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ORIGINAL PAPER

Site-specific pre-evaluation of bioremediation technologies for chloroethene degradation

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Abstract Groundwater systems are important sources of water for drinking and irrigation purposes. Unfortunately, human activities have led to widespread groundwater contamination by chlorinated compounds such as tetrachloroethene (PCE). Chloroethenes are extremely harmful to humans and the environment due to their carcinogenic properties. Therefore, this study investigated the potential for bioremediating PCE-contaminated groundwater using laboratory-based biostimulation (BS) and biostimulation–bioaugmentation (BS-BA) assays. This was carried out on groundwater samples obtained from a PCE-contaminated site which had been unsuccessfully treated using chemical oxidation. BS resulted in complete dechlorination by week 21 compared to controls which had only 30 % PCE degradation. BS also led to a approximately threefold increase in 16S rRNA gene copies compared to the controls. However, the major bacterial dechlorinating group, *Dehalococcoides (Dhc)*, was undetectable in PCE-contaminated groundwater. This suggested that dechlorination in BS samples was due to indigenous non-*Dhc* dechlorinators. Application of the BS-BA strategy with *Dhc* as the augmenting organism resulted in complete dechlorination by week 17 with approximately twofold to threefold increase in 16S rRNA and *Dhc* gene abundance. Live/dead cell counts (LDCC) showed 70–80 % viability in both treatments indicating active growth of potential dechlorinators. The LDCC was strongly correlated with cell copy numbers ($r > 0.95$) suggesting its potential use for low-cost monitoring of bioremediation. This study also shows the dechlorinating potential of indigenous non-*Dhc* groups can be successfully exploited for PCE decontamination while demonstrating the applicability of microbiological and chemical methodologies for preliminary site assessments prior to field-based studies.

Keywords Chlorinated compounds · Biostimulation · Bioaugmentation · Quantitative PCR · Cell viability

Introduction

Improper disposal and storage of chlorinated compounds has led to widespread contamination of subsurface resources by chlorinated aliphatic contaminants such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) (SCRD 2007). Due to their toxicity and suspected carcinogenic properties, monitoring the effective remediation of this group of contaminants has gained wide public and academic interest. Standard remedial approaches such as in situ chemical oxidation (ISCO) and pump-and-treat methods have proven to be ineffectual and costly in terms of removing these substances from the environment. To date, enhanced in situ bioremediation has proved to be a promising technique for chloroethene bioremediation (Aulenta et al. 2006; Ernst 2009). This strategy involves the delivery of organic substrates into the groundwater for the purpose of stimulating

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growth and development of native microbial populations (biostimulation) by creating an anaerobic groundwater treatment zone generating hydrogen through fermentation reactions (ESTCP 2004, 2005). In some cases, specific microorganisms known for their dechlorinating capabilities may be added (bioaugmentation), but only if the natural microbial population is incapable of efficiently performing the required transformations (Löffler and Edwards 2006; Schaefer et al. 2010).

To stimulate the activity of desired indigenous dechlorinating microbes at contaminated sites, the redox conditions need to be created (especially in wells which are not completely anoxic) or maintained. This can be achieved by the addition of easily oxidizable organic carbon substrates such as acetate, lactate, butyrate, propionate, hydrogen releasing compounds (HRCs), vegetable oils and molasses (Ballapragada et al. 1997; Fennell et al. 1997; He et al. 2002; Ibbini et al. 2010; Lee et al. 2000). For bioaugmentation of chloroethene-contaminated sites, various members of the *Chloroflexi* phylum such as *Dehalococcoides ethenogenes strain* 195, GT, BAV—1, FL2, CBDB1, KB1/VC are commercially available, e.g. KB-1, Pinellas, Bio-Dechlor, SDC-9 cultures and are well known for completely reducing PCE to the environmentally safer ethene (Cichocka et al. 2010; Cheng et al. 2010; Cupples et al. 2003; Duhamel et al. 2004; Ellis et al. 2000; He et al. 2003, 2005; Hendrickson et al. 2002; Ibbini et al. 2010; Lendvay et al. 2003; Major et al. 2002; Müller et al. 2004; Schaefer et al. 2010; Sung et al. 2006). For site owners and bioremediation consulting companies, estimation of the degradation potential, the regulatory requirements and economics of the overall process at a contaminated site are important for selecting the appropriate remediation strategy. If biostimulation reduces the time and leads to meeting key bioremediation endpoints within the desired timeframe, it may well reduce the cost as less monitoring is required. On the other hand, some regulators like the Australian EPA discourage an introduction of foreign organisms to any environment that could stand a chance of causing mutation in indigenous organisms and adversely affecting the biome (Ball 2012). In such cases (as in this study), application of a bioaugmentation strategy can increase the time required for permits, inoculum and remediation costs.

This study was carried out to assess the remediation of PCE-polluted environments using biological strategies. Specifically, this study evaluated the impact of biostimulation alone (BS) and biostimulation combined with bioaugmentation (BS-BA) on the degradation of chloroethene in groundwater enrichment cultures. This involved assessment of the dechlorinating potential using laboratory-based experiments prior to future in situ anaerobic bioremediation. It is believed that the data obtained from this study would provide a better understanding of the feasibility of each treatment for site-specific applications. The bioremediation strategies were applied to PCE-contaminated groundwater obtained from a study site located in Victoria, Australia, which had been previously unsuccessfully treated using ISCO by injecting modified Fenton reagents such as hydrogen peroxide with iron chelate catalysts. To successfully apply the bioremediation approach over the failed ISCO attempt, we chose electron donors such as acetate for biostimulating dechlorinating organisms. A consortia of *Dehalococcoides* (*Dhc*) strains FL2, BAV-1 and GT was used for bioaugmentation. Time-intensive studies were performed intermittently to give a more detailed picture of comparative culture performance during which the biodegradation of electron donors and the formation of dechlorination products were documented. 16S rRNA dechlorinator-targeted quantitative real-time PCR (qPCR) was used to monitor the abundance of dechlorinating populations throughout the treatments to determine the extent of their growth in relation to the rate of chloroethenes removal. In addition, a live/dead cell count (LDCC)-based assay was conducted for quick monitoring of dechlorinating microbial cell viability during dechlorination.

