NOTE

Rapid Reductive Dechlorination of Trichloroethene in Contaminated Ground Water Using Biostimulation Agent, BD-1, Formulated from Canola Oil

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Abstract: A case study of the bioremediation of groundwater contaminated with trichloroethene (TCE) was conducted using the biostimulation agent, BD-1. TCE levels were monitored by gas chromatography-mass spectroscopy. Total organic carbon (TOC) and volatile fatty acids (VFAs) were analyzed to investigate the environmental fate of BD-1. The effects of BD-1 on microbial activity were investigated using 16S rRNA gene-based quantitative polymerase chain reaction (qPCR) analysis. The biodegradation of BD-1 was accompanied by a reduction in TCE, and the initially high TOC levels decreased rapidly as BD-1 was transformed into VFAs. qPCR analysis showed that the genus *Dehalobacter* became progressively dominant through the experiment. These results suggested that BD-1 might dechlorinate TCE by activating dechlorinating bacteria.

Key words: trichloroethene, dechlorination, *Dehalobacter*, agent BD-1

1 INTRODUCTION

Organochlorines such as tetrachloroethene and trichloroethene (TCE) were used intensively in industry until the discovery of their adverse effects on humans and the environment¹⁾. The US EPA National Priorities List contains numerous reports on cleaning up TCE contamination in groundwater; these reports emphasize the need for efficient and cost-effective remediation strategies to remove this probable human carcinogen²⁾. The remediation of organochlorines in groundwater is generally conducted by aerating contaminated water pumped up through wells. However, organochlorines are easily absorbed by soil, which makes this method unsuitable for the remediation of organochlorines in soil. A promising strategy for TCE removal is biological reductive dechlorination involving the sequential reduction of TCE to ethylene via *cis*-1,2-dichloroethene (*cis*-DCE) or *trans*-dichloroethene (*trans*-DCE) and vinyl chloride (VC). Biological reductive dechlorination is an attractive method that has been successfully used for TCE removal from contaminated fields, but its success and

efficiency can be greatly influenced by microbes, insufficient supply of donor substrate, or kinetic limitations on the dechlorination reactions³⁾. A cost-effective alternative is biostimulation, which involves treating contaminated soil or water with low concentrations of soluble organic substrates that activate indigenous bacteria to degrade contaminants. These organic substrates include commercial products, alcohols, and fatty acids from edible oils⁴⁻⁶⁾. However, it is risky to use alcohols as a carbon source because they are flammable and readily diffuse in soil and water. In addition, fatty acids with aliphatic chain lengths exceeding ten carbons (C10) cannot penetrate soil particles. Fatty acids are also unsuitable for extensive use in the reduction of contaminants because they form emulsions containing particles that distribute heterogeneously and aggregate readily. Therefore, the physical properties of these substrates make them impractical for use in biostimulation. The ability of bacteria to reductively dechlorinate organochlorines to their hydrocarbon counterparts is widely exploited in the biostimulation of organochlorine-contaminat-

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ed environments. Various materials have been developed and used to stimulate dechlorinating bacteria. BD-1, a biostimulation agent, is formulated from canola oil and is mainly composed of fatty acids, especially oleate and linoleate. BD-1 was developed to overcome the problem of organochlorine contamination⁷⁾. BD-1 has been shown to stimulate the microbial dechlorination of organochlorines at contaminated sites in Japan, even after only short incubation times⁸⁾.

The objectives of this study were to clarify the effects of BD-1 on TCE dechlorination, to elucidate the environmental fate of this agent, and to determine the changes in the community structure of bacteria involved in the reductive dechlorination of an organochlorine-contaminated site.

