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Review

Enhanced anaerobic bioremediation of chlorinated solvents: environmental factors influencing microbial activity and their relevance under field conditions

Federico Aulenta,[1] Mauro Majone[1]* and Valter Tandoi[2]

[1]*Department of Chemistry, University of Rome 'La Sapienza', P. le Aldo Moro 5, 00185, Rome, Italy*
[2]*Water Research Institute, National Research Council (IRSA-CNR), Via Reno 1, 00198, Rome, Italy*

Abstract: Enhanced *in situ* anaerobic bioremediation of chlorinated solvents is a cost-effective, expanding technology for the clean-up of chlorinated solvent-contaminated sites. However, this technology is knowledge-intensive and its application requires a thorough understanding of the microbiology, ecology, hydrology and geochemistry of chlorinated solvent-contaminated aquifers. This review summarises current knowledge and future perspectives in the area of microbial anaerobic dechlorination of chlorinated solvents, particularly chloroethenes. Main attention is paid to the discussion of environmental factors and conditions that influence microbial activity under field conditions. Approaches to stimulate and manipulate the activity of native dechlorinating populations in order to meet target remediation goals for both plume management and source treatment are reviewed in detail. Possible research efforts needed to increase the likelihood of success of this technology are finally presented.

Keywords: anaerobic fermentation; DNAPL; electron donors; microbial competition; reductive dechlorination

INTRODUCTION

Perchloroethylene (PCE), trichloroethylene (TCE) and other highly chlorinated aliphatic hydrocarbons (CAHs) such as 1,1,2-trichloroethane (TCA) and chloroform (CF) are widely used in various industrial processes, mainly as cleaning solvents in dry-cleaning operations and semiconductor manufacture. Careless storage, handling and disposal as well as their high chemical stability have contributed to the status of CAHs as most frequently encountered subsurface contaminants. They are highly toxic as well as known or suspected carcinogens, so their presence in the environment poses important health risks and has prompted investigations concerning their fate in the subsurface. CAHs are transformed in the subsurface through a range of biotic and abiotic reactions.[1] Most abiotic transformations are slow, but they can still be significant within the time scales commonly associated with groundwater remediation. Biotic reactions are typically much faster provided that appropriate conditions are present such as sufficient substrate, nutrients and suitable microbial populations. CAHs such as PCE, TCE, TCA and CF do not support microbial growth in an aerobic environment.[2] However, many of them are anaerobically transformed to less chlorinated or non-chlorinated compounds through dechlorination reactions. Such dechlorination reactions can be a threat to living organisms if, under uncontrolled conditions, daughter compounds more toxic than the parent compound are formed, such as the carcinogen vinyl chloride from PCE.[1] On the other hand, reductive dechlorination has also received considerable attention as a reliable and cost-effective strategy for the removal of chlorinated solvents from contaminated environments,[3–5] because either less chlorinated daughter compounds formed are more biodegradable under aerobic conditions or non-chlorinated, harmless end-products are obtained. Within the last 20 years, basic research on reductive dechlorination has suggested that the transformation of chlorinated contaminants into harmless non-chlorinated end-products can be achieved practically by enhancing bacterial dechlorination reactions in the field. Enhanced *in situ* reductive dechlorination has been successfully applied for remediation of chlorinated solvent-contaminated sites.[6,7]

The scope of this paper is to review current knowledge on and recent advances in the anaerobic microbial dechlorination of chlorinated solvents, particularly chloroethenes. Special attention is given to the factors and conditions that may influence microbial activity under field conditions and in turn limit the efficacy of this process for *in situ* bioremediation of contaminated aquifers.

* Correspondence to: Mauro Majone, Department of Chemistry, University of Rome 'La Sapienza', P. le Aldo Moro 5, 00185, Rome, Italy
E-mail: mauro.majone@uniroma1.it
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DECHLORINATION REACTIONS AND PATHWAYS

Because of the electronegative character of chlorine substituents on aliphatic compounds, polychlorinated aliphatic compounds often behave as electron acceptors (oxidants) that are reductively dechlorinated in the process. The two main microbially mediated reductive dechlorination reactions are hydrogenolysis and dichloroelimination (Fig. 1). Hydrogenolysis, often simply known as reductive dechlorination (RD), involves the replacement of chlorine with hydrogen, with a net input of one proton and two electrons. The RD of PCE to ethene proceeds through a series of hydrogenolysis reactions, where TCE, *cis*-dichloroethene (cDCE) and vinyl chloride (VC) are typical intermediates. Dichloroelimination[8] has been observed only on sp^3-hybridised vicinal carbon atoms (e.g. chloroethanes) carrying a halogen substituent each. The reaction results in the replacement of the chlorine substituents and the formation of a double bond between the two carbon atoms, with a net input of two electrons. Chloroethanes also undergo dehydrochlorination (Fig. 1), an abiotic reaction involving the removal of a halogen from one carbon atom and the concomitant removal of a hydrogen atom from an adjacent carbon. This reaction converts a chlorinated alkane into a less chlorinated alkene. Dehydrochlorination is not a reductive reaction and does not require the input of electrons.