Materials and methods

Chemicals

All chlorinated ethenes, ethene and other chemicals for microcosm preparation and analytical measurements were purchased from Sigma-Aldrich (NSW, Australia) with a minimum purity of 99.5 %. All gases were ordered from Coregas (VIC, Australia).

Groundwater sample collection

Since 1935, the study site located in Victoria, Australia, has a history of commercial industrial activities. The latest commercial activity at the site was foam manufacturing. Initial investigations identified chemicals of concern including PCE which has formed dense non-aqueous phase liquid (DNAPL) pools acting as a long-term source of contamination. Based upon varying PCE concentrations at different locations, groundwater samples from four different monitoring wells (MWs 1–4) were collected using the protocol suggested by Ritalahti et al. (2010). Temperature, pH, redox potential, specific conductance and dissolved oxygen were measured in groundwater that was pumped through a flow cell (YSI, VIC, Australia) onsite, with a pH/mV/EC/T/O_2 multi-parameter and corresponding probes (YSI) (Table 1).

Table 1 Field characteristics of groundwater samples at the time of collection

Monitoring well	Initial PCE concentration (μg/l)	Temp (°C)	pH	Eh (mV)	Dissolved oxygen (ppm)	EC (uS/cm)	Alkalinity (ppm)	Soluble Iron (ppm)
MW 1	146.0	19.8	6.90	184.9	9.97	12,698	960.0	0.0
MW 2	3,540.0	19.1	6.93	247.4	10.02	12,665	880.0	0.0
MW 3	130.0	18.2	7.29	160.6	7.71	17,214	820.0	3.0
MW 4	5.0	18.0	7.68	110.3	1.16	19,006	260.0	2.0

Enrichment culture development

Two sets of enrichment cultures, A and B, were set up as per the guidelines presented by Löffler et al. (2005). Set 'A' designates biostimulation only (BS), while set 'B' designates biostimulation (BS) and bioaugmentation (BA) combined approaches for dechlorinating PCE. Both culture sets were prepared in Wheaton serum bottles (125 ml nominal volume) containing 75 ml of growth medium and 20 ml of groundwater as an inoculum which were sealed with Teflon-coated butyl rubber septa and aluminium crimp caps (Alltech, VIC, Australia). Anoxic mineral salt medium was prepared as per ATCC guidelines (American Type Culture Collection; www.atcc.org) and was amended with acetate (5 mM) as an electron donor and PCE (5 μl) as an electron acceptor. Hydrogen (5 in 95 % nitrogen) was added in the headspace (5–10 % of the headspace volume of a bottle) of acetate-fed cultures at a low partial pressure of 9 kPa (He et al. 2003). Cultures were prepared under strict anaerobic conditions and maintained in an anaerobic glove box (La-Petite, Thermo Fisher Scientific Australia, VIC) using N_2:CO_2 at the ratio of 80:20 %. The mixed consortia of well-known *Dhc* strains FL2 (ATCC® BAA-2098), GT (ATCC® BAA-2099) and BAV-1 (ATCC® BAA-2100) was injected only into set B (1.0×10^4 cells/ml); however, all chlorinated compounds, vitamins and electron donors were injected into both sets from anoxic, sterilized stock solutions using a Hamilton gas tight syringe (Alltech, VIC, Australia). To minimize the contact of the inoculum with the air present in the syringes during transfers, syringes were reduced with freshly prepared, filter-sterilized aqueous sulphide solution (0.5 mM). All experiments were repeated in duplicate and appropriate controls (without inoculum and/or electron donor, autoclaved) accompanied each experiment. Immediately upon setup, all enrichment cultures turned clear from pink tint (given by resazurin redox indicator added to the groundwater) indicating establishment of reduced conditions. Culture bottles were incubated statically at room temperature (22–25 °C) in the dark, and samples were analysed over 21 weeks (147 days). Since maintaining the optimum concentration of nutrients was essential for successful dechlorination, nutrient levels were monitored at predetermined time intervals by gas chromatography–mass spectrometry (GC/MS). Every 28 days, the samples from the cultures were analysed using GC/MS in order to measure the concentration of electron donors in addition to monitoring the conversion of PCE to secondary and tertiary products. Nutrient replenishments were carried out every time analyses indicated they were exhausted.

Analytical methods

Analytical procedures for both sets A and B were conducted as described by Zaan et al. (2010) with a few modifications. Chlorinated hydrocarbons were analysed in 1 ml gas headspace using a 5975C gas chromatographic (GC) system equipped with a mass spectrometry (MS), flame ionizing detector (FID) detector and a Porabond Q column (0.32 mm × 25 m) (Agilent Tech, Australia). The GC settings were: injector temperature 200 °C; detector temperature 300 °C; oven temperature 3 min at 40 °C, followed by an increase of 10 °C min^{-1} to 70 °C, followed by an increase of 15 °C min^{-1} to 250 °C for 7 min; and carrier gas (He) with a flow rate of 2 ml min^{-1}. External standards at six different concentrations from 0 to 30 μM were used for calibration. Chloride ion analyses on 1 ml diluted cultures were performed using a Chloride Analyser 926 (Ciba-Corning, Essex, England) as per the manufacturer's protocol.

