Hydrocarbon-degrading bacteria in the heavy oil polleted soil and seawater sfter 5 years bioremediation

Hydrocarbon-degrading bacteria in the heavy oil polluted soil and seawater after 5 years bioremediation

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Abstract

A five years bioremediation study on the aquatic and sedimentary/soil hydrocarbons in the Sea of Japan has been carried out since 1997. A large amount of heavy oil spilled from a Russian oil tanker "*Nakhodka*" on 2 January 1997 in the Sea of Japan and caused a serious impact on the seashores from Mikuni, Fukui Prefecture to Noto Peninsula, Ishikawa Prefecture. The chemical composition of drifted heavy oil was mainly aliphatic hydrocarbons namely n-alkanes of C_9-C_{30}, in which n-eicosane (n-$C_{20}H_{42}$) was the most abundant compound. In the current study, we have performed laboratory examination for bioremediation process of heavy oil with the two different treatments, namely the inside building examination and the outside building one to investigate the significantly role of hydrocarbon-degrading bacteria on them. Observation of hydrocarbon-degrading bacteria was employed by using optical microscope with DAPI staining and scanning electron microscope. In addition, measurements of pH, dissolved-oxygen (DO), temperature, electrode potential *versus* the standard hydrogen electrode (Eh), and electrical conductivity (EC) was performed to investigate the bioremediation process taken place. The chemical composition of heavy oil was estimated by using NCS analyzer. Optical and scanning electron microscopic observation

revealed that a large number of hydrocarbon-degrading bacteria still existed in the sites consisting of a variety of morphological form of bacteria such as coccus (*streptococcus* and *staphylococcus*), bacillus (*streptobacillus*) and filamentous. There was no significant bacterial activity differentiation in the two treatments. In addition, removal of heavy oil by hydrocarbon-degrading bacteria in the outside building examination was slightly greater than in the inside one. The values of DO, pH and temperature in all of the treatments indicated that the bioremediation process took place under aerobic condition (4.8 - 11.1 mg/l), neutral-alkaline condition (7.3 - 8.6) and under condition of low temperature (6.5 - 16.5 ℃).

Key words: *Nakhodka* Russian oil tanker, Bioremediation, Heavy Oil, Hydrocarbon-degrading bacteria

INTRODUCTION

The Russian tanker "*Nakhodka*" spilled approximately 6,240 kl of C-heavy oil into the Japan Sea occurring on January 2, 1997. The heavy oil spill led to a serious impact to the surrounding environment, particularly the heavy oil pollution of the shoreline from Mikuni, Fukui Prefecture to Noto Peninsula, Ishikawa Prefecture (Tazaki 1998). In Ishikawa Prefecture, 202,947 people worked to recover the spilled oil and yielded the emulsified oil of 212,199 kl with seawater from the beach. In Kanazawa City, 14,083 citizens worked to recover the heavy oil and 5,574 people cleaned up the polluted seashore (Itagaki and Ishida 1999). Nevertheless, the heavy oil remained in place and contaminated of soil and seawater in the seashore.

Microbial degradation of petroleum and petroleum product is of considerable economic and environmental importance where microbial degradation is the conversion process that dissolves and disperses hydrocarbons into oxidized products by microorganisms (Tazaki 1998). Since petroleum is a rich source of organic compound and the hydrocarbons within it are readily attacked aerobically by a variety of microorganisms, it is prevalent that petroleum is subject to microbial attack. Under some circumstances such as in heavy oil spills, microbial utilization of oil is desirable

and may even be enhanced by the addition of inorganic nutrients. Bioremediation technologies have increasingly been proposed to decontaminate the polluted sites. The term bioremediation has been coined to refer to the cleanup of oil or other pollutants by microorganisms and in recent years the importance of bioremediation in oil spills has been amply demonstrated in several major heavy oil spills in the marine environment (Vila et al. 2001). However, bioremediation can be time-consuming, but is a safe technique that can be performed at a low cost and without any adverse affect on the environment. A successful bioremediation process is dependent on the ability to create and maintain environmental conditions necessary for microbial growth. Microorganisms are sensitive to temperatures, pH, contaminant toxicity and concentration, moisture content, nutrient concentrations, and oxygen concentration (Eweis et al. 1998).

Most studies on the microbial activity of hydrocarbon-degrading bacteria have been performed with utilizing hydrocarbon under study as the sole source of carbon and energy or a growth substrate. It was reported that most bacterial petroleum hydrocarbon degraders have been isolated from heavily contaminated coastal areas under study (Tazaki 1998; Itagaki and Ishida 1999; Shutsubo 2001).

In the present study, we have studied microbial activity of hydrocarbon-degrading bacteria in their role in the bioremediation process of heavy oil polluted soil and seawater. Since 1997 we have carried out laboratory examination for bioremediation process of heavy oil with the two different treatments, namely the inside building examination and the outside building one to investigate the significantly role of hydrocarbon-degrading bacteria on them.

MATERIALS AND METHODS

Field sites and sampling conditions

Field sampling was performed twice. Samples of heavy oil, seawater, and sand were firstly collected from "Nakhodka" tanker on February 21, 1997, and were secondly collected on November 21, 2001 in Ozawa and Atake at Wajima seashore in Ishikawa, Japan. In Atake, sands were sampled at two previously heavy oil-polluted fields, that is, from the top layer (0 to 30 cm) about 3 - 4 m outside the shoreline and from the top layer (0 to 60 cm) about 5 - 6 m outside the shoreline. These were undertaken to

investigate the difference of microbial activity of hydrocarbon-degrading bacteria, and also as the control sample in isolating of hydrocarbon-degrading bacteria. In addition, heavy oil samples were collected at three differently polluted field sites namely on inshore, nearshore, and away from outside shorelines. All samples were stored at 4 ℃ until required for analysis. Field parameters including seawater temperature, pH, dissolved-oxygen (DO), electrode potential *versus* the standard hydrogen electrode (Eh), and electrical conductivity (EC) were measured in situ using portable meter. These showed that seawater temperatures ranged between 14 and 16.5 ℃; pH from 7.9 to 8.6; dissolved oxygen from 4.8 to 11.1 mg/l; EC from 10.4 to 48.6 mS/cm, and Eh between 20 and 118 mV.

