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INFLUENCE OF ESCHERICHIA COLI ON BIOAUGMENTATION AND BIOSTIMULATION OF TETRACHLOROETHYLENE AND CIS-1,2-DCE CONTAMINATION

WPŁYW ESCHERICHAI COLI NA BIOAUGUMENTACJĘ I BIOSTYMULACJĘ ZANIECZYSZCZENIA TETRACHLOROETYLENEM CIS-1,2-DCE CONTAMINATION

Bioremediation is a technique to rehabilitate contaminated subsurface using power of microorganisms. Principally, some bacteria which have activity to decompose targeting organic pollutants such as oil, halogenated ethenes, herbicides, etc play the major role for remediation. Adequate number of pollutant degrading bacteria and faster rehabilitation should be available. In this connection, growth and activation of bacteria in situ are the key factors for faster act. Usually some nutrients like glucose, yeast extracts, lactic acid, starch, etc, are supplemented to subsurface directly for growth and activation of the useful bacteria. But, in the subsurface, there are many hygiene bacteria and there is a common fear of the chance of hygiene bacterial growth as well, once nutrients are added. We would like to propose our findings to this concern. In this regard, we did some bioaugumentation and biostimulation experiments using Escherichia coli as a hygiene indicator.
For bioaugumentation experiment, we used Clostridium sp. DC-1 as a degrader of cis-1,2-DCE and monitored the profile of cis-1,2-DCE degradation and behavior of Clostridium sp. DC-1 and E. coli by pour plate and DGGE (Denaturing Gradient Gel Electrophoresis) analyses. We found that E. coli did not inhibit the degradation of cis-1,2-DCE and its growth was also suppressed. For biostimulation experiment, we used actual groundwater contaminated with chlorinated ethene. E. coli was added to the groundwater and its growth was chased with the changes in the bacteria flora. Dechlorination of tetrachloroethylene to trichloroethylene and cis-1,2-DCE within 14 days took place similarly both with and without the addition of E. coli. E. coli was detected only at day 0 by DGGE. From these experiments, it is considered that E. coli does not inhibit dehalogenation of chlorinated ethene and its growth was shown to be suppressed by indigenous bacteria.

1. Introduction

Chloroethenes like tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), and vinyl chloride (VC) are widely used as solvents in dry cleaning and metal degreasing industries [1-3]. Utilization of chloroethenes sometimes results in wide-range environmental contamination in the nearby anaerobic environmental compartments of ground waters, soils and sediments [4]. This infectivity is a big alarm because of the toxic and carcinogenic traits of chloroethenes [5]. Various studies have shown that under anaerobic conditions, chloroethene compounds can be reductively dehalogenated by some microbial community followed by dehalorespiration [6, 7]. In case of complete reductive dechlorination, PCE and TCE are anaerobically dechlorinated via *cis*-1,2-DCE and VC to the dehalogenated end-products ethene and ethane through halorespiration or co-metabolic degradation [8, 9]. The procedure of microbial reductive dechlorination usually can efficiently reduce the concentrations of PCE and TCE in anaerobic groundwater environments [10, 11].

Important thing to be considered is the possible growth of pathogenic bacteria in the contaminated site when bioremediation takes place. As long as groundwater is concerned pathogens are also important issue to think about. Because groundwater posses huge number of pathogens. In this connection *E. coli* has been reported as a pathogen in many water bodies along with some other groundwater pathogen like *Helicobacter pylori*, *Salmonella typhimurium* and *Vibrio cholera* [12 - 15]. If the harmful microbial community develops in the site, it could be a threat by causing inhibition in bioremediation and it could be a hazard to the environment as well. It is important to study about the influence of pathogenic bacteria during bioremediation. In this connection we selected *E. coli* as a model for pathogen and observed its behavior during the dechlorination of *cis*-1,2-DCE with *Clostridium* strain DC-1. To use a poisonous bacterium in laboratory is severely harmful and restricted. So in this study, we used *E. coli* strain NIHZ (NBRC 14237), categorized in biosafety level 2 and conventionally used for antibiotic activity test, as a model for pathogen.

Reductive dechlorination requires additional electron donors and nutrients augmentation to the contaminated site in order to stimulate microbial activity [16]. Several liquid electron donors, such as acetate, pyruvate, glucose, and lactate have been shown to support reductive dechlorination [17]. However, augmenting these liquid electron donors into the subsurface may significantly shift the microbial ecosystem. Some studies on the microbial ecology of chlorinated ethene in contaminated sites have been reported where both incubating in the laboratory and direct sampling technique from contaminated sites have been performed. [18 - 22].

Dechlorinating bacteria have been reported to become the dominant species during bioremediation [23]. However, most of the reported studies focused on the bacterial dechlorination, but studies focusing on pathogenic bacteria such as opportunistic bacterium have not been reported yet. If electron donor is introduced into the contaminated site, they stimulate not only the enhancement of dechlorinating bacterial growth but also stimulate the enhancement pathogenic microbial growth. Additionally, if pathogenic bacteria exist in chlorinated ethene contaminated site, they may inhibit the dechlorination caused by the corresponding dechlorinating bacteria. And in recent times several studies showed the presence of pathogens while exploring the groundwater quality [24].

The behavior of pathogenic bacteria while biostimulation is very important. Therefore, we conducted *in vitro* experiment for exploring the consequences of *E. coli* during the biostimulation of chlorinated ethene in the real contaminated groundwater and

survivability of *E. coli* was examined by using desoxycholate culture media. Shift in microbial community was determined by DGGE and clone library [25]. The objective of this study was to determine whether after conducting biostimulation the percentage of *E. coli* is increased or decreased in association with other microbial community in the original contaminated groundwater and hence to imply practical application to ensure the safe and productive implementation of biostimulation. In this experiment lactate which has been studied as a very potential nutrient was used for the biostimulation. The lactic acid is a potential donor to be metabolized by the indigenous microbes to produce hydrogen which is used in the reductive dechlorination process. Recently lactic acid is being utilized as a credible nutrient in exploring biostimulation [26]. This manuscript is summary and review of related articles [27, 28].

