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Microbial controls on As-mineralogy in pH 7~8 natural solutions

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Abstract: A series of simple experimental systems combining seven different As-minerals with natural Fe-rich underground water (pH 7) was established in search of clues to arsenic bioremediation. After one year of aging, arsenic-tolerant microorganisms were detected by means of an epifluorescence microscope, SEM-EDX and TEM. Direct observations revealed that a mutual relationship between bacterial colonies and As-minerals occurred in the biofilm. The most arsenic-tolerant microorganism was found on the surface of realgar (AsS) and the second one was found on the surface of arsenolite. A symbiotic relationship between free living and adhering bacillus, coccus, and filamentous types of bacteria occurred under conditions of pH 8, Eh 200~400 mV, 25~28℃, and high concentration of dissolved As after one year of aging. These microorganisms survived and proliferated in the As-mineral-water systems without any kind of supplementary nutrients. There were two methods of staining used in this study: DAPI and CFDA stains. The DAPI stain method, in which DNA or RNA fluorescences blue under ultra violet light (365 nm), indicated the total bacterial abundance with in the colony. The CFDA stain method indicated enzymatically active bacteria on the mineral surface. The significant roles of symbiotic arsenic tolerant bacteria in the system were enhanced by their adaptation to conditions of pH 7~8 and high concentration of dissolved As ion. SEM images illustrated very high cell densities on the surface of realgar. Colonization occurred on AsS surfaces in natural solutions during a one-year culture period. These results might be important for solutions at circum-neutral pH in which abundant cell-mineral surfaces are able to adsorb metals from solution. The results might also indicate some of the effective factors for arsenic detoxification in non-marine or brackish waters.

Key words: Arsenic, As-minerals, Electron microscopy, Epifluorescence microscope, Tolerant Microorganisms.

Introduction

Arsenic, an undesirable element, is commonly present in processing solutions as arsenite (i.e., As^{3+}) or arsenate (i.e., As^{5+}) under differing chemical conditions.

Inorganic arsenic is subject to biomineralization and the possibility of bioremediation has been widely studied. Arsenite and arsenate are toxic themselves, and indeed are more toxic than some methylarsenic compounds. Monomethylarsine (CH_3AsH_2), dimethylarsine ($(CH_3)_2AsH$), and arsine (AsH_3) are formed in soils as well as in marine and fresh waters, and in microbial cultures (Alexander, 1994).

The ground water in some parts of Bangladesh contains high levels of arsenic. Arsenic concentration shows good correlation with vanadium and copper, which is suggestive of porphyrin compounds in the sediments. Total sulfur measurements from both Samta

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and Dauli villages in Bangladesh, show lower TS/TOC ratios than the average for normal marine samples. The ratios suggest that these sediments were deposited under non-marine or brackish conditions (Ishiga *et al*., 1999).

Furthermore, in Hazigonj, Bangladesh, vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$), an iron phosphate mineral, has been identified in biomats forming around an As polluted tube-well, which is used for drinking water (Islam *et al*., 2003). The water chemistry, pH 7.2~7.4, EC 1.7~2.8 mS/cm, and high concentration of NaCl (33~40 wt%), suggests brackish conditions. The biomats are composed mainly of metabolically active microorganisms such as coccus, bacillus and filamentous types of bacteria and algae. Vivianite may contain a small amount of As by isomorphic substitution for P, resulting in a solid solution of symplesite (Islam *et al*., 2003).

Microbial densities can be as high as 10^{12}cells/g in slimes and biofilms. Populations in soils typically range from approximately 10^6 to 10^9 microbial cells per cm^3. Mineral surface may be populated by cells at densities that approach, and even exceed, one cell/μm^2. Banfield and Welch (2000) provided SEM image illustrating very high cell densities (>1/μm^2) on an arsenopyrite surface. Colonization occurred on FeAsS surfaces in natural solutions after a period of a few months.

Microbial reactions may mobilize arsenic from the solid to the aqueous phase, resulting in contaminated drinking water (Oremland and Stolz, 2003). That condition resulting from microbially-controlled process confirms the previous finding that the microbial mats around groundwater tube wells in Bangladesh accumulated high concentration of As due to microbial activity. Likewise, a mineralogical study revealed that bacteria can accumulate Fe and As to form lollingite ($FeAs_2$) though the type of bacteria is not yet known (Nagai *et al*., 2001). Some microorganisms can survive in highly toxic environments and can reduce the concentration of aqueous heavy metals in well water to better than acceptable levels through biogeochemical processes, such as arsenic oxidizing, respiring, mobilizing and metabolizing. Hence, it is reasonable to assume that bacteria may be able to purify As-polluted water. Certain prokaryotes utilize arsenic oxyanions to generate energy, either by oxidizing or by respiring arsenate. These microbes are phylogenetically diverse and occur in a wide range of environments, such as marine, non-marine, and brackish conditions.

In this study, arsenic-tolerant microorganisms were found in simple experimental systems using seven kinds of As-minerals in batch cultures consisting of Fe-rich natural ground water as the liquid medium. After one year of aging, the As-tolerant microbial colonies could be observed by epifluorescence microscopy, SEM-EDX and TEM. These microbes may be useful in bioremediation. The purpose of this paper was to provide some insights into how microorganisms affect the chemical and biological characteristics of their surroundings to illustrate in more detail the intimate connections between biological and mineralogical processes.

Materials and Methods

Experimental design

Seven As-minerals (arsenolite, native arsenic, realgar, orpiment, arsenopyrite, enargite and tennantite), were employed for the experiments (Table 1 and 2). The laboratory experiments were designed in a static batch culture. There were seven experimental systems (with the introduction of As-minerals) and one experimental system as control (without the introduction of As-minerals). A series of 100 ml carboys with caps were used for this experiment (Fig. 1). Each carboy contained 80 ml of ground water with high Fe content (total Fe 4.7 ppm) which was collected from the Onma Formation in Kanazawa University Campus, Ishikawa, Japan. Cultures were then inoculated with 1 g of coarse grain of As-minerals and incubated at room temperature for one year. Samples were removed and cultures were further monitored during the experimental period, and pH, Eh (electrical potential versus the standard hydrogen electrode), and WT (water temperature) were measured for experimental water samples using a portable inspection meter made by HORIBA. The ground water in the experimental systems with As-minerals showed pH 3.1~8.7, Eh 65~549 mV, and WT 25~28℃ during the one-year experimental period. The chemical composition of the ground water is shown in Table 1.

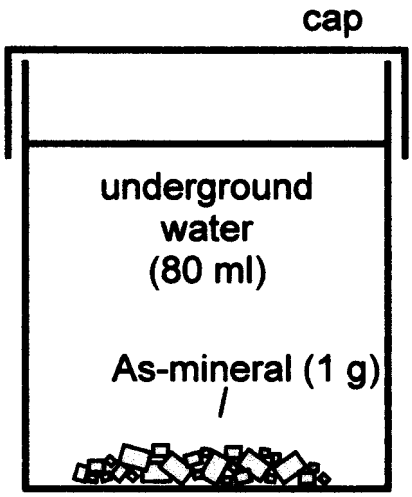

Fig. 1. Biomineralogical experimental system using As-minerals (1 g) and ground water (80 ml) in 100 ml carboy with a cap.

Table 1. Water chemistry of As-mineral experimental systems. Note that pH values after one year are related to the logarithm of dissolved As in the ground water after one year of aging (Fig. 9). As concentrations were analyzed by ED-XRF, showing integrated intensity.

