, and weathering conditions. Depending on the situation, samples are taken of sediments, rocks, water, and other materials. Where microbial mats themselves are collected, appropriate samples handling of the study material is critical.

2.Water chemistry

Since microorganisms absorb materials into cells as ions, water conditions affect the microorganisms living in microbial mats. Water chemistry data are important in identifying the species and properties of the microorganisms present. Water temperature, pH, Eh, DO, and EC are measured in the field to obtain data on water conditions.

Seasonal changes in these indexes should be taken into consideration. The chemical compositions of eater in the field are also essential data.

3.Observation of microbial mats

During field observations, the following information is recorded in detail.

1. Colors of microbial mats reflects the species of microorganisms and biominerals present,
2. Exposure of microbial mats to solar radiation,
3. Volume and flow rate of surrounding water,
4. Sediments and rock type on which the microbial aggregate,
5. Thickness, hardness and any other physical characteristics of the microbial mats,
6. It is desirable to take photographs of field occurrence to record information for further analyses in laboratory.

< Laboratory work >

1.Sample preparation

1-1. Thin section preparation using resin

Optical microscopic observations of thin sections are essential to determine the spatial relationship between the microorganisms and clay minerals in the microbial mats. As microbial mats contain water and are often fragile, thin section preparation is difficult. Accordingly, to prevent breakage, it is often necessary to coat samples with a resin; thin sections are made by the following procedure.

1. Dying to eliminate water,
2. Embed the inner micro texture with cyanobond,
3. Resin coat the microbial mats with epoxy or polyester resin,
4. Thin section preparation is completed in the same way as for ordinary preparation.

1-2. Ultra thin section preparation

Ultra thin section produces a section about 0.1 μm thick, which are used to observe the inner micro texture of the cells. Ultra thin section is, therefore, useful for observing

the distribution of minute mineral grains. The procedures for preparation of ultra thin sections are as follows.

1. Fixing; to preserve the micro texture of the samples with glutaldehyde or similar fixative,
2. Dehydration; to substitute for water by means of a water soluble synthetic resin,
3. Resin coating; to coat the samples with a synthetic resin,
4. Cutting; to cut the samples using an ultra microtome,
5. Dyeing or coating; to increase the contrast of TEM image.

2.Optical microscope

2-1. Bright field image

Observation by a bright field image is a standard observation method in transmitted light. As a first step in laboratory studies of microbial mats, this observation method is usually used in conjunction with stereoscopic and differential interference imagery. Distribution, form, color and texture of microorganisms and minerals ranging in size from a few to several hundred microns can be observed at the level of magnification of an optical microscope.

2-2. Polarizing microscopic image

Observation by polarizing microscope is essential to identify the minerals present through observation in plane polarized light, crossed Nichols and conoscopic illumination. The optical properties of minerals in microbial mats can determine the minerals identified.

2-3. Fluorescence microscopic image

Sample fluorescence can be observed by mean of a fluorescence microscope. When exposed to ultraviolet rays excitation, some minerals and microorganisms in microbial mats fluorescence. For example, chlorophyll or bacterio-chlorophyll exhibits red fluorescence. In case of same microorganism, which is hard to distinguish from other minerals, fluorescence before and after dyeing can be a determining factor. Nucleic acids such as DNA and RNA singularly combine with a fluorescing stain to

change into a fluorescent material. For example when exposed to ultraviolet rays, 4' 6-diamidino-2-phenylindole (DAPI) -stained DNA displays blue fluorescence. Cells were also stained with the RNA specific dye acridine orange (AO) which is excited with light at a wavelength of 436 or 490 nm. The Indian ink, ruthenium red, and vinegar are also used for identifying the existences of S-layers or extra cellular microbial polymers surrounding microbial cells.

3.X-ray powder diffraction (XRD)

X-ray powder diffraction is a relatively simple method of identifying clay minerals. According to the type of clay minerals, the diffraction pattern in the chart is different, and the clay minerals can be identified by its characteristic diffraction pattern to quantify mixture, crystallization, and other parameters. For XRD analysis of microbial mats, powdered samples or the mats themselves are set on a non-refracting glass slide surface.

4.X-ray fluorescence spectroscopy (XRF)

When X-rays excite an atom, characteristic X-rays are emitted, the wavelength of which is dependent on the element. The elements present in microbial mats can be determined qualitatively and quantitatively by this method.

5.Scanning electron microscope (SEM)

Observations by SEM are used to produce a magnified image on the monitor. Microbial mats must be dried before observation under high-vacuum conditions. Where this is a problem, other method, such as freeze drying or observation under low-vacuum conditions can be used.

6.Energy dispersive X-ray spectrometer (EDX)

By detecting the characteristic X-ray, the elements contained in the sample are identified. The use of EDX in combination with SEM and TEM allows qualitative and quantitative analysis of specific sites within the image of the sample under observation.

7.Transmission electron microscope (TEM)

When an electron beam strikes a very thin sample, part of the electron beam is transmitted. TEM images can be divided into bright field images (high-resolution images, crystal lattice images), dark field images, and electron diffraction patterns. Ordinarily, selected-area electron diffraction patterns which can be obtained only from a selected mineral within the TEM image. As microbial mats contain very fine mineral grains, which can scarcely be seen under an optical microscope, TEM is very useful in the study of microbial mats.

8.Microorganism culture methods

Supplying water and sediments collected from the field to living microbial mat samples can replicate the field environmental conditions. This natural means of culture retains the properties of the mats. Other environmental parameters such as pH, temperature, light, and partial pressure of oxygen are controlled and altered during culture in the laboratory to determine which factors are essential in controlling microorganism behavior.

These culture methods are effective, but difficulties can be encountered, when culturing microbial mats by such methods over a long period. It should also to taken into account that the phenomena observed in the microbial mats containing plural species of microorganisms result from interactions between the microorganisms. An alternative method is to culture a particular species of microorganism separated from the microbial mats in an artificial medium. This method is useful for studying the properties of individual types of microorganisms, but the range of microorganisms is very limited.

All data obtained from both field and laboratory works are invaluable to understand collectively to correlation between microorganisms and clays in the natural environmental system. Characteristics of microbial mats and the bio-clays formation at various localities are introduced in this paper as follows.

BIO-AMORPHOUS / LAYER SILICATE CLAY FORMATION

Microbial mats in pond

Red-brownish microbial mats occur in solid or colloidal states at various locations,

such as a cut cliff in the Omma Formation and drains and settling ponds in and around Kakuma Campus of Kanazawa University (Tazaki 1997; Yoshizu and Tazaki 1997). Water flowing through the Omma Formation into settling ponds tends to range from weakly acidic to neutral (pH 6.4 - 7.1). The dispersion of Eh and DO data shows seasonal variation (-40 - 80 mV, 6.3 - 9.7 mg/l). The water chemistry of freshwater is as follows: Mn 1.03 ppm, Fe 2.50 - 10.00 ppm, Ca 76.00 ppm and K 18.00 ppm at pH 6.5 - 6.8 and the temperature from 12 - 20 ℃. Optical and fluorescence microscopic observations revealed inhabitant of the bacillus, spiral, and filamentous microbes in the microbial mats (Figure 1A).

SEM micrographs show doughnut-shaped construction contains the capsule of *Siderocapsa* sp. in the center. EDX analysis identified that such construction composed of mainly Al, Si, Ca and Fe suggesting layer silicates (Figure 1B, C, D). XRD patters showed strong diffraction of crystalline calcite (3.0 Å) and illite associated with large amounts of amorphous and/or organic materials because of the high background. Natural culture experiment revealed the production of Fe-Mn rich construction after 2 month aging. SEM micrographs of the holdfasts on the cover glass slide showing successive formation processes of mixtures of layer silicates and hydrated Fe/Mn oxides. Doughnut-shaped holdfast with hole in center (Figure 1B) and the side views (Figure 1C) are the same as transmitted views (Figure 2A) showing coccus typed bacteria living in the center (arrows). Closed-up photograph of the bacteria revealed high electrical dense granular materials on the cell wall (Figure 2B).

