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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.

for 24 h. Samples were stored at −80 °C until DNA extraction (<2 months). DNA extractions were carried out using the UltraClean soil DNA isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. DNA extracts were stored at −20 °C until analysis by real-time qPCR (<1 month).

2.5. Quantitative real time PCR

The DNA extracts were used as a template in the qPCR targeting DHC 16S rRNA genes and the functional rdh (*tceA*, *vcrA* and *bvcA*) genes. Quantitative real time PCR was performed in an I-cycler (Bio-Rad, Hercules, CA, USA). PCR reagents used were 7.5 μl Maxima™ SYBR Green master mix (Fermentas, St. Leon-Rot, Germany), 0.4 mM of the primers (Table 2) (MWG, Ebersberg, Germany), 1 μg bovine serum albumin (New England Biolabs, Ipswich, MA, USA), 1 μl extracted DNA and PCR grade water to a final volume of 15 μl. For each DNA sample, three replicate real-time PCR were performed. For the standard curve (see below) three replicates of four 10-fold diluted concentrations were run for each PCR plate. The gene copy numbers of the standards were chosen to cover the range of the samples analyzed. The PCR efficiency of the real-time PCR measured as recommended by Bio-Rad and was in the range 94.4–101.1% for all plates.

The qPCR protocol for DHC 16S rRNA genes was as follows: 95 °C for 10 min (enzyme activation), 50 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 45 s (extension), and 82 °C for 10 s (quantification), after the 50 cycles a final 72 °C extension step for 6 min was performed. Amplicon specificity was confirmed by a melting curve analysis consisting of 80 cycles of a 0.5 °C increase starting at 55 °C. For the three functional genes *tceA*, *vcrA*, and *bvcA* the same protocol was used except the annealing temperature was 58 °C and quantification was performed at 72 °C.

Standards for real time PCR quantification were prepared by insertion of the PCR products of the respective genes into pCR 2.1-TOPO cloning vectors which were then propagated in competent *E. coli* cells (Invitrogen, Carlsbad, CA, USA). PCR products were obtained using the primers given in Table 2 on DNA extracted from the KB-1 culture. After propagation of transformed *E. coli*, the plasmids were extracted using an UltraClean 6 min mini plasmid prep kit (MOBIO, Carlsbad, CA, USA) and the plasmid concentrations were measured using a Nanodrop spectrophotometer (Wilmington, DE, USA) and plasmid copy numbers were calculated as described elsewhere (Park and Crowley, 2005). The quantification limit for the DHC 16S rRNA genes, *vcrA*, and *bvcA* genes were ~10^3 genes per mL sample and ~10^4 genes per mL sample for the general 16S rRNA genes.

2.6. Denaturing gradient gel electrophoresis (DGGE)

PCR for DGGE was performed in 50 μl reactions with 0.4 μM of each of the 341–518 16S rRNA gene primers for DGGE (Table 2) and with a reaction mixture consisting of 1.25 u DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany), 0.8 mM dNTP mix, 2.5 μg bovine serum albumin (New England Biolabs, Ipswich, MA, USA), and 5 μl extracted DNA. The thermal cycler protocol was as follows: 95 °C for 3 min (enzyme activation), 35 cycles of 95 °C for 60 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 60 s (extension), after the 35 cycles a final 72 °C extension step for 20 min was performed. The DGGE gels contained 8% acrylamide and a urea-formamide gradient of 35–70%. The 100% denaturant contained 7 M urea and 40% formamide.

Bands of interest were excised using a pipette tip and incubated in PCR grade water allowing the DNA fragments to diffuse out. A second PCR was then performed on the samples as previously described, but using the primers 341-forward and 518-reverse without employing a GC-clamp. The PCR products were then purified using the UltraClean PCR Clean-up DNA Purification Kit as described by the manufacturer (MO BIO, Carlsbad, CA, USA). The purified PCR products were sequenced (Eurofins MWG Operon, Ebersberg, Germany) and the sequences matched to known sequences using the NCBI (National Center for Biotechnology Information, Bethesda, Maryland, USA) nucleotide BLAST (basic local alignment search tool) (Altschul et al., 1990). All sequence data is provided in Supplemental material.

2.7. Kinetic modeling

Dechlorination kinetics was modeled using a Monod kinetic based model, where two dechlorinating biomass populations are considered, one responsible for TCE to cis-DCE degradation and the other (*Dehalococcoides*) responsible for cis-DCE to ethene dechlorination (Haest et al., 2010). This means that *Dehalococcoides* are assumed to grow on cis-DCE and VC dechlorination only, while TCE degradation is performed by other bacteria, mainly *Geobacter* in the KB-1 culture (Haest et al., 2010). Equilibrium between gas, liquid and sorbed phases was assumed, and degradation was assumed to take place only in the aqueous phase (Yu and Semprini, 2004; Schaefer et al., 2009):

$$\frac{dC_i}{dt} = (-r_i + r_{i+1})\frac{1}{1+\frac{\rho}{\theta}K_{d,i}+\frac{\theta_a}{\theta_w}K_{h,i}} \quad (1)$$

$$r_i = \frac{k_{m,i}X_jC_i}{K_{s,i}(1+C_{i+1}/K_{inh,i+1}+C_{i+2}/K_{inh,i+2})+C_i} \quad (2)$$