2. Materials and methods

2.1. Bioaugumentation experiments

2.1.1. Soil collection and DNA extraction

Soil has been collected from PCE uncontaminated mountainous area and has been sieved for experimental use. 2.5 g of soil was taken for each preparation of DNA extraction. Mo Bio ULTRACLEAN^{TM} soil DNA kit (Carlsbad, USA) has been used for DNA extraction according to the directions of the manufacturer.

2.1.2. Preparation of *Clostridium* strain DC-1 and *Escherichia coli* cultures

DC-1 culture which dehalogenates PCE and TCE to *cis*-1,2-DCE and was originated from a sediment sample collected at a landfill site in Nanji-do, Seoul, Korea, has been prepared [29] with the culture medium containing g/l of; K_2HPO_4, 3.0; KH_2PO_4, 0.8; $MgSO_47H_2O$, 0.2; L -asparagine, 5.0; D-glucose, 10; ferric citrate, 1.0 *p*-aminobenzoic acid, 0.6; and biotin 0.01. The medium was adjusted with pH 7.2. Prior to the addition of the glucose, ferric citrate, vitamin, *p*-aminobenzoic acid, and biotin, the 10 ml volume of culture media was prepared with the other components in 20 ml glass vials. The headspace of the vials were aseptically flushed with pure nitrogen in an anaerobic chamber and sealed with teflon-lined rubber septa, aluminum crimped caps and autoclaved. After the addition of the glucose, ferric citrate, vitamin, *p*-aminobenzoic acid, and biotin, DC-1 was added in the vials and maintained at its optimal growth temperature 30 °C. Bacterial growth was monitored via optical density readings at a wavelength of 660 nm (OD 660), using a spectrophotometer (UV-1600; Shimadzu, Japan). The bacterial culture has been transferred to the experimental setups, once its optical density reached at around 1.8. At the same optical density state, DNA of DC-1 was extracted from one vial for DGGE experiment using alkaline-lysis method.

For conducting the experiment in association with *E. coli*, *E. coli* strain NIHJ (NBRC 14237) was taken. Colonies were grown on desoxycholate agar plates "DAIGO" (Nihon Seiyaku, Nagoya, Japan) at 37 °C. The mentioned medium contained in gram per liter of Peptone, 10.0; lactose, 10; sodium desoxycholate, 1; NaCl, 5; K_2HPO_4, 2; ammonium

iron(III) citrate, 2; and neutral red, 0.033. The medium was adjusted with pH in between 7.0-7.4. For making the working state of *E. coli*, a colony was transferred into liquid LB media which contained in gram per liter of peptone, 10; yeast extract, 5; and NaCl, 10. Then the liquid culture was kept at 30 °C shaking for 24 hours. Cell count was done by calculating with optical density. Dilution of cell was conducted using autoclaved 0.8% NaCl solution. For the DGGE experiment DNA was extracted from the liquid LB media of *E. coli* using alkaline-lysis method.

2.1.3. Experimental set up

For this study culture media preparation was the same as for the preparation of *Clostridium* strain DC-1 culture. After the addition of glucose, ferric citrate, vitamin, *p*-aminobenzoic acid, and biotin, vials were spiked with *cis*-1,2-DCE (TCI-GR, Tokyo, Japan), to make it's concentration 5 mg/L, in 10 ml volume solution of each vial. Finally 1 ml of DC-1 culture with optical density of around 1.8 was added to make the final 10 ml volume and vials were incubated at 30 °C.

Four preparations of cultures were examined. In preparation 1, the culture was prepared with the collected soil, *Clostridium* strain DC-1, and *cis*-1,2-DCE. In preparation 2, the culture was prepared with the collected soil, *cis*-1,2-DCE, *Clostridium* strain DC-1, and $1.5x10^3$ cells/ml of *E. coli*. In preparation 3, the culture was prepared with the collected soil, *cis*-1,2-DCE without addition of *Clostridium* strain DC-1 and *E. coli.* In preparation 4, the culture was prepared with the collected soil being autoclaved, and *cis*-1,2-DCE without addition of *Clostridium* strain DC-1 and *E. coli*.

For each preparation, in an anaerobic chamber, 2.5 g [wet wt.] of soil was taken for each vial of 10 ml liquid solution of (g/l): K_2HPO_4 , 3.0; KH_2PO_4 , 0.8; $MgSO_47H_2O$, 0.2; L -asparagine, 5.0; D -glucose, 10; ferric citrate, 1.0 *p*-aminobenzoic acid, 0.6 ; and biotin 0.01 (pH 7.2). The teflon caps of the vials were opened and soil was taken in the vials. After taking soil, vials were flushed with nitrogen one more time and again sealed with teflon-lined rubber septa, aluminum crimped caps. *cis*-1,2-DCE was spiked at 5 mg/L of final concentration. For preparation 1, vials were added with 1 ml of DC-1 culture at its O.D of around 1.8. For preparation 2, vials were added with 1 ml of DC-1 culture at its O.D of around 1.8 and $1.5x10^3$ cells/ml of *E .coli*.