Clearly, elucidation of factors controlling the occurrence of these different reaction pathways under field conditions is crucial because of differing toxicity, mobility and persistence of the intermediate daughter products. For instance, TCE, DCEs and VC were the predominant and persistent daughter products of 1,1,2,2-TeCA biodegradation occurring in wetland soil microcosms[9] and in the presence of inocula originating from anaerobic digestion of activated sludge.[10,11] In the above-mentioned studies, only minor formation of ethene (ETH) or ethane (ETA), which are the desired end-products of 1,1,2,2-TeCA dechlorination, was observed. On the other hand, in a recent microcosm study[12] we observed the almost complete dechlorination of 1,1,2,2-TeCA into ETH by using yeast extract as primary electron donor. In contrast, only partial dechlorination to TCE, cDCE and VC was observed with other electron donors such as lactate, butyrate or molecular hydrogen. Interestingly, the dechlorination of 1,1,2,2-TeCA also led to the formation of tDCE, whereas this isomer is not usually produced – although exceptions have been reported[13] – during dechlorination of PCE. The *trans* isomer turned out to be much more recalcitrant to anaerobic dechlorination than the corresponding *cis* isomer.[12]

MICRO-ORGANISMS RESPONSIBLE FOR CHLOROETHENE DECHLORINATION

Several bacteria have been isolated that can couple the reductive dechlorination of chlorinated aliphatic compounds to energy conservation and therefore are of great interest for bioremediation of chlorinated solvent sites.[2,14] These micro-organisms differ in their electron donor requirements, kinetics, end-points of dechlorination and maximum concentrations of chlorinated solvent tolerated (Table 1). Several strains are quite restrictive in terms of electron donor requirements, such as *Dehalobacter* and *Dehalococcoides* that can only utilise H_2. On the other hand, other strains (*Dehalospirillum, Desulfitobacterium*) are quite versatile, utilising a broad spectrum of electron donors. *Desulforomonas* spp. are unique since they are the only strains that can utilise acetate as electron donor for PCE dechlorination. A detailed description and characterisation of dechlorinating micro-organisms is beyond the scope of this review and can be found elsewhere.[34,35] However, it is interesting to note that only members of the genus *Dehalococcoides* seem to be able to drive the dechlorination of chloroethenes to harmless ethene. For this reason, many research efforts have been carried out to understand the prevalence of *Dehalococcoides* strains in the environment, their geographic distribution and their functional roles in the dechlorination processes. Hendrickson *et al.*[36] tested samples from 24 chloroethene-dechlorinating sites in North America and Europe for the presence of members of the *Dehalococcoides* group by using PCR

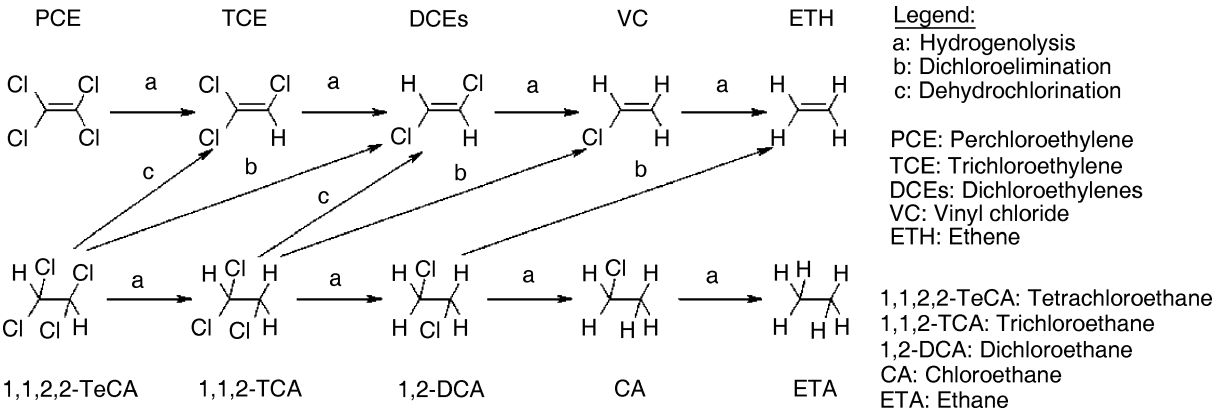

Figure 1. Anaerobic dechlorination pathways for chloroethenes and chloroethanes.

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Table 1. Some properties of PCE- and TCE-dehalorespiring bacteria

Micro-organism	Ultimate electron donor[a]	End product of dechlorination	PCE tolerated (mmol L^{-1})
Desulfitobacterium Viet1[15]	H_2	TCE	NR
Desulfitobacterium PCE1[16]	H_2	TCE	NR
Desulfitobacterium frappieri TCE1[17]	H_2	cDCE	NR
Desulfitobacterium PCE-S[18]	H_2	cDCE	NR
Desulfitobacterium Y51[19]	H_2	cDCE	SAT
Clostridium bifermentans DPH-1[20]	H_2	cDCE	SAT
Dehalobacter restrictus PER-K23 and TEA[21–23]	H_2	cDCE	<0.2
Enterobacter agglomerans MS-1[24]	Ac[g]	cDCE	SAT
Desulfuromonas chloroethenica TT4B[25]	Ac	cDCE	<0.1
Desulfuromonas michiganensis BB1 and BRS1[26]	Ac	cDCE	SAT
Sulfurospirillum multivorans[27b]	H_2	cDCE	<0.16
Sulfurospirillum halorespirans PCE-M2^T[28]	H_2	cDCE	NR
Dehalococcoides ethenogenes 195[29,30c]	H_2	ETH	<0.7
Dehalococcoides FL2[31d]	H_2	ETH	NR
Dehalococcoides BAV[32e]	H_2	ETH	NR
Dehalococcoides VS[33f]	H_2	ETH	NR

NR, not reported; SAT, saturation.

