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The impact of bioaugmentation on dechlorination kinetics and on microbial dechlorinating communities in subsurface clay till

Jacob Bælum [a,b,c], Charlotte Scheutz [d], Julie C. Chambon [d,1], Christine Mosegaard Jensen [e], Rikke P. Brochmann [a], Philip Dennis [f], Troels Laier [a], Mette M. Broholm [d], Poul L. Bjerg [d], Philip J. Binning [d], Carsten S. Jacobsen [a,g,h,*]

[a] The Geological Survey of Denmark and Greenland (GEUS), Øster Voldgade 10, DK-1350 Copenhagen, Denmark
[b] Technical University of Denmark (DTU), Novo Nordisk Foundation Center for Biosustainability, Fremtidsvej 3, DK-2970 Hørsholm, Denmark
[c] The Technical University of Denmark (DTU), Center for Biological Sequence Analysis, Kemitorvet – Building 208, DK-2800 Kgs. Lyngby, Denmark
[d] The Technical University of Denmark (DTU), Department of Environmental Engineering, Miljøvej – Building 113, DK-2800 Kgs. Lyngby, Denmark
[e] Capital Region of Denmark, Kongens Vænge 2, DK-3400 Hillerød, Denmark
[f] SiREM, 130 Research Lane Suite 2, Guelph, ON N1G 5G3, Canada
[g] University of Copenhagen, Thorvaldsensvej 40, DK-1958 Frederiksberg C, Denmark
[h] Center for Permafrost, Department of Geosciences and Natural Resource Management, University of Copenhagen, Øster Voldgade 10, DK-1350 Copenhagen, Denmark

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ABSTRACT

A molecular study on how the abundance of the dechlorinating culture KB-1 affects dechlorination rates in clay till is presented. DNA extracts showed changes in abundance of specific dechlorinators as well as their functional genes. Independently of the KB-1 added, the microbial dechlorinator abundance increased to the same level in all treatments. In the non-bioaugmented microcosms the reductive dehalogenase gene *bvcA* increased in abundance, but when KB-1 was added the related *vcrA* gene increased while *bvcA* genes did not increase. Modeling showed higher vinyl-chloride dechlorination rates and shorter time for complete dechlorination to ethene with higher initial concentration of KB-1 culture, while *cis*-dichloroethene dechlorination rates were not affected by KB-1 concentrations. This study provides high resolution abundance profiles of *Dehalococcoides* spp. (DHC) and functional genes, highlights the ecological behavior of KB-1 in clay till, and reinforces the importance of using multiple functional genes as biomarkers for reductive dechlorination.

1. Introduction

The extensive use of tetrachloroethene (PCE) and trichloroethene (TCE) in industrial activity has resulted in high concentrations of chlorinated ethenes in sediment and groundwater at numerous sites worldwide (Moran et al., 2007). Various chemical, physical and biological strategies have been applied to remediate chlorinated ethenes at contaminated sites. Biostimulation of indigenous bacterial communities with organic and inorganic substrates is typically applied to overcome substrate and nutrient limiting conditions (Lendvay et al., 2003), while bioaugmentation with cultures having the potential to perform reductive dechlorination has been used in cases where indigenous microbial communities lack the required dechlorination activity (Ellis et al., 2000, Major et al., 2002, Scheutz et al., 2008). Though other bacterial species, such as *Geobacter* spp. and *Desulfitobacterium* spp. (Gerritse et al., 1995; Duhamel and Edwards, 2006) mediate incomplete TCE dechlorination, *Dehalococcoides* spp. (DHC) are the only microorganisms currently known to perform the entire dechlorination process from TCE to ethene (Maymo-Gatell et al., 1997). KB-1 is a bacterial consortium shown to possess the potential for complete respiratory dechlorination of PCE and TCE to ethene (Duhamel et al., 2002). KB-1 contains a number of different bacterial species including DHC, *Geobacter* spp., and *Sulfurospirillum* spp. (Duhamel et al., 2002; Duhamel and Edwards, 2006), and has been used successfully for in situ bioremediation at numerous sites (Major et al., 2002, Scheutz et al., 2008). While the role of KB-1 in reductive dechlorination is well characterized, its ability to survive and

* Corresponding author.
E-mail address: csj@geus.dk (C.S. Jacobsen).
[1] Now at Geosyntec Consultants, 1111 Broadway, 6th Floor, Oakland, CA 94607, USA.