Vials were prepared for a 14-days-experimental study. Triplicate was made for each day's measurement. All the vials were shifted to a water bath (Taitec, Nishikata, Saitama, Japan). Rotation was fixed at 80 rpm and temperature was fixed at 30 °C. *cis*-1,2-DCE reduction was analyzed by gas chromatography. Headspace samples, taken in 100 µl, were quantified using a model GC- 14B gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector and a glass column (i.d. 3.2 × 2.1 m, silicone DC-550 20% Chromosorb W [AWDMCS] 80/100) according to the Japanese Standard Method JIS K0102 [30]. The injection and detection temperatures were maintained at 100 °C and 160 °C, respectively, while the column temperature was held at 40 °C. The carrier gas was pure nitrogen at a flow rate of 50 ml/min. Identification and quantification were calculated by preparing the calibration curve with laboratory reagents *cis*-1,2-DCE (TCI-GR, Tokyo, Japan). The reagent was diluted with 99% ethanol: 3.94 µl was added to 5 ml of ethanol in small vial. Anaerobic culture medium was prepared in other vials followed by the addition of 2.5 g of autoclaved soil and the ethanol diluted *cis*-1,2-DCE were taken in the vials containing media at the concentration of 5 mg/L in solution used to make a calibration curve for the measurement of *cis*-1,2-DCE reduction.

2.1.4. 16S rDNA amplification and DGGE analysis

Mo Bio ULTRACLEAN™ soil DNA kit (Carlsbad, USA) was used for DNA extraction according to the directions of the manufacturer. For Bacteria-specific PCR of 16S rDNA fragments, the primers PRBA341-FGC (*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′) (Base number 40 +17) and PRUN517-R (*E. coli* position 517-534) (5′-ATT ACC GCG GCT GCT GG -3′) (Base number 17) were used for the amplification of V3 target region of *E. coli* position. The primers were purchased from *FASMAC* Co., Ltd. (Kanagawa, Japan). 10 µl volume of the PCR mixture contained 0.5 µl of 12.5 pM of GC 341 F and 517 R primers, 2.6 µl of 10 x PCR buffer (Applied Biosystem, USA), 1 µl of 2 mM dNTP (TAKARA, Japan), 1 µl of DNA template and 0.05 µl of Go Taq HS (5 U/µl) (Promega, USA). PCR amplification was carried out with TAKARA PCR Thermo Cycler. After initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 15 sec, primer annealing at 50 °C for 30 sec and extension at 72 °C for 30 sec was performed followed by a final extension step at 72 °C for 7 min. DGGE analysis of 16S rDNA fragments was performed using DCode™ universal mutation detection system (BIO-RAD, Japan). Gels (16 cm x 16 cm) consisted of 40% bisacrylamide (37:5:1) and a denaturant gradient of 35%-65%. After the PCR, DNA of each sample was equalized in 15 ng/µl of concentration. Electrophoresis was performed in 1 X TAE buffer at 60 °C and 80 V for 8 hours 30 min. Gels were stained for 30 min with a 1:10000 dilution of SYBR gold (Invitrogen, USA) and analyzed using UV Transilluminator (BIO-RAD, Japan).

2.1.5. Sequence analysis

From the DGGE gel, the central portions of the bands of interest were excised and washed with 99% ethanol and autoclaved Milli-Q water, respectively. The DNA was reamplified with the same primers as for the initial PCR reaction. The only exception was for the forward primer, where the GC clamp was not added. The reamplified products were again purified and sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Sequence comparisons were performed through a BLAST [31] search against the Gene Bank database from the site National Center for Biotechnology Information. CLUSTAL W program [32] was used for multiple sequence alignment.

2.2. Biostimulation experiments

2.2.1. Groundwater sample

Groundwater sample was collected from TCE and *cis*-DCE contaminated site by Panasonic Environmental Systems & Engineering Co., Ltd. during March 2010. The sample was labeled as MF09-06 (MF). The sample was immediately transferred to airtight bottles and stored at 4 °C until the analytical measurement and DNA extraction had been started.

2.2.2. Incubation

Approximately 9.5 ml of groundwater was transferred into 20 ml glass vials with 0.5 ml of lactate (89 mM) as electron donor. The vials were inoculated with 100 µl of 4.0×10^6 cells/ml *E. coli* strain NIHJ (NBRC 14237), cultured in LB medium. The vials are sealed with teflon-lined rubber septa, and aluminum crimped caps. Then they were kept in 30 °C in shaking state for 14 days. Similar kind of culture preparation with the same ratio of corresponding components was prepared in 120 ml bottle where the total culture volume was 60 ml. 5 bottles of 60 ml volume culture was prepared for day 0 and day 4 as well. Number of *E. coli* used for the inoculation was calculated from the measured number of bacteria by nucleic acid staining method using fluorescence microscope (BX-50, OLYMPUS, Tokyo, Japan) and optical density at wavelength of 600 nm using a spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan). Nucleic acid staining method was performed using Phosphate Buffered Saline (PBS) contained g/l of NaCl, 80.0; KCl, 2.0; Na_2HPO_4, 11.5; KH_2PO_4, 2.0, and 5×SYBR Gold. *E. coli* culture was not added to some vials and used as negative control. All the vials were spiked with 30 µM PCE using a syringe. PCE (30 µM) was made with laboratory reagent of PCE (Kanto Chemical Co., Tokyo, Japan).

2.2.3. Analytical methods

Suspended solid (SS) and volatile suspended solid (VSS) obtained from groundwater were analyzed in YAMATO DVS-400 (Japan). Total organic carbon (TOC) analysis was performed using TOC-L analyzer (Shimadzu, Kyoto, Japan).

Chlorinated ethene was analyzed by GC-14B (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a column (30 m length × 0.25 mm i.d., VOCOL ™). Injection was performed using 250 µl syringes from headspace gas. The injector and detector temperatures were maintained at 100 °C and 160 °C, respectively. The column temperature was held at 40 °C. Identification and quantification were calculated by preparing the calibration curve with laboratory grade chlorinated ethene.

2.2.4. *E. coli* detection

Detection of *E. coli* was same to the description in 2.1.3.

