Oil fixation by diatom cell in oily hoto spring water -A clue bioremediation Part ?-

OIL FIXATION BY DIATOM CELL IN OILY HOT SPRING WATER

– A CLUE TO BIOREMEDIATION PART I –

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ABSTRACT

Oil fixation of diatom cell has been found in the reddish brown microbial mats at the Toyotomi Hot Springs, Hokkaido, Japan. The oily hot spring water showed a neutral pH and anaerobic condition with methane gas. The crude oil in hot spring water contains Fe, S, and Si except petroleum hydrocarbons. The reddish brown microbial mats were mainly composed of Fe ion to form ferrihydrite. The organic carbon gleams light-yellow in color under the fluorescence ultraviolet-ray. Optical and epifluorescence microscopic observation revealed diatom fixed crude oily droplets within/on the cell wall. The results suggest that diatoms have the ability to remediate petroleum hydrocarbons in an oil-polluted area.

INTRODUCTION

Crude oil used to be regarded as toxic to microorganisms. However, in recent years, many researchers have reported microorganisms that are resistant to oil and can degrade it (e. g. Swannel and Heas, 1994). Bioremediation of crude oil pollutant or its derivates is a technology based on activity of living organisms and especially that of microorganisms, which have the ability to use petroleum hydrocarbons as source of energy

and carbon (Allen, 1988).

Basically, bioremediation or the so-called biodegradation uses microorganisms to remove pollutants from an environment. The term of bioremediation is accompanied by a human factor, whereby conditions are manipulated to affect the rate. It is one of the most common treatments of non-toxic liquid with solid wastes, contaminated ground water, toxic hazardous wastes, and grease decomposition. It is known to be a practical and cost-effective method to remove hydrocarbons from contaminated areas (Bragge *et. al.*, 1994). Populations of hydrocarbon -degraders normally constitute less than 1% of the total microbial communities, but when oil pollutants are present, these hydrocarbon-degrading populations increase, typically to 10% of the community. With regard to rates of natural degradation, these typically have been found to be low and limited by environmental factors (Walker *et. al.*, 1993).

Altogether, more than 70 microbial genera are known to degrade petroleum components (U. S. Congress Report, 1991). Since any given oil would have a diversity of compounds, obviously there is a need for the variety.

The biodegradation of hydrocarbons by microorganisms is one of the primary ways by which crude oil is eliminated from contaminated sites (Suominen *et. al.*, 2000). Bioremediation by microorganisms was applied when crude oil spilled from the wrecked American tanker "*Exxon Valdez*" attacked the coasts of Alaska district in 1989 (Swannel and Head, 1994). For removing spilled oil covering the Hokuriku coastline and petroleum pollutant on soil, similar technology was applied (Tazaki *et al.*, 1997). Degradation of aromatic hydrocarbon by microorganisms likewise indicated the close relationship between heavy oil and microorganisms (Bragg *et al.*, 1994). One genus of diatoms found in seawater, *Phaesdactylum tricornutum,* a petroleum plankton, is known to contain oil within its cells (Ceron *et al.*, 2000). This diatom contains an enzyme which decomposes fatty acid like the eicosapentaenoic acid in the oil, and utilizes

it as a nutrition source (Iwasa, 1976).

In this study, we observed diatoms found in the oily hot spring water to clarify the process of hydrocarbon accumulation and to understand a clue to bioremediation. Our study area is the Toyotomi Hot Springs contain a gush of methane gas and crude oil with fossil seawater.

LOCATION AND GEOLOGY

The Toyotomi Hot Springs are located in the northern part of Hokkaido (45°N, 142°E), Japan (Fig. 1). The study area is located in the Teshio sedimentary basin of the Neogene period. Sedimentary rock formations from the mid Miocene to the Pleistocene periods known as the Soya coal bearing formation, Masuporo, Koetoi, Yuuchi, and Sarobetu formation overlie on the bedrock dating from the Cretaceous and Paleogene periods (Nagao, 1960). There are many folds and faults running in the north-south direction. The Toyotomi Hot Springs pump up water from the fracture zone, 950m in depth, in the mudstone of Miocene Masuporo formation which consists of black sandstone, conglomerate, silt and thick mudstone.

