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THE USE OF DNA TECHNIQUES IN IDENTIFYING TARGET MICROORGANISMS IN SOIL AND GROUNDWATER REMEDIATION

ZASTOSOWANIE TECHNIK DNA DO IDENTYFIKACJI MIKROORGANIZMÓW ODPOWIEDNICH ZA BIOREMEDIACJĘ GRUNTÓW I WÓD PODZIEMNYCH

In situ bioremediation can efficiently remove toxic and potentially carcinogenic pollutants such as volatile organic chlorinated compounds (VOCs); perchloroethylene, trichloroethylene, dichloroethylene and vinyl chloride, or oil from groundwater as well as subsurface. Typically, treatability test is performed before bioremediation to find if it would be effective or not. It is however difficult to find the right microbial community by one treatability test alone and as a result, many trials are necessary for selection of adequate nutrients for activation of microorganisms to degrade target compounds. It is desirable to know the structure of microbial community of contaminated site before bioremediation. For this purpose, DNA techniques are very powerful tools and I would like to introduce the DNA microarray and PCR-DGGE techniques applied to rehabilitations of VOC and Kerosene polluted sites, respectively.

We have designed a 40-mer microarray based on the internal transcribed spacer sequence of the 16S-23S ribosomal DNA of 22 well-known strains of anaerobic as well as aerobic bacteria that are capable of partial or complete degradation of VOCs. Additionally, two kinds of tetrachloroethylene dehalogenase genes are designed and spotted on special slide glass. Using the DNA microarray, distribution of VOCs degraders in 76 contaminated points from 27 sites were monitored with analyses of VOCs. Dehalococcoides ethenogenes 195, which dehalogenates completely tetrachloroethylene to ethene, was detected in 13 samples from 5 sites and other partial degraders of tetrachloroethylene were found in about half points tested. Bioremediation was applied to the sites where VOC degraders were detected. VOC-degrading bacteria were remarkably increased by addition of adequate supplements, resulting in reduction of VOCs in those sites.

Active microorganisms for degrading petroleum hydro-carbon were detected by the PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analyses of the Kerosene contaminated soils sampled from the site before starting the remediation. The results were evaluated by degradation rate of the total petroleum hydro-carbon, human response to smell and observation of membranous floating oil. Nutrients were added starting July 25, 2006 by using 46 wells being drilled to the top of the aquifer. The addition was applied once a week in the first stage during two months since the start and twice a month in the second stage from September to July 2007. DGGE analyses were also made several

times during the remediation work to check the change of bacterial flora being active at this site. The concentration of Kerosene was decreased to less than 1000 mg/l at the observation wells of 1, 2 and 3 by the measurements on February 17, 2007. While conducting bioremediation, PCR-DGGE pattern suggested that Pseudomonas sp. thought to a petroleum degrader increased and after bioremediation, the bands disappeared and PCR-DGGE pattern turned to those of non-contaminated subsurface close to previously polluted sites.

DNA techniques are becoming popular in the environmental engineering field and the methods are useful studying fundamentals of bioremediation of groundwater and soil and to develop practical cost-effective application to real-life cases.

1. Introduction

Governments say that 1,800,000 sites of subsurface and groundwater in EU, 2,000,000 sites in USA, and 440,000 sites in Japan are contaminated by chemicals and oil. There are many methods for rehabilitation of contaminated sites. Among them, *in situ* bioremediation is considered to be the best cost-effective method.

In situ bioremediation can efficiently remove toxic and potentially carcinogenic pollutants such as volatile organic chlorinated compounds (VOCs); perchloroethylene (PCE), trichloroethylene(TCE), dichloroethylene(DCE) and vinyl chloride(VC), or oil from groundwater as well as subsurface. Typically, treatability test is performed before bioremediation to find if it would be effective or not. It is however difficult to find the right microbial community by one treatability test alone and as a result, many trials are necessary for selection of adequate nutrients for activation of microorganisms to degrade target compounds. It is desirable to know the structure of microbial community of contaminated site before bioremediation. For this purpose, DNA techniques are very powerful tools and I would like to introduce the DNA microarray and PCR-DGGE techniques applied to rehabilitations of VOC and Kerosene polluted sites in Japan, respectively.

2. DNA microarray

2.1. Methods

DNA microarray for the detection of 22 kinds of bacteria associated with PCE dehalogenation (Figure 1) using their sequenced internal transcribed spacer (ITS) sequences in the 16S and 23S rDNA. 3 to 7 unique 40-mer probes per bacterium were constructed (1). To simplify probe selection, probes which have 50 % GC sense-oriented and more than 4 mismatches with each other and no hairpins were chosen. The printing of the probes of the microarray on highly-sensitive Takara Hubble Slides was custum-ordered to Takara Bio, Inc., Japan.

