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Alkane-degrading bacteria and heavy metals from the *Nakhodka* oil spill-polluted seashores in the Sea of Japan after five years of bioremediation

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Abstract This paper describes the isolation of alkane-degrading bacteria from the *Nakhodka* oil spill-polluted seashores in the Sea of Japan. Seven representative strains were identified using 16S rDNA sequence analysis as *Bacillus* spp., *Pseudomonas* sp., and *Paracoccus* spp. All bacterial strains showed their ability to grow well on aliphatic hydrocarbons, but not on aromatic hydrocarbons. In addition, elemental levels in heavy oil showed wide ranges in all the heavy oil samples consisting of Si, S, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, and Pb. Compounds of Si, S, and Cr were observed at high levels, while those of Ti, Mn, Fe, Co, Ni, Cu, Zn, and Pb were observed at low levels. Of all heavy metals, Co appeared to be toxic for all bacterial growth at concentrations of >1 ppm, while the presence of Ti, Cr, and Cu at 0.01 to 10 ppm were found not to inhibit growth of all bacterial strains. It is suspected that the presence of heavy metals may have a significant effect on the composition of the bacterial community, (i.e., alkane-degrading bacterial isolates), as well as on the biodegradative processes of the *Nakhodka* oil spill during the 5-year bioremediation.

Keywords : Alkane-degrading bacteria, *Nakhodka* oil spill, Bioremediation, Heavy oil, Heavy metal.

1. Introduction

Pollution of petroleum hydrocarbons, in particular oil spills, has attracted much attention in the past and recent decades. In general, oil spills, no matter of their sizes, influence natural microbial community, and physical and chemical properties of the affected sites (e.

g., Ijah and Antai, 2003 ; Tazaki, 2003). Oil spills have also created an interest in the rate and course of oil removal by microbial communities of oil spill-impacted areas under both aerobic and anaerobic conditions. Many approaches to oil spill clean up attempted to accelerate natural biodegradation processes in promoting the activity of oil degrading microorganisms. For most heavily oiled sites, failure to remove the bulk oil will result in it remaining for a considerable length of time (Kingston, 2002). There are several instances where oil spill has persisted for many years. One such spill was that of the Russian tanker the *Nakhodka* that spilled heavy oil into the Sea of Japan on January 2, 1997. The impact of the *Nakhodka* oil spill resulted in a viscous sticky fluid fouling the shores in the Sea of Japan and affected wildlife and marine and coastal ecosystems (Sawano, 2003 ; Tazaki, 2003).

Studies on bacterial strains degrading aliphatic and aromatic hydrocarbons attributable to oil spills received much attention as well. However, only few studies have been conducted on the 1997 *Nakhodka* oil spill in relation to the long-term bioremediational study, as well as hydrocarbon degraders (Shibata et al., 1997 ; Itagaki and Ishida, 1999 ; Ishiyama et al., 1999 ; Hozumi et al., 2000a ; Hozumi et al., 2000b ; Kasai et al, 2001). Hence, our group has carried out a careful bioremediational research of the *Nakhodka* oil spill along seashores in Sea of Japan since 1997 (Tazaki et al., 1997a ; Tazaki et al., 1997b ; Tazaki et al., 1997c ; Chaerun et al., 2002a ; Chaerun et al., 2002b ; Chaerun et al., 2002c ; Chaerun and Tazaki, 2003 ; Asada et al., 2003 ; Tazaki, 2003).

In general, both types of contamination (i.e., organics and heavy metals) are often present in the environment. Many metallic compounds occur in petroleum in extremely small concentrations such as inorganic salts, metal soaps, and organic metal-complex compounds. Petroleum also contains heavy metal complexes (Neumann et al., 1981). Major metal contaminants in petroleum oil are usually aluminium (Al), sodium (Na), iron (Fe), nickel (Ni), and vanadium (V), with, frequently, smaller amounts of magnesium (Mg), tin (Sn), barium (Ba), zinc (Zn), molybdenum (Mo), calcium (Ca), copper (Cu), manganese (Mn), lead (Pb), chromium (Cr), and titanium (Ti) (Wrightson, 1949). Metals in crude oil may enhance or inhibit the degradation of oil by microorganisms. Some metals may be toxic (Gadd and Griffiths, 1978 ; Kamel et al., 1989), and many metals are essential trace nutrients for microbial growth (Doelman, 1986). Long-term exposure to heavy metals (Zn, Cu, and Ni) has been found to alter microbial structure as assessed from total soil PLFA (phospholipids fatty acids) profiles (Baath et al., 1998). Heavy metal contamination of soil reduced fungal biomass and altered the composition of the fungal community (Nordgren et al., 1983 ; Nordgren et al., 1985).

Therefore, it is hypothesized that the presence of metals in heavy oil may be the important factor in the bioremediation/biodegradation of the *Nakhodka* oil spill during the five years of bioremediation. During that period, some metals may be essential for oil-degrading microorganisms and promote the biodegradation processes, while others may not be necessary for biological functions and were toxic. Some metals (e.g., Cu, Fe, Mn,

and Zn) are required in trace concentrations (micronutrients). Some metals (e.g., Cr and Pb) are considered to be important pollutants (Kovalick, 1991). Correspondingly, little or no attention has been paid to the effect of environmental factors (e.g., heavy metals) on the degradation of *Nakhodka* oil spill in contaminated sites. Since environmental contamination with oil is often accompanied by the presence of high levels of heavy metals that are known to be toxic for organisms, the presence of heavy metals in the environment, can therefore, negatively influence the effectiveness of bioremediation processes.

The major objective of this study was to isolate and describe hydrocarbon-degrading bacteria from the *Nakhodka* oil spill-contaminated seashores in the Sea of Japan after five years of bioremediation. Representative bacterial strains were identified by 16S rDNA sequence analysis and characterized for their use and ability to grow on the different hydrocarbons as the sole carbon and energy source. In addition, the metallic contents of heavy oil and their effect on the growth of bacterial isolates were evaluated in comparison, since the indigenous microorganisms at *Nakhodka* oil spill-polluted sites may be exposed to the metals-contaminated oil spill, which may affect their growth and ability to degrade heavy oil during the 5-year bioremediation. To our knowledge, there are no previous reports on the influence of heavy metals on bioremediation of *Nakhodka* oil spill.

2. Materials and methods

2.1. Field sites and sample collection

The study site was the *Nakhodka* oil spill-contaminated coastal areas in the Sea of Japan (Fig. 1). Heavy oil, sand, and seawater contaminated with the *Nakhodka* oil spill were sampled in February 1997 at the *Nakhodka* tanker, in December 1999 at Katano coast in Fukui Prefecture, and in November 2001 at Osawa and Atake coasts in Ishikawa Prefecture, Japan. Samples were stored at 4℃ prior to use.