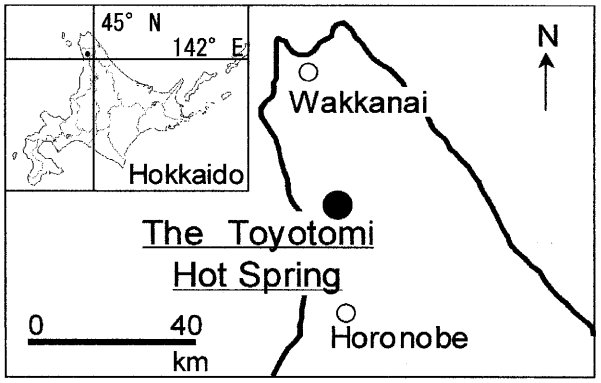

Figure 1 Locality map of the Toyotomi Hot Springs in Hokkaido, Japan

MATERIALS AND METHODS

Materials

Crude oil, oily hot spring water and microbial mats were collected from the Toyotomi Hot Springs in Hokkaido, Japan, on March 16～18, 2002. The hot spring water gushes out together with crude oil and methane gas (Fig. 2A). The hot spring water is stored in a tank (Fig. 2B), then distributed to individual hot baths through pipes. The drainage system of the field

established nearby river (Fig. 2C), where reddish brown microbial mats are formed in the drain (Fig. 2D).

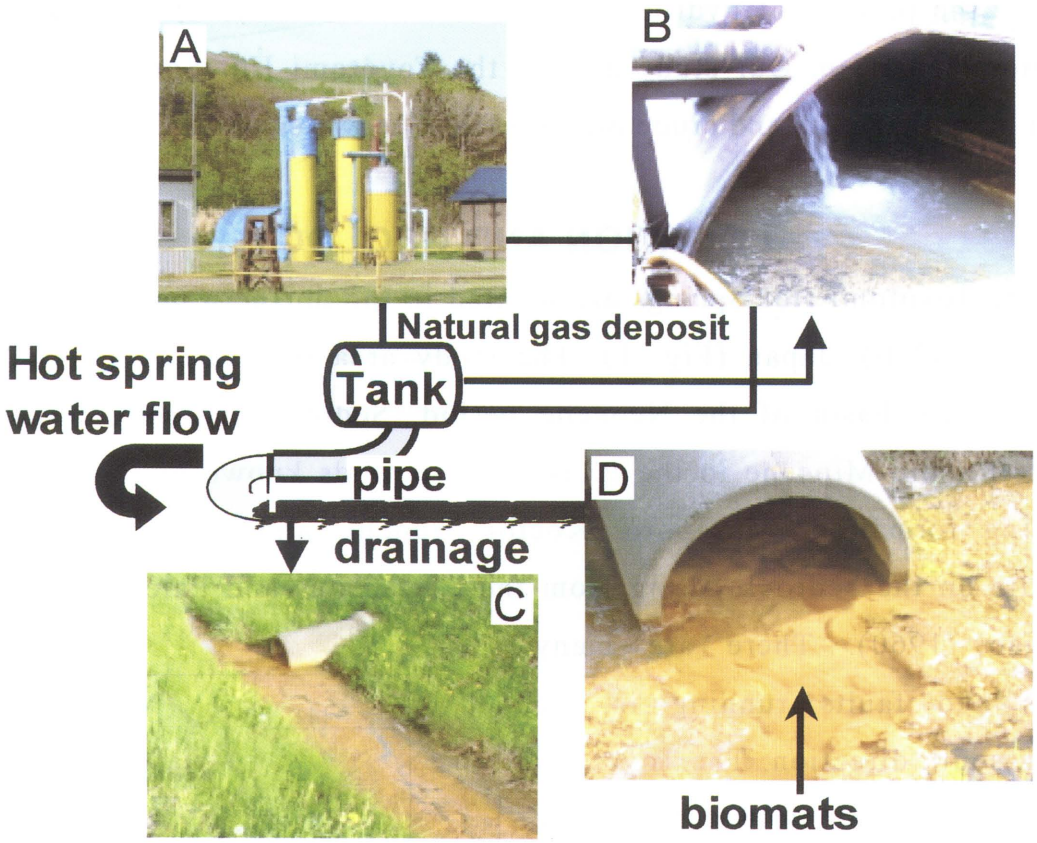

Figure 2 Schematic diagram of sampling points at the Toyotomi Hot Springs. A; The hot springs water is gushes out together with crude oil and methane gas, B; Hot spring water storage tank, C; The drainage pipe established nearby river, D; The reddish brown microbial mats are formed in the drainage.

Water chemistry

Water quality of the hot springs was measured and compared with the river water at the Toyotomi Hot Springs, using by pH; D-12, Eh; D-13, EC; ES-12, DO and WT; OM-12 made by HORIBA equipments. The chemical composition of water (Na, K, Mg, and Ca) was analyzed by atomic absorption photometer (SEIKO, SAS-727). Concentrations of P, Mn, and Fe were assessed by Laboratory Spectrophotometer (HACH, ODYSSEY DR/2500).