300 ml of water or 50 g of soil from sites polluted by VOCs were collected. Total genomic DNA was extracted by bead-beating method (Ultraclean Soil DNA Extraction

Kit, Mo Bio, USA). A regular ITS forward and a Cy-dye-labeled reverse primer for PCR reaction was used. After PCR-amplification of total genomic sample, the PCR products was cleaned using AutoSeq G-50 spin-columns and then evaporated the samples. The evaporated samples were re-dissolved in 5X SSC 0.3% SDS boiled for 3 min, placed in ice for no more than 1 min, added to the microarray, and the n allowed to hybridize for 4-12 hrs. The microarray was washed with 0.2 X SSC+ 0.3% SDS for 5 min, 0.2 X SSC for 5 min and then 0.05X SSC briefly it was spun dry. Analysis was done on a ScanArray 4000 (Perkin Elmer, Japan) laser scanner and results were evaluated visually for the presence of signals to each probe.

VOCs were analysed using a GC equipped with a flame ionization detector by the headspace method. 250µL headspace samples were quantified using a model GC-14B

Tab. 1 Bacteria associated with PCE bioremediation used in the study.

Code	Bacterium	Original VOCs	Final product
Q	*Dehalococcoides ethenogenes* 195	PCE	Ethene
B	*Desulfitobacterium frappieri*	PCE	*cis*DCE
I	*Desulfitobacterium hafniense*	PCE	*cis*DCE
H	*Desulfitobacterium dehalogenans*	PCE	*cis*DCE
N	*Desulfitobacterium* sp. strain PCE1	PCE	*cis*DCE
O	*Desulfitobacterium frapperi* TCE1	PCE	*cis*DCE
R	*Desulfomonile tiedjei* DCB-1	PCE	*cis*DCE
K	*Desulfuromonas chloroethenica*	PCE	*cis*DCE
M	*Dehalobacter restricus*	PCE	*cis*DCE
A	*Sulfospirillum multivorans*	PCE	*cis*DCE
G	*Desulfomicrobium norvegicum*	PCE	*cis*DCE
J	*Clostridium formicoaceticum*	PCE	*cis*DCE
L	*Acetobacterium woodii* A	PCE	TCE
P	*Acetobacterium woodii* B	PCE	TCE
C	*Rhodococcus* sp. Sm-1	*cis*DCE	CO_2
D	*Rhodococcus rhodococcus*	*cis*DCE	CO_2
E	*Xanthobacter flavus*	*cis*DCE	CO_2
F	*Mycobacterium* sp. L1	*cis*DCE	CO_2
	Clostridium bifermentans DPH-1	VC	CO_2
	Desulfitobacterium hafniense Y-51	PCE	*cis*DCE
	Clostridium sp. KYT-1	*cis*DCE	?
	Clostridium sp. DC-1	*cis*DCE	CO_2

gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector and a glass column (i.d 3.2Ø x 2.1 m; Silicone DC-550 20% Chromosorb W [AWDMCS]80/100) according to the Japanese Standard Method JIS K0102. The injection and detection temperatures were maintained at 100ºC and 160ºC, respectively. The column temperature was held at 40ºC. The carrier gas was pure nitrogen at a flow rate of 50 ml/min. Identification and quantification have been calculated preparing the calibration curve with laboratory reagent of PCE (Kanto Chemical Co., Japan), TCE (Nacalai Tesque, Japan) and *cis*-1,2- DCE (TCI-GR, Japan), respectively. Laboratory reagent of PCE, TCE and *cis*-1,2- DCE were diluted with 99% ethanol. 3 µl of PCE, TCE and *cis*-

1,2- DCE were added individually with 5 ml of ethanol in individual small vials. Anaerobic culture media was prepared in other vials and the ethanol diluted PCE, TCE and *cis*-1,2- DCE were taken in the culture media containing vials in 1 ppm (parts per million) of concentration, ie, one milligram per liter in solution. 250µL headspace samples were quantified using gas chromatograph. This quantification was used to make a calibration curve for the measurement of PCE, TCE and *cis*-1,2- DCE from the samples those were collected from the PCE contaminated sites.

The site for actual rehabilitation work of PCE contaminated site is shown in Figure 1. Groundwater flows from upside to bottom in Figure 1 and nutrient used was "AMTECLEAN" composed of glycerin, stearic acid, and myristic acid. Three injection wells, 1.5 m interval each, were used and samples were collected from upstream (before bioremediation) and downstream (after bioremediation) wells. The site is electric device manufacturing factory previously making home electric appliances and several decades had passed since the factory started to use PCE and related solvents.

