**Arpita BHOWMIK[1], Akane ASAHINO[2], Takanori SHIRAKI[2], Kohei NAKAMURA[2], Kazuhiro TAKAMIZAWA[1.2]**

*United Graduate School of Agriculture*[1]
*Gifu University, Japan*

*Faculty of Applied Biological Science*[2]
*Gifu University, Japan*

# DYNAMIC BEHAVIOR OF BACTERIA IN PCE CONTAMINATED SURFACE DURING BIOREMEDIATION

## DYNAMICZNE ZACHOWANIE SIĘ BAKTERII W CZASIE BIOREMEDIACJI OBSZARÓW SKAŻONYCH TETRACHLOROETYLENEM

*Among the most ubiquitous chlorinated compounds as major environmental pollutants, tetrachloroethylene (PCE) is a common groundwater contaminant due to its widespread use as solvent. A potential method for managing PCE contaminated sites is to evaluate the intrinsic and enhanced bioremediation. In this study we characterized the microbial community in the PCE contaminated soil. DNA has been extracted in soil samples from PCE-contaminated sites. Variable behavior of microbes has been observed between natural attenuation and biostimulation that has been mediated by the addition of nutrients. Results of Denaturing Gradient Gel electrophoresis (DGGE) of amplified bacterial 16S rDNA in case of biostimulation showed that the microbial community was dominated by species phylogenetically related to the β proteobacteria. In case of natural attenuation, sequences have been found belonging to the multiple species of different phyla. Interestingly, we have found sequences matched to the species belonging to the Firmicutes which contains bacteria capable of reductive dehalogenation. According to this finding we are assuming the possibility of the presence of some Clostridium like PCE degraders within the microbial community in either bioremediation or biostimulation.*

## 1. Introduction

Anthropogenic chlorinated organic compounds such as tetrachloroethylene (PCE) and trichloroethylene (TCE) are common groundwater contaminants due to their widespread use as solvents which can cause even liver cancer in human. PCE is not soluble in water and resistant to biodegradation in aerobic subsurface environment. Several studies

showed that under anaerobic conditions, these compounds can be reductively dehalogenated to less chlorinated ethenes or innocuous ethane by microorganisms through dehalorespiration [1]. Because this process has the potential of completely detoxifying chlorinated ethenes, it is recognized as a promising approach for the cleanup of contaminated sites and has been extensively studied for in situ bioremediation.

The factors those affect reductive dehalogenation are the metabolic capabilities of microorganisms and interactions among the microorganisms involved. To date a number of bacteria have been cultivated that are capable of reductive dehalogenation of chlorinated ethenes [2] In this study we report on phylogenetic identification of organisms in a microbial community pursuing both biostimulation and natural attenuation with the samples collected from PCE contaminated sites In this connection, for extracted DNA from soil samples, we have carried out experiments by amplifying 16S rRNA genes (16S rDNA) using universal primers [3] pursuing Denaturing Gradient Gel Electrophoresis (DGGE) since it is an electrophoretic separation method based on differences in melting behavior of double stranded DNA fragments for even a singe base pair change and DGGE can detect the presence of particular bacterial genera that is present in large portion of the population [4].

## 2. Methodology

Ground water polluted soils have been collected from PCE contaminated sites belonging to a company and PCE, TCE, *cis*-dichloroethylene (*cis*-DCE), vinyl chloride (VC) were quantified using 250μL head space sample in gas chromatography system equipped with a flame ionization detector according to the Japanese Standard Method JIS K0102. Then Mo Bio ULTRACLEAN$^{TM}$ soil DNA kit has been used for DNA extraction from the contaminated soil sample according to the directions of the manufacturer.

For Bacteria-specific PCR of 16S rDNA fragments, the primers GC 341F (E-coli position 341-358) (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCT ACG GGA GGC AGC AG-3′) and 517 R (E-coli position 517-534) ( 5′-ATT ACC GCG GCT GCT GG -3′) were used.

DGGE analysis of 16S rDNA fragments was performed using DCode$^{TM}$ universal mutation detection system (BIO-RAD). Gels (16cm x 16cm) consisted of 40% bis-: acrylamide (37:5:1) and a denaturant gradient of 30%-60%. Electrophoresis was performed in 1xTAE buffer at $60^0$C and 130V for 5h. Gels were attained for 30 min with a 1:10000 dilution of SYBR gold (Invitrogen) and analyzed using TOYOBO Electronic U, V, Transilluminator and Dark Reader Transilluminator (Clare Chemical Research).