Laboratory examination

We have been performing laboratory examination to investigate the role of hydrocarbon-degrading bacteria in bioremediation process of heavy oil since 1997. We have employed the two different treatments, viz. treatments of outside building and inside building. At the outside building, it can be assigned that not only heavy oil is as a sole energy source but also one another energy source is sunlight that is directly used by *phototrophs*. Further, an experimental design of the laboratory examination was as follows: for the inside building treatment, the medium composition was composed of *Nakhodka* oil spill (collected from Mikuni seashore, Fukui Prefecture), seawater and sand (collected from Shioya seashore, Ishikawa Prefecture), while for the outside one, the medium composition was composed of *Nakhodka* oil spill (collected from Mikuni seashore), seawater (collected from Togi seashore, Ishikawa Prefecture), moss and peat as supplement.

Culture and cultivation of hydrocarbon-degrading bacteria

Hydrocarbon-degrading bacteria were cultured in nutrient agar plate. Heavy oil samples were extracted using n-hexane. A heavy oil sample was taken and added n-hexane to separate heavy oil from others. Furthermore, n-hexane containing heavy oil was evaporated to get the residue of the heavy oil. Then, the 1 ml of the heavy oil solution was added to 9.0 ml of sterile 0.85 % NaCl (w/v) solution. In addition to sand

samples, bacteria were extracted by adding 1.0 g of the sand to 9.0 ml of sterile 0.85 % NaCl (w/v) solution and vigorously shaking the mixture. All agar plates were incubated in an inverted position for 2 to 3 days at 27 ℃.

Chemical and physical parameters

Temperature, pH, Eh, EC, and Dissolved Oxygen were measured during sampling to observe the bioremediation process taken place. In addition, the chemical composition of heavy oil in N (Nitrogen), C (Carbon), and S (Sulphur) to determine biodegradation of hydrocarbon was estimated by using NCS analyzer.

Microbiological parameters

Investigation of hydrocarbon-degrading bacteria was observed by using optical microscope (Nikon NTF2A) with DAPI (4'6-diamidino-2-phenylindole) staining and scanning electron microscope (SEM; JEOL JSM-5200LV) equipped with energy dispersive X-ray analyzer (EDX) for natural and cultured samples.

RESULTS

Observation of hydrocarbon-degrading bacteria by using optical and scanning electron microscopy

The hydrocarbon-degrading bacteria in association with heavy oil droplets were observed under optical microscopy by means of DAPI stain. The bacteria are concentrated in large numbers at the heavy oil-water interface but are not within the droplet (Fig. 1A and Fig. 1B), where A is a differential interference microscopic image, and B is an episcopic fluorescence microscopic image. Hydrocarbon-degrading bacteria develop rapidly on oil films and slicks. Furthermore, the findings revealed that after 5 years bioremediation, a large number of hydrocarbon-degrading bacteria still exist in the sites consisting of a variety of morphological form of bacteria such as coccus (*streptococcus* and *staphylococcus*), bacillus (*streptobacillus* and *staphylobacillus*) and filamentous (Bitton 1994; Tortora et al. 1994; Chaerun and Wisjnuprapto 2000). The filamentous bacteria were surrounded by a tube-like structure called the sheath. The bacterial cells inside the sheath become flagellated (swarmer cells) when they leave the

sheath, and the swarmer cells produce new sheaths at a relatively rapid rate (Bitton 1994). The cells of bacteria tend to remain together in cluster, that is, cocci (spherical-shaped) or bacilli (rods-shaped) occur in long chains (Fig. 2, Fig. 3, Fig. 4 and Fig. 5). Some cocci and bacilli form slime layer of cells, while others occur in three-dimensional cubes or irregular grape-shaped or irregular star-shaped clusters (Fig. 6 and Fig. 7). Point-staining within cells with DAPI led to a blue fluorescence under ultraviolet radiation (wavelength 365 nm) indicating that cells of hydrocarbon-degrading bacteria contain DNA (Deoxyribonucleic acid) molecules. The sizes of bacterial cells at all treatments indicated the approximately similar one ranging between 2 - 3 μm in length and 0.5 - 1 μm in width for rods bacteria, and 0.5 - 1 μm in diameter for spherical bacteria. Bacteria have a high surface-to-volume ratio, a critical factor in substrate uptake, because of the relatively small size.