[a] Indicated is the ability to use H_2 or acetate (Ac) as electron donor for reductive dechlorination. However, *Dehalococcoides* and *Dehalobacter* are the only species that are restricted to the use of H_2. Other species can also use a variety of simple organic compounds.

[b] Formerly known as *Dehalospirillum*.

[c] VC dechlorination to ETH is a cometabolic process.

[d] PCE dechlorination to TCE and VC dechlorination to ETH are cometabolic processes.

[e] PCE and TCE are dechlorinated only cometabolically.

[f] PCE is not dechlorinated and TCE is dechlorinated only cometabolically.

[g] H_2 was not tested.

(polymerase chain reaction). A positive correlation between ethene formation and the presence of *Dehalococcoides* was observed.

However, as reported in Table 1, different *Dehalococcoides* species exhibit distinct dechlorinating abilities, and none of the isolated strains can gain energy from dechlorination of all chloroethenes (i.e. PCE, TCE, DCEs, VC). For instance, *D. ethenogenes* strain 195[29] can metabolically dechlorinate PCE to VC, but VC conversion to ethene is a slow cometabolic process; strain BAV1[32] can use all DCE isomers and VC as electron acceptors for growth, but it dechlorinates PCE and TCE only cometabolically; strain VS[33] uses cDCE and VC as growth-supporting electron acceptors, but it dechlorinates TCE only through cometabolism and cannot use PCE. In other words, the 'perfect PCE dechlorinator' has not been identified so far.

Moreover, He *et al.*[31] observed that *Dehalococcoides* FL2 share a 99.9% similar 16S rRNA gene sequence with strain BAV1 and an identical 16S rRNA gene sequence with strain CBDB1,[37] an isolate that dechlorinates chloroaromatic compounds but cannot dechlorinate chloroethenes. The authors concluded that 16S rRNA gene-based analyses are insufficient to predict dechlorination activity and distinguish between some members of the *Dehalococcoides* group. This information is relevant for bioremediation practitioners, because the detection of *Dehalococcoides* 16S rRNA gene sequences is often interpreted as evidence that native populations are capable of full dechlorination of PCE to ethene. Hopefully, in the near future, additional information about the dechlorination potential of a contaminated site could be provided by detection of genes directly involved in the dechlorination process of interest.[38–40]

In addition, more studies aimed at understanding the distribution and relative importance of dechlorinating micro-organisms other than *Dehalococcoides* are needed. Indeed, in some emerging *in situ* bioremediation approaches such as bioenhanced DNAPL (dense, non-aqueous phase liquid) dissolution the partial conversion of PCE to cDCE can be highly beneficial without the need to drive the dechlorination to completion, as discussed in the following subsections.

Electron donor requirements and influence of electron donor concentration

The two main reactions involved in the biodegradation of chlorinated solvents, i.e. hydrogenolysis and dichloroelimination, require the input of electrons from an external donor. Because of the oligotrophic nature of many chlorinated solvent subsurface ecosystems, electron donor addition is necessary for enhancing RD processes. Most isolated bacterial populations that metabolically use CAHs as terminal electron acceptors require hydrogen as the terminal electron donor (Table 1). Thus *in situ* enhancement of RD reactions is usually accomplished through the subsurface addition of selected carbon sources that can be fermented to H_2.

In field and laboratory studies, several different types of organic fermentable substrates such as glucose, formate, yeast extract, methanol, lactate, propionate, butyrate, benzoate and ethanol have been used as sources of hydrogen for dechlorination.[7]

Even though hydrogen is produced in the anaerobic decomposition of all the above-mentioned substrates, the rate and extent of H_2 formation and eventually of dechlorination may differ significantly.

Dechlorination rates have shown a Monod or Michaelis–Menten dependence on H_2 concentration.[41,42] Smatlak *et al*.[43] found that the half-velocity coefficient with respect to H_2 for conversion of PCE to VC in a mixed PCE-dechlorinating (from which *D. ethenogenes* strain 195 was later isolated), suspended growth culture was $100 \pm 50\,nmol\,L^{-1}$; however, the authors did not quantify the half-velocity coefficient of H_2 utilisation for conversion of VC to ETH, which is usually the slowest step in the RD of chlorinated ethenes. Ballapragada *et al*.[41] calculated the half-velocity coefficient of H_2 utilisation for each step of PCE RD in a fluidised bed reactor. They found that values ranged from 9 to $21\,nmol\,L^{-1}$. More recently, Cupples *et al*.[42] found that half-velocity coefficients of H_2 utilisation for conversion of cDCE and VC by *Dehalococcoides* strain VS were equal to $7 \pm 2\,nmol\,L^{-1}$.

