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Thermally enhanced in situ bioremediation of groundwater contaminated with chlorinated solvents – A field test

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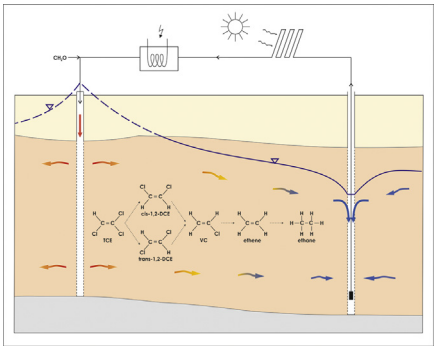
HIGHLIGHTS

- The technology was tested at a site contaminated by chlorinated ethenes.
- A groundwater circulation system was used for heating the aquifers.
- Hydrochemical and molecular genetic methods were used to analyse the processes.
- The application of heat and whey resulted in a fast removal of chlorinated ethenes.
- Temperature alone did not create favourable conditions for dechlorination.

GRAPHICAL ABSTRACT



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ABSTRACT

In situ bioremediation (ISB) using reductive dechlorination is a widely accepted but relatively slow approach compared to other technologies for the treatment of groundwater contaminated by chlorinated ethenes (CVOCs). Due to the known positive kinetic effect on microbial metabolism, thermal enhancement may be a viable means of accelerating ISB.

We tested thermally enhanced ISB in aquifers situated in sandy saprolite and underlying fractured granite. The system comprised pumping, heating and subsequent injection of contaminated groundwater aiming at an aquifer temperature of 20–30 °C. A fermentable substrate (whey) was injected in separate batches.

The test was monitored using hydrochemical and molecular tools (qPCR and NGS). The addition of the substrate and increase in temperature resulted in a rapid increase in the abundance of reductive dechlorinators (e.g., *Dehalococcoides mccartyi*, *Dehalobacter* sp. and functional genes *vcrA* and *bvcA*) and a strong increase in CVOC degradation. On day 34, the CVOC concentrations decreased by 87% to 96% in groundwater from the wells most affected by the heating and substrate. On day 103, the CVOC concentrations were below the LOQ resulting in degradation half-lives of 5 to 6 days. Neither an increase in biomarkers nor a distinct decrease in the CVOC concentrations was observed in a deep well affected by the heating but not by the substrate.

NGS analysis detected *Chloroflexi* dechlorinating genera (*Dehalogenimonas* and GIF9 and MSBL5 clades) and other genera capable of anaerobic metabolic degradation of CVOCs. Of these, bacteria of the genera *Acetobacterium*, *Desulfomonile*, *Geobacter*, *Sulfurospirillum*, *Methanosarcina* and *Methanobacterium* were stimulated by the

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substrate and heating. In contrast, groundwater from the deep well (affected by heating only) hosted representatives of aerobic metabolic and aerobic cometabolic CVOC degraders.
The test results document that heating of the treated aquifer significantly accelerated the treatment process but only in the case of an abundant substrate.

1. Introduction

Chlorinated ethenes are among the most abundant pollutants of groundwater and soil due to their frequent use in industrial applications. Since the mid-1990s, in situ biodegradation (ISB) has been a widely used remedial technology for the treatment of groundwater contaminated by these pollutants. Generally, it relies on sequential reductive dechlorination with each step in the process removing one chlorine atom and replacing it with a hydrogen atom (Bradley and Chapelle, 2010). Thus, tetrachloroethene (PCE) is converted stepwise to trichloroethene (TCE), dichloroethene (DCE), primarily to *cis*-1,2-DCE, then to vinyl chloride (VC) and finally to ethene (Stroo et al., 2014). In this process, chlorinated ethenes serve as the electron acceptor and molecular hydrogen and acetate, both released as by-products of organic substrate fermentation reactions, are used by dechlorinating bacteria as an electron donor and as a carbon source, respectively (Bradley and Chapelle, 2010).

The ability of reductive dechlorination of chlorinated ethenes is restricted to only a few bacterial genera. Microorganisms capable of sequential dechlorinating PCE down to *cis*-1,2-DCE include *Dehalobacter restrictus* (Holliger et al., 1998, 1993), *Desulfuromonas* sp. (Krumholz et al., 1996; Sung et al., 2003), *Geobacter lovleyi* (Sung et al., 2006), *Sulfurospirillum multivorans* (Luijten et al., 2003) and *Desulfitobacterium* sp. (Maillard et al., 2005). *Dehalococcoides mccartyi* is the only one known to be capable of gaining energy through dechlorination of DCE to VC and eventually to ethene (Löffler et al., 2013; Maymó-Gatell et al., 2001).

As in situ availability of electron donors is an important factor limiting the efficiency of microbial reductive dechlorination of chlorinated ethenes to ethene (e.g. Bouwer et al., 1994; Gibson and Sewell, 1992; McCarty, 1996), most practical applications of ISB lie in the delivery of a fermentable substrate (electron donor source). Various types of electron donor sources have been used including soluble, semi-soluble and solid substances (AFCEE, 2004). At sites where microorganisms that can perform full reductive dechlorination are absent or sparse, bioaugmentation i.e., the addition of exogenous cultures, may be utilized to enhance degradation (Ellis et al., 2000; ESTCP, 2010).

The role of temperature on reductive dechlorination was studied in laboratory experiments, which found that the growth of dechlorinating bacteria and the corresponding dechlorination activity depended highly on temperature (Zhuang and Pavlostathis, 1995; Friis et al., 2007; Ni et al., 2015). Friis et al. (2007) showed that degradation rates of lactate-amended TCE dechlorination increased approximately by a factor of 10 when the temperature was increased from 10 to 30 °C. Ni et al. (2015) tested a combination of underground storage energy systems (UTES) and ISB in batch experiments. They concluded that the overall removal rates of *cis*-1,2-DCE under UTES conditions (~25 °C) were from 8.5 to 13 times higher than under natural conditions (10 °C) depending on the configuration of the UTES. Reductive dechlorination of chlorinated ethenes at temperatures of 10, 20, 30 and 40 °C has previously tested in laboratory batch experiments (Najmanová et al., 2016). The highest degradation rates for chlorinated ethenes and the highest abundance of dechlorinating bacteria *Dehalococcoides* sp. were observed at 20 and 30 °C, whereas at 40 °C only incomplete dechlorination was observed. Therefore, increased temperatures can accelerate the bioremediation of sites contaminated by chlorinated ethenes and lessen the drawbacks of slow IRB observed at many sites.

To our knowledge, there are almost no articles published in the literature describing field testing of thermally enhanced IRB. Therefore, the present study was performed to assess the feasibility of this method at a real contaminated site with complex (hydro)geological settings. The remediation process was monitored using a combination of techniques ranging from physical and chemical analysis to molecular biology approaches. This enabled us to describe the effect of increased temperatures on a microbial consortium and to elucidate the treatment process in general.

2. Materials and methods

2.1. Test site

The pilot test was performed at a manufacturing site in the Czech Republic contaminated by chlorinated ethenes originating from historical degreasing activities. The hydrogeological system at the site comprises three aquifers. The upper phreatic aquifer is developed in Quaternary loams and has a saturated thickness of approximately 2 m. The middle aquifer is developed in a sandy eluvium of the granite massive. It is separated from the upper aquifer by a 1.0–1.5 m thick clay layer. The thickness of the confined middle aquifer is 1.5 to 2 m. Irregularly fractured granite holds the lower aquifer. The groundwater discharges into a local river located approximately 300 m from the site.

The groundwater of the upper and middle aquifers is of the Ca-Cl^- type and has elevated mineralization - total dissolved solids from 0.7 g/l (the middle aquifer) to 1.4 g/l (the shallow aquifer) and is slightly acidic (pH 6.4–6.8). The groundwater of the lower aquifer is of the Ca-SO_4^{2-} type and is characterized by low mineralization (total dissolved solids from 0.3 g/l to 0.5 g/l) and slightly acidic pH (6.2–6.5).

Historical use of TCE at the site as a degreasing agent has resulted in contamination of the subsurface at the manufacturing plant. Previous remediation of aquifers using pump and treat and in-situ reductive bioremediation, which terminated in 2012, reduced the amount of CVOCs. However, significant residual contamination remained. Whereas in the upper aquifer concentrations of chlorinated ethenes were below their respective limit of quantification (LOQ; 1.0 μg/l), in the middle and the lower aquifers the total concentration of CVOCs ranged from 249 to 1213 μg/l and from 5340 to 7004 μg/l, respectively.