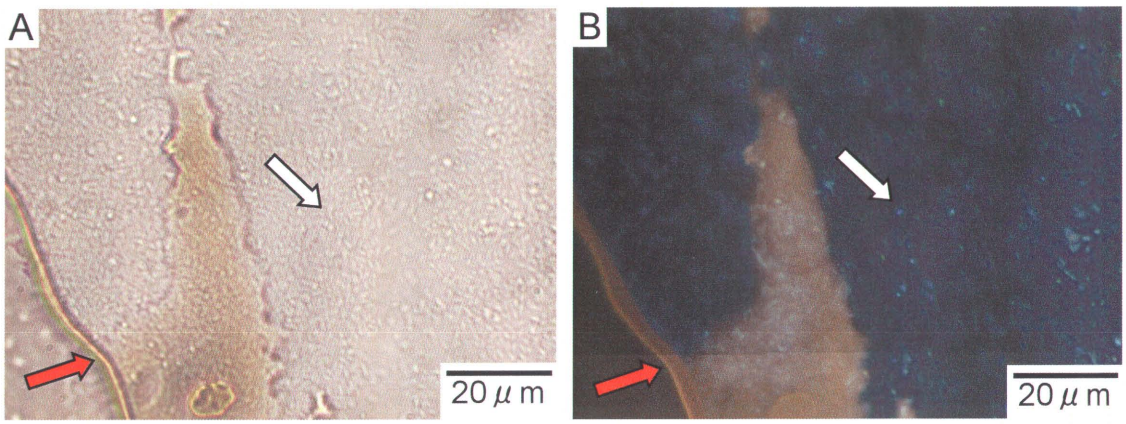

Figure 1 Optical micrographs of hydrocarbon-degrading bacteria in association with oil droplets at the inside building treatment. The sample was collected from Shioya seashore, Ishikawa Prefecture. The white arrow indicates bacteria, and the red arrow indicates heavy oil droplet designated by the brown color in the figure. (A) A differential interference microscopic image. (B)

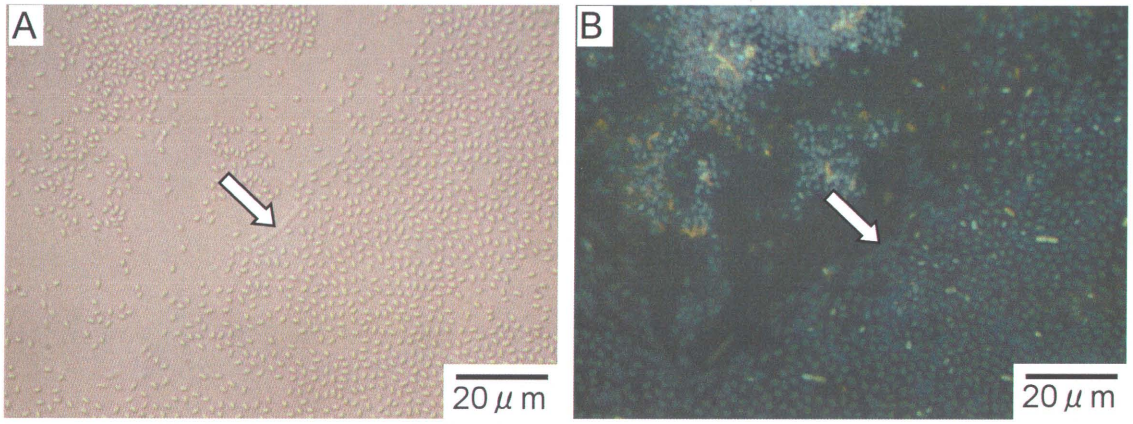

Figure 2 Optical micrographs of hydrocarbon-degrading bacteria in heavy oil at the outside building treatment showing various morphological forms (spherical and filamentous bacteria). The sample was collected from Togi seashore, Ishikawa Prefecture. The arrows indicate filamentous bacteria. (A) A differential interference microscopic image. (B) An episcopic fluorescence microscopic image.