2.2. Isolation and screening of hydrocarbon-degrading bacteria

Bacteria capable of degrading hydrocarbons were isolated directly from samples of heavy oil, sand, and seawater contaminated with *Nakhodka* oil spill by selective enrichment using Bushnell Hass Mineral Salts medium (BHMS) modified with 2% NaCl and supplied with heavy oil 1% (v/v) from *Nakhodka* oil spill as the sole added carbon and energy source. BHMS contained (per liter of distilled water) 0.2 g of $MgSO_4.7H_2O$, 0.02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NH_4NO_3, 2 drop of $FeCl_3$ 60%. The pH was adjusted to 7 to 7.8 with 1N NaOH. The BHMS medium was sterilized by autoclaving (121℃ for 20 min). Heavy oil was sterilized by filtration through 0.2 μm membrane filters. Enrichment of bacterial culture was carried out in 300 ml Erlenmeyer flasks containing 100 ml of BHMS. Samples of heavy oil, sand, or seawater (10% w/v or v/v) were added to 100 ml of BHMS supplemented with 2% NaCl and 1% (v/v) heavy oil from the *Nakhodka* oil spill. All flasks were incubated at room temperature (24-25℃) by shaking at 125 rpm. Bacteria were isolated by plating serial dilutions on ZOBELL agar. All plates were incubated

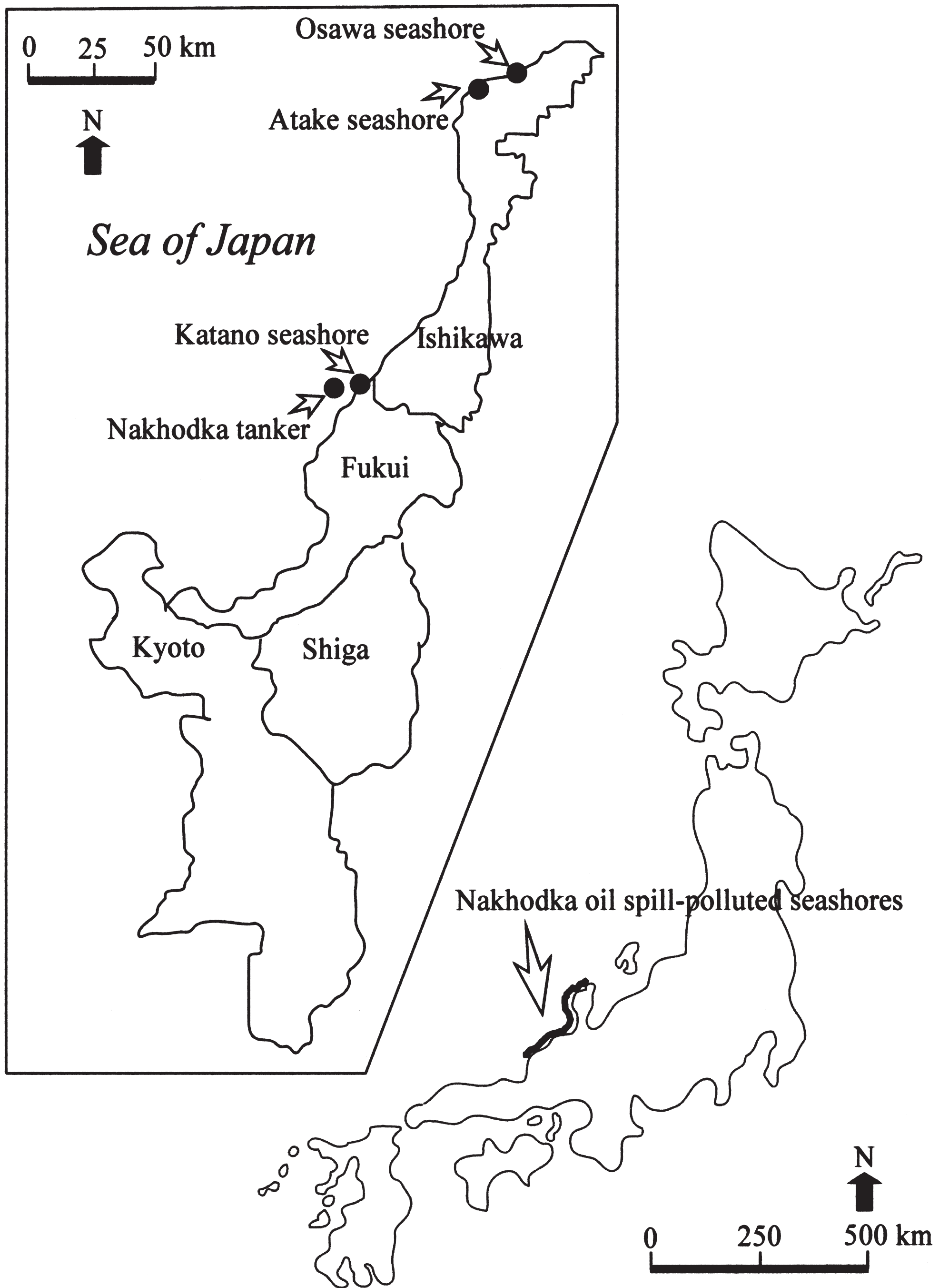

Fig. 1. Map of the study area and sampling stations. The map of Japan on the right shows the coastal sites (as indicated by a solid black line) in the Sea of Japan contaminated with the *Nakhodka* oil spill occurring on January 2, 1997. The magnified map of contaminated coastal areas (on the left) shows the sampling stations indicated by arrows.

at 25℃ for 2-3 days. Colonies were purified by repeated transfer on the isolation medium, and then screened for growth on BHMS amended with 0.85% (w/v) NaCl and 0.5 g/l yeast extract, and supplied with 1% (v/v) heavy oil as carbon source. The isolates that grew well on heavy oil were selected for detailed study, whereas those failed to grow on heavy oil were excluded from further screening. Of the 39 bacterial isolates obtained, seven isolates were assayed for their use and ability to grow on different individual hydrocarbons other than heavy oil (*Nakhodka* oil spill).

2.3. Characterization of hydrocarbon degrading bacteria

Seven isolates were characterized for their use and ability to grow on the different hydrocarbons as the sole carbon and energy source. They were tested in test tube containing 10 ml of BHMS amended with 0.85% (w/v) NaCl which was sterilized by autoclaving (121℃ for 20 min), and supplied with the following hydrocarbons as sole carbon source : hexane, octane, tetradecane, hexadecane, octadecane, eicosane, octacosane, paraffin, pristine, cyclohexane, toluene, biphenyl, naphthalene, phenanthrene, and fluoranthene. Hydrocarbons were sterilized by filtration (membrane filter 0.2 μm), while solid hydrocarbons (octadecane, eicosane, octacosane, and aromatic hydrocarbons) were added directly to BHMS medium before autoclaving. The pH of the medium was adjusted to 7.8 with 1N NaOH. A loopful of each of the tested isolates from the purity plates was inoculated in test tubes containing sterile BHMS supplemented with the sterile hydrocarbons mentioned above. The tubes were incubated with agitation (150 rpm) at 25℃ for 15 days. After 15 days of incubation, the bacterial growth response was determined immediately by the increase in both optical density (OD_{600}) and colony forming units (CFU) in nutrient agar plates supplemented with 0.85% (w/v) NaCl and 0.5 g/l yeast extract.

2.4. 16S rDNA sequencing analysis

Sequence analyses of 16S ribosomal DNA (16S rDNA) were performed on the isolates by amplifying the 16S rRNA genes by PCR using the bacterial universal primers 27f and 1492r (Lane, 1991). The PCR mixture contained 1 μl of template DNA, 2.5 μl of 10 X reaction buffer, 2 μl of 2.5 mM of deoxynucleoside triphosphate (dNTPs), 0.5 μl of 10 pmol of each primers, and 0.25 μl of 2.5 U of Z-*Taq* DNA polymerase (TaKaRa Bio Inc, Shiga, Japan) in a final volume of 25 μl. DNA amplification was performed in a model PTC-100 Thermal cycler (MJ Research Inc, Watertown, Mass. USA) with following profile : 30 cycles of 96℃ for 1 sec, 55℃ for 10 sec, and 72℃ for 20 sec. The PCR products purified with EXOSAP-IT kit (USB corp., USA) were sequenced directly using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, USA). After purification of sequencing products by ethanol precipitation, they were run on an ABI 3100 Genetic Analyser (Applied Biosystems, USA). The closest related sequences were found using the BLAST programs (Altschul et al., 1997).