As a consequence of the remedial activities, parent contaminants were significantly degraded to less chlorinated metabolites. The chlorine number (weighted average number of Cl atoms per molecule of ethene (Bewley et al., 2015)) in the groundwater ranged from 1.1 (the middle aquifer) to 1.8 (the lower aquifer) prior to the pilot test. Therefore, vinyl chloride (VC) and *cis*-1,2-dichloroethene (*cis*-1,2-DCE) were the dominant chlorinated contaminants.

2.2. Pilot application of the technology

The pilot test focused on the middle and lower aquifers. The treated zone was heated via groundwater circulation. Groundwater was abstracted from well HV-64 screened to both of the aquifers (average rate of 0.13 l/s), pre-heated in solar absorbers, then heated to the predefined temperature of 35 to 40 °C in an electrical heater and finally injected back via injection well IN-1, which was also screened to both of the aquifers. Heating and groundwater circulation lasted 3 months with

the temperature of the injected water being 40 °C between day 10 and day 35 and then 35 °C until day 103.

During this period, liquid cheese whey (supplied by the local dairy Mlékárna Čejetičky) was dosed as a fermenting substrate to the injection well in three batches on days 0, 48 and 78. In total, 5.2 m^3 of whey was injected over the circulation/heating period. After this period, groundwater monitoring continued for another 2 months.

As a reference, the existing well HV-53D, situated approximately 90 m to the west, cross-gradient of the heated area, was dosed with a corresponding amount of whey (also applied in three batches) but without heating and circulation. The well was chosen due to its similar geological profile, concentration and composition of CVOCs and groundwater flow.

2.3. Monitoring

Groundwater samples were collected during the pilot test from the following wells (Figs. 1 and 2): two monitoring wells screened to the middle aquifer (HML-4S, HV-8), two monitoring wells (HML-1S and HML-4D) screened to the lower aquifer and two wells (monitoring wells AP-2 and pumping well HV-64) screened to the both middle and lower aquifers.

The reference well HV-53D (with the dosing of whey but without heating) was also screened to both the middle and lower aquifers. The groundwater samples were collected after well purging using a Gigant submersible sampling pump (Ekotechnika, Czech Republic). Field parameters including pH, oxidation-reduction potential, electrical conductivity and temperature were recorded during the sampling. Prior to sampling, approximately 3 borehole casing volumes of groundwater were removed from the groundwater monitoring well to follow the standard procedure (ČSN ISO 5667-11, 1993).

The groundwater samples from all of the monitoring wells were analysed and monitored for the following parameters (Section 2.6): chlorinated ethenes, ethene, ethane, methane, sulphate, hydrogen sulphide, nitrate, ammonium, manganese, total organic carbon (TOC), relative abundance of specific bacteria and functional genes using real-time PCR (Section 2.4). The groundwater samples from the reference well HV-53D were analysed for chlorinated ethenes, ethene, ethane, methane and TOC. Prior to (day −1), in the middle of (day 68) and 2 months after (day 166) the heating period, groundwater samples were collected from wells HV-8, AP-2, HML-4S and HML-4D for microbial community analysis using Next Generation Sequencing (Section 2.5).

2.4. qPCR

Prior to analysis, water samples (0.35–0.5 L) were concentrated by filtration through a 0.22 μm membrane filter (Merck Millipore, Germany). DNA was extracted from the filter using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's protocol. A Bead Blaster 24 homogenisation unit (Benchmark Scientific, NJ, USA) was employed for cell lysis. Extracted DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies, CA, USA).

Relative qPCR was employed to assess the relative abundance of *Dehalobacter* sp. (Dre), *Dehalococcoides mccartyi* (DHC-RT), *Desulfitobacterium* spp. (Dsb), *Geobacteraceae* (Geo). and the VC reductase genes *vcrA* and *bvcA*. In addition, qPCR was employed to monitor the relative level of sulphate-reducing bacteria by detecting levels of genes for the adenosine-5′-phosphosulphate reductase *apsA* gene, and denitrifying bacteria by detecting relative levels of the nitrite reductase gene *nirK* (Braker et al., 1998). The relative abundance of 16S rDNA (U16SRT total bacteria marker) was determined as a control marker. All primers used for qPCR are listed in the Supplementary material, Table S1.

All qPCR reactions were performed on a LightCycler® 480 (Roche, Switzerland) with the same reaction conditions as described in our previous study (Němeček et al., 2017). Crossing point values were obtained using the 'second derivative maximum' method included in the

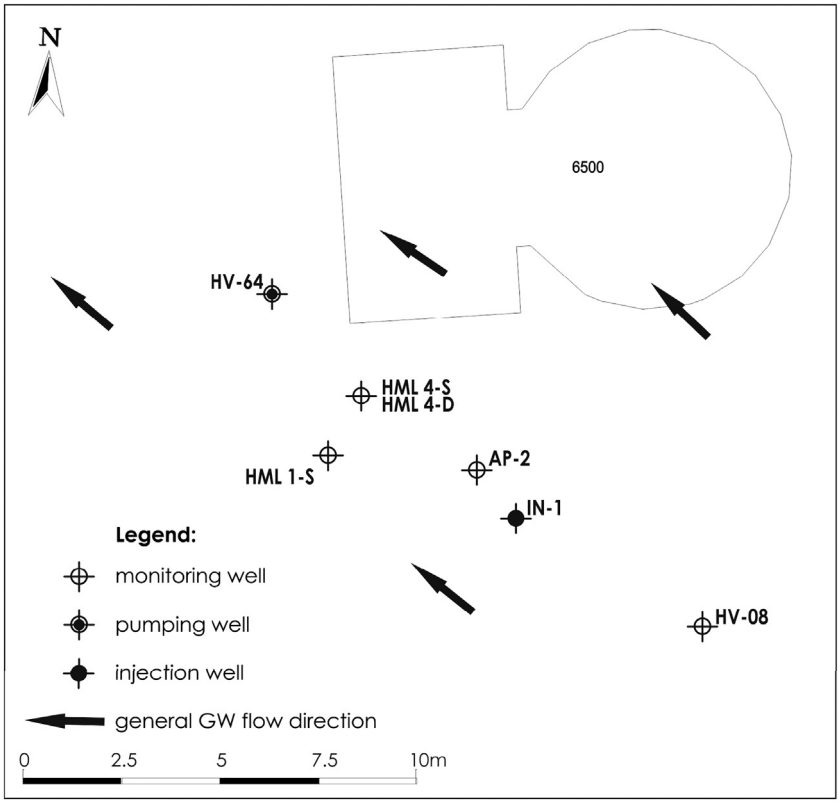

Fig. 1. Layout of the pilot test wells.

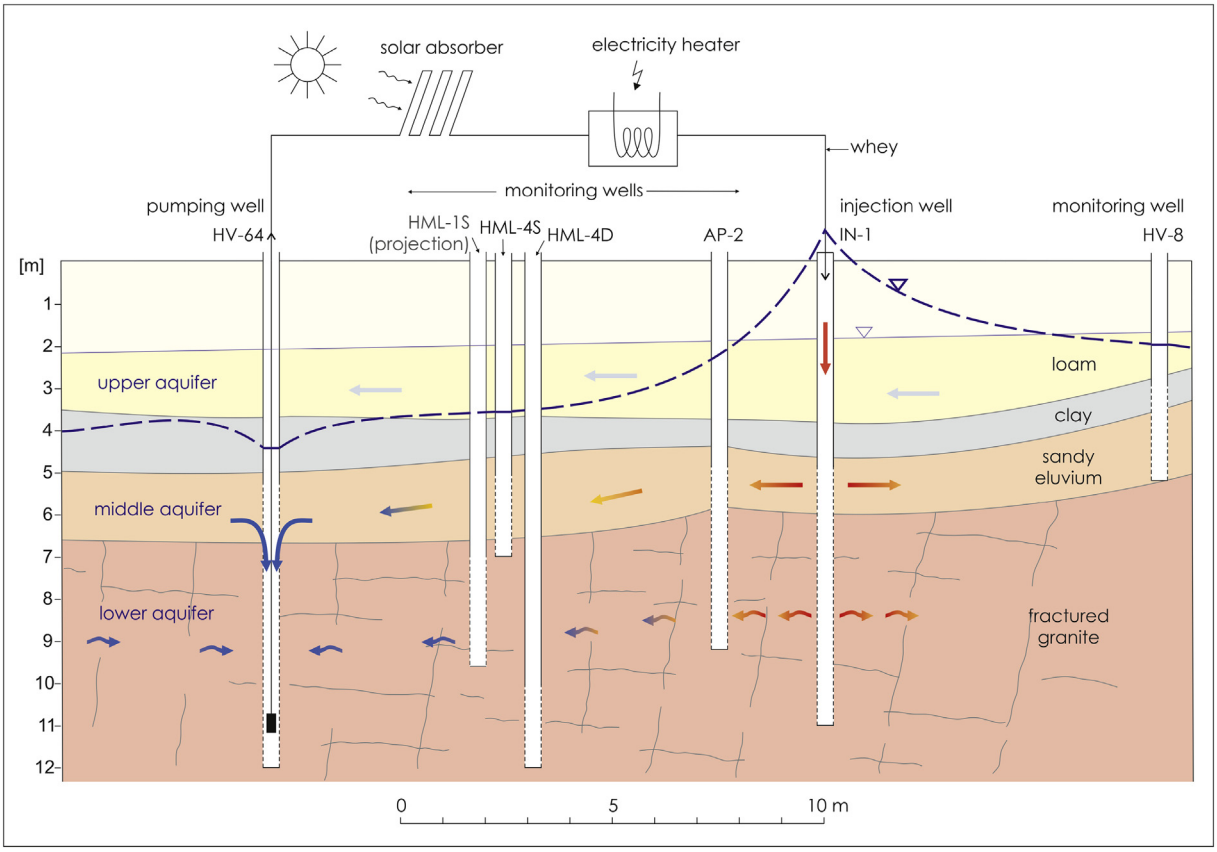

Fig. 2. Pilot test set up.

