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ANALYSIS OF DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) PATTERN OF BACTERIAL 16S RDNA PROVIDES INFORMATIVE MICROBIAL COMMUNITY CHANGES IN CONTAMINATED GROUNDWATER

ANALIZA MODELI DGGE RDNA BAKTERII 16S ZMIENIA ŚRODOWISKO MIKROBIOLOGICZNE W SKAŻONYCH WODACH PODZIEMNYCH

Zbadano środowisko mikrobiologiczne zmieniające się przy dodatku tetrachloroetylenu (PCE), trichloroetylenu (TCE), cis-1,2-dichloroetylenu (cDCE) i chlorku winylu (VC) do konsorcjum wody gruntowej skażonej PCE wykorzystując klon biblioteki oraz metodę DGGE w oparciu o genom bakterii 16S. Świeża woda zebrana w obszarze skażonym PCE wykazywała dehalogenację PCE rzędu 5 μM w okresie 6 dni, jednakże woda z dodatkiem PCE wykazała zdolność redukcji 25-30 μM PCE w ciągu 2-3 dni. Przystosowujące się konsorcjum utrzymało aktywność dehalgonującą po 51 powtórzeniach subkultury. Wykorzystując to konsorcjum porównano środowiska mikrobiologicznego różnych pokoleń subkultury stosując ekologiczne techniki molekularne .Wygenerowano klon biblioteki czternastego pokolenia oraz model DGGE środowiska, następnie poprzez OTU zanalizowano process przystosowawczy i klon pokolenia 14.
Z 47 klonów, 28 należało do OTU 1 i OTU 2, których nie wiązano z dehalogenującymi mikrobami. Jednakże 5 klonów (OTU 3) było spokrewnionych ze szczepem P4 Enterobacter radicincitans, którego niektóre gatunki DNA są wiązane z dehalogenacją PCE do cDCE. Wykryto gatunki rodzaju Propionicimonas (OTU 6) i Desulfovibrio (OTU 7), które są znanymi dehalogenatorami lub tolerują halogenowane rozpuszczalniki.. Te wyniki wskazują, że bakterie wykazujące zdolność dehalgonujące nie były dominujące w konsorcjum 14-ego pokolenia. Modele DGGE pierwotnej wody gruntowej, pokoleń: 8, 14, 27, 51, 78 zanalizowano i porównano z klonami powyższych. Co zdumiewające pasma 5 I 6 zostały wykryte w pierwotnej wodzie, pokoleniach 8, 14, 27 i 51 jednakże zniknęły z pokolenia 78. Te pasma mają podobne rozmieszczenie do OTU 7, które było związane z rodzajem Desulfovibrio, którego niektóre gatunki są znanymi dehalogenatorami. W rzeczy samej ich brak powodował brak aktywności dehalogenującej w 78 pokoleniu. Podobnie OTU 1 zniknęło w 78 pokoleniu. OTU 1 wykazuje 99% podobieństwo do śniegu rzeki Elbe (SeqSRB5) i 91% podobieństwa do dehalogenującego Sporomusa ovate H1. Te badania wskazują, że analiza DGGE skażonej wody gruntowej jest istotnym narzędzie dla przewidywania obecności reduktorów na badanym obszarze.

1. Introduction

Analysis of microbial community dynamics while rehabilitation of contaminated subsurface is a major concern for development of better bioremediation process. We have been reporting the importance of microbial community shifting of contaminated sites during natural attenuation and biostimulation using DNA microarray of ITS region of DNA of tetrachloroethylene (PCE)-degrading bacteria and denaturing gradient gel electrophoresis (DGGE) pattern of amplified bacterial 16S rDNA (1, 2, 3).
There are many kinds of methods for analyzing bacterial community. We would like to know the best method for bioremediation work. Accurate, rapid, informative, cost effective, and easy method is applicable and desirable. Recent development of DNA sequencing is amazing and the meta genome analysis seems to be ideal but most developed DNA sequencing machine is necessary. In this report, conventional DGGE method and clone analysis is applied to know bacterial community shifting during acclimatization of PCE-degrading consortia to supplemental its spiking. As a result, molecular studies indicate that DGGE analysis of contaminated groundwater is a powerful tool for anticipating the existence of degraders in the site.