$$\frac{dX_j}{dt} = \sum_i (Y_{j,i}r_i) - b_jX_j \quad (3)$$

Where C_i is the aqueous concentration of the compound i (μmol L^{-1}). Compounds indexed by $i+1$ and $i+2$ are the parent compounds of species i and are included in Eq. (2) to account for competitive inhibition with competitive inhibition constants $K_{inh,i+1}$ and $K_{inh,i+2}$. (μmol L^{-1}). X_j is the concentration of microbial dechlorinators j (j depends on the reaction and denotes either *Geobacter* or *Dehalococcoides*) (cell L^{-1}), $k_{m,i}$ is the maximum utilization rate of compound i (μmol $cell^{-1}$ d^{-1}), $K_{S,i}$ is the half-saturation constant of compound i (μmol L^{-1}), $Y_{j,i}$ is the specific yield of microbial dechlorinators j on substrate i (cell μmol^{-1}), b_j is the decay rate of microbial dechlorinators j (d^{-1}), $K_{d,i}$ is the distribution coefficient of compound i (L kg^{-1}), $K_{h,i}$ is the Henry's constant of compound i (−), ρ is the bulk density in the bottle (kg L^{-1}), θ_w is the water content in the bottle (−) and θ_a is the air content in the bottle. The physical parameters (ρ, θ_w and θ_a) are updated during the simulation, as they change at each sampling time (water and clay till are removed from the bottles). The parameters for each microcosm are estimated using the Shuffled Complex Evolution Metropolis algorithm (Vrugt et al., 2003).

Table 2
Primers used for PCR.

Oligo name	Target gene	Oligo (5′–3′)[a]	Fragment size	Annealing temp.	Ref.
Primers for real time PCR					
Dhc 16S	Dhc 16S	F: GGGAGTATCGACCCTCTC R: GGATTAGCTCCAGTTCACACT	438	55	Hendrickson et al., 2002
tceA	tceA	F: ATCCAGATTATGACCCTGGTGAA R: GCGGCATATATTAGGGCATCTT	66	58	Holmes et al., 2006
vcrA	vcrA	F: CTCGGCTACCGAACGGATT R: GGGCAGGAGGATTGACACAT	64	58	Holmes et al., 2006
bvcA	bvcA	F: GGTGCCGCGACTTCAGTT R: TCGGCACTAGCAGCAGAAATT	67	58	Holmes et al., 2006
Primers for DGGE					
341f clamp-518r	Universal 16S	F:GCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGGCCTACGGGAGGCAGC R: ATTACCGCGGCTGCTGG	231	55	Muyzer et al., 1993
Amplification of cloning products					
27F-1492R	Universal 16S	27F: GAGTTTGATCMTGGCTCAG[b] 1492R: GGYTACCTTGTTACGACTT[b]	1501	55	Lane, 1991
tceA-CDS	tceA	F: CATTCTACAGTCACAAGGCG R: TCTACGCTTGCGCCGCGTAT	1209	58	This study
vcrA-CDS	vcrA	F: TGAATGGCTCGAGGGAAAGA R: CAACGGTACTACAGCTAACA	1886	56	This study
bvcA-CDS	bvcA	F: ATGCATAATTTCCATTGTAC R: TTACCAAGGCTTGTGTGATT	1551	51	This study

[a] F, Forward primer, R, Reverse primer.
[b] Y, T/C; M, A/C.

3. Results

3.1. DNA extraction optimization

Using a variety of different DNA extraction protocols (MoBio PowerSoil and UltraClean kits, the Bio101 FastDNA SPIN kit for soil, and a previously published method (Nicolaisen et al., 2008)) we were not able to obtain detectable (using qPCR) amounts of DNA. Therefore, the addition of G2 blocking solution prior to the extraction protocol was tested (Table 3). The addition of 500 μl G2 blocking solution increased the recovery of DHC 16S rRNA genes by 3000-fold, reaching similar values as extraction without the clay till (Table 3).

3.2. TCE dechlorinating microcosms

Dechlorination of TCE through cis-DCE and VC to ethene was observed in all microcosms where TCE was added (Fig 1). The most rapid dechlorination occurred within 210 days in MC8 (1 mg L^{-1} TCE, 10^6 cells of KB-1). In MC3, MC5, MC6, and MC9 dechlorination was slower with complete dechlorination occurring in 576, 371, 576, and 330 days, respectively. TCE was dechlorinated to *cis*-DCE rapidly in all of the microcosms, however, it occurred faster in microcosms with higher concentrations of KB-1 (i.e., MC8 and MC9 were faster than MC5 and MC6, which were faster than MC2 and MC3). Initially (within the first two weeks) a notable decrease in TCE was observed with no resulting production of *cis*-DCE, suggesting sorption of TCE and *cis*-DCE to the clay particles. Accumulation of *cis*-DCE in the microcosms persisted for varying periods of time, the shortest observed in bottles with the highest initial KB-1 addition. The VC to ethene step was rapid and occurred concurrently with the *cis*-DCE to VC step (Fig. 1). In the microcosms with no addition of TCE, low concentrations of chlorinated compounds (~0.2 μmol $bottle^{-1}$ in total) were detected, most likely due to desorption of residues from the clay till. In MC4 and MC7 with KB-1 culture and no TCE addition, these residues were dechlorinated.