LightCycler® 480 Software. The individual amplification efficiency was determined by measuring the slope of curves constructed from a serial dilution of a template DNA from five environmental internal standards. In the evaluation process, a water sample was normalized to dilution and the total amount of DNA was extracted. The relative abundance of bacterial 16S rDNA and functional genes was calculated and expressed as a fold change between two states in the particular well (at a given sampling time and at an initial time) using the delta Cp method. Based on the approach used, it is necessary to consider each molecular biological marker separately and observe the trends in the relative abundance over time.

2.5. Next Generation Sequencing

All samples for microbial community analysis were sequenced in duplicate, with two consecutive PCR reactions performed per sample to amplify the DNA from the V4 region (normal and barcode fusion primers used). In silico analysis of the primers was performed in order to cover as much diversity as possible, while keeping the amplicon size below 400 bp. Amplification of the V4 region of the bacterial 16S rDNA was performed with primers 515F (5′–TGCCAGCMGCNGCGG–3′; Dowd et al., 2008) and barcode one 802R (5′–TACNVGGGTATCTAATCC–3′; Claesson et al., 2010). An in-house prepared synthetic mock community comprising 12 known bacterial genomes was used to determine whether the used NGS measurement and data evaluation process accurately capture exact information about the studied communities. PCR conditions were the same as in our previous study (Němeček et al., 2017). The concentration of purified PCR product was measured with a Qubit 2.0 fluorometer (Life Technologies, CA, USA). Barcode-tagged amplicons from different samples were then mixed in equimolar concentrations. Sequencing of bacterial amplicons was performed on the Ion Torrent platform (Life Technologies, CA, USA).

The raw Ion Torrent reads were processed with Mothur software (Schloss et al., 2009). Low quality reads were removed and sequences were assigned to each sample. Chimeric sequences were identified using UCHIME (Edgar et al., 2011) and subsequently removed. Sequences exceeding 400 bases were trimmed and sequences shorter than 170 bases were removed. Sequences were classified against the Silva database version 128 with a bootstrap value set at 80%. A cut-off value of 97% was used for clustering of sequences into operational taxonomic units (OTUs). Sequence data were normalized to sample with the least amount of sequences (7188 sequences) by randomly selecting a number of sequences from each sample. Cluster analysis was performed using the Vegan package in the R statistical package (Oksanen et al., 2012). Phyla/genera exceeding a relative abundance of 3% were visualized on graphs.

For a description of the dissimilarity (1-similarity) among the samples (normalized to all 7188 OTUs identified without a filter on the minimal abundance) we used dendrogram visualization based on the Yue & Clayton measure.

Two different metrics were used for estimating diversity – (i) inverse Simpson index (Lande, 1996) and (ii) tail statistic (Li et al., 2012). Inverse Simpson diversity calculations were carried out using the diversity function in the Vegan package (Oksanen et al., 2012). The tail statistic was calculated according to the equation developed by Li et al. (2012).

2.6. Physical and chemical parameters of the groundwater

Ca, Mg, Na, K, Fe and Mn dissolved in groundwater were analysed using ICP-OES (Optima 2100, Perkin Elmer, USA) according to ČSN EN

ISO 11885 (2007). The groundwater samples were filtered through a 0.45 μm membrane filter prior to the metal analyses.

Ammonium (NH_4^+) and sulphane were determined spectrophotometrically according to ČSN ISO 7150-1 (1994) and ČSN 83 0530-31 (1980), respectively.

Total organic carbon (TOC) was determined according to ČSN EN 1484 (1998) using a TOC analyser (MULTI N/C 2100S, Analytik Jena, Germany).

The oxidation reduction potential (ORP), pH, temperature and conductivity of groundwater were measured in a flow-through cell by a Multi 350i Multimeter (WTW, Germany).

Chloride, nitrate and sulphate were analysed by ion chromatography according to ČSN EN ISO 10304-1 (2007) and bicarbonates and carbonates by titration according to ČSN EN ISO 9963-1 (1994).

Volatile organic carbons including chlorinated ethenes, ethene, ethane and methane were analysed by GC–MS (CP 3800, Saturn 2200, Varian, USA) using a VF-624 ms column (Varian, USA), a CTC Combipal injector (CTC Analytics, USA) and a headspace agitator.

2.7. Data analysis

The biodegradation rate was fitted by the first order kinetic:

$$C = C_0 \cdot e^{-kt} \quad (1)$$

where:

C – biodegraded concentration of CVOCs at time t
C_0 – initial concentration of CVOCs
k – degradation rate constant ($time^{-1}$)

The calculation was performed using Excel software. A graph of the concentrations of CVOCs against time was fit to an exponential curve and degradation rate constant – k was calculated. The calculated degradation rate was then converted to degradation half-life $t_{1/2}$ according to:

$$t_{1/2} = \ln 2/k \quad (2)$$

Principal component analysis (PCA) was performed using the Vegan package (Oksanen et al., 2012) and FactoMineR package (Le et al., 2008). A dataset comprising physical and chemical parameters together with qPCR data for wells HV-8, AP-2, HML-4S and HML-4D was used as input data. The dataset of samples collected on days −22, −1, 34, 68, 103, 132 and 166 comprised the following parameters: pH, temperature, ORP, concentrations of ethane, ethene, methane, CVOCs, sulphate, TOC, Fe, H_2S, TCE, *cis*-1,2-DCE, VC, the chlorine number (Cl no.) and biological markers 16S rDNA, *bvcA*, *vcrA*, DHC-RT, Dsb, Dre, *apsA*, *nirK*, Geo.

3. Results and discussion

3.1. Groundwater temperature

Prior to the start of the pilot test, the temperature of the groundwater was 12–13 °C. During the pilot test, heat propagation reflected the subsoil permeability, heterogeneity and distance from the heated water-injecting well. After starting the circulation and heating of the groundwater, the temperature increased in wells AP-2 and HML-4S situated 2.3 m and 7.0 m from the injection well IN-1, respectively, see Figs. 1 and 3. In both wells that were screened to the middle aquifer (well AP-2 also to the lower aquifer), the temperature exceeded 25 °C after 30 days of heating. After a decrease in the temperature of the injected groundwater from 40 to 35 °C (day 35), the temperatures in these wells exhibited a decreasing trend. However, they remained within the target temperature range (20–30 °C). A significantly slower increase in temperature (to 17–21 °C) was observed in wells HML-4D and HML-1S screened to the lower aquifer and in the well (HV-8) located upgradient from the injection well under a natural groundwater flow regime. After termination of the heating and groundwater circulation, the temperature of the groundwater decreased slowly with the exception of well AP-2, where the observed decrease was steep due to a higher temperature increase in the first step. Two months later, the groundwater temperature was still 1 °C (well HML-1S) to 8 °C (AP-2) higher than the initial temperature.

3.2. TOC in the groundwater

Prior to the first dose of whey, TOC varied from 3.7 mg/l (well HV-64) to 11.6 mg/l (well HV-8) (see Fig. 3, right). The injected substrate migrated in a radial direction and on day 34 a significant increase in TOC to a level of 10 to 18 mg/l was observed in most of the monitoring wells (AP-2, HML-4S, HV-64, HV-8, HML-1S). After repeated substrate applications, TOC further increased to 15–43 mg/l in the second half of the heating period.

No significant increase in TOC during the pilot test was observed in the deep well HML-4D screened solely to the lower aquifer. Obviously, the local irregularly fractured granite is not an optimal medium for the distribution of the substrate.