2.5. Metallic content of heavy oil

Metallic content of heavy oil was estimated using the ED-XRF (Energy Dispersive-X-Ray-Fluorescence) analyzer according to Obiajunwa et al. (2002) with modifications. 0.5 g of heavy oil sample was dissolved in 5 ml of n-hexane, shaken vigorously, and allowed to stand for 20 min. 50 µl of the oil-solvent mixture was mounted on Mylar films, and the solvent was allowed to evaporate overnight. The heavy oil was then analyzed by an energy dispersive X-ray fluorescence spectroscopy (ED-XRF ; JEOL JSX-3201), using Rh-Ka generated at 30 kV under vacuum. Measurement was conducted in triplicate. Three replicates of each heavy oil sample were prepared, and the metal content was estimated in all replicates.

2.6. Effects of heavy metals on the growth of bacterial strains

To determine whether the presence of metals in heavy oil affect the isolated strains of alkane-degrading bacteria, four representative strains (selected based on sampling sites) as well as *P. aeruginosa* PAO1 and *E. coli* K-12 MG 1655 (as controls) were tested for their ability to grow on nutrient broth (NB) medium plus 1g/l yeast extract and 0.85% w/v NaCl (except for *E. coli* K-12, which was grown on LB medium) amended with various concentrations (0.01, 0.1, 0.5, 1, 2, 5, and 10 ppm) of metals (Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd, and V). They were tested in test tube containing 10 ml of NB medium plus 1g/l yeast extract amended with 0.85% (w/v) NaCl which was sterilized by autoclaving (121℃ for 20 min), and supplied with the following sterile concentrated metal salt stock solutions of Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd, and V. pH of the medium was adjusted to 7.8 with 1N NaOH. 10% v/v of stock culture of each of the tested isolates was inoculated in test tubes containing sterile NB medium supplemented with the sterile metals mentioned above. The tubes were incubated with agitation (150 rpm) at 25℃ for 24 hours. After 1 day of incubation, the bacterial growth response was determined immediately by the increase in optical density (OD_{600}). Test cultures, control cultures without added metals, and uninoculated control cultures were tested in duplicate.

2.7. Light and Scanning electron microscopy

For light microscopy, bacterial cells were wet mounted on glass slides, stained with 4',6-diamidino-2-phenylindole (DAPI), then observed under epifluorescence microscope (Nikon NTF2A). For scanning electron microscopy, bacterial cells were fixed with 2% (vol /vol) glutaraldehyde in phosphate buffer at room temperature for 1 h, post-fixed in 1% osmium tetroxide at room temperature for 1 h, dehydrated in a graded ethanol series (50, 75, 95, and 100%), mounted on carbon-coated copper stubs, and viewed on a JEOL JSM-5200 LV scanning electron microscope.

3. Results and discussion

3.1. Isolation and identification of bacterial strains able to use the heavy oil as carbon source

Seven bacterial strains isolated showed an ability to use the heavy oil from the *Nakhodka* oil spill as the sole source of carbon and energy. They were coded as D1, D2, D3, D4, D5, D6, and D7. Strains D1 and D2 were isolated from the *Nakhodka* oil tanker, strain D3 from Katano seashore, strain D4 from Osawa seashore, and strains D5, D6, D7 from Atake seashore. Using 16S rDNA sequence analysis, the strains D1, D2, D3, D4, D5, D6, and D7 were affiliated to those of *Pseudomonas aeruginosa* (99% similarity), *Bacillus cereus* or *Bacillus thuringiensis* (99%), *Pseudomonas aeruginosa* (98%), *Pseudomonas aeruginosa* (99%), *Pseudomonas aeruginosa* (98%), *Pseudomonas aeruginosa* (99%), and *Paracoccus seniphilus* or *Paracoccus marcusii* (97%), respectively.

Sequence analysis of 16S rDNA showed that the bacterial communities of the *Nakhodka* oil spill-contaminated coastal sites were dominated by genus *Pseudomonas* during the 5-year bioremediation. The appearance of *Pseudomonas* strain as a major component of the bacterial community was common to all samples and therefore related to the hydrocarbon-polluted sites (Rosenberg, 1992 ; Janiyani et al., 1993). It is likely that the *Pseudomonas* sp. isolated from four locations, and shown to be abundant in those seashores, also became a predominant member of the bacterial community at the site under the *Nakhodka* oil spill-polluted seashores. This organism appears to be well adapted to hydrocarbon biodegradation in seashores and survival during bioremediation process. Hydrocarbon-degrading communities must adapt to the *Nakhodka* oil spill that consists mainly of alkane hydrocarbons. To date, the majority of the PAH-degrading bacteria reported belongs to the genus *Pseudomonas* (Cerniglia, 1993). Microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilizing and/or surviving toxic contamination (Macnaughton et al., 1999). As a result, genus *Pseudomonas* obtained as the most predominant bacteria in bacterial community of contaminated sites seems to be capable of utilizing the *Nakhodka* oil spill as well. The bacterial diversity may be influenced by the complexity of chemical mixtures present and the exposure (Macnaughton et al., 1999). *Pseudomonas* strain may be tolerant and resilient to the metabolic products that are formed during degradation/bioremediation of the *Nakhodka* oil spill, so that this species was predominant among the bacterial communities at all the three coastal sites and *Nakhodka* tanker. Another study also showed that after exposure to toluene, *Pseudomonas* strain was the dominant community capable of toluene mineralization (Hubert et al., 1999).

3.2. Growth of bacterial isolates on different hydrocarbons

Seven selected isolates were assayed for their suitability and ability to grow on both aliphatic and aromatic hydrocarbons, as well as the heavy oil (Table 1). All the tested isolates grew very well on the aliphatic hydrocarbons (C_{14}-C_{28}) and the heavy oil from *Nak-*

Table 1. Growth and ability of strains isolated to use different organic compounds as carbon source

Organic compounds	Strain	D1	D2	D3	D4	D5	D6	D7
Alkanes	Hexane (C_6)	+	+	+	+	+	+	+
	Octane (C_8)	+	+	+	+	+	+	+
	Tetradecane (C_{14})	+ +	+ +	+	+ +	+ + +	+ +	+ +
	Hexadecane (C_{16})	+ +	+ +	+	+ +	+ + +	+ +	+ +
	Octadecane (C_{18})	+ +	+ +	+ +	+ +	+ +	+ + +	+ +
	Eicosane (C_{20})	+	+	+ +	+ +	+ +	+ +	+ +
	Octacosane (C_{28})	+	+	+	+	+	+	+
	Paraffin	+	+	+	+	+	+	+
	Pristane (branched C_{19})	+	+	+	+	+	+	+
	Cyclohexane	+	+	+	+	+ +	+	+
Aromatics	Toluene (1 ring)	−	−	−	−	−	+	−
	Biphenyl (2 rings)	−	−	−	−	−	−	−
	Naphthalene (2 rings)	−	−	−	−	−	−	−
	Phenanthrene (3 rings)	−	−	−	−	+	−	−
	Fluoranthene (4 rings)	−	−	−	−	+	−	−
Heavy oil	the *Nakhodka* oil spill	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+ +