The kinetics of fermentation of lactate was similar in all microcosms and occurred within 50 days after addition (Fig. S1). In general fermentation of the products acetate and propionate was slower than lactate and in the non-KB-1 amended microcosms where propionate was detected throughout the experiment. In contrast, propionate and acetate were depleted after 300 days of incubation in the KB-1 amended microcosms.

The patterns of the redox conditions were similar in all the microcosms (Fig. S2), with the exception that the sulfate concentration decreased more rapidly in microcosms with the highest amount of KB-1 added (MC7, MC8, and MC9). The general trend was sulfate decreased throughout the initial 151 days, nitrate was never detected, and methane was detectable after 220 days and during the remaining incubation period.

3.3. Quantitative PCR

The initial abundance of general 16S rRNA genes in microcosms with no KB-1 added was $\sim 2 \times 10^5$ genes mL^{-1}, while after addition of the 10^5 and 10^6 KB-1 cells mL^{-1} it was equivalently higher. Throughout the experiment, the general 16S rRNA gene abundance increased to $\sim 10^8$ genes mL^{-1} in all microcosms (Fig. 2).

A background of DHC specific 16S rRNA genes and *bvcA* and *vcrA* genes was detected originating from the clay till microbial community, while the *tceA* gene was not detected in the KB-1 culture, nor within the clay till microbial community. Despite the absence of added KB-1 in MC1, MC2 and MC3, increasing abundance of DHC and RDase genes was observed (originating from indigenous DHC in the clay till microbial community). In MC2 and MC3 increasing abundance of the three gene markers was detected, and the amount of DHC 16S rRNA genes and *bvcA* genes followed the trend of *cis*-DCE dechlorination, while the increase in *vcrA* genes was less pronounced. The abundance of DHC 16S rRNA genes and *vcrA* genes also increased slightly in MC4 and MC7 where no TCE was added. This could be attributed to residues of chlorinated solvents in the clay till, which may have been sufficient to support DHC growth. In the microcosms with added KB-1 (MC4, MC5, MC6, MC7, MC8, and MC9) a different pattern was observed. In these microcosms the abundance of *bvcA* genes never increased, but remained constant at $\sim 10^4$ genes mL^{-1} throughout the experiment. In contrast, the *vcrA* genes increased together with the abundance of DHC 16S rRNA genes in all of these microcosms, and substantial increases over 2–3 orders of magnitude were observed in MC4, MC5, and MC6, while in MC7, MC8 and MC9, with the highest initial KB-1 concentration, a less notable increase was observed.

Table 3
Recovery of *Dehalococcoides* spp. specific 16S rRNA genes from the Rugårdsvej sediment measured by qPCR as a function of G2 addition.

Sediment	KB-1 (μl)	G2 (μl)	Recovery (genes/assay)
−	200	−	$2.3 \times 10^7 \pm 1 \times 10^6$
+	200	−	$1.1 \times 10^4 \pm 7 \times 10^2$
+	200	10	$1.2 \times 10^4 \pm 5 \times 10^2$
+	200	100	$4.7 \times 10^4 \pm 7 \times 10^2$
+	200	500	$3.1 \times 10^7 \pm 9 \times 10^5$

3.4. Dechlorination kinetics

The model described by Eqs. (1)–(3) was used to simulate the chemical and microbial (DHC) data in the six experiments where TCE was added. The estimated kinetic parameters for the six experiments are summarized in Table 4, and the modeling results are shown in Fig. 3. Some of the parameters are not very well constrained (decay rate and cis-DCE inhibition constant), but in this paper we focus on the maximum utilization rates and specific yield, which are estimated with narrow confidence intervals (see Table 4). The maximum utilization rates vary between 1 and 10×10^{-10} and between 0.06 and 75×10^{-10} μmol × $cell^{-1} \times d^{-1}$, for cis-DCE and VC respectively. The estimated kinetic parameters can be used to compare the dechlorination efficiency in the different treatments. Increasing KB-1 concentration from 10^5 to 10^6 cell × mL^{-1} results in a decrease of the DHC growth per μmol of cis-DCE (specific yield in MC5 vs. MC8 and MC6 vs. MC9), and an increase of growth of DHC per μmol of VC. The same is observed for the maximum utilization rates estimated for cis-DCE and VC. This shows that increasing KB-1 concentration does not increase the cis-DCE dechlorination performance, while VC dechlorination rate is greatly improved. This is of importance as it can mean that VC accumulation can be limited in the field if the correct amount of KB-1 is used. Furthermore the performance of the indigenous community (MC2 and MC3) can be compared with the bioaugmented microcosms, by comparing the parameters estimated of the kinetic models. Both the indigenous *Dehalococcoides* (carrying the *bvcA* gene) and the *Dehalococcoides* added with KB-1 culture (carrying the gene *vcrA*) performed cis-DCE degradation, as shown by the similar specific yield and maximum utilization rates estimated for MC2 and MC5, and MC3 and MC6. In contrast, VC degradation is enhanced by the addition of KB-1, as reflected by the increased maximum utilization rate in MC5 compared to MC2 (two orders of magnitude), and in MC6 compared to MC3 (1 order of magnitude). The addition of KB-1, even in the presence of an indigenous dechlorinating population, can enhance VC degradation and thus reduce the risk of VC accumulation.

