Microbes in Oily Hot Spring Water

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Microbes in Oily Hot Spring Water

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Abstract – Microbial mats in oily hot springs were collected from Toyotomi hot springs in Hokkaido, and Tsukioka hot springs in Niigata, Japan, for biomineralogical study. Ferrihydrite and digenite(Cu_9S_5) were found in microbial mats. Diatoms and filamentous bacteria inhabit under the anaerobic oily water condition. The microbes absorbed crude oil to the internal cells to form hydrocarbon minerals.

I. Introduction

Crude oil is one of the most important resources all over the world. Geophysically, crude oil forms under the conditions of high pressure and temperature. Likewise, biological process is also able to create the significant amounts of hydrocarbons [1]. Typical crude oil reservoirs, which contain seawater, are mostly sandstone or limestone in which crude oil is trapped. Since crude oil and gas are lighter than water, they float on top of seawater. When crude oil wells are drilled, sometimes seawater gushes together with crude oil [2].

Furthermore, the pollution of crude oil in soils, sediments and seawater is a matter of an international concern due to the toxicity and refractory character of the aromatic components in the absence of oxygen [3]. The biodegradation of hydrocarbons by microbes is one of the primary ways by which crude oil is eliminated from contaminated sites [4]. Several studies about hydrocarbon mineralization with nitrate, Fe (III), sulphate or CO_2 as alternative electron acceptors, in sediments or seawater have been reported [5]. Moreover, Yushkin (1998) proposed a concept of hydrocarbon crystallization of life because their origin is supposed to be the reworking of initially biogenic hydrocarbons [6].

On the other hand, most of the heavy metals and toxic materials are accumulated by microbes and forming biominerals, after precipitation of insoluble metals [7]. Especially, there have been many studies demonstrating an active biomineralization in hot springs which contain a large amount of heavy metals [8]. However, there are few reports of the biomineralization of hydrocarbon and habits of microbe under the special environment in which crude oil is mixed in hot spring water.

In this study, microbial mats with diatom and filamentous bacteria were found at two oily hot springs, Toyotomi hot springs in Hokkaido, and Tsukioka hot springs in Niigata, Japan. The gush of methane gas and crude oil with fossil seawater is recognized in Toyotomi hot springs [9]. Previously, Tsukioka hot springs well was used for pump crude oil, and this hot spring water is also fossil seawater [10]. Hence, we focus on investigation of the process of hydrocarbon mineralizaton by microbes especially in oily hot springs environment.

II. Materials and methods

Crude oil, oily hot spring water and microbial mats were collected from Toyotomi hot springs in March 2002, and Tsukioka hot springs in September 2002. Toyotomi hot springs located at the northern part of Hokkaido, while Tsukioka hot springs located at the northwestern part of Niigata prefecture in Japan (Fig. 1). Both hot springs water are gush out together with crude oil. The reddish brown microbial mats are formed on the surface of the river near Toyotomi hot springs. The green colored microbial mats were collected from the resource well of Tsukioka hot