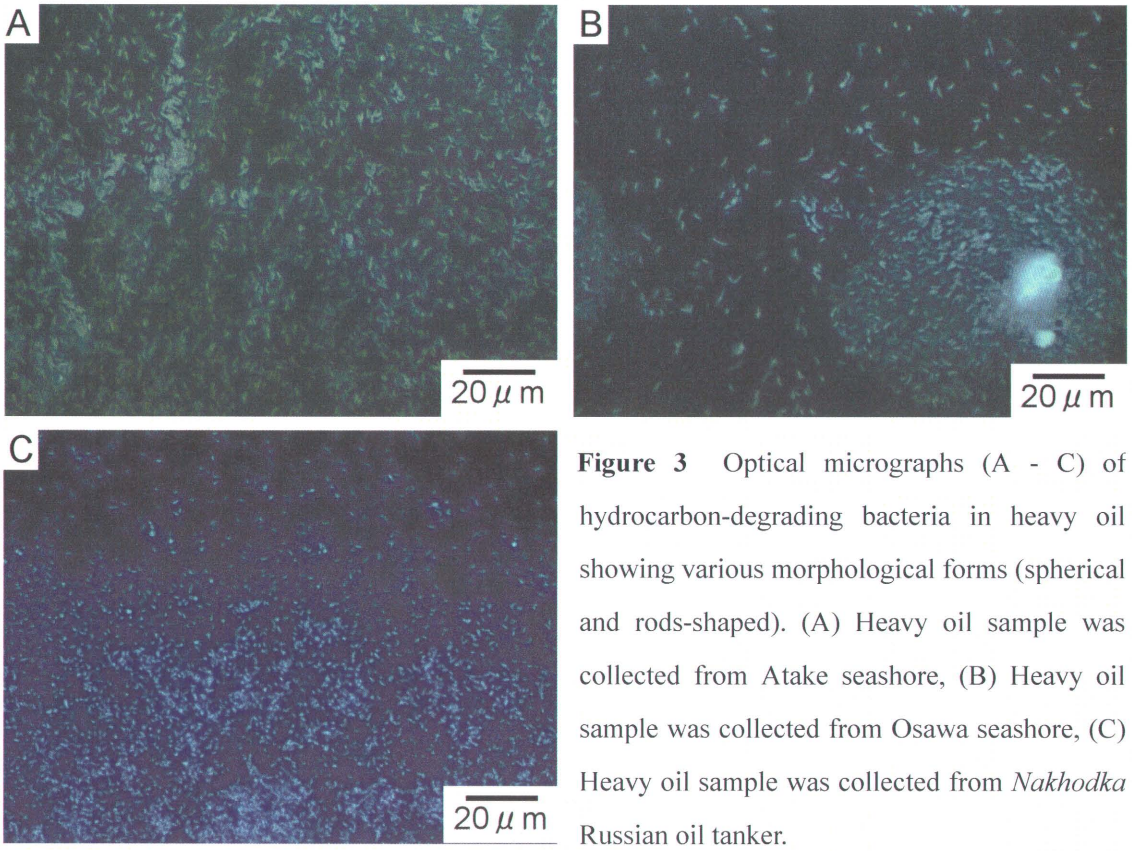

Figure 3 Optical micrographs (A - C) of hydrocarbon-degrading bacteria in heavy oil showing various morphological forms (spherical and rods-shaped). (A) Heavy oil sample was collected from Atake seashore, (B) Heavy oil sample was collected from Osawa seashore, (C) Heavy oil sample was collected from *Nakhodka* Russian oil tanker.

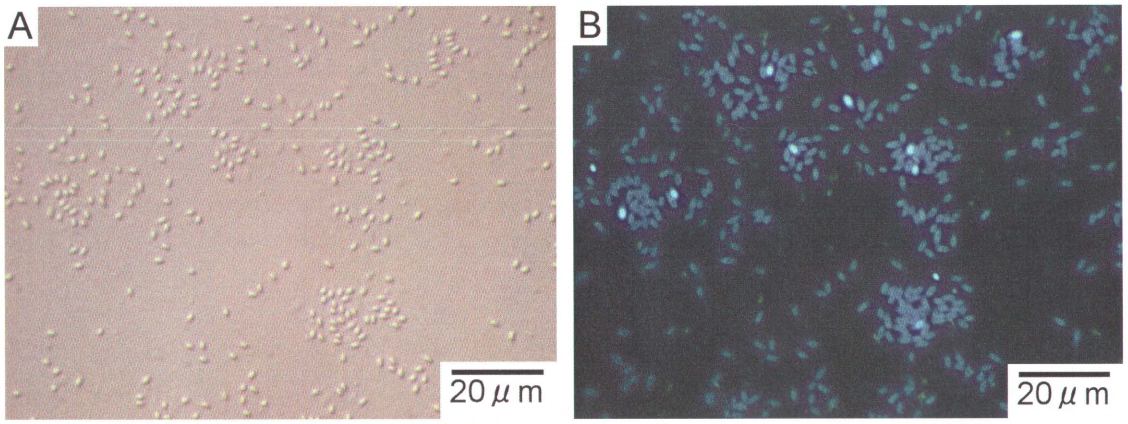

Figure 4 Optical micrographs of hydrocarbon-degrading bacteria of heavy oil polluted sand collected from Atake seashore at the top layer (0 - 30 cm) about 3 - 4 m outside the shoreline (bacterial cell shapes are predominant in short rods-shaped). (A) A differential interference microscopic image. (B) An episcopic fluorescence microscopic image.

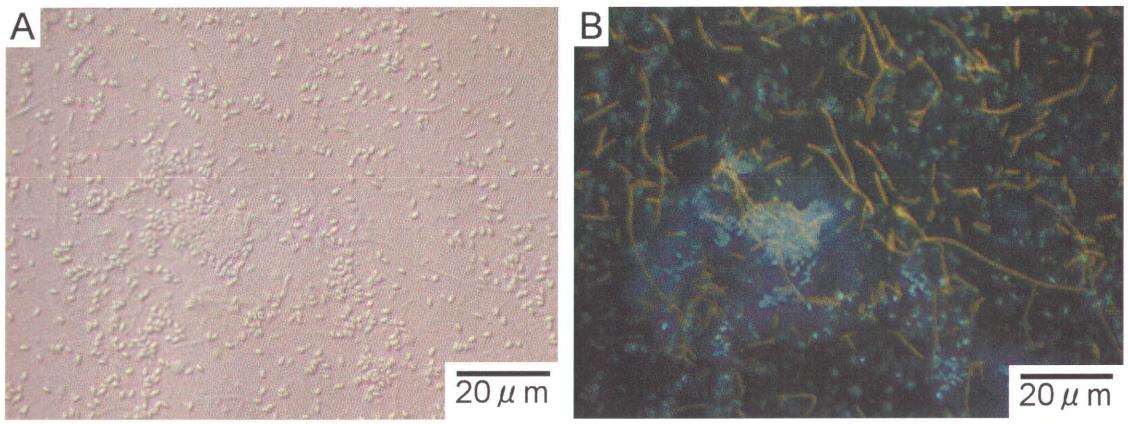

Figure 5 Optical micrographs of hydrocarbon-degrading bacteria of heavy oil polluted sand collected from Atake seashore at the top layer (0 - 60 cm) about 5 - 6 m outside the shoreline. Cells of bacteria form clusters (spherical or rods in long chains), while their shapes are short rods associated with filamentous. (A) A differential interference microscopic image. (B) An episcopic fluorescence microscopic image.