Collected underground water on June 28, 2002					
pH	6.7	Total Fe	4.7	Na^+	14.4
Eh	24 mV	Ca^{2+}	14.5	K^+	1.5
EC	0.47 mS/cm	Mg^{2+}	10.6	PO_4^-	0.2
WT	16 ℃	Mn^{2+}	1.5		(ppm)

Started experiment on June 28, 2002
underground water 80ml + As-mineral 1g

pH

Sample No.	1 control	2 Arsenolite	3 Native As	4 Realgar	5 Orpiment	6 Arsenopy	7 Enargite	8 Tennan
2002/7/1	8.4	8	8.1	7.6	7.6	7	6.5	7.9
2002/7/5	8.6	8.4	8.5	8.4	7.7	7.1	7.7	7.4
2002/7/9	8.6	8.5	8.6	8.6	7.7	8.3	7.9	8.5
2002/7/12	8.6	8.4	8.7	8.6	7.7	8.4	8	8.6
2003/5/6	8.4	5.9	7.4	7.7	3.1	8.2	4.8	8.4

Eh(mV)

Sample No.	1 control	2 Arsenolite	3 Native As	4 Realgar	5 Orpiment	6 Arsenopy	7 Enargite	8 Tennan
2002/7/1	336	191	280	105	74	351	462	373
2002/7/5	366	155	185	116	261	265	377	316
2002/7/9	388	121	156	84	251	192	373	323
2002/7/12	346	95	192	65	251	225	354	319
2003/5/6	531	376	381	193	549	364	536	356

WT(℃)

Sample No.	1 control	2 Arsenolite	3 Native As	4 Realgar	5 Orpiment	6 Arsenopy	7 Enargite	8 Tennan
2002/7/1	25	25	25	25	25	25	25	25
2002/7/5	26	26	26	26	26	26	26	26
2002/7/9	28	27	26	28	27	27	27	26
2002/7/12	27	27	27	27	27	27	27	27
2003/5/6	25	25	25	25	25	25	25	25

Dissolved As ion in the underground water after one year (by ED-XRF)

Sample No.	1 control	2 Arsenolite	3 Native As	4 Realgar	5 Orpiment	6 Arsenopy	7 Enargite	8 Tennan
Integrated Intensity	n.d.	350000	440000	30000	50000	6100	n.d.	190

n.d.; not detected

As-minerals (initial conditions)

Seven kinds of As-minerals were purchased from Iwamoto Mineral Company in Tokyo, Japan. The mineralogical composition of As-minerals were analyzed by X-ray powder diffractometer (XRD; Rigaku RINT 2000) with CuKα generated at 40 kV and 30 mA using the $2\theta / \theta$ method with a scan speed of 2°/min. The chemical composition of As-minerals prior to the experiment was determined by energy dispersive X-ray fluorescence spectrometer (ED-XRF; JEOL JSX-3201

Table 2. XRD mineral composition and ED-XRF chemical analysis of the seven As-minerals used as starting materials for the experimental systems.

Sample No.	2	3	4	5	6	7	8
Mineral Name	Arsenolite	Native Arsenic	Realgar	Orpiment	Arsenopyrite	Enargite	Tennantite
Mineral Composition by XRD	Arsenolite(◎) [As_2O_3] Native Arsenic(▲) [As]	Native Arsenic(◎) [As] Stibarsen(▲) [AsSb] Arsenolite(▲) [As_2O_3]	Realgar [AsS]	Orpiment [As_2S_3]	Arsenopyrite(◎) [FeAsS] Quartz(▲)	Enargite(○) [Cu_3AsS_4] Pyrite(○) [FeS_2] Quartz(▲)	Tennantite(◎) [$(Cu, Fe)_{12}(AsS_3)_4S_{0-10}$] Quartz(▲) As-Sb alloy (?)
Chemical composition by ED-XRF							
Al	–	–	–	–	–	0.6	–
Si	–	–	–	–	1.7	3.1	3.9
S	–	–	22.6	31.8	13.6	32.2	16.3
Ca	–	–	1.0	–	0.1	–	–
Cr	–	–	–	–	tr.	tr.	–
Fe	0.1	–	–	0.4	32.8	23.3	3.6
Cu	–	–	–	–	1.0	27.2	47.6
Zn	–	–	–	–	0.2	–	2.5
As	97.4	91.0	76.5	66.8	50.6	12.2	9.4
Sb	2.5	9.0	–	1.1	–	1.3	14.6
Hg	–	–	–	–	–	–	2.1
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

(wt%)

◎; abundant, ○; common, ▲; a little, ?; possible, tr.; trace, –; not detected.

Table 3. Optical light and epifluorescence microscopic observation of the experimental As-minerals after one year of aging showing fluorometric enumeration of total bacteria and their diversities in groundwater, using DAPI and CFDA stains.

Sample No.	1 control	2 Arsenolite	3 Native Arsenic	4 Realgar	5 Orpiment	6 Arsenopyrite	7 Enargite	8 Tennantite
Morphology of mineral particles and precipitation	Brown precipitates	Brown mineral particles and precipitates	Brown mineral particles and precipitates	Red mineral particles and precipitates	Rounded aggregates of about 50 μm in diameter	Black mineral particles in the co-aggregation with the precipitates	Rounded aggregates of about 50 μm in diameter	Dark green mineral particles and precipitates
Diversity of microorganisms	Small cocci	Cocci, short and curved rods	Short rods and small cocci	Cocci, short and curved rods, filaments (10–20 μm in length)	Small cocci	Short rods and filaments (10–12 μm in length)	Short rods	Short rods and filaments (10 μm in length)
Total bacterial abundance (DAPI)	tr.	++	tr.	+++	tr.	+	+	+
Abundance of active bacteria (CFDA)	tr.	++	tr.	+++	tr.	+	+	tr.
Solble As after one year	n.d.	350000	440000	30000	50000	6100	n.d.	190

Frequency of occurrence: n.d.; not detected, tr.; trace, +; rare, ++; common, +++; abundant.

using RhKα) (Table 2). Therefore, Cl concentration could not identified, because of overlap Rh peak each other. The samples No. 4 and 5 were single components, whereas the other samples were associated with quartz, pyrite and others (Fig. 2).

Determination of concentration of As in liquid culture after one year of aging

Relative concentration of As in liquid culture after one year of aging was determined by ED-XRF analysis. 100 μl of each solution was mounted and dried on mylar film. Approximate concentration of As was determined by comparing the peak areas based on the graphical integration of intensity. Samples No. 2, 3, 4 and 5 were soluble in the ground water and showed integrated intensity from 50000 to 350000 (Table 1 and 3, below).

Optical microscopic observation

To examine microorganisms in solution, an aliquot of the culture liquid was transferred to a glass slide, stained with DAPI (4', 6-diamidino-2-phenylindole), and observed with an optical microscope, using a standard phase-contrast and epifluorescence microscope (Nikon EFD 3) to identify the presence and variety of microorganisms in the liquid cultures. Additionally, CFDA (5-carboxyfluorescen diacetate) stain was also employed to observe the presence of enzymatically active bacteria on the mineral surfaces. The function of CFDA stain was to detect esterase activity and show viability of the bacterial cells via enzymatic activity and membrane integrity seen as yellow fluorescence under blue light (450~490 nm), while DAPI stained the DNA in bacterial cells yielding blue fluorescence under ultraviolet light (365 nm).