Genomic DNA extraction

Every 28 days, cells for DNA extraction were collected from enrichment cultures under sterile and anaerobic conditions with a gas tight syringe. These samples (1 ml) were centrifuged for 30 min at 16,000*g*, 4 °C with pellets being re-suspended in 1x phosphate-buffered saline (PBS) buffer and stored at −20 °C for 1 h to enhance cell lysis. Genomic DNA was extracted with a Qiagen DNeasy Tissue Kit (Qiagen, NSW, Australia) according to the manufacturer's protocol with the following modifications: For improved cell lysis, 20 μl of lysozyme (100 mg/ml) and 180 μl enzymatic lysis buffer (20 mM Tris–Cl, pH 8.0, 2 mM sodium EDTA, 1.2 % Triton X-100) were added and the mixture was incubated for at least 30 min at 37 °C (Löffler

et al. 2005). The quality of the genomic DNA extracts was evaluated by electrophoresis at 110 V for 25 min on a 2 % w/v agarose gel stained with SYBR Safe (Invitrogen, Australia) and visualized by UV transillumination (Chemi Doc^{TM} MP, BioRad, NSW).

Quantitative real-time PCR (qPCR)

Copy numbers of bacterial 16S rRNA genes from both sets A and B were quantified using universal bacterial 341F and 518 R primer set (Muyzer et al. 1993), while *Dhc*-specific genes were quantified using *Dhc*1F and *Dhc* 259R primers (Kim et al. 2010). Amplification was carried out using a MJ Mini Opticon^{TM} real-time PCR detection system (BioRad, NSW) in reactions (25 µl) containing: Sybr Green Supermix (12.5 µl) (BioRad, NSW), distiled MilliQ water (8 µl), forward and reverse primers (1.25 µl; 6 pmol/µl) and DNA template (2 µl). The thermocycling program for 341F and 518 R was performed as described by Patil et al. (2010), while *Dhc* genes were quantified as per Smits et al. (2004). External standard curves showing the relationship between *Dhc* and 16S rRNA copy numbers, and *C(T)* values were generated with 3 × 10 fold serial dilutions. Amplification efficiencies were calculated from the slopes of the standard curves according to the formula: $E = 10^{-1/\text{slope}}$ (Rebrikov and Trofimov 2006). PCR efficiencies for both 16S rRNA and *Dhc* genes were between 1.8 and 1.95 with R^2 of 0.98 and 0.99, respectively (for standard curves see Fig. S1 in supplementary material). The *Dhc* target was normalized to the 16S rRNA target of the same sample using the following calculations: $\Delta C(T)_{\text{sample}} = \text{average } C(T)_{Dhc} - \text{average } C(T)_{16S\ RNA}$. For the $2^{-\Delta\Delta C(T)}$ analysis, the normalized sample values were referenced to the values obtained for time point week 1 to study the x-fold increase in *Dhc* target, with the following equation: $\Delta\Delta C(T)_{\text{sample}} = \Delta C(T)_{\text{sample}} - \Delta C(T)_{\text{week 1}}$. The ratio of *Dhc* genes relative to the week 1 was estimated using $2^{-\Delta\Delta C(T)}$ (Livak and Schmittgen 2001; Treusch et al. 2005; Erkelens et al. 2012) (Table S1 in Supplementary material).

Cell viability test

Cell viability (live, dead and total cells) within sets A and B was measured periodically using Countess^{TM} Automated Cell Counter (Invitrogen, Australia). Enrichment culture (10 µl) was mixed with 0.4 % trypan blue stain (10 µl), and then the sample mixture (10 µl) was loaded into the cell counting chamber slides to calculate cell count and viability as per the manufacturer's guidelines. Statistical significance was determined between different samples by *t* test analyses. Linear regression analysis was carried out to assess the relationship between cell concentration/ml obtained from LDCC analyses and cell copy numbers obtained from qPCR analyses. All statistical analyses were carried out in SPSS version 20.

Results and discussion

Reductive dechlorination of chlorinated compounds

Groundwater from MW 1–4 in set A (BS) and set B (BS-BA) was used to assess the biodegradative potential of natural microbiota upon stimulation by addition of nutrients or combined with the addition of microorganisms (Fig. 1). Monitoring of electron donor levels was carried out to ensure that optimum levels of these nutrients were maintained during the experimental period. Nutrient replenishments were carried out at week 9 (BS and BS-BA) and week 17 (BS only) as required (indicated with arrows in Fig. 1). Figure 1a shows the time course of 21 weeks (147 days) for the anaerobic PCE dechlorination by the enrichment cultures in MW 1–4. Although each of these enrichments displayed different dechlorination rates, PCE conversion began sequentially in all enrichments and the secondary products of dechlorination (TCE, *cis*-DCE and VC) coexisted until ethene was formed (Fig. 1a). The conversion of PCE to TCE commenced after about 25 days lag and complete conversion to *cis*-DCE occurred by week 13. The intermediate *cis*-DCE was completely dechlorinated to VC by week 17, and the complete dechlorination of VC to ethene was achieved by week 21. Dechlorination intermediates, *cis*-DCE and VC, were temporarily accumulated in all cultures. In contrast to the rapid conversion of PCE to *cis*-DCE, the dechlorination of *cis*-DCE to VC and ethene was relatively slow.