X-ray powder diffraction analyses (XRD)

The mineralogical properties of microbial mats with diatom 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 2θ/θ method with a scan speed of 1°/min. Dried powder sample was placed on the square concavity of a slide.

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

The crude oil sample (50 μl) for ED-XRF analysis was set up between Mylar films, whereas the microbial mats were air-dried at room temperature and ground to fine powder for ED-XRF analyses. The powder samples were pressed to make pellets and mounted on Mylar film. Chemical analyses of both crude oil and microbial mat samples were carried out by an energy dispersive X-ray fluorescence spectrometer (JEOL JSX-3201), using Rh Kα, operated at an accelerating voltage of 30 kV under a vacuum condition.

Optical light and epifluorence microscopy of diatoms in microbial mats

To identify the presence and variety of diatoms in microbial mats, optical microscopic observation was carried out. The microbial mats were fixed with 2.5 % glutaradehyde. The fixed samples were stained with DAPI (5 μg/ml) for 3 minutes for observation under epifluoresence microscope (Nikon EFD-3). The DNA of the bacterial cell and of the crude oil resulted in blue or yellow under the fluorescence ultraviolet-ray (365 nm). Identification method of diatoms was referred to the practice of Yanagisawa (1999).

Scanning electron microscopy (SEM)

Freeze-dried method was used for microbial mats referred to Suzuki *et al.*(1995). One drop of fixed microbial mats with 1 % glutaraldehyde was mounted onto a JEOL filter, washed by deionized water, and fixed with t-butyl alcohol. The frozen samples in liquid nitrogen were dried in low-vacuum SEM. After freeze-drying, the samples were transferred on a

carbon stub with double-sided adhesive carbon tape. After carbon coating, the samples were observed with a scanning electron microscope (JEOL JSM-5200LV), equipped with an energy dispersive X-ray spectrometer (Philips-EDX PV9800 STD).

Transmission electron microscopy (TEM)

Transmission electron microscopy (JEOL JEM-2000EX) were used for the observation of diatom cells. One drop of the suspension of microbial mats was mounted on micro grids. After air-drying, the uncoated samples were observed at an accelerating voltage of 120 kV. Ultramicrotomy method was used for sample preparation referred to Horita (1992).

RESULTS AND DISCUSSION

Water chemistry

Both the Toyotomi Hot Spring water and river water nearby the study area show a neutral (pH 6.4 ~ 7.7) and anaerobic condition of Eh -14 ~ 37 mV (Table 1). Atomic absorption analysis and Laboratory Spectrophotometer analysis showed an extremely high Na concentration (3200 ppm) suggesting a fossil seawater origin. The concentration of Ca and K in the hot spring water also was much higher than that in the river water. On the other hand, the Fe concentration in the river water (6.4 ppm) was higher than that in the hot spring water (0.1 ppm), and the results of Mn concentration analysis similarly resembled the results of Fe concentration analysis (namely, 0.7 ppm for the river water; and 0.1 ppm for the hot spring water;). The concentrations of P and Mg were almost the same for both the hot spring water and the river water (Table 1).

Table1. Physical and chemical characteristics of hot spring water and river water.

Samples	pH	Eh (mV)	EC (mS/cm)	DO (mg/l)	WT (℃)	P (ppm)	Mn (ppm)	Fe (ppm)	Na (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)
Hot Spring water	7.7	37	18.5	2.6	31.7	0.4	0.1	0.1	3200.0	31.0	31.0	40.0
River water	6.4	-14	0.6	2.8	13.9	0.4	0.7	6.4	91.4	2.9	30.0	11.0

Chemical and mineralogical analyses of oil and microbial mats

The ED-XRF analyses of crude oil detected high concentration of S associated with Si, Fe and Ca (Fig. 3A). The reddish brown microbial mats contain abundant Fe with Si, Ca, S, Ba, Al, K, and Mn (Fig. 3B). XRD patterns of reddish brown microbial mats identified the presence of quartz (3.34 Å) and ferrihydrite (2.5 Å and 2.2 Å) minerals with a broad background, suggesting that organic materials or amorphous materials are present (Fig. 3C).