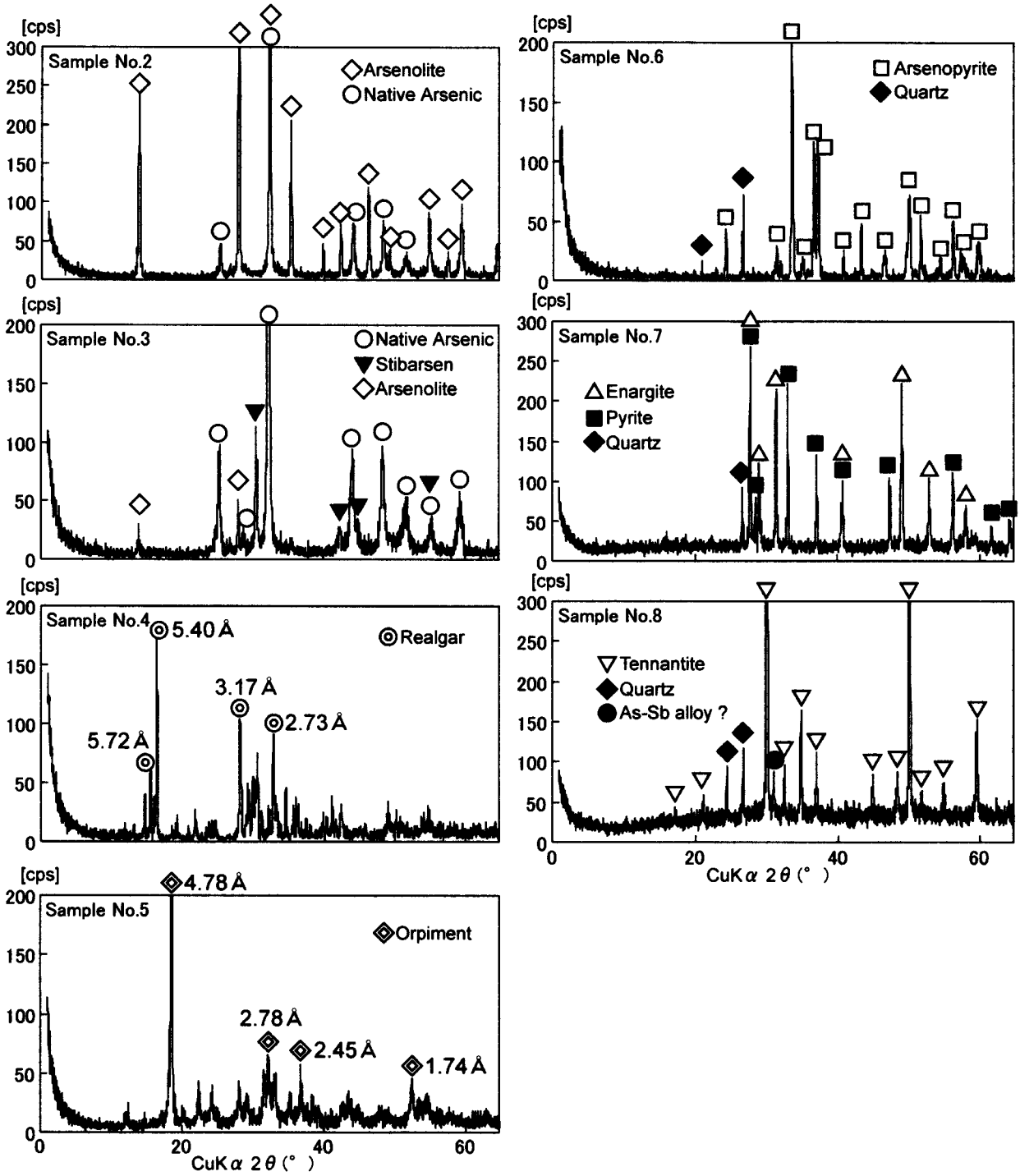

Fig. 2. X-ray powder diffraction plots of the initial As-mineral samples. The mineral compositions agreed with ED-XRF chemical data in Table 2.

Scanning electron microscopic observation

A scanning electron microscope equipped with an energy dispersive X-ray spectrometer (SEM-EDX) was used to observe the microorganisms on the surface of As-minerals and analyze their chemical composition. A freeze-drying method was used for sample preparation (Suzuki *et al*., 1995). The suspension samples were fixed using 2.5 % (vol/vol) glutaraldehyde, pipette-drawn, and mounted on a 0.22 μm membrane filter, further washed and fixed with t-butyl alcohol, frozen in liquid nitrogen, and dried using low-vacuum SEM. After freeze-drying, the samples were transferred to a brass-stub with double-sided adhesive carbon tape, coated with carbon and then observed using a scanning electron microscope (JEOL JSM-5200 LV), equipped with an energy dispersive X-ray spectrometer (PHILLIPS EDAX PV 9800 EX).

Transmission electron microscopic observation

Transmission electron microscopy (TEM; JEOL JEM-2000 EX) was used for the observation of free-living microorganisms and As-mineral grains. Selected area electron diffraction (SAED) analysis was also used to identify minerals. One drop of the suspension was pipette -drawn and mounted on the micro grid for observation. The accelerating voltage used was 120 kV.

Results

Characteristics of the starting materials

X-ray powder diffraction analysis (XRD)

The seven kinds of As-minerals (arsenolite, native arsenic, realgar, orpiment, arsenopyrite, enargite, and tennantite) were identified by XRD (Fig. 2). Samples No. 4 and 5 mostly showed a single mineralogy, whereas the other samples contained small amounts of other minerals, such as native arsenic, stibarsen, arsenolite, quartz, pyrite, and As-Sb alloy, respectively. The results of mineralogical composition by XRD confirmed the results of chemical composition by ED-XRF (Table 2).

Energy dispersive X-ray fluorescence analysis (ED-XRF)

The chemical compositions of As-minerals in this study are summarized in Table 2. It can be noted that As dominated the elemental compositions of As-minerals, except for enargite and tennantite, which contained only 9~12 wt% As associated with Sb (1.3~14.6 wt%). Generally, these As-minerals contained high amounts of As ranging from 50.6 to 97.4 wt%, and most of them contained high amounts of sulfur, ranging from 14 to 32 wt%. Arsenolite and native arsenic did not contain sulfur.

Optical microscopy, SEM-EDX and TEM observations of bio-mineralogical experimental products after one year of aging

Optical microscope observation

Optical micrographs of the As-mineral samples showed the presence of high-density bacterial colonies on As-mineral grains (Fig. 3), except for the control (No. 1). Epifluorescence micrographs revealed that numerous living bacterial cells were present. DAPI stained epifluorescence micrographs fluoresced blue, indicating the presence of DNA in bacterial cells. A large number of colonies of various morphologies were observed after one year of aging in the experimental system including the control. However, the greatest total bacterial abundance (by DAPI stain) and enzymatically active bacteria (by CFDA stain) was observed in experimental sample No. 4 (realgar), showing numerous enzymatically active bacteria on the mineral surface (Fig. 4), followed by samples in the following sequence: No. 2 (arsenolite), No. 6 (arsenopyrite), No. 7 (enargite), No. 8 (tennantite), No. 3 (native arsenic), No. 5 (orpiment), and No. 1 (control) (Table 3). Under optical microscopic observation, the colored precipitates in association with the aggregation of these As-minerals and ground water was as follows: arsenolite and native arsenic precipitate which appeared to be brown mineral particles; realgar also formed a precipitate, which appeared to be red mineral particles; arsenopyrite was associated with a black mineral aggregation; orpiment and enargite had rounded aggregates of approximately 50 μm in diameter; tennantite also precipitated which appeared to be dark green mineral particles; while the control was associated with a brown precipitate. Diverse bacteria were associated with the cultures; they were predominantly composed of cocci, short rods, curved rods and filaments with dimensions of 10~20 μm in length (Figs. 3, 4, and Table 3).

SEM-EDX observations

SEM observation of As-mineral grains in the experimental system after one year of aging revealed the presence of bacterial colonies on the surface of realgar (Fig. 5A). The colony was composed of coccus, bacillus and filamentous types of bacteria, and clearly appeared to be a biofilm attached to the surface of the realgar (Fig. 5A). The lamella structure of realgar was also seen in the background (Fig. 5Ab), while the exopolymer matrix of biofilms (Fig. 5Aa) containing large amounts of polysaccharides occurred as a monolayer between the bacterial colonies and realgar. EDX analysis of this matrix in Fig. 5A (arrow b) confirmed this result, showing the matrix to be inorganic, mainly composed of As and S. Biofilms contained Al, Si, P, Ca, Mn, and Fe with a large amount of As and S (Fig. 5Aa). Furthermore, the EDX spectrum of the lamella structure of realgar indicated a low background with chemical compositions of As and S (Fig. 5Ab). These observations suggest that enzymatically active adhering-bacteria were capable of accumulating As compounds. In addition, a microphotograph of biofilms on the surface of realgar also showed an entangled colony of various microorganisms (Fig. 5B). It seemed that nm-sized uniform granules of secondary by-product of realgar which were composed of As, Si, Cl, Ca, and Fe were mixed with microorganisms (Fig. 5C). It is important to note that the nm-sized uniform granules were composed of large amounts of Cl, Si, and Fe.