Cells were incubated in aerated tubes containing BHMS medium plus 0.85% (w/v) NaCl supplemented with the appropriate carbon source. Growth was assessed by the increase in both optical density (OD_{600}) and colony forming units (cfu) in NA plates supplemented with 1 g/l yeast extract and 0.85% w/v NaCl during a period of 15 days. (+++) OD_{600} increased above 1.0, corresponding to more than 10^7 cfu ml^{-1}; (++) OD_{600} ranged between 0.5 and 1 (between 10^6 and 10^7 cfu ml^{-1}); (+) OD_{600} did not increase above 0.5; (-) OD_{600} reproducibly failed to increase over a value of 0.1 after 15 days of incubation. All carbon sources were supplied at 1% (w/v or v/v), except that toluene, paraffin, and hexane were provided at 2%, 2%, 3% (w/v), respectively.

hodka oil spill. They were also able to grow on other alkane hydrocarbons such as hexane (C_6), octane (C_8), paraffin (a mixture of alkanes), pristine (branched alkane C_{19}) which is rather recalcitrant in nature (Leisinger and Brunner, 1986), and cyclohexane (one of the most abundant natural cycloalkanes). Most of the strains appeared to be unable to use aromatic hydrocarbons as carbon source. Nevertheless, strains D5 and D6 were isolates which had little ability to grow on aromatic hydrocarbons : strain D5 was able to grow on phenanthrene and fluoranthene, while strain D6 could use toluene as a carbon source. Alkane hydrocarbons (in particular C_{14}-C_{28}) and heavy oil appeared to be their good growth substrates. All strains could grow on linear n-alkanes ranging from at least 6 or 8 to above 28 carbon atoms in length, as well as branched alkanes. However, alkanes from 14 to 20 carbon atoms allowed all strains to grow abundantly. These results suggested that all the strains tested could predominantly utilize alkane hydrocarbons as carbon source, but not aromatic hydrocarbons. In addition, SEM images showed the rod-shaped bacterial strain isolated from Katano seashore (Fig. 2A), the spherical bacterial strain isolated from Osawa

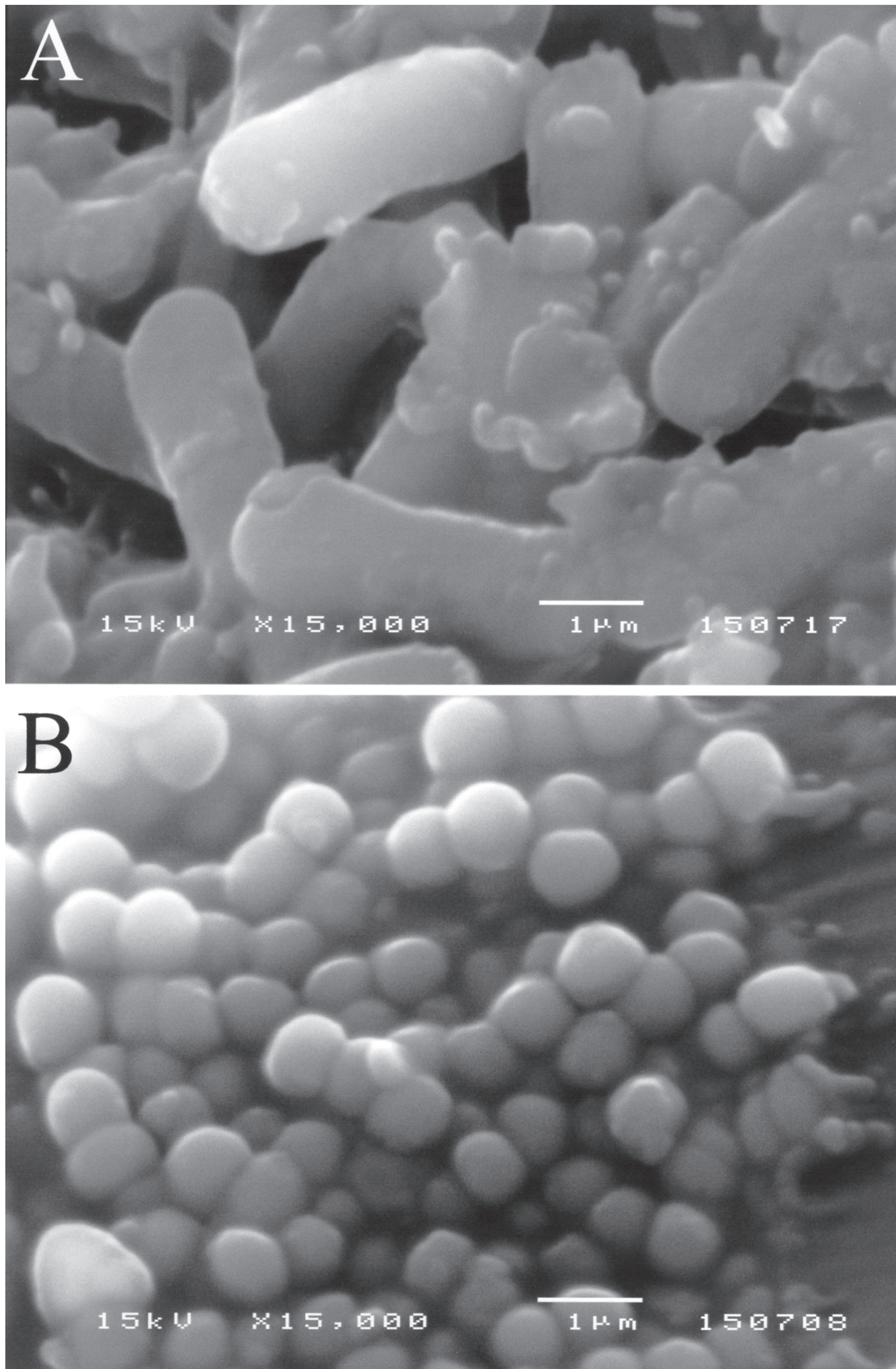

Fig. 2. Scanning electron micrographs of bacterial strains isolated from Katano seashore (A) and Osawa seashore (B).