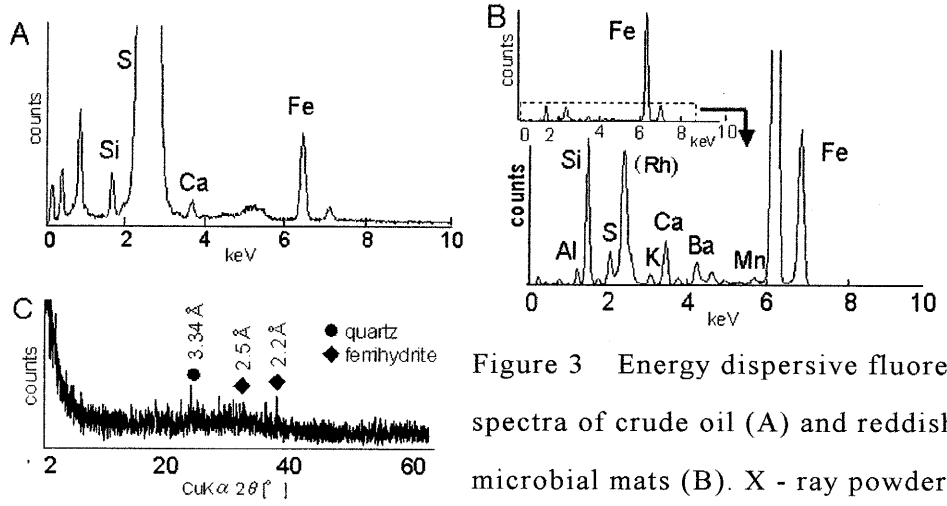

Figure 3 Energy dispersive fluorescent X-ray spectra of crude oil (A) and reddish brown microbial mats (B). X - ray powder diffraction patterns of bulk sample of reddish brown microbial mats (C).

Optical microscopic, SEM and TEM observations of diatom

Optical light micrograph and epifluorescence microscopic observation of the reddish brown microbial mats clearly showed the presence of various kinds of bacteria; mainly iron bacteria, diatom, high-density brown materials, and oil droplets (Fig. 4A, B; arrows pointed oil droplet in Fig. 4B). Epifluorescence micrographs show colonies of microorganisms in the microbial mats, are composed of diatoms (about 40～50 μm in length), algae which has chlorophyll (10 μm in diameter), and coccus and bacillus typed bacteria (2～4 μm in length) (Fig. 4C). Epifluorescence micrographs revealed diatom cells to emit fluorescent blue under the DAPI stain.