For DGGE, as a control, DNA from the Clostridium sp. KYT-1 was extracted and was amplified. Since Clostridium sp. KYT-1 gene for 16S ribosomal RNA have been already characterized and sequenced [5].

Sequence comparisons were performed through a BLAST [6] search against the Gene Bank database from the site National Center for Biotechnology Information.

## 3. Results and discussion

The concentration of the intermediate products of PCE degradation has been measured with nine samples (Fig .1) those have been collected from some PCE contaminated sites. The samples were named as a, a´, b, c, d, e, f, g and h. Among them five samples (a´, c, f, g, h) were subject to natural attenuation and the other three samples (a, b, d) were subject to biostimulation. In case of sample e, both natural attenuation and biostimulation have been performed.

For sample a, PCE was not observed and 2.4mg/l of *cis*-1,2- DCE and 12mg/l of VC were found. For sample a´, PCE was not found and 1.7mg/l of *cis*-1,2- DCE and 2.3 mg/l of VC were found. For sample b, striking dechlorination was observed finding no PCE, TCE and *cis*-1,2- DCE and only 0.75mg/l of VC. For sample c, 0.046mg/l of PCE, 4.7mg/l of TCE, 25 mg/l of *cis*-1,2- DCE and 0.05mg/l of VC were found. For sample d, PCE was not found, TCE was found only in 0.001mg/l of amount and 32 mg/l of *cis*-1,2- DCE and 14 mg/l of VC were found. For sample f, 0.045 mg/l of PCE, 0.015 mg/l of TCE, 4.30 mg/l of *cis*-1,2- DCE and 9.90 mg/l of VC were found. For sample g, PCE was not found and 0.016 mg/l of TCE and 0.25 mg/l of *cis*-1,2- DCE were found. VC was also not found for sample g. For sample h, 0.26mg/l of PCE, 0.082 mg/l of TCE, 0.18 mg/l of *cis*-1,2- DCE and 0.002 mg/l of VC were found. For sample e, before adding nutrient 0.002 mg/l of PCE and zero amount of TCE, 36 mg/l of *cis*-1,2- DCE and 46 mg/l of VC were found. But after adding nutrient PCE and TCE were not found and only 0.022 mg/l of *cis*-1,2- DCE and 0.18 mg/l of VC were found. So for sample e, the corresponding site became almost completely dechlorinated after being experimented with biostimulation.

In each case for the nine samples, it has been observed that significant dechlorination of PCE has been occurred in the contaminated sites.

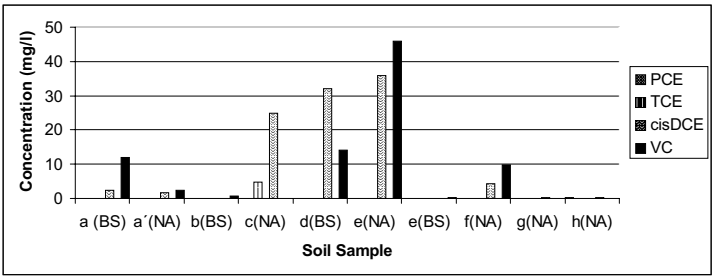


*Fig.1. PCE-dechlorinating activities in soil samples under biostimulation (BS) for sample a, b and d and under natural attenuation (NA) for sample a´, c, f, g and h. For sample e, both experiments have been performed. Every case showed the almost absolutely degradation of PCE and for sample e, after biostimulation, dechlorination rate has been accelerated more.*

All samples corresponding to the natural attenuation and biostimulation were subject to DGGE analysis to assess changes in the bacterial community throughout the research study. Because the main purpose of the study was to identify the dominant microorganisms involved in dehalogenation.

DGGE was performed for the samples after their analytical measurement followed by the extraction of DNA from the samples and PCR amplification of 16SrDNA. For every sample DGGE analysis revealed distinct banding patterns (Fig.2). In DGGE, each band in a lane corresponds to a different organism. In this case, for nine individual samples the DGGE profile showed bands in the individual lanes those are almost dissimilar to each other indicating the distinctiveness and diversity of the microbes corresponded with the bands. Even for the sample e, band pattern altered for the bands obtained through biostimulation from the bands obtained through natural attenuation. It indicates the growth of diverse microorganisms those were responsible for dechlorination, are site specific for individual samples and the microbial community could be shifted with the addition of nutrient.