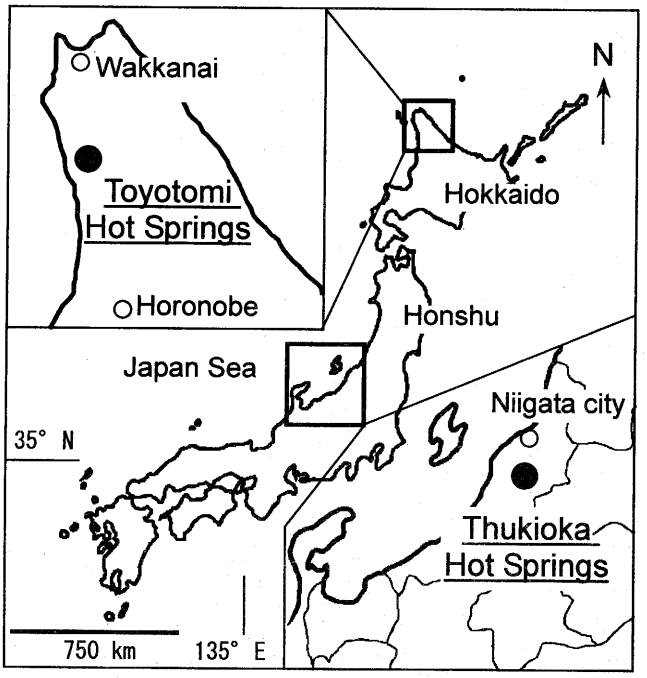

Fig. 1. Locality map of Toyotomi hot springs at Hokkaido and Tsukioka hot springs at Niigata prefecture in Japan. Both hot spring water are mixed with crude oil.

springs.

Water quality was measured for hot spring water and river water in the field of Toyotomi hot spring (HORIBA: pH; D-12, Eh; D-13, EC; ES-12, DO and WT; OM-12). In the case of Tsukioka hot springs, measurement of water quality was conducted in the laboratory. The mineralogical properties of microbial mats were also analyzed by X-ray powder diffractometer (XRD), using a Rigaku RINT 2000 with CuKα radiation. It was generated at 40 kV and 30 mA using the $2\theta/\theta$ method with a scan speed of 1° /min. Dried microbial mats powder was taken on the square concavity of the slide and fixed them up. After fixation the slide was set up on the stage of XRD for analysis. Further, 50 μl of crude oil sample were taken on the Mailer film, the microbial mats were air-dried up at room temperature and ground to fine powder for ED-XRF analysis. The powder samples were pressed to make pellet and mounted on the Mylar film. Analyses of crude oil and microbial mats samples were carried out by an energy dispersive X-ray fluorescence spectrometer (JEOL JSX-3201), using Rh Kα, which operated at an accelerating voltage of 30 kV under a vacuum condition. To identify the presence and variety of bacteria, optical microscopic observation was carried out. Both of the episcopic and DAPI (4', 6-diamidino-2-phenylindole) stained samples were observed through episcopic fluorescence microscope (Nikon EFD-3). The DNA of bacterial cell and crude oil shows the fluorescence blue or yellow under the ultraviolet ray (365 nm). Freeze-dried method was used for sample preparation [11]. One drop of fixed microbial mats with 1 % glutaraldehyde was mounted onto JEOL filter, sample was then washed and fixed with t-butyl alcohol, frozen in liquid nitrogen and dried up with low-vacuum SEM. After the completion of freeze-drying, the sample was transferred on carbon stub with double-sided adhesive carbon tape, coated with carbon and observed with a scanning electron microscope (JEOL JSM-5200LV), equipped with an energy dispersive X-ray spectrometer (Philips-EDX PV9800 STD). Identification method of diatom was referred to the practice of Yanagisama [12].

III. Results and Discussion

Toyotomi hot springs

A. Water chemistry

Both hot spring water and river water near Toyotomi hot springs show neutral and anaerobic condition showing Eh −14 ~ 37 mV. Water characteristic of hot spring water indicated pH 7.7, EC 18.5 mS/cm, Eh 37 mV, DO 2.6 mg/l and WT 31.7 ℃, whereas the river water indicated pH 6.4, EC 0.6 mS/cm, Eh -14 mV, DO 2.8 mg/l and WT 13.9 ℃. Water quality of Tsukioka hot springs also indicated neutral pH (7.2) with anaerobic condition (Eh -280mV). The water characteristic data are in agreement with the observation of microbe in the both springs.

B. Chemical and mineralogical analyses of oil and microbial mats collected from Toyotomi hot springs

The ED-XRF analysis of crude oil collected from Toyotomi hot springs showed the presence of Fe, S, Si and Ca with the traces of K, Ti, Mn and Zn. The high concentration of Fe, S, Cl, Si and Ca with traces of Al, K, Ba and Mn is also observed for the reddish brown microbial mats. While XRD patterns of reddish brown microbial mats confirmed the presence of quartz (3.34 Å) and ferrihydrite (2.5 Å and 2.2 Å) minerals with a broad back ground suggested that sulfur and organic materials are present as amorphous materials.