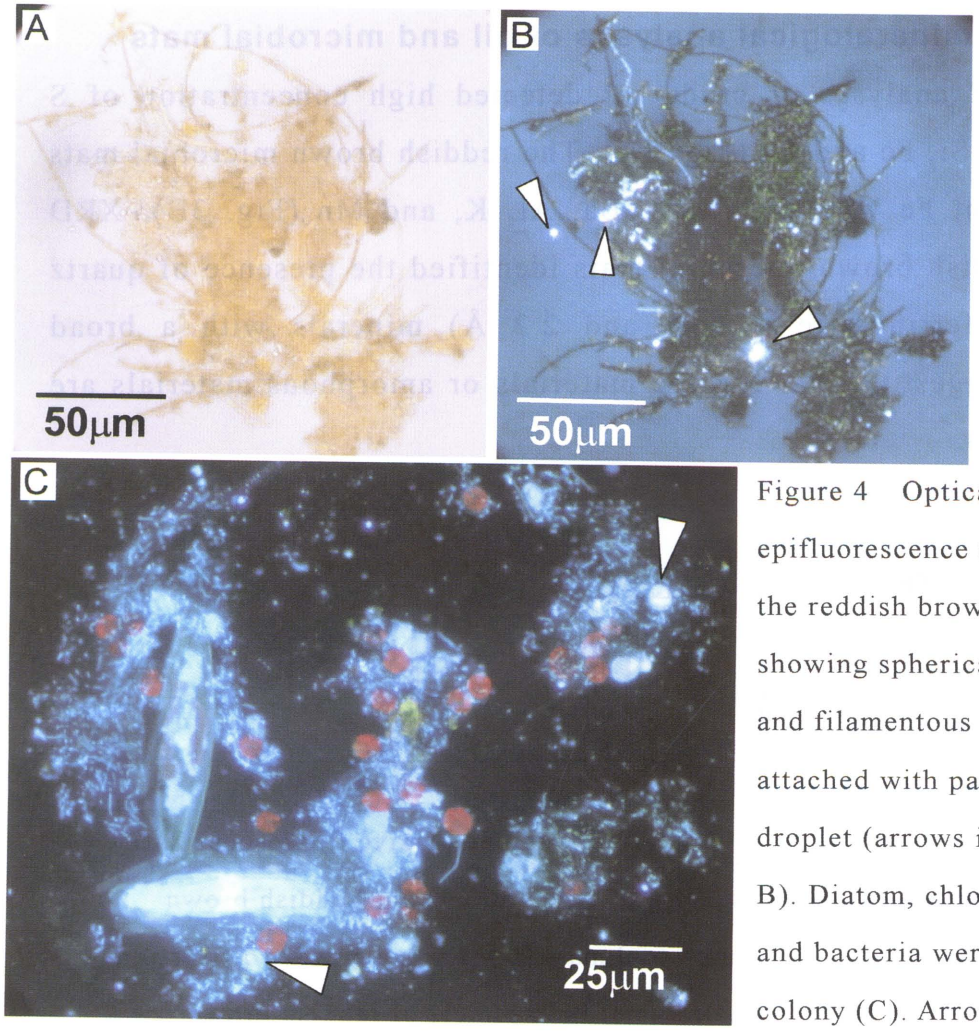

Figure 4 Optical and epifluorescence micrographs of the reddish brown microbial mats showing spherical, rod-shaped, and filamentous iron bacteria attached with particles and oil droplet (arrows in Fig. 4B) (A, B). Diatom, chlorophyllose algae, and bacteria were inhabited in the colony (C). Arrows indicated oil droplets (in Fig. 4C).

Epifluorescence microscopic observation indicates the presence of a diatom, identified as *Pinnularia* spp. (150 ~ 200 μm in length) and *Frustulia rabenhorst*. (100 ~ 250 μm in length). These diatoms were encrusted with crude oil around the cells (Fig. 5A,B). Another diatom identified as *Achnanthes* sp. or *Navicula* sp., of about 10 ~ 20 μm in length, showed an accumulation of crude oil droplet inside the cells (Fig. 5C). Epifluorescence microscopic image of nonluminous diatoms cell which encrusted with a large amount of crude oil after DAPI staining (Fig. 5D). The results suggest that a thick encrustation of crude oil bring dyspnea of diatoms and stunts their growth.