3.3. Physical and chemical parameters of the groundwater

Prior to the pilot test, ORP ranged from 55 mV to 214 mV (see Supplementary material Fig. S1). During the heating period and application of whey, no significant changes in ORP were recorded. ORP decreased preferentially in wells with higher initial redox values: HV-64, HML-4D and HML-1S. On the contrary, a slight increase was observed in wells AP-2 and HV-8.

Application of the substrate, its fermentation (production of volatile fatty acids) and reductive dechlorination caused only a slight decrease in the pH of the groundwater (Fig. S1). Values of pH were above 6.0, which is the limit beneath which a rapid decrease in metabolic activity of dechlorinating bacteria can be observed (Eaddy, 2008; Rowlands, 2004).

Dechlorinating microorganisms competed with other anaerobic hydrogenotrophic microorganisms for utilization of H_2 under sulphate-reducing, methanogenic and homoacetogenic conditions. The competitiveness of these microorganisms decreases in the presence of more oxidative terminal electron acceptors (Vogel et al., 1987; Yang and McCarty, 1998). Therefore, the redox conditions of the aquifer were also monitored by analysing terminal electron acceptors (nitrate, sulphate) and acceptor reduced forms (dissolved Fe and Mn, sulphide, methane).

An increase in dissolved iron and sulphide and a decrease in sulphate concentrations indicated favourable conditions for dechlorinating microorganisms due to substrate application and/or increased temperature.

In groundwater from the wells that were impacted by the substrate (whey or its metabolites) and by heating (AP-2, HML-4S, HV-64, HV-8, HML-1S), an increase in the concentrations of dissolved iron up to 57.58 mg/l (HML-4S on day 68) was observed as a consequence of the activity of the iron reducers, see Fig. S1. A slight increase in temperature in well HML-4D did not cause an increase in the dissolved iron due to the absence of the substrate.

Wells affected by both the substrate and heating exhibited a decrease in sulphate concentrations from tens of mg/l to below the respective LOQ (1.0 mg/l). The rate of decrease was proportional to the distance of the respective wells from the injection well IN-1 (the fastest decrease was observed in wells AP-2 and HML-4S). Except for well AP-2, a slight increase in sulphate concentrations was ascertained in groundwater from these wells after the end of the heating period. On the contrary, a clear decrease was observed after the heating period in well HML-4D, which was affected by heating but not by substrate.

Sulphate was microbially reduced to hydrogen sulphide as depicted in Fig. S1. Except for well HML-1S, hydrogen sulphide concentrations

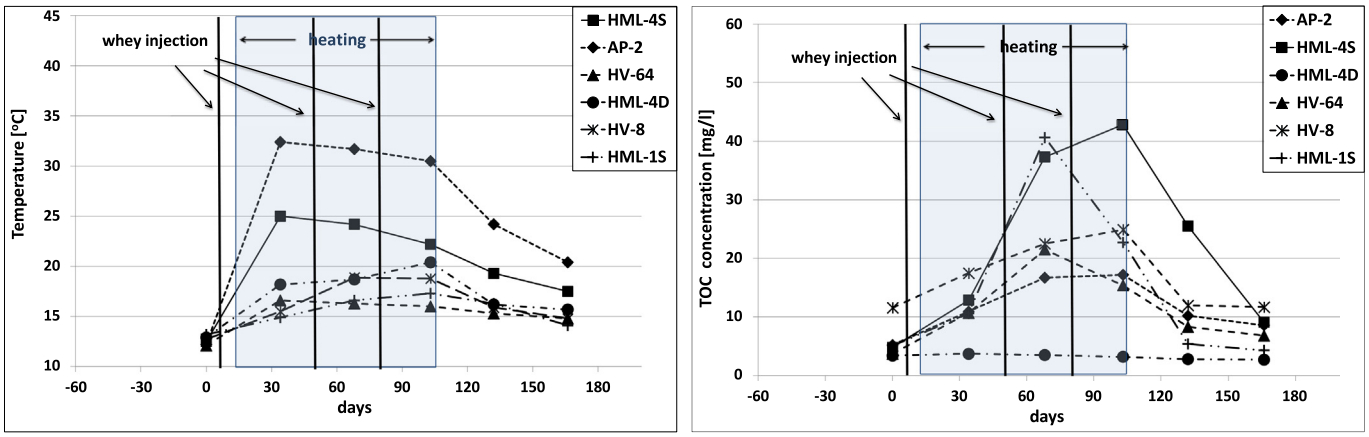

Fig. 3. Temperature of groundwater (left), TOC concentration in the groundwater (right).

above the respective LOQ (0.01 mg/l) were found before the start of the pilot test. After the first substrate application and start of heating, hydrogen sulphide concentrations increased in the groundwater from all of the affected wells. In the second half of the heating period, the concentrations of hydrogen sulphide in wells HV-8 and AP-2 decreased, probably due to a depletion of sulphate. An increase in hydrogen sulphide concentrations continued in the other affected wells (HML-4S, HML-1S and HV-64).

Substrate application and heat enhanced microbial methanogenesis caused an increase in methane concentrations in the groundwater from all of the affected wells, but with different rates. An instant increase was observed in well HV-8, in which the methanogenesis was significant before the pilot test. On the contrary, a gradual increase was found in well HV-4S where the initial concentration of methane was low.

It can be concluded that the measured ORP alone did not characterize the real geochemical conditions and related processes. Due to macro- and microheterogeneities of the aquifer system, there were zones of various conditions and related spectra of terminal electron-accepting processes from iron-reducing to methanogenesis.

3.4. Concentrations of chlorinated ethenes and their metabolites in the groundwater

The aim of the pilot test was to thermally enhance complete biological reductive dechlorination of chlorinated ethenes via less chlorinated hydrocarbons to nontoxic products ethene and ethane.

The substrate injection and increase in temperature stimulated sequential reductive dechlorination of the parent TCE to less chlorinated or dechlorinated ethenes (Figs. 4 and 5). The fastest decrease in TCE concentrations was observed in groundwater from wells that were the most affected by the heating and the substrate: AP-2, HML-4S and HV-8 (Fig. 4). On day 34, TCE concentrations dropped to an order of magnitude of units and tens of μg/l (decrease by 70% to 90%). On day 103, TCE concentrations further decreased below it respective LOQ (1.0 μg/l) in these wells and remained below or close to LOQ for the following 2 months of the pilot test. A less intensive decrease in TCE concentrations (by 45% on day 103) was also observed in well HML-1S, which was not so significantly affected by temperature and was screened to the lower aquifer (see Section 3.1).

On the contrary, a temporal but prominent increase in TCE concentrations (by a factor of 1.6) was observed in the groundwater from well HML-4D, which was affected by the heating but not by the substrate. This increase can be explained by the temperature enhanced desorption and increased solubility of TCE at higher temperatures. In pumping well HV-64, the concentrations of TCE initially decreased (by 57% on day 34) but exhibited an increasing trend to higher than the baseline level for the remainder of the pilot test. As well HV-64 was screened to both the middle and lower aquifers, the results reflected an inflow of groundwater with higher TCE concentrations from the lower aquifer with fractured granite and with an inefficient distribution of the substrate. This explanation is supported by the decrease in TOC concentrations in the pumped groundwater after day 68, whereas in the nearby well HML-4S located upgradient the TOC concentrations increased until day 110.

The concentration trends of the TCE transformation products *cis*-1,2-DCE and VC in the affected wells AP-2, HML-4S, HV-8 and HML-1S did not show the typical pattern for sequential dechlorination (temporal accumulation), but they exhibited a continuous decrease. The courses of *cis*-1,2-DCE and VC concentrations in the groundwater of wells HML-4S and HV-64 were similar to that of TCE in these wells. Ethene and ethane are nontoxic products of the biological reductive dechlorination of chlorinated ethenes. Before the pilot test, the concentrations of ethene in the groundwater were elevated (45 to 572 μg/l) as a result of natural microbial processes. After the substrate application and increase in temperature, ethene concentrations increased in all of the monitored wells (by a factor of 1.2 to 3.0 on day 34), except for in well HML-4D, which was not affected by the substrate, see Figs. 3 and 5. During the remaining time of the pilot test, ethene concentrations decreased obviously due to the very low residual concentrations of chlorinated ethenes. On the contrary, ethane concentrations increasing overall as ethene was further reduced to ethane.

The total concentration of CVOCs provides an overall picture of the effect of the thermally enhanced biological reductive dechlorination. From the graph in Fig. 4 (lower right) it is evident that there was a significant decrease in CVOCs in the wells most affected by the heating and substrate (AP-2, HML-4S and HV-8). On day 34, the concentrations of CVOCs had decreased by 87% to 96%. On day 103 (end of the heating period) the concentrations of CVOCs were below their respective LOQ. An apparent decrease in the concentrations of CVOCs was also observed in the groundwater from well HML-1S. This decrease was less intensive but continuous, reaching a reduction of 57% by day 132. Subsequently, a rebound in the concentrations to 55% of the initial level was observed after the substrate was depleted and the temperature decreased.