TEM observations

Transmission electron microscopy provided more details and revealed the precise location of the As and Fe precipitation or accumulation on cell surfaces, and throughout the extrapolymeric slime of the biofilm. TEM

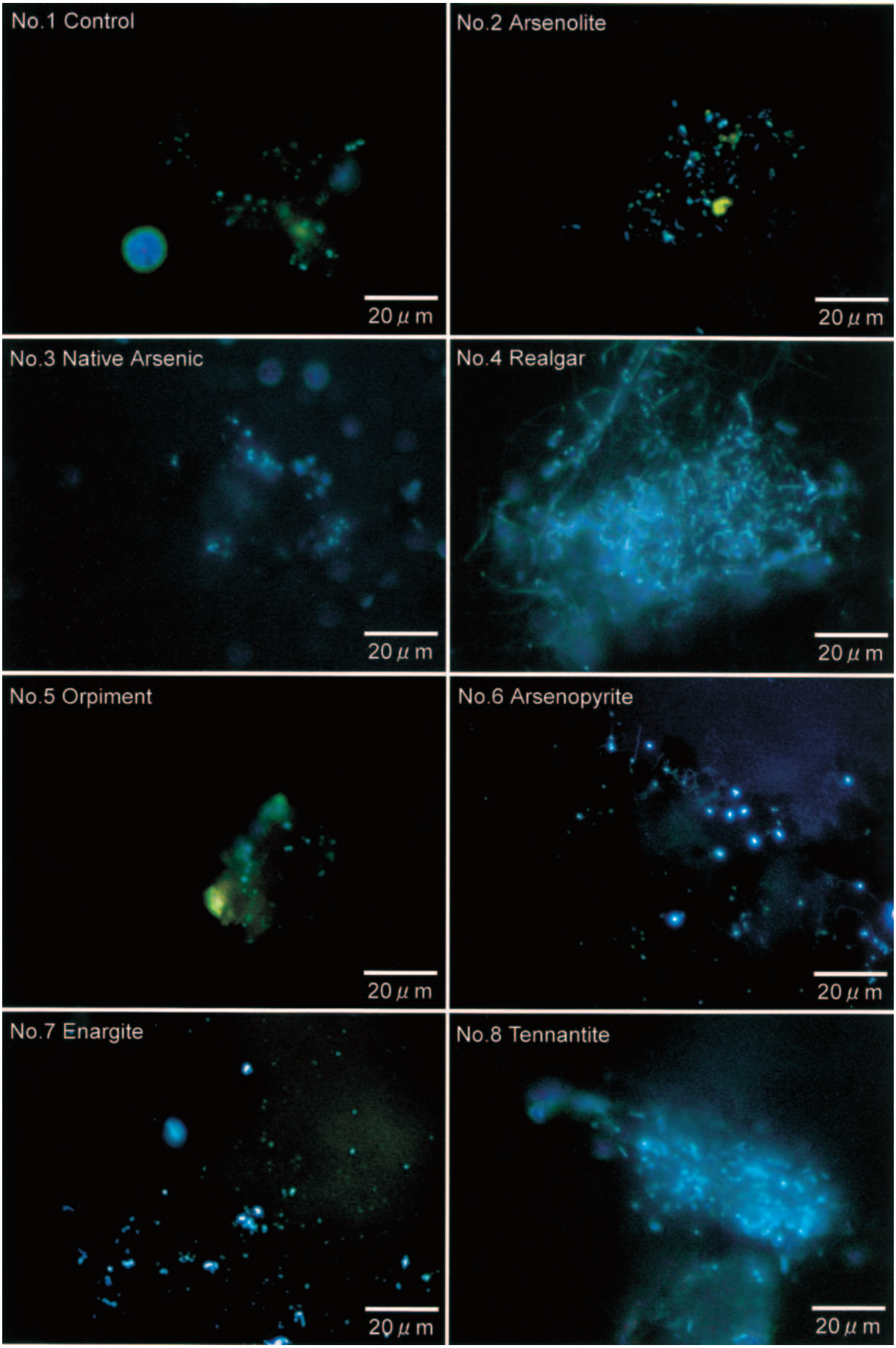

Fig. 3. Optical epifluorescence micrographs of As-minerals after one year of aging showing numerous living bacteria on the surface of As-minerals. The cells show blue fluorescence in DAPI-stained microbial films.

observations of arsenolite (sample No. 2) showed filamentous bacteria attached to abundant opaque granules (Fig. 6A), while around the granules, thin biofilms were connected to each other like a spider web (Fig. 6B). The spider web consisted of an envelope of slime made up of extracellular polymeric substances (EPS), often polysaccharides, surrounding and was excreted by the consortium of microorganisms that made up the biofilms (Figs. 6A and B). The dominance of coccus and bacillus types of bacterial colonies on the surface of realgar was also observed by TEM (Fig. 7). The free-living bacteria (short and long rods) were observed in realgar. A great abundance of organic materials were also observed around the bacteria, apparently coating the cell wall to form a fibrous matrix (Figs. 7A, B and C). Along the side of one cell, a cluster of mineral particles could be seen attached to the surface of the cell (Fig. 7A arrow), while another cell was seemingly surrounded by mineral particles (Figs. 7B and C). It was difficult to differentiate between mineral granules and coccus type bacteria by using SEM images (see Fig. 5C). Yet, by TEM images, the differentiation of coccus type bacteria and granules of Fe−As appeared to be clear (see Fig. 7C). Furthermore, the size of the coccus type bacteria was about 500 nm in diameter. The thin film with a high-density of bacillus type of bacteria was about 1~5 μm in length and 100~500 nm in width (Fig. 7B). Most of the bacteria possessed a thin film of about 50 nm in thickness, forming a coating membrane that can be seen on the surface. On the surface of As-minerals, coccus type bacteria formed spherules ranging between 100 and 500 nm in diameter. The spherules were electron opaque (Fig. 7C). Coccus type bacteria were also heavily encapsulated with spherules. Filamentous microorganisms grew up to 200 μm in length and 5 μm in width (Figure not shown). The surface of realgar was covered with a network structure of granular materials (Fig. 7D), suggesting the alteration of the As-minerals into As−Fe biominerals during the culture period (Fig. 7E). These filamentous micro-organisms were enzymatically active bacteria, fluorescing yellow after CFDA staining. The EDX analysis indicated not only As content but also Si, S, Ca, and Fe contents, associated with minor amounts of Cl.