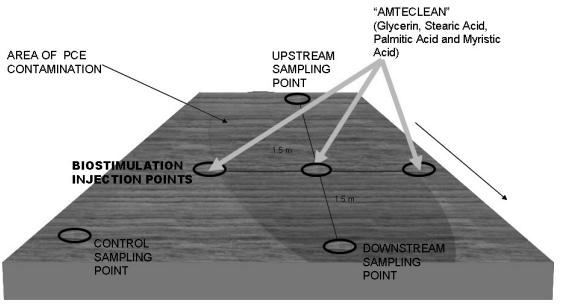

Fig. 1. Outline of the VOCs contaminated site for rehabilitation work

2.2. Results and Discussion

Time-course changes in analytical results of upstream and downstream water are shown in Figure 2. Environmental quality standards of PCE, TCE, *cis*DCE, and 1,1-dichloroethylene in Japan are 0.01, 0.03, 0.04, and 0.02 mg/l, respectively and we don't have standard value of VC.

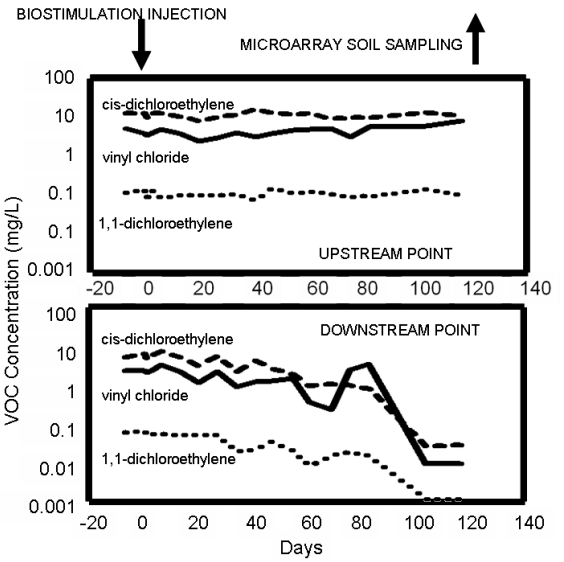

Fig. 2. Time course changes in VOC concentrations after injection of nutrient to the site originally contaminated by PCE and TCE

As the history of pollution by PCE and TEC in the site is very old, natural attenuation of PCE and TCE has been happened, resulting in accumulation of *cis*DCE and VC in the site. After injection of nutrient, the detected levels of VOCs gradually decreased to close to environmental quality standard values.

Figure 3 shows the result of DNA microarray detection of bacteria associated with VOC dehalogenation in soil and groundwater before and after biostimulation. Interestingly, *Sulfospirillum multivorans* (A)*, Desulfomicrobium norvegicum* (G), *Clostridium formicoaceticum* (J), and *Dehalobacter restricus* (M) are found in both water and soil controls without pollution by VOCs. Probably, they are naturally distributed in the area. Comparing the result obtained from water control, higher detection levels were found in the sample of water up. Almost all VOCs degraders genes loaded on microarray were detected. After injection of nutrient, relative bacterial amount of each degrader became extremely higher than the values of water up. Similar result was obtained in the case of soil extract. From these result, it can be thought that bacteria associated with VOC dehalogenation has activated by supplementation of nutrient, resulting in decrease of VOCs concentration at the site.

One more fact I must mention here is the absence of *Dehalococcoides ethenogenes* 195 (Q) in the site. *Dehalococcoides ethenogenes* 195 is a well-known super strain which dehalogenates PCE completely to ethene by single strain. Without the exsistence of the strain, complete dehalogenation happens at the site, suggesting the existence of unknown bacteria harboring dehalogenation activity.

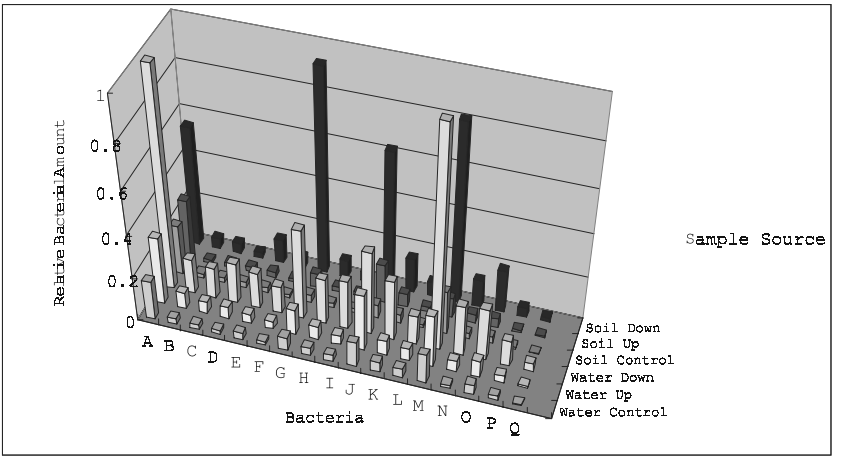

Fig.3. Microarray detection of bacteria associated with VOC dehalogenation in soil and groundwater before and after biostimulation