C. Optical microscopic and SEM observations

Optical light micrograph of the reddish brown microbial mats showed the presence of diatom with high-density brown materials. Epifluorescence microscopic observation indicates the presence of diatom, identified as *Pinnularia* spp., approximately 150 ~ 200 μm in size. The diatom were encrusted with crude oil around their cell (Fig. 2A). While diatom identified as *Achnanthes* sp. or *Navicula* sp., about 10 ~ 20 μm in size show the accumulation of crude oil internal cells (Fig. 2B).

SEM observation of reddish brown microbial mats showed the diatoms colony aggregated with granular particles and crude oil droplet (Fig. 2C). EDX spectrum of crude oil droplet (arrow ① in Fig. 2C) showed the presence of Si, P, S, K, Ca and Fe with a hilly back ground, suggesting the presence of organic materials. Diatom cells (arrow ② in Fig. 2C) were mainly composed of Si and Fe, with traces of Al, P, K and Ca. Existence of small crude oil droplet about 0.3 μm in size in the diatom cells are also observed in SEM image (arrows in Fig. 2D). These observations suggest that the size of crude oil droplet less than 0.3 μm in which diatom is able to take to the inside cells and the crude oil droplet was accumulated in the globular form in the cells.

Diatoms used organic compound as energy source [13]. They have the enzyme which decompose crude oil in the cells, and its degradation ability decrease continuously with water salinity [14]. The chemistry of hot spring water of Toyotomi hot springs is similar to seawater chemical composition. However, it seems that the enzymatical activity of diatom is greater in degrading and accumulating crude oil in the reddish brown microbial mats, since the high salinity hot spring water diluted with fresh water. Moreover, microbial mats of Toyotomi hot springs contain marine diatoms cells identified as *Denticulopsis* sp., *Melosira albicans* and *Neodenticula kamtschatica*, which is extinct species. Existences of marine diatoms demonstrate that the fossil seawater gush out together with crude oil and gas.