2.2.5. DNA extraction

For DNA extraction with the aim to carry out DGGE and cloning, DNA was extracted from the experiment conducted with biostimulation of PCE contaminated MF with lactate addition and *E. coli* augmentation. In this connection DNA extraction was carried out from MF on day 0 and day 14 samples those prepared with the addition of lactate. For DNA extraction, the bigger culture preparation, as mentioned in "Incubation", was taken. From 5 bottles, 300 ml of corresponding samples were centrifuged (12000 × g) for 15 min. Total DNA was extracted using ISOIL for Beads Beating DNA Extraction Kit (NIPPON GENE, Tokyo Japan) for the pellet of MF centrifuged following the manufacture's protocol. Extracted DNA was dissolved in TE buffer, and stored at -30 °C.

2.2.6. 16S rDNA amplification and DGGE analysis

Methods for 16S rDNA amplification and DGGE analysis were similar to the description in 2.1.5.

2.2.7. 16S rDNA amplification and clone library analysis

Bacterial 16S rDNA gene was amplified by PCR with a set of universal primers 10F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500R (5'-GGYTACCTTGTTACGACTT-3'). PCR mixtures (50 µl of total) contained 2.5 µl of 10 µM of 10F and 1500R primers, 13 µl of 5 × PCR Green Go Taq Flexi Buffer, 5.0 µl of 2.0 mM dNTP, 21.75 µl of sterile Milli Q water, 5.0 µl of DNA template, and 0.25 µl of Go Taq Hot Start Polymerase. Initial denaturation at 95 °C for 2 min, 21 cycles of denaturation at 95 °C for 30 sec, primer annealing at 50 °C for 1 min and extension at 72 °C for 1.5 min were performed followed by a final extension step at 72 °C for 10 min.

The PCR products were purified using a PCR purification kit (GL Science, Japan) before amplicons were inserted into the pCR4-TOPO cloning vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) according to the manufacture's recommendations. Clones were picked at random and grown overnight in LB plate media containing 50 µl/ml of kanamycin. Twenty clones were picked for day 0 sample and 23 for day 14 sample, respectively. Colony direct PCR was done using primers T3 (5'-AACCCTCACTAAAGGGAA-3') and T7 (5'-TAATACGACTCACTATAGGG -3'). The PCR products were purified before sequencing.

2.2.8. Sequence analysis

Method for sequence analysis was similar to the description in 2.1.6.

3. Results and discussion

3.1. Bioaugumentation experiments

3.1.1. Biodegradation profile of *cis*-1,2-DCE

As regards the analytical study, in case of preparation 1, where the culture was prepared with the collected soil, *cis*-1,2-DCE, and *Clostridium* strain DC-1; *cis*-1,2-DCE degradation was observed from day 3 and degradation reached at its maximum point at day 5. From day 6 on, analytical graph showed a steady line until day 14 (Fig 1).

In case of preparation 2, where the culture was prepared with the collected soil, *cis*-1,2-DCE, *Clostridium* strain DC-1, and $1.5x10^3$ cells/ml of *E. coli*, similar degradation profile was observed like that of preparation 1, that is degradation was observed from day 3 and degradation reached at its maximum point at day 5. From day 6 on, analytical graph showed a steady line until day 14.

In case of preparation 3, where the culture was prepared with the collected soil, *cis*-1,2-DCE without addition of *Clostridium* strain DC-1 and *E. coli,* the analytical data showed no degradation with a steady state curve from day 0 to day 14 [Data not shown].

In case of preparation 4, where the culture was prepared with the collected soil being autoclaved, and *cis*-1,2-DCE without addition of *Clostridium* strain DC-1 and *E. coli*, analytical profile showed similar data as observed in case of preparation 3 [Data not shown].

This finding suggested, it was the *Clostridium* strain DC-1 that caused the *cis*-1,2-DCE degradation in presence with other microbial community.

In the analytical study, preparation 2 where the culture was prepared with the collected soil, *cis*-1,2-DCE, *Clostridium* strain DC-1, *E. coli*, the *cis*-1,2-DCE degradation profile was as same as in case of preparation 1 (Fig 1). This result suggested that the degrading activity of *Clostridium* strain DC-1 was not inhibited by the presence of *E coli*.

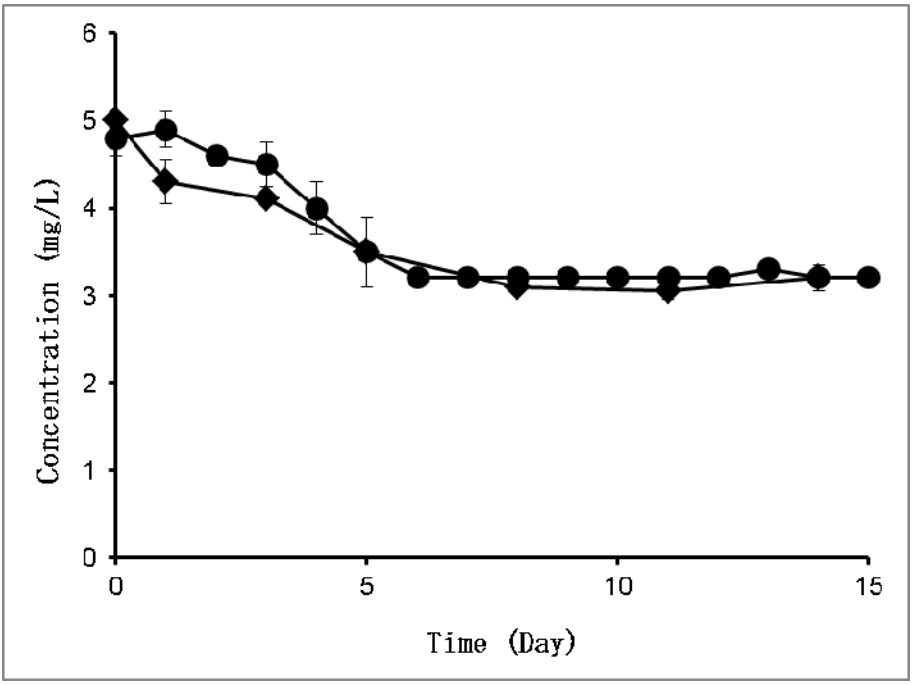

Fig. 1 *Biodegradation profile of cis-1,2-DCE by Clostridium strain DC-1 in the presence of indigenous microorganisms and with or without Escherichia coli.*
Filled circle represents soil + cis-1,2-DCE + Clostridium strain DC-1 and filled diamond means soil + cis-1,2-DCE + Clostridium strain DC-1 + Escherichia coli.