Microbial mats in hot springs

The coccus and bacillus typed bacteria in the microbial mats collected from hot springs (pH 6.8, 39 ℃) in Laugarvatn, Iceland, showing incipient stage of formation of granular layer silicates preservation on the cell wall. TEM of ultra thin section of the cells clearly distinct cell wall (a), surface array (b), capsule (c), and plasma membrane (d) are covered by layer silicate with high electrical dense (Figure 3A, B) (Tazaki and Ishida 1996).

XRD analysis of the bulk microbial mats shows hilly background of amorphous pattern with 3 - 4 Å peak top (Figure 3A', B'). The TEM and EDX of thin-sectioned

specimens revealed the remains of living bacterial internal cell structure with the accumulations of Si, Al, and Fe.

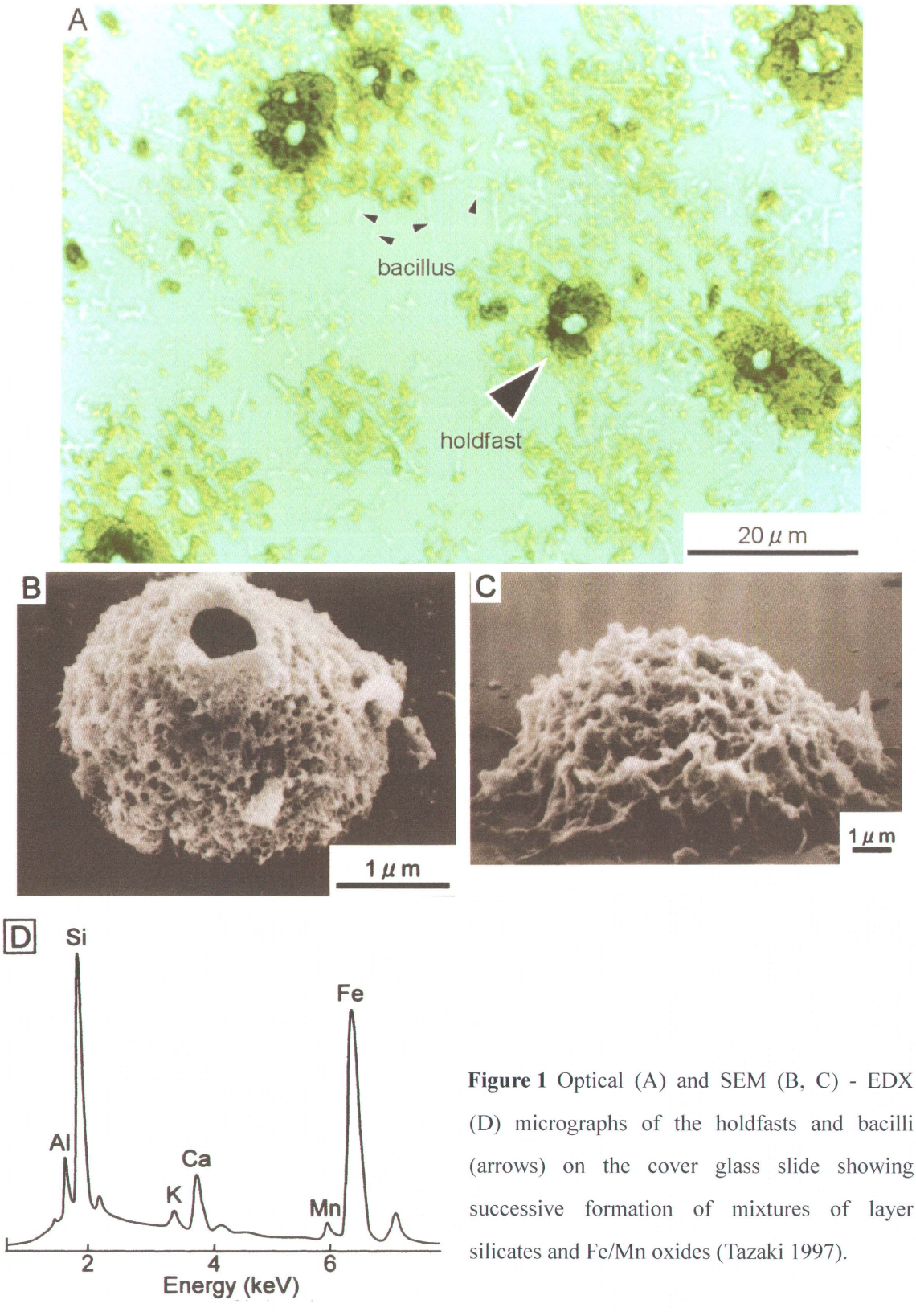

Figure 1 Optical (A) and SEM (B, C) - EDX (D) micrographs of the holdfasts and bacilli (arrows) on the cover glass slide showing successive formation of mixtures of layer silicates and Fe/Mn oxides (Tazaki 1997).