Using the DNA microarray, distribution of VOCs degraders in 76 contaminated points from 27 sites were monitored with analyses of VOCs. The result is shown in Table 2. *Dehalococcoides ethenogenes* 195, which dehalogenates completely tetrachloroethylene to ethene, was detected in 13 samples from 5 sites and other partial degraders of tetrachloroethylene were found in almost all points tested. Bioremediation was applied to the sites where VOC degraders were detected. VOC-degrading bacteria were remarkably increased by addition of adequate supplements, resulting in reduction of VOCs in those sites.

Tab. 2 The relationship between treatability test and DNA microarray pre-monitoring of the bacteria associated with VOCs dehalogenation

VOCs detected by treatability test	Dehalogenators detected by DNA microarray	Numbers of sites detected degraders by microarray
PCE/TCE/DCE/VC	Not Detected	2
	PCE/E, PCE/DCE, DCE/?, VC/CO2	2
	PCE/E, PCE/DCE	1
	PCE/TCE, PCE/DCE	1
	PCE/DCE, DCE/CO2	1
	PCE/DCE, DCE/?	1
	PCE/DCE	3
PCE/TCE/DCE	PCE/DCE, VC/CO2	2
PCE/TCE	PCE/DCE, DCE/?	1
	PCE/DCE, VC/CO2	1
	PCE/DCE	1
TCE/DCE/VC	PCE/E, PCE/DCE, DCE/?, DCE/CO2, VC/CO2	1
	PCE/TCE, PCE/DCE, DCE/CO2, DCE/?	1
	PCE/DCE, DCE/CO2	1
	PCE/DCE	2
TCE	PCE/E, PCE/DCE	1
	PCE/E	1
	Not Detected	1
DCE/VC	PCE/DCE	1

3. PCR-DGGE

3.1. Methods

Ground water polluted soils have been collected from oil contaminated sites in the time of April 2006 to March 2007.

Nucleic acid was extracted directly from duplicate 5-10g composite samples. PCR amplification of the 16S rDNA fragments prior to DGGE was performed as described by Muyzer *et al* (2). Briefly, thermocycling consisted of 35 cycles at 94°C for 45 s, 53°C for 30 s, and 72°C for 45 s, with 0.5 U of AmpliTaq Gold (AppliedBiosystems) and 10 pmol of each of the primers described in the work of Muyzer *et al*. (the forward primer carried the 40-bp GC clamp) in a total volume of 25 µl. Thermocycling was performed with a PCR block (TaKaRa). The primers targeted eubacterial 16S regions corresponding to *Escherichia coli* nucleotide positions 341 to 517.

DGGE was performed by using a D-Code 16/16-cm gel system with a 1.5-mm gel width (Bio-Rad, Hercules, Calif.) maintained at a constant temperature of 60°C in 7 liters of 1× TAE buffer. Gradients were formed between 30 and 60% denaturant (with 100% denaturant defined as 7 M urea plus 40% [vol/vol] formamide). Gels were run at 130 V for 5 h. Gels were stained in purified water (Milli-Ro; Millipore, Bedford, Mass.) containing SYBR gold for 15 min each.

The central 1-mm^2 portions of strong DGGE bands were excised with a razor blade and soaked in 50 µl of purified water overnight. A portion (15 µl) was removed and used as the template in a PCR as described above. The products were purified by electrophoresis through a 1% agarose–TAE gel followed by glass-milk extraction (Gene-Clean kit; Bio 101). Purified DNA was sequenced using Dye Terminator Cycle Sequencing Kit ver. 3.1 (Perkin Elmer, USA) with an ABI-Prism model 373 automatic sequencer. Sequence identification was performed by use of the BLASTN of the National Center for Biotechnology Information and the Sequence Match of the Ribosomal Database Project.

Analysis of oil was followed by the Japanese Standard Method JIS K0102 and also human response to smell and observation of membranous floating oil were recorded.

The site for the field study was located at Nagano, Japan. Polluted area were 4,500 m^2 and maximum concentration of oil of subsurface was 28,000 mg/l.

