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Bioremediation Potential in Recalcitrant Areas of PCE in Alluvial Fan Deposits

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***Abstract*—**In the transition zone between aquifers and basal aquitards, the perchloroethene (PCE)-pools are more recalcitrant than those elsewhere in the aquifer. Although biodegradation of chloroethenes occur in this zone, it is a slow process and a remediation strategy is needed. The aim of this study is to demonstrate that combined strategy of biostimulation and *in situ* chemical reduction (ISCR) is more efficient than the two separated strategies. Four different microcosm experiments with sediment and groundwater of a selected field site where an aged pool exists at the bottom of a transition zone were designed under i) natural conditions, ii) biostimulation with lactic acid, iii) ISCR with zero-value iron (ZVI) and under iv) a combined strategy with lactic acid and ZVI. Biotic and abiotic dehalogenation, terminal electron acceptor processes and evolution of microbial communities were determined for each experiment. The main results were: i) reductive dehalogenation of PCE-pools occurs under sulfate-reducing conditions; ii) biostimulation with lactic acid supports more pronounced reductive dehalogenation of PCE and trichloroethene (TCE), but results in an accumulation of 1,2-cis-dichloroethene (cDCE); iii) ISCR with ZVI produces a sustained dehalogenation of PCE and its metabolites iv) combined strategy of biostimulation and ISCR results in a fast dehalogenation of PCE and TCE and a sustained dehalogenation of cisDCE. These findings suggest that biostimulation and ISCR with ZVI are the most suitable strategies for a complete reductive dehalogenation of PCE-pools in the transition zone and further to enable the dissolution of dense non-aqueous phase liquids.

***Keywords*—**Aged PCE-pool, anaerobic microcosm experiment, biostimulation, in situ chemical reduction, natural attenuation.

I. Introduction

CHLORETHENES are chlorinated solvents that belong to the group of dense non-aqueous phase liquids (DNAPLs) and have been detected in numerous contamination events [1]. These compounds have an elevated toxicity [2], and in the case of PCE, TCE and vinyl chloride (VC), the risk of cancer increases under exposure [3].

The transition zones between granular aquifers and basal aquitards were described by [4] as a reasonable paradigm for the DNAPL source area architecture in granular aquifers. Such zones are characterized by the presence of numerous thin silty-clay layers interstratified with coarser-grained layers (i.e., sands and gravels). The low contaminant mobility in transition zones should be noted because it implies that DNAPL sources in these zones are much more recalcitrant than those in the rest of the aquifer, which has far-reaching implications for the environment.

Chloroethenes may be recalcitrant under certain conditions over long periods (several decades or longer). However, they can be biotransformed under anoxic conditions by biotic reductive dehalogenation [5]-[7] carried out by organohalide-respiring bacteria (OHRB) [8]. Reductive dehalogenation of chloroethenes occurs sequentially from PCE to TCE to cDCE, which is the most common metabolite in TCE biodegradation [9], to VC, and to ethene or ethane [1], [10]. Reductive dehalogenation of PCE and TCE to cDCE can be carried out by a wide range of microorganisms such as *Dehalococcoides*, *Geobacter*, *Dehalobacter*, *Desulfitobacterium*, *Sulfurospirillum*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio*, *Desulfuromonas* and *Dehalogenimonas* spp. [11], [12]. However, only *Dehalococcoides* spp. have been described as capable of the complete reductive dehalogenation of PCE to ethene [13], [14].

The reductive dehalogenation of PCE and TCE may occur under nitrate- [15], Mn- and Fe-reducing conditions and under sulfate-reducing and methanogenic conditions [6], [9], [16], especially if an excess of electron donors is supplied to achieve substantial dehalogenation [17]. The reductive dehalogenation may be wholly or partially inhibited by competition for electron donors depending on environmental conditions. This competition occurs between communities of OHRB and communities of anaerobic hydrogenotrophic (including reducers of NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-}), autotrophic methanogenic, and homoacetogenic microorganisms [18].

High concentrations of chloroethenes in the contaminant source may inhibit microbial activity [19], [20], causing a decrease in the microbial richness of the population due to their toxicity [21]. This potential inhibition of microbial activity does not affect all chloroethene-biodegrading microorganisms equally, inducing a specialization in the microbial community, which decreases the microbial richness of the population [22]. For example, *Dehalobacter restrictus* PER-K23 [23], *Desulfuromonas chloroethenica* TT4B [24], *Sulfurospirillum halorespirans* DSM 12446 T [25], and *Dehalococcoides mccartyi* [26] are completely inhibited by high concentrations of PCE. By contrast, other species such as *Desulfitobacterium* Y51 [27], *Clostridium bifermentans* DPH-1 [28], *Enterobacter*

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agglomerans MS-1 [29], and *Desulfuromonas michiganensis* BB1 and BRS1 can dehalogenate PCE and/or TCE even at saturation concentrations [30]. In addition, high concentrations of chloroethenes may inhibit the activity of microorganisms that potentially compete with OHRB, such as, for example, methanogenic populations [31].

ISCR with ZVI has been proven an efficient strategy to dehalogenate chloroethenes [32]. The reaction mechanisms of ZVI to reductively dehalogenate chloroethenes are complex and produce different end-products depending on the conditions [10]. For example, [33] found that 80% of TCE was dehalogenated to ethene and ethane (in a ratio of 2:1), together with other subproducts, such as methane, propane, propene, 1-butene and butane. Other investigations detected similar products during abiotic reductive dehalogenation of PCE and TCE with ZVI [34], e.g. VC, cDCE, 1,1DCE, methane, chloroacetylene, acetylene, ethene, ethane. The great variety of abiotic degradation pathways by ZVI potentially avoids the accumulation of toxic by-products, in contrast with biodegradation, where by-products such as cDCE and VC may accumulate in the aquifer [35].

Each remediation strategy presents several limitations. For example, the accumulation of metabolites in the case of biostimulation and the relatively long time of application in the case of monitored natural attenuation (MNA) and ISCR with ZVI. Consequently, different remediation strategies can be applied sequentially [34] or combined [35]. It is common to sequentially apply a remediation strategy in the source (such as biostimulation or injection of chemicals) and subsequently apply a MNA in the plume. In other cases, when the biostimulation of chloroethenes with lactic acid or lactate leads to an accumulation of cDCE [36], a second strategy is needed to completely dehalogenate this compound, such as bioaugmentation [37], ISCR or oxidative biostimulation. Otherwise, ISCR technologies are usually applied in an oil emulsion [38]. This process increases the disponibility of ZVI; therefore, it also increases the abiotic dehalogenation of chloroethenes, while OHRBs are stimulated.