On the other hand, the hydrogen level reached during the fermentation of electron donors may differ by orders of magnitude, depending on both the kinetics and thermodynamics of the particular fermentation reaction.[44] In general, degradation of volatile fatty acids (VFAs) to acetate and hydrogen, or in the case of propionate to acetate, H_2 and CO_2, is an endergonic reaction under standard conditions (i.e. reactants and products at $1\,mol\,L^{-1}$ or 1 atm and pH 7) (Table 2). Therefore micro-organisms that bring about these fermentations are synthrophically dependent upon hydrogenotrophic micro-organisms to maintain the H_2 partial pressure low enough (<10 Pa, corresponding to about 10^{-4} atm or $80\,nmol\,L^{-1}$ liquid phase concentration at 25 °C) to achieve negative free energies.[44] These low H_2 production ceilings compared with the half-velocity coefficients with respect to H_2 for dechlorination suggest that, in the presence of VFAs, dechlorination could possibly be rate-limited by H_2 availability. This hypothesis is supported by recent studies conducted in our laboratory.[45] When, in a butyrate-fed, PCE-dechlorinating culture, butyrate ($0.3\,mmol\,L^{-1}$) was replaced by hydrogen (spiked at initial liquid phase concentration $>100\,\mu mol\,L^{-1}$), the PCE dechlorination rate increased fourfold. This suggests that, with butyrate as electron donor, PCE dechlorination was rate-limited by butyrate fermentation and in turn by H_2 availability.

Table 2. Gibbs free energy changes of H_2-releasing fermentation reactions of organic electron donors relevant in biostimulation of microbial dechlorination processes under standard conditions (reactants and products at $1\,mol\,L^{-1}$ or 1 atm, pH 7, 25 °C)

Reaction	$\Delta G^{o'}$ ($kJ\,mol^{-1}$)
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.5
$Butyrate^- + 2H_2O \rightarrow 2Acetate^- + H^+ + 2H_2$	+48.3
$Methanol + 2H_2O \rightarrow HCO_3^- + H^+ + 3H_2$	+23.0
$Ethanol + H_2O \rightarrow Acetate^- + H^+ + 2H_2$	+9.6
$Lactate^- + 2H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	−3.9

In contrast to VFAs, fermentation of alcohols such as ethanol is thermodynamically favourable under higher hydrogen partial pressures (<100 Pa).[46] Therefore, provided that in an alcohol-fed culture a high number of H_2-producing, fermentative bacteria are present (compared with the number of H_2-utilising micro-organisms), H_2 concentrations higher than half-velocity coefficients for dechlorination can be maintained during fermentation. As an example, in a previous study[47] we observed that methanol fermentation resulted in H_2 liquid concentrations higher than $400\,nmol\,L^{-1}$ and PCE dechlorination to ETH proceeded at its maximum rate (i.e. was not H_2 rate-limited).

Notably, as for acetotrophic dechlorination, there is virtually no information available in the literature on the influence of acetate concentration on the rate of reductive dechlorination.

Competition for electron donors

The addition of an electron donor may stimulate not only the activity of dechlorinating micro-organisms but also the activity of other micro-organisms (such as methanogens, homoacetogens, sulfate reducers and nitrate reducers) that utilise the added electron donor (primary substrate) or its degradation products such as H_2 or acetate (ultimate electron donors) (Fig. 2). Because in the field the cost of the electron donor that must be supplied to achieve an effective enhancement of dechlorination processes may be significant, it is always desirable to minimise competition for added reducing equivalents between dechlorinators and competing organisms.

Pioneering studies by Smatlak *et al*.,[43] Ballapragada *et al*.,[41] Fennell *et al*.[46] and Yang and McCarty[48] addressed the issue of competition for H_2 between dechlorinators and methanogens in contaminated subsurface environments. The authors found that dechlorinators have the potential to out-compete methanogens when H_2 is present at low concentration owing to their higher affinity for H_2 (low half-velocity coefficient for H_2 use). Smatlak *et al*.[43] reported a tenfold higher half-velocity constant for H_2 use for methanogenesis ($960 \pm 180\,nmol\,L^{-1}$) compared with PCE dechlorination to VC ($100 \pm 50\,nmol\,L^{-1}$). Moreover, the threshold for H_2 utilisation (i.e. the minimal H_2 concentration that can be consumed under defined reducing conditions by a micro-organism[42,49]) by mixed dechlorinating cultures and pure cultures of dechlorinating bacteria is reported to be lower than $0.3\,nmol\,L^{-1}$, while that for methanogens is reported to be in the range $5–95\,nmol\,L^{-1}$.[50–54] Based on these observations, the addition of electron donors such as butyrate