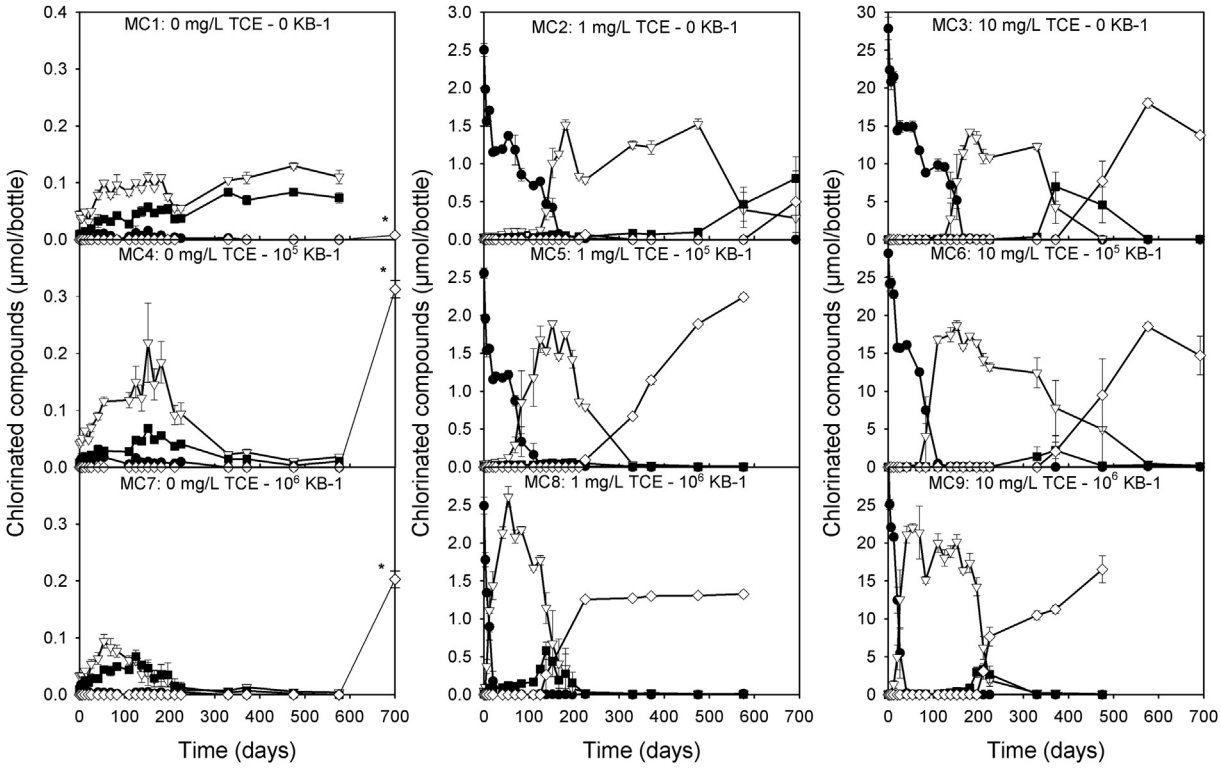

Fig. 1. Measured amount of chlorinated ethenes in the nine microcosms (MC), with error bars indicating the standard error between three replicate microcosms (● TCE, ▽ *cis*-DCE, ■VC, □ ethene). * These data points were measured with GC-FID after the experiment was ended and can only be regarded semi-quantitative.