Figure 1b summarizes the results from the enrichment culture set B treated with nutrients and the *Dhc* consortia. In set B, PCE dechlorination commenced within 30 days and resulted in the rapid accumulation of TCE. Between weeks 5 and 9, accumulation of TCE and *cis*-DCE was observed. Its subsequent disappearance was coupled to VC formation. The hydrogenolysis of chloroethenes started at week 9 and resulted in a significant decrease in TCE and *cis*-DCE and a corresponding increase in VC and ethene concentrations by week 13 and 17, respectively. With set B cultures, the wells had different responses to dechlorination; however, in all cultures, PCE was dechlorinated sequentially to ethene by week 17 (119 days). In both sets of culture, the subsequent increase in chloride ion concentration observed during reductive dechlorination confirm biodegradation, since the chloride ion is a product of reductive dechlorination (Fig. 1a, b). PCE dechlorination did not progress beyond TCE and *cis*-DCE in the uninoculated and electron donor less autoclaved controls for both sets (data not shown).

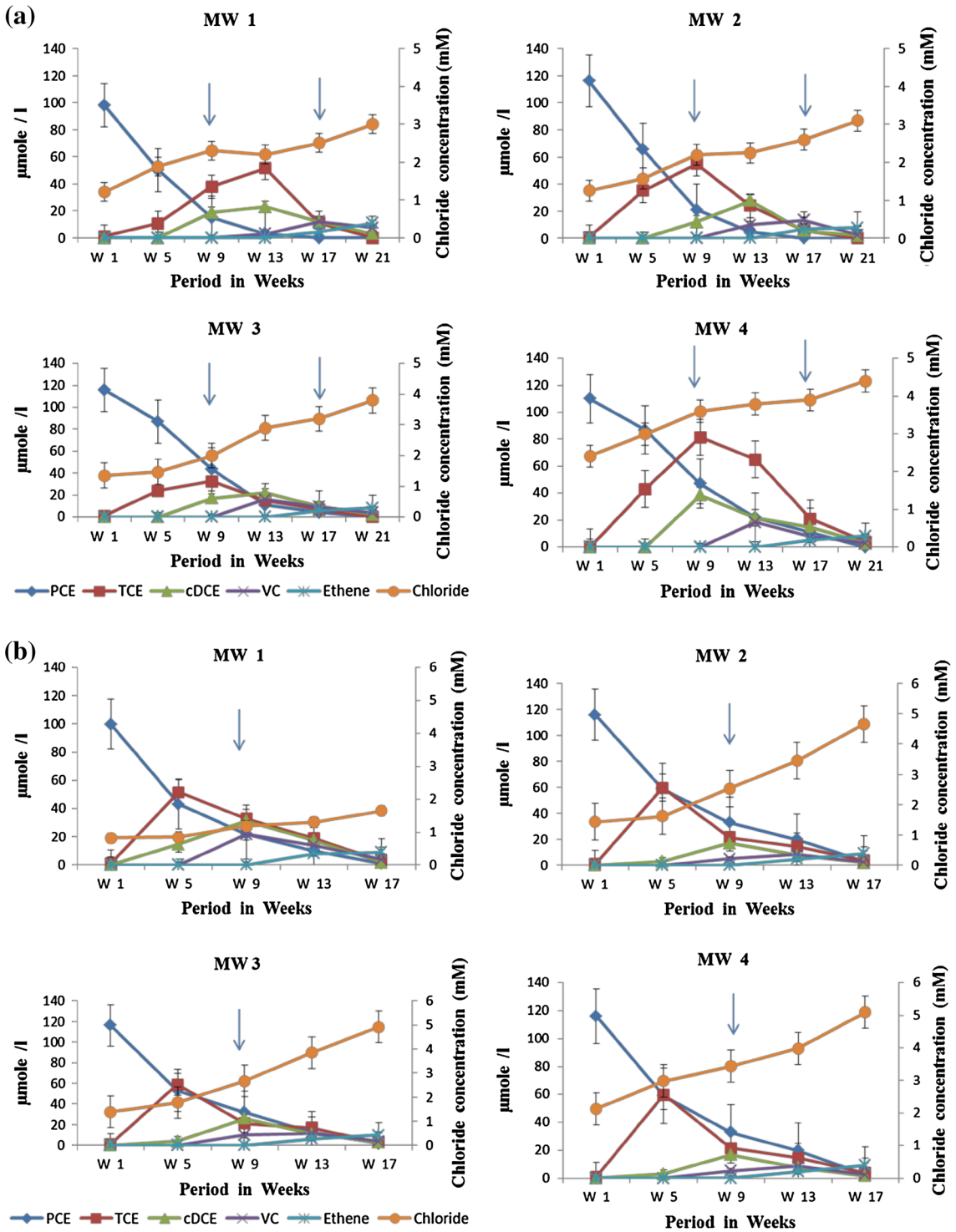

Fig. 1 PCE degradation and secondary product transformation in **a** BS only experiment (set A) and **b** BS-BA (set B) for MW 1–4 enrichment cultures. The *arrow* indicates the addition of electron donors. Data are the average of the duplicates (duplicates differed by <10 %). *Error bars* indicate standard error ($n = 2$)

The detection of some initial mass imbalance with PCE to TCE conversion in some wells is not unusual as this has been observed in other similar studies (Daprato et al. 2007; Yang et al. 2005). However, in this study, the system was stabilized after 20 days of incubation. In BS cultures, the electron donors enhanced complete dechlorination, indicating the presence of a native dechlorinating population whose dechlorinating activity was probably limited by the