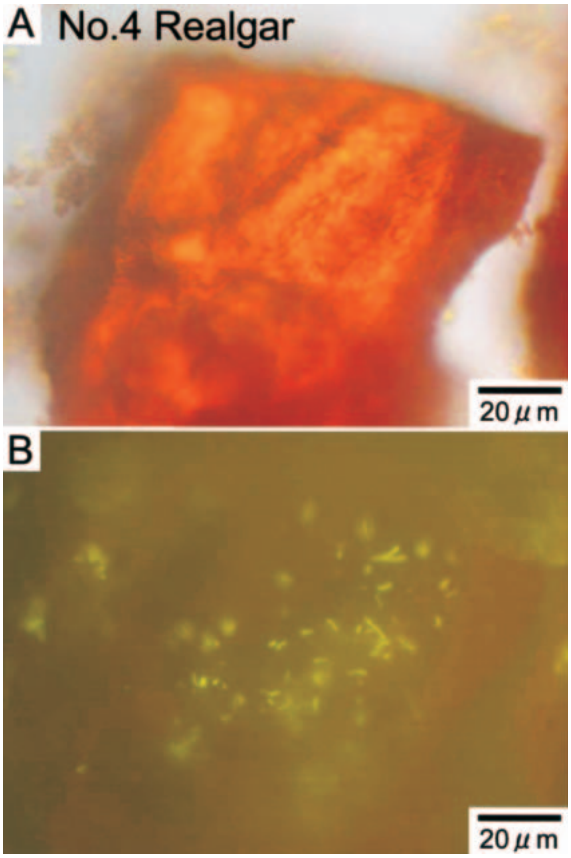

Fig. 4. Optical light (A) and epifluorescence (B) microscopic images of realgar (sample No. 4) after one year of aging using CFDA stain, showing numerous enzymatically active bacteria on the mineral surface.

Discussion

Bacterial growth in the As-mineral-water system

Exposure to different As-minerals for one year had different effects on bacterial abundance (observed by DAPI staining) and on the enzymatically active bacteria (observed by CFDA staining) (Figs. 3, 4 and Table 3). Realgar-As-mineral (No. 4) was the most appropriate experimental system for measuring either microbial diversity or abundance of active bacteria in this study. Not only the greatest total abundance of microorganisms and active bacteria but also the greatest microbial colonization of As-mineral surfaces were found in experimental system No.4 (Realgar) (Fig. 4A). Likewise, the presence of As-minerals aided bacterial growth in all experimental systems, with the exception of the control (No. 1). Thus, prolonged (one year) exposure to As-minerals appeared to affect the growth of microorganisms. This implies that prolonged exposure to As-minerals does not inhibit the bacterial respiratory function, which is a reasonable conclusion in light of the fact that As-minerals are used in oxidative phosphorylation (Nriagu, 1994). Our results, therefore, suggest that As-minerals were not toxic or a limiting factor for bacterial growth under conditions of oxidation redox potential (Eh; 200~400 mV), neutral-alkaline pH (7~8), and moderate temperature (25~28℃) (Table 1, Figs. 8 and 9). Previous work by Cullen and Reimer (1989) demonstrated that bacterial growth can take place in the pH range of 6.1~9.4, and the drop in pH we observed during bacterial growth as a result of the formation of arsenate also corroborated our results.

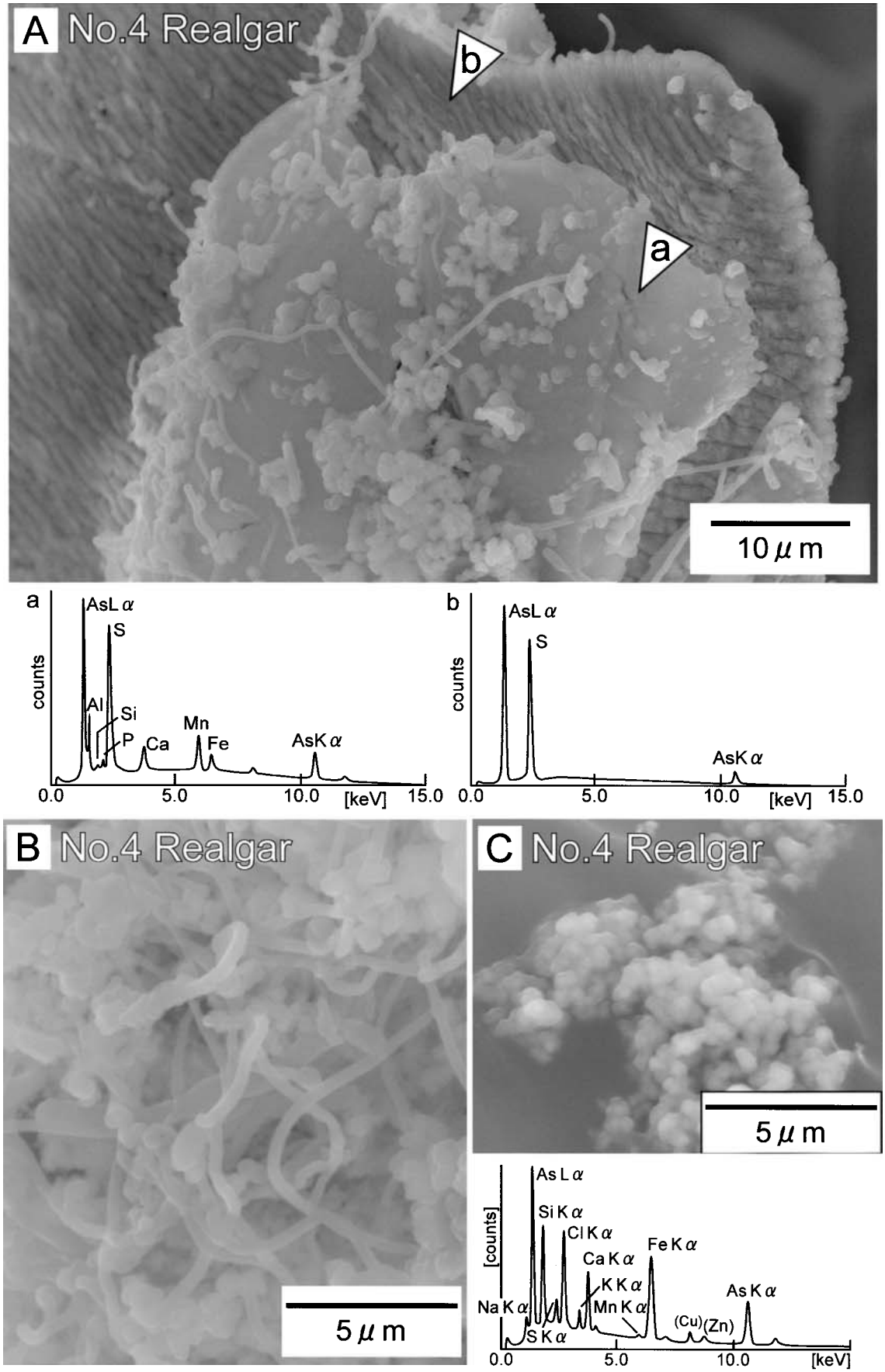

Fig. 5. SEM-EDX micrographs of realgar (sample No. 4) after one year of aging showing biofilms (A-a) on the surface of the original lamellar structure of realgar (A-b). The abundant microorganisms inhabited the biofilms after one year of aging (B). The uniform sized granules contain mainly As, Si, Cl, Ca, and Fe, with traces of Na, S, K, and Mn (C and inset).

Chemical composition of mineral surface

On the basis of the elemental and mineral compositions (Table 2), realgar (AsS) (No. 4) and arsenolite (As_2O_3) (No. 2) were the most favorable for microbial growth and colonization (Table 3). A possible explanation for this was that the availability of certain elements (such as Fe and S) enhanced bacterial activity which resulted in arsenic detoxification. This result agreed with previous findings, demonstrating that the rate of arsenate reduction in cultures having both arsenate and sulfate was always faster than that of sulfate reduction, independent of the relative concentrations of arsenate and sulfate or of the substrate history of the inoculum (Newman *et al*., 1997). Another study also showed that greater bacterial growth on 1 and 5 mM As (V) occured in the presence of S(VI) than without (Newman *et al*., 1997). In addition, DIRB Shewanella alga BrY, an organism that cannot respire As(V), was able to mobilize As(V) from the solid-phase ferric arsenate mineral scorodite by reducing Fe(III) to Fe(II) (Cummings *et al*., 1999). As(V) reduction is therefore not a prerequisite to arsenic solubilization from $FeO(OH)_x$, even though an increase in soluble As(V) concentrations occurs, provided DIRB were not also capable of reducing As(V) (Cummings *et al*., 1999). Arsenic is commonly present in processing solutions as arsenite (As^{3+}) or arsenate (As^{5+}). The larger arsenate ions seem to be accommodated in the tetrahedral sites by the expansion of the unit cell and by deficiencies in adjacent Fe−O(OH) octahedral sites (Paktunc and Dutrizac, 2003).