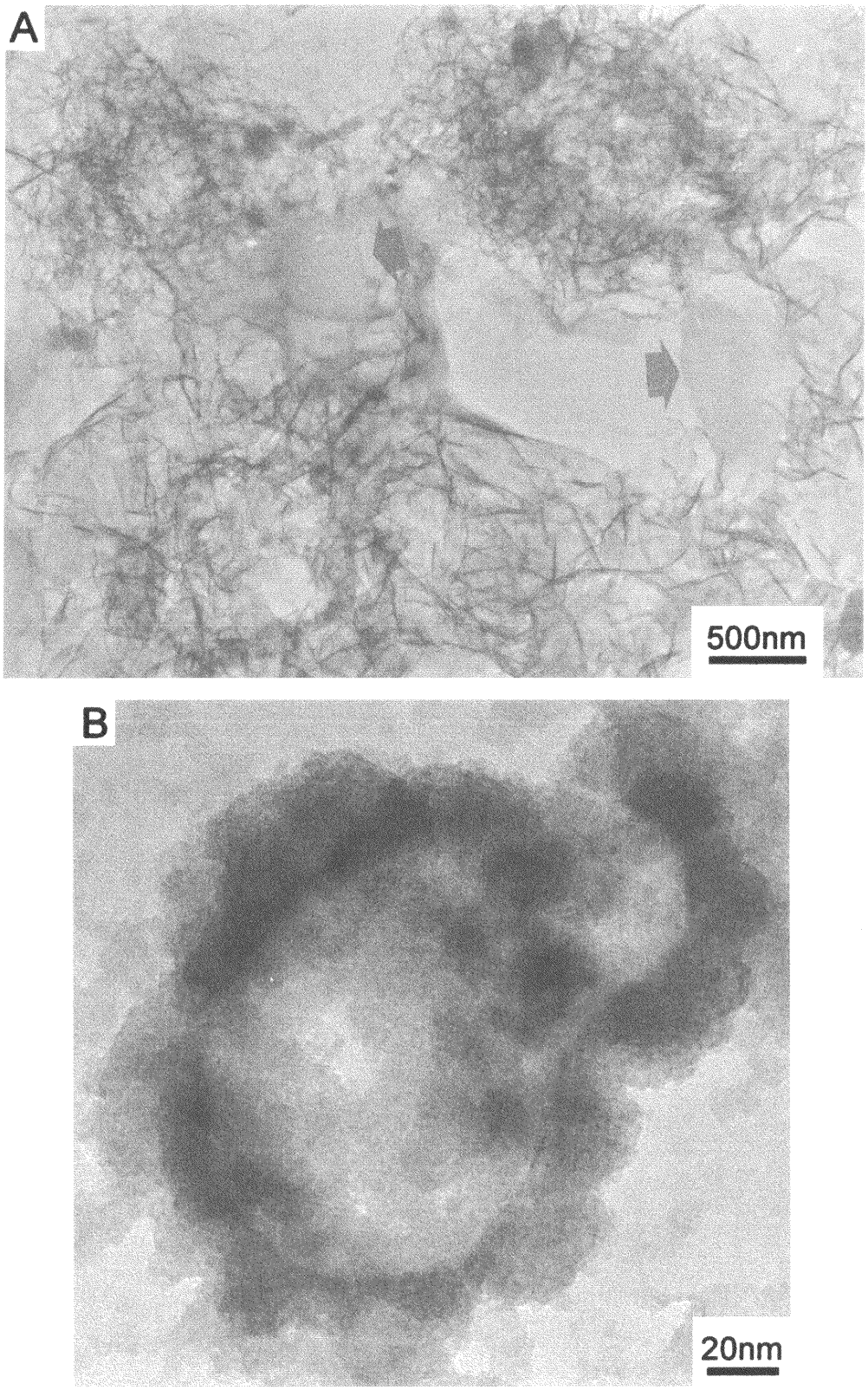

Figure 2 TEM micrographs of clay films attached to doughnut-shaped holdfasts with bacillus and coccus bacteria (A, arrows) and of coccus bacteria with granular coating of amorphous materials (Tazaki 1997).

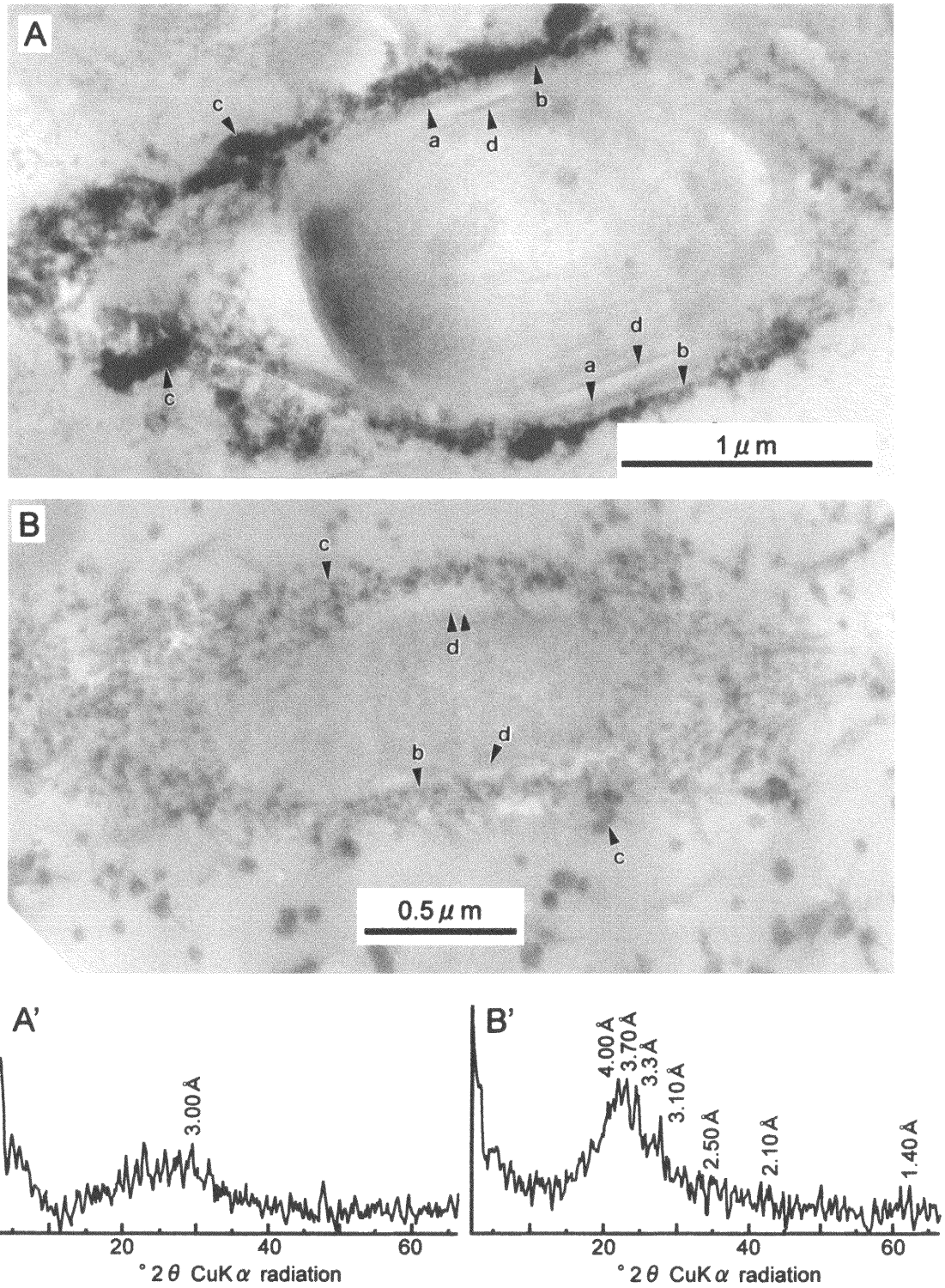

Figure 3 TEM ultra-thin sectioned micrographs of amorphous layer silicate with Fe (A) and Ca (B) concentrated spherules showing internal cell structures. a; cell wall, b; surface array, c; capsule, d; plasma membrane. XRD patterns of A and B samples indicated presence of amorphous materials (A', B') (Tazaki and Ishida 1996).

BIO-7 Å CLAY FORMATION: BIO-HALLOYSITE

Green microbial mats in Kutani glaze

Bio-weathering processes of K-feldspar were studied on the Kutani Glaze suspension in Kanazawa Prefecture, Japan, as the model of microbial clays from silicate minerals of major components of rocks. Green microbial mats occurred in the container with water and precipitates composed mainly K-feldspar under pH 9.0 - 9.4 and EC 0.38 - 0.65 mS/cm for 3 years in a bright room (Figure 4A). The chemical composition of the K-feldspar by using XRF analysis is as follows; SiO_2 69.03, Al_2O_3 17.37, Fe_2O_3 0.06, CaO 0.89, MgO 0.06, K_2O 8.94, Na_2O 3.33, Ig. loss, 0.31 wt%, respectively (Ueshima and Tazaki 1997).

The optical micrographs of green microbial mats show chained microbes with capsule of fine- and coarse-particles surrounded sheath of cyanobacteria, *Phormidium* sp. (Figure 4B, C) indicating microbial chlorophyll due to strong red auto fluorescence. The weak red pseudo fluorescence presumably shows the presence of clays. The green microbial mats stained with Indian ink clearly visualized existence of *Phormidium* sp. with extra cellular polymeric substance layer of microbes (Figure 5A). More over ruthenium red stained *Phormidium* sp. indicated the presence of acidic polysaccharide polymers in pink color (Figure 5B). SEM and TEM images also