3.1.2. DGGE profile

As regards the DGGE result, in case of preparation 1 (Fig 2) followed by the set up with the culture containing the collected soil, *cis*-1,2-DCE, and *Clostridium* strain DC-1, few dominant bands have been obtained for day 1, day 3, day 5, and day 14 as indicated in lane 2, lane 3, lane 4, lane 5 and lane 6 respectively (Fig 2). For each of these days, band 5, band 10, band 15, band 20, and band 25 were exactly at the same position as compared with the band 26 in lane 7 which represented the band for the *Clostridium* strain DC-1. It indicated that these bands represented DC-1 and the existence of DC-1

was observed throughout the experimental period. The band pattern of DC 1in lane 2 (band 5) was found to be similar with the band pattern of DC 1 in lane 6 (band 25). Band 10 was detected as in little more dominant pattern and band 15 was detected as in most prominent and dominant pattern. Lane 1 that represented the indigenous microbial community in the collected soil without being added in the experimented culture media did not provide any bands as dominant.

As regards the other bands, in case of lane 2, lane 3, and lane 4, that were represented as day 1, day 3, and day 5 respectively, four dominant bands were observed in same position in each lane. They are band 1, 2, 3, and 4 (Lane 2); band 6, 7, 8, and 9 (Lane 3); and band 11, 12, 13, and 14 (Lane 4). In case of lane 5 and lane 6, represented as day 10 and day 14 respectively, different dominant band pattern was observed from the other lanes. In these two lanes also, four dominant bands were observed in same position in each lane. They are band 16, 17, 18, and 19 (Lane 5); band 21, 22, 23, and 24 (Lane 6).

DGGE was also conducted in case of preparation 2, where the culture was prepared with the collected soil, *cis*-1,2-DCE, *Clostridium* strain DC-1, and $1.5x10^3$ cells/ml of *E. coli* (Fig 3). DGGE in this case was conducted with the sample of day 1, day 3, day 5, day 10, and day 14 of preparation 2, as represented in lane 1, lane 2, lane 3, lane 4, and lane 5 respectively. In Fig 3, lane 6 represented as *Clostridium* strain DC-1 that was in similar position as in the other lane where the DC-1 band pattern was observed. The band patterns representing DC 1 in Fig 3 are similar with the band patterns representing DC1 in Fig 2.

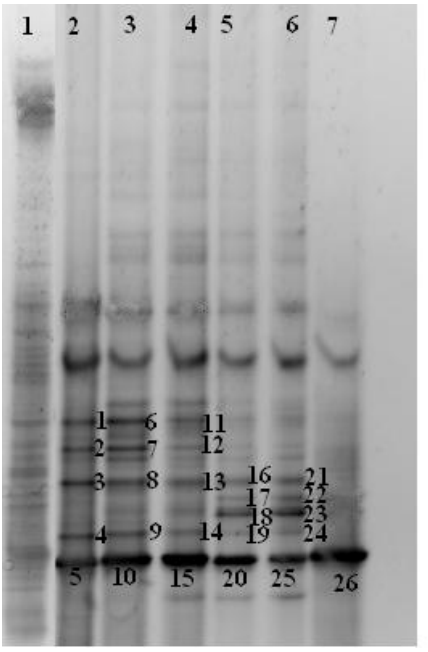

Fig. 2 *DGGE analysis for amplified DNA extracted at individual sampling days of the experimental period for preparation 1 where the examined culture was prepared with the collected soil, cis-1,2-DCE, and Clostridium strain DC-1.*
Lane 1: The collected soil, Lane 2: day 1, Lane 3: day 3, Lane 4: day 5, Lane 5: day 10, Lane 6: day 14, and Lane 7: Clostridium strain DC-1

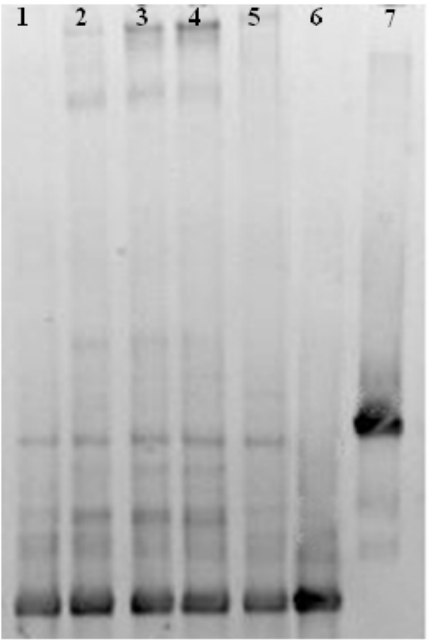

Fig. 3 *DGGE analysis for amplified DNA extracted in individual day of the experimental period for preparation 2 where experimented culture was prepared with the collected soil, cis-1,2-DCE, and Clostridium strain DC-1 and E. coli.*
Lane 1: day 1, Lane 2: day 3, Lane 3: day 5, Lane 4: day 10, Lane 5: day 14, Lane 6: Clostridium strain DC-1, and Lane 7: E. coli.

3.1.3. Sequence analysis

Sequence analysis was conducted with the dominant bands obtained from the DGGE of preparation 1 (Fig 2), and sequences of the dominant bands are shown in Table 1.