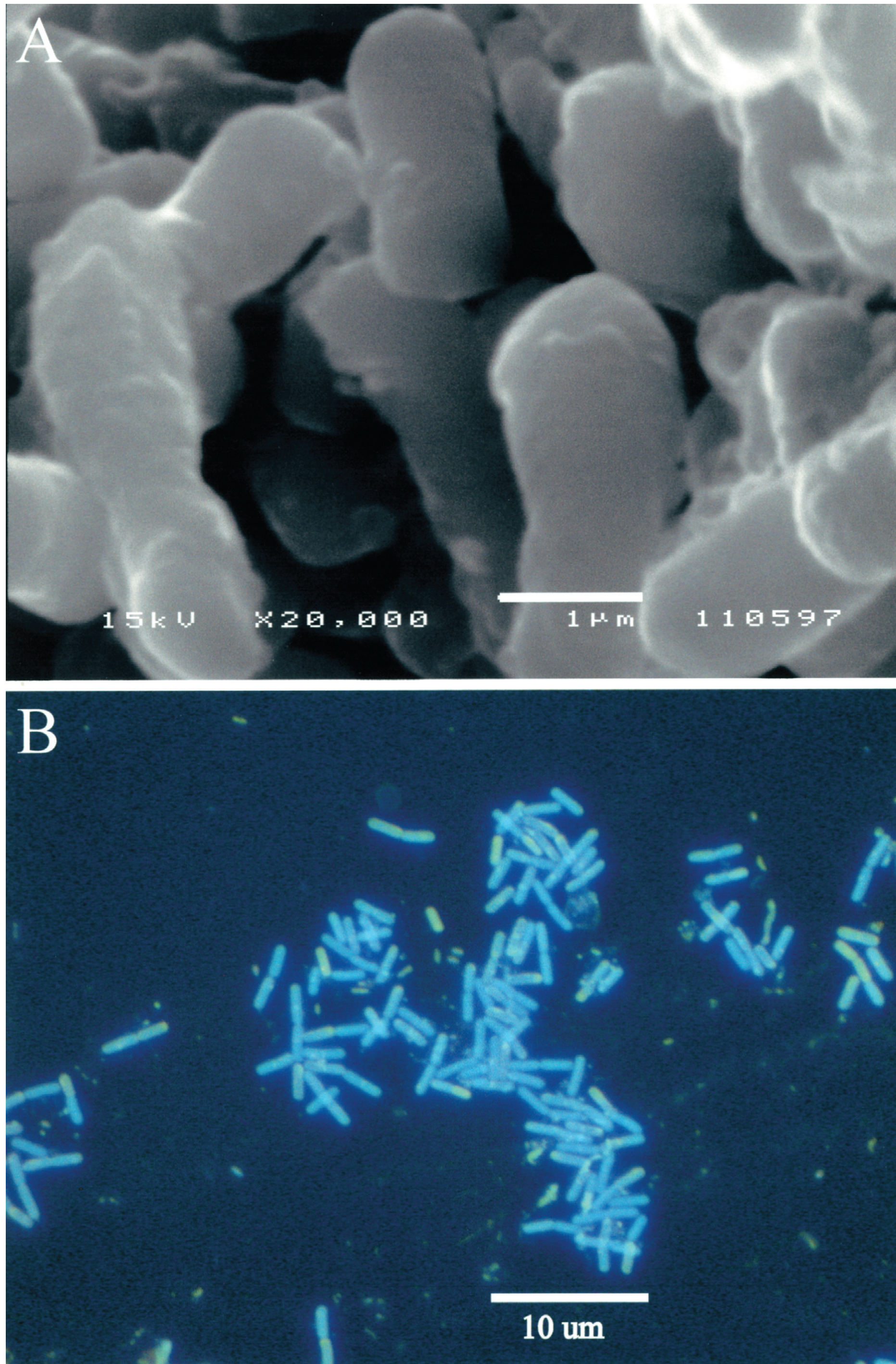

Fig. 3. Scanning electron micrograph (A) and epifluorescence photomicrograph (B) of bacterial strain isolated from the *Nakhodka* tanker.

seashore (Fig. 2B), and the rod-shaped bacterial strain isolated from the *Nakhodka* tanker (Fig. 3A). Epifluorescence microscopy showed DAPI-stained cells isolated from the *Nakhodka* tanker (Fig. 3B).

The incapability of our strains to use PAHs as sole carbon source is not surprising, since the strains were isolated from seashores contaminated with the *Nakhodka* oil spill which consisted mainly of aliphatic hydrocarbons. That is, n-alkanes of C_9-C_{30}, in which n-eicosane (n-$C_{20}H_{42}$) was the most abundant compound, while the contents of aromatic compounds were low (Tazaki et al., 1997b ; Tazaki et al., 1997c ; Shibata et al.,1997 ; Itagaki and Ishida, 1999 ; Sampei and Tazaki, 2003 ; Sampei et al., 2003 ; Chaerun et al., 2003 ; Chaerun and Tazaki, 2003 ; Tazaki, 2003). Microorganisms capable of degrading PAHs are often isolated from environments with a history of contamination by PAHs (Herbes and Schwall, 1978 ; Heitkamp and Cerniglia, 1988 ; Kastner et al., 1994 ; Trzesicka-Mlynarz and Ward, 1995). Bacteria which are capable of degrading alkanes commonly do not degrade PAHs (Foght et al., 1990), although metabolic pathway for both types of compounds is compatible (Whyte et al., 1997). Also, degradation of PAHs occurs much more efficiently by means of mixed microbial consortia than by single strains (Venkateswaran and Harayama, 1995 ; Komukai-Nakamura et al., 1996).

3.3. Metal content of heavy oil

Elemental levels in heavy oil showed wide ranges in all the heavy oil samples which consisted of Si, S, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, and Pb (Table 2). Si, S, and Cr were ob-

Table 2. Average elemental concentrations (wt%) in heavy oil for each sampling site

Element	Nakhodka tanker	Katano seashore	Osawa seashore	Atake seashore
Si	11.37 ± 0.69	93.70 ± 1.52	65.05 ± 26.63	29.66 ± 5.19
S	88.00 ± 0.79	n.d.	46.59 ± 13.02	68.51 ± 5.88
Ti	0.05 ± 0.07	1.11 ± 0.99	0.48 ± 0.26	0.28 ± 0.41
Cr	0.48 ± 0.20	4.60 ± 0.74	2.78 ± 1.33	1.15 ± 0.41
Mn	n.d.	0.01 ± 0.02	0.08 ± 0.07	n.d.
Fe	n.d.	n.d.	0.15 ± 0.16	0.24 ± 0.03
Co	n.d.	0.30 ± 0.10	0.08 ± 0.09	n.d.
Ni	0.06 ± 0.03	0.17 ± 0.04	0.16 ± 0.07	0.09 ± 0.06
Cu	0.01 ± 0.01	n.d.	0.01 ± 0.13	0.04 ± 0.01
Zn	0.03 ± 0.02	0.07 ± 0.01	n.d.	0.03 ± 0.03
Pb	n.d.	n.d.	0.06 ± 0.05	n.d.

Note : mean ± standard deviation ; n = 3, except for S concentration of Osawa seashore, which was calculated using n = 2. Samples of heavy oil were taken on February 21, 1997 (for *Nakhodka* tanker), on December 10, 1999 (for Katano seashore), and on November 21, 2003 (for Osawa and Atake seashores). n.d. : not detected.

served at high levels, while Ti, Mn, Fe, Co, Ni, Cu, Zn, and Pb were at low levels. Average levels of all metals except Mn in heavy oil were highest in Katano seashore. Mn concentration was higher at Osawa seashore. Concentrations of S, Fe, Cu, and Pb were not detected at Katano seashore, whereas the most predominant elements in heavy oil at all seashores were Si, S, and Cr, except in heavy oil at Katano seashore, which did not contain S. It is important to note, however, that the high S content in heavy oil might affect the oxidation rate of oil by bacteria during the bioremediation processes (Brock, 1970 ; Jeffry, 1980). Sulfur is the third most abundant element in crude oil (Speight, 1980), and sulfur heterocycles are common constituents of petroleum and liquid derived from coal (Blessler and Fedorak, 2001).

Elements originating from the *Nakhodka* oil spill were Si, S, Ti, Cr, Ni, Cu, and Zn (at *Nakhodka* tanker), and so it could be stated that the other metals (i.e., Mn, Fe, Co, and Pb) which were found at Katano, Osawa, and Atake seashores did not originate from *Nakhodka* oil spill. Distinct metallic contents of heavy oil among three seashores (Katano, Osawa, and Atake) may have been attributable to both natural (erosion, fires, leaching, volcanic activity, and microbial transformation) and anthropogenic (industrial waste, dumping of sewage, burning of fossil, etc.) sources (Babich and Stotzky, 1980 ; Wong, 1993 ; Kuo and Genthner, 1996 ; Benka-Coker and Ekundayo, 1998 ; Baldrian et al., 2000).