For a better understanding of the processes affecting the fate of chloroethenes, an integrative set of chemical and biological methods is needed. For instance, there is a need to monitor the different terminal electron acceptor processes (TEAPs) [39]. Moreover, compound-specific stable isotope analysis (CSIA) has been applied efficiently as direct proof of the biological degradation of chlorinated solvents and to distinguish the different processes affecting the fate of these pollutants [40].

Different molecular techniques have been used to investigate the complexity of the reductive dehalogenation processes in microbial systems. To characterize the microbial consortia in the presence of chlorinated solvents, terminal restriction fragment length polymorphism (T-RFLP) has been used efficiently in laboratory experiments [41]-[43] and in field studies [44]-[47].

The aim of this article was to define an efficient bioremediation strategy to treat a source of chloroethene in the context of alluvial fan deposits. Moreover, we tested a combination of biological and chemical strategies to achieve better efficiency. Four different microcosm experiments were designed under i) natural conditions, ii) biostimulation with lactic acid, iii) ISCR with ZVI and under iv) a combined strategy with lactic acid and ZVI. Microcosm experiments have been successfully used to choose the most efficient remediation strategy [5], [48], [49] and to determine how geochemical conditions would change and microbial communities would adapt [50], [39].

The working hypothesis of this article is that toxicity and electron supply are the main limiting factors of the biotic reductive dehalogenation of chloroethenes in presence of DNAPL phase.

II. Methods and Materials

A. Site Description

Reference [51] have characterized that the source of the PCE is located at the contact of the transition zone to the basal aquitard (lower part of the aquifer) and that there is evidence that reductive dehalogenation is active. Moreover, it has been proven that MNA is not a viable strategy in the middle term and that the source should be treated.

B. Design of Microcosm Experiments

Four remediation strategies were studied: Natural attenuation, biostimulation with lactic acid, ISCR with ZVI and a combined strategy with lactic acid and ZVI. Each experiment consisted of two life and two abiotic (autoclaved) controls. An autoclave (Selecta Model Autester 75 E DRY-PV) was used (for four periods of 30 minutes reaching a temperature of 121 °C, a pressure of 1 atm, and saturated vapor conditions) to sterilize the control microcosm bottles containing 1200 mL of groundwater, 250 mL of sediment, and 50 mL of stock solution with 147 mM $HgCl_2$ (Riedel-de Haën, CAS 7487-94-7) as a bactericide, following [52]. The remaining materials were cleaned and sterilized with methanol (MeOH, Merck, ISO Pro analysis). Experiments were conducted in an anaerobic chamber (Glove-type box, Coy Laboratory Products Inc.).

The sediment used in the experiments was obtained from cores recovered from B-F2UB between 6.77 and 7.46 m [51]. Sediment cores, corresponding to the transition zone to the basal aquitard (foc = 0.016%, Mn = 5.7 mmol/g and Fe = 174.1 mmol/g [51]) were homogenized. Groundwater for the experiments was pumped from conventional well S3 (located 3 m from B-F2UB) and collected in Pyrex bottles (1 L). Sediment and groundwater were stored in a cold room at 4 °C in total darkness until usage.

Groundwater initially showed oxidizing conditions, with dissolved oxygen, NO_3^- and SO_4^{2-} concentrations of 1.55 mg/L, 100 and 60 mg/L, respectively, and an absence of Mn^{2+} and Fe^{2+} [53]. Dissolved oxygen content was reduced to 0.10 mg/L by purging with N_2 gas (as described by [54]) for 60 min to promote the most favorable conditions for the reductive dehalogenation of chloroethenes.

TABLE I
ADDITION AND SAMPLING TIMES ALONG MICROCOSM EXPERIMENTS

Addition				Sampling					
Nº days	$HgCl_2$	Lactic acid	Chloroethenes	$\delta^{13}C_{CE}$	Anions	Metals	Acetate	Bacteria.	Gases
Previous	Ad	Ad	-	-	-	-	-	-	-
0	-	-	Sp	-	Sp	Sp	Sp	Sp	-
14	-	-	Sp	Sp	Sp	Sp	Sp	Sp	-
28	Ad	Ad	Sp	-	-	-	-	-	-
58	-	-	Sp	Sp	Sp	Sp	Sp	Sp	-
77	Ad	-	Sp	Sp	-	-	-	-	-
98	-	-	Sp	Sp	-	Sp	Sp	Sp	-
113	-	-	-		-	-	-	-	Sp
176	Ad	Ad	Sp	Sp	Sp	Sp	Sp	Sp	-
259	Ad	-	Sp	Sp	Sp	Sp	Sp	Sp	Sp

Ad=Addition, Sp= sampling, CE = chloroethenes,

Each bottle was filled with 850 g of homogenized sediment and 1100 mL of groundwater, which represents 17% for sediment and 55% for groundwater of the total volume of the bottle. As the bottles had a capacity of 2000 mL, the remaining 28% was the anaerobic atmosphere of the chamber (95% N_2 and 5% H_2). In ISCR and the combined strategy microcosm experiments, a total of 5 g of granular ZVI (Panreac Quimica, iron metal fine granulated QP 99%) was added. Due to the loss of PCE during the purge of dissolved oxygen, pure PCE (Sigma-Aldrich, reagent grade, 99.9%) was added to a final concentration of 100 µM. Bottles were sealed with Mininert® valves (SUPELCO analytical) and insulating tape. Furthermore, in the anaerobic chamber, all bottles were arranged horizontally on shelves and covered by a thick black cloth to be preserved in complete darkness.

Periodically, 2 mL of lactic acid (Sigma-Aldrich, 85%, Table I) was injected in the microcosm experiments of biostimulation and combined strategy. Additionally, periodically, 8 mL of stock solution 6% $HgCl_2$ (Riedel – de Haën, 99.5%, 31005, Table I) was injected in all control experiments.

Water samples from the microcosm experiments (Table I2) were collected to study the time evolution of 1) concentrations of the main inorganic electron acceptors (SO_4^{2-}, NO_3^- and NO_2^-), acetate, chloroethenes (PCE, TCE, isomers of DCE, and VC), ethine, ethene, ethane, methane, Mn^{2+} and Fe^{2+}; 2) isotope values of chloroethenes; and 3) microbial communities. Sodium azide (N_3Na Fluka, purum pa) was added to the microcosm water samples immediately after being collected to inhibit bacterial activity. Before analysis, vials containing water and gas samples were stored at 4 °C in total darkness. In the case of microbial analysis, a total of 20 mL of aqueous phase was taken with a sterile syringe. Then, water was filtered with a filter system (Swinnex, Millipore) and 0.2 µm filters (IsoporeTM membrane filters, Millipore). Filters were kept in sterile Eppendorf cones and stored at - 20 °C until further extraction and analysis.