2. Materials and methods

2.1. Groundwater sample

Groundwater sample was collected from a PCE contaminated site (named ME) and biostimulation was performing at the day for sampling.

2.2. Acclimatization to aliphatic chlorinated compounds

Groundwater kept in 4 ^{o}C cold room was inoculated to previously autoclaved medium shown in Figure 1 followed by spiking respective aliphatic chlorinated compounds. Cultivation was performed at 30 ^{o}C dark and static condition. 20 ml vials, with Teflon sealed butyl rubber stoppers and aluminum caps, containing 1 ml media were autoclaved and 9 ml stocked groundwater were inoculated followed by addition of each aliphatic compounds to become initial concentration 1 mg/l. At the first acclimatization (generation), inoculation yield was 90 % then reduced to 10 %. Also from 2nd generation, 0.22 □m filtered groundwater was added to medium at the ratio of 40 % as animating materials.

2.3. Analytical method

PCE, TCE, *cis*-1,2- DCE, and VC were analyzed by gas chromatography. Headspace samples taken in 250 µl were quantified using a model GC-14B gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector and a glass column (i.d. 0.25 mm × 30 m, d_f 1.50 □m, VOCOL^{TM}) according to the Japanese Standard Method JIS K0102. The

injection and detection temperatures were maintained at 100ºC and 160ºC respectively, while the column temperature was held at 40ºC. Identification and quantification were calculated preparing the calibration curve with laboratory reagent of PCE (Kanto Chemical Co.), TCE (Nacalai Tesque) , *cis*-1,2- DCE (TCI-GR), and VC (Kanto Chemical Co.), respectively.

2.4. DNA Extraction

Cells in groundwater and cultures were disrupted by bead-beating method to isolate genomic DNA. Extracted DNA was qualified and quantified by Nanodrop (ND-1000, Thermo Fischer).

2.5. 16S rDNA amplification and clone library analysis and DGGE analysis

To amplify bacterial 16S rDNA fragments, primer pairs Eu10F (5′-AGA GTT TGA TCC TGG CTC AG -3′) and Univ1500R (5′-GGY TAC CTT GTT ACG ACT T -3′) and PRBA GC341F (5'-GCclump-CCTACGGGAGGCAGCAG-3') and PURN 517R (5'-ATTACCGCGGCTGCTGG-3') were used for the amplification of whole length and V3 region, respectively. PCR mixture contained 1 µl of 10 µM of each of primers, 2 µl of 10 × PCR buffer (Applied Biosystems, USA), 2 µl of 2 mM dNTP (TAKARA BIO, Shiga, Japan), 1 µl of DNA extract and 0.2 µl of Ampli *Taq* gold™ (5 U/µl) (Applied Bio system, USA) in a final volume of 20 µl. PCR amplification was carried OTU with TAKARA PCR Thermo Cycler. After initial denaturation at 95ºC for 10 min, 35 cycles of denaturation at 95 ºC for 15 sec, annealing at 50 ºC for 30 sec and extension at 72ºC for 1.5 min (or 30 sec for V3 region amplification) was performed followed by a final extension step at 72ºC for 10 min.

PCR products of almost full length of 16S rDNA were directly cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) and One Shot TOP 10 competent cell (Invitrogen, USA) according to the manufacture's protocol. Clones were picked at random and grown overnight using LB plate media containing 50 □g/ml of Kanamycin. Colony direct PCR was performed using primers M13F (5'- TGT AAA ACG ACG GCC AGT-3') and M13RV (5'-GGA AAC AGC TAT GAC CAT G-3'). The PCR products were purified before sequencing. Sequencing was performed by using a primer 907R (5'- CCG TCA ATT CMT TTG AGT TT -3′), BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), and 3130 genetic analyzer (Applied Biosystems, USA) according to the manufacture's instruction. Partial sequences of 16S rDNA (abOTU 600 bp) were obtained.