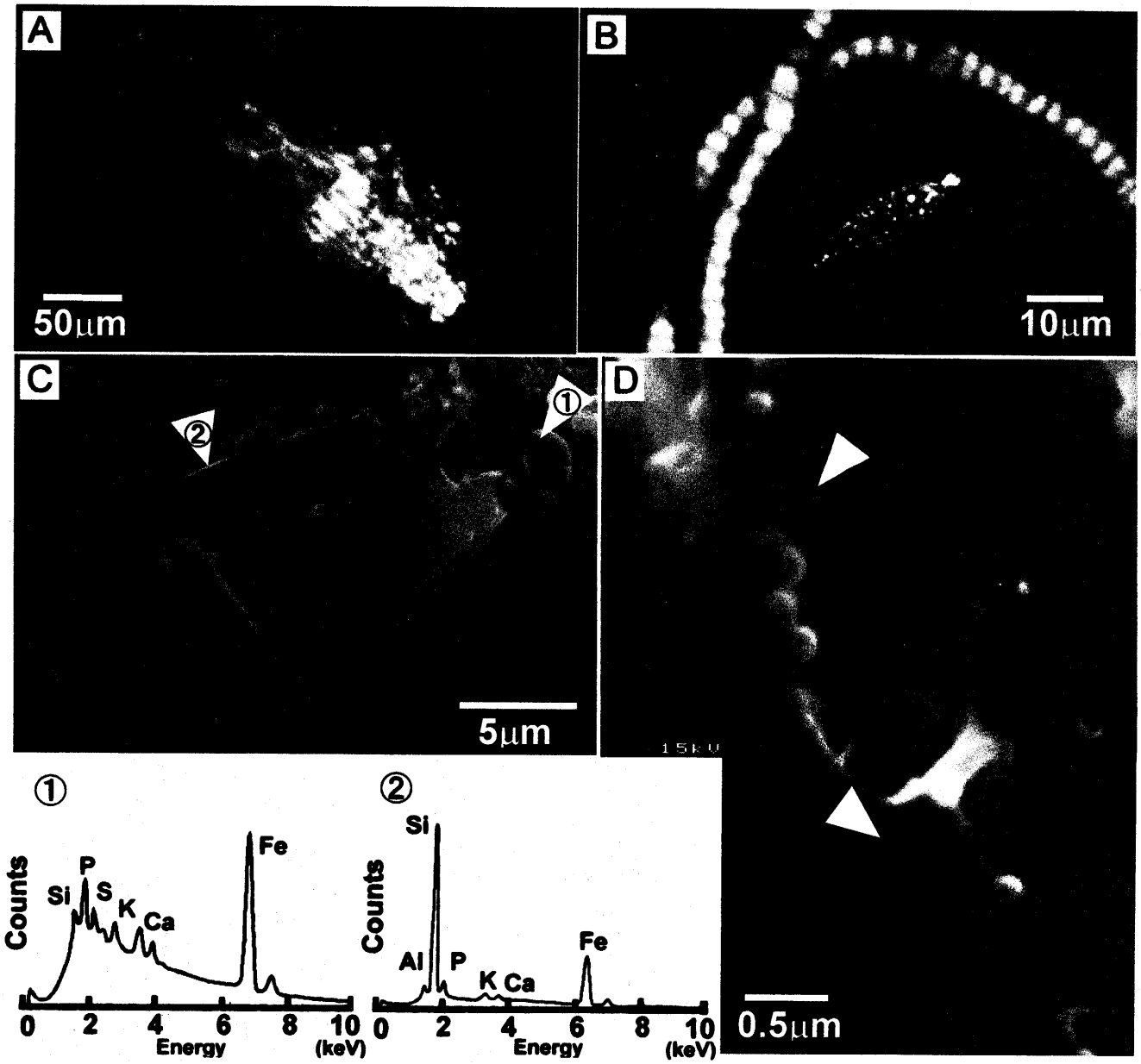

Fig. 2. Epifluorescence micrographs and SEM images of reddish brown microbial mats were collected from Toyotomi hot springs. Epifluorescence micrographs showed crude oil around external cells of diatom (A). Image of diatom cells about 10 ~ 20 μm in size show accumulation of oil inside of cells (B). SEM image showing the diatom colony (mainly *Achnanthes* sp. or *Navicula* sp.) aggregated with granular particles and crude oil droplet (C). EDX spectrum of crude oil droplet (analyzed in the arrow point ①) showed the presence of mainly Fe with a hilly back ground, suggesting the presence of organic materials. Diatom (Analytical point is marked by arrow ②) was mainly composed of Si and Fe, with traces of Al, P, K and Ca. SEM image showed that small oil droplets about 0.3 μm in size exists inside of diatom (arrows in Fig. 2D).

Tsukioka hot springs

A. Water chemistry

Tsukioka hot spring water also shows neutral pH and anaerobic condition. Water characteristic of hot spring water indicated pH 7.2, EC 5.3 mS/cm, Eh -280 mV and DO 2.8 mg/l. This is quite anaerobic water condition, suggesting the gush of spring water from deep geological strata.

B. Chemical and mineralogical analyses of oil and microbial mats collected from Tsukioka hot springs

The ED-XRF analysis of the crude oil collected from Tsukioka hot springs showed the presence of high concentration of S (97.6 %) and traces of Si and Cu. On the other hand, the green colored microbial mats contained high concentration of Cu (53.2 %) with traces of S, Fe, Ca, Si, P, Pb, K, Zn, Cr, Mn and Sr. XRD patterns revealed the