lack of electron donors in the groundwater. Both BS and BS-BA treatments resulted in complete PCE conversion to ethene, but augmentation with *Dhc* consortia followed by stimulation was necessary to accelerate the rate and time of dechlorination in set B. Previously, a few studies carried out using only the biostimulation approach failed to attain complete dechlorination of PCE under laboratory and field conditions (Ibbini et al. 2010; Major et al. 2002; Lendvay et al. 2003). Incomplete dechlorination poses a major threat as accumulation of the intermediate dechlorination product VC is more toxic and carcinogenic than PCE and TCE (ASTDR 2007). Partial dechlorination is usually observed if the organisms that reductively dechlorinate ethene are not present or active at the contaminated site. However, this study reports the complete dechlorination of PCE via biostimulation, suggesting the presence of active indigenous dechlorinators in the groundwater collected from contaminated site. Fluxes of both H_2 and acetate might have controlled microbial redox processes in the biostimulation cultures that can synergistically sustain the complete reduction in PCE to benign ethene. Biostimulation increases the flux of H_2 and acetate (He et al. 2002), but competition for reducing equivalents, in particular for H_2 often limits its success (Sung et al. 2006). However, native dechlorinators stimulated in this study showed greater electron donor versatility towards acetate and H_2, indicating that an ecological niche in a well-controlled laboratory environment existed for organisms that could derive energy from the complete dechlorination of chlorinated solvents to ethene. To date, there is limited evidence using laboratory mixed cultures indicating that there might be organisms other than *Dhc* that can synergistically sustain complete reduction in PCE to ethene (Dong et al. 2011; Lee et al. 2011).

Growth-linked reductive dechlorination of chlorinated ethenes

16S rRNA gene-targeted qPCR was performed on sets A and B to study the correlation between rate of reductive dechlorination and growth of dechlorinating population in response to the BS and BS-BA treatments. Dechlorination of all four chlorinated ethenes was accompanied by an increase in 16S rRNA gene copies (Fig. 2). During BS, cell copies of indigenous microorganisms in enrichments MW 1–4 increased from week 1 to 21 from 2.01×10^4 to 1.43×10^5; 1.53×10^4 to 1.66×10^5; 2.63×10^4 to 1.79×10^5 and 2.27×10^4 to 1.77×10^5 cells/ml, respectively (Fig. 2a). Cell density in cultures MW 1 and 3 dropped in week 9 but increased by week 13. Most likely, the electron donors had become depleted as both dechlorination and growth accelerated by week 13, when nutrients were replenished. This suggests that the growth of dechlorinators is dependent on electron donors and dechlorination can slow down if these substrate are depleted (Cichocka et al. 2010). For the BS-BA experiment in set B, a mixed consortia of *Dhc* strains (1×10^4 cells/ml) was added into PCE-contaminated groundwater enrichment cultures followed by stimulation with electron donors. In enrichments MW 1–4, initial total cell concentration at week 1 was 3.65×10^4, 3.89×10^4, 3.08×10^4, 7.21×10^4 cells/ml which increased significantly to 7.98×10^5, 7.43×10^5, 6.98×10^5, 8.09×10^5 cells/ml by the end of dechlorination in week 17 (Fig. 2b). Similar to set A, a decrease in total cell copies in set B was observed for enrichments MW 3 and 4 during week 9 which increased in week 13 after nutrient replenishment. This emphasizes the fact that nutrients should be added to sustain native dechlorinators and *Dhc* activity throughout the process to achieve complete dechlorination. There was a distinct difference in the cell numbers between the two sets. The BS-BA culture contained at least an order of magnitude greater numbers of cells than in BS only cultures which is consistent with the higher ethene production rates in the augmented cultures (Fig. 2). However, over the experimental period, the number of cell copies did not increase significantly (week 1; 2.0×10^4 cell/ml and week 21 3.21×10^4 cells/ml) in the control sets where inoculum and electron donors were omitted from the system.

Analysis of groundwater from the BS enrichment culture with universal bacterial primers amplified 16S rRNA genes from indigenous dechlorinators; however, *Dhc*-specific 16S rRNA gene primers failed to detect *Dhc* population (Fig. 3a). This indicated the absence of *Dhc* species in the groundwater collected from PCE-contaminated site, although it was also possible that they could have been below the detection threshold of the primers used. Upon stimulation, enrichment cultures MW 1–4 showed ~3.86-, 2.58-, 2.96- and 3.73-fold increase in the abundance of 16S rRNA genes in set A at week 21 compared to week 1, respectively (Fig. 3a). As the absolute amounts of 16S rRNA copies measured in the same cultures stayed constant at week 1, the increase in the gene copies by the end of week 21 was found to be statistically significant (*t* test, 95 % confidence level). In order to analyse whether the *Dhc* species might be induced in set B, the *Dhc* target gene was normalized to the 16S rRNA target of the same culture (Treusch et al. 2005). Figure 3b displays the relative increase in *Dhc* genes normalized to 16S rRNA over the period of 17 weeks. While the abundance of 16S rRNA and *Dhc* genes was largely the same at week 1, bioaugmentation followed by stimulation resulted in approximately twofold and threefold increase in the abundance of 16S rRNA and *Dhc* genes by week 17, respectively (Fig. 3b). The comparison of the