2 EXPERIMENTAL PROCEDURES

2.1 Chemicals

TCE and *cis*-DCE were purchased from Wako (Japan) and used as standards for gas chromatography (GC) analysis. The novel microbial biostimulation agent, BD-1, was supplied by Miyoshi Oil & Fat Co. Ltd. BD-1 was composed of canola oil (melting point $\leq 0^{\circ}\text{C}$), polyoxyl (10) oleyl ether as a nonionic surfactant, glycerol, and water. BD-1 was prepared as follows: 60 wt.% canola oil, 3.0 wt.% polyoxyl (10) oleyl ether, glycerol with 200 wt.% of polyoxyl (10) oleyl ether, and 5.4 wt.% water were agitated. The resulting mixture was emulsified after addition of 29.2 wt.% water⁷⁾. After emulsification, BD-1 formed an oil-in-water emulsion with a mean oil droplet diameter of 10 μm . The mean oil droplet diameter was demonstrated by the ratio of the droplet volume to all dispersoid presenting in the sample solution and measured using a laser scattering particle size distribution analyzer (LA-910, Horiba, Japan).

2.2 *In vitro* dechlorination of TCE by BD-1 treatment

The effects of BD-1 on electron donor supporting TCE reductive dechlorination were tested by adding 0.1% (w/w) BD-1 to 5 L of unsterilized organochlorine-contaminated groundwater that was supplemented with an additional 500 mg of TCE ($3.81 \mu\text{mol L}^{-1}$) as an electron acceptor. BD-1 was added from an aqueous, anoxic stock solution, using a syringe. The 5 L samples of treated groundwater were transferred to plugged aluminum bags, which were incubated in the dark at room temperature with no shaking. TCE and *cis*-DCE were quantified by using the methods described in section 2.4 (Analytical procedures). Potential hydrogen production from BD-1 was investigated by transferring 30 mL aliquots of the treated groundwater samples to glass vials. The vials were sealed with sterilized Teflon-coated butyl rubber stoppers and incubated at 20°C for 35 days. Hydrogen was measured by taking a 500 μL sample of the headspace gas and injecting it into a packed GC

column equipped with a thermal conductivity detector (TCD) (GC-2014, Shimadzu, Japan). The GC-TCD operated with a stainless steel packed column ($6.0 \text{ m} \times 3.00 \text{ mm}$ ID, Shincarbon, Shimadzu) and the following program: injector temperature, 100°C ; detector temperature, 200°C ; splitless injection system; argon carrier flow rate, 30.0 mL min^{-1} . The GC temperature program was as follows: 40°C rising to 200°C at a rate of $25^{\circ}\text{C min}^{-1}$, and 200°C held for 8.6 min. Quantification was based on external calibration curves.

2.3 Site description and sampling

The sampling site was located in Aichi, Japan by a machinery manufacturer that had been contaminated with TCE from industrial use. The specific site was referred to as well-47 and it was located 2 m from a plume of chlorinated contamination. On June 18, 2007, 34 kg of BD-1 was applied through a 50 cm diameter injection well at 10 m intervals, to a depth of 4.7–7.0 m below the surface. Sequential aquifer samples were collected between June 18 and July 2, 2007 from groundwater approximately 6 m below the surface of well-47. Samples were identified by the number of days after BD-1 treatment. Samples were anaerobically collected in 2 L glass bottles, and stored at 4°C . These samples were analyzed for chlorinated contaminants, volatile fatty acids (VFAs), chloride ions, and TOC using the methods described in section 2.4 (Analytical procedures).

Approximately 0.25 g of the microbial cells collected from each sample was subjected to DNA extraction within 24 h of collection. Bacterial DNA was extracted using a PowerSoil™ DNA isolation kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. The purity and quantity of the DNA preparations were determined⁹⁾ by measuring their UV absorption spectra (BioSpec-mini, Shimadzu).