The course of the concentrations of CVOCs in pumping well HV-64 reflects the changes in the mixing ratio of the groundwater abstracted from different depths and are discussed above. In groundwater from HML-4D, which was affected by heat but not by the substrate, the concentrations of CVOCs increased by a factor of 1.7 on day 68, mainly due to the above-discussed significant increase in TCE concentrations. Subsequently, the concentrations of CVOCs exhibited a decreasing trend below the baseline at the end of the pilot test. As the concentrations of

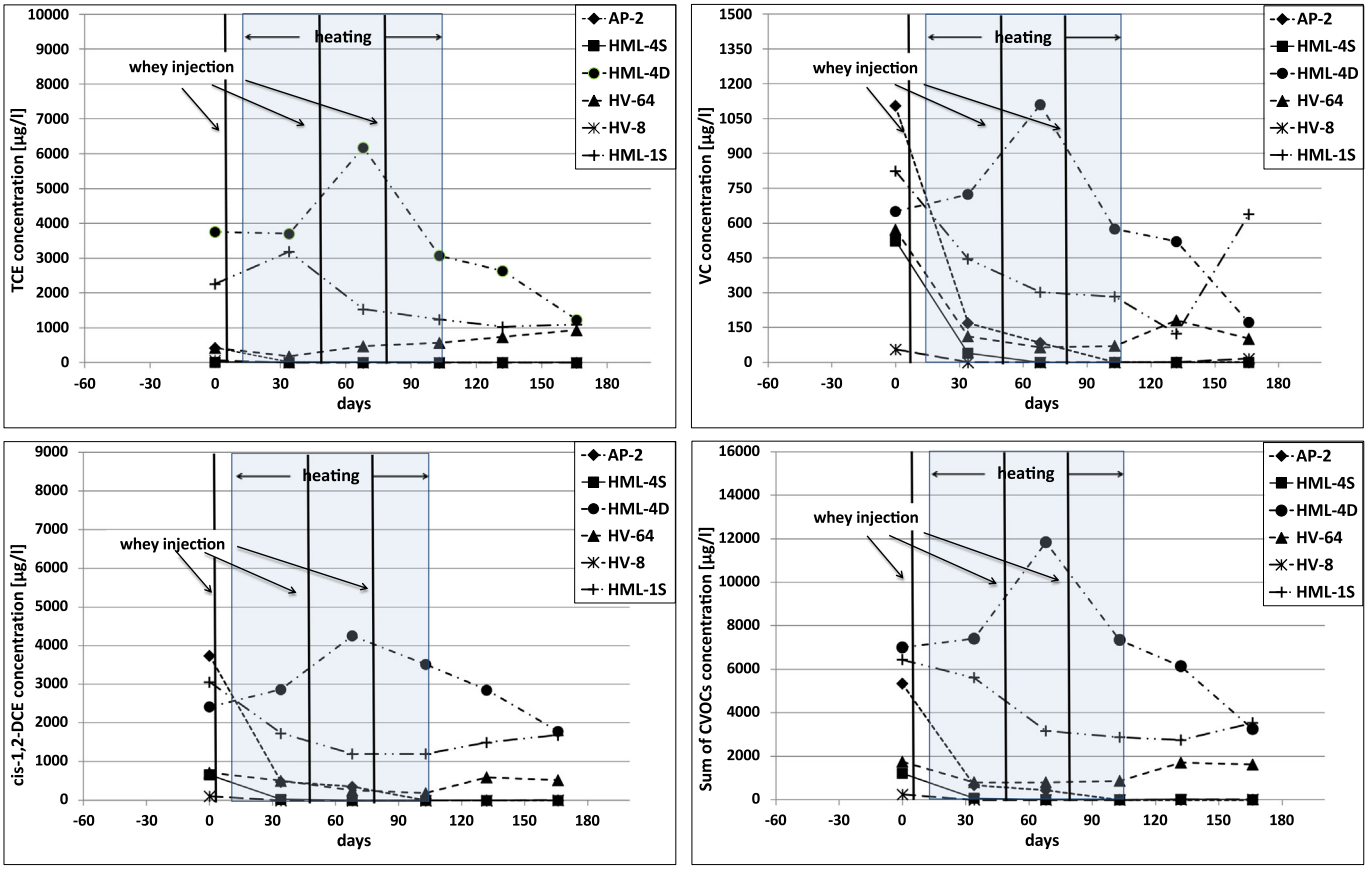

Fig. 4. Concentrations of TCE (upper left), *cis*-1,2-DCE (lower left), VC (upper right) and total concentration of chlorinated ethenes (lower right) in the groundwater during the pilot test.

ethane were low compared to the values in the remaining monitoring wells, other mechanisms (e.g. dilution) probably contributed to this decrease.

A measure of the dechlorination of parent TCE via less chlorinated ones to non-chlorinated ethene can be expressed by the chlorine number. Soon after the first substrate application and start of heating, a distinct decrease of the chlorine number was observed in wells with the highest rate of decrease in concentrations of CVOCs: AP-2, HV-8 and HML-4S (Fig. 6). This implies that the decrease in concentrations of CVOCs was primarily due to sequential biological dechlorination and not abiotic or physical processes. A rebound of the chlorine number in wells HV-8 and HML-4S was caused by a one-shot increase in the concentration of the parent contaminant. No general decrease in the chlorine number was found in wells HV-64 and HML-1S. This was probably due to changes in the mixing ratio of groundwater from different depths in these wells as discussed above and a rapid reduction of ethene to ethane, respectively.

The degradation rate constants were calculated according to the 1st order kinetic (Section 2.7). The resulting degradation rate constants and degradation half-lives calculated from data of the wells affected by both heat and the substrate (AP-2, HML-1S, HML-4S, HV-8) and from reference well HV-53D dosed by whey only are given in Table 1.

Wells AP-2, HML-4S, and HV-8, which were the most affected by heating and the substrate, exhibited the shortest degradation half-

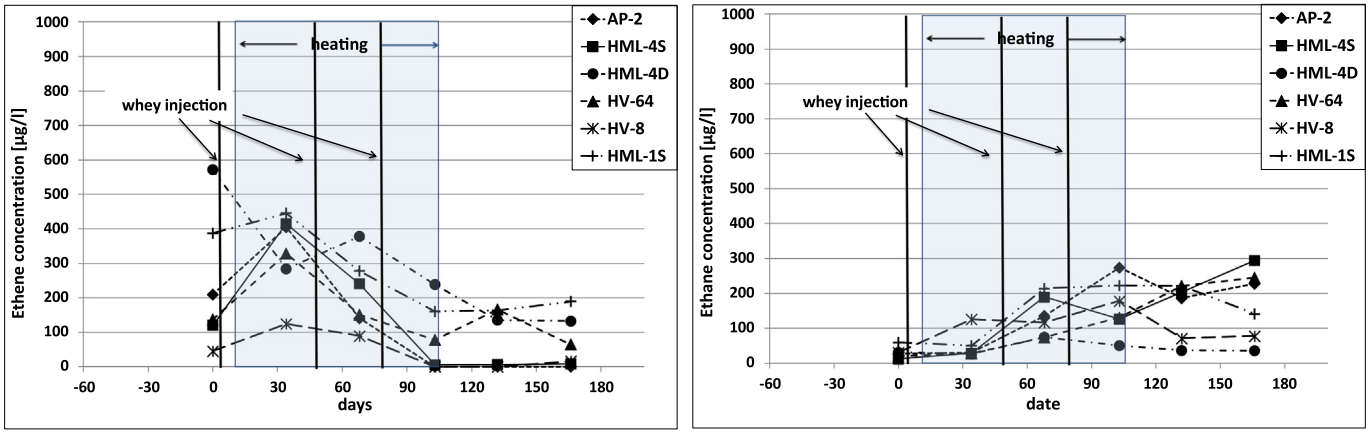

Fig. 5. Concentrations of ethene (left) and ethane (right) in the groundwater during the pilot test.