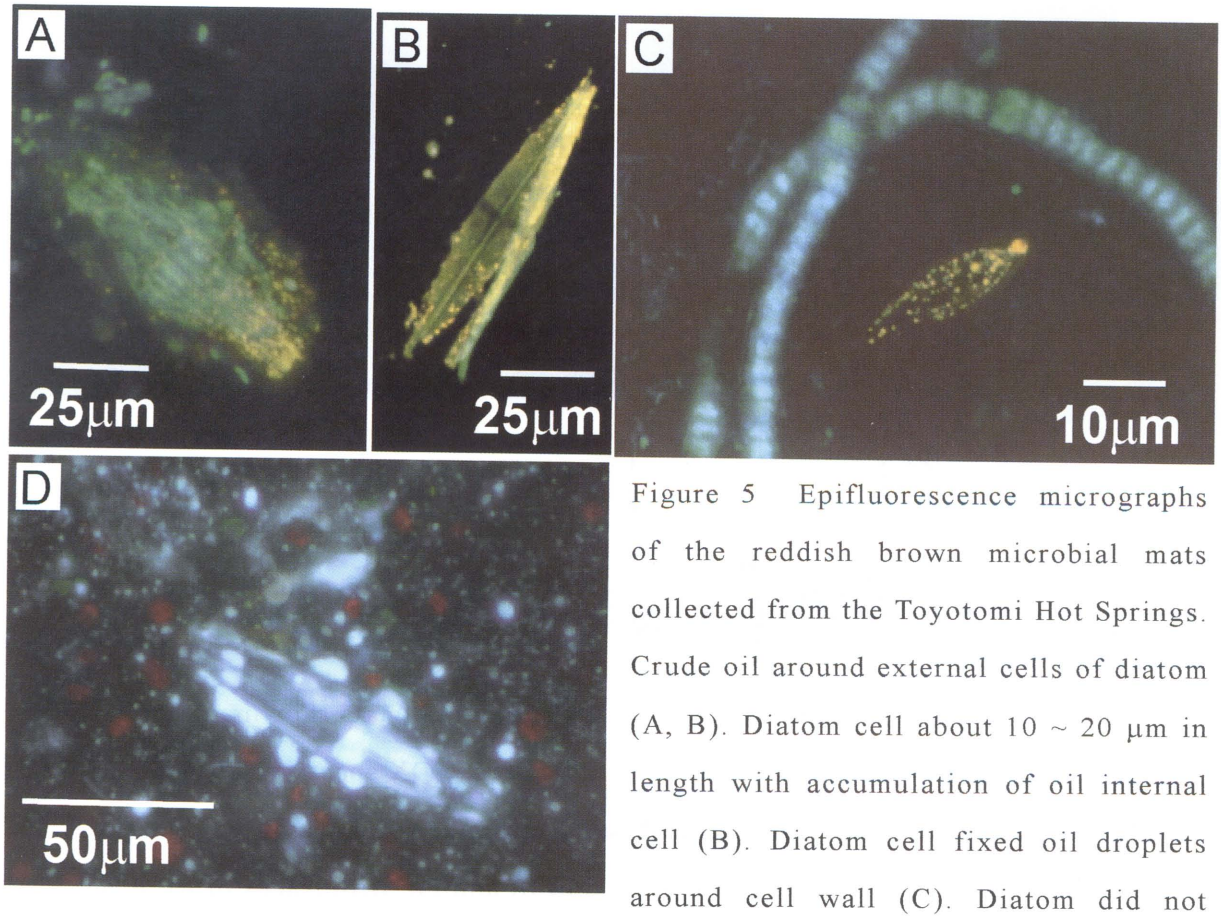

Figure 5 Epifluorescence micrographs of the reddish brown microbial mats collected from the Toyotomi Hot Springs. Crude oil around external cells of diatom (A, B). Diatom cell about 10 ~ 20 μm in length with accumulation of oil internal cell (B). Diatom cell fixed oil droplets around cell wall (C). Diatom did not gleam when a large amount of oil droplets encrusted cell wall (D).

SEM observation of reddish brown microbial mats showed the diatoms in a colony and iron bacteria aggregated with granular particles cling to the crude oil droplets (Fig. 6A). EDX spectrum of a crude oil droplet (arrow ① in Fig. 6A) showed a hilly background and high Fe contents associated with traces of Si, P, S, K, and Ca, suggesting the presence of petroleum. Diatom cells (arrow ② in Fig. 6A) were mainly composed of Si and Fe, with traces of Al, P, K, and Ca. Coccus typed bacteria, 1 μm in diameter were found around the oil droplets, indicating the process of metabolic transformation of crude oil is taking place (arrows in Fig. 6B).

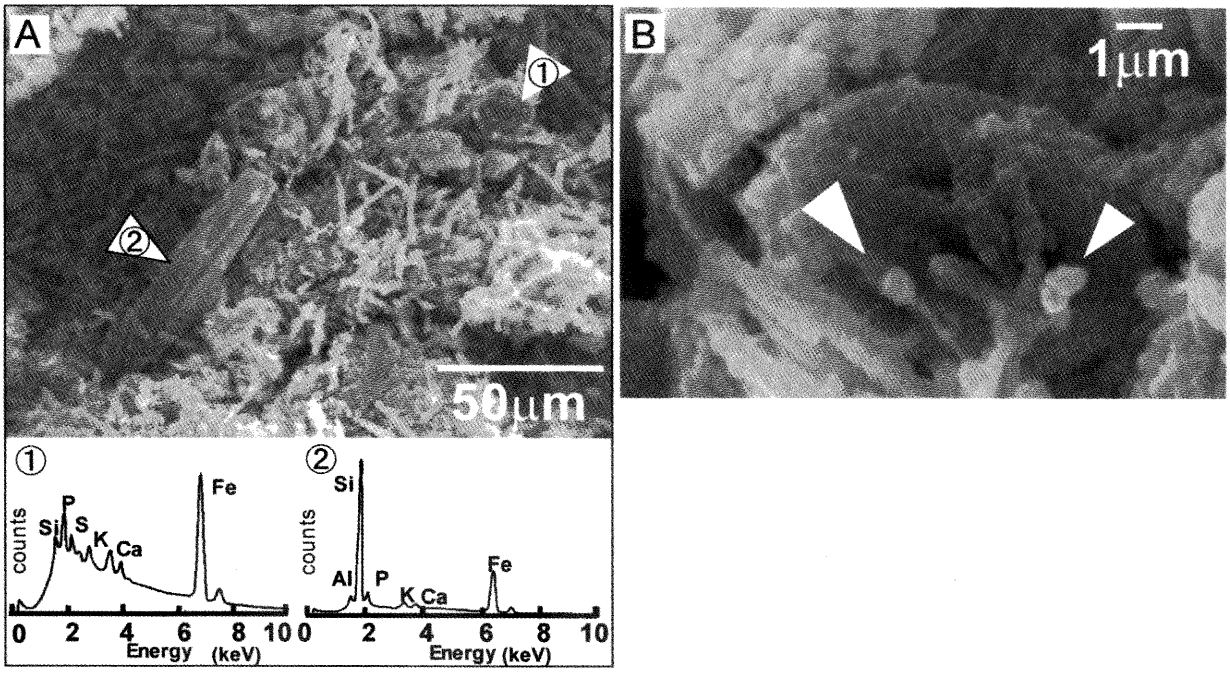

Figure 6 SEM maicrographs and EDX spectra of microbial mats showing colonies of iron bacteria together with diatoms and oil droplets (A). EDX spectrum of crude oil droplet (arrow ①) with a hilly background, suggesting the presence of organic materials. Diatom (arrow ②) mainly composed of Si and Fe, with traces of Al, P, K and Ca. Coccus typed bacterial cells with oil droplets (arrows in Fig. 6B).