C. Chemical Analysis

All chemical analyses were conducted in the laboratories of Scientific-Technical Services at the University of Barcelona. Gas chromatography-mass spectrometry (GC-MS) was used to determine chloroethenes in water samples. The limits of quantification of PCE, TCE, cDCE, transDCE, 1,1-DCE and VC were 2.16, 1.92, 1.68, 1.68, 1.62 and 1.31 µg/L, respectively (i.e., 0.0130, 0.0146, 0.0173, 0.0173, 0.0167 and 0.0210 µmol/L). CSIA was performed using GC combustion isotope ratio MS (GC-C-IRMS, Delta Plus XP model, Finnigan) to determine $\delta^{13}C$ values in the chloroethenes of the water samples. These analyses were performed in duplicate and followed a protocol that involved removal of VOCs by direct adsorption from the aqueous phase [55]. Extraction of each sample was performed by inserting an adsorbent fiber (Supelco; SPME Fiber Assembly 75 µm Carboxen PDMS) into 20 mL of water stored in a glass vial (SUPELCO analytical) closed with a septum of silicone and with the water sample in continuous agitation for 30 min to adsorb the VOCs on the fiber. The standards used were PCE, TCE, and cDCE (Sigma-Aldrich) determined using Elemental Analyzer Flash EA 1112 coupled to an IRMS delta C Thermo Finnigan. NO_3^-, NO_2^- and SO_4^{2-} were analyzed using ion chromatography (IC) following EPA protocol 9056. Fe^{2+} and Mn^{2+} were analyzed using absorbance spectrophotometry (Reactive tests 14761 and 14770 for Fe^{2+} and Mn^{2+}, respectively, using Spectroquant NOVA60, Merck). Acetate was analyzed using HPLC (Agilent 1100) following the protocol for organic acids. CO_2 was removed by a $CaCO_3$ trap to determine the concentrations of methane, ethane, ethene and ethine. Semiquantitative concentration of gases was determined using GC.

D. Molecular Analysis

Molecular analyses were conducted to verify the presence of bacterial communities in water samples and to analyze their role in the biotransformation of chloroethenes. The analyses were performed at the laboratories of Helmholtz Centre for Environmental Research–UFZ (Leipzig-Germany). Genomic DNA was extracted from filters using Kit Ultra Clean Soil DNA (MoBio) following the manufacturer's protocol to perform T-RFLP and clone library analysis.

PCR was used to amplify part of the 16S rRNA genes from Eubacteria. The PCR mix per reaction contained 10 µL de GoTaq® Green Master Mix (Promega), 0.5 µL (each) forward and reverse primers (10 µM, Promega), 1.5 µL from the template and 7.5 µL molecular-grade water (Promega, Madison, WI, USA). Eubacterial primers 27f [56] and 1492r

[56] were used to amplify nearly the complete 16S rRNA gene using the following scheme: 95 °C (15 min); followed by 25 cycles of 95 °C (45 s), 52 °C (45 s) and 72 °C (120 s); and completed with an additional 15 min at 72 °C. If there was a positive signal, the same conditions of PCR were repeated with fluorescent primer 27FAM in order to perform T-RFLP analysis. If there was a negative sign, a second round of PCR for T-RFLP analysis employing universal primer 1378r [57] and fluorescent primer 27FAM was completed. The same master mix was used with the addition of 1 µL from the PCR product. The PCR scheme was 95 °C (15 min); followed by 30 cycles of 95 °C (45 s), 52 °C (45 s) and 72 °C (120 s); and completed with an additional 15 min at 72 °C. The PCR product was purified using purification Kit Wizard® for Genomic DNA (Promega). A total of 50 ng of purified DNA was restricted twice for each sample with three different restriction enzymes (HaeIII, HhaI and MspI, Thermo Scientific) and their respective buffers. Dry DNA was dissolved with Hi-DiTM Formamid (Applied Biosystems) using the standard GeneScan™ 500 ROX™ and was analyzed using an ABI 3100 Genetic Analyzer (Applied Biosystems) and the Genemapper 3.7 Software (Applied Biosystems).

Clones of three water samples were sequenced to characterize the microbial communities responsible of reductive dehalogenation. These samples correspond to a 1) bacterial community of a natural attenuation experiment of T5 (267 days) where reductive dehalogenation of PCE and TCE was detected; 2) a bacterial community of the combined strategy experiment of T1 (22 days) where reductive dehalogenation of PCE and TCE was detected; and 3) a bacterial community of a combined strategy experiment of T5 (267 days) where reductive dehalogenation of cDCE and VC was detected. The PCR products obtained with primers 27f and 1492r and extracted genomic DNA as previously described were ligated into the pGEM-TEasy™ vector (Promega, Madison, WI, USA) and were transformed into competent *E. coli* JM109 cells. Procedures of plasmid extraction, amplification, grouping into OTUs, purification, and sequencing were performed following the protocol from [58].

E. Molecular Analysis

T-RFLP results were used to determine the microbial diversity (microbial richness). Microbial diversity was assessed with the number of RF greater than 50 bp and greater than 1% of the total area. From the three different results obtained (one for each restriction enzyme), the larger was taken as valid. The actual microbial diversity is 3 or 4 times higher than the number of RFs, according to [59], [60].

The density of the microbial community (degree of development) was estimated qualitatively by checking the presence or absence of a signal in the first round of PCR with primers 27f-1492r. Therefore, bacterial communities were characterized by a high degree of development if there was a sign in the first round and with a low degree of development if there was only a sign in the second round of PCR.

Sequences from clone library analysis were compared to sequences from databases using the BLASTN search tool [61] and mapped onto the NCBI taxonomic hierarchy using the metagenome analyzer (MEGAN) to define the most likely ancestor for each query sequence (version 5.2.3) [62]. Sequences were virtually digested with restriction enzymes HaeIII, HhaI and MspI. When there was a 100% positive match between T-RFLP results and virtual digestion, RFs were positively identified. If there was no positive match, RFs were identified through the T-RFLP database or remained as unknown RF.

III. Results and Discussion

A. Natural Attenuation

Under natural conditions, biotic reductive dehalogenation processes were active from day 98, with a substantial increase in TCE, cDCE and 1,1DCE (Fig. 1 (a)) and a slight shift of $\delta^{13}C_{PCE}$ and $\delta^{13}C_{TCE}$ to more positive values (Fig. 1 (c), from -26.5‰ to -22.7‰). These reductive dehalogenation processes are effective when there are sulfate-reducing conditions (Fig. 1 (e), after day 58). Biotic reductive dehalogenation led to a small isotopic fractionation of PCE (ε < -1‰, Table II), similar to isotopic fractionation at the site studied [53] and in other studies [63]. Prior to the start of these processes, the only process that reduced the content of PCE is abiotic dehalogenation (degradation of 12% of total PCE). Products of biotic dehalogenation of cDCE (VC, ethane, ethene and ethine) were not detected (Table I).