3.4. Effect of heavy metals on bacterial growth

Effects of added metals (i.e., Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd, and V) at 0.01 to 10 ppm on the growth of bacterial isolates are presented in Table 3. Strains D1 (isolated from the *Nakhodka* oil tanker) and D5 (isolated from Atake seashore) were able to grow in the presence of all added metals at concentrations from 0.01 to 10 ppm, except Co, which inhibited growth at 2 ppm or greater. All bacterial strains were able to grow in the presence of low concentrations of all these metals (0.01 to 1 ppm), with the exception of strains D3 (isolated from Katano seashore) and D4 (isolated from Osawa seashore), which were very sensitive even to low concentration of Cd at $\geq$0.5 and $\geq$0.1 ppm, respectively. These are not surprising, since Cd is not an element found in heavy oil samples (Table 2). Compared with strains D1 and D5, strains D3 and D4 tended to be sensitive to all metals tested at 2 ppm or greater. The presence of Ti, Cr, and Cu at 0.01 to 10 ppm did not inhibit growth of all bacterial strains, while Co was inhibitory at concentrations more than 1 ppm, the sublethal concentrations reducing growth by approximately $\geq$50%. Little or no growth was observed at >2 ppm Co for all bacterial strains, with the exception of *E. coli* K-12 control strain. Other metals (i.e., V, Mn, Fe, Ni, Zn, Cd, and Pb) also appeared not to inhibit growth of all bacterial strains, except strain D3 which was very sensitive to $\geq$5 ppm Fe, $\geq$5 ppm Zn, and $\geq$0.5 ppm Cd, as well as strain D4 sensitive to 10 ppm V, 10 ppm Ni, 10 ppm Zn, $\geq$0.1 ppm Cd, and $\geq$2 ppm Pb. However, there was no significant difference in resistance to metals tested between bacterial isolates and control strains such as *P. aeruginosa* PAO1 or *E. Coli* K-12. They showed almost similar sensitivities to added metals.

Table 3. Effect of added metals on the growth of four representative strains

Metal and conc.	%V[a]					
(ppm)	Strain D1	Strain D3	Strain D4	Strain D5	PAO1	*E. coli*
Ti						
0.00[b]	100	100	100	100	100	100
0.01	108	98	80	87	102	93
0.1	102	94	100	87	96	94
0.5	87	96	71	94	101	88
1	98	94	106	95	95	94
2	85	79	106	89	77	95
5	83	71	97	76	91	73
10	68	79	102	93	89	85
V						
0.00[b]	100	100	100	100	100	100
0.01	106	94	71	98	101	88
0.1	95	98	74	104	101	82
0.5	88	96	91	79	102	91
1	82	96	85	82	102	89
2	94	95	103	75	96	84
5	107	98	67	113	100	97
10	102	97	42	92	81	89
Cr						
0.00[b]	100	100	100	100	100	100
0.01	108	83	101	118	93	88
0.1	97	98	100	100	86	95
0.5	96	100	106	88	102	73
1	98	84	107	116	98	83
2	85	100	106	106	102	84
5	109	99	102	113	102	89
10	95	96	95	87	89	84

Table 3. (*Continued*)

Metal and conc. (ppm)	%V[a]					
	Strain D1	Strain D3	Strain D4	Strain D5	PAO1	*E. coli*
Mn						
0.00[b]	100	100	100	100	100	100
0.01	82	98	102	92	92	69
0.1	85	98	108	78	102	74
0.5	83	98	88	80	104	96
1	83	98	106	93	100	94
2	89	100	75	111	88	67
5	108	100	109	83	99	77
10	107	98	97	96	100	98
Fe						
0.00[b]	100	100	100	100	100	100
0.01	106	95	101	104	88	87
0.1	105	97	103	110	102	86
0.5	101	102	101	86	84	86
1	99	102	81	100	96	92
2	106	78	76	124	102	98
5	87	23	83	103	92	69
10	104	14	80	112	109	84
Co						
0.00[b]	100	100	100	100	100	100
0.01	89	98	96	99	100	98
0.1	103	98	62	96	100	72
0.5	94	99	97	84	93	86
1	105	54	79	97	88	85
2	77	17	55	101	83	82
5	55	11	43	37	44	91
10	25	9	16	18	19	85

Table 3. (*Continued*)

Metal and conc. (ppm)	%V[a]					
	Strain D1	Strain D3	Strain D4	Strain D5	PAO1	*E. coli*
Ni						
0.00[b]	100	100	100	100	100	100
0.01	102	97	106	99	93	81
0.1	105	88	106	101	101	84
0.5	105	92	85	89	100	64
1	96	98	104	97	87	67
2	78	98	106	109	97	90
5	97	98	75	80	96	89
10	96	86	51	101	85	98
Cu						
0.00[b]	100	100	100	100	100	100
0.01	104	99	104	86	78	67
0.1	105	96	101	78	89	83
0.5	103	98	103	85	101	71
1	91	132	107	102	95	67
2	109	99	107	78	94	87
5	109	101	108	94	96	95
10	96	99	107	79	95	69
Zn						
0.00[b]	100	100	100	100	100	100
0.01	95	98	100	100	101	96
0.1	106	91	73	76	103	97
0.5	91	99	102	117	92	98
1	98	97	99	111	102	98
2	91	88	72	79	102	98
5	88	11	70	92	103	99
10	104	10	44	75	105	100

Table 3. (*Continued*)

Metal and conc. (ppm)	%V[a]					
	Strain D1	Strain D3	Strain D4	Strain D5	PAO1	*E. coli*
Cd						
0.00[b]	100	100	100	100	100	100
0.01	104	98	98	101	87	84
0.1	109	90	58	91	101	96
0.5	89	41	50	120	103	95
1	96	23	49	77	98	94
2	83	13	55	89	96	90
5	99	10	50	120	103	82
10	99	9	51	98	100	99
Pb						
0.00[b]	100	100	100	100	100	100
0.01	102	97	97	111	85	95
0.1	96	95	99	119	95	87
0.5	106	94	65	98	104	80
1	103	92	84	102	102	88
2	107	92	32	56	46	49
5	101	94	23	56	104	54
10	99	95	40	119	49	110

[a] %V = growth with added metals ÷ growth in controls lacking metals x 100
[b] Control lacking added metal
E. coli K－12 and P. aeruginosa PAO1 were used as control bacterial strains.
Strains D1, D3, D4, and D5 were isolated from the *Nakhodka* tanker, Katano seashore, Osawa seashore, and Atake seashore, respectively.

Furthermore, although V is not an element found in heavy oil samples but is the most abundant heavy metal in oil residues (Sasaki et al., 1998), our bacterial strains were able to grow in the presence of V at 0.01 to 10 ppm. Since Cr was the most abundant heavy metal in heavy oil samples, capability of our strains to grow well in the presence of Cr was not surprising, indicating that bacterial strains may have adapted to the toxicity of Cr. However, their ability to grow at 5 to 10 ppm was extraordinary. Thus, the ability of our some strains to grow in the presence of somewhat higher concentration of some heavy metals (V, Cr, Ni, Zn, Cd, and Pb) indicated that bacterial acclimation periods ensued in metal-containing heavy oil, resulting from induction of a protein required for metal precipitation or detoxifi-

cation, a genetic change, or selection of a tolerant or detoxifying bacterial species or population. These, thereby, resulted in precipitation and/or detoxification of added metals (Kuo and Genthner, 1996).

Our present results showed that metal contents of heavy oil occurred at low levels, thereby enabling these metals as trace elements (micronutrients) needed for microbial growth, in particular to alkane-degrading bacteria, and affected their growth and ability to degrade heavy oil. The results may be related to the findings of Kuo and Genthner (1996), that the presence of metals can be a way of predicting the outcome of anaerobic bioremediation of an organic pollutant as well as the possibility of using anaerobic bacterial species to bioremediate a site that was contaminated with heavy metals. Several anaerobic species may be used to bioremediate both heavy metals and organic pollutants either individually or in combination. It is also possible, however, that the indigenous microorganisms inhabiting the *Nakhodka* oil spill (i.e., alkane-degrading bacteria) which are being exposed to metals may also become resistant to or capable of transforming and detoxifying heavy metals (Compeau and Bartha, 1985 ; Hughes and Poole, 1989 ; Hughes and Poole, 1991 ; Lovley, 1993). The presence of miscellaneous pollutants in any of the oil spills may present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community (Shi et al., 2002). However, it is suggested that both heavy metals and hydrocarbons would influence the composition of microbial community which genus *Pseudomonas* was dominant member at all the contaminated coastal sites in this study. Genus *Pseudomonas* is apparently well-equipped to cope with aliphatic hydrocarbon toxicity and may, therefore, be a primary catalyst for aliphatic hydrocarbon degradation close to the origin of the *Nakhodka* oil spill during the 5-year bioremediation.