DGGE analysis of V3 region of 16S rDNA fragments was performed using DCode™ universal mutation detection system (BIO-RAD, Japan). Gels (16 cm x 16 cm) consisted of 40% bis- acrylamide (37:5:1) and a denaturant gradient of 30-60%. Electrophoresis was performed in 0.5 × TAE buffer at 65 ºC and 80 V, 400 mA for 9 hours. Gels were stained for 30 min with a 1:10000 dilution of SYBR gold (Invitrogen, USA) and visualized by Gel Doc XR Plus (BIO-RAD, California, USA). DGGE bands were excised, re-amplified by use of the same PCR conditions for V3 region amplification followed by applying into DGGE until excised bands became homogeneous. Subsequently, purified bands were sequenced as described above except for use of primer, PURN 517R .

2.6. Sequence analysis

Sequences obtained from clone library and DGGE analyses were directly uploaded to on-lined gapped BLAST search algorism (4) in the National Center for Biotechnological Information website to find relative taxa. Sequences of clone library analysis were aligned by CLUSTAL W (5) implemented in MEGA 4 (6). Aligned sequences were analyzed by a 'compute pairwise distances' program in MEGA 4 using Jukes-Canter model (7) to determine evolutionary distances among sequences. An operational taxonomic unit (OTU) was compiled as sequences with 3 % or less distances (97 or more similarities).

3. Results and discussion

3.1. Acclimatization to aliphatic chlorinated compounds

The microbial consortium of groundwater ME was active for PCE spike and trichloroethylene (TCE) was detected as the dehalogenated product. At 6^{th} day, supplemented PCE was almost converted to TCE (Figure 2). The consortium was responded to neither spikes of TCE, *cis*-1,2-dichloroethylene(cDCE), nor vinyl chloride(VC), respectively (data not shown). Subculturing with PCE spike was repeated approximately 1 week interval and at 8^{th} generation, PCE reduction rate at 3^{rd} day increased from 14 % at the 1^{st} generation to 91 % at 8^{th} generation. Nevertheless, further dehalogenation of PCE was not recorded (Figure 3). The acclimatized consortium has continued its dehalogenation activity by 51^{st} repetition of acclimatization. But unfortunately, at 78^{th} generation, due to incubation trouble, temperature rose up to 50 ^{o}C, resulting in losing PCE reduction activity of the consortium.

3.2. Clone library analysis

Using the stable consortium, its microbial community was studied. Clone library of 16S rDNA at 14^{th} generation of acclimatization and DGGE pattern of 16S rDNA by 51^{st} generation were compared. We obtained 47 clones and 22 clones were belonged to OTU 1 and most relative sequence was uncultured bacterium clone TCE33 (Table 1). For cultured strains, Elbe River snow SeqSRB5 and *Anaerovibrio burkinabensis* DSM 6283^{T} were listed. OTU 2 contains 6 clones; uncultured *Acetotacterium* sp. clone A4 and *Acetobacterium wieringae* DSM 1911^{T}. There were no clones directly concerning to dehalogenation in OTU 1 and OTU 2. But, the consortium including uncultured bacterium clone TCE33 had ability to dehalogenate TCE to cDCE (8) and that of uncultured *Acetotacterium* sp. clone A4 could dehalogenate trichlorodibenzo-p-dioxin (9). On the other hand, 5 clones belongs to OTU 3 were most relative to *Enterobacter radicincitans* strain P4 and within this genus a certain species having dehalogenateion of PCE to cDCE is reported (10). *Propionicimonas* species of OTU 6, relative to *Propionicicella* genera, halogenated solvent tolerant strain *Propionicicella superfundia* is recently isolated (11). *Desulfovibrio* of OTU 7 includes known dehalogenators (12). These results suggest that existence ratio of bacteria having wide range activity of dehalogenation was not high in the consortium.