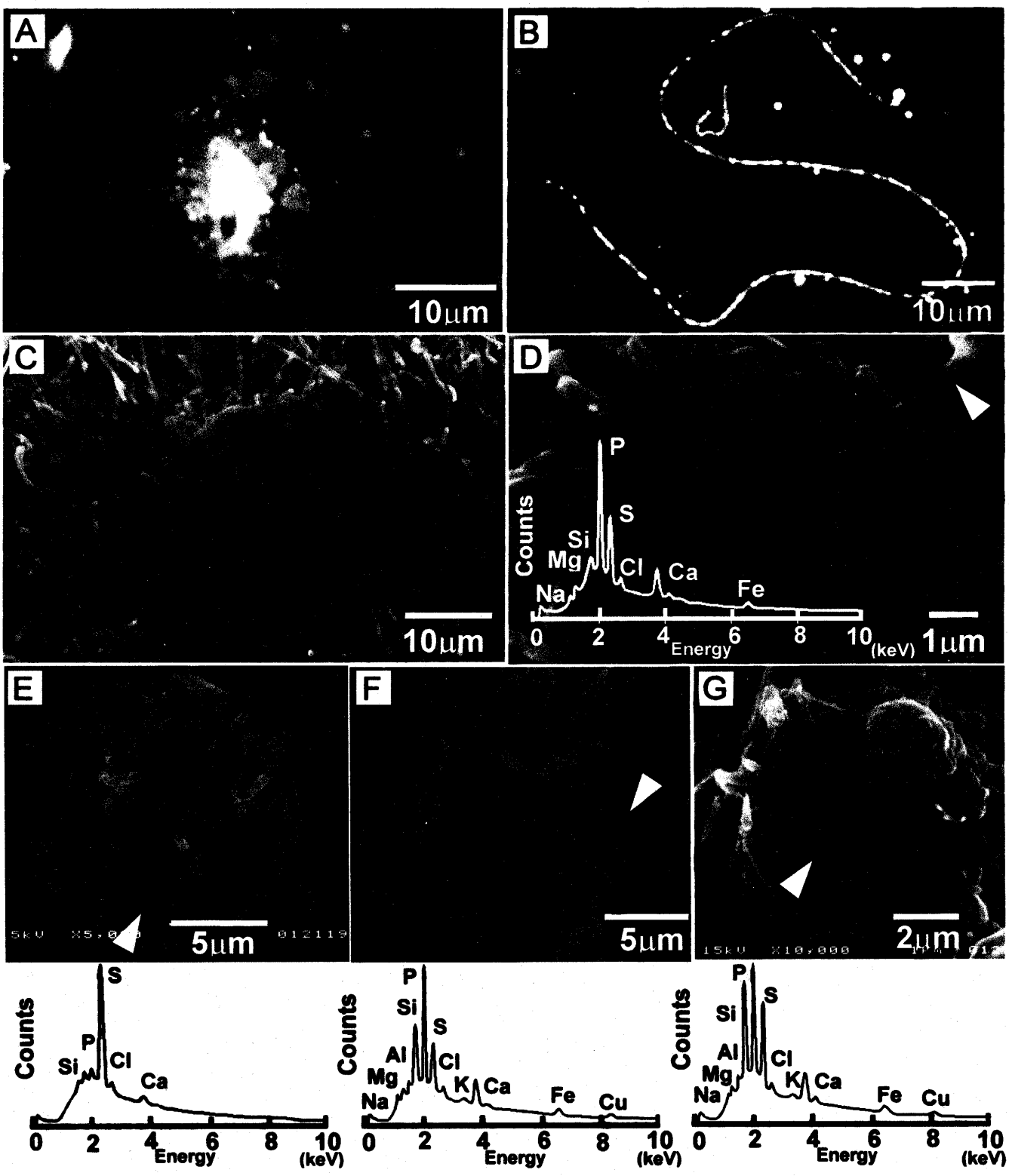

Fig. 3. Epifluorescence micrographs and SEM images of green colored microbial mats were collected from Tsukioka hot springs. Filamentous bacteria aggregated together with bacteria and yellow color mineral particles (A). Filamentous bacteria attach with the yellow color particles in the cells (B). SEM image showing the filamentous bacterial colony and bacterial agglutination (C). This agglutination was formed by bacteriolytic processes (D). EDX spectrum of filamentous bacteria (analytical point: arrow in D) showed the presence of P, S, Si, Ca, Cl, Mg, Na and Fe. SEM image of oil droplet within green colored microbial mats showing the successive process of mineralization of oil droplet. Oil droplet contains a high S with the traces of Si, P, Cl and Ca (E). The filamentous bacteria gather around oil droplet (F). EDX spectrum indicated the high concentration of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu. The oil droplet is completely covered with biofilm (G). EDX spectrum designated by arrow mark show the high concentration of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu. Analytical points are marked by arrows.