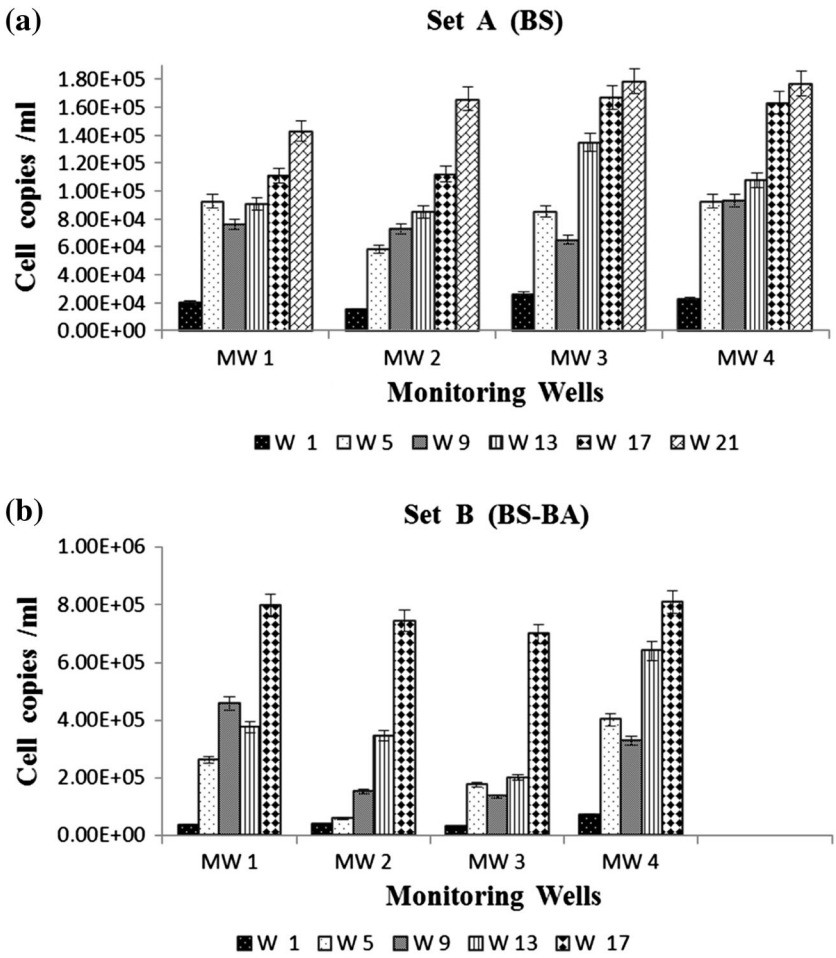

Fig. 2 Quantitative estimation of population abundance by qPCR in two PCE-dechlorinating enrichment sets of **a** BS only and **b** BS-BA over the period of 21 weeks. *Error bars* indicate standard error ($n = 2$)

bioremediation treatments and the quantitative analyses showed that rapid dechlorination in the BS-BA cultures could be enhanced due to simultaneous increase in *Dhc* and native dechlorinating microorganisms. The outcome of competition between *Dhc* and other chlorinated ethene respirers over electron donors, especially H_2 could possibly influence the rate and extent of PCE dechlorination (Becker 2006). The results obtained therefore suggested a syntrophic association between *Dhc* species and other native dechlorinators existed to carry out PCE reduction. Previously, an increase in *Dhc* gene copy numbers has been associated with enhanced reductive dechlorination of chlorinated compounds (Cichocka et al. 2010; Cupples 2008; Duhamel and Edwards 2006; Smits et al. 2004; Sung et al. 2006). Therefore, a approximately threefold increase in *Dhc* gene abundance over a approximately twofold increase in 16S rRNA genes might have synergistically contributed to the complete and rapid dechlorination of PCE in set B. BS and BS-BA treatments in enrichment cultures MW 1–4 enhanced dechlorination at different rates (Fig. 2) which could be due to varying abundance and response level of dechlorinators present at respective wells (Fig. 3).

Cell viability assay

The dechlorination process depends upon the presence and viability of an appropriate microbial population to facilitate the reaction (Major et al. 2002). The higher the viability of cells, the greater the ability of dechlorinating cells to reproduce in controlled anaerobic environment and therefore to reduce PCE by substrate utilization. Therefore, in addition to qPCR, we conducted cell viability assays to quantitatively distinguish between live and dead bacterial cells based on a total cell sample in a mixed population. For biostimulation, total cell concentration in enrichments MW 1–4 increased from weeks 1 to 21 from 2.05×10^4, 1.52×10^4, 2.69×10^4, 2.23×10^4 to 1.41×10^5, 1.67×10^5, 1.8×10^5, 1.76×10^5 cells/ml, respectively (Fig. 4a). The overall cell viability (live/dead cells based on total cell counts) throughout the dechlorination process was higher in MW 1 and 2 (80 %) than in MW 3 (75 %) and MW 4 (70 %).

For the BS-BA enrichments MW 1–4, initial total cell concentration at week 1 was 3.64×10^4, 3.87×10^4, 3.1×10^4, 7.24×10^4 cells/ml which increased to 7.96×10^5, 7.42×10^5, 6.98×10^5, 8.1×10^5 cells/ml