Bacterial communities associated with the sulfate reduction and reductive dehalogenation of PCE and TCE are characterized by a well-developed bacterial community (Fig. 1 (g)), a mid-to-high richness (Fig. 1 (g)) and the predominance of the Firmicutes phylum. Specifically, these bacterial communities (Fig. 2) were characterized by the presence of fermenting microorganisms of the Peptococcaceae family [64] and bacteria related to *P. propionicus* [65], [66], sulfate reducers *D. lacus* and *D. adipica* [67] and Fe reducers in the Gallionellaceae family [68]. No OHRBs were detected; thus, it can be assumed that these microorganisms were a proportionally smaller part of the bacterial community, as described by [69], although some of the sulfate-reducing bacteria may degrade TCE and PCE, as described by [70]-[72].

The biotic reductive dehalogenation process does not occur or is not significant prior to sulfate reduction because NO_3^-, Mn^{4+} and Fe^{3+} (Fig. 1 (e)) compete with PCE and TCE as electron acceptors. Therefore, denitrification and Fe and Mn reduction processes override the reductive dehalogenation process. During denitrification and the reduction of Fe and Mn (Fig. 1 (e)), bacterial communities are well developed (Fig. 1 (g)), and several populations have been identified whose functions are unknown, with the exception of the fermenting bacteria of the Peptococcaceae family. Subsequently, a bacterial community undergoes a lag phase (a less-developed bacterial community, Fig. 1 (g)), with a variation in its structure and a predominance of metal-reducing Gallionellaceae and sulfate-reducing *D. lacus*.

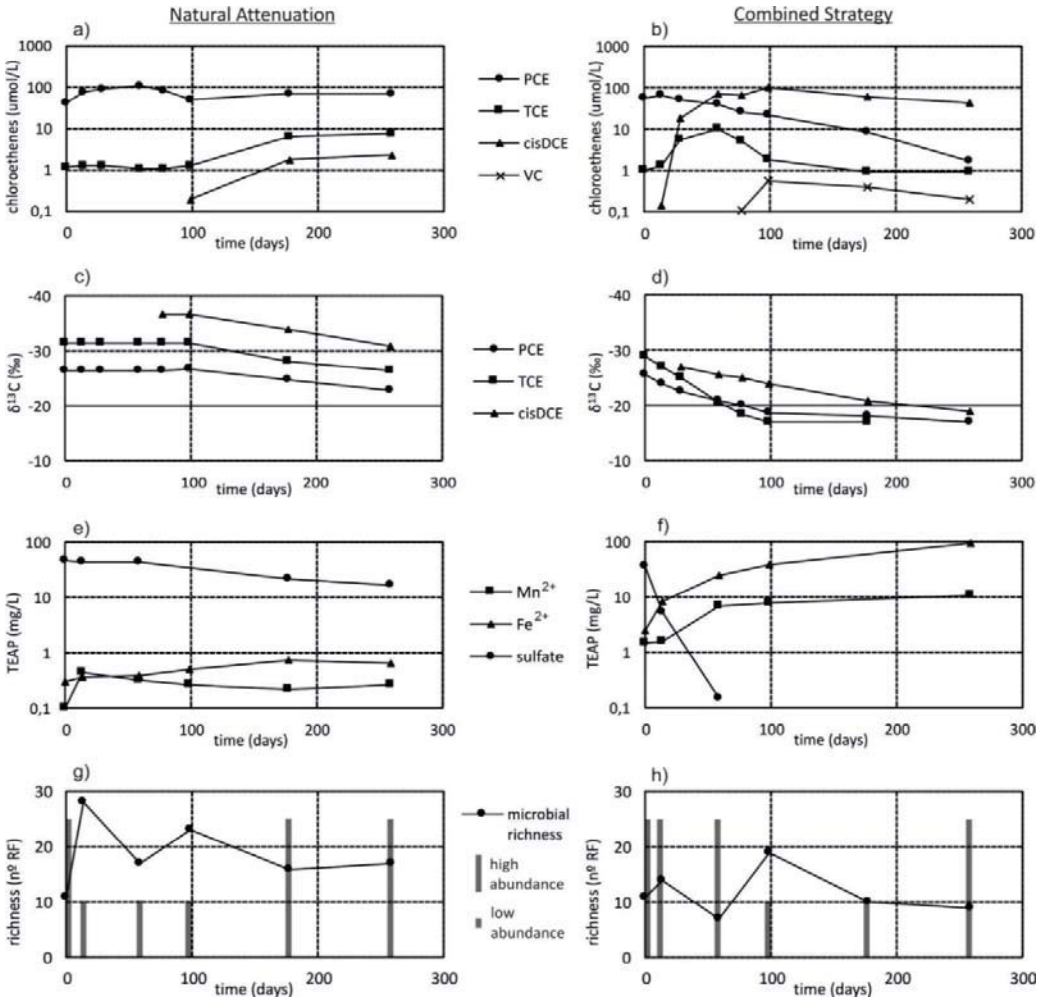

Fig. 1 Evolution of concentration of chloroethenes (a) and (b), isotopic composition of chloroethenes (c) and (d), Mn^{2+}, Fe^{2+} and sulfate as TEAP indicators (e) and (f) and richness and abundance of microbial communities (g and h) of microcosm experiments of natural attenuation (a), (c), (e) and (g) and combined strategy of acid lactic and ZVI (b), (d), (f), (h)

B. Limiting Factors of Dehalogenation

The main limiting factors of reductive dehalogenation that have been characterized are competition and overriding of reductive dehalogenation by TEAP, a lack of bioavailable electron donors and displacement of a potentially dehalogenating bacterial community by a fermenting bacterial community.

One of the main limiting factors is competition on electron donors. The OHRBs characterized in these experiments are active under sulfate-reducing conditions; thus, the processes of denitrification and Fe and Mn reduction are energetically more favorable than reductive dehalogenation and sulfate reduction, as has been seen in other site studies, such as [6], [9], [16].

Another limiting factor is the lack of organic substrate. This limitation means that denitrification; Mn, Fe and sulfate reduction; and PCE reductive dehalogenation are slower and start later in the natural attenuation experiments compared to biostimulation experiments.

Lactic acid injection in biostimulation experiments involves the accumulation of cDCE and a bacterial community with exclusive acetogenic and fermenting metabolism (data not shown). This is a common problem for bacterial dehalogenating communities [14], [30], [73]. The absence of reductive dehalogenation of cDCE may be due to toxicity, although it is possible that there is strong competition between acetogenic microorganisms and OHRBs for the use of H_2, and, in the absence of lactic acid, this competence could be displaced in favor of OHRBs.