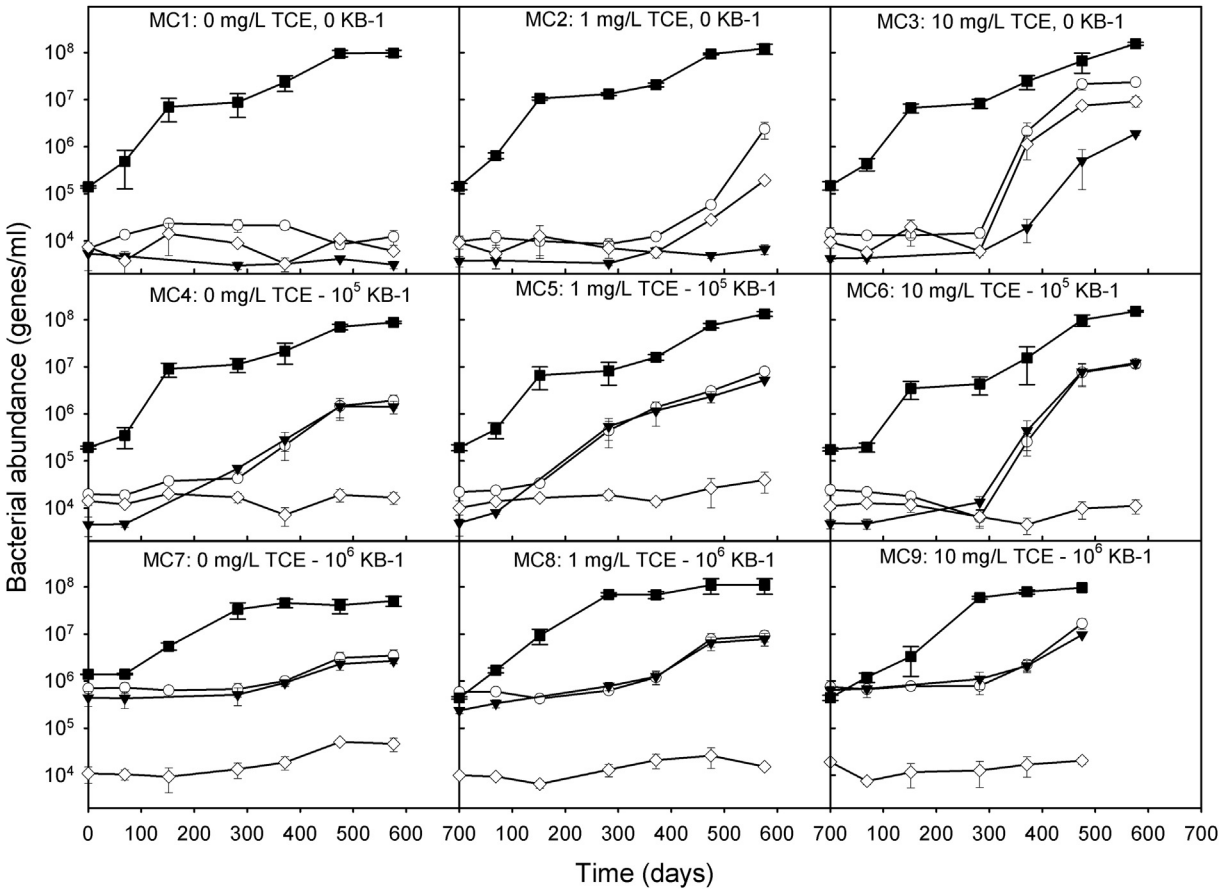

Fig. 2. Microbial abundance of specific *Dehalococcoides* spp 16S rRNA genes (○), *vcrA* (▼) and *bvcA* genes (□), and general 16S rRNA genes (■) in the nine microcosms (MC), with error bars indicating the standard error between three replicate microcosms.

Table 4
Kinetic parameters for Monod kinetics for the six microcosms. The numbers in brackets are the 95% confidence intervals.

Microcosm	KB-1	0		10^5		10^6		(Haest et al., 2010)[a]	(Schaefer et al., 2009)[b]
	TCE	1	10	1	10	1	10		
		MC2	MC3	MC5	MC6	MC8	MC9		
Max utilization rate 10^{-10} μmol cell^{-1} d^{-1}	$k_{m,TCE}$	6.99						1560	312
	$k_{m,DCE}$	2.40 (1.07–2.87)	9.36 (5.54–9.69)	1.92 (1.06–2.34)	9.91 (8.55–9.97)	0.83 (0.55–2.50)	2.77 (2.13–5.86)	208	125
	$k_{m,VC}$	0.06 (0.08–4.45)	0.51 (0.35–1.01)	5.94 (2.77–9.50)	7.95 (2.30–8.84)	73.83 (4.84–84.04)	20.91 (2.73–55.41)	5	336
Specific yield 10^7 cell μmol^{-1}	Y_{TCE}	9.93						0.09	0.44
	Y_{DCE}	8.61 (5.88–25.02)	3.84 (3.65–6.81)	17.07 (11.3–28.8)	4.88 (4.28–7.29)	0.73 (0.49–12.97)	0.12 (0.11–1.07)	1.56	
	Y_{VC}	8274 (121–6875)	75.18 (18.0–108.5)	1.15 (1.06–8.24)	1.05 (1.03–2.54)	20.18 (6.69–30.28)	3.37 (2.59–6.29)	20	
Half-saturation constant μmol L^{-1}	$K_{S,TCE}$	10						4.19	3.2
	$K_{S,DCE}$	4.61 (1.11–4.65)	1.25 (1.24–8.44)	4.71 (1.37–8.60)	147.5 (131–196)	2.86 (1.29–27.75)	12.82 (4.71–99.49)	99.7	2
	$K_{S,VC}$	32.02 (32.0–118.6)	1.01 (1.0–4.75)	1.13 (1.06–3.54)	1.32 (1.07–1.71)	68.81 (6.68–113.8)	58.28 (2.36–196.6)	2.6	14
Inhibition constant μmol L^{-1}	$K_{inh,TCE}$	100						370	
	$K_{inh,DCE}$	559 (167–943)	125 (37–200)	317 (113–796)	3.8 (2.5–51.4)	3.1 (1.3–112.6)	936 (74–951)	4.79	5.2
Decay rate d^{-1}	$k_{d,Geo}$	0						0.029	0
	$k_{d,Dhc}$	0.0064 (0.004–0.012)	0.0189 (0.009–0.018)	0.0010 (0.001–0.002)	0.0010 (0.001–0.002)	0.0010 (0.001–0.002)	0.0016 (0.001–0.005)	0.05	

[a] KB-1 culture, initial *Dhc* 10^4 cell mL^{-1}.
[b] SD-9 culture, initial *Dhc* 10^6 cell mL^{-1}, assuming a unique "aggregate" dechlorinating biomass.