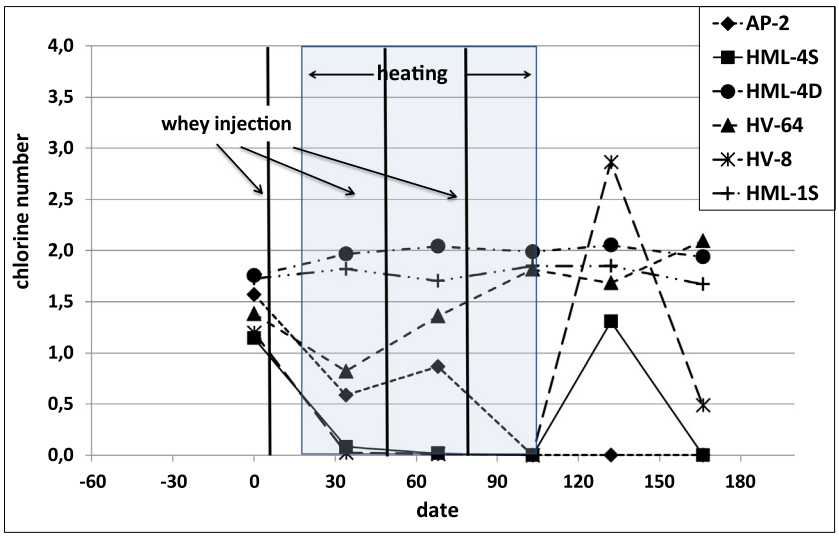

Fig. 6. Chlorine number (the chlorine number was set to zero when concentrations of CVOCs were below their respective LOQ).

lives of CVOCs ($t_{1/2}$ = 5 to 6 days). In well HML-1S, which was screened to the lower aquifer, the degradation half-life of CVOCs was significantly longer ($t_{1/2}$ = 77 days). This is similar to the reference well HV-53D ($t_{1/2}$ = 69 days), to which substrate was applied but the groundwater was not heated. These results indicate that the proper distribution of heat and fermentive substrate resulted in accelerated biodegradation of chlorinated ethenes.

3.5. Specific bacteria and functional genes

Based on the relative quantity of 16S rDNA (Fig. 7), the total bacterial biomass increased in the groundwater from all analysed wells affected by both heating and the substrate application (AP-2, HML-4S, HV-64 and HV-8). The maximum relative increase in biomass by a factor of 161 was encountered in well HV-64 on day 68. When the substrate was depleted and the groundwater temperature started to decrease, the biomass in these wells exhibited a decreasing trend. On the contrary, the total bacterial biomass in the groundwater from well HML-4D, which was affected by heating only, showed a general decrease during the whole course of the pilot test. A similar pattern was also observed for *Dehalococcoides mccartyi*, and the related reductive dehalogenase genes (*vcrA* and *bvcA*). The abundance of *Dehalococcoides mccartyi* increased by a factor of 2 (well HV-8) to 72 (well HV-64) during the heating period and then decreased below the original level, whereas in well HML-4D a continuous decreasing trend was observed. The relative abundance of *Dehalobacter* sp. increased in groundwater from wells AP-2, HML-4S and HV-64 (by a factor of 14 to 43) but not in well HV-8. As the total bacterial biomass increased in this well, the environmental conditions were obviously not optimal for *Dehalobacter* sp. to outcompete other microorganisms. The relative abundance of another dechlorinating bacteria belonging to *Desulfitobacterium* spp. varied during the heating period (mainly in wells HV-64 and HML-4S) and increased insignificantly (by a maximum factor of 14 in well AP-2 on day 34). As in well AP-2 on day 34, the highest groundwater temperature of all of the wells was found (see Fig. 3), heating was obviously not the cause of the overall poor stimulation of these bacteria.

The course of the sulphate-reducing gene *apsA* was similar to the total bacterial biomass. An increase in the relative abundance by a factor of 2 (well HV-8) to 18 (well HV-64) was found in wells affected by both heating and the substrate. On the contrary, a decrease in this gene during the whole pilot test was found in groundwater from well HML-4D affected by heating only.

The course of relative abundance of bacteria of the *Geobacteraceae* family can be characterized by an insignificant initial increase in the groundwater from wells AP-2 and HV-8 followed by a decrease below the initial levels at the end of the heating period. Other wells affected by both heating and the substrate (HV-64 and HML-4S) exhibited an elevated abundance during the whole heating period.

Data on the physical and chemical parameters, concentrations of chlorinated ethenes, ethene, ethane and the calculated chlorine number and results of qPCR were subjected to a principal component analysis (PCA), see Fig. 8. Temperature and TOC (parameters representing thermally enhanced bioremediation) showed a positive correlation with the concentrations of dissolved iron and hydrogen sulphide and a negative correlation with the redox potential and sulphate (see the correlation coefficients and p-values in Table S2). This indicates that both the elevated temperature and the applied substrate created iron-reducing to sulphate-reducing conditions, under which dechlorinating microorganisms can be competitive (Yang and McCarty, 1998; Löffler et al., 1999).

PCA also showed a positive correlation of temperature and TOC with total microbial biomass (16S rDNA) and other qPCR biomarkers including dechlorinating bacteria *Dehalococcoides mccartyi*, *Dehalobacter* sp. (Dre) and dehalogenase genes *vcrA* and *bvcA*. This implies that both heating and the substrate had a positive effect on the growth of the target microorganisms. On the contrary, there was no statistically important correlation between temperature, TOC and *Desulfitobacterium* spp. (Dsb).

Parameters comprising chlorinated ethenes and the chlorine number are grouped along the negative section of the first component axis (45% of variability), which indicates a negative correlation with temperature, TOC, ethane and abundance of the above-mentioned dechlorinating qPCR biomarkers. This implies that an increase in temperature and TOC

Table 1
Degradation rate of CVOCs.

Well	Degradation rate constant - k	Degradation half-life – $t_{1/2}$	Coefficient of determination R^2
	(day^{-1})	(day)	(−)
AP-2	0.137	5	0.74
HML-1S	0.009	77	0.91
HV-8	0.113	6	0.92
HML-4S	0.129	5	0.89
HV-53D	0.01	69	0.78

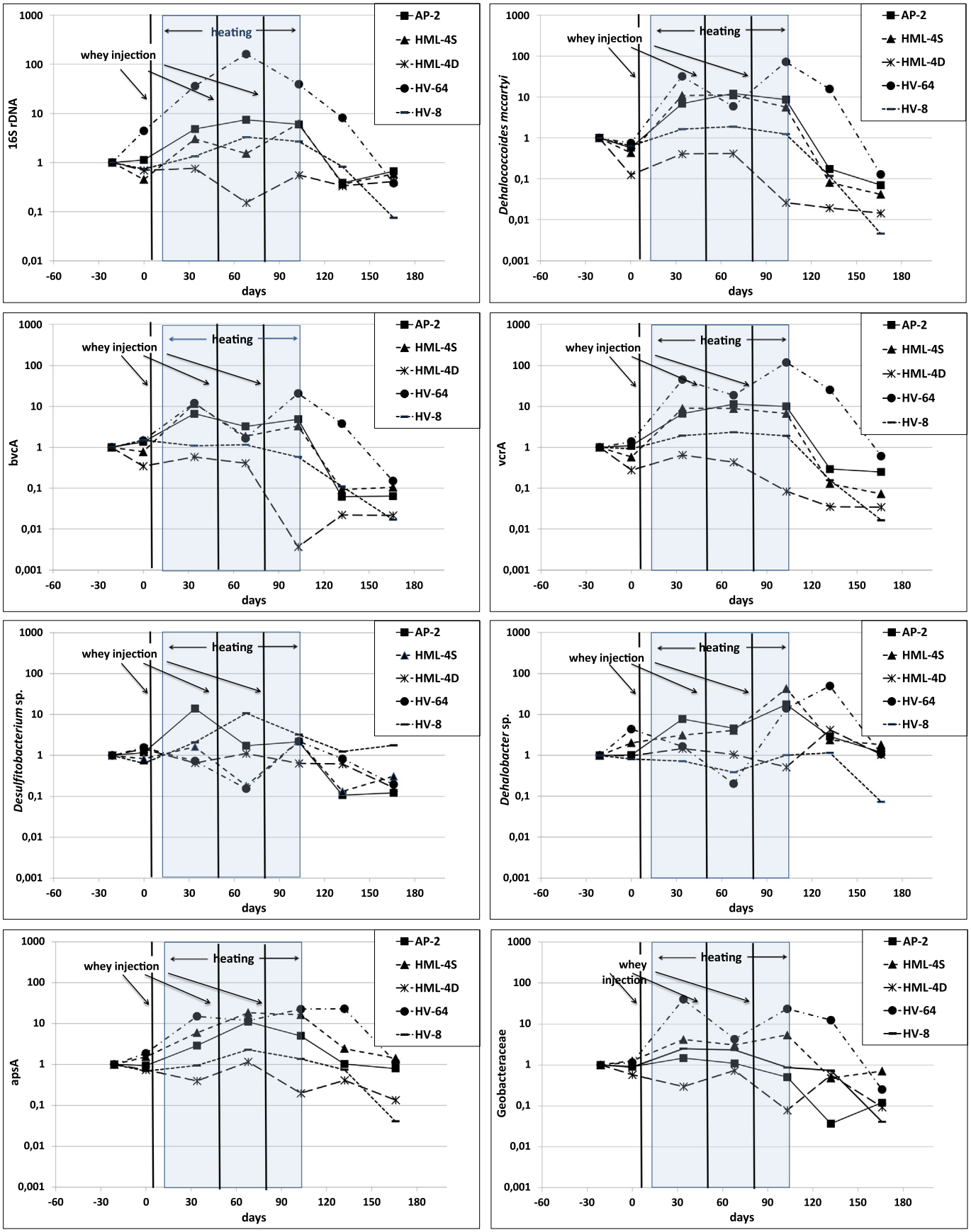

Fig. 7. Changes in total bacterial (prokaryotic) biomass (a rough estimate based on the quantity of 16S rDNA), dechlorinating bacteria (*Dehalobacter* sp., *Dehalococcoides mccartyi*, *Desulfitobacterium* spp.), bacteria family *Geobacteraceae* and functional genes.