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perform reductive dechlorination when added in low concentrations to sediment-containing environments containing a well-established microbial community is not well investigated. A better understanding of the behavior of KB-1 in the environment would improve our ability to manage in situ bioaugmentation projects more efficiently.

Even though numerous reductive dehalogenases (RDase) have been shown to be involved in the different dechlorination steps of PCE (Holscher et al., 2004), only a few have been investigated in detail. The most intensively studied are the PceA, TceA, BvcA, and the VcrA RDases, for which the genes have also been described and PCR primers developed (Krajmalnik-Brown et al., 2004, Muller et al., 2004, Johnson et al., 2005, Holmes et al., 2006). The RDase genes along with DHC 16S rRNA genes have been used as biomarkers for the determination of the dechlorination potential both in laboratory and in field scale studies (Johnson et al., 2005, Scheutz et al., 2008, Scheutz et al., 2010). Different strains of DHC have been shown to carry varying compositions of RDase genes (McMurdie et al., 2009) and both the *vcrA* gene and the *bvcA* gene can be found within the confirmed strains of DHC present in KB-1. The *bvcA* gene is less abundant, being present in only 5–20% of the KB-1 DHC cells, while the *tceA* gene is not present at high concentrations in the culture (SiREM unpublished data).

The objective of this paper was to investigate the effect of inoculation concentrations of the DHC containing mixed culture KB-1 on the timeframe for TCE degradation, and survival and growth of DHC in a clay till media. Schaefer et al. (2009) have previously investigated the effect of different inoculation concentrations under less controlled field-scale conditions and found no relationship between reductive dechlorination rates and different amounts of bacteria inoculated. As environmental conditions are difficult to control in field-scale experiments, this finding required verification under controlled laboratory conditions. Even though most in situ field scale bioaugmentation experiments have been performed in sediment rich environments, very few controlled laboratory studies have been performed in presence of sediment. Due to its large and highly reactive surface, clay rich matrixes are known to have a large effect on chemical bioavailability and microbial behavior, and therefore this topic requires further attention. The presence of clay also complicates analysis, for example the extraction of DNA from clay rich matrixes has been shown to be especially difficult, mainly because after cell lysis the DNA is strongly sorbed to clay particles (Yankson and Steck, 2009; Paulin et al., 2013).

In this paper bioremediation in a natural clayey till groundwater ecosystem was simulated in microcosms. The effect of different TCE concentrations and different levels of addition of bioaugmentation culture (KB-1) was investigated allowing us to present dose–response relationships between the number of specific degraders and dechlorination rates for nine different treatments. A DNA extraction protocol, not sensitive to presence of clay, was optimized for regular quantification of DHC 16S rRNA genes and three functional genes (*vcrA*, *bvcA*, and *tceA*) throughout reductive dechlorination in the nine experiments. This allowed high-resolution identification of the proliferation of the main bacterial species and functional dehalogenase genes during sequential degradation of TCE to ethene.

2. Materials and methods

2.1. Cell enumeration

The KB-1 culture was used as received from SiREM (Guelph, ON, Canada). The cell density was estimated by staining an aliquot of the culture with the selective fluorescent cationic dye acridine orange and "quantification under an" Olympus microscope (Center Valley, PA, USA). Using direct counts the total microbial density was estimated to be 4×10^8 cells mL^{-1} and included both *Dehalococcoides* sp. (DHC) and other microorganisms. Furthermore, the culture contained 1.1×10^8 DHC 16S rRNA gene copies mL^{-1} (based on real time PCR quantification – see qPCR section) meaning that approximately 25% of the cells were DHC.