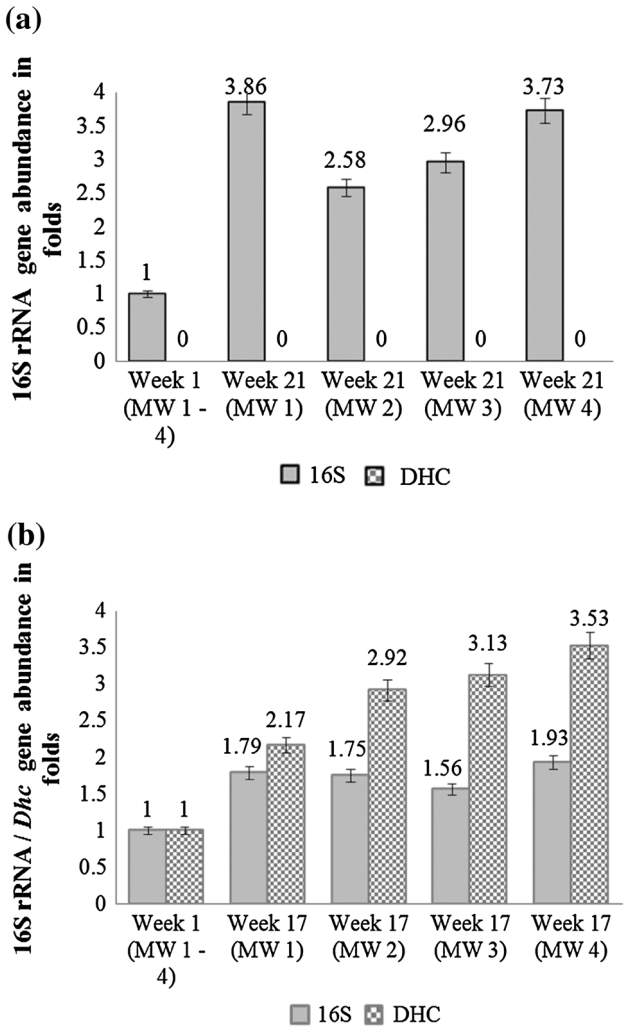

Fig. 3 Relative abundance of *Dhc* gene copies referenced to 16S rRNA of indigenous bacteria in the **a** set A with BS only and **b** set B with BS-BA microcosm experiments using qPCR analysis. *Dhc*-specific 16S rRNA genes failed to amplify in set A, indicating its absence in the groundwater. The fold increase over 21 weeks was calculated with normalized sample values and referenced to the week 1 values. *Error bars* indicate standard error ($n = 2$)

by the end of dechlorination in week 17 (Fig. 4b). Cell viability during the incubation period was approximately 75, 82, 78 and 79 % in cultures MW 1–4, respectively. Although total cell numbers of native cells increased in the BS, the numbers were at least an order of magnitude lower than BS-BA. In control samples (week 1; 2.0×10^4 cells/ml and week 21; 3.69×10^4 cells/ml), there was no significant increase in cell count and viability over the experimental period. In general, the total cell concentration within both sets increased with increasing cell viability as dechlorination progressed. Overall, the data indicated that higher cell viability can be correlated with active dechlorination and presumably growth within a microbial community.

We compared our total cell concentration results within both sets obtained using cell counter and qPCR (Figs. 2, 4). The comparative analyses demonstrated similar trend of increased total cell copies as dechlorination progressed, showing a good correlation between the LDCC and qPCR quantitative data ($r > 0.95$). In terms of methodology, this study therefore demonstrates the usefulness of using alternative low-cost quantification technique such as LDCC for monitoring total cell numbers and viability of organisms involved in reductive dechlorination. The LDCC assay provided insights into dechlorinating microbial viability which was highly similar to that obtained from qPCR analyses. LDCC could be a cheaper and faster alternative to the more rigorous qPCR method for quantitative analysis as it requires substantially less financial outlay compared to qPCR. It is also more portable and bioremediation practitioners can easily be trained in its use without the need for them to have a scientific background unlike in qPCR. Therefore, this could be of particular interest during field trials, where quick monitoring of microbial activities needs to be done to assess the progress of bioremediation.

Feasibility of bioremediation treatments

Bioremediation, both natural and enhanced, has proven to be a powerful approach for remediating chlorinated solvents (Cupples et al. 2004; Lee et al. 1997; Maymó-Gatell et al. 1997). But in recent years, there has been considerable debate over whether bioaugmentation is beneficial over biostimulation, particularly when it comes to commercial site application (ESTCP 2005). The decision to bioaugment or biostimulate is a function of several factors including economic, political and technical considerations. Within the last decade, basic research on natural microbial dechlorination mechanisms has shown that degradation of chlorinated compounds can be practically achieved by stimulating microbial reductive dechlorination (Ellis et al. 2000). However, conditions in some groundwater wells may not be completely anoxic, as observed in MW1-MW3 (Table 1) in this study. In order to ensure efficient microbial reductive dechlorination, low-H_2-generating organic substrates such as acetate, lactate or molasses could be added to create the required anaerobic conditions (Aulenta et al. 2006; ESTCP 2004; Fennell et al. 1997; He et al. 2002). Some researchers have stated that at the vast majority of sites, the desired activities will occur by stimulating existing environment and it is simply a matter of more time and more electron donors (Koenigsberg et al. 2003; Suthersan et al. 2002). Some environmental regulatory bodies are also wary of adding organisms, particularly mixed cultures in which not all of the organisms are fully characterized (Ball 2012). In such scenarios, bioremediation needs to be carried out by stimulating already present