TABLE II
EVIDENCES RELATED TO DEHALOGENATION PROCESSES

	Natural Attenuation		Biostimulation and ISCR	
	biotic	abiotic	biotic	abiotic
Total degraded mass (%)	34.5	11.3	97.8	94.9
ε_{PCE}(‰)	<-1	-	-2.5±	-3.1±0.6
Isotope balance	B	B	UB	UB
Methane	+	-	+	+
Ethane	-	-	+	+
Ethene	-	-	+	-
Ethine	-	+	-	+

B= balance; UB = unbalanced; + = presence; - = absence; ε given with ± interval of confidence of 95%.

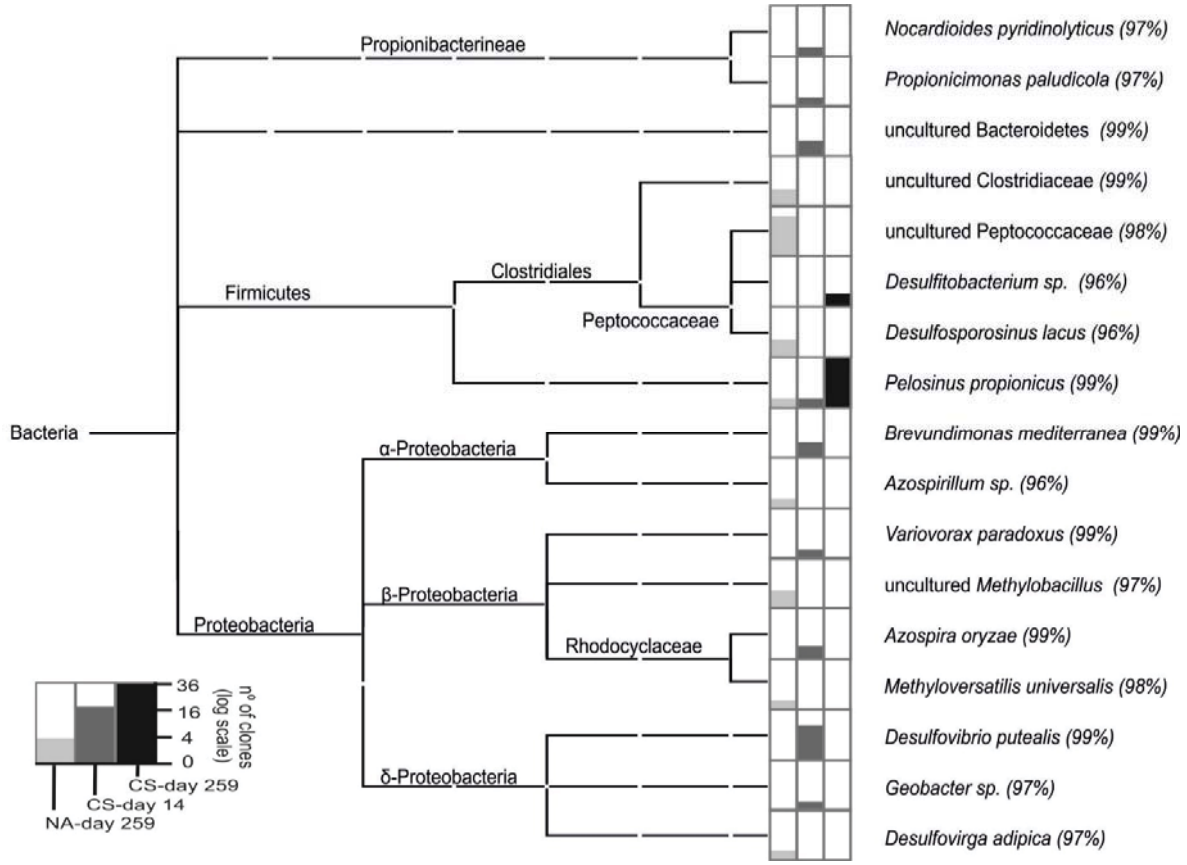

Fig. 2 Phylogenetic tree of the sequenced samples. Percentage refers to the similitude degree with database sequences. NA = natural attenuation; CS = combined strategy

One possible limiting factor that occurs in many dehalogenating bacterial communities is the absence of OHRBs capable of mineralizing PCE [74]. This seems not to be the case here because *Dehalococcoides* and complete reductive dehalogenation (based on the presence of VC) have been detected in the pollutant source of the study area [51].

C. ISCR with Biostimulation Conditions

Reducing conditions pass quickly to acetogenic and methanogenic (day 28 concentration of NO_3^- and SO_4^{2-} is practically zero, the concentration of Mn^{2+} and Fe^{2+} is already high and the concentration of acetate is near 1200 mg/L, Fig. 1 (f)). Therefore, these conditions allow the reductive dehalogenation of PCE to TCE, and later to cDCE and 1,1DCE, to be very fast (Fig. 1 (b)). The evolution of isotopic composition in these experiments is higher than in the biostimulation experiments (data not shown), with isotopic fractionation of PCE, TCE and cDCE (Fig. 1 (d)), and ε values of -2.5 ±0.5‰ (Table II). However, unlike the biostimulation experiments, chloroethenes are isotopically unbalanced toward more positive values (Table II) due to the formation of a non-chlorinated product. In the control experiments, PCE has a ε value of -3.1 ± 0.6‰ and is also isotopically unbalanced toward more positive values (Table II).

The bacterial community responsible for the reductive dehalogenation of PCE and TCE is characterized by a low richness and a high degree of development (Fig. 1 (h)), and predomination of Proteobacteria phylum (Fig. 2). This bacterial community has a predominance of sulfate-reducing bacteria related to *Desulfovibrio putealis* (Fig. 2) and the presence of several microorganisms, among them, metal and sulfate reducer (and potential OHRB) *Geobacter* sp., fermenting bacteria *Propionicimonas paludicola*, *Pelosinus propionicus* and uncultured Bacteroidetes (Fig. 2). This microbial community shares the same characteristics as the dehalogenating bacterial community of PCE and TCE of biostimuation experiments.

Once the majority fraction of chloroethenes is cDCE (Fig. 1 (b)), abiotic dehalogenation and biotic reductive dehalogenation of cDCE and VC simultaneously occur and are not differentiable as it has not been determined what the percentage of each process is. Nevertheless, there is a presence of ethene, ethane and methane in the active experiments (Table II), while in the control experiments, ethine and methane are present (Table II). During dehalogenation of cDCE, the bacterial community evolves, similar to the biostimulation experiments, to a fermenting bacterial community formed by the Firmicutes phylum and dominated by bacteria related to *P. propionicus* (Fig. 2). Nonetheless, and unlike biostimulation experiments, this community has less permanence during the experiment and allow more diversity. Moreover, there is the presence of the sulfite reducer and potential OHRB *Desulfitobacterium* sp. (Fig. 2).

