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Stimulation of trichloroethene biodegradation in anaerobic three-phase microcosms

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ABSTRACT

Three-phase microcosm experiments were set up to investigate the enhancement of trichloroethene (TCE) biodegradation and to identify the most promising electron donor for *in situ* bioremediation. Acetate as carbon and energy source and hydrogen as electron donor were tested in microcosm experiments. Previous studies showed only partial dechlorination of TCE in a two-phase system due to the absence of adhesion surface and the difficulty of biofilm formation. Therefore soil was used to ensure adequate surface for the settlement of bacteria. The dynamics of biodegradation was monitored by using gas-chromatography. Microbial community structure and function were characterized by molecular biological methods (Terminal restriction fragment length polymorphism—T-RFLP, clone library), and through PCR-based group specific detection of key taxa as well as key metabolic genes both at DNA and RNA level. TCE was degraded to vinyl-chloride (VC) with added acetate, while in the case of the biotic control ethene production was detected by day 220. T-RFLP revealed that TCE enrichment resulted in different dechlorinating communities with the dominance of *Sulfurospirillum halorespirans* in the microcosms where VC was the end product, and with the dominance of *Dehalococcoides* sp. where the dechlorination ended in ethene. Using PCR-based techniques, key community members and dechlorinating bacteria were detected in the effectively dechlorinating microcosms. Scanning electron microscopy results provided evidences of biofilm formation on the surface of soil particles.

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1. Introduction

Chlorinated aliphatic compounds such as TCE are among the most abundant aquifer contaminants (Vogel, 1994). They have been widely used in industry, agriculture and households as degreasing agents, biocides and solvents (Doherty, 2000; Aulenta et al., 2005; Löffler and Edwards, 2006). Improper handling resulted in serious accumulation of halogenated compounds in anaerobic subsurface environments (Häggblom and Bossert, 2003; McCarty, 2010). Under anaerobic conditions, TCE can be degraded to non-toxic ethene through the reductive dechlorination of halorespiring microorganisms (Holliger et al., 1999). Several bacteria are capable of dechlorinating TCE to *cis*-DCE—e.g. *Desulfuromonas chloroethenica*, *Desulfitobacterium* sp., *Dehalobacter restrictus*, *Desulfomonile tiedjei* (DeWeerd et al., 1990; Krumholz 1997; Villemur et al., 2006; Holliger et al., 1998)—but remediation requires complete dechlorination to ethene (Löffler et al., 2000; Smidt and de Vos, 2004; Tas et al., 2009). *Dehalococcoides ethenogenes* strain 195 was the first isolate described to dechlorinate tetrachloroethene (PCE) to ethene (Maymo-Gatell et al., 1999). However, not all *Dehalococcoides* strains are able to achieve the complete dechlorination of PCE or TCE (Maymo-Gatell et al., 1997; Hendrickson et al., 2002; Duhamel et al., 2004; Krajmalnik-Brown et al., 2004; He et al., 2005; Adrian et al., 2007). The presence of *Dehalococcoides* (determined by 16S rRNA gene detection) in itself does not explain the complete dechlorination process in the investigated samples. One alternative is to complement *Dehalococcoides* specific detections with catabolic enzyme examinations concerning the dehalogenation of TCE or VC to ethene (Krajmalnik-Brown et al., 2004; Ritalahti et al., 2006; Daprato et al., 2007).

Dechlorination by *Dehalococcoides* has been observed in numerous laboratory experiments (Fennell et al., 2001; Révész et al., 2006; Nijenhuis et al., 2007), reactors (Chung et al., 2008) and enrichment cultures (Holmes et al., 2006; Lee et al., 2006, 2008; Cichocka et al., 2010). Several research papers have demonstrated that many different electron donors are able to sustain reductive dehalogenation of TCE to ethene; however, hydrogen is the intrinsic electron donor and complex electron donors have to be

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transformed to hydrogen first via fermentation pathways (Maymo-Gatell et al., 1995). Current biostimulation methods focus on stimulating H_2-consuming dechlorinators (He et al., 2002); however, these groups have to compete for H_2 with other microbes (e.g. methanogens, acetogens and sulphate-reducing bacteria).

The goal of this study was to stimulate complete degradation of TCE and to determine the most auspicious electron donor for application during *in situ* bioremediation. Three-phase microcosms were set up by addition of soil, substrates and trace elements, because previous investigations (Mészáros et al., 2011; Sipos et al., 2011) showed only partial dechlorination of TCE in a two-phase system, possibly due to the absence of adequate surface and biofilm formation capability. We applied a complex chemical and molecular biological approach, including mRNA analyses and catabolic gene tests, to assess the existing dechlorination potential, to follow the changes in the bacterial community with molecular fingerprinting techniques and to determine the presence and activity of halorespiring bacteria under different microcosm conditions.

2. Material and methods

2.1. Sample origin

Groundwater samples were taken from an industrial contaminated site, where *in situ* pilot tests had taken place previously. This site is located in an area where TCE was formerly stored in above ground tanks and where careless handling and leakage from storage tanks resulted in accumulation of halogenated compounds in the subsurface environment. *In situ* biostimulation involved the injection of an organic substrate (sour whey solution) into the wells. Groundwater samples were collected one year after *in situ* bioremediation was completed using a low-flow technique, in anaerobically sterilized 2 l bottles filled up without headspace under nitrogen atmosphere and closed by PTFE-lined silica septa. Groundwater samples were kept at 4 °C during transfer to the laboratory and were processed immediately upon arrival.