3.5. DGGE-profiles

In the original clay till material only 4-5 distinct bands were visible with an *Arthrobacter* sp. and a *Sulfuricella* sp. being the two most abundant (Fig. 4 and Table 5). In MC7, MC8, and MC9 with the highest concentration of KB-1, bands corresponding to DHC from the KB-1 culture were also visible at day 0 (Fig. 4). As the incubation period proceeded, more bands became visible, suggesting that initially rare species proliferated and became more dominant in the microbial community. The bands representing the most abundant bacteria were relatively persistent in all microcosms, and only the band representing DHC (# 15, 18, 26, 30, and 39) could be directly related to the dechlorination process. Interestingly, the only species originating from the KB-1 culture that became substantially abundant in both the microcosms with low and high initial concentrations of KB-1 were the DHC, while band 33 seemed to increase in intensity only in the microcosms with the high initial KB-1 concentration. As band 33 did also increase in abundance in MC7 without addition of TCE this increase cannot be directly related to TCE dechlorination. By comparison to the pattern of the KB-1 culture and to bands appearing in the control microcosm (MC1), other bands that consistently increased in intensity throughout the incubation period did not seem to originate from the KB-1 culture, but rather from the clay till material. DNA sequencing of bands that proliferated during the incubation period primarily revealed high abundance of anaerobic, sulfur-reducing, and iron-reducing bacteria, some of which have previously been shown to be involved in reductive dechlorination processes (Table 5).

4. Discussion

In this study we investigated how the concentration of the dechlorinating microbial KB-1 culture affects the rate of reductive dechlorination of TCE and the potential of its microbes to become dominant within a general microbial community. An in-depth quantitative approach was applied to study the abundance of specific degraders and their functional genes throughout the different phases in the dechlorination process. Our study was performed in microcosms containing a water/clay till mixture in order to investigate how clay till particles and the abundance of microbial communities foreign to the KB-1 culture affect degradation rates.

4.1. DNA extraction optimization

Molecular microbial studies in clay materials are rare, due in part to challenges associated with difficulties in extracting DNA from clay rich material (Novinscak and Filion, 2011). It is well known that DNA tends to sorb to clay especially in low biomass sediments, and this has a negative influence on extraction efficiency (Barton et al., 2006; Yankson and Steck, 2009). This was confirmed in this study. Only through the addition of the G2 blocking solution were we the expected quantities of DNA from DHC recoverable. Without addition of G2 we were unable to detect DHC or any of the functional genes and the G2 solution. With the addition of the G2 solution, increases in DNA recovery of over several orders of magnitude were observed. As TCE contaminated sites are often located in low biomass sediment, this approach could improve field monitoring accuracy of DHC abundance and thereby our understanding of sediment groundwater partitioning and total DHC mass at contaminated sites.

4.2. TCE dechlorination

It widely reported that the addition of KB-1 culture has a positive effect on dechlorination of chlorinated ethenes both in