For day 1, band 1, 2, 3, 4, and 5 showed 98%, 99%, 98%, 98%, and 100% homology respectively to *Clostridium* species. For day 3, bands 6, 7, 8, 9, and 10 showed 98%, 99%, 100%, 99%, and 100% homology respectively to *Clostridium* species. For day 5, bands 11, 12, 13, 14, and 15 showed 98%, 99%, 100%, 96%, and 100% homology respectively to *Clostridium* species. For day 10, bands 16, 19, and 20 showed 98%, 98%, and 100% homology respectively to *Clostridium* species and bands 17 and 18 showed 96% and 97% homology to *Bacillus* species and *Eubacterium* species, respectively.

For day 14, bands 21, 24, and 25 showed 98%, 98%, and 100% homology respectively to *Clostridium* species and bands 22, and 23 showed 95% and 97% homology to *Bacillus* species and *Eubacterium* species, respectively.

Tab. 1 Phylogenetic affiliation of 16S rDNA fragments retrieved in this study (band numbering refers to the DGGE gel in Fig. 2)

Band number	Sample	Microorganism	Homology (%)	Phylum
1	Day 1	*Clostridium* sp.	98%	*Firmicutes*
2		*Clostridium* sp.	99%	*Firmicutes*
3		*Clostridium butyricum*	98%	*Firmicutes*
4		*Clostridium* sp.	98%	*Firmicutes*
5		*Clostridium beijerinckii*	*100%*	*Firmicutes*
6	Day 3	*Clostridium* sp.	98%	*Firmicutes*
7		*Clostridium* sp.	99%	*Firmicutes*
8		*Clostridium butyricum*	100%	*Firmicutes*
9		*Clostridium* sp.	99%	*Firmicutes*
10		*Clostridium beijerinckii*	100%	*Firmicutes*
11	Day 5	*Clostridium* sp.	99%	*Firmicutes*
12		*Clostridium* sp.	98%	*Firmicutes*
13		*Clostridium* sp.	99%	*Firmicutes*
14		*Clostridium* sp.	96%	*Firmicutes*
15		*Clostridium beijerinckii*	100%	*Firmicutes*
16	Day 10	*Clostridium* sp.	98%	*Firmicutes*
17		*Bacillus* sp.	96%	*Firmicutes*
18		*Eubacterium tarantellae*	97%	*Firmicutes*
19		*Clostridium* sp.	98%	*Firmicutes*
20		*Clostridium beijerinckii*	100%	*Firmicutes*
21	Day 14	*Clostridium* sp.	98%	*Firmicutes*
22		*Bacillus* sp.	95%	*Firmicutes*
23		*Eubacterium tarantellae*	97%	*Firmicutes*
24		*Clostridium* sp.	98%	*Firmicutes*
25		*Clostridium beijerinckii*	100%	*Firmicutes*
26	DC-1	*Clostridium beijerinckii*	100%	*Firmicutes*

Sequence analysis (Table 1) from DGGE (Fig 2) mostly provided dominance of some other *Clostridium* species. It might due to the *Clostridium* supporting culture medium that was utilized in the study. But since the control preparation 3 showed no degradation of *cis*-1,2-DCE, we are assuming that these dominant ones might not contribute in degradation in this regard.

3.1.4. Pour plate study

For the three preparations of pour plate three different profiles of *E. coli* growth was obtained (Table 2).

Pour plate result was observed with no *E. coli* growth when the preparation was conducted in association with *Clostridium* strain DC-1 and the soil indigenous microbes, at around day 4. From this result we hypothesized that DC-1 might have contributed in decreasing the *E. coli* growth along with the indigenous microbial community. Results from the other two pour plate preparations; pour plate 2 and pour plate 3 supported this finding.

Tab. 2 Result of pour plate study

Preparations of pour plate	Growth of *E. coli,* cfu/m*l*				
	Day 0	**Day 4**	**Day 7**	**Day 10**	**Day 14**
Plate 1: *cis*-1,2-DCE, *E. coli*, soil, and *Clostridium* sp. DC-1.	4×10^{2}	No colony			
Plate 2: *cis*-1,2-DCE, *E. coli*, and soil	1.2×10^{2}	2×10	No colony		
Plate 3: *cis*-1,2-DCE and *E. coli*	5.8×10	1.7×10^{5}	3.2×10^{5}	5.4×10^{3}	No colony

3.2. Bioaugumentation experiments

3.2.1. Chemical characters of groundwater

Chlorinated ethylene concentration at the time provided after sampling was detected in TCE 34.9 mg/Land *cis*-DCE 12.6 mg/L. The pH was within the range of water quality standard at 7.82. TOC was slightly higher with the value at 4.57 mg/l. SS and VSS were shown to be less organic matter in groundwater at 2753 mg/l and 255 mg/l, respectively but with higher turbidity. Even though this groundwater sample initially was thought to be in an unfavorable condition for the growth of microorganisms, but later the growth of microbes during the experiment opposed the initial thinking.