Small oil droplets about 0.3～0.5 μm in diameter are also observed on the surface of diatom cell wall according to the SEM image (arrows in Fig. 7A, B). The size of oil droplets are less than 0.3～0.5 μm in diameter on the warty surface of diatom cell wall .

TEM observation of ultra thin-sectioned diatoms, *Frustulia rabenhorst,* showed pores on the cell wall, 200 nm in diameter (arrows in Fig. 8). Microscopic observation indicated that this genus adhere to oil droplets around the external cell (Fig. 5B). Usually, these pores have a role of substance circulation from external to internal diatom cell, and release mucus to revitation in water (Iwasa, 1976).

Various kinds of microorganism to take crude oil within their cells as nutrients and energy source have been reported (Iwasa, 1976 ; Souitham *et. al.*, 2001). The enzyme of microorganisms decomposes hydrocarbon of

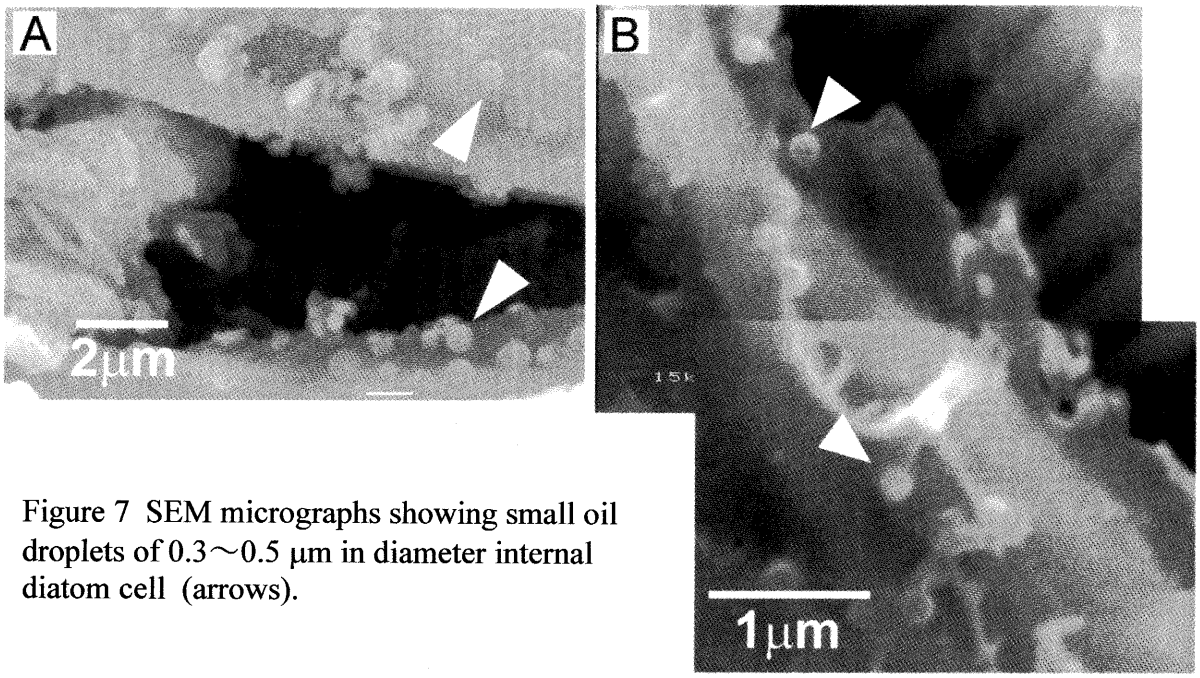

Figure 7 SEM micrographs showing small oil droplets of 0.3～0.5 μm in diameter internal diatom cell (arrows).