2.4 Analytical procedures

To determine organochlorine content, a 0.5 mL aliquot was collected from treated groundwater samples after a specified period and immediately extracted with an equal volume of hexane. The resulting 1 μL aliquot of the prepared sample was subjected to capillary GC using a GC-17A equipped with a mass spectrometer (MS), GC-QP5050A (Shimadzu). The GC-MS operated with a stainless steel capillary column ($60 \text{ m} \times 0.25 \text{ mm ID} \times 1 \mu\text{m}$, Aquatic GL Science, Japan) and it was programmed as follows: injector temperature, 120°C ; detector temperature, 180°C ; injection system split ratio, 1:2; helium carrier flow rate, 29.5 kPa. The GC temperature program ran as follows: 30°C for 5 min, 30°C rising to 70°C at a rate of $4^{\circ}\text{C min}^{-1}$, 70°C rising to 200°C at a rate of $8^{\circ}\text{C min}^{-1}$, and 200°C held for 3 min. Quantification was based on external calibration curves. VFAs were analyzed by sampling a further 0.5 mL volume

and washing with an equal volume of chloroform to remove impurities. Analytes were collected from the water layer. VFAs were quantified by high-pressure liquid chromatography (HPLC) using a Prominence system equipped with an electrical conductivity detector, CDD10A_{VP} (Shimadzu). The HPLC system was operated with a Shim-pack SCR column (300 mm × 7.9 mm ID; Shim-pack SCR-102H). A 10 µL aliquot of analyte was loaded and eluted isocratically using 5 mM *p*-toluenesulfonate (pH 2.7) at a flow rate of 0.8 mL min⁻¹. The oven temperature was 40°C. A TOC analyzer (TOC-V CPH, Shimadzu) was used to determine the TOC values of groundwater treated with BD-1. Chloride ion concentration in the groundwater was determined by ion chromatography (DX-300, Dionex, USA) in an anion exchange column (IonPaC AS4A-SC, 4 × 250 mm), and the analysis was performed isocratically with 1.8 mM Na₂CO₃/1.7 mM NaHCO₃, at a flow rate of 1.0 mL min⁻¹.

2.5 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE)

PCR primers 341F-GC (forward primer) and 534R (reverse primer) were used to amplify the variable V3 region of the bacterial 16S rRNA gene (corresponding to position 341-534 in the *E. coli* 16S rRNA gene sequence) connected to a GC clamp¹⁰. PCR was performed using Premix Taq (Ex version) polymerase (TaKaRa, Japan), according to the manufacturer's instructions (100 ng template DNA and 25 pmol of each primer in a 50 µL reaction volume). The reaction conditions are described elsewhere¹⁰. DGGE analysis was performed with a DCode™ instrument (Bio-Rad, USA), according to the manufacturer's instructions. Gels were made with a gradient of denaturants from 30% (containing 2.1 M urea and 18.3% formamide) to 60% (containing 4.2 M urea and 24% formamide). Next, 500 ng of each PCR amplicon was subjected to electrophoresis on a 10% (w/v) polyacrylamide gel at 160 V for 5 h at a running temperature of 58°C. After electrophoresis, the gel was stained with SYBR Gold (Invitrogen, USA) for 30 min, according to the manufacturer's instructions. A DNA band was excised from the gel and its DNA sequence was cloned and determined by Hosoda *et al.*'s method using an automated sequence analyzer (ABI PRISM 3130xl Genetic Analyzer; Applied Biosystems, USA). The sequence analysis procedure is described elsewhere¹⁰.

2.6 Quantitative PCR (qPCR)

qPCR was conducted to enumerate 16S rRNA gene copies of the genus *Dehalobacter* using a StepOne Plus™ real-time PCR system (Applied Biosystems, USA). The reaction conditions were described previously¹⁰. *Dehalobacter*-specific qPCR was performed with the primers DhbF2 (5'-CAATAAGCATTCGCGCTGGG-3') and DhbR2 (5'-TTGGTAAGGTTCTTCGCGTTG-3') with an annealing

temperature of 60°C. These primers were slightly modified from Smits *et al.*¹¹. The fragment size of the DhbF2-DhbR2 primer set was 120 bp. A calibration was performed using serial dilutions of a known quantity of T7W/SP6W-amplified fragments of *Dehalobacter* 16S rRNA gene-containing plasmids, which were generated by TA cloning with the pGEM-T easy vector system (Promega, USA). The dynamic range of the target 16S rRNA genes by qPCR was 7.6×10^3 to 3.1×10^8 16S rRNA gene copies per reaction. DNA copy numbers were calculated following a previously described procedure¹⁰.