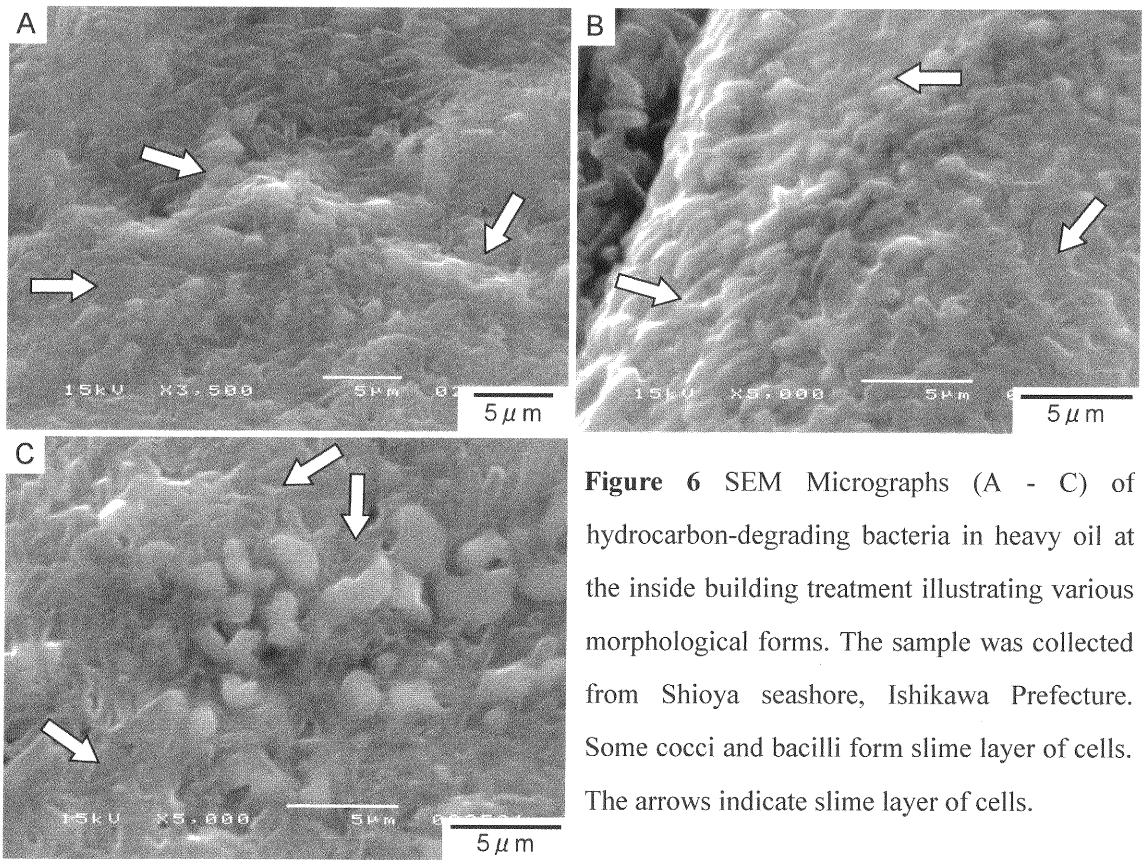

Figure 6 SEM Micrographs (A - C) of hydrocarbon-degrading bacteria in heavy oil at the inside building treatment illustrating various morphological forms. The sample was collected from Shioya seashore, Ishikawa Prefecture. Some cocci and bacilli form slime layer of cells. The arrows indicate slime layer of cells.