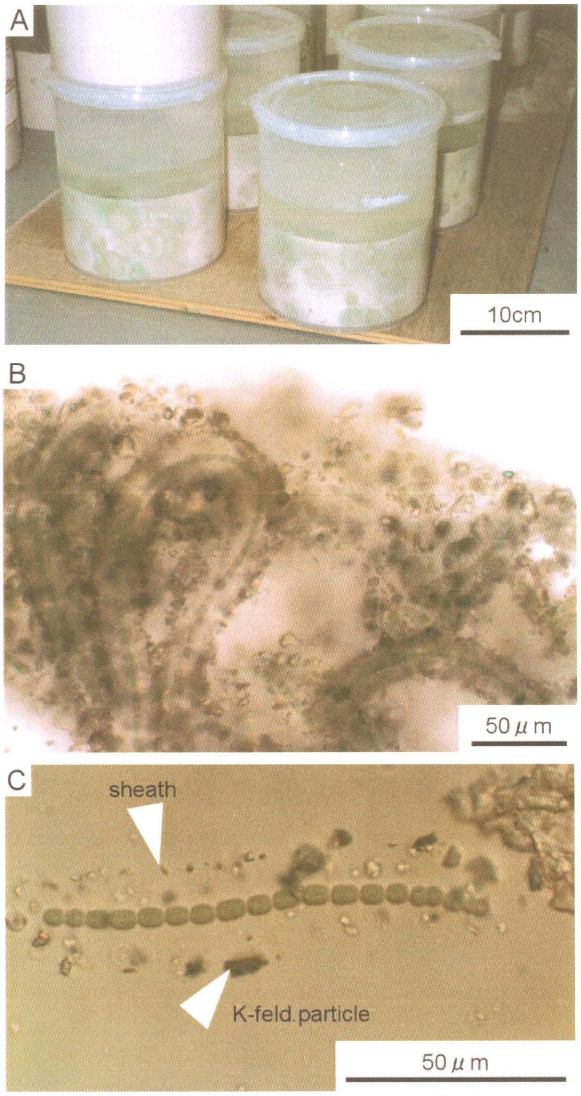

Figure 4 Green microbial colonies occurred in Kutani glaze solution (A) and optical micrographs of a green cyanobacterial colony (B) and K-feldspar particles on the sheath (arrows in C) of cyanobacteria surrounding capsule (Ueshima and Tazaki 1997).

revealed adhesion materials and fine grained particles indicate main components of Al, Si and K suggesting K-feldspar fragments are digested to form halloysite on the surface of the cell (Figure 6A, B). The primitive clay precursors and halloysite were formed in extra cellular polymeric substance layer of *Phormidium* sp. composed of Si and Al with trace amount of K. The fragments were apparently transformed to halloysite, which separated from the surface of coarse-grained particles.

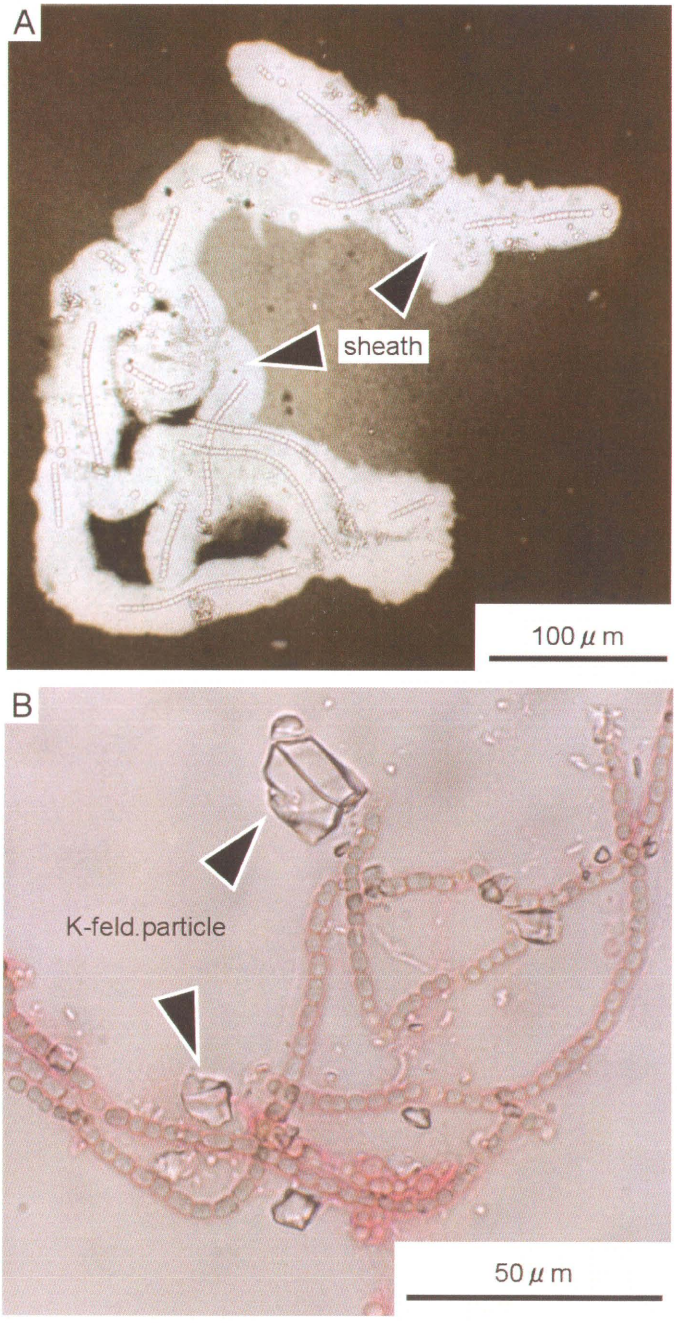

Figure 5 Optical micrograph of *Phormidium* sp. in green microbial colony stained with Indian ink (A) and stained with ruthenium red (B), indicating the presence of sheath (arrows in A) and acidic polysaccharide polymers on the cell wall with K-feldspar particles (arrows in B).