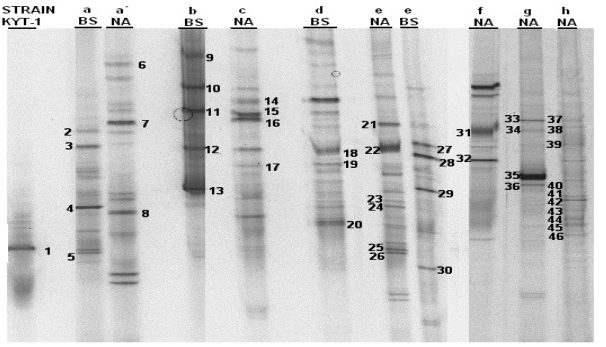


*Fig.2. DGGE patterns of 16S rDNA fragments from the corresponding samples. Strain KYT-1 has been used as a control, since its sequence is known previously. Among the ten samples biostimulation for a, b, d (As shown in the gel BS) and natural attenuation for a´, c, f, g, h. (As shown in the gel NA) For sample e, both experiments (NA and BS) results have been shown in the gel. For each sample distinct bands have been found. Bands subject to sequence analysis are labeled with numbers. (Refer to table 1 and table 2).*

For the study of phylogenetic affiliations of predominant community members, the bands (45 bands and 1 for KYT-1 as control) in the DGGE fingerprints were excised, reamplified, purified, and sequenced. All the bands yielded sequences without ambiguous positions and were included in phylogenetic analysis. Most of the bands in our analysis yielded a clean sequence, indicating that each band represented a different microbial population.

Results from DGGE analysis as in Table 1 and Table 2 shows, for sample a, band number 5 and for sample e, band number 21,22,23,24,25,26,27,28 have given significant percent homology with the genera *Burkholderia*, *Clostridium* and *Nitrosomonas* respectively, Since these genera are corresponding to the reported PCE degraders, it indicates the presence of some distinctive PCE degraders in the site from where samples have been collected. For the other samples, bands didn't show homology to the genera corresponding to the reported PCE degraders. But since the analytical data as in Figure 1 showed that bioremediation has been occurred for every sample, we are hypothesizing that, some PCE degraders must be developed in those sites those have not been reported so far. Our interest in this study was to experiment whether we find the dynamic behav-

ior pattern of bacteria, that is whether the different microbial community related to dechlorination develops in the PCE contaminated sites and whether the biostimulation stimulate the growth of PCE degraders. The results showed bioremediation has been taken place for the samples and for sample e, we found the acceleration of PCE dechlorimation after the biostimulation experiment. Another finding was that in each sample major strains belong to the same phylum, indicating that the sites could be unfavorable for the other phyla. So the development of microbial community responsible for PCE degradation varies with the individual contaminated sites.

Despite some limitations [7][8], the DGGE technique allowed us to monitor community changes that were consistent with the shift in environmental conditions [9]. As regards the PCE contamination and the growth of PCE degraders, our concern was to follow the bioremediation profile and the dynamic diversity of microbial population in the corresponding sites. Therefore, instead of preparing and examining enrichment culture in vitro, [10][11] we emphasized upon the in situ bioremediation followed by naturally developed degraders in some of our experimenting sites and for the rest of the sites to be experimented we applied the addition of nutrients directly in those sites. And in this connection the Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes provided us the profile for studying this dynamic behavior of complex microbial assemblages that carried out in the sites of PCE contamination.

*Tab. 1. Phylogenetic affiliation of 16SrDNA fragments retrieved in this study (Band numbering refers to the DGGE gel in Fig. 2)*