The presence of *D. lacus* at day 176 (Fig. 2) highlights the

presence of ZVI conditions and stimulates the bacterial community, and these microorganisms perform a fermentative metabolism due to the absence of sulfate. However, lactic acid is the most important conditioning factor according to the degree of similarity between the bacterial communities of the biostimulation and combined strategy experiments. The results demonstrate that this strategy is the most efficient.

IV. Conclusions

As seen in the field [51], [53], natural attenuation is not an efficient strategy. Microcosm experiments prove that the main limiting factors are the lack of electron donors and toxicity due to the high concentration of PCE in the source area.

Desulfovibrio putealis is an OHRB capable of reductively dehalogenating PCE and TCE in high concentrations when electron donors are supplied to the environment [71], [75]. However, there is no OHRB at the site capable of degrading cDCE. Therefore, there is a need to use a chemical reagent to reduce the total amount of chloroethene without diminishing the microbial activity.

ZVI is a reducing reagent that effectively reduces all chloroethenes. This reagent demonstrated that under a lower total amount of chloroethenes, OHRBs can dehalogenate reductively all chloroethenes more efficiently. This finding is in line with evidence of dehalogenation in the source area, where, in areas with lower concentrations due to heterogeneities, OHRBs can degrade cDCE and VC. However, this approach is not optimal.

Under stable reductive conditions, there is an increase in the proportion of fermenting bacteria, and it is higher in the experiments in which lactic acid is injected. These microorganisms have a key role in supporting reductive dehalogenation.

A combined strategy of biostimulation with lactic acid and ISCR with ZVI is the most efficient strategy to completely remediate the source area. In this strategy, *D. putealis* rapidly dehalogenates PCE and TCE to cDCE, and ZVI slowly reduces the total amount of chloroethenes, reducing the toxicity and allowing other OHRBs to dehalogenate the rest of chloroethenes.

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References

[1] A. Tiehm and K. R. Schmidt, "Sequential anaerobic/aerobic biodegradation of chloroethenes--aspects of field application.," *Curr. Opin. Biotechnol.*, vol. 22, no. 3, pp. 415–21, Jun. 2011.

[2] M. J. Moran, J. S. Zogorski, and B. L. Rowe, "Approach to an assessment of volatile organic compounds in the nation's ground water and drinking-water supply wells," *Open-File Rep.*, 2006.

[3] USEPA, "National Primary Drinking Water Regulations. U.S. Environmental Protection. EPA 816-F-09-004.," in *National Service Center for Environmental Publications*, 2009.

[4] B. L. Parker, J. A. Cherry, S. W. Chapman, and M. A. Guilbeault, "Review and analysis of chlorinated solvent dense nonaqueous phase liquid distributions in five sandy aquifers," *Vadose Zo. J.*, vol. 2, no. 2, pp. 116–137, 2003.

[5] T. H. Wiedemeier *et al.*, "Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water," *Natl. Risk Manag. Res. Lab. EPA*, no. September, p. EPA/600/R-98/128.pag, 1998.

[6] P. M. Bradley, "History and Ecology of Chloroethene Biodegradation: A Review," *Bioremediat. J.*, vol. 7, no. 2, pp. 81–109, Apr. 2003.

[7] P. M. Bradley and F. H. Chapelle, "Microbial Mineralization of Dichloroethene and Vinyl Chloride under Hypoxic Conditions," *Ground Water Monit. Remediat.*, vol. 31, no. 4, pp. 39–49, Nov. 2011.

[8] L. Adrian and F. E. Löffler, "Organohalide-Respiring Bacteria—An Introduction," in *Organohalide-Respiring Bacteria*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2016, pp. 3–6.

[9] E. Bouwer, "Bioremediation of chlorinated solvents using alternate electron acceptors," *Handb. bioremediation*, 1994.

[10] T. M. Vogel, C. S. Criddle, and P. L. McCarty, "ES Critical Reviews: Transformations of halogenated aliphatic compounds," *Environ. Sci. Technol.*, vol. 21, no. 8, pp. 722–36, Aug. 1987.

[11] S. Atashgahi, Y. Lu, and H. Smidt, "Overview of Known Organohalide-Respiring Bacteria—Phylogenetic Diversity and Environmental Distribution," in *Organohalide-Respiring Bacteria*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2016, pp. 63–105.

[12] I. Nijenhuis and K. Kuntze, "Anaerobic microbial dehalogenation of organohalides — state of the art and remediation strategies," *Curr. Opin. Biotechnol.*, vol. 38, pp. 33–38, Apr. 2016.

[13] S. H. Zinder, "The Genus *Dehalococcoides*," in *Organohalide-Respiring Bacteria*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2016, pp. 107–136.

[14] X. Maymó-Gatell, Y. Chien, J. M. Gossett, and S. H. Zinder, "Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene," *Science (80-.).*, vol. 276, no. 5318, pp. 1568–1571, 1997.

[15] B. van der Zaan *et al.*, "Correlation of *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenase genes with geochemical conditions in chloroethene-contaminated groundwater.," *Appl. Environ. Microbiol.*, vol. 76, no. 3, pp. 843–50, Feb. 2010.

[16] P. M. Bradley and F. H. Chapelle, "Anaerobic mineralization of vinyl chloride in Fe (III) -Reducing , aquifer sediments," *Env. Sci Technol.*, vol. 30, no. 6, pp. 2084–2086, 1996.

[17] F. Aulenta, A. Pera, S. Rossetti, M. Petrangeli Papini, and M. Majone, "Relevance of side reactions in anaerobic reductive dechlorination microcosms amended with different electron donors.," *Water Res.*, vol. 41, no. 1, pp. 27–38, Jan. 2007.

[18] N. Wei and K. T. Finneran, "Influence of ferric iron on complete dechlorination of trichloroethylene (TCE) to ethene: Fe (III) reduction does not always inhibit complete dechlorination," *Environ. Sci. Technol.*, vol. 45, no. 17, pp. 7422–7430, 2011.

[19] National Research Council, "Improving management of persistent of contaminants. Groundwater and soil cleanup," *Natl. Acad. Press.*, pp. 113–174, 1999.

[20] J. Philips, P. J. Haest, D. Springael, and E. Smolders, "Inhibition of *Geobacter* Dechlorinators at Elevated Trichloroethene Concentrations Is Explained by a Reduced Activity Rather than by an Enhanced Cell Decay," *Environ. Sci. Technol.*, p. 130115145641003, Jan. 2013.

[21] S. K. Haack and B. A. Bekins, "Microbial populations in contaminant plumes," *Hydrogeol. J.*, vol. 8, no. 1, pp. 63–76, Mar. 2000.

[22] B. E. Sleep *et al.*, "Biological enhancement of tetrachloroethene dissolution and associated microbial community changes," *Environ. Sci. Technol.*, vol. Environmen, no. 40, pp. 3623–3633, 2006.