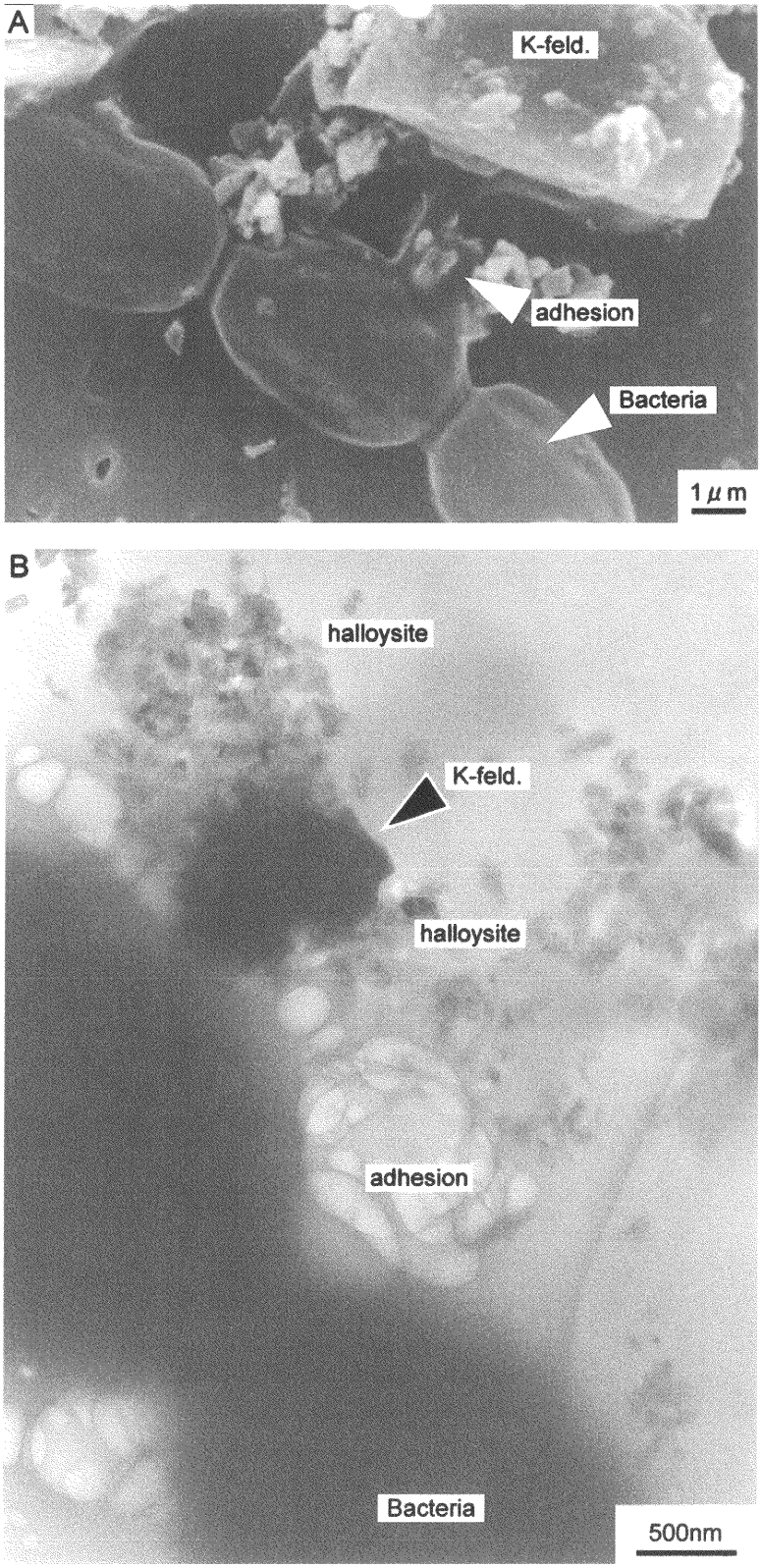

Figure 6 SEM image of green colony showing the resides of adhesion materials (A) and TEM image of *Phormidium* sp. adhering K-feldspar particles transform into halloysite (B).

Halloysite ball from dam sediments

Interaction between clays and microorganisms has investigated at microbial films of the cultured solution with natural dam sediments collected from Portalegre, Brazil (Asada and Tazaki 2000; Tazaki and Asada 2001). The microorganisms were cultured for a few months to two years under pH 6.0 to 7.4 in order to make clear the clay mineralization process and to understand the role of microorganisms living in microbial mats at room temperature. A schematic figure of cultivation system shows formation of microbial films on a glass slide, container wall, and top surface of sediments (Figure 7A). Optical microscopic observation of cultured microorganisms with DAPI staining revealed that ultra thin films covered on the microbes with spherical clays (Figure 7C arrows).

TEM observation of the thin films clearly distinct that spherical, hollow halloysite (halloysite ball) associated with coccus and bacillus typed bacteria was formed (Figure 8A). The electron diffraction of spherical hallow halloysite (halloysite ball) shows 4.43, 2.56, 2.49, 2.22 and 1.48 Å d-spacing (Figure 8B). The chemical composition of the thin films is mainly Al, Si, S and Fe with traces of Mg, P, K, Ca, Ti, and Mn (Figure 8C). XRD of the thin films indicated 7.13 Å d-spacing which is the almost same diffraction pattern as standard kaolin minerals (Figure 8D). Atomic force micrograph using contact mode showed formation of bio-halloysite cultures on the surface of bacterial cell wall, shows the clusters with 50 - 500 nm in width and 2 - 20 nm in thickness, has an orientation along with the same direction. The clusters developed to spherical halloysite through hollow halloysite in diameter from 800 nm to 1 μm.

Bio-kaolin minerals / imogolite-like clays from weathered feldspar

Bio-weathering experiments were carried out using thin section of granite in freshwater with iron bacteria (*Toxothrix* sp. and *Gallionella* sp.) at Omma Formation, Ishikawa Prefecture, Japan. Microbial mats were formed on the surface of the thin section after a 3 - 10 days aging. Cavities and chaps with bacilli and filamentous bacteria were observed on the surface of feldspars. Si content was reduced at flake materials (Si : Al = 3 : 2) associated with bacilli on the surface of K-feldspar immersed in freshwater. K and Si ions release from K-feldspar were recognized after a 2 months

aging. TEM observation and electron diffraction analyses confirmed that kaolinite formed on the surface of K-feldspar (Ueshima and Tazaki 1998).

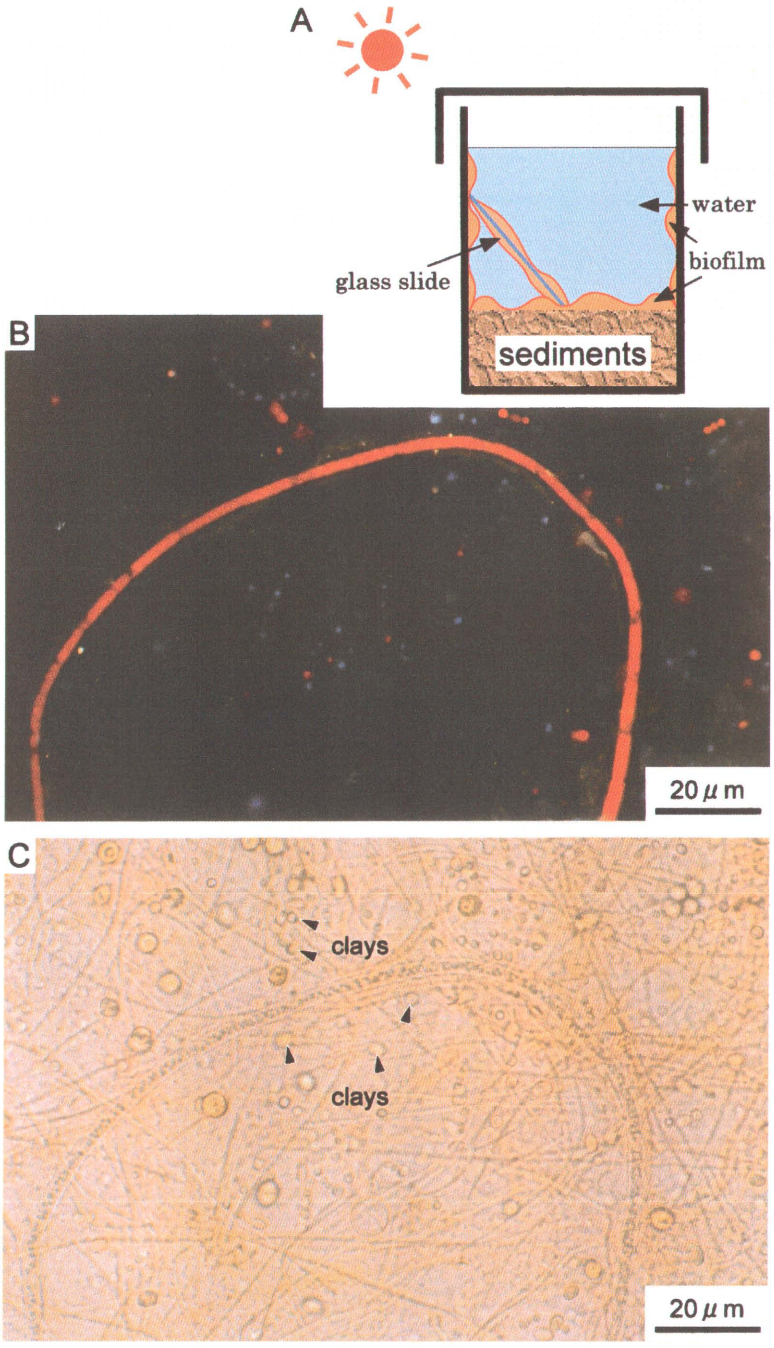

Figure 7 A schematic diagram of a natural cultivation system showing biofilm formation on a glass slide, a wall of beaker and surface of sediments (A) and the optical micrographs of DAPI stained microorganisms associated with spherical halloysite (B, C arrows) (Asada and Tazaki 2000).