| BAND Number | Sample | Microorganism | Homology (%) | Phylum |
|---|---|---|---|---|
| 1 | KYT-1 | *Clostridium* sp. KYT-1 | 95 | *Firmicutes* |
| 2 | a (BS) | *Massilia timonae* | 91 | *Proteobacteria* (β) |
| 3 | | *Petrobacter succinimandens* | 85 | *Proteobacteria* (β) |
| 4 | | *Dyganella sp. clone* | 92 | *Proteobacteria* (β) |
| 5 | | *Burkholderia* sp. | 92 | *Proteobacteria* (β) |
| 6 | a´(NA) | *Dysgonomonas gradei* | 93 | *Bacteroidetes* |
| 7 | | *Bacteroides sp.* | 95 | *Bacteroidetes* |
| 8 | | *Prevotera sp.* | 91 | *Bacteroidetes* |
| 9 | b(BS) | *Thauera sp.* | 92 | *Proteobacteria* (β) |
| 10 | | *Denitratisomo* sp. | 98 | *Proteobacteria* (β) |
| 11 | | *Massilia timonae* | 90 | *Proteobacteria* (β) |
| 12 | | *Methyloversatilis universalis* | 94 | *Proteobacteria* (β) |

| BAND Number | Sample | Microorganism | Homology (%) | Phylum |
|---|---|---|---|---|
| 13 | | *Nitrosospira* sp. | 89 | *Proteobacteria* (β) |
| 14 | c(NA) | *Bacteroides* sp. | 90 | *Bacteroidetes* |
| 15 | | *Prevotella* sp. | 96 | *Bacteroidetes* |
| 16 | | *Bacteroides* sp.strain | 88 | *Bacteroidetes* |
| 17 | | *Prevotella* sp. oral clone | 88 | *Bacteroidetes* |
| 18 | d(BS) | *Treponema sp.clone* | 96 | *Spirochaetes* |
| 19 | | *Treponema sp.clone* | 93 | *Spirochaetes* |
| 20 | | *Clostridium sp.* | 94 | *Firmicutes* |

*Tab. 2. Phylogenetic affiliation of 16SrDNA fragments retrieved in this study (Rest of the bands) (Band numbering refers to the DGGE gel in Fig. 2)*

| BAND Number | Sample | Microorganism | Homology (%) | Phylum |
|---|---|---|---|---|
| 21 | e(NA) | *Clostridium* sp.clone | 97 | *Firmicutes* |
| 22 | | *Clostridium* sp. | 96 | *Firmicutes* |
| 23 | | *Clostridium* sp. | 87 | *Firmicutes* |
| 24 | | *Clostridium* sp.clone | 97 | *Firmicutes* |
| 25 | | *Clostridium* sp. | 96 | *Firmicutes* |
| 26 | | *Clostridium sp.* | 96 | *Firmicutes* |
| 27 | e(BS) | *Nitrosomonas* sp. | 96 | *Proteobacteria (β)* |
| 28 | | *Nitrosomonas* sp. | 95 | *Proteobacteria (β)* |
| 29 | | *Zoogloea* sp. | 92 | *Proteobacteria (β)* |
| 30 | | *Azoarcus denitrificans* | 92 | *Proteobacteria (β)* |
| 31 | f(NA) | *Prevotella* sp. | 92 | *Bacteroidetes* |
| 32 | | *Prevotella* sp. | 93 | *Bacteroidetes* |
| 33 | g(NA) | *Thiobacillus aquaesulis* | 93 | *Proteobacteria (β)* |
| 34 | | *Nitrosospira sp.* | 95 | *Proteobacteria (β)* |
| 35 | | *Nitrosospira* sp. | 95 | *Proteobacteria (β)* |

| BAND Number | Sample | Microorganism | Homology (%) | Phylum |
|---|---|---|---|---|
| 36 | | *Nitrosospira* sp. | 96 | *Proteobacteria (β)* |
| 37 | h(NA) | *Rhodobacter* sp. | 100 | *Proteobacteria (α)* |
| 38 | | *Rhodobacter* sp. | 99 | *Proteobacteria (α)* |
| 39 | | *Rhodobacter* sp. | 99 | *Proteobacteria (α)* |
| 40 | | *Rhodobacter* sp. | 99 | *Proteobacteria (α)* |
| 41 | | *Methylocystis heyerii* | 94 | *Proteobacteria (α)* |
| 42 | | *Nitrosospira* sp. | 90 | *Proteobacteria (β)* |
| 43 | | *Nitrosospira* sp. | 93 | *Proteobacteria (β)* |
| 44 | | *Nitrosospira* sp. | 95 | *Proteobacteria (β)* |
| 45 | | *Nitrosospira* sp. | 94 | *Proteobacteria (β)* |
| 46 | | *Nitrosospira* sp. | 95 | *Proteobacteria (β)* |

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