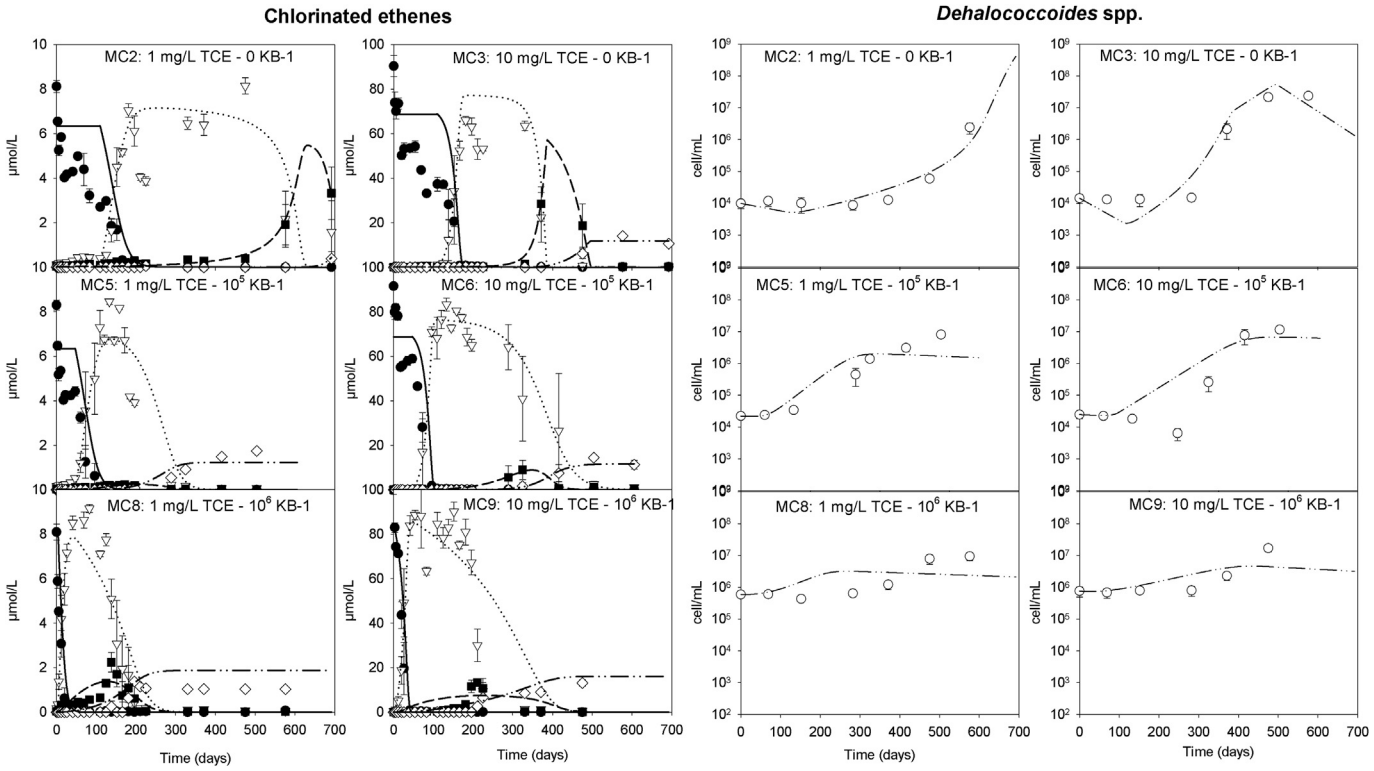

Fig. 3. Modeling results (lines) and observation (symbols) for the six microcosms with TCE addition. Left: Aqueous concentration of chlorinated ethenes (● TCE, ▽ *cis*-DCE, ■VC, □ ethene). Right: Concentration of *Dehalococcoides* spp.

laboratory experiments (Friis et al., 2007a) and at field sites (Major et al., 2002, Scheutz et al., 2008, Scheutz et al., 2010). Our results support this knowledge, and further clarify the benefit of adding increased volumes of KB-1 culture. This contrasts with the previously reported results of Schaefer et al. (2009) who observed no effect of increasing DHC concentration, above a certain threshold, in a field scale experiment. This might either be due to the uncontrolled conditions present at field scale experiments or because KB-1 has a tendency to proliferate differently under in-situ conditions in the field. Laboratory studies performed with KB-1 have in many cases been carried out in the absence of sediment (Duhamel and Edwards, 2007; Friis et al., 2007a, Friis et al., 2007b; Heimann et al., 2007), only a few studies (Friis et al., 2006, Aulenta et al., 2007a, Friis et al., 2007b, Schaefer et al., 2009) have included sediments. The presence of sediment could influence the dechlorination process dramatically not only by surface interactions but also through the introduction of a well established microbial community that could compete with the KB-1 microbes for nutrients, carbon sources, etc. In this study the only organism originating from the KB-1 culture that sufficiently increased in abundance to become visible on a DGGE gel was DHC.

Compared to other microcosm studies with KB-1, the dechlorination process reported herein is relatively slow. This is probably due to the presence of clay till, a foreign microbial community, a

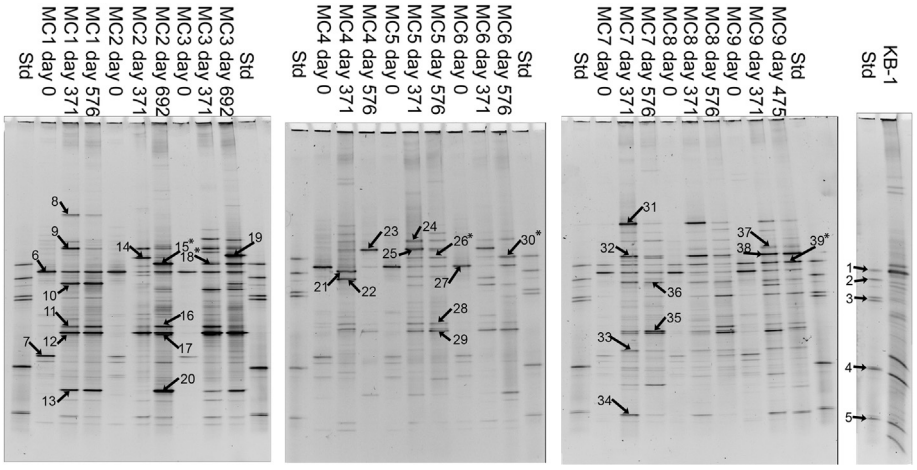

Fig. 4. Bacterial community structure using PCR-DGGE analysis of the amplified 16S rRNA genes. MC1–MC9 refers to the different microcosms (see Table 1). Std are the PCR products of 5 reference strains. Numbers 1–39 are picked bands that have been successfully re-amplified and sequenced (see Table 4 for BLAST match).

Table 5
Best BLAST hits for the 16S rRNA sequences amplified from the stapped bands from the DGGE gel (see Fig. 3).

Band#	Closest GenBank match (Genbank accession number)	Identity (%)
1	*Dehalococcoides* sp. BAV1 (AY165308)	99
2	*Dehalobacter* sp. (JQ918083)	99
3	*Geobacter lovleyi* SZ (NR074979)	99
4	*Spirochaetes* sp. (HM532366)	99
5	Uncultured *Bacteroidetes* (FJ718689)	98
6, 27	Uncultured *Sulfuricella* (KC171228)	97
7, 33	*Arthrobacter* sp. (KF150451)	99
8, 31	Uncultured *Desulfobulbus* (HE974837)	100
9, 24, 37	*Desulfosporosinus* sp. BSREI1 (JF810424)	99
10, 22, 36	*Desulfobulbus rhabdoformis* (AB546248)	96
11, 16, 28	*Clostridium* sp. (EF059534)	98
12, 17, 29, 35	*Rhodoferax* sp. (JQ977500)	99
13, 20	*Actinobacterium* CH9 (FN554394).	96
14, 19, 23, 25, 32, 38	*Sulfurospirillum deleyianum* DSM 6946 (CP001816)	100
15, 18, 26, 30, 39	*Dehalococcoides* sp. GT (NR102515)	99
21	*Psychrosinus fermentans* (DQ767882)	97
34	*Segniliparus rotundus* DSM 44985 (NR074426)	99