Micromorphology of mineral surface

In terms of bacterial morphology, the experimental system with realgar (No. 4) had greater abundance of bacteria than other minerals, and tended to have more filaments (Fig. 5). Bacteria found in the experimental systems using As-minerals included a variety of rods, cocci and filaments. Conditions favoring the growth of filamentous bacteria were especially present in experimental systems No. 2 and 4. In these experiments, as shown in Figs. 3~7, the relative abundance of filamentous and nonfilamentous bacteria (such as cocci and rods) presumably related to their relative growth rates when exposed to different As-minerals. According to the SEM-EDX analysis (Fig. 5A), the formation of thick biofilms occurred in systems containing As, Al, Si, P, Ca, Mn, and Fe. In this case, P was a significant factor in the formation of biofilms adhering to the As-mineral as a substratum. The finding confirmed a previous study which argued that phosphate could act as a protecting agent against arsenate (but not arsenite) toxicity (Cullen and Reimer, 1989).

SEM and TEM images showed that greater numbers of bacteria occurred when they were organized into colonies in a biofilm bound to surfaces of mineral particles (Figs. 5, 6 and 7). Under extreme conditions (especially under unfavorable environment, for example that with highly toxic contaminant like arsenic), the bacteria are positioned in a heterogeneous environment with gradients of nutrients and waste products controlled by diffusion and mass transport processes, and it is therefore to be expected that this heterogeneity would be reflected in the physiology of the individual cells. In response to these conditions, colonies in biofilms often appear as rather complex and heterogeneous assemblies consisting of clusters of bacteria embedded in polymeric substances, which are separated by void regions (cell-free channels) (Zahid and Ganzarczyk, 1994). Our results also showed a similar tendency in the formation of biofilms, indicating that the bacterial cells tended to be attached to the surface of minerals (Figs. 5, 6 and 7).

Mechanisms of As-mineral transformation

The possible mechanisms of As-minerals transformation in the presence of Fe can be explained by chemical, physical, and biological processes. Firstly, As-minerals underwent chemical weathering or biological modification by bacteria, resulting in dissolved or soluble As in the medium (Table 3). This process was shown by SEM images, illustrating that the surface of As minerals (realgar in this example) appeared as serrated structures (Fig. 5A). Secondly, bacteria attached to As minerals were, therefore, likely to be exposed to higher concentrations of arsenic than those in the liquid phase. In this step, bacteria were presumably able to accumulate As cations by means of two processes; (1) the bacteria sorbed heavy metals (As) on the surface of cell through physicochemical interactions and also showed energy-dependent uptake of heavy metals into the cytoplasm (Gadd, 1992); (2) Metal cations bound to cell surface polymers through a variety of mechanisms, such as cation exchange, complexation, coordination and precipitation reactions. These may act singly or in cooperation (Gadd, 1992). Finally, the bacteria produced exopolysaccharide polymers to form the biofilm. Based on the TEM images (Figs. 6 and 7), we suggest that these heavy metals (As and Fe), enmeshed within a biofilm, might be incorporated into As-minerals. It was also likely that dissolved As in culture solutions (see Tables 1 and 2) resulted from detachment, desorption, or leaching processes during biofilm formation as well as dissolution of As-minerals by microorganisms. It has also been shown that the accumulation of metal cations by bacteria in biofilms is higher than that of their free-living counterparts (Scot and Karanjkar, 1992). These factors agree with our finding that the greatest abundance of bacteria and bacterial colonies were in the dense biofilm

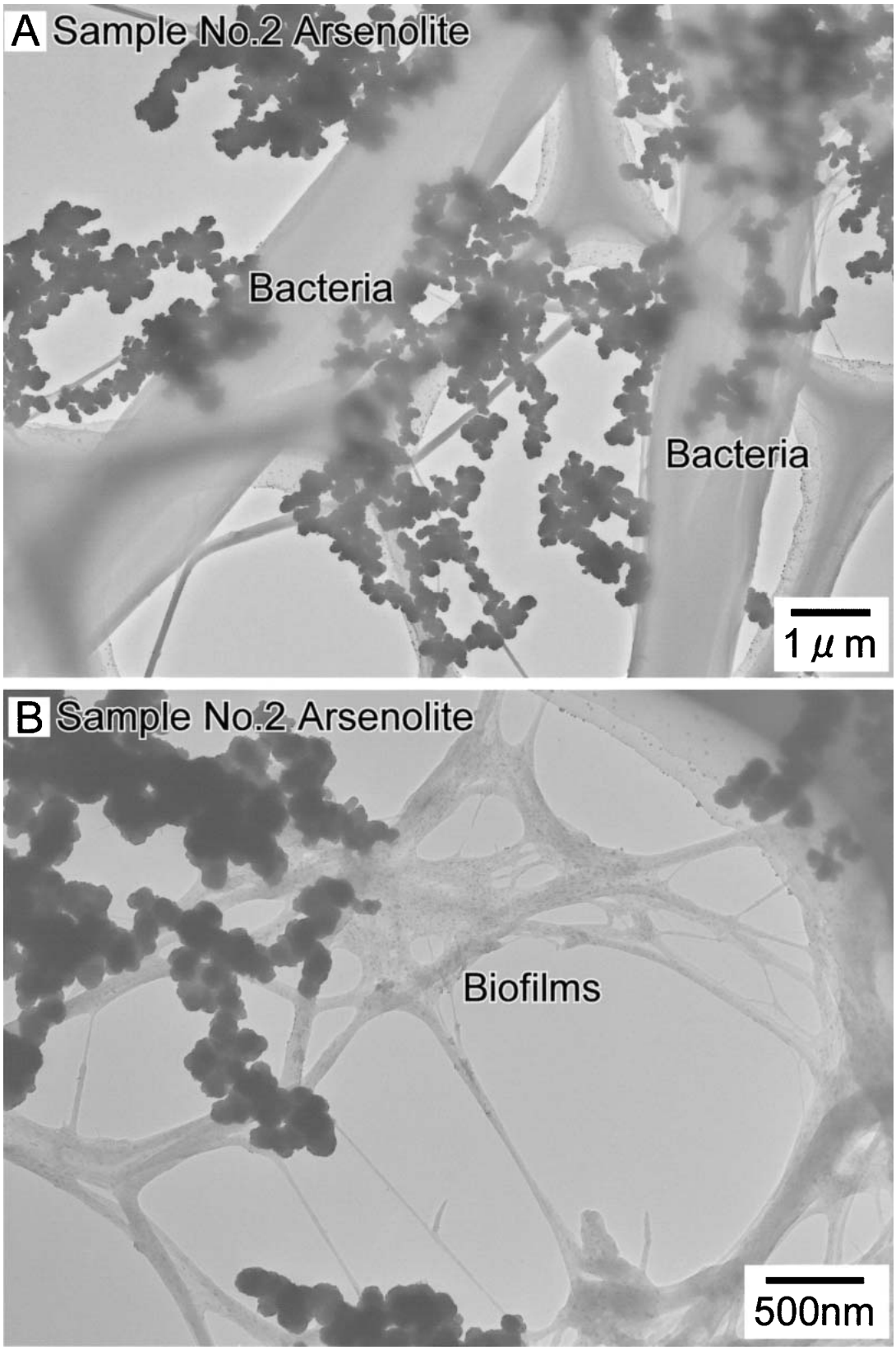

Fig. 6. TEM micrographs of arsenolite (sample No. 2) showing filamentous bacteria (upper) and biofilms (below) with uniform sized As−Fe granules in the solution after one year of aging, suggesting the formation of As-biominerals.