exsistence of halite (2.82, 1.99, and 1.63 Å), gypsum (7.63, 4.27, and 3.06 Å) and digenite (Cu_9S_5) (3.08, 2.81, and 1.90 Å) in the microbial mats. The high concentration of Cu might originate from digenite. As well, precipitation of NaCl from seawater was responsible for halite. However, the organic crystal is not found by XRD analysis.

Crude oil in Tsukioka hot springs contains large amounts of S and Cu, on the other hand, crude oil in Toyotomi hot springs contain S and Fe. These differences of chemical composition of crude oil depend on the geological conditions of their reservoir rocks [15].

C. *Optical microscopic and SEM observations*

Filamentous bacteria with orange colored crude oil are found in epifluorescence micrographs of the green colored microbial mats collected from Tsukioka hot springs. Filamentous bacteria forms colony aggregating together with the yellow colored mineral particles were shown in Fig. 3A. Furthermore, yellow color droplets were recognized inside of bacterial cells (Fig. 3B). This yellow colored droplets was observed in diatom cells.

SEM images of the filamentous bacterial colony are shown in Fig. 3C. This image showing a high density bacterial agglutination which an individual filament form is not able to distinguish, suggesting that this agglutination was formed by bacteriolytic process (Fig. 3D). EDX spectrum of filamentous bacteria (arrow in Fig. 3D) shows the presence of P, S, Si, Ca, Cl, Mg, Na and Fe with a hilly background, suggesting the presence of organic materials. The series of SEM images in Fig. 3 of green colored microbial mats suggest the mineralization process from crude oil droplet. At first stage, filamentous bacteria gather around the crude oil droplet, which contains high a S (Fig. 3E analytical point is marked by arrow). With concentration of bacteria, the surface of the crude oil droplet is coated by the bacteriolitic material (Fig. 3F). The EDX spectrum of the material surface indicated the high concentration of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu (analytical point is marked by arrow). A high concentration of P was responsible for biological materials. Finally, the crude oil droplet is completely covered with the bio-film resulting in high concentrations of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu in the microbial mats (Fig. 3G analytical point is marked by arrow).

Hunkeler *et al.* (1998) have reported that reduction condition was an important factor for a hydrocarbon mineralization [16]. Additionally, Pallasser (2000) has reported that the biodegradation of crude oil is active under the anaerobic condition [17]. Tsukioka hot springs show the quite anaerobic condition (Eh -280 mV), suggesting the active anaerobic bacterial biodegradation of crude oil and expedite of hydrocarbon mineralization by the filamentous bacteria. This observation suggests that microbe has an ability of crude oil accumulation in the cells and affecting the mineralization of hydrocarbon.

IV. Conclusions

The reddish brown microbial mats have been found on Toyotomi hot springs, Hokkaido which contains crude oil and gas. Whereas the green colored microbial mats have been found on Tsukioka hot springs, Niigata, which contain crude oil. Water quality of both hot springs showed neutral pH (6.4 ~ 7.7) and anaerobic condition (Eh: -280 ~ 37 mV). The reddish brown microbial mats contain a high concentration Fe, while the green colored microbial mats contain a large amount of Cu originated from digenite (Cu_9S_5).

Crude oil of Toyotomi hot springs, which consists of Fe, S, Si and Ca with the traces of K, Ti, Mn and Zn, shows the yellow fluorescence under the ultraviolet ray. On the other hand, the crude oil of Tsukioka hot springs, which contains a high concentration of S and traces of Si and Cu, shows the orange fluorescence under the ultraviolet ray.