2.2. Microcosm setup

The clay till for the microcosms was obtained from a TCE-contaminated site located at Rugårdsvej, Odense, Denmark, where concentrations of 25–100 μmol kg^{-1} (dw) *cis*-DCE and 50–200 μmol kg^{-1} (dw) VC were found (Scheutz et al., 2008, Scheutz et al., 2010). The till sample was taken from a core 8 m below surface (mbs) in the saturated zone and was composed of 44% sand, 20% silt, and 36% clay. The site is a former manufacturing facility contaminated 40–50 years ago. The clay till was homogenized and residues of chlorinated ethenes were allowed to volatilize overnight in a fume hood. No precautions were taken to prevent oxygenation of the clay till. Microcosm experiments were carried out in 320 mL serum bottles sealed with butyl rubber stoppers. Each bottle contained 100 g (dw) of clay till and 200 mL of sterile tap water and was flushed with nitrogen for 30 min in order to remove O_2. Thereafter, lactate (60%, VWR International, Radnor, PA, USA) was added to obtain a concentration of 6 mM in all bottles. To ensure O_2-residue removal, the bottles were incubated overnight to allow microbial respiration. The following day, the microcosms were spiked with KB-1 culture yielding concentrations of 0, 10^5, and 10^6 KB-1 cells mL^{-1} (based on total number of bacteria estimated by microscopic quantification) and TCE (Merck, Darmstadt, Germany) yielding concentrations of 0, 7.6 and 76 μM (Table 1), corresponding to 0, 1, and 10 mg L^{-1} (Table 1). Each bottle was equipped with a 1.8 mm needle (Acufirm®, Dreieich, Germany) and a multidirectional stopcock (Discofix, B. Braun Melsungen AG, Switzerland) to ensure easy access for sampling of water and solids. After 110 days of incubation a second addition of 12 mM of lactate was performed. Triplicate microcosms were set up for each combination of KB-1 and TCE. The microcosms were incubated in the dark at 10 °C for 692 days.

2.3. Chemical analysis

Chlorinated ethenes and ethene were quantified using GC/MS (Agilent Technologies, Santa Clara, CA, USA). Aqueous phase samples (1 mL) were placed in sealed 20 mL vials containing chloroform as an internal standard. Samples were introduced into the gas chromatograph after pre-heating to 80 °C. Separation was performed on a HP plot Q 30 m × 0.32 mm × 30 μm column (Agilent Technologies, Santa Clara, CA, USA) with helium as carrier gas. Detection limits were as follows: TCE 0.5 μg L^{-1}, *cis*-DCE 0.3 μg L^{-1}, 1,1-DCE 0.3 μg L^{-1}, *trans*-DCE 0.4 μg L^{-1}, VC 1.0 μg L^{-1}, ethene 0.9 μg L^{-1}, ethane 2.6 μg L^{-1}. For details see Scheutz et al. (2010). Aqueous concentrations were converted to concentrations per microcosm using Henry's law constants at 10 °C (Heron et al., 1998) and sorption constants from Lu et al. (2011).

Methane was measured throughout the experiment using GC-FID (Shimadzu GC-9A, Kyoto, Japan) at 100 °C with a silica gel 70/80 mesh column of 4 m and helium as carrier gas. 0.2 mL headspace was sampled from the bottles and injected into the gas chromatograph with injection intervals of three minutes. Detection limit for methane was 0.5 ppm.

For the analysis of anions (chloride, bromide, nitrate, and sulfate), a 1 mL aqueous phase sample was taken and filtered through 0.45 μm Acrodisc® nylon filters (Pall Life Sciences, Ann Arbor, MI, USA) and analyzed by ion chromatography (Dionex, Sunnyvale, CA, USA) using 3.5 mM Na_2CO_3/1 mM $NaCO_3$ as eluent. The detection limit of all compounds was between 0.02 and 1.32 ppm.

1 mL aqueous phase sample was sampled for analysis of volatile fatty acids (formate, acetate, propionate, and lactate), filtered through 0.45 μm Acrodisc® nylon filters, and acidified with 50 μl 20% H_2SO_4. Samples were analyzed by suppressed ion exclusion chromatography (Dionex, Sunnyvale, CA, USA) on a Suppressor (Dionex AMMS-IEC2) and ion exclusion column (ICE-AS1 9 × 250 mm). 4 mM heptafluorobutyric acid was used as eluent and 10 mM tetrabutylammonium hydroxide was used as chemical suppressor. Detection limits were between 0.1 and 10 mM.

2.4. DNA extraction

To ensure homogenous slurry samples, the microcosms were shaken vigorously prior to sampling of 1 mL clay till and water for DNA extraction. In order to prevent sorption of DNA to the highly phosphate adsorbing clay till during the extraction procedure, 500 μl G2 blocking solution (GEUS, Copenhagen, Denmark) was added followed by a 5 s vortex. To reduce the water content, the samples were freeze-dried

Table 1
Microcosm setup and abbreviations.

Conc. of TCE (μM)	Conc. of KB-1 (cells/mL^{-1})		
	0	10^5	10^6
0	MC1	MC4	MC7
7.6	MC2	MC5	MC8
76	MC3	MC6	MC9

MC: Microcosm.