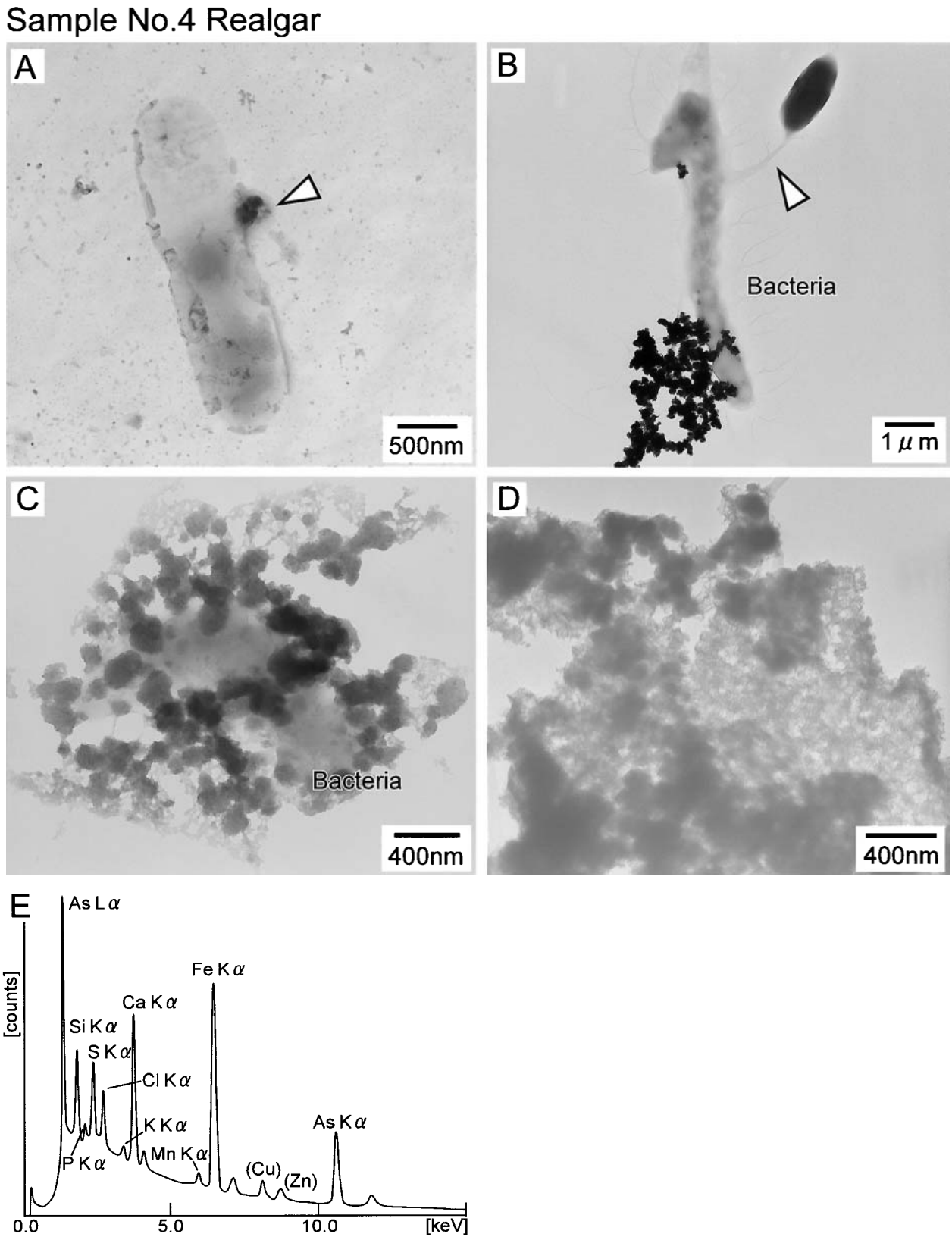

Fig. 7. TEM micrographs of realgar (sample No. 4) associated with bacillus (A), filamentous (B), and coccus (C) types of bacteria with uniform sized granules of realgar (C, D) in the ground water after one year of aging, showing the cohesive materials with granules around the cell wall (C). High density of nm-sized granules (50-100 nm in diameter) with flaky films are existent on the altered surface of realgar (D) mainly composed of As, Si, S, Cl, Ca, and Fe (E). Electron diffraction (ED) pattern of the granules around the bacterial cell wall indicates amorphous or poorly crystallized materials.

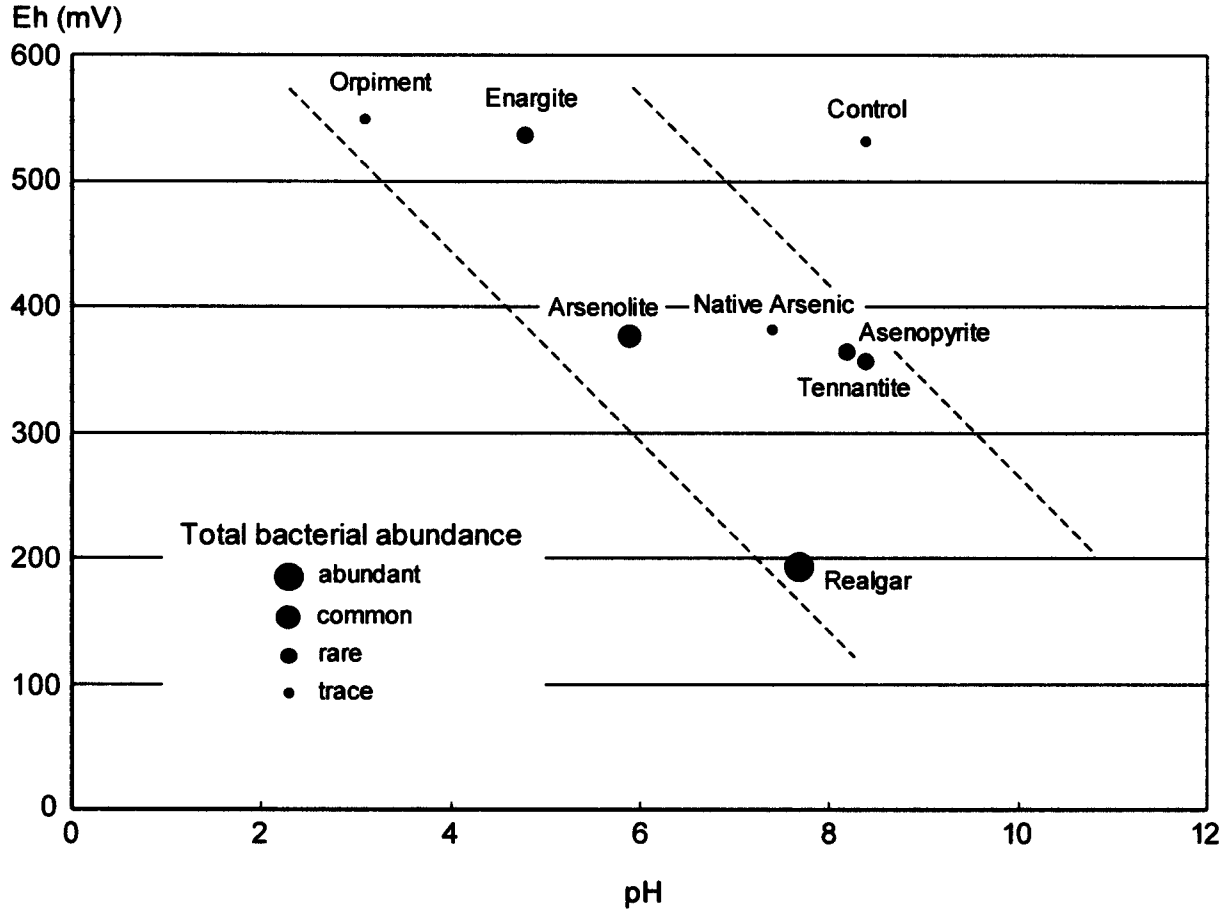

Fig. 8. Diagram of pH-Eh-total bacterial abundance of As-mineral experimental systems. Dissolved As ion from As-minerals was affected by the pH of ground water after one year of aging.