[23] C. Holliger *et al.*, "*Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra-and trichloroethene in an anaerobic respiration," *Arch. Microbiol.*, vol. 169, no. 4, pp. 313–321, 1998.

[24] L. R. Krumholz, "*Desulfuromonas chloroethenica* sp. nov. uses tetrachloroethylene and trichloroethylene as electron acceptors," *Int. J. Syst. Bacteriol.*, vol. 47, no. 4, pp. 1262–1263, 1997.

[25] M. L. G. C. Luijten *et al.*, "Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov.," *Int. J. Syst. Evol. Microbiol.*, vol. 53, no. 3, pp. 787–793, 2003.

[26] X. Maymó-Gatell, I. Nijenhuis, and S. H. Zinder, "Reductive dechlorination of cis-1,2-dichloroethene and vinyl chloride by '*Dehalococcoides ethenogenes*'.," *Environ. Sci. Technol.*, vol. 35, no. 3, pp. 516–21, Feb. 2001.

[27] A. Suyama, R. Iwakiri, K. Kai, T. Tokunaga, N. Sera, and K. Furukawa, "Isolation and characterization of *Desulfitobacterium* sp. strain Y51 capable of efficient dehalogenation of tetrachloroethene and polychloroethanes.," *Biosci. Biotechnol. Biochem.*, vol. 65, no. 7, pp. 1474–81, Jul. 2001.

[28] Y. C. Chang, M. Hatsu, K. Jung, Y. S. Yoo, and K. Takamizawa, "Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1," *J. Biosci. Bioeng.*, vol. 89, no. 5, pp. 489–491, 2000.

[29] P. K. Sharma and P. L. McCarty, "Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1, 2-dichloroethene.," *Appl. Environ. Microbiol.*, vol. 62, no. 3, pp. 761–765, 1996.

[30] Y. Sung *et al.*, "Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. nov.," *Appl. Environ. Microbiol.*, vol. 69, no. 5, pp. 2964–2974, 2003.

[31] Y. Yang and P. L. McCarty, "Comparison between donor substrates for biologically enhanced tetrachloroethene DNAPL dissolution," *Environ. Sci. Technol.*, vol. 36, pp. 3400–3404, 2002.

[32] R. Gillham and S. O'Hannesin, "Enhanced degradation of halogenated aliphatics by zero-valent iron," *Ground Water*, vol. 32, pp. 958–967, 1994.

[33] W. S. Orth and R. W. Gillham, "Dechlorination of trichloroethene in aqueous solution using Fe 0," *Env. Sci Technol.*, vol. 30, no. 1, pp. 66–71, 1996.

[34] T. J. Campbell, D. R. Burris, A. L. Roberts, and J. R. Wells, "Trichloroethylene and tetrachloroethylene reduction in a metallic iron-water-vapor batch system," *Environ. Toxicol. Chem.*, vol. 16, no. 4, pp. 625–630, 1997.

[35] R. A. Brown, "Chemical oxidation and resuction for chlorinated solvent remediation," in *In situ Remediation of Chlorinated Solvent Plumes*, no. 1, 2010.

[36] B. M. Henry, "Biostimulation for anaerobic bioremediation of chlorinated solvents," in *In situ Remediation of Chlorinated Solvent Plumesemediation of chlorinated solvent plume*, 2010.

[37] M. M. Lorah, E. H. Majcher, E. J. Jones, and M. a Voytek, "Microbial consortia development and microcosm and column experiments for enhanced bioremediation of chlorinated volatile organic compounds, West Branch Canal Creek wetland area, Aberdeen Proving Ground, Maryland.," 2008.

[38] D. E. Ellis *et al.*, "Bioaugmentation for accelerated in situ anaerobic bioremediation," *Environ. Sci. Technol.*, vol. 34, no. 11, pp. 2254–2260, Jun. 2000.

[39] D. Puigserver *et al.*, "Temporal hydrochemical and microbial variations in microcosm experiments from sites contaminated with chloromethanes under biostimulation with lactic acid," *Bioremediat. J.*, vol. 20, no. 1, pp. 54–70, Jan. 2016.

[40] D. Hunkeler and R. Aravena, "Investigating the origin and fate of organic contaminant in groundwater using stable isotope analysis," in *Environmental isotopes in biodegradation and bioremediation*, no. 8, C. M. Aelion, P. Höhener, D. Hunkeler, and R. Aravena, Eds. Boca Raton, Fla.: CRC Press, 2010, p. 450.

[41] D. Hunkeler, R. U. Meckenstock, B. Sherwood Lollar, T. C. Schmidt, and J. T. Wilson, "A guide for assessing biodegradation and source identification of organic ground water contaminants using compound specific isotope analysis (CSIA)," *PA 600/R-08/148*, no. December. 2008.

[42] S. J. Flynn, F. E. Löffler, and J. M. Tiedje, "Microbial community changes associated with a shift from reductive dechlorination of PCE to reductive dechlorination of cis-DCE and VC," *Environ. Sci. Technol.*, vol. 34, no. 6, pp. 1056–1061, 2000.

[43] S. Révész *et al.*, "Bacterial community changes in TCE biodegradation detected in microcosm experiments," *Int. Biodeterior. Biodegradation*, vol. 58, no. 3, pp. 239–247, 2006.

[44] É. Mészáros, R. Sipos, R. Pál, C. Romsics, and K. Márialigeti, "Stimulation of trichloroethene biodegradation in anaerobic three-phase microcosms," *Int. Biodeterior. Biodegradation*, vol. 84, pp. 126–133, Oct. 2013.

[45] R. E. Richardson, V. K. Bhupathiraju, D. L. Song, T. A. Goulet, and L. Alvarez-Cohen, "Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques," *Environ. Sci. Technol.*, vol. 36, no. 12, pp. 2652–2662, 2002.

[46] J. M. Lendvay *et al.*, "Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation," *Environ. Sci. Technol.*, vol. 37, no. 7, pp. 1422–1431, 2003.

[47] T. W. Macbeth, D. E. Cummings, S. Spring, L. M. Petzke, and K. S. Sorenson, "Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture," *Appl. Environ. Microbiol.*, vol. 70, no. 12, pp. 7329–7341, 2004.

[48] ITRC, *Strategies for Monitoring the Performance of DNAPL Source Zone Remedies*, Technical/Regulatory Guidelines, Interstate Technology and Regulatory Council, 206 pages, August 2004, Washington, DC.

[49] J. J. Morse *et al.*, "A treatability test for evaluating the potential applicability of the reductive anaerobic biological in situ treatment technology (RABITT) to remediate chloroethenes," *Draft Tech. Protoc. Environ. Secur. Technol. Certif. Program. Arlington, VA Environ. Secur. Technol. Program.*, 1998.