3.3. DGGE analysis

DGGE patterns of original groundwater, 8th, 14th, 27th, 51st and 78th were analyzed and compared with representative clones retrieved from the clone library of 14th generation. The 8th, 14th, 27th, and 51st generations have stable PCE dehalogenation activity but 78th has lost its activity by elevation of incubator temperature. Bands 5 and 6 are remarkably detected from original groundwater, 8th, 14th, 27th, and 51st generations, respectively, but disappeared at 78th generation (Figure 4). As the DNA sequence of respective bands 5 and 6 were coincided with those of OTU 7, *Desulfovibrio* sp., a dehalogenator, loss of bands 5 and 6 resulted in shortage of PCE dehalogenation activity at the 78th generation. A similar shifting of DGGE pattern was found in OTU 1. The OTU 1 has 99 % identity with Elbe River snow SeqSRB5 and 91 % identity with a dehalogenator, *Sporomusa ovate* H1 (13). These molecular studies indicate that DGGE analysis of contaminated groundwater is a powerful tool for anticipating the existence of degraders in the site.

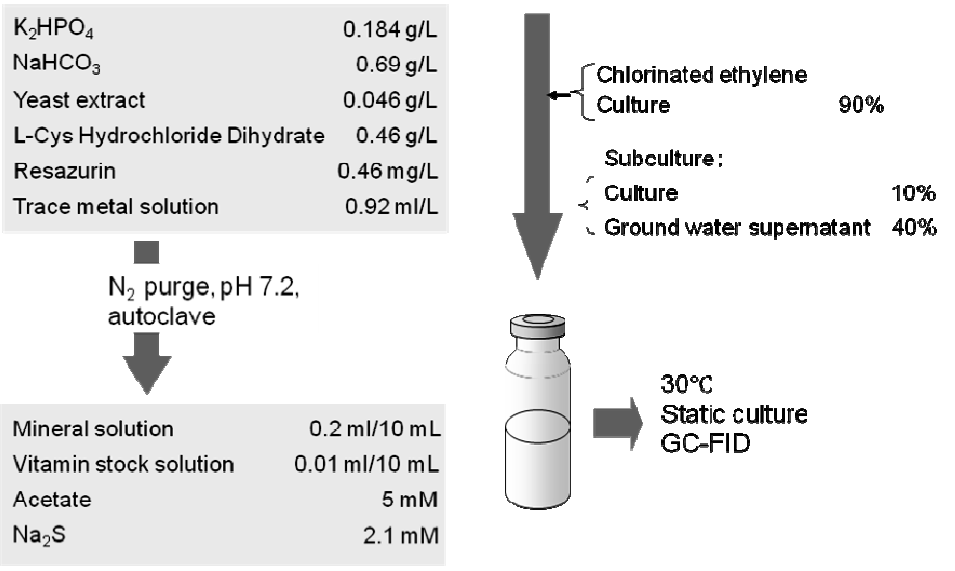


Fig. 1. Scheme of acclimatization.

Rys. 1. Schemat dodatków

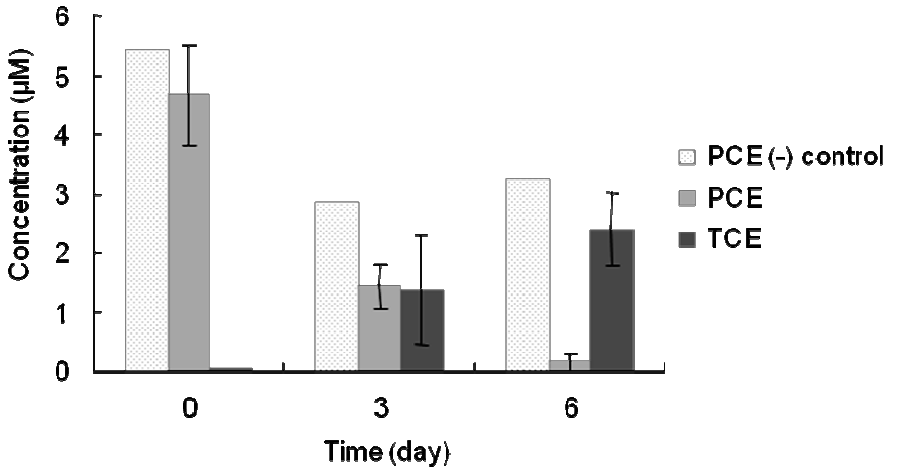


Fig. 2. Profile of PCE reduction by the microbial consortium in groundwater ME. 5 □M of PCE was spiked at day 0. PCE (-) control means no-addition control of PCE into cultures.