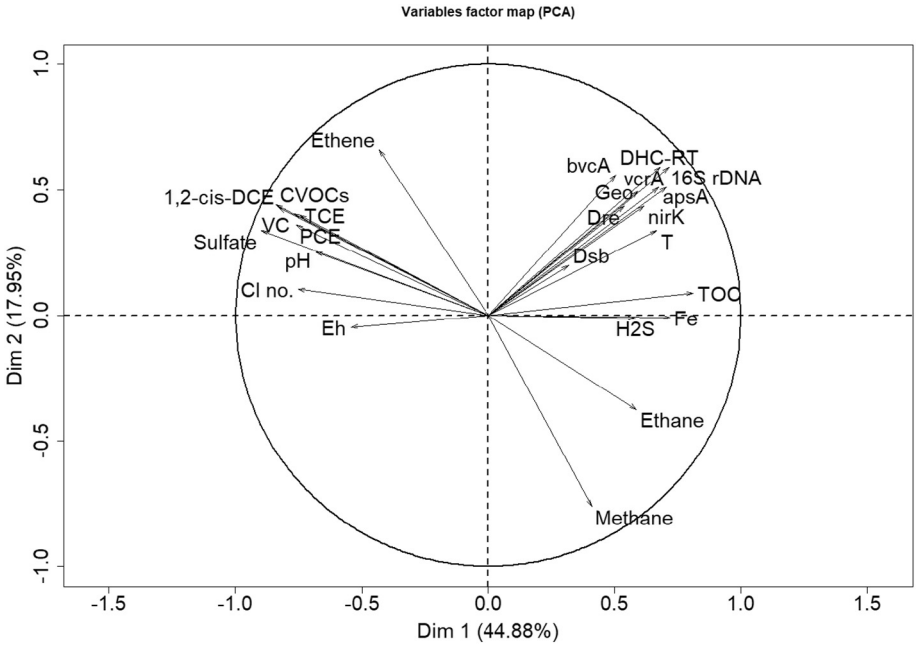

Fig. 8. Principal component analysis of the qPCR, physical and chemical parameters, concentrations of chlorinated ethenes, ethene, ethane. Refer to the text for the abbreviations used.

resulted in an increase in the abundance of dechlorinating biomarkers and a decrease in the concentrations of chlorinated ethenes and production of the dechlorination metabolite ethane.

3.6. Microbial community analysis

NGS was used to characterize bacterial communities in groundwater in relation to the stimulation of chlorinated ethene reductive dechlorination. Groundwater samples for NGS were collected from four wells in three monitoring campaigns: prior to the start of the pilot test (day −1), during heating and substrate application (day 68), and two months after the period of heating and three months after the last substrate application (day 166).

In total, NGS sequencing yielded 231,995 sequence reads of a sufficient quality and length. The number of sequences for each of samples was in the range of 7188–16,868. A total of 3029 OTUs (1842 excluding singleton and doubleton OTUs) were retrieved from our dataset.

Microbial communities present in the groundwater in the four sampling wells before the start of the pilot test (day −1) differed significantly from each other (Fig. S2). *Deltaproteobacteria* dominated in wells AP-2 (23%) and HML-4S (22%), whereas *Betaproteobacteria* (54%) and *Omnitrophica* (13%) were the most abundant bacterial groups in wells HML-4D and HV-8, respectively.

The microbial community before the start of the pilot test was characterized by a high abundance of genera involved in geochemical cycles of iron (e. g. *Gallionella* or *Rhodoferax*) and sulphur (e. g., *Sulfuritalea*, *Sulfuricurvum*, *Desulfobacca*, *Desulfomonile*, *Desulfovirga*, or *Desulfovibrio*) in the case of wells AP-2, HML-4S and HV-8. Methanotrophic genera (e. g., *Methylomonas* or *Methylobacter*) were typical for deep well HML-4D. *Brevundimonas*, *Pseudorhodobacter* or *Undibacterium* represented the other common genera detected in HML-4D, but they were very rare or even absent in the other sampling wells.

The microbial communities present in wells AP-2, HML-4S and HV-8 before the start of the pilot test had a higher taxonomic richness than the communities retrieved from later sampling times (Table 2).

After the whey application, the diversity dramatically dropped (day 68). This was followed by an increase in diversity during the final sampling (Table 2). In the case of groundwater from deep well HML-4D, which was treated by heating only, we observed opposite trends in the microbial diversity evolution compared to the other sampling wells. From these results, we can conclude that the addition of whey, which acts as an easily fermentable organic substrate, caused a reduction in the microbiome diversity in accordance with the findings of Matturro et al. (2016). In contrast, heating only led to a greater diversification of the microbial community because it affected the availability of organic carbon and terminal electron acceptors (e.g., Westphal et al., 2017).

After substrate application and heating the structure of microbial community significantly changed. Some changes were common for all of the sampling wells, namely a decrease in *Betaproteobacteria* and *Omnitrophica*, and a rapid increase in *Epsilonproteobacteria* and *Firmicutes*. A decrease in *Actinobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* was observed in wells AP-2, HML-4S and HV-8 after heating and substrate application. In contrast, these groups were stimulated by heating only in the case of deep well HML-4D. In general, the impact on the microbial community was less dramatic in the case of deep well HML-4D compared to the other sampling wells, which can be attributed to the fact that this well was treated by heating only. Samples collected from well HML-4D at different sampling times clustered together (Fig. 9), suggesting unique and relatively stable conditions present in this deep well.

Table 2
Diversity comparison of the samples.

Well	Time	Richness	Inv. Simpson	Tail
AP-2	Day −1	210 ± 70	19	38
	Day 68	131 ± 97	10	16
	Day 166	194 ± 70	19	33
HML-4D	Day −1	157 ± 107	8	22
	Day 68	221 ± 57	28	41
	Day 166	183 ± 90	11	29
HML-4S	Day −1	227 ± 62	24	41
	Day 68	153 ± 88	12	22
	Day 166	223 ± 59	26	40
HV-8	Day −1	245 ± 57	28	49
	Day 68	151 ± 143	4	19
	Day 166	215 ± 107	8	30

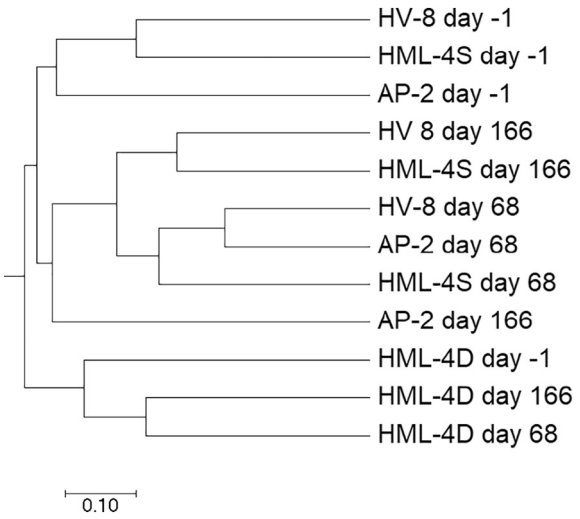

Fig. 9. Dendrogram showing dissimilarities between all 12 NGS samples based on the Yue & Clayton measure (calculated from all 3029 OTUs without any filter on abundance).

Microbial communities retrieved from the final sampling time (day 166, i.e., two months after the end of the heating period) were characterized by a gradual return to the original state. Most of the bacterial groups, which were stimulated by the substrate applications and heating at the second sampling time, decreased in their abundance (*Epsilonproteobacteria*, *Firmicutes*, *Parcubacteria*, *Spirochaetes*). Bacteria that decreased after substrate/heating treatment started to proliferate again (e.g., *Actinobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Omnitrophica*). However, the abundancies of the influenced bacterial groups at the end of the experiment still did not reach the original values.