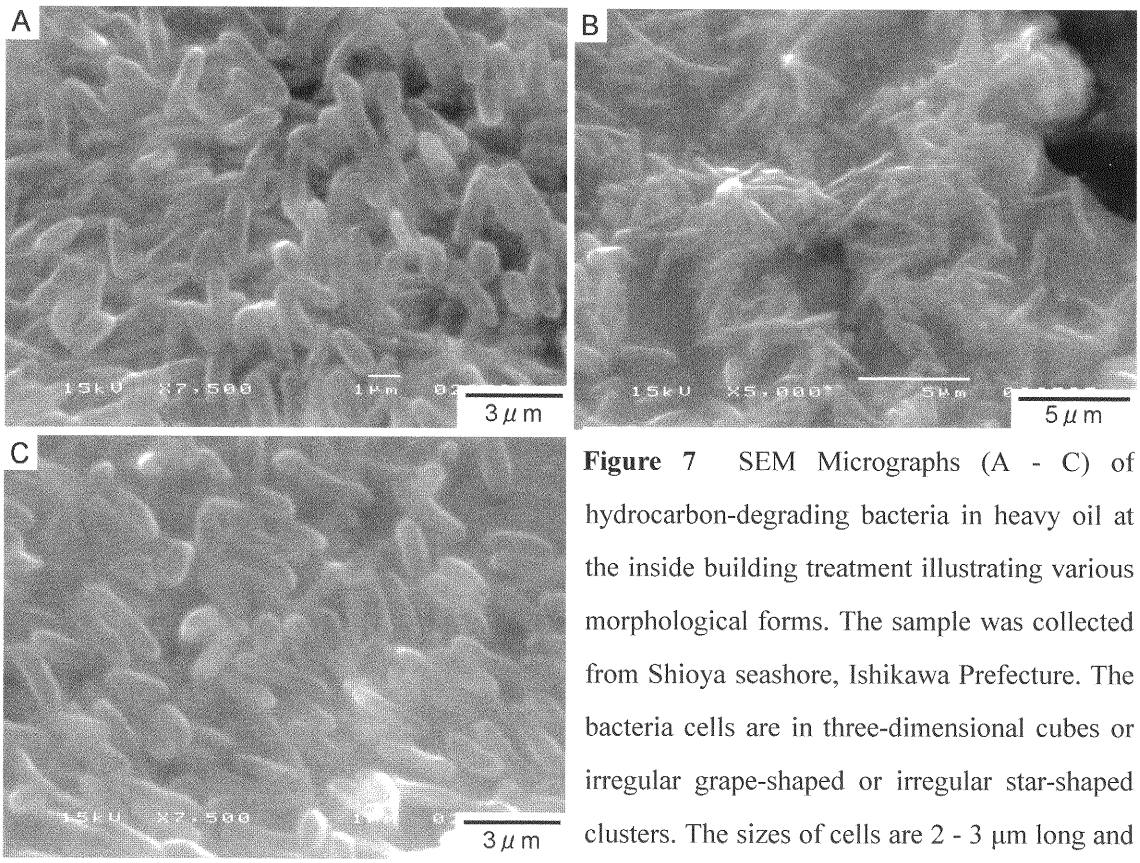

Figure 7 SEM Micrographs (A - C) of hydrocarbon-degrading bacteria in heavy oil at the inside building treatment illustrating various morphological forms. The sample was collected from Shioya seashore, Ishikawa Prefecture. The bacteria cells are in three-dimensional cubes or irregular grape-shaped or irregular star-shaped clusters. The sizes of cells are 2 - 3 μm long and 0.5 - 1 μm wide.

Analysis of physical characteristics of seawater

The field measurements of seawater either with heavy oil slick or without heavy oil slick of each sampling site are given in Table 1. The data showed that seawater pH ranged between 7.3 and 8.6 having the tendency to neutral-alkaline condition; DO ranged from 4.8 to 11.1 mg/l; temperature ranged from 6.5 ℃ to 16.5 ℃; Eh ranged from 12 mV to 138 mV, and EC ranged between 10.4 mS/cm to 199.9 mS/cm (measured in November 2001 and January 2002). Furthermore, comparison of physical characteristics between inside and outside building at laboratory examination during 5 years bioremediation is given in Figure 8. During sampling at all treatments, there were no significantly changes in pH, whereas EC increased rapidly. Conversely, Eh decreased sharply at all treatments, while DO concentration showed bioremediation process took place under aerobic condition. On the other hand, at inside building treatment, at the second measurement (October 20, 1997) DO concentration decreased abruptly to anaerobic condition (0.6 mg/l).

Table 1 Physical characteristics of seawater for each sampling site.

Site	Sample	pH	Eh (mV)	EC (mS/cm)	DO (mg/l)	WT (℃)
Osawa	Seawater	7.9	20	10.4	11.1	16.0
Atake	Seawater without heavy oil	8.6	75	48.6	9.6	16.5
	Sea water with heavy oil	8.3	118	19.0	4.8	14.0
Laboratory examination (inside building)	Seawater with heavy oil	7.7	12	169.1	5.5	15.0
Laboratory examination (outside building)	Seawater with heavy oil	7.3	27	199.9	5.6	6.5
"*Nakhodka*" tanker	Seawater with heavy oil	8.0	138	50.2	9.3	16.5

Eh: electrode potential vs. standard hydrogen electrode,

EC: electric conductivity, DO: dissolved oxygen, WT: water temperature.