2.7 Nucleotide sequence accession numbers

The sequences reported in this paper have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under GenBank/EMBL/DDBJ accession Numbers AB517889-AB517919 (DGGE bands).

3 RESULTS AND DISCUSSION

3.1 Biostimulatory effects of BD-1 on reductive dechlorination in microcosms

Before injecting BD-1 to treat organochlorines in the contaminated site, we evaluated the relationship between hydrogen production and the biostimulatory effects of BD-1 on organochlorine-dechlorinating bacteria in batch culture. Hydrogen production was observed between 11 (114 µM) and 35 days (1,200 µM). Concentrations of VFAs (initially 1,520 µM of acetate and 190 µM of butyrate) were reduced to 36 µM and 4.3 µM, respectively. That no methane was detected was a significant finding because it suggested that methane fermentation did not occur simultaneously with hydrogen production (Fig. 1A). The initial TCE concentration of 4.1 µM decreased to 0.020 µM after 35 days of incubation, whereas no TCE dechlorination was observed in the control sample (Fig. 1B). The ratio of TCE to *cis*-DCE was comparable to those reported by other studies⁴. The conversion rate of BD-1 to hydrogen was equivalent to 31.4 moles of hydrogen per 1 kg of BD-1, assuming that the average molecular weight of BD-1 was the same as that of edible canola oil¹². The initial TCE level of 21 µmol was reductively dechlorinated to 8.4 µmol of *cis*-DCE after 21 days of incubation by the activity of anaerobic dechlorinating bacteria using hydrogen as an electron donor. This was based on the stoichiometric amount of hydrogen generated during incubation with BD-1, which was estimated to be 94 µmol. This theoretical amount of hydrogen was sufficient for TCE dechlorination. These results indicated that treatment with BD-1 might have supplied hydrogen to activate dechlorinating bacteria. Hydrogen production was also confirmed by the production of VFAs (mainly acetate and butyrate), a result consistent with microbial bioconversion of oleate and linoleate from

Fig. 1 Hydrogen, carbon dioxide, and VFA production from BD-1 (A). Reductive dechlorination of TCE supplemented with and without BD-1 in microcosm experiments (B).

The five symbols in panel (A) represent hydrogen (solid circles with dashed line), carbon dioxide (crosses with dashed line), methane (solid squares with dashed line), acetate (open squares with dashed line), and butyrate (open diamonds with dashed line). The four symbols in panel (B) represent TCE with BD-1 (solid circles), *cis*-DCE with BD-1 (solid triangles), TCE without BD-1 (open circles), and *cis*-DCE without BD-1 (open triangles).

BD-1. Furthermore, increases in the concentration of *cis*-DCE correlated with similar decreases in TCE concentration after 35 days of BD-1 treatment, suggesting that BD-1 could have enhanced reductive TCE dechlorination.

3.2 On-site effects of BD-1 on organochlorine dechlorination

BD-1 effectively dechlorinated TCE to *cis*-DCE in a microcosm and induced the production of sufficient hydrogen without methane fermentation. Therefore, BD-1 was applied to the dechlorination of a contaminated site that was still used for industrial TCE disposal. The concentra-

Fig. 2 Reductive dechlorination of TCE after a 14-day incubation period (A), and accumulated VFAs and total organic carbon (TOC) concentration after degradation by BD-1 (B). The five symbols in panel (A) represent TCE (solid circles), *cis*-DCE (solid triangles), VC (solid squares), ethylene (solid diamonds), and chloride ions (open circles with dashed line). The five symbols in panel (B) represent TOC (asterisks), acetate (open squares with dashed line), propionate (plus signs with dashed line), butyrate (open diamonds with dashed line), and bicarbonate ions (open triangles with dashed line).