(in the realgar and arsenolite cultures). The presence of As-tolerant microorganisms and the factors leading to optimal growth conditions (see Figs. 8 and 9) will serve as a guide to bioremediation processes.

As-polluted geo-aqueous system

These results might also be important for solutions of near-neutral pH in which abundant cell-mineral surfaces are able to adsorb metals from solution. Hazigonj, Bangladesh, lies on an estuary of old Meghna River alluvium that is deeply flooded by rainwater during the monsoon. Flooding is caused by the Meghna River and by the Dakatia River. As a result, a large amount of sediment from the upper stream has been carried down towards the Bay of Bengal. Hazigonj is typically inundated annually and is frequently flooded due to its very low water table (1~2 m). At the same time ground water is affected by the mobilization of As from minerals or clay minerals at a depth of 23~29 m. Geologic conditions at Hazigonj are favorable for the formation of minerals, with high Fe^{2+} and NaCl concentrations in ground water (pH 7.2~7.4) (Islam *et al*., 2003), very similar conditions to the experiments in this study. Furthermore, high fluctuation of the water table (6~7 m) occurs between the dry and wet seasons, which transports a lot of alluvial sediment within the lowlands of Hazigonj during the rainy seasons. Generally, the alluvial sediment and weathered clay minerals contain high As. Ishiga *et al*. (1999) reported that the organic black mud also contain high concentrations of As in Bangladesh. Total Sulphur (TS) is also detected in samples from both Samta and Dauli Villages showing high sulfur concentration suggesting marine or brackish water. Paktunc and Dutrizac (2003) reported that Fe affects the amount of arsenate substitution, which is limited to about 17 mole % $AsO_4/(AsO_4 + SO_4)$. The charge imbalance caused by the substitution of arsenate is compensated by the partial protonation of the AsO_4 and SO_4.

As polluted ground waters are found to be capable of accumulating As along with other elements. This accumulation of elements into the microbial cellular system takes place by biogeochemical processes. Newman *et al.* (1997) reported that the main compounds of As in aqueous solution are $HAsO_4^{2-}$, H_2AsO^{4-}, H_3AsO_3, As, and AsH_3 at pH 7 or near-neutral pH. These

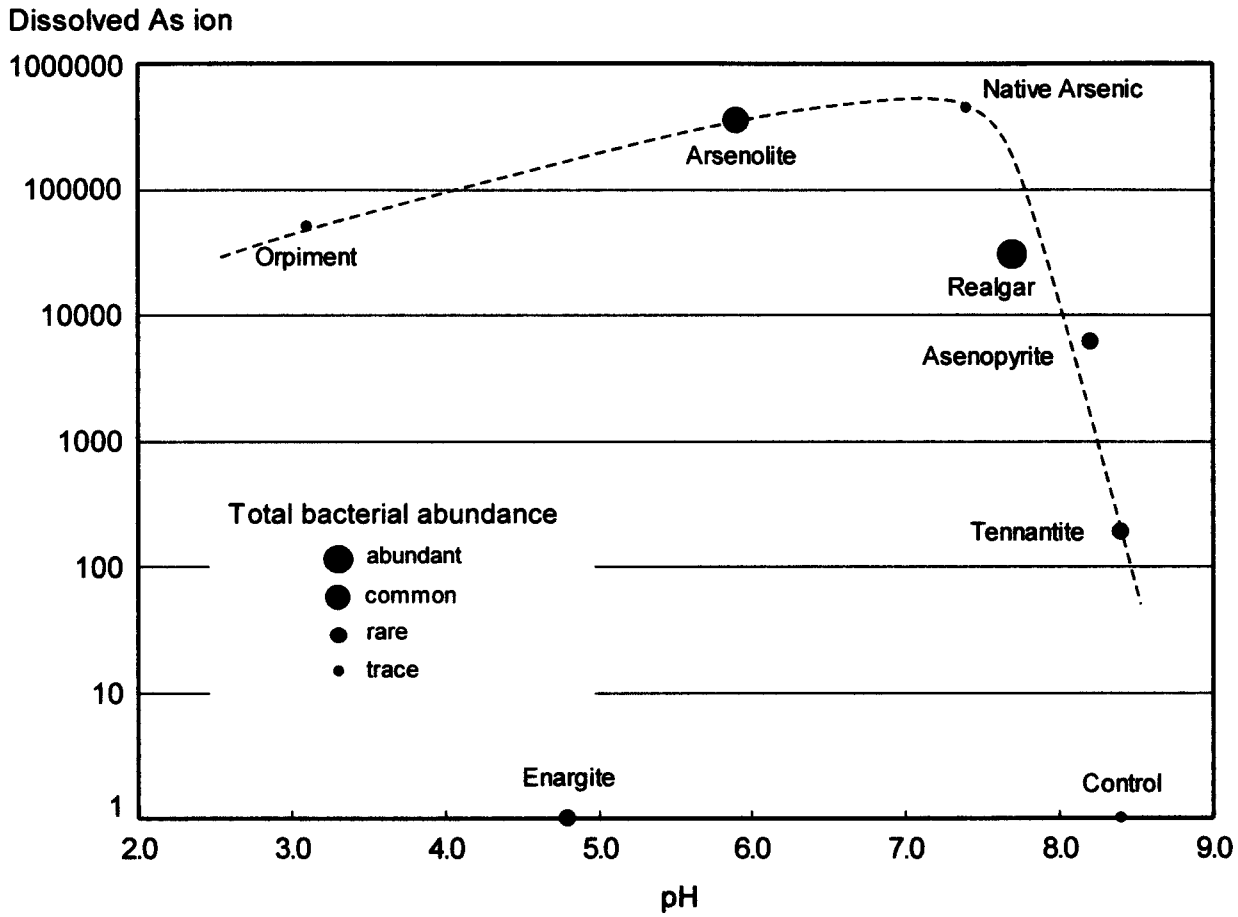

Fig. 9. Diagram of pH-dissolved As-total bacterial abundance of As-mineral experimental systems. Dissolved As ion is greatest in the pH range 7−8 and associated with high total bacterial abundance. pH is related to the logarithm of concentration of dissolved As ion in the ground water after one year of aging. Note that pH 7~8 and Eh 200~400 mV allows the survival of microorganisms under high As conditions.

conclusions are also important for this study, as most of the pH values of the solutions are pH 7~8. The results of this study could be implemented in the purification or bioremediation of As-polluted geo-aqueous ecological systems. Accumulation takes place by adsorption, precipitation, complexation, and transportation at non-marine or brackish conditions in sandy low lands, such as in Bangladesh and in peninsular sandbars.

Conclusions

By means of optical, SEM, and TEM microscopy, seven kinds of As-minerals in Fe-rich ground water (pH 7) were studied. After one year of aging, a variety of microorganisms such as bacillus, coccus, and filamentous types of bacteria survived in low-nutrient and high As concentration environments. These bacteria appeared to live in clumps, bound to As-mineral surfaces in association with As and S. From this, it was concluded that these microorganisms were resistant and/or tolerant to arsenic compounds and consumed them as energy sources for their growth. Likewise, symbiotic bacteria may have a metal-accumulating character even in these toxic environments, because they could endure the extremely high As concentrations. The results of these experimental systems show that these As-mineral - bacteria associations could be utilized in the purification or bioremediation of As-polluted geo-aqueous ecological system.

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