3.2.2. Dechlorination activity

Ground water sample that was subject to natural attenuation and later incorporated with PCE after collection showed dechlorination from PCE to *cis*-DCE for 14 day (Fig. 4). *cis*-DCE was increased gradually from 1.0 mg/L to 2.3 mg/L, but VC and ethene was not detected even after 96 day (data not shown). MF added with lactate was also showed

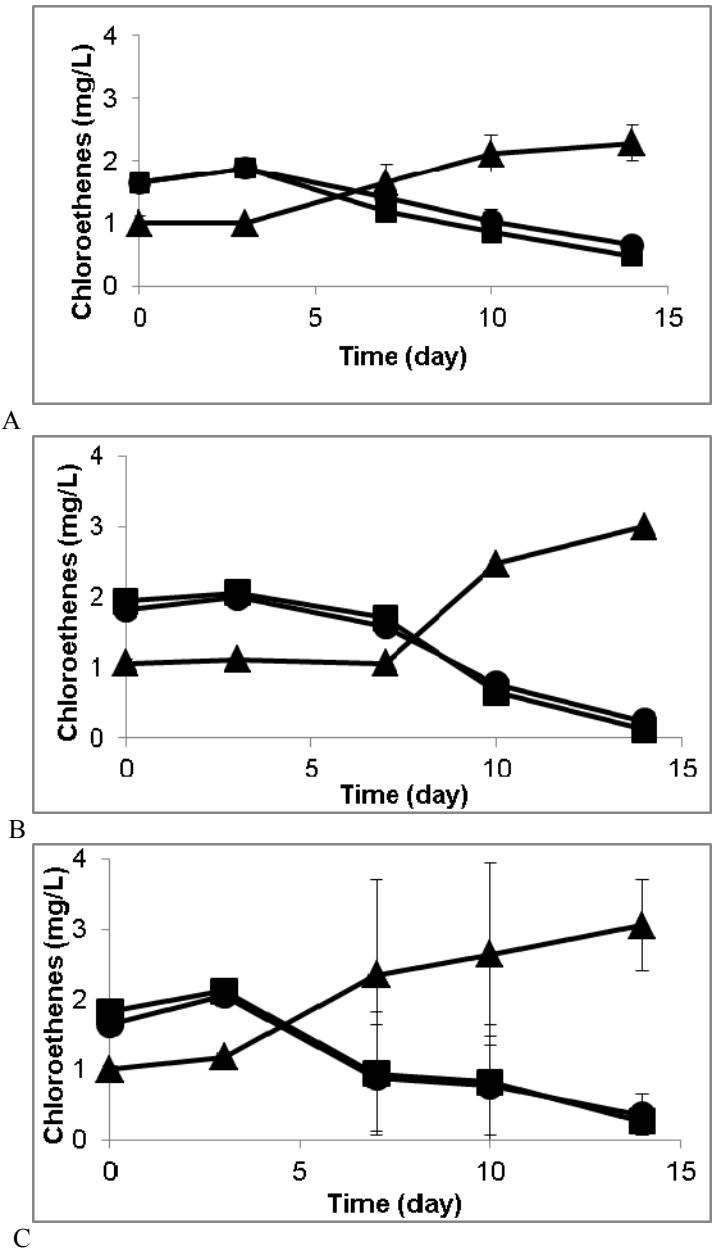

Fig. 4 *Reductive dechlorination of PCE (filled circles) and TCE (filled squares) to cis-1,2-DCE (filled triangles) in the groundwater.*
A: natural attenuation, B: lactate addition, and C: lactate and E. coli. Data are shown as averages (symbols) and ranges (bar) of duplicate results.

dechlorination from PCE to *cis*-DCE for 14 day (Fig. 4). *cis*-DCE was increased from 1.1 mg/L to 3.0 mg/L. Dechlorination activity of MF was slightly higher than natural attenuation due to the addition of lactate. But VC and ethene was not detected even after 96 days (data not shown). MF inoculated with *E. coli* was showed dechlorination from PCE to *cis*-DCE for 14 days, but dechlorination rate is different (Fig. 4).

Inoculated *E. coli* has reached at final concentration of 4.7×10^6 cells/ml by calculated from the OD 600 and nucleic acid staining method when *cis*-DCE was increased from 1.0 mg/L to 3.1 mg/L. It indicated that, inhibition of dechlorination activity by *E. coli* was not happened. These results suggested, activity inhibition of contaminated groundwater in presence of hygiene indicator bacteria was proved not to be happened.

3.2.3. Detection of *Escherichia coli*

MF in the state of natural attenuation was detected with *E. coli* of 2.2×10^6 cells/ml at day 0. At day 14, *E. coli* was not detected (Table 3). This result showed during the dechlorination of groundwater contaminated chlorinated ethene followed by natural attenuation, hygiene indicator bacteria cannot survive. In case of the experiment conducted with addition of lactate was detected with *E. coli* of 2.3×10^6 cells/ml at day 0. At day 14, 6.0×10^3 cells/ml of *E. coli*, at day 23, 2.0×10^3 cells/ml of *E. coli* and at day 32, 1.6×10^3 cells/ml of *E. coli* were detected (Table 3). This result showed, during the dechlorination of groundwater contaminated chlorinated ethene followed by biostimulation, hygiene indicator bacteria decline gradually.

Tab. 3 Colony measurement of E. coli by desoxycholate media of MF sam ples which were inoculated with E. coli of 4.7 x 106 cells/ml

Conditions of sample	Time (day)	Number of colonies (cfu/ml)
Sample without *E. coli* inoculation	0	0
Natural attenuation	0	2.2×10^6
	14	0
Addition of lactate	0	2.3×10^6
	14	6.0×10^3
	23	2.0×10^3
	32	1.6×10^3

3.2.4. DGGE and sequence analysis

In DGGE, one band was observed for the sample from day 0 and eight bands were observed for the sample from day 14 (Fig. 5). Microbial community prior to inoculation of *E. coli* detected genus such as *Comamonas*, *Polaromonas Variovorax*, *Geobacter*, *Rhodoferax*, *Acidovorax* with more than 97% homology. Band pattern of day 0 detected