Rys. 2. Profil redukcji PCE przez mikrobiologiczne konsorcjum w wodzie z ME. 5 μM PCE zostało wprowadzonych w dniu 0. PCE (-) control oznacza próbę porównawczą bez dodatku PCE do kultur.

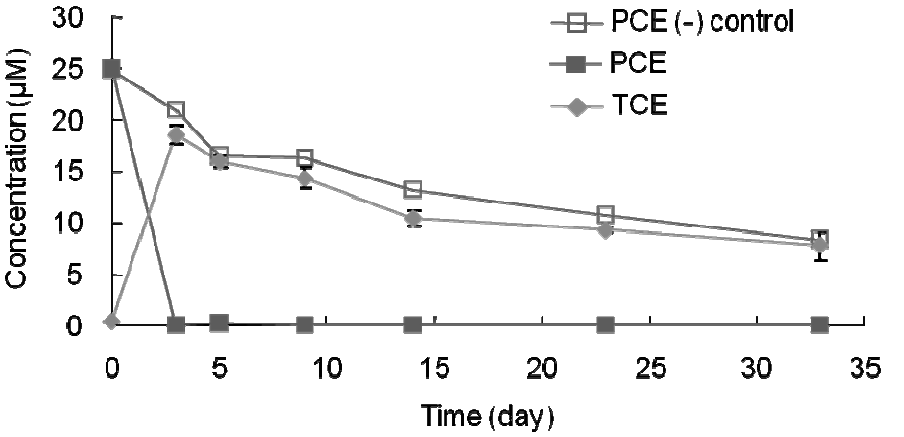


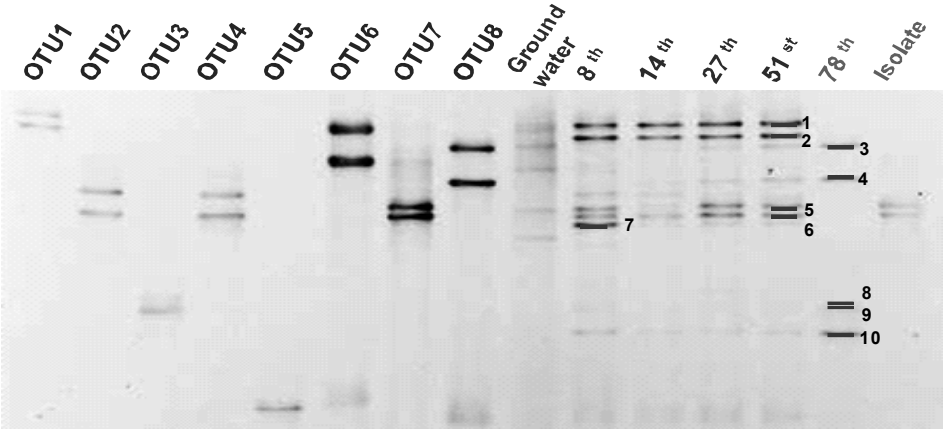
Fig. 3. Typical PCE reduction profile of acclimatized consortium.

Rys. 3. Typwoy profil redukcji konsorcjum z dodatkiem.