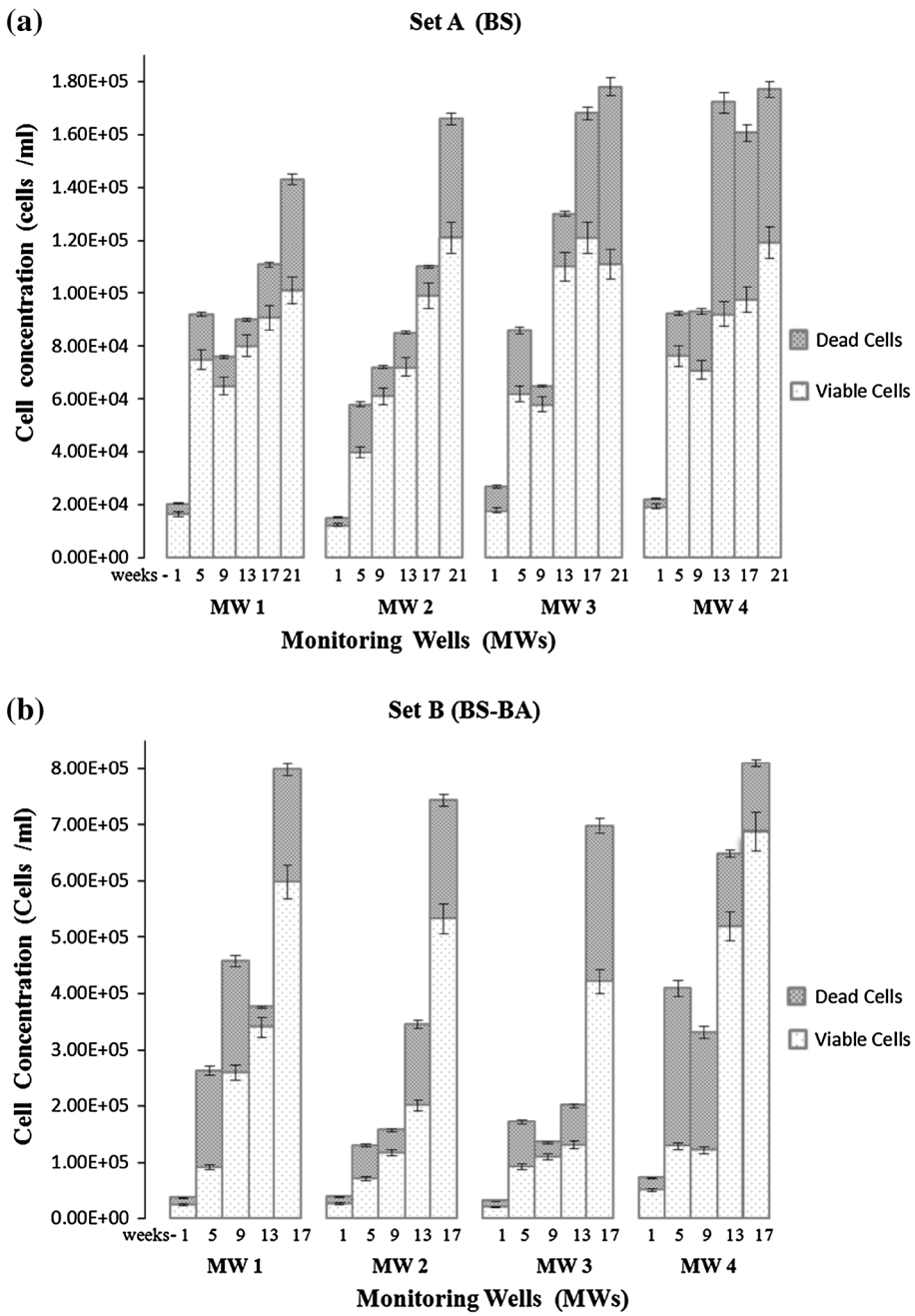

Fig. 4 Cell viability assay of **a** BS only (set A) and **b** BS-BA experiment (set B) for MW 1–4 enrichment cultures over the period of 21 weeks. *Error bars* indicate standard error ($n = 2$)

native microorganisms that are well suited for subsurface environments and well distributed spatially within the subsurface.

Studies demonstrating biostimulation followed by bioaugmentation with an enrichment culture capable of complete dechlorination of PCE to ethene indicated some benefits of this strategy over a biostimulation only approach (Ellis et al. 2000; He et al. 2003; Sung et al. 2006; Cichocka et al. 2010; Ibbini et al. 2010; Major et al. 2002). Bioaugmentation may offer a solution for contaminated sites where dechlorination is not occurring naturally (where the appropriate organisms are lacking) or where it is too slow to be practical (very low numbers of dechlorinating organisms) (Major et al. 2002). Even at sites where competent *Dhc* are present, bioaugmentation may decrease the lag time prior to the onset of dechlorination. This is particularly true for sites desiring rapid remediation due to an impending property transaction and stringent regulatory or commercial deadlines. Although costs for the culture solutions needed for bioaugmentation are decreasing, the inoculum itself can still be a significant expense at relatively large sites. The choice of electron donor can also affect the decision whether to or not to bioaugment. Bioaugmentation with *Dhc*-containing cultures may not

always ensure that complete dechlorination of PCE can be sustained in the presence of PCE to *cis*-DCE dechlorinating specialist unless adequate electron donor can be specifically delivered to *Dhc* populations (Becker 2006). In order for bioaugmentation to succeed, a niche must be created that is specifically available for augmented microorganisms (Ellis et al. 2000). The fate of electron donors and their fermentation products including not only H_2 but also other intermediates is of critical importance for understanding the response of dechlorinating communities. Using high-strength soluble donors (such as lactic acid, molasses) added at frequent intervals may make bioaugmentation more attractive, because it is relatively expensive to operate the system for even a few months without achieving complete dechlorination (ESTCP 2005). On the other hand, bioaugmentation may be less attractive when using long-lasting, less soluble donors (such as chitin, HRC™ or vegetable oil), because the time and additional operational and maintenance needed to achieve complete dechlorination may represent a relatively small incremental cost (Ibbini et al. 2010). Overall, this fundamental information on the ecology and biophysical interaction of community members involved in the partial and complete dechlorination process should help to better understand and design appropriate remediate strategies for chloroethene-contaminated sites.

Conclusion

This study reported a comparative pre-evaluation of BS and BS-BA approaches for the PCE remediation on groundwater samples obtained from a PCE-contaminated site. Both approaches resulted in complete dechlorination in samples, with the BS-BA approach resulting in a shorter dechlorination time frame. However, even when bioaugmentation cannot be applied either due to costs or legislative difficulties, biostimulation strategy can still be effectively applied leading to remediation of site. 16S rRNA-based qPCR and LDCC analyses were used to assess the dechlorinating community potential during PCE dechlorination. Regression analysis showed that LDCC represents a low-cost and 'low-tech' approach to monitoring the dechlorinating potential of community resulting in substantial time and financial savings. Given the variety of environmental factors expected in different contaminated sites, this study has demonstrated the value of site-specific pre-evaluation of PCE-contaminated sites using a combination of microbiological and chemical approaches; a crucial step to design a successful in situ field-based bioremediation strategy.

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