lower initial KB-1 concentration and a realistic incubation temperature of 10 °C. While dechlorination was slow, we observed a rapid consumption of lactate. Clay till contained sulfate, iron, and a well-established microbial community, and on-going microbial sulfate and iron reduction processes require lactate or H_2 to occur and so these processes compete with reductive dechlorination. The importance of the clay till matrix for the availability of hydrogen, carbon, essential nutrients and electron donors in other comparable studies have shown that only a minor fraction of the added donor and available nutrients are used for dechlorination (Aulenta et al., 2007, Azizian et al., 2010, Malaguerra et al., 2011). Quantification of general 16S rRNA genes in our study supports this hypothesis, as a substantial increase in numbers of general bacteria occurred independently of the dechlorination process.

An interesting finding of this study is the observed ability of DHC to grow even at very low concentrations of chlorinated compounds (~0.01 μM). Growth with the support of such low chlorinated solvent concentration has not previously been reported. A Monod kinetic model (Cupples et al., 2004) has previously been used to suggest a decrease in the number of DHC if the concentration of chlorinated compounds fell below 0.7 μM, but this result is not confirmed by our experimental result. Our results therefore contribute to the on-going discussion on the lowest solvent concentration that can be reached using reductive dechlorination as remediation technology at contaminated sites. Furthermore, we note that KB-1 greatly enhanced VC degradation, even in the presence of an indigenous dechlorinating community, as demonstrated by the increased specific yield and maximum dechlorination rate on VC for the bioaugmented microcosms (MC5-MC6 and MC8-MC9) compared to the non-bioaugmented microcosms (MC2-MC3). However, the results reported herein are not sufficient to determine a proper correlation.

In addition to DHC, there is evidence that bacteria such as *Geobacter* spp., *Desulfitobacterium* spp., and others, might also be involved in dechlorination of TCE to cis-DCE (Gerritse et al., 1999, Sung et al., 2006). Due to this fact, the quantification of microorganisms involved in the TCE dechlorination pathway becomes rather complicated. To the best of our knowledge it is not possible to establish a qPCR based quantification assay, which is able to specifically target and measure all the organisms involved in the dechlorination and at the same time exclude organisms that are not able to perform the task. For example; the *Geobacter* genus contain quite a broad range of species, of which only a couple have been shown to perform TCE dechlorination, Based on the current knowledge of functional genes involved in dechlorination in *Geobacter* spp., a qPCR based assay would have to be based on 16S rRNA genes and thereby the chance of amplifying false positive would be substantial. Contrary to this, the substrate specificity seems to be much more narrow for DHC and the fact that our knowledge of functional genes, such as *vcrA*, *bvcA*, *tceA*, is substantial (Richardson, 2013), makes the combination of DHC 16S rRNA and functional gene quantification a more reliable assay for detection and relative quantification of the TCE dechlorination potential. Such considerations become even more essential if the matrix contains natural bacterial communities, as the case is in the present study.

4.3. Microbial community changes

In this study, the dechlorinating microbial community already present in the clay till was clearly different from the dechlorinating community introduced with the addition of KB-1 culture. In the KB-1 culture the functional *vcrA* RDase gene was more abundant than the two other well-known RDase genes *bvcA* and *tceA* (*tceA* was not detectable). Organisms harboring the *vcrA* gene were also the only organisms able to proliferate during the dechlorination process. Even though organisms harboring *vcrA* genes were present, and increased in abundance during dechlorination in the non-bioaugmented clay till, increases in abundance of *bvcA* genes were observed only in microcosms where no KB-1 culture was added. Once the KB-1 culture was introduced, even at relatively low doses, the dechlorinating organisms harboring *bvcA* genes introduced with the clay till were restrained. *vcrA* genes are often more abundant in the environment, and depending on the geochemical conditions, are more tightly correlated to DHC 16S rRNA genes than *bvcA* and *tceA* genes (van der Zaan et al., 2010). This demonstrates the complexity and the genomic variation of dechlorinating communities and the functional genes involved (McMurdie et al., 2009). Numerous RDases have been identified (Holscher et al., 2004), with the *vcrA*, *bvcA*, and *tceA* genes being the most intensively studied. But it is clear that not all of them are critical for the occurrence of chlorinated ethene dechlorination. Often when engineers wish to determine the potential for dechlorination at a contaminated site, qPCR based methods targeting DHC 16S rRNA genes and the *vcrA* gene are used. This may be sufficient when studying a site bioaugmented with a culture with a known functional gene structure such as KB-1. However, a broader spectrum of functional genes should be taken into account when monitoring the indigenous potential for dechlorination.

5. Conclusion

We have demonstrated that a higher initial abundance of KB-1 results in faster reductive dechlorination of TCE to ethene, and that the DHC strains in KB-1 have a suppressing effect on the indigenous DHC population in this specific clay till even at low concentrations. We report reductive dechlorination and growth of DHC even at very low chlorinated solvent concentrations (~0.01 μM). Finally we demonstrated the importance of using multiple functional genes as biomarkers to assess the potential for microbial dechlorination at contaminated sites, especially at sites where commercial dechlorinating cultures are not applied.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.11.013

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