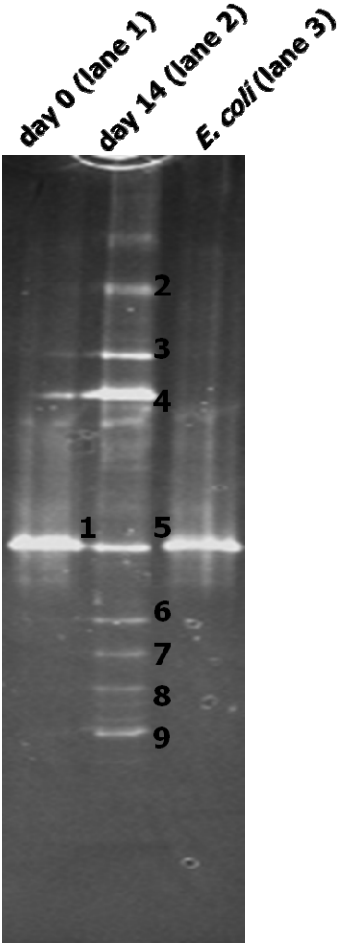

Fig. 5 DGGE analysis for amplifies DNA extracted in MF inoculated for E. coli of cultivation day 0 and day 14.Sequences data are shown in Table 4.

strongly one band with the same position as well as band representing *E. coli* in lane 3. This is due to much amount of *E. coli* inoculation in day 0. For lane 3, DNA of *E. coli* was extracted from LB medium culture of 100 µl. Sequence analysis showed band number 1 has 100% homology to *E. coli* (Table 4). Band pattern of day 14 was detected seven bands other than the band in position of *E. coli* (Band 5). Fluorescence intensity of the band of *E. coli* (band number 5) was quite thin in comparison with band of day 0. Band number 4 showed strongest fluorescence intensity was affiliated with the *Pseudomonas putida*. The most dominant species at before or after dechlorination of MF was changed from *E. coli* to *Pseudomonas putida*. Band numbers 6, 7, 8 and 9 showed a significant percent homology with the *Clostridium* species. Since some species of genus *Clostridium* were reported as dechlorinating bacteria [29, 33, 34], these genus *Clostridium* grown as a dominant species during dechlorination were considered possibility

degrading bacteria for PCE and TCE. Consequently, *E. coli* showed to have less impact upon the growth of other microorganisms, at the same time, number of *E. coli* was found to be reduced.

Tab. 4 Phylogenetic affiliation and homology to the closest relative of amplified DNA sequence excised from DGGE gel

Time (day)	Band no.	Microorganisms	Homology (%)
0	1	*Escherichia coli*	100
14	2	*Anaerosinus glycerini*	96
	3	*Pseudomonas putida*	100
	4	*Pseudomonas putida*	99
	5	*Escherichia coli*	100
	6	*Clostridium crotonatovorans*	99
	7	*Clostridium baratii*	98
	8	*Clostridium vincentii*	98
	9	*Clostridium magnum*	100

3.2.5. Clone library analysis

Result of clone library also supported DGGE result by providing the data where only *E. coli* was detected in case of day 0 sample. Sample of day14 after dechlorination was detected with the dominance of *Pseudomonas putida*, *Desulfosporosinus meridiei*, and *Clostridium* species in addition to *E. coli* (Table 5). Previously *Pseudomonas putida* has been studied as a microbe containing dehalogenase [35]. *Pseudomonas putida* is also being explored as dehalogenator in several recent studies [36, 37]. Besides members of the genus *Desulfosporosinus* are sulfate-reducing bacteria, often found in microbial communities associated with mining environments and involved in the bioremediation of metal-contaminated water and sediments [38, 39]. In these environments, enriched with SO_4^{2-}, sulfate reduction contributes to precipitation of metal sulfides and thereby the immobilization of toxic metals.

Tab. 5 Phylogenetic affiliation and homology to the closest relative of amplified DNA sequence selected from clone library

Time (day)	OTU (>97%)	Clone no.	Microorganisms	Homology (%)
0	1	24	*Escherichia coli*	100
14	2	19	*Pseudomonas putida*	100
	3	2	*Escherichia coli*	100
	4	1	*Desulfosporosinus meridiei*	99
	5	1	*Clostridium crotonatovo-rans*	98
	6	1	*Clostridium populeti*	96

4. Conclusion

Public concern if local government or company try to remediate contaminated subsurface using the power of microorganisms, both in cases of bioaugmentation and biostimulation, is what happens on pathogen in soil or groundwater. We would like to answer of the query using *E. coli* as a typical model hygienic microorganism and performed bioaugmentation and biostimulation experiments in laboratory scale and finally obtained the following conclusion.

Experiment with addition of *E. coli* showed that dechlorinating activity of DC-1 was not inhibited by the presence of *E. coli*. Pour plate experiment with DC-1 and *E. coli* revealed that the dominance of *Clostridium* species caused the decrease of *E. coli* growth in a bioremediation state. Experiment conducting with *E. coli* suggested that the strain in the contaminated site did not inhibit the degradation of *cis*-1,2-DCE and during the degradation period, rather some other *Clostridium* species became dominant and the growth of *E. coli* was decreased.

In biostimulation experiment, we showed the state of dechlorination activity and behavior of microbial structure by the addition of *E. coli*-as hygiene indicator bacteria in contaminated groundwater sample. Dechlorination took place similarly both with and without addition of *E. coli*. It indicated that, inhibition of against dechlorinating activity of corresponding dechlorinating bacteria was not happened by *E. coli*. Structural change of bacterial community was analyzed both before and after dechlorination using denaturing gradient gel electrophoresis (DGGE) and clone library. Result of DGGE detected *E. coli* only at day 0. Sample of day 14 after dechlorination detected *Pseudomonas putida*, *Anaerosinus glycerini*, and *Clostridium* genus but no *E. coli*. Result of clone library also showed the identical profile. Detection of *E. coli* using desoxycholate media was decrease from 2.3×10^6 cells/ml to 6.0×10^3 cells/ml during day 14. These results suggest that biostimulation of groundwater contaminated by chlorinated ethene in presence of hygiene bacteria caused the dechlorination without activity inhibition and decrease of dechlorinating bacteria in association with a suppress in hygiene bacterial growth.

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