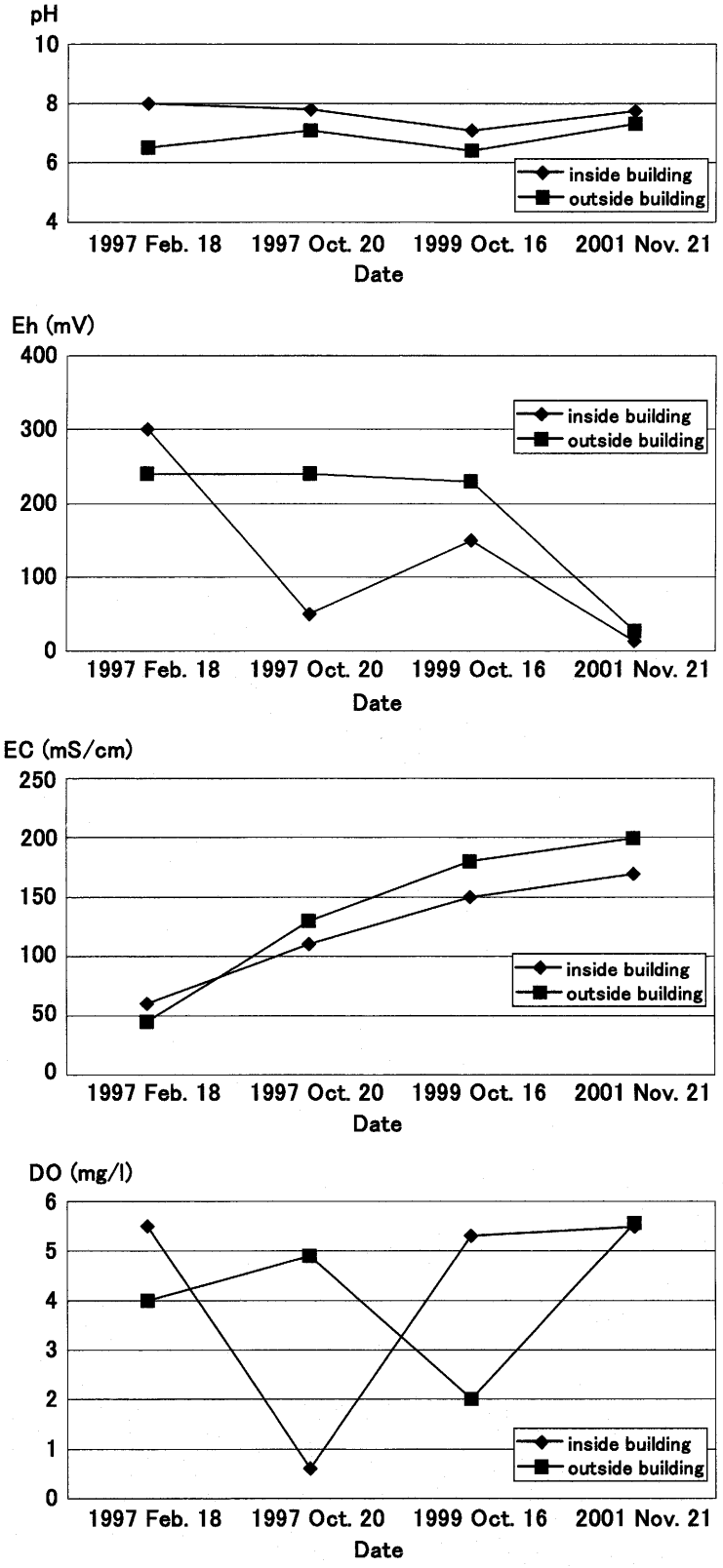

Figure 8 pH, Eh, EC, and DO in the inside and outside building at laboratory examination during 5 years bioremediation. The sample was collected from Shioya seashore, Ishikawa Prefecture (for the inside building treatment) and Togi seashore, Ishikawa Prefecture (for the outside one).

NCS Analysis of heavy oil contents

The chemical compositions of heavy oil of each sample detected in N, C, and S are given in Table 2, demonstrating the contents of C, N, S. The unit of contents was shown in percentage of weight. The contents of C ranged from 59.68 to 87.20 wt% (for *Nakhodka* tanker, laboratory examination inside building, Osawa, and Atake), 24.43 wt% C (for heavy oil inshore in Atake), 0.1 wt% C (for seawater with heavy oil slick), 0.03 wt% C (for seawater without heavy oil slick), 0.06 - 0.2 wt% C (for sand at different depths). Compared with the content of carbon, the contents of N and S were relatively low, that is, 0 - 0.22 wt% N, and 0 - 2.44 wt% S. The "*Nakhodka*" tanker site was highest in proportion to the C content of those of other sites (i.e., 87.20 wt%), while the lowest one was obtained at seawater sample without heavy oil in Atake (i.e., 0.03 wt%). This provided that the biodegradation rate of heavy oil at "*Nakhodka*" tanker site was relatively low if compared with those of other sites. Compared with the inside building treatment, the outside building treatment had a relatively high biodegradation rate of heavy oil shown by the low C content (0.054 wt%). In Atake seashore, the biodegradation rate of heavy oil climbed slightly where it reached a peak on the inshore and gradually dropped into the nearshore and outside shoreline designated by declining the C contents (83.40 wt%, 82.90 wt%, 24.40 wt%, respectively). Whereas C content of sands at all depths elucidated that there was no significantly difference among them indicated by the low contents if compared with those of other samples.

Table 2 Chemical composition of heavy oil in N, C, S (wt %) for each sampling site analyzed by using NCS analyzer.

Site	**Sample**	**N (wt%)**	**C(wt%)**	**S(wt%)**
***"Nakhodka"* tanker**	Seawater with heavy oil	n.d.	87.20	1.44
Laboratory Examination (inside building)	Seawater with heavy oil	n.d.	59.68	1.83
Laboratory Examination (outside building)	Seawater with heavy oil	n.d.	0.05	1.61
Osawa	Heavy oil	0.19	83.08	1.12
Atake	Heavy oil (outside shoreline)	n.d.	83.40	1.38
	Heavy oil (inshore)	n.d.	24.43	0.18
	Heavy Oil (nearshore)	0.22	82.90	1.20
	Seawater with heavy oil	n.d.	0.10	2.44
	Seawater Without heavy oil	n.d.	0.03	2.27
Atake *Sand of 30 cm in depth*	Sand At the top layer	n.d	0.14	0.01
	Sand In the middle layer	n.d.	0.10	n.d
	Sand At the bottom layer	n.d.	0.14	0.01
Atake *Sand of 60 cm in depth*	Sand At the top layer	n.d.	0.20	n.d.
	Sand In the second layer	n.d.	0.16	n.d.
	Sand In the third layer	n.d.	0.26	0.004
	Sand In the fourth layer	n.d.	0.08	n.d.
	Sand At the bottom layer	n.d.	0.06	n.d.

N:Nitrogen, C:Carbon, S: Sulphur, n.d.: not detected.

Bacterial activities during 5 years bioremediation

Investigation of bacterial activity during 5 years bioremediation demonstrated that at all sampling sites ("*Nakhodka*" tanker, laboratory examination, and Osawa-Atake), there was no change in morphology and size of hydrocarbon-degrading bacteria during observation (Table 3). Hydrocarbon-degrading bacteria occured in two basic shapes namely cocci (spherical) and bacilli (rods-shaped), and one unusual shape namely filamentous (sheathead bacteria). Bacterial sizes at all sampling sites were in the range of 2 - 3 ì m illustrating the approximately similar one from 1997 to 2001. In addition to the bacterial number, there was a drop in the bacterial number at heavy oil samples in Osawa- Atake in 2001 (10^6 - 10^8 cells/ml declined to 10^4 - 10^6 cells/ml). Conversely, there was a rise in the bacterial number at seawater and sand samples, that is, 10 - 10^2 cells/ml increased to 10^3 - 10^4 cells/ml for seawater samples and 10^3 cells/ml increased to 10^3 - 10^4 cells/ml for sand samples. In addition, bacterial colonies rose vastly in 2001.