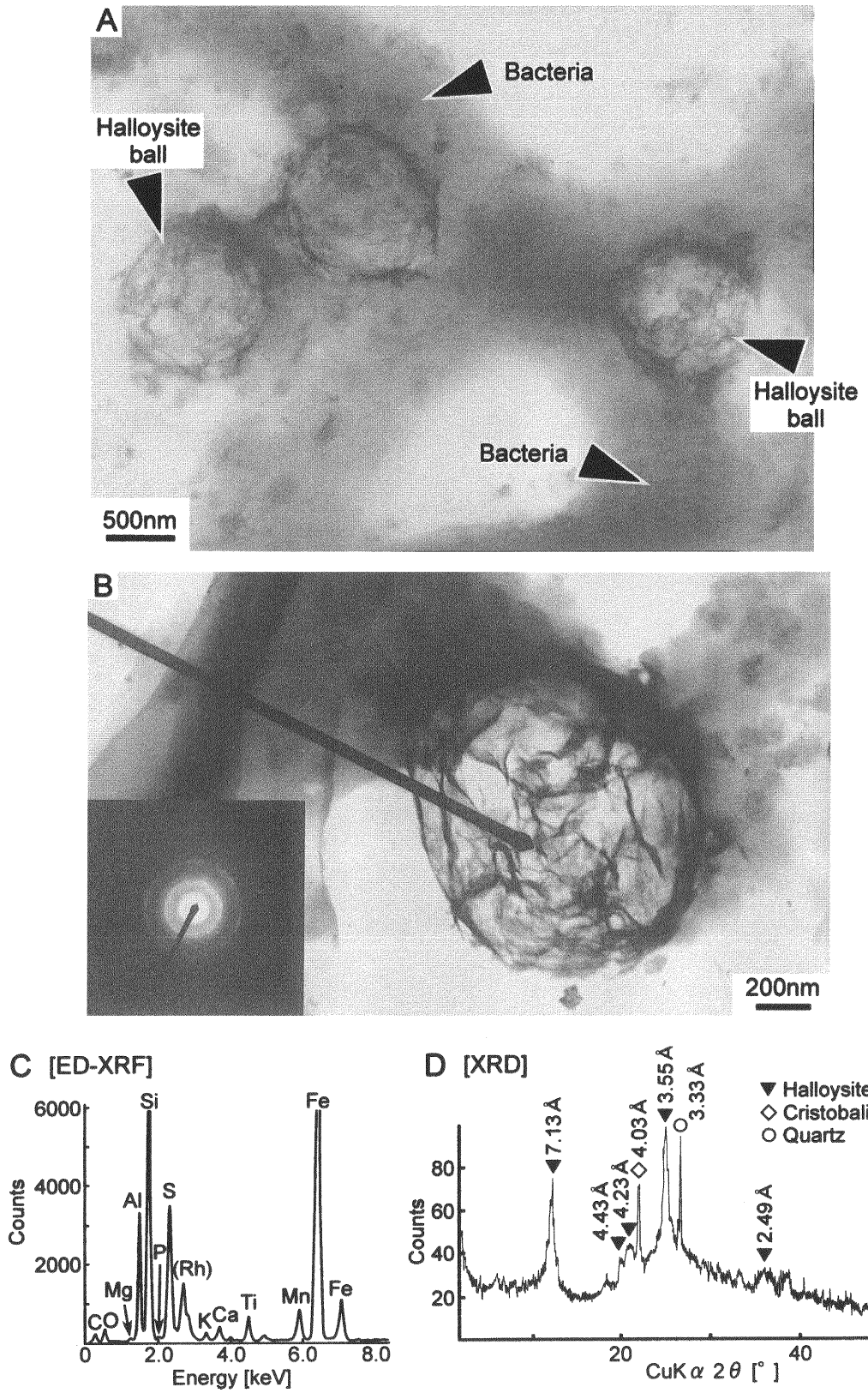

Figure 8 TEM micrographs of bio-halloysite ball associated with bacillus type bacteria (A) with electron diffraction spots and rings (B). ED-XRF (C) and XRD (D) analyses of a cultured biofilms indicated main components of Al, Si and Fe, identified halloysite (7.13 Å) formation (Tazaki and Asada 2002).

Further more bio-weathering experiment of feldspar particles of granite was conducted for 55 days, using natural river water collected from Kurobe River, Toyama, Japan, to compare with ion exchange water. The starting material of feldspars also collected from the same riverside, composed of Al and Si with traced of Na and Ca (Figure 9A, D). Characteristics of the river water as follows: pH 7.3, Eh 313 mV,

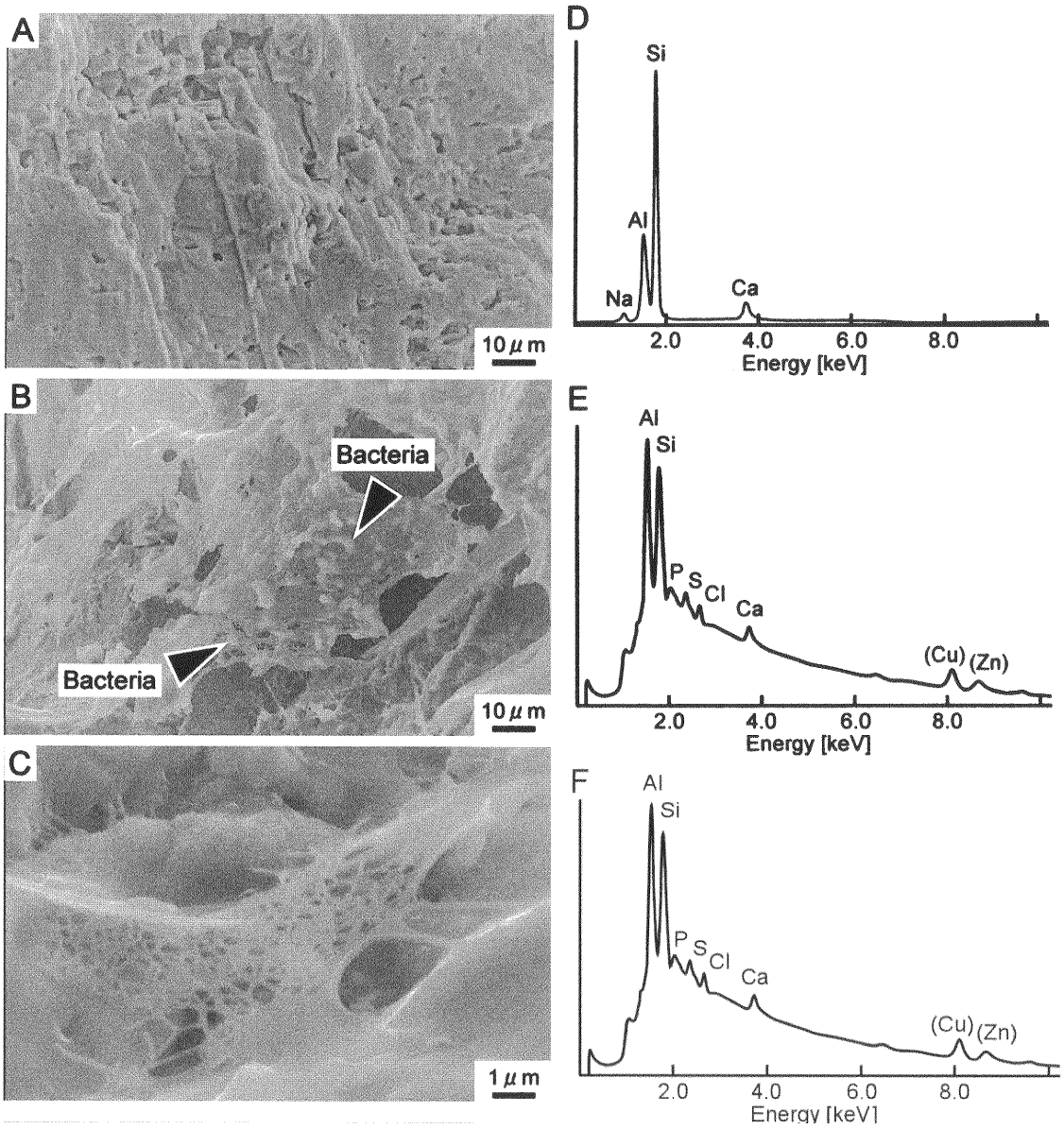

Figure 9 SEM-EDX micrographs of bio-weathering of feldspar grain with etch pits at initial stage (A), weathered surface with biofilms associated with abundant bacteria (B, arrows), formation of imogolite-like network structure covering on the surface (C). The EDX analyses of initial stage (D) and imogolite-like net work (E, F) indicate the ratio of Al to Si 1 : 3 to 1 : 1 (Morikawa 2001 unpublished).

