氏　　　名	SITI KHODIJAH
学位の種類	博士（理学）
学位記番号	博甲第690号
学位授与の日付	平成16年9月30日
学位授与の要件	課程博士（学位規則第4条第1項）
学位授与の題目	Bioremediation of coastal areas 5 years after the *Nakhodka* oil spill in the Sea of Japan （"ナホトカ号"によるオイル流出事故後5年間にわたる日本海沿岸のバイオレメディエーション）
論文審査委員(主査)	田崎　和江（自然科学研究科・教授）
論文審査委員(副査)	奥野　正幸（自然科学研究科・教授），加藤　道雄（自然科学研究科・教授），古本　宗充（自然科学研究科・教授），中村　省吾（富山大学・教授）

学位論文要旨

Abstract

This study deals primarily with the isolation and characterization of hydrocarbon bacteria indigenous to the *Nakhodka* oil spill after five years of bioremediation, as well as what environmental factors influencing the bioremediation of the *Nakhodka* spill. Seven bacterial strains, which were identified using 16S rDNA sequence analysis as *Bacillus* spp., *Pseudomonas* sp., and *Paracoccus* spp., showed their ability to grow well on aliphatic hydrocarbons, but not on aromatic hydrocarbons, and all of them were able to grow well on heavy oil. The benevolent interaction of bacteria with fungi occurred during the 429-day bioremediation of the *Nakhodka* oil spill, even though the environmental parameter (i.e., pH) was not maintained. Hydrocarbon bacteria had tendency to play greatest role as the solution pH was neutral-alkaline condition. On the contrary, when the pH solution changed to be acidic, the fungi took over to degrade heavy oil. Fungal succession was predominated by *Rhinocladiella* sp., while all fungi which survived in degrading heavy oil were identified as *Rhinocladiella* sp., *Aspergillus* sp., *Acremonium* sp., and *Penicillium* sp. During that period, aliphatic hydrocarbons were reduced significantly, whereas aromatic hydrocarbons remained relatively constant, and there was the production of metabolites after 429 days of bioremediation. Of all metals contained in the heavy oil, seawater, and beach sands, Co appeared to inhibit hydrocarbon bacterial growth, while Si tended to enhance hydrocarbon bacterial growth. In addition, the clay minerals present in the marine and coastal sites tended to stimulate

hydrocarbon bacterial growth. These results provide a significant insight into how bioremediation of the *Nakhodka* oil spill take place and what environmental factors (i.e., Si compound and clay minerals) influence that bioremediation, thereby giving more fruitful information for the bioremediation of the oil spill-contaminated marine and coastal environments.

Key Words: *Nakhodka* oil spill, bioremediation, heavy oil, heavy metals, clay minerals, montmorillonite, kaolinite, *Bacillus* spp., *Pseudomonas* spp. *Paracoccus* spp., *Rhinocladiella* sp., *Aspergillus* sp., *Acremonium* sp., *Penicillium* sp.

Studies on bacterial strains degrading aliphatic and aromatic hydrocarbons attributable to oil spills received much attention. 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. Hence, a careful bioremediational research of the *Nakhodka* oil spill along seashores in Sea of Japan as well as the investigation of what environmental factors influencing that bioremediation were carried out since 1997.

The primary 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 as well as to investigate what environmental factors influencing the bioremediation of the *Nakhodka* oil spill.

Seven bacterial strains capable of utilizing the heavy oil spilled from the *Nakhodka* Russian oil tanker were isolated from three coastal areas (namely Katano Seashore of Fukui Prefecture, Osawa and Atake seashores of Ishikawa Prefecture) and the *Nakhodka* Russian oil tanker after five years of bioremediation. All bacterial strains showed their ability to grow well on aliphatic hydrocarbons, but not on aromatic hydrocarbons, and all of them were able to grow well on heavy oil. They were identified using 16S rDNA sequence analysis as *Bacillus* spp., *Pseudomonas* sp., and *Paracoccus* spp.

The benevolent interaction of bacteria with fungi occurred during the 429-day bioremediation of the *Nakhodka* oil spill at laboratory scale, even though the environmental parameter (i.e., pH) was not maintained or uncontrolled. Hydrocarbon bacteria had tendency to play greatest role as the solution pH was neutral-alkaline condition (7~7.8). On the contrary, when the pH solution changed to be acidic (pH 2~4), the fungi took over to degrade heavy oil. Fungal succession during the 429-day bioremediation was predominated by *Rhinocladiella* sp., while all fungi which survived in degrading heavy oil were identified as *Rhinocladiella* sp., *Aspergillus* sp., *Acremonium* sp., and *Penicillium* sp. During that period, aliphatic hydrocarbons were reduced significantly, whereas aromatic hydrocarbons remained relatively constant, and there was the production of metabolites after 429 days of bioremediation.

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.