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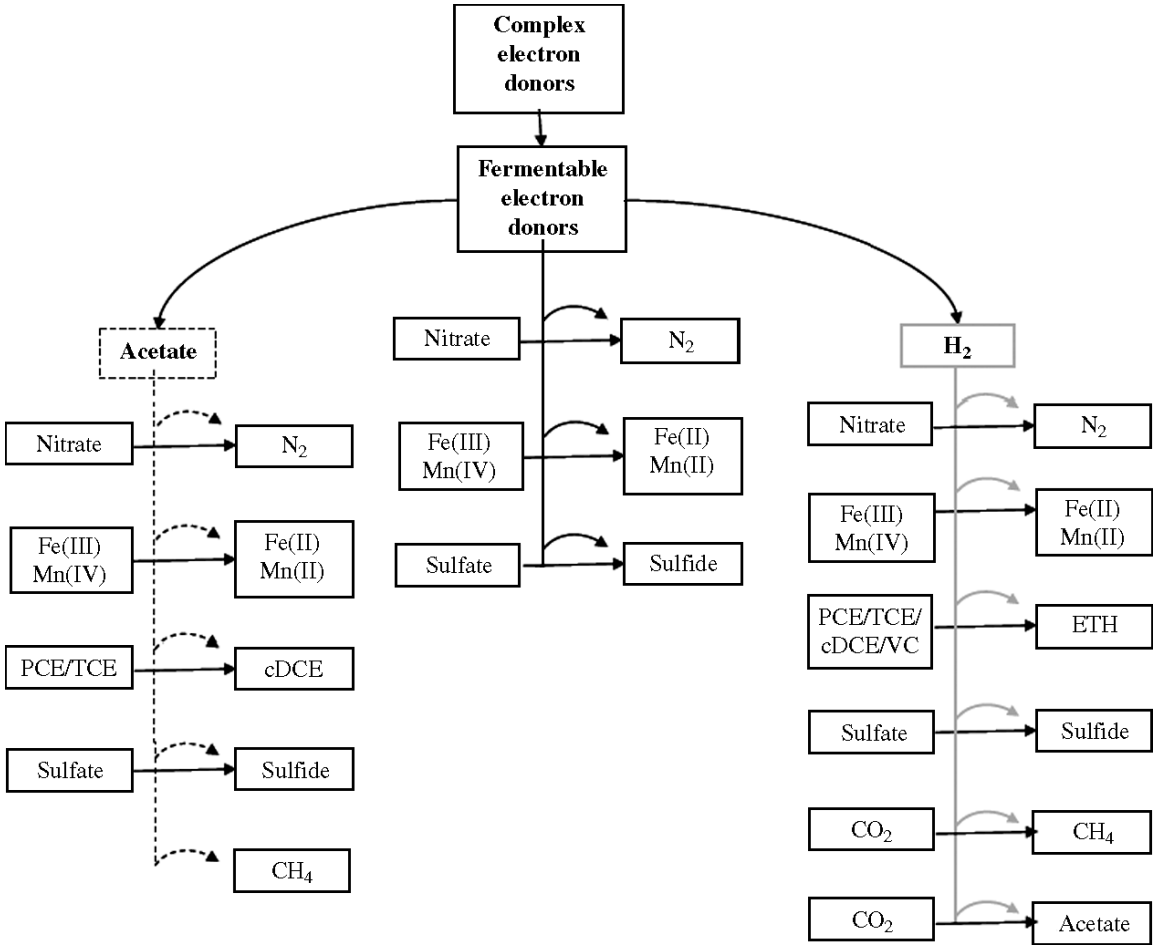

Figure 2. Simplified scheme showing the main metabolisms in competition for fermentable electron donors, acetate and hydrogen in a mixed complex microbial community.

or propionate, which are slowly fermented and are thermodynamically limited to release hydrogen at low concentrations, should channel most of the H_2 released to dechlorination reactions. There are, however, conflicting results on whether all the steps of PCE dechlorination are characterised by the same H_2 threshold concentration ($\sim 0.3\,nmol\,L^{-1}$) or whether the reduction of cDCE and VC requires higher H_2 levels.[51,52,55] Moreover, taking into consideration the influence of H_2 level on the rate of dechlorination (see previous subsection), dechlorination reactions will also proceed quite slowly at such low H_2 levels.

One approach to slowly deliver low concentrations of H_2 into an aquifer in support of reductive dechlorination is to use low-solubility or solid electron donors such as polylactate esters, edible oils, chitin, decaying biomass, wood chips, cane molasses, peat, mulch, etc.[56–61] These substrates, once injected and/or immobilised in the subsurface (e.g. within permeable barriers), slowly hydrolyse, biotically or abiotically, into more soluble compounds (e.g. vegetable oils are hydrolysed into soluble long-chain VFAs) which are carried along the contaminated plume and act as persistent, slow-fermenting sources of H_2. In this case the slow H_2 release in the aquifer is achieved because of the slow hydrolysis rate of these donors rather than because of thermodynamic regulation. One major advantage of using low-solubility or solid substrates is that a single injection has the potential to sustain dechlorination reactions for a long time.[7] In contrast, *in situ* bioremediation with soluble substrates typically entails injection and recovery pumping in order to achieve effective distribution and mixing with the contaminated groundwater.[7]

Zero-valent iron (ZVI), a reactive medium commonly utilised in permeable reactive barriers (PRBs), could also be a source of H_2 for stimulating biological dechlorination reactions in the subsurface. ZVI catalyses the abiotic conversion of a variety of pollutants such as chlorinated aliphatics, chlorinated aromatics, nitroaromatics, nitrates and redox-sensitive, high-valency toxic metals (e.g. Cr^{6+}). In addition to chemical reactions, ZVI can also serve as an electron donor to support the microbial reductive conversion of oxidised contaminants such as chlorinated solvents.[62–64] The most probable mechanism of electron transfer from ZVI to micro-organisms is via cathodic hydrogen.[65] Under anaerobic conditions, H_2 is produced from the chemical reaction of ZVI with water. A recent study[64] has shown that ZVI is a readily utilised electron donor for methanogenesis and sulfate reduction in anaerobic sludge. In some cases, however, ZVI was inhibitory to dechlorinating bacterial activity;

inhibition was due to encapsulation of bacterial cells by iron precipitates.[66]