[50] X. Lu, J. T. Wilson, and D. H. Kampbell, "Comparison of an assay for *Dehalococcoides* DNA and a microcosm study in predicting reductive dechlorination of chlorinated ethenes in the field.," *Environ. Pollut.*, vol. 157, no. 3, pp. 809–15, Mar. 2009.

[51] D. Puigserver *et al.*, "Reductive dechlorination in recalcitrant sources of chloroethenes in the transition zone between aquifers and aquitards," *Environ. Sci. Pollut. Res.*, vol. 23, no. 18, pp. 18724–18741, Sep. 2016.

[52] J. T. Trevors, "Sterilization and inhibition of microbial activity in soil," *J. Microbiol. Methods*, vol. 26, pp. 53–59, 1996.

[53] . Herrero, D. Puigserver, I. Nijenhuis, K. Kuntze, and J. M. Carmona, "Evolution of degradation of chloroethenes as a function of the biogeochemical interactions taking place in the source zone-plume zone," *unpublished*.

[54] Y. D. Chen, J. F. Barker, and L. Gui, "A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: a microcosm study.," *J. Contam. Hydrol.*, vol. 96, no. 1–4, pp. 17–31, Feb. 2008.

[55] J. Palau, A. Soler, P. Teixidor, and R. Aravena, "Compound-specific carbon isotope analysis of volatile organic compounds in water using solid-phase microextraction," *J. Chromatogr. A*, vol. 1163, no. 1, pp. 260–268, 2007.

[56] D. J. Lane, "16S/23S rRNA Sequencing," in *Nucleic acid techniques in bacterial systematics*, E. Stackebrandt and M. Goodfellow, Eds. New York: Wiley, 1991.

[57] H. Heuer, M. Krsek, P. Baker, K. Smalla, and E. Wellington, "Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients," *Appl. Envir. Microbiol.*, vol. 63, no. 8, pp. 3233–3241, Aug. 1997.

[58] G. Imfeld *et al.*, "Characterization of microbial communities in the aqueous phase of a constructed model wetland treating 1,2-dichloroethene-contaminated groundwater.," *FEMS Microbiol. Ecol.*, vol. 72, no. 1, pp. 74–88, Apr. 2010.

[59] W.-T. Liu, T. L. Marsh, H. Cheng, and L. J. Forney, "Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA.," *Appl. Environ. Microbiol.*, vol. 63, no. 11, pp. 4516–4522, 1997.

[60] T. L. Marsh, P. Saxman, J. Cole, and J. Tiedje, "Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis," *Appl. Environ. Microbiol.*, vol. 66, no. 8, pp. 3616–3620, 2000.

[61] http://www.ncbi.nlm.nih.gov/blast/.

[62] D. H. Huson, S. Mitra, H. J. Ruscheweyh, N. Weber, and S. C. Schuster, "Integrative analysis of environmental sequences using MEGAN 4," *Genome Res.*, vol. 21, pp. 1552–1560, 2011.

[63] D. Hunkeler and B. Morasch, "Isotope fractionation during

transformation processes," in *Environmental isotopes in biodegradation and bioremediation*, C. M. Aelion, P. Höhener, D. Hunkeler, and R. Aravena, Eds. CRC Press, 2010, pp. 79–125.

[64] S. S. Patil, E. M. Adetutu, A. Aburto-Medina, I. R. Menz, and A. S. Ball, "Biostimulation of indigenous communities for the successful dechlorination of tetrachloroethene (perchloroethylene)-contaminated groundwater.," *Biotechnol. Lett.*, vol. 36, no. 1, pp. 75–83, Jan. 2014.

[65] H. I. Boga, R. Ji, W. Ludwig, and A. Brune, "*Sporotalea propionica* gen. nov. sp. nov., a hydrogen-oxidizing, oxygen-reducing, propionigenic firmicute from the intestinal tract of a soil-feeding termite," *Arch. Microbiol.*, vol. 187, pp. 15–27, 2007.

[66] E. S. Shelobolina *et al.*, "*Geobacter pickeringii* sp. nov., *Geobacter argillaceus* sp. nov. and *Pelosinus fermentans* gen. nov., sp. nov., isolated from subsurface kaolin lenses," *Int. J. Syst. Evol. Microbiol.*, vol. 57, pp. 126–135, 2007.

[67] W. Robertson, J. Bowman, P. Franzmann, and B. Mee, "*Desulfosporosinus meridiei* sp. nov., a spore-forming sulfate-reducing bacterium isolated from gasolene-contaminated groundwater," *Int J Syst Evol Microbiol*, vol. 51, no. 1, pp. 133–140, Jan. 2001.

[68] L. Hallbeck and K. Pedersen, "Autotrophic and mixotrophic growth of *Gallionella ferruginea*," *Journal of General Microbiology*, vol. 137. pp. 2657–2661, 1991.

[69] B. Sercu *et al.*, "The influence of in situ chemical oxidation on microbial community composition in groundwater contaminated with chlorinated solvents," *Microb. Ecol.*, vol. 65, no. 1, pp. 39–49, 2013.

[70] W. W. Mohn and K. J. Kennedy, "Reductive dehalogenation of chlorophenols by *Desulfomonile tiedjei* DCB-1.," *Appl. Environ. Microbiol.*, vol. 58, no. 4, pp. 1367–1370, 1992.

[71] F. Löffler, J. Cole, K. Ritalahti, and J. Tiedje, "Diversity of dechlorinating bacteria," in *Dehalogenation: microbial processes and environmental application*, M. Haggblom and I. Bossert, Eds. 2003, pp. 53–87.

[72] D. M. Bagley and J. M. Gossett, "Tetrachloroethene transformation to trichloroethene and cis-1,2-dichloroethene by sulfate-reducing enrichment cultures," *Appl. Environ. Microbiol.*, vol. 56, pp. 2511–2516, 1990.

[73] N. Yoshida, K. Asahi, Y. Sakakibara, K. Miyake, and A. Katayama, "Isolation and quantitative detection of tetrachloroethene (PCE)-dechlorinating bacteria in unsaturated subsurface soils contaminated with chloroethenes," *J. Biosci. Bioeng.*, vol. 104, no. 2, pp. 91–97, 2007.

[74] K. Dowideit *et al.*, "Spatial heterogeneity of dechlorinating bacteria and limiting factors for in situ trichloroethene dechlorination revealed by analyses of sediment cores from a polluted field site," *FEMS Microbiol. Ecol.*, vol. 71, pp. 444–459, 2010.

[75] J. F. Heidelberg *et al.*, "The genome sequence of the anaerobic, sulfate-reducing bacterium Desulfovibrio vulgaris Hildenborough.," *Nat. Biotechnol.*, vol. 22, no. 5, pp. 554–9, May 2004.