The substrate application and heating stimulated the growth of bacteria that are able to metabolise sugars and other carbon sources effectively (e.g., *Arcobacter*, *Trichococcus*). On day 68, these taxa outcompeted several bacteria that dominated on day −1 (e.g., *Desulfobacca*, *Desulfomonile*, or *Sulfuricurvum*) in wells AP-2, HML-4S and HV-8. However, most of these bacteria had recovered by the time of the last sampling (day 166). Heating, which was the only treatment of deep well HML-4D, caused an increase in OTUs affiliated to genera *Geobacter*, *Methylobacter*, *Methylomonas* or *Sulfuritalea* in this deep well. In contrast, OTUs affiliated to genera *Aquabacterium*, *Gallionella*, *Pseudorhodobacter* or *Undibacterium* reacted to elevated temperatures by decreasing.

The number of detected dechlorinating bacteria was relatively low, which is in accordance with findings from other sites contaminated by chlorinated ethenes (e.g., Kotik et al., 2013; Němeček et al., 2017). Members of the *Chloroflexi* phylum were detected in groundwater from all of the sampled wells, although they were present in very low quantities in deep well HML-4D in all sampling times. The genus *Dehalogenimonas* represented the most common member of *Chloroflexi* in our dataset, being most abundant in wells AP-2 and HML-4S. This genus comprises only three recently recognised species (*D. lykanthroporepellens*, *D. alkenigignens*, and *D. formicexedens*) that are strict anaerobes capable of dihaloelimination of polychlorinated aliphatic alkanes (Bowman et al., 2013; Maness et al., 2012; Moe et al., 2009; Key et al., 2017). A novel reductive dehalogenase (*tdrA*), which catalyses the dechlorination of tDCE to VC, was recently reported from this genus (Molenda et al., 2016). Other OTUs belonging to *Chloroflexi* were mostly affiliated with GIF9 and MSBL5 clades that were recently reported from sites contaminated by polychlorobiphenyls (Matturro et al., 2016). No OTUs from our dataset were assigned to the genus *Dehalococcoides*, which includes strains well known for their ability of complete reductive dechlorination from PCE to ethene (*D. mccartyi*). However, the absence of these bacteria at sites contaminated by chlorinated compounds is not unusual (Kotik et al., 2013; Lowe et al., 2002; Miller et al., 2007) and may be related to an accumulation of *cis*-1,2-DCE and/or VC (Hendrickson et al., 2002; Kranzioch et al., 2015; Rossi et al., 2012). Nevertheless, the presence of *D. mccartyi* as well as vinyl chloride reductase genes (*vcrA* and *bvcA*) was confirmed by qPCR. This discrepancy between NGS and qPCR data for *D. mccartyi* detection was previously reported by Matturro et al. (2016) and can probably be explained by the above-mentioned very low relative abundance of members of *Chloroflexi* in the bacterial community. Another factor may be the more efficient PCR amplification with the highly specific primers used for qPCR compared to the universal primers used for NGS.

The number of *Chlorflexi* affiliated bacteria was significantly lower on days 68 and 166 compared to day −1. However, this does not necessarily mean that heating and substrate application had a negative effect on this group. The described trend is likely to be based on limiting factors for the growth of organohalide respiring bacteria. While before the substrate application (which served as a source of electrons) the limiting factor was the absence of electron donors, it was subsequently terminal electron acceptors (inorganic compounds in a high oxidation state and chlorinated hydrocarbons). Therefore, an increase in bacterial activity was followed by a decrease.

Members of the genera *Acetobacterium*, *Desulfomonile*, *Desulfitobacterium*, *Geobacter*, *Methanobacterium*, *Methanosarcina*, and *Sulfurospirillum*, which include species capable of anaerobic metabolic degradation of chlorinated ethenes (Dolinová et al., 2017; Mattes et al., 2010), were also retrieved from our dataset. The number of sequences affiliated to the most abundant genus *Desulfomonile* decreased in samples collected on day 68, but increased again on day 166, when most of the applied substrate has been consumed. Bacteria belonging to the genera *Acetobacterium*, *Geobacter*, *Sulfurospirillum*, *Methanosarcina* and *Methanobacterium* were stimulated by the application of the substrate and heat. In general, the bacteria capable of anaerobic dechlorination were mostly present in wells AP-2, HML-4S and HV-8, but were absent or rare in deep well HML-4D, with the exception of OTU55 affiliated to *Geobacter* sp. On the other hand, well HML-4D hosted bacterial genera including strains with a recognised ability to degrade chlorinated ethenes by an aerobic (metabolic or co-metabolic) pathway. These taxa were much rarer or even absent in the other sampling wells, suggesting different mechanisms of dechlorination processes ongoing in deep well HML-4D compared to the other wells. Members of the genera *Rhodoferax*, *Brevundimonas*, *Rhodococcus*, *Mycobacterium* with different types of metabolisms and methanotrophic *Methylomonas* and *Methylobacter* were the most common representatives of the detected aerobic degraders of chlorinated ethenes. Interestingly, a strain affiliated to the genus *Polaromonas* was also detected in deep well HML-4D. The *Polaromonas* sp. strain JS666 is the only bacterium that has been shown so far to biodegrade cDCE completely by an aerobic metabolic pathway (Coleman et al., 2002). The abundance of most strains with possible aerobic dechlorinating activity increased after the start of treatment (e.g., *Rhodoferax*, *Methylobacter*, *Methylomonas*, *Mycobacterium*). In contrast, only a few aerobic degraders decreased in the later sampling times (e.g., *Polaromonas* sp.). Since well HML-4D was not affected by whey application, we attribute these changes to the increased temperatures only.

4. Conclusions

The aim of this study was to assess the feasibility of thermally enhanced in situ reductive bioremediation at a real contaminated site with complex (hydro)geological settings.

The main findings of this study were as follows:

1. qPCR results proved an increase in total microbial biomass, in dechlorinating bacteria *Dehalococcoides mccartyi* and *Dehalobacter* sp. and in reductive dehalogenase genes (*vcrA* and *bvcA*) after the addition of a fermenting substrate and heating of a treated zone to 17–30 °C. The abundance of dechlorinating bacteria *Desulfitobacterium*

spp. varied during the heating period and increased only insignificantly. On the contrary, groundwater from deep well HML-4D, which was affected by heating and not by the substrate application, showed a general decrease in the above-mentioned biomarkers during the whole course of the pilot test.

2. Along with an increase in dechlorinating biomarkers, a strong increase in dechlorination of CVOCs was observed. By day 34, concentrations of CVOCs decreased by 87% to 96% in the groundwater from the wells most affected by heating and the substrate (AP-2, HML-4S a HV-8), and on day 103 they were below their respective LOQ. For the same wells, very short degradation half-lives of CVOCs of 5 to 6 days were calculated compared to almost no decrease in the concentrations of CVOCs in the well affected by heating only (HML-4D).
3. PCA analysis confirmed a positive correlation between temperature, TOC, abundance of dechlorinating biomarkers and the concentration of the dechlorination metabolite ethane and a negative correlation between temperature, TOC and concentration of CVOCs.
4. Community analysis by NGS revealed members of the *Chloroflexi* phylum in groundwater from all of the sampled wells, although they were present in very low quantities in deep well HML-4D. Specifically, the genus *Dehalogenimonas* and clades GIF9 and MSBL5, all known for their dechlorination activities, were found. In addition, the genera *Acetobacterium*, *Desulfomonile*, *Desulfitobacterium*, *Geobacter*, *Methanobacterium*, *Methanosarcina*, and *Sulfurospirillum*, which include species capable of anaerobic metabolic degradation of chlorinated ethenes, were also retrieved from our dataset. Of these genera, bacteria belonging to the genera *Acetobacterium*, *Desulfomonile*, *Geobacter*, *Sulfurospirillum*, *Methanosarcina* and *Methanobacterium* were stimulated in the wells affected by the substrate and heat. On the contrary, groundwater from deep well HML-4D (affected by elevated temperature only) hosted a different bacterial community, in which representatives of aerobic metabolic or aerobic cometabolic degraders of chlorinated ethenes were found. Several genera exhibited an increase in their abundance (namely *Rhodoferax*, *Methylobacter*, *Methylomonas*, *Mycobacterium*). However, their activity did not significantly degrade chlorinated ethenes.

The results of this study document the feasibility and high efficiency of the thermally enhanced in situ bioremediation of groundwater contaminated by chlorinated solvents at the contaminated site. Heating of the treated aquifer to 20–30 °C can significantly accelerate the treatment process, but only in the case of an abundance of fermentable substrate.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.047.

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