Tab. 1. Affiliation of clones obtained from 16S rDNA of 14th generation consortium acclimated to tetrachloroethylene

Tab. 1. Powiązanie klonów uzyskanych z pokolenia 14 16S rDNA konsorcjum z dodatkiem tetrachloroetylenu

OTU	Number	Cultured strains	Most relative sequence
1	22	Elbe River snow SeqSRB5	Uncultured bacterium clone TCE33
		Anaerovibrio burkinabensis DSM 6283^T	
2	6	*Acetobacterium wieringae* DSM 1911^T	Uncultured *Acetobacterium* sp. clone A4
3	5	*Enterobacter radicincitans* strain P4	
4	4	*Lactobacillales* bacterium HY-36-1	
5	3	Iron-reducing bacterium	
6	3	*Propionicimonas paludicola* strain Wf	
7	2	*Desulfovibrio desulfuricans* strain F28-1	
8	2	*Dysgonomonas mossii* strain CCUG 43457	



Band No.	Most relative strain	Band No.	Most relative strain
1	Elbe River snow isolate SeqSRB5	6	*Desulfovibrio desulfuricans* strain Ser-2
2	*Staphylococcus* sp. SACB25	7	*Sulfurospirillum deleyianum*
3	*Dysgonomonas wimpennyi*	8	*Enterobacter radicincitans* strain P4
4	*Desulfovibrio desulfuricans*	9	*Klebsiella pneumoniae*
5	*Desulfovibrio desulfuricans* strain Ser-2	10	*Enterobacter cloacae* strain MJ2

Fig. 4. Denaturing gradient gel electrophoresis (DGGE) of amplified bacterial 16S rDNA of consortium acclimatized to PCE and the representative clones from the clone library of 14th generation of acclimatization. Bands were excised and sequenced. Affiliation of bands are shown in the table below the DGGE profile.

Rys. 4. DGGE wzmocnionego 16S rDNA konsorcjum z dodatkiem PCE o reprezentatywne klony z klonu biblioteki 14 pokolenia. Pasma wycięto I sekwencjonowano. Powiązania pasm są przedstawione w tabeli poniżej profile DGGE.

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OCENA JAKOŚCI WÓD STUDZIENNYCH NA OBSZARACH WIEJSKICH NA PRZYKŁADZIE GMINY GRABICA

EVALUATION OF WELL WATER QUALITY IN RURAL AREAS ON THE EXAMPLE COMMUNITY GRABICA

Determination of the quality of water which is for immediate consumption by farmers who have not very deep wells is a very important problem. The aim of the research was to study the quality of drinking water in the rural region Grabica. Depht of wells was from 5 to 20m. The control tests were made for different 10 wells in February, April and June 2011. The following parameters were taken into account: nitrates, nitrites, chlorides, chromium, copper, iron, manganese, hardness, pH, conductance, etc. Analysis were carried out by means of Spectrophotomer DR/2010 (Hach). The results were compared with the current Polish Standards and EU Directive.

1. Wprowadzenie

Woda do celów przemysłowych i potrzeb ludności musi odpowiadać ściśle określonym wymogom. Możliwy jest monitoring wód z sieci wodociągowych, natomiast wody pochodzące ze studni gospodarczych nie podlegają wymogom kontroli. Rolnicy użytkując taką wodę zwykle opierają się na wrażeniach organoleptycznych: kolorze, smaku i zapachu. Nie zawsze pożądane efekty zmysłowe świadczą o dobrej jakości wody studziennej. Jakość wód gruntowych, płytko zalegających, jest funkcją wielu parametrów, których udział co do wielkości jest zmienny w czasie. Skład wód jest zależny od charakterystyki obszaru (budowy geologicznej, właściwości sorpcyjnych podłoża, procesów wietrzenia i rozpuszczania), procesów zachodzących w samej wodzie, głębokości studzien i ich lokalizacji w stosunku do źródeł zanieczyszczeń [1]. W wodach studziennych można spodziewać się tzw. zanieczyszczeń rolniczych, na które w głównej mierze składają się środki ochrony roślin, nawozy sztuczne pochodzące ze spływu z pól uprawnych i łąk, związki pochodzące z gnojowisk i obszarów hodowli zwierząt [2,3]. Studnie cembrowane ujmują wodę z pierwszego, najpłytszego poziomu wodonośnego. Obecność zanieczyszczeń może skutkować zauważalnymi zmianami organoleptycznymi jak nieprzyjemny smak, zapach czy kolor wody i wtedy łatwiej zareagować na zmianę