EC 84 μS/cm, and water temperature 20 ℃ at the initial stage of experiment. The river water contains elements of Na 14.7, Mg 14.4, Al 1.6, Si 31.3, K 1.4 and Ca 36.8 wt% respectively, whereas an ion exchange water shows pH 5.5, Eh 322 mV, EC 0.7 μS/cm, and water temperature 21 ℃ (Morikawa 2002). After a 55 days aging, both experiments occurred adhesive substances of thin films composed of mainly Al and Si associated with Na, P, S, K and Ca, existing abundant microorganisms which form on the surface of feldspar (Figure 9B, E). The Al and Si ratio indicated about 1:1 suggest that kaolin minerals formed. More over imogolite-like minerals are also occurred as shown in Figure 9C, F. TEM of adhesive substances associated with thin thread network structure identified imogolite-like minerals after a few minutes ultrasonic treatment.

BIO-14Å CLAY MINERAL FORMATION

Bio-nontronite from Iheya deep-sea sediments

Okinawa Trough, Northwest of Iheya-Jima Island, Okinawa Prefecture, Japan, is one of the back arc basins developed along the western part of the Eurasian Plate associated with subduction of the Philippine Sea Plate along the Ryukyu Trench. Hydrothermal venting was observed at the Natsushima Seamount in the Iheya Basin of the Middle Okinawa Trough. Black manganese oxide covers the mound and yellowish sediments are distributed along the ridge. The yellowish sediments are composed of iron hydroxide, amorphous silica, and nontronite (Tawara et al. 1997).

The temperature of the discharge water is 2 - 3 ℃ higher than that of ambient seawater. A 40 cm long thermometer inserted into the mound recorded temperatures ranging from 20 to 50 ℃ at pH 5, Eh -110 mV and EC 47 mS/cm. Analysis of water showed methane content of about 200 ml/kg. Tubular and granular nontronite was identified by XRD, SEM-EDX, and TEM. The sediments contain mainly Si and Fe. Tubular materials collected from the vicinity of deep-sea smokers, uniformly coated with a film nontronite (Figure 10). After a 3 minutes ultrasonic treatment, bacilli bacterial colonies were observed. TEM micrographs of ultra thin-section sample show that flaky thin films of nontoronite covered on living bacteria (Figure 11). Nontronite of the flaky thin films has an orientation along with the direction of lipopolysaccharide exudation from surface of the cell wall. Nontronite crystallization occurs on the lip

polysaccharide molecules due to accumulation of Fe and Si from ambient seawater. Microbial polysaccharides would appear to have affected layer silicate formation (Ueshima and Tazaki 2001).

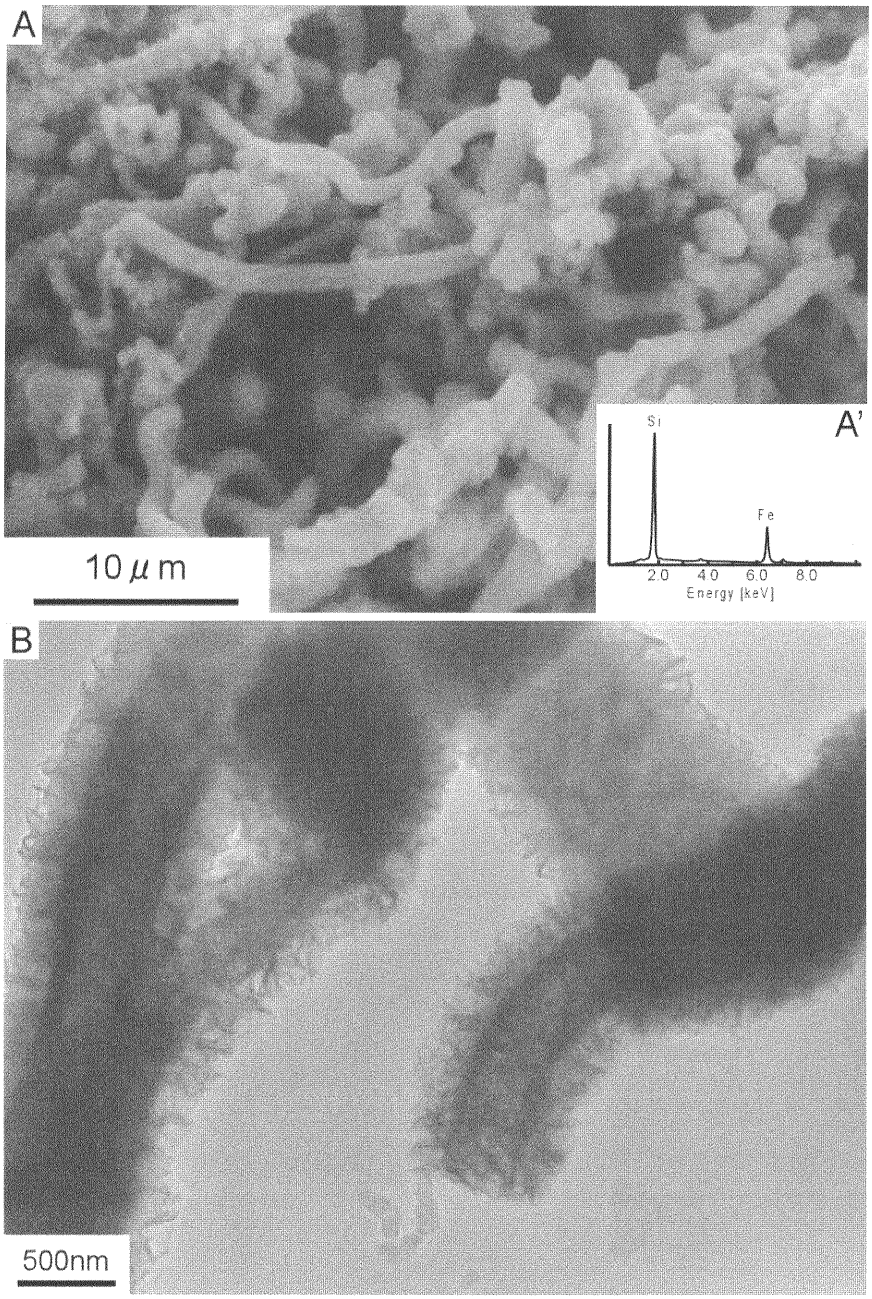

Figure 10 SEM-EDX (A, A') and TEM (B) micrographs of filamentous bacteria collected from Iheya deep sea floor. The sediments composed of Si and Fe whereas XRD pattern shows the 13.4 Å peak shifted to 17.2 Å by ethylene glycol treatment, identified as nontronite (Ueshima and Tazaki 1997).