Tabel 3 Comparison of bacterial activity at "*Nakhodka*" tanker, lab. Examination (the sample was collected from Shioya and Togi seashores, Ishikawa Prefecture), and Osawa-Atake seashores during 5 years bioremediation.

Bacteria	Year 1997	Year 2001	Year 2001
	"*Nakhodka*" tanker	**Lab. Examination**	**Osawa-Atake**
Morphology	**Cocci Bacilli Filamentous**	**Cocci Bacilli Filamentous**	**Cocci Bacilli Filamentous**
Size	**2 - 3 μm**	**2 - 3 μm**	**2 - 3 μm**
Number (Cells/ml)	**Heavy oil: 10^6-10^8 Seawater: 10-10^2 Sand: 10^3**	**Seawater: 10^2-10^3**	**Heavy oil: 10^4-10^6 Seawater: 10^3-10^4 Sand: 10^3-10^4**
Colony	**Little**	**Abundance**	**Abundance**

DISCUSSION

Based on the data of field measurements, it can be pointed out that temperature has a strong effect upon bacterial growth and the important parameter to control biological degradation and transformation of petroleum hydrocarbons in soil and water system. If possible, treatments of organic pollutants such as petroleum derivatives and aromatic

hydrocarbons, are performed at moderate temperatures (20 to 37 ℃) in order to facilitate metabolic activity, diffusion, and mass transfer. A higher pollutant degradation rate is usually obtained at moderate than at lower temperatures (Leahy et al. 1990; Zhou et al. 1995; Eriksson et al. 2001). However, a given temperature does not affect different kinds of bacteria in the same way, since they have different optimum temperatures for growth.

There is a very close relationship among temperature, DO, pH, Eh, and EC. Rates of biological oxidation increase with temperature, and oxygen demand increases accordingly, high-temperature conditions, where dissolved oxygen is least soluble. The solubility of oxygen is less in salt-containing water (seawater) than it is in clean water. With the addition of oxidizable pollutant (i.e. heavy oil), the bacterial population rises, and the dissolved-oxygen level drops. An indication of the available electron acceptors within a site can be estimated from Eh measurements expressing the electron availability as it affects oxidation states. Eh usually decrease with depths below the surface because of the limited diffusion of oxygen. Microbial activity is normally greater near the surface and decreases to low levels at depths of over 200 ft (Cookson, 1995).

Referring to data of NCS analyzer, there was a sharp decrease in the content of carbon inshore in Atake. A strong positive correlation exists here between the amount of organic matter (i.e. carbon content) and the abundance of hydrocarbon-degrading bacteria. Kennish (1995) reported that the abundance of bacteria peaks in the estuaries and coastal waters and gradually declines into offshore areas and the open ocean. Additionally, at the outside building treatment, bioremediation process was also remained in effect by the action of phototropic bacteria utilizing sunlight as an energy sources known as photosynthesis. Most phototropic bacteria are capable of growing on CO_2 as sole carbon source. Energy from sunlight is thus utilized in the reduction of CO_2 to organic compounds. The light reactions bring about the conversion of light energy into chemical energy in the form of ATP. Bacteria utilize light primarily to form ATP and then produce NADPH from reducing materials present in their environment such as organic compound (Abeles et al.1992).

Furthermore, the previous research result has been obtained that the chemical

composition of drifted heavy oil was mainly aliphatic hydrocarbon, that is, n-alkanes of C_9-C_{30}, in which n-eicosane (n-$C_{20}H_{42}$) is the most abundant compound, while the contents of aromatic compounds were low (Shibata et al.1997; Itagaki and Ishida 1999). Aliphatic hydrocarbons are not fermentable. Thus, only in the presence of O_2, significant aliphatic hydrocarbon degradation occurs, and if the heavy oil gets carried into anaerobic sediments, it will degrade very slowly and may remain in place for many years. Instead, in aerobic environments, only if other environmental condition, such as temperature, pH, and inorganic nutrients, are sufficient, hydrocarbon-degrading bacteria can play a role. Since heavy oil is insoluble in water and is less dense, it floats to the surface and forms slicks. Hydrocarbon-degrading bacteria are able to attach to insoluble heavy oil droplets, and can often be seen there in large number. The action of these bacteria eventually leads to degradation of the heavy oil and dispersal of the slick. In this case, bacteria participate in heavy oil spill cleanups by oxidizing the oil to CO_2 (Brock 1970).

In addition, microbial utilization of heavy oil is desirable and may even be enhanced by adding inorganic nutrients such as phosphorous and nitrogen to heavy oil spill areas able to increase bioremediation rates significantly. On the other hand, a decrease in microbial activity will slow down degradation and extend the bioremediation period. Cleanup goals may not be achievable employing bioremediation because some pollutants are nonbiodegradable or only partially biodegradable or because the levels of pollutant removal cannot be attained microbially. As the pollutant levels diminish, biodegradation slows down and the bacteria may switch to other energy sources or cease to proliferate altogether. Finally, bioremediation is often relatively time-consuming. The time required to bioremediate a site generally depends on the rate at which the pollutants are degraded (Eweis et al.1998).

As a conclusion, in heavy oil spills where careful bioremediational study has been performed, it has been shown that hydrocarbon-degrading bacteria are the main agents responsible for degradation of heavy oil. In addition, a wide variety of bacteria are capable of heavy oil biodegradation or oxidizing hydrocarbons, and appear to be the prevalent hydrocarbon degraders in aquatics and terrestrial ecosystems, that is, soil/sedimentary and are widespread in them. This study also confirms that a diverse

microbial community exists capable of utilizing hydrocarbon as an electron donor and carbon source. Thereby, hydrocarbon-degrading bacteria obviously play an interesting and important role in the bioremediation process.

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