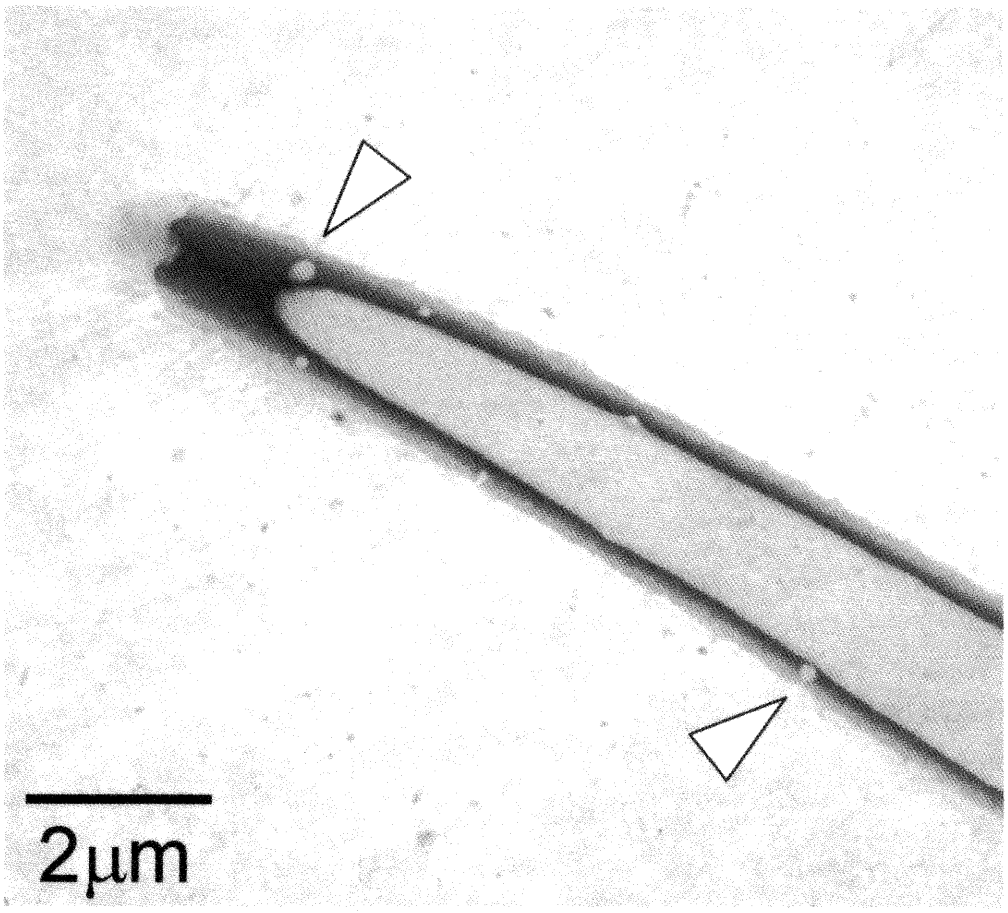

Figure 8 TEM micrograph of ultra thin-sectioned diatoms, *Frustulia rabenhorst,* existence of small pores (200 nm in diameter) on the cell walls (arrows).

crude oil in the cells, and oil degradation ability tend to decreases. High water salinity bring the decreases of enzymatical activity of decomposes crude oil (Shudo *et. al.*, 1985). The chemical composition of the hot spring water at Toyotomi is similar to seawater. In case of the Toyotomi Hot Springs, dilution of hot spring water with fresh water bring salinity decrease, and increase diatom's enzymatical activity of degrading and accumulating crude oil.

Analyses of ED-XRF and XRD indicate the microbial mats to consist mainly of amorphous Fe. Based on the observation by SEM confirming the existence of iron oxidizing bacteria forming microbial mats. The results suggest that diatoms are effective for bioremediation in a crude oil polluted area.

CONCLUSIONS

Electron microscopical observations revealed crude oil fixation of diatoms in microbial mats at the Toyotomi Hot Springs. Crude oil contains Fe, S, Si, and Ca emitting a yellow fluorescence light under the ultraviolet-ray. Optical and epifluorescence microscopic observations show two typed oil fixations; *Pinnularia* spp. and *Frustulia rabenhorst* are encrusted with oil on the surface of cells, whereas *Achnanthes* sp. or *Navicula* sp. accumulate oil internal cells. The *Frustulia rabenhorst* formed abundant pores (200 nm in diameter) on the cell walls where the oil droplets were filled. The observation still remains to be further analyzed as to the role of the pores not only substance circulation but also oil fixation, and whether such pores are recognized in diatoms other than the genus *Frustulia rabenhorst*.

The oil fixation of diatom in the oily hot spring water confirms that crude oil is degraded as nutrients and energy source for diatoms. This biodegradation of oil droplets suggests that diatoms have the ability to remediate petroleum hydrocarbons in an oil-polluted area.

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