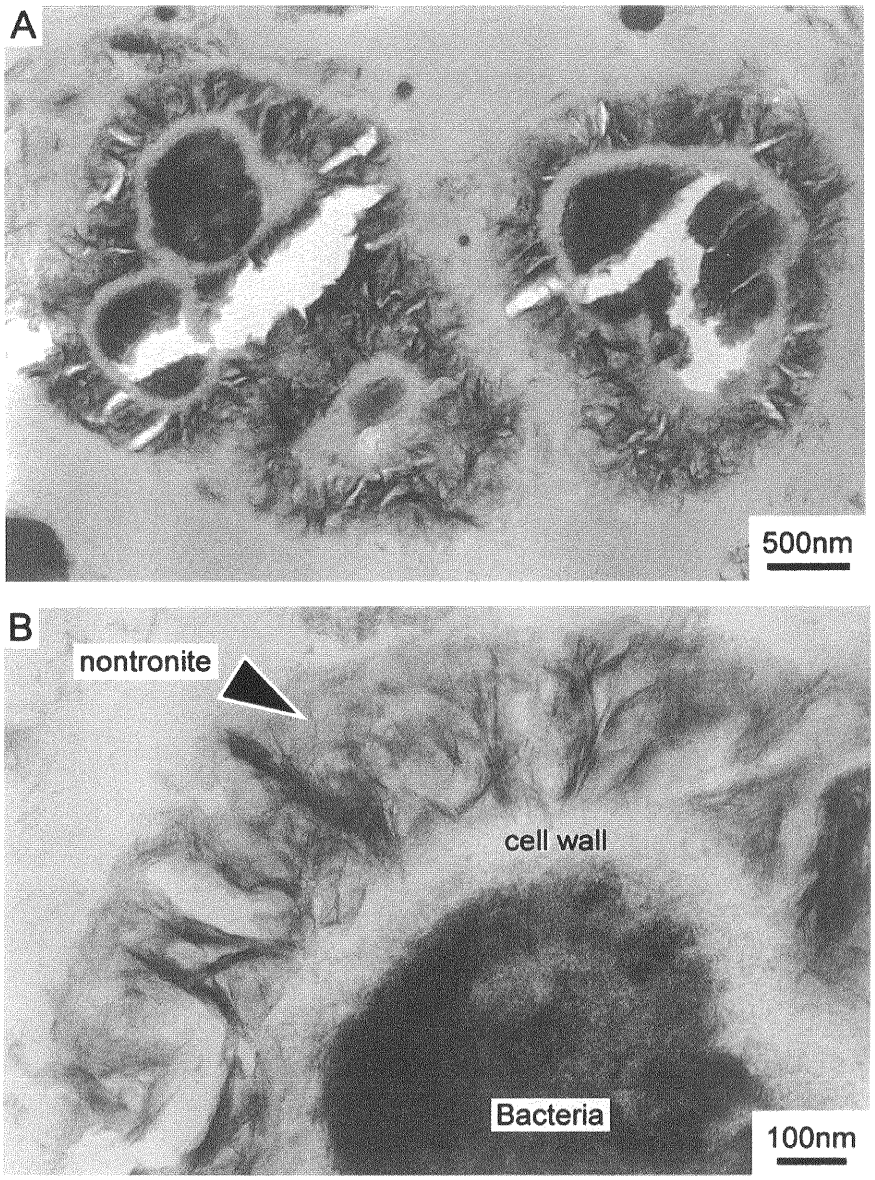

Figure 11 Ultra thin sectioned TEM micrographs of nontronite collected from Iheya deep sea floor, showing cross-sectioned filamentous bacteria covered by nontronite with radial growth (B arrow) surrounding cell wall of acidic polysaccharides.

Bio-smectite at Kasaoka bentonite pond

Interaction between bentonite and microorganisms can be observed at floc collected from the pond of Kasaoka bentonite mine, Okayama, Japan. The floc consists of bentonite, algae, diatom and bacteria under pH 7.7, EC 120 μS/cm, DO 12 mg/l, and water temperature 27 ℃ conditions. XRD data of the floc show the 15.3 Å peak expanded to 17.2 Å by ethylene-glycol treatment (Ueshima et al. 2000). Epifluolescence micrograph of the thin-sectioned floc with DAPI staining shows yellow bentonite, blue

DNA, and red chlorophyll of living algae. SEM-EDX of the floc after a 30 minutes ultrasonic treatment indicated the elements of Al and Si associated with P, S, Cl, K and Fe in algae (Figure 12A, A'). TEM image of the algae after a 30 minutes ultrasonic treatment and the electron diffraction are corresponded to smectite (Figure 12B and inset).

The biosynthesis and biomineralization studies open up the possibility of a new world, such as bioremediation of polluted environments, new clean energy production, new mineral deposition and bio-medical technologies (Tazaki 2001).

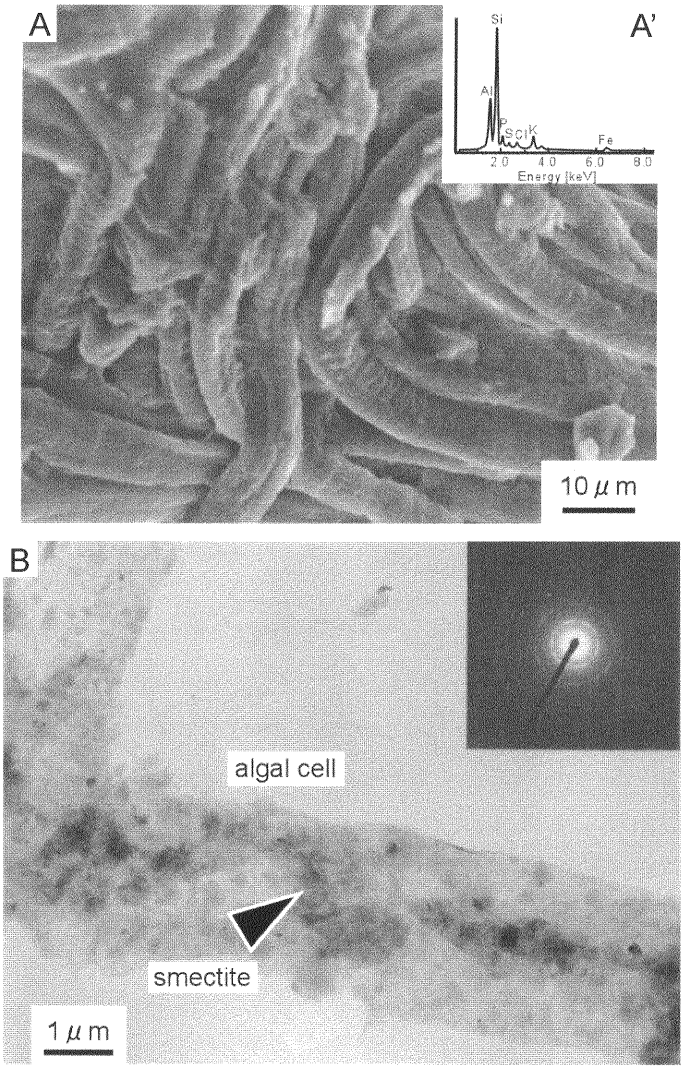

Figure 12 SEM-EDX (A, A') and TEM (B) micrographs of bio-bentonite in the floc after a 30 minutes ultrasonic treatment. The sample was collected from the pond of Kasaoka bentonite mine, Okayama, Japan. Electron diffraction pattern of the algal cell is correspondent to smectite (inset in B) (Ueshima et al. 2000).

CONCLUSIONS

We now know that, in the Earth's surface environment, microorganisms are universal in biofilms and microbial mats. And we now know that clay minerals are synthesized in complex bio-mediated processes. Understanding microbial mineral synthesis is essential to understanding our environment. For example, in pond, hot springs, weathered feldspar, deep sea floor, and mining area, we have found many kinds of clay minerals being simultaneously synthesized, outside and inside the living bacterial cells with polysaccharides. The biofilms that consisted of clay aggregates, each one of which contained bacteria, phyllosilicates and grains of iron oxides, are developed. The clays may serve as carbon shuttles. We have various micro techniques available to study small microorganisms and clay minerals supporting our life environmental systems. Biosynthesis and biomineralization of clays lead to the understanding of bioremediation of polluted environment. Moreover microbial mats will certainly play an important role in future ecosystems.

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