tions of TCE and *cis*-DCE were 1.7 and 1.6 μM , respectively, before BD-1 leached into the groundwater 6 m below the surface. The detection of *cis*-DCE was considered to be carry-over contamination because other organochlorines were found at the sampling site. BD-1 was introduced into the groundwater, and the concentrations of TCE and *cis*-DCE decreased to 0.12 and 0.04 μM , respectively, after 14 days, while the dechlorination of TCE started right from the first day without any lag time over the course of 14 days (Fig. 2A). In addition, ethylene production was found to occur without any VC accumulation. TCE was dechlori-

nated at a rate of approximately $0.11 \mu\text{M day}^{-1}$ over the 14-day incubation period. The decrease in TCE did not match the increase in *cis*-DCE because we sampled an open, flowing groundwater system where the flux of TCE and *cis*-DCE did not allow us to account for every mole. The rate of TCE dechlorination in the field treated with BD-1 was twofold faster than simple application of edible oil¹³. The TOC level (initially 4,700 ppm) and chemical oxygen demand level (initially 6,300 ppm) decreased rapidly with BD-1 treatment to 680 ppm and 50 ppm, respectively, over the 14-day incubation period. The rapid degradation of BD-1 was verified by VFA analysis. BD-1 was converted to VFAs such as acetate (116 ppm) and propionate (21 ppm) after the 14-day incubation period. The concentration of butyrate increased to 36 ppm after 10 days and finally decreased to 19 ppm (Fig. 2B). Our results demonstrated that TCE levels decreased rapidly after the addition of BD-1 and there was no accumulation of VC shortly after BD-1 application, which is important because VC would inhibit further dechlorination to ethylene^{14, 15}. The accumulation of acetate from BD-1 degradation could be useful for reductive dechlorination because He *et al.* reported that several TCE-containing microcosms amended with acetate as an electron donor were completely dechlorinated to ethylene¹⁶. In addition, the rapid decrease in TOC suggested that BD-1 application did not result in further environmental contamination. The initial pH value (6.3) was slightly reduced to 6.1 after 14 days of incubation with BD-1. The sulfate ion concentration was also reduced from 1.5 mM to 0.1 mM. In contrast, the manganese concentration increased from 0.036 mM to 0.056 mM. The nitrate ion concentration was undetectable. Furthermore, the dissolved oxygen (1.9 ppm) and oxidation-reduction potential levels (-34 mV) in the aquifer were reduced to 0.7 ppm and -170 mV , respectively. The hydrogen concentration could not be measured at this site because the application of a general sampling method for gaseous hydrogen was difficult due to the sampling well structure. These results indicated that BD-1 addition made the aquifer environment more suited to anaerobic dechlorinating bacteria, which significantly stimulated rapid dechlorination compared with other studies that used an emulsified oil barrier⁵. Thus, the application of the relatively high-TOC agent, BD-1, led to high levels of VFAs, including acetate, butyrate, and propionate, which provided great potential for the rapid reductive dechlorination of organochlorines without VC accumulation.

3.3 Quantitative analysis of dechlorinating bacteria

As BD-1 proved to efficiently induce dechlorination, we investigated whether BD-1 influenced microbial diversity by performing 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE). Sequence analysis of the DGGE bands from 2 to 21 days showed that many of the

predominant bands were affiliated with known fermentative bacteria, including the genera *Trichococcus*¹⁷ (bands #10 and 11), *Sporotalea*¹⁸ (band #17), *Clostridium* (bands #8 and 19) and *Klebsiella*¹⁹ (bands #9 and 16). These bacteria are known to produce hydrogen from fatty acids with VFA accumulation²⁰. The other predominant bands were also affiliated with the genera *Desulfovibrio* (band #1), *Azovibrio* (band #2), *Thiobacillus* (band #3), *Propionivibrio* (bands #4 and 5), *Enterobacter* (bands #12, 13, 14, 15, and 20), and *Pantoea* (band #18). The genera *Propionivibrio* and *Enterobacter* might be involved in degradation of BD-1; however, it remains unclear how the genera *Desulfovibrio*, *Azovibrio*, *Thiobacillus*, and *Pantoea* can serve to degrade BD-1 or produce hydrogen. Furthermore, the detection of a sequence affiliated with the genus *Dehalobacter*, which can use TCE as an electron acceptor¹⁴, suggested the presence of *Dehalobacter* spp. (band #35) on the site after the addition of BD-1 (Fig. 3). Therefore, we enumerated the *Dehalobacter*-specific 16S rRNA genes using quantitative real-time PCR. The *Dehalobacter*-specific 16S rRNA gene copy numbers found in the collected groundwater increased by as much as 1,000-fold after 14 days (Fig. 4). Moreover, an increase in the chloride ion concentration derived from TCE was correlated with the increased abundance of *Dehalobacter*-specific genes (Fig. 2A). Thus, we suggested that bacteria in the genus *Deha-*