4. Conclusion

This study has shown that after long-term exposure to both hydrocarbons and heavy metals of the *Nakhodka* oil spill, alkane-degrading bacteria isolated from three seashores and the *Nakhodka* tanker, identified as *Bacillus* spp., *Pseudomonas* sp., and *Paracoccus* spp., may also tolerate heavy metals other than toxic levels of hydrocarbons. Results of this study suggest that *Pseudomonas* species which was a dominant organism in the bacterial community of the *Nakhodka* oil spill-contaminated coastal sites during the 5-year bioremediation may have been associated with the hydrocarbon degradation at those sites. In addition, the bacterial composition of community in those sites may have played a key role in hydrocarbon degradation and that *Pseudomonas* strain, in particular, was associated with hydrocarbon-degrading activity as well as the bioavailability of heavy metals.

Heavy metals obtained in all the heavy oil samples of three seashores in the Sea of Japan showed that there was a considerable heavy metal pollution which could be correlated not only with the *Nakhodka* oil spill but also with biogenic and anthropogenic sources. Results indicated that Si, S, Ti, Cr, Ni, Cu, and Zn were predominantly indigenous to the *Nakhodka* oil spill, whereas Mn, Fe, Co, and Pb were present as the result of contamina-

tion associated with other sources. Our data also have a significant insight into how these metals in the *Nakhodka* oil spill affect the biodegradation of heavy oil during the 5-year bioremediation, thereby giving more fruitful information for such kinds of oil spills. In contrast, these results also provided information that the *Nakhodka* oil spill resulted in not only organic pollutants (i.e., hydrocarbons) but also heavy metal pollutants (i.e., Cr) along the coastal areas of Sea of Japan.

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References

Altschul SF, Madden TL, Scha”ffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST : a new generation of protein database search programs. Nucleic Acids Res 25 : 3389-3402

Asada R, Tanaka Y, Chaerun SK, Tazaki K (2003) Bioremediation of oil contaminated soils : cultivation experiments using an oil adsorption-resolution item “sponge”. In : Tazaki K (ed) Heavy oil spilled from Russian tanker “Nakhodka” in 1997 : towards eco-responsibility, earth sense. 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan, pp 337-351

Baath E, Diaz-Ravina M, Frostegard A, Campbell CD (1998) Effect of metal-rich sludge amendments on the soil microbial community. Applied and Environmental Microbiology 64 : 238-245

Babich H, Stotzky G (1980) Environmental factors that influence the toxicity of heavy metal and gaseous pollutants to microorganisms. Crit Rev Microbiol 8 : 99-145

Baldrian P, Wiesche C, Gabriel J, Nerud F, Zadrazil F (2000) Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by Pleurotus ostreatus in soil. Applied and Environmental Microbiology 66 : 2471-2478

Benka-Coker MO, Ekundayo JA (1998) Effects of heavy metals on growth of species of *Micrococcus* and *Pseudomonas* in a crude oil/minerals salts medium. Bioresource Technology 66 : 241-245

Bressler DC, Fedorak PM (2001) Identification of disulfides from the biodegradation of dibenzothiophene. Applied and Environmental Microbiology 67 : 5084-5093

Brock TD (1970) Biology of microorganisms. Prentice-Hall, New Jersey, pp 575-674

Cerniglia CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 4 : 331-338

Chaerun SK, Tazaki K, Asada R (2002a) Microbial activities of hydrocarbon-degrading bacteria in the

heavy oil contaminated soil and seawater after 5 years of bioremediation. Memoirs of Division of Global Environmental Science and Engineering, Graduate School of Natural Science and Technology, Kanazawa University, Japan, 7, pp 11-27

Chaerun SK, Tazaki K, Asada R (2002b) Role of Hydrocarbon-degrading bacteria in the bioremediation of heavy oil polluted coastal area. In : Abstracts of the Japan Earth and Planetary Science Joint Meeting, Japan, pp B006-008

Chaerun SK, Tazaki, K, Asada R (2002c) Microbial degradation of heavy oil by pure and mixed culture under aerobic condition. In : Abstracts of the 106th Annual Meeting of the Geology Society of Japan, Japan, pp 33

Chaerun SK, Tazaki K (2003) Hydrocarbon-degrading bacteria in the heavy oil polluted soil and seawater after 5 years of bioremediation. In : Tazaki K (ed) Water and soil environments : microorganisms play an important role. 21st century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan, pp 187-204

Chaerun SK, Tazaki K, Asada R (2003) Double function of bentonite and kaolinite as adsorbents and “microbial growth-support media” for degradation of crude oil. In : Tazaki K (ed) Heavy oil spilled from Russian tanker “Nakhodka” in 1997 : towards eco-responsibility, earth sense. 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan, pp 253-277

Compeau GC, Bartha R (1985) Sulfate-reducing bacteria : principal methylators of mercury in anoxic estuarine sediment. Applied and Environmental Microbiology 50 : 498-502

Doelman P (1986) Resistance of soil microbial communities to heavy metals. FEMS Symp 33 : 369-384

Foght JM, Fedorak, PM, Westlake DW (1990) Mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene in crude oil : specificity among bacterial isolates. Can J Microbiol 36 : 169-175

Gadd GM, Griffiths AJ (1978) Microorganisms and heavy metal toxicity. Microb Ecol 4 : 303-317

Heitkamp MA, Cerniglia CE (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Applied and Environmental Microbiology 54 : 1612-1614

Herbes SE, Schwall LR (1978) Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. Applied and Environmental Microbiology 35 : 306-316

Hozumi T, Tsutsumi H, Kono M (2000a) Bioremediation on the shore after an oil soil from the *Nakhodka* in the Sea of Japan. I. chemistry and characteristics of heavy oil loaded on the *Nakhodka* and biodegradation tests by a bioremediation agent with microbiological cultures in the laboratory. Marine Pollution Bulletin 40 : 308-314

Hozumi T, Hirota Y, Hirashima A (2000b) Bioremediation on the shore after an oil soil from the *Nakhodka* in the Sea of Japan. II. Toxicity of a bioremediation agent with microbiological cultures in aquatic organisms. Marine Pollution Bulletin 40 : 315-319

Hubert C, Shen Y, Voordouw G (1999) Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. Applied and Environmental Microbiology 65 : 3064-3070

Hughes MN, Poole RK (1989) Metal toxicity. In : Hughes MN, Poole RK (eds) Metals and Microorganisms. Chapman & Hall, New York, pp 252-302

Hughes MN, Poole RK (1991) Metal speciation and microbial growth-the hard (and soft) facts. J Gen Microbiol 137 : 725-734

Ijah UJJ, Antai SP (2003) Removal of Nigerian light crude oil in soil over a 12-month period. International Biodeterioration & Biodegradation 51 : 93-99

Ishiyama M, Matsui N, Kunii K, Kubo K, Sato C, Nakamura S (1999) Degradation of heavy oil by bacteria isolated from the *Nakhodka* oil spill. In : Proceedings of the International Symposium of the Earth-Water-Humans, Kanazawa, Japan, pp 97-103.