The salient kinetic and thermodynamic differences between hydrogenotrophic dechlorination and methanogenesis made it possible to develop a strategy for addressing, in favour of dechlorinators, the problem of competition for added H_2. In contrast, much more controversial is the problem of competition between dechlorination and sulfate reduction. Studies aimed at investigating the effect of sulfate on dechlorination reported either no inhibition (or even sulfate reducers out-competed by dechlorinators at H_2 concentrations lower than 2.5 nmol L^{-1}),[67–69] partial inhibition,[70,71] or complete inhibition.[72,73] Fennell and Gossett[74] suggested that, when observed, the lack of dechlorination under sulfate-reducing conditions may be the result of (1) direct competition for supplied primary electron donor by sulfate-reducing micro-organisms, (2) inhibition by sulfate of enzymes involved in dechlorination, (3) preferential use of sulfate as terminal electron acceptor, instead of chlorinated compounds within the same organism (e.g. *Desulfitobacterium* spp.), or (4) larger predominance and faster growth kinetics of sulfate-reducing bacteria compared with dechlorinators in sulfate-rich environments.

Even though the potential for simultaneous dechlorination and sulfate reduction exists, the recommended approach[75] for enhancing dechlorination reactions in sulfate-rich environments typically consists in first depleting all the sulfate present in the groundwater (soluble and associated with the aquifer soil matrix) by initially dosing an excess of electron donor. Similar considerations also apply to the competition for H_2 between dechlorination and nitrate and Fe(III) reduction. While this approach was proven successful at some sites,[7] it may not be applied at sites where aquifer solids contain large amounts of $Fe(OH)_3$ or $CaSO_4$, which can serve as virtually inexhaustible reservoirs of low-solubility electron acceptors.

A possible drawback with the use of fermentable organic substrates to stimulate *in situ* the hydrogenotrophic RD of chlorinated solvents is the production and possible accumulation in the subsurface of large amounts of fermentation products (e.g. acetate or propionate), Fe(II) and Mn(II) (from the reduction of Fe(III) and Mn(IV) respectively), with resulting deterioration of groundwater quality. In principle, direct hydrogen addition may offer some advantages over the use of slowly fermentable organics. Indeed, the dechlorination rate can be greatly accelerated by directly providing dechlorinating micro-organisms with hydrogen.[45] In addition, hydrogen does not leave any environmentally harmful residue in the subsurface, is generally less expensive than most organic compounds, is less likely to increase the dissolution of iron and manganese (Aulenta F, unpublished) and generates less biomass in the subsurface, so its use is less likely to modify groundwater flow patterns.[7] Thus direct hydrogen addition to the subsurface has recently been proposed, based on passive dissolution through membranes,[76–78] low-pressure sparging[79] or hydrogen-generating electrodes.[80] On the other hand, several factors may still limit the use of direct hydrogen addition to support the RD of chlorinated solvents: its poor solubility in water (about 1 mmol L^{-1}) and its tendency, once injected in the subsurface, to rapidly escape from the contamination plume. Moreover, the presence of high levels of H_2 could also provide a selective advantage to methanogens and eventually result in the marginalisation of dechlorinators. As an example, Ma *et al*.[78] utilised a polyethylene hollow fibre membrane to deliver hydrogen in soil columns. Even though the membrane-supplied H_2 effectively stimulated PCE dechlorination, the system was very inefficient in that only 5% of the supplied H_2 was used for dechlorination. Most of the remainder was used to support methanogenesis (94%). In addition, extensive growth of methanogens eventually resulted in excessive foulant accumulation on the outside of the membrane. Aside from issues of hydrogen competition and direct biomass-induced fouling, bubble formation from excessive subsurface methanogenesis could significantly diminish the hydraulic conductivity of an aquifer and result in explosive levels of methane, thus posing safety concerns.[74]

There are conflicting results about the possibility of sustaining long-term dechlorination with H_2 as the sole electron donor.[45,81] Early studies with a mixed methanol-fed, PCE-dechlorinating culture[81] indicated that, while H_2 could serve as an electron donor in the RD of PCE over periods of 14–40 days, the addition of filtered supernatant from a methanol-fed culture was necessary to support dechlorination for more extended periods. This result suggested a nutritional dependence of hydrogen-utilising dechlorinators on the metabolic products of other organisms that were present in the more diverse methanol-fed culture. In a recent study[45] we compared the ability of different electron donors (hydrogen, methanol, butyrate and yeast extract) to sustain long-term (500 days) reductive dechlorination of tetrachloroethene (PCE) in anaerobic fill-and-draw bioreactors operated at 3:1 donor/PCE ratio. The H_2-fed bioreactor showed the best ability to completely dechlorinate the dosed PCE (0.5 mmol L^{-1}) to ethene. As the study progressed, however, the H_2-fed reactor experienced a diminishing ability to dechlorinate, while more stable dechlorinating activity was maintained in the reactors that were fed organic donors. This diminished ability of the H_2-fed reactor to dechlorinate was partially explained in terms of increased competition for H_2 between dechlorinators and methanogens, but other factors such as growth factor limitation and/or accumulation of toxic and/or inhibitory metabolites were also shown to play a role for longer periods (>500 days). In spite of decreasing activity with time, the H_2-fed reactor