2.2. Laboratory microcosm preparation

On two occasions, 2 l water samples were concentrated for the microcosm experiments by filtering through a sterile membrane with pore size of 0.2 μm (Millipore, Billerica, MA, USA) under anaerobic conditions. The bottles containing groundwater samples were closed with HPLC screw caps. Gas-tight PTFE tubing was fixed into the holes of these caps. During filtration, the collected groundwater was purged with N_2 through one PTFE tube in order to produce overpressure in the bottle, and forced the groundwater through another tube into the vacuum filtration funnel unit which contained a sterile 0.2 μm pore size filter. The upper part of the vacuum filtration unit was kept under CO_2 overflow to keep out any O_2. Each membrane containing concentrated autochtonous bacteria (the cell suspension retained on sterile membrane) were stored in 60 ml sterile, anaerobic medium (Zinder, 1998) and the bottles were shaken overnight. 120 ml bottles were filled with 65.8 ml medium (Zinder, 1998) and 5 g 0.3 mm sifted soil, sealed and crimped with Teflon-coated butyl rubber septa (Wheaton Science Products, Millville, NJ, USA). The vials were autoclaved for 50 min (121 °C, 1 atm), then the headspace was changed to nitrogen gas. Each microcosm was amended with $NaHCO_3$ (1 g l^{-1}), Na_2S (25 mg l^{-1}), and vitamins (Zinder, 1998). Acetate (3 mM) served as carbon source and hydrogen (overpressure) served as electron donor. Biotic control was amended with no carbon source and electron donor. TCE (417 μmol pure solvent) was added as electron acceptor. Except for the abiotic culture, all microcosms were randomly inoculated with 8.5 ml anaerobic medium containing the filtrate derived from the groundwater sample (concentrated groundwater sample) following the protocol of Fennell and Gossett (2003). All types of microcosm were prepared in three parallels. Cultures were incubated upside down in the dark at 12 °C without shaking. Abiotic control was autoclaved on three consecutive days. Samples from all parallel microcoms were retrieved at day 6, 20, 50, 58, 62, 87, 91, 115, 122, 143, 177, 203 and 220 for chemical analysis and at day 0 (initial inoculant) and 220 for biological analysis. All chemicals were purchased from Sigma–Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany) at the highest purity available.

2.3. Analytical methods

The dynamics of biodegradation was monitored by using gas chromatography (gas chromatograph: YL6100 GC, YL Instrument Co., Ltd., Hogye-dong Anyang, South Korea) using flame ionization detection (FID) equipped with a 15 m × 0.530 mm HP-PLOT Q column (Agilent Technologies Inc., Santa Clara, CA, USA). The carrier gas was 5.0 helium (Linde, Munich, Germany). The injector was operated in split mode at a ratio of 1:20, constant carrier flow was 3.8 ml min^{-1}. The temperature program used was as follows: 2 min at 60 °C, 25 °C min^{-1} to 250 °C, hold 0.4 min. The FID was operated at 250 °C. Air and hydrogen flow were set at 300 ml min^{-1} and 30 ml min^{-1}, respectively. This method was able to separate methane, ethene, ethane, vinyl chloride, *cis*-DCE and TCE. Authentic standards were prepared for identification by adding known volumes of chemicals (Sigma–Aldrich and Supelco, Bellefonte, USA). The calibration standards were prepared with the same headspace, liquid and soil ratio as the microcosm bottles and were sampled and stored in exactly the same way. The injected volume was 100 μl of the headspace during manual injection using a sterile, gas-tight Hamilton syringe. Before sampling, microcosms were incubated for 12 h at ambient laboratory temperature (c.a. 25 °C) without disturbance until the equilibrium of the volatile compounds developed among the three phases. The data were recorded using the YL-Clarity Chromatography Data System (YL Instrument Co., Ltd.) software.

2.4. Nucleic acid extractions

The bottles were vigorously shaken to obtain a “uniform composite” sample, and then 1.5 ml samples were removed from each microcosm for the extraction of community DNA. The cells were collected by centrifugation at 14,000 *g* for 30 min at 4 °C. DNA was isolated from the pellet using the MoBio Soil Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer’s instructions in the alternative lysis protocol. DNA was eluted in 45 μl RNase-free distilled water and stored at −20 °C until further processing.

Total RNA was extracted from 1.5 ml microcosm sample after centrifuging at 14,000 *g* for 30 min at 4 °C, following an alternative protocol. The pellet was dissolved in 1.5 ml Trizol (Gibco BRL/Life Technologies, Breda, the Netherlands) and transferred to a 2 ml bead beating tube containing Lysing Matrix B (MP Biomedicals, Illkirch Cedex, France), chilled on ice and lysed using mixer mill MM301 (Retsch, Haan, Germany) for 30 s at 11 rev s^{-1} frequency. The samples were incubated at room temperature for 20 min, then centrifuged at 12,000 *g* for 14 min at 4 °C. The supernatant was transferred to a new tube and 300 μl chloroform was added. The tubes were incubated at room temperature for 15 min, then centrifuged again at 12,000 *g* for 14 min at 4 °C. The upper aqueous phase was transferred to a new tube and 600 μl ethanol 96% was added. The mixture was transferred onto an RNeasy column and from this step on we followed the RNA cleaning protocol of the

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