3.2. Results and Discussion

PCR-DGGE pattern of the contaminated site is shown in Figure 4. Ten clear bands were found. In DGGE, each band in a lane corresponds to a different organism. The band pattern altered for the bands obtained through biostimulation from the bands obtained without treatment. It indicates the growth of diverse microorganisms those were responsible for oil decomposition and the microbial community could be shifted with the addition of nutrient (Figure 4).

For the study of phylogenetic affiliations of predominant community members, the bands in the DGGE fingerprints were excised, reamplified, purified, and sequenced. All the bands yielded sequences without ambiguous positions and were included in phylogenetic trees. Most of the bands in the analysis yielded a clean sequence, indicating that

each band represented a different microbial population. The sequence analysis shows band number 2 and 3 gave significant percent homology with the genera *Pseudomonas* belonging to the phylum *Proteobacteria* and band number 8 and 10 showed significant percent homology with the genera *Novosphingobium* belonging to the phylum *Bacteroides and Cytophaga*. It has been already reported that these mentioned genera corresponds to some distinct Kerosene degraders. Interestingly, band number 2 was extremely grown by nutrient addition and act as degraders despite no clear proliferation of oil degraders, *Novosphingobium* spp. Supplemented chemical was mainly composed of stearic acid and it is considered that bacteria which acclimatize new environment and/or utilize glycerin, stearic acid, and myristic acid grew.

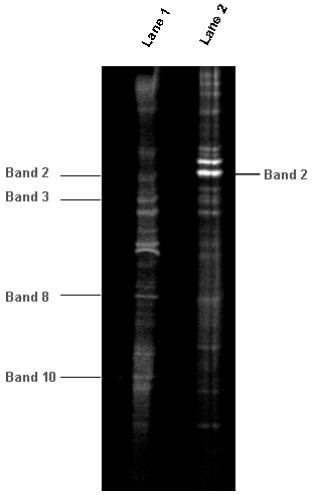

Fig.4. DGGE patterns of 16SrDNA fragments of extracted DNA from Kerosene contaminated soil and its rehabilitated soil. Lane 1: Kerosene contaminated soil, Lane 2: Rehabilitated soil

Concentration of Kerosene in several observation wells are shown in Figure 5. Nutrients were added starting July 25, 2006 by using 46 wells being drilled to the top of the aquifer. The addition was applied once a week in the first stage during two months since the start and twice a month in the second stage from September to July 2007. After the first spike of nutrient, concentration of each well decreased gradually below 1,000 mg/l and also oily smell and membranous floating oil disappeared completely.

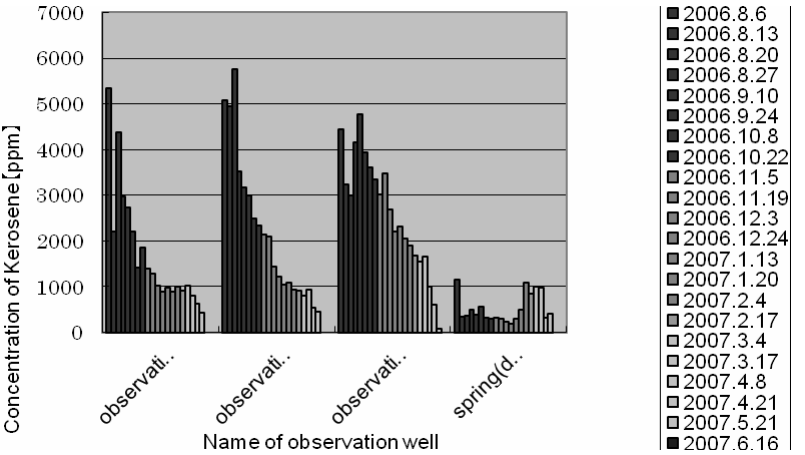

Fig.5. Time course changes in decreasing Kerosene concentration in each monitoring well after nutrient supplementation

4. Conclusion

One of most important knowledge before application of *in situ* bioremediation is to understand the structure of microbial community of contaminated site and if distribution of degraders of target chemicals is found, treatability test for selection of adequate nutrients turns easier and faster rehabilitation work will be anticipated. For this purpose, DNA techniques are considered very powerful tools and I have introduced the DNA microarray and PCR-DGGE techniques applied to rehabilitations of VOC and Kerosene polluted sites, respectively. These are typical success specimen. DNA techniques are becoming popular in the environmental engineering field and the methods are useful studying fundamentals of bioremediation of groundwater and soil and to develop practical cost-effective application to real-life cases.

References

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