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proved to be the most effective for PCE dechlorination: after about 500 days, more than 65% of the added PCE was dechlorinated to ethene in the H_2-fed reactor, *versus* 36, 22 and <1% in the methanol-fed, butyrate-fed and control reactors respectively. Therefore direct H_2 supply can be considered as a possible alternative when remedial action can be or has to be performed in a shorter time frame and/or can be concentrated in an engineered system such as a biological permeable barrier.

Recent studies suggest that acetate can also be an important electron donor for dechlorination.[12,25,26,82] This is an important feature, because acetate is the prevalent by-product of fermentation reactions. However, the distribution and relative importance of acetotrophic dechlorinators in the environment are still largely unexplored. Competition for acetate between acetotrophic dechlorinators and competing organisms such as Fe(III) reducers, sulfate reducers and methanogens has been far less studied. Recently, He and Sanford[83] determined acetate threshold concentrations under dechlorinating and Fe(III)-reducing conditions for *Anaeromyxobacter dehalogenans* strain 2CP-C, a chlorophenol-dechlorinating model micro-organism. The acetate threshold concentrations measured were 69 ± 4, 19 ± 8 and $<1\,\mathrm{nmol\,L^{-1}}$ for dechlorination, amorphous Fe(III) reduction and Fe(III) citrate reduction respectively. Threshold concentrations for acetate use by methanogens *Methanosarcina* spp. and *Methanosaeta* spp. were found to be 190–1180 and 7–69 $\mu\mathrm{mol\,L^{-1}}$ respectively.[84] Loeffler *et al*.[35] observed that *Desulforomonas* spp. reduced the acetate concentration below the level that supported acetoclastic methanogenesis in *Methanosarcina barkeri*. Overall, these studies suggest that acetotrophic dechlorinators are good competitors for acetate and can use acetate at concentrations that are too low to support methanogenic activity. Further studies are needed to verify the ability of dechlorinators to compete for acetate under other terminal electron acceptor conditions such as nitrate, sulfate and Fe(III) reduction. Typically, bioremediation approaches that result in increased H_2 fluxes, such as addition to the subsurface of fermentable substrates or, directly, H_2, also often result in increased acetate fluxes, through acetogenic fermentation reactions or H_2/CO_2 homoacetogenesis, and therefore may be used to stimulate the activity of both hydrogenotrophic and acetotrophic dechlorinators.[85]

MICROBIAL DECHLORINATION OF CHLORINATED SOLVENT DNAPLs

A major problem associated with the contamination of groundwater systems by PCE, TCE or other highly chlorinated solvents stems from the formation of dense, non-aqueous phase liquids (DNAPLs).[86,87] PCE and TCE form DNAPLs that sink through permeable groundwater aquifers until a non-permeable zone is reached. Substantial DNAPL volumes can also be retained because of the presence of non-uniform soil texture, which may result in DNAPL pooling above layers or lenses of lower-permeability media. During DNAPL migration, hysteretic capillary forces cause retention of a portion of the liquid within the pores as discontinuous globules or ganglia. Hence the typical resulting distribution of the DNAPL is highly complex and non-uniform.[88] Entrapped DNAPL mass tends to dissolve slowly into flowing water, serving as a long-term source of groundwater contamination. Remedial actions aimed at removing an appreciable contaminant mass from such DNAPL source zones have the potential to significantly reduce the life span of a contamination plume. Therefore a number of innovative technologies have recently been developed to enhance contaminant removal from source zones. For example, alcohol and surfactant flushing have been demonstrated to recover significant contaminant mass from a source zone.[88–91] The ability of bioremediation to achieve substantial mass reduction has been given little attention in the past, mainly because saturation concentrations of chlorinated solvents were believed to be toxic to micro-organisms.

The abilities of dechlorinating micro-organisms to tolerate saturation concentrations of chlorinated solvents differ significantly (Table 1). As an example, *D. ethenogenes* could apparently grow at high PCE concentrations ($0.7\,\mathrm{mmol\,L^{-1}}$, close to the saturation concentration of about $0.9\,\mathrm{mmol\,L^{-1}}$) in pure culture, but these concentrations adversely impacted its ability to dechlorinate, particularly from VC to ethene.[30] PCE dechlorination by *S. multivorans*[27] and *D. restrictus*[21] was inhibited when the PCE concentration was higher than 0.3 and $0.2\,\mathrm{mmol\,L^{-1}}$ respectively. In contrast, *D. michiganensis*,[26] *C. bifermentans* strain DPH-1,[20] *Desulfitobacterium* strain Y51[19] and *E. agglomerans* strain MS-1[24] were all reported to tolerate PCE concentrations close to saturation (Table 1). In addition, dechlorination of saturation concentrations of PCE or other chlorinated solvents has also been reported for several mixed cultures.[92–94]