Fig. 3 16S rRNA gene-based DGGE profiles of bacterial populations in the groundwater treated with BD-1. Lanes 1-6 are indicated in the sampling data. The numbers show the bands excised for DNA sequencing analysis.

Fig. 4 Changes in *Dehalobacter* 16S rRNA gene copy number. Error bars represent the standard deviation of copy numbers measured for triplicate samples.

lobacter were involved in TCE dechlorination. Given the results of the microcosm and on-site experiments as well as the DGGE analysis, it was concluded that BD-1 was degraded to acetate, butyrate, and propionate by the genera *Trichococcus*, *Sporotalea*, *Clostridium*, and *Klebsiella*. Furthermore, we believe these bacteria produced hydrogen from acetate, butyrate, and propionate, along with other sulfate-reducing bacteria¹⁸⁾. Butyrate made a particular contribution to hydrogen production because it decreased after 10 days (Fig. 2B). Populations of the genus *Dehalobacter* increased during the experiment, which demonstrates that hydrogen derived from fatty acids or VFAs acted as an electron donor for the dechlorination of TCE by bacteria related to the genus *Dehalobacter*. Trace nutrients essential for the growth of *Dehalobacter* spp. were probably supplied by the degradation of BD-1. Our results suggested that BD-1 directly induced fermentative bacteria by providing carbon and energy sources for the production of hydrogen, which acted as an electron donor during the dechlorination of organochlorines. Moreover, qPCR analysis enabled us to better understand the changes in community structure of bacteria involved in organochlorine dechlorination in groundwater after the application of BD-1.

4 CONCLUSIONS

Previous reports have documented the application of biostimulation agents such as commercial products, alcohols, and fatty acids from edible oils to enhance the dechlorination of organochlorines^{4–6)}. These studies, in which edible oils were applied to approximately 10 μ M TCE with 500 ppm TOC over at least a 400-day incubation period, showed the effectiveness of these substrates but did not investigate the effects of microbial activity. This study clari-

fied the effects of BD-1, a triglyceride formulation, on the dechlorination of TCE (initially 2 μ M) and the levels of VFA and TOC (initially 4,700 ppm). It was also clear that there were changes in the bacterial community structure involved in reductive dechlorination. Therefore, we conducted a molecular analysis to evaluate TCE and VFA levels and the diversity of microbes involved in dechlorination using BD-1. A microcosm study indicated that BD-1 was degraded into acetate, butyrate, and propionate to yield sufficient hydrogen for TCE dechlorination. An on-site study of organochlorine dechlorination using BD-1 showed that *Dehalobacter* spp. were involved in dechlorination because their abundance increased over a 14-day incubation period, and this increase correlated with a decrease in TCE concentration. Investigation of the environmental fate of BD-1 showed that it was readily degraded into VFAs (mainly acetate, butyrate, and propionate), along with a decrease in TCE. This result was due to the canola oil in the formulation, which enhances dispersal in the environment and availability for bacterial growth. Moreover, the high TOC level with BD-1 was not affected by dechlorination, despite its rapid decrease. Thus, BD-1 indirectly activated dechlorinating bacteria that use VFAs or hydrogen as an electron donor during the dechlorination of organochlorines via the direct stimulation of fermentative or hydrogen-producing bacteria that can assimilate VFAs and produce hydrogen by BD-1 degradation. Although high TOC levels were not consistent with results from well-known biostimulation agents, high TOC levels were a prominent feature of BD-1 characterized by the present study. This novel result will aid in the discovery of new biostimulation agents.

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