Optical and epifluorescence microscopic observation show that two kinds of diatoms with yellow colored oil inhabit the reddish brown microbial mats. One kind of diatom, *Pinnularia* spp., encrusted with oil around their cells. Whereas others, *Achnanthes* sp. or *Navicula* sp., accumulate oil to internal of cells.

Hydrocarbon minerals were covered with the biofilm, which formed by the bacteriolytic process. EDX spectrum indicated the high concentrations of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu. The observations suggest that microbe have not only the ability of oil accumulation to the internal cells but also affecting the mineralization of hydrocarbon.

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References

[1] J. Heider, A. M. Spormann, H. R. Beller, F. Widdel, "Anaerobic bacterial metabolism of hydrocarbons," *FEMS Microbiology Reviews*, Vol. 22, pp. 459-473, 1999.

[2] M. Tezuka, *Real Oil (In Japanese)*, TEIKOKU Co., Ltd., 1990.

[3] R. Müller, I. Kleeberg, W. D. Deckwer, "Biodegradation of polyesters containing aromatic constituents," *Journal of Biotechnology*, Vol. 86, pp. 87-95, 2001.

[4] M. Itävaara, S. Karjomaaa, J. F. Selinb, "Biodegradation of polylactide in aerobic and anaerobic thermophilic conditions," *Chemosphere*, Vol. 46, pp. 879-885, 2002.

[5] J. D. Coates, R. Chakraborty, J. G. Lack, S. M. Oconnor, K. Y A. Cole, K. S. Bender, L. A. Achenbach, "Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas," *Nature*, Vol. 411, pp. 1039-1043, 2001.
[6] N. P. Yushkin, "Hydrocarbon crystals as protoorganisms and bological systems prodecessors," Invited Paper, Part of the SPIE Conference on Instruments, Methods, and Missions for Astrobiology, 1998.
[7] J. R. Lloyd, L.E. Macaskie, "Bioremediation of Radionuclide-Containing Wastewater," *Environmental Microb-Metal Interaction*, American Society of Microbiology, pp. 277-327, 2000.
[8] K. Nagai, A. R. Islam, K. Tazaki, "Bacterial Fe-As mineralization," *The Science Reports of Kanazawa University*, Vol. 46, pp. 49-66, 2001.
[9] S. Nagao, *EXPLANATORY TEXT OF THE GEOLOGICAL MAP OF JAPAN – TOYOTOMI – (In Japanese)*, Geological Survey of Hokkaido, 1960.
[10] M. Shinmazu, *The Topographical Record of Hot Springs, Niigata Prefecture, Japan (In Japanese)*, Nojima Pcblishing Co., Ltd., 2001.
[11] T. Suzuki, M. Shibata, K. Tanaka, K. Tsuchida, T. Toda, "A new drying method: low vacuum SEM freeze drying and its application to plankton observation." *Contribution to the Bulletin of Planktonic Society of Japan*, Vol. 42, pp. 53-62, 1995.
[12] Y. Yanagisawa, "Fossil diatoms biostratigraphy of the Miocene in the southern part of Kanazawa sity, Ishikawa prifecture, in central Japan," *Bulletin of the Geological Survey of Japan*, Vol. 50, pp. 49-65, 1999.
[13] K. Iwasa, *Biology of Diatoms*, Tokyo university Press, Inc., 1976.
[14] Y. Shdo, T. Nakamura, M. Ri, E. Oshima, "Separation of lipids in Phaeodactylum tricoenutum by enzym reaction," *Tokyo industry technical college research report*, Vol. 17, pp. 119-122, 1985.
[15] S. Konishi, *Introduction to Fuel Engineering (In Japanese)*, Shokado, 1991.
[16] D. Hunkeler, D. Jorger, K. Haberli, P. Hohener, J. Zeyer, "Petroleum hydrocarbon mineralization in anaerobic laboratory aquifer columns," *Journal of Contaminant Hydrology*, Vol. 32, pp. 41-61, 1998.
[17] R. J. Pallasser, "Recognising biodegradation in gas/oil accumulations through the 13C compositions of gas components," *Organic Geochemistry*, Vol. 31, pp. 1363-1373, 2000.