Clay minerals, which are usually present in the marine or coastal environments contaminated with high concentration of oil spills, were capable of promoting microbial growth and allowed microorganisms to proliferate favorably above levels of non-clay-containing control at an extremely high concentration of heavy oil (150 g/l) (Fig.1). Clays at low to moderate concentrations (0.5 to 10 g/l) tended to enhance bacterial growth. On the contrary, clays at high concentrations (> 10 g/l) appeared to inhibit growth of hydrocarbon degrading bacteria, resulting from the low redox potential values of the liquid phase and not from the toxicity of elements (such as Si and Al) contained in clays, since dissolved Si and Al were observed at low concentrations in the aqueous phase. The presence of clays at high concentrations generally yields the low dissolved oxygen levels in the bulk solution, thus being considered as pollutants. Inversely, if clay minerals are present at low concentrations that remain sustainable oxygen levels for bacterial growth, they become stimulator for hydrocarbon bacterial growth by forming the C-O-Na-Si complexes on the surface of hydrocarbon bacterial cells. However, the uptake of Si element by bacterial cells appeared to be more predominant than Al element. These results indicate that the enhancement of hydrocarbon bacterial growth may be the results of clays likely serving as trace elements required for bacterial growth as well as growth supporting carrier. In addition, these results showed that Al element contained in clays did not result in the toxic effect on hydrocarbon bacterial growth. The toxicity of Al were not observed at levels of clays (0.5 to 30 g/l) used in this study. Hydrocarbon bacteria used in this study tended to be favorable to Si element of clays. It is suspected that Si is an element facilitating the high hydrocarbon bacterial growth, and the C-O-Na-Si complexes on the surface of bacterial cell walls are the stimulator for hydrocarbon bacterial growth.

This study provides evidence that after five years of bioremediation, the bacterial community of the *Nakhodka* oil spill-contaminated coastal sites is predominated by alkane-degrading bacteria among which *Pseudomonas* species is a dominant organism. However, not only do hydrocarbon bacteria play significant role, but hydrocarbon fungi predominated by *Rhinocladiella* sp. also participate in the degradation of heavy oil, where their role is dependent upon the pH of the aqueous phase. In addition, the elements (in particular Si) and the clay minerals are of environmental factors enhancing the hydrocarbon bacterial growth in the bioremediation of the *Nakhodka* oil spill.

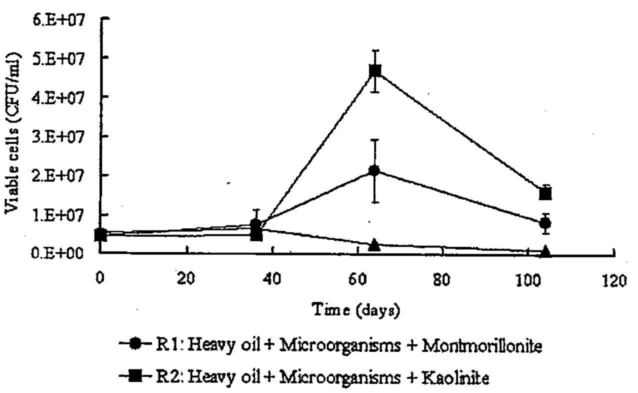

Fig. 1. Microbial growth (bacteria and fungi) as viable cells in colony forming unit (CFU) per ml in the experimental systems containing clays and in the control experimental system containing non clays. Error bars represent standard deviations.

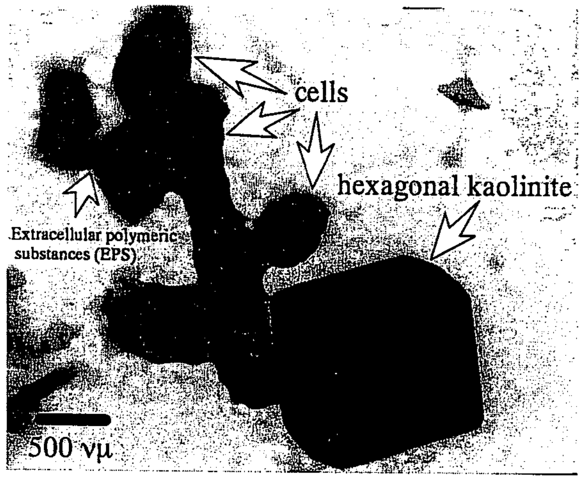

Fig. 2. Transmission electron micrograph of the attractive network formation of bacterial cells consisting of short and long rods-shaped bacteria and spherical bacteria attached to the edges of hexagonal kaolinite. The cells appear to connect with one another and are attached to the edges of hexagonal kaolinite by an adhesive agent, assumed to be extracellular polymeric substances (EPS).

学位論文審査結果の要旨

平成 16 年 7 月 15 日に第 1 回学位論文審査委員会を開催し，7 月 28 日に口頭発表を行い，同日最終の審査委員会を開催した．協議の結果以下のとおり判定した．本学位論文は，ナホトカ号重油流出後 5 年間の重油の分解に寄与した炭化水素分解細菌の単離と特性評価を行い，バイオレメディエーションに影響する要因について研究した。ナホトカ号から抜き取った重油や海岸（片野，大沢，アタケ）の漂着重油・砂・海水の試料から 39 菌株が単離され，7 菌株が炭化水素（n- アルカン）を炭素源として培養できることを明らかにした．

この 7 菌株について ^{16}S rDNA 塩基配列解析を行い，*Bacillus* spp.，*Pseudomonas* sp.，*Paracoccus* spp. を同定した．429 日間の C 重油分解実験では，炭化水素分解細菌は共存する菌類と pH 範囲に応じて炭化水素を分解する働きがあることを明らかにした．すなわち，pH7.0 ～ 7.8 の領域では細菌が主に炭化水素分解を担い，pH2.0 ～ 4.0 では菌類が分解する．さらに，重油と粘土鉱物が共存する系での培養実験を行った結果，粘土鉱物の存在は分解細菌の成長を助長する効果があることも明らかになった．本研究結果はナホトカ号重油流出事故後に自然環境下で土着の微生物による重油の分解過程やバイオレメディエーションに影響する環境要因ついて重要な知見を提供し、バイオレメディエーションの実用化へ向けた有用な情報を与えた．参考論文は国際誌 2 編、国内誌 1 編が既に受理されている。

5 名の審査員はいずれも博士（理学）に相当する質の高い論文であると評価した．