Itagaki E, Ishida H (1999) Drift of C-heavy oil spilled from a Russian tanker "NAKHODKA" on Kanazawa seashore and its bioremediation by marine hydrocarbon degrading bacteria. Coastal Engineering Journal 41 : 107-119

Janiyani KL, Wate SR, Joshi SR (1993) Morphological and biochemical characteristics of bacterial isolates degrading crude oil. Environ Sci Health A28 : 1185-1204

Jeffrey LM (1980) Petroleum residues in the marine environment. In : Geyer RA (ed) Marine environmental pollution : 1-hydrocarbon. Elsevier, Amsterdam, pp 163-179

Kamel Z, Khalil MS, Shalaby AM (1989) Calcium and the biological activities of two *Streptomyces* species isolated from the rhizosphere of soy bean plants. J Basic Microbiol 29 : 31-39

Kasai Y, Kishira H, Syutsubo K, Harayama S (2001) Molecular detection of marine bacterial populations on beaches contaminated by the *Nakhodka* tanker oil-spill accident. Environ Microbiol 3 : 246 -255

Kastner M, Breuer-Jammali M, Mahro B (1994) Enumeration and characterization of the soil micro-flora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl Microbiol Biotechnol 41 : 267-273

Kingston PF (2002) Long-term environmental impact of oil spills. Spill Science & Technology Bulletin 7 : 53-61

Komukai-Nakamura S, Sugiura K, Yamauchi-Inomata Y, Toki H, Venkateswaran K, Yamamoto S, Tanaka H, Harayama S (1996) Construction of bacterial consortia that degrade Arabian light crude oil. J Ferment Bioeng 82 : 570-574

Kovalick W (1991) Perspectives on risks of soil pollution and experience with innovative remediation technologies, In Strategies 2000. In : Proceedings of the 4th World Congress Chemical Engineering. pp 281-295.

Kuo C, Genthner BRS (1996) Effect of added heavy metal ions on biotransformation and biodegradation of 2-chlorophenol and 3-chlorobenzoate in anaerobic bacterial consortia. Applied and Environmental Microbiology 62 : 2317-2323

Lane DJ (1991) 16S/23S rRNA sequencing. In : Stackebrandt E, Goodfellow M, (eds) Nucleic acid techniques in bacterial systematics. A wiley-Interscience publication, John Wiley & Sons, pp 115-175

Leisinger T, Brunner W (1986) Poorly degradable subatances. In : Rehm HJ, Reed G (eds) Biotechnology. VCH, Germany, Vol. 8, pp 475-513

Lovley DR (1993) Dissimilatory metal reduction. Annu Rev Microbiol 47 : 263-290

Macnaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang Y, White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. Applied and Environmental Microbiology 65 : 3566-3574

Neumann HJ, Paczynska-Lahme B, Severin D (1981) Composition and properties of petroleum. In : Beckmann H (ed) Geology of Petroleum. Halsted Press, Wiley Ltd., New York, pp 16-18

Nordgren A, Baath E, Soderstrom B (1983) Microfungi and microbial activity along a heavy metal gradient. Applied and Environmental Microbiology 45 : 1829-1837

Nordgren A, Baath E, Soderstrom B (1985) Soil microfungi in an area polluted by heavy metals. Can J Bot 63 : 448-455

Obiajunwa EI, Pelemo DA, Owolabi SA, Fasasi MK, Johnson-Fatokun FO (2002) Characterisation of heavy metal pollutants of soils and sediments around a crude-oil production terminal using EDXRF. Nuclear Instruments and Methods in Physics Research B 194 : 61-64

Rosenberg E (1992) The hydrocarbon-oxidizing bacteria. In : Procaryotes. 2nd ed. Springer-Verlag, Berlin, pp 446-459

Sampei Y, Tazaki K (2003) Compositional changes of heavy oil and hydrocarbon from Nakhodka oil : primary change in spilled oil during first month in seawater. In : Tazaki K (ed) Water and soil environments : biological and geological perspectives. International Symposium of the Kanazawa University 21st-Century COE Program, Japan, pp 271-274

Sampei Y, Tazaki K, Obara Y, Yoshimura T, Sawano N, Takayasu K, Iizumi S, Tokuoka T (2003) Compositional changes of heavy oil and aliphatic hydrocarbon from the spilled Nakhodka-oil washed ashore at Fukui, Ishikawa and Niigata Prefectures, Japan. In : Tazaki K (ed) Heavy oil spilled from Russian tanker "Nakhodka" in 1997 : towards eco-responsibility, earth sense. 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan, pp 174-191

Sasaki T, Maki H, Ishihara M, Harayama S (1998) Vanadium as an internal marker to evaluate microbial degradation of crude oil. Environmental Science and Technology 32 : 3618-3621

Sawano N (2003) What kind of shoreline is the most dangerous for oiling?. In : Tazaki K (ed) Heavy oil spilled from Russian tanker "Nakhodka" in 1997 : towards eco-responsibility, earth sense. 21st Century COE Kanazawa University, Kanazawa University, Kanazawa, Japan, pp 47-58

Shi W, Becker J, Bischoff M, Turco RF, Konopka AE (2002) Association of microbial community composition and activity with lead, chromium, and hydrocarbon contamination. Applied and Environmental Microbiology 68 : 3859-3866

Shibata Y, Hatano H, Kimura Y (1997) In term report on the environmental study of the accidental spillage of C-Heavy oil from Nakhodka. National Institute of Environment Research, Japan, pp 1-12 (in Japanese)

Speight JG (1980) The chemistry and technology of petroleum. Marcel Dekker Inc., New York.

Tazaki K, Sawano N, Nagasaka M, Aoki A, Matsumoto K, Nishida S, Tawara K, Ueshima M (1997a) Heavy oil spilled from the wrecked Russian tanker "*Nakhodka*" attacked the coast of Hokuriku district, and remarkable microbial remediation is advancing. J Geol Soc Japan 103 : VII-VIII (in Japanese)

Tazaki K, Tawara K, Watanabe H, Matsumoto Ka, Azuma M, Kizu R, Hayakawa K, Akai J, Chiba H, Nakamura S, Sawano N, Yajima T, Matsumoto Ke (1997b) The accidental pollution of heavy oil in the Sea of Japan. Kaiyo Monthly 29 : 567-633 (in Japanese).

Tazaki K, Kamiya T, Hasegawa S, Okuno M, Ishiwatari A, Kato M, Yajima T, Matsumoto K, Sasayama

Y, Hasegawa K (1997c) Progress report of specific research of the accidental heavy oil spill and its remediation. Kanazawa University, Japan (in Japanese).

Tazaki K (ed) (2003) Heavy oil spilled from Russian tanker "*Nakhodka*" in 1997 : towards eco-responsibility, earth sense. 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan.

Trzesicka-Mlynarz D, Ward OP (1995) Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its components pure cultures, obtained from PAH-contaminated soil. Can J Microbiol 41 : 470-476

Venkateswaran K, Harayama S (1995) Sequential enrichment of microbial populations exhibiting enhanced biodegradation of crude oil. Can J Microbiol 41 : 767-775

Whyte LG, Bourbonniere L, Greer CW (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. Applied and Environmental Microbiology 63 : 3719-3723

Wong CK (1993) Effects of chromium, copper, nickel and zinc on longevity and reproduction of the cladoceran *Moina macrocopa*. Bull Environ Contam Toxicol 50 : 633-639

Wrightson FM (1949) Determination of traces of iron, nickel and vanadium in petroleum oils. Anan Chem 21 : 1543-1545