Reductive dechlorination can enhance DNAPL dissolution because it acts as a reaction sink near the DNAPL and increases the concentration gradient. As an example, because TCE, cDCE, VC and ethene all have much higher solubilities than PCE, the PCE dissolution rate can be increased. In a laboratory study on PCE DNAPL dechlorination, Yang and McCarty[94] found that the PCE DNAPL dissolution rate was increased about fivefold when coupled with biological RD. The major degradation product was cDCE, but significant amounts of VC and ethene were also formed. Adamson *et al*.[95] found that TCE and cDCE were the main dechlorination products detected in the proximity of the DNAPL source zone after inoculation with a *Dehalococcoides* culture. They concluded that dechlorination beyond cDCE was probably inhibited by the high PCE levels. Incomplete PCE DNAPL dechlorination to mainly cDCE has also been reported by other authors[94,95] for cultures

that contained *Dehalococcoides* spp. as the putative dechlorinating micro-organism.

It is noteworthy that the primary goal of bioenhanced DNAPL dissolution and source treatment is not typically to reduce contaminant concentrations to regulatory levels but rather to achieve and maintain a high flux of contaminant from the DNAPL to the aqueous phase. In this context, achieving complete dechlorination to the non-toxic ethene may not be the ultimate criterion to assess the success of a source zone treatment,[96] considering also that any full-scale source zone remedial action will likely include a down-gradient polishing treatment step (either *in situ* or pump and treat). It has to be considered that, differently from PCE, less chlorinated compounds such as VC or cDCE are more amenable to aerobic biodegradation.[97,98] A possible source zone treatment scheme may then include bioenhanced anaerobic DNAPL dissolution coupled to an aerobic polishing step. Alternatively, Christ *et al*.[88] proposed to combine an aggressive physicochemical process that removes significant contaminant mass with a bioremediation 'polishing' step to control the contaminant mass flux emanating from the remaining DNAPL.

ELECTRON DONORS FOR MICROBIAL DECHLORINATION OF CHLORINATED SOLVENT DNAPLs

Various electron donors and delivery strategies have recently been proposed in order to achieve enhanced PCE DNAPL biodissolution.[58,94] As an example, Yang and McCarty[58] evaluated, in laboratory-scale column reactors, different substrates to bioenhance DNAPL dissolution, namely pentanol (soluble substrate, fed continuously), calcium oleate (insoluble substrate, placed in column initially) and olive oil (mixed with PCE and placed in column initially).

Interestingly, these studies indicated that the problem of competitive utilisation of added electron donor substrate is greatly reduced in the presence of saturation concentrations of PCE. This was largely due to the inhibitory effect of high concentrations of PCE and produced cDCE on competing methanogens and homoacetogens. Hence the resulting electron donor utilisation efficiency for dechlorination is very high, overcoming therefore one of the greatest cost factors for reductive dechlorination. Based on this evidence, slow hydrogen-releasing substrates, which often result in continued but slow dechlorination, could in principle be replaced by direct hydrogen addition, which was often found to sustain higher dechlorination rates.[45,99,100] On the other hand, its relatively low solubility in water could be a significant limitation, especially in the case of DNAPL dechlorination.

Differently from plume treatment, hydrochloric acid released during DNAPL dechlorination can be a significant problem, necessitating a high buffer to prevent adverse pH conditions.[96] In addition, when a fermentable substrate is used as electron donor, not only is hydrochloric acid produced but also acetic acid. The latter requires additional buffer and can lead to further degradation of water quality through iron, manganese or sulfate reduction and methane formation.[101] To overcome these limitations, McCarty *et al*.[101] have recently proposed the use of formate as an ideal electron donor for DNAPL dechlorination. Formate is enzymatically converted into bicarbonate and hydrogen. The hydrogen is used for reductive dechlorination and the hydrochloric acid produced is neutralised by the bicarbonate. The net result is the production of less chlorinated compounds, sodium chloride and carbon dioxide gas. Carbon dioxide is a highly soluble weak acid gas, and some bicarbonate must be present to buffer its impact. Table 3 reports the reactions involved in the use of formate as electron donor for PCE DNAPL dechlorination,[101] assuming the formation of ethene as the ultimate RD product. Indeed, the researchers also suggested that *Dehalococcoides* spp. could produce ethene by using the H_2 rapidly formed from formate (even though they cannot use formate directly as electron donor).

Table 3. Reactions involved in the use of formate as electron donor for PCE DNAPL dechlorination

Formate dehydrogenation	$4HCOONa + 4H_2O \rightarrow 4NaHCO + 4H_2$
Reductive dechlorination of PCE	$C_2Cl_4 + 4H_2 \rightarrow C_2H_4 + 4HCl$
Acid neutralisation	$4NaHCO_3 + 4HCl \rightarrow 4NaCl + 4CO_2 + 4H_2O$
Net reaction	$C_2Cl_4 + 4HCOONa \rightarrow C_2H_4 + 4NaCl + 4CO_2$

Other interesting electron donors for dechlorination of chlorinated solvent DNAPLs are surfactants. Indeed, recent studies[102,103] demonstrated that polysorbate surfactants (such as Tween 60, 61 and 65) are partially and slowly degraded under anaerobic conditions and may be used to simultaneously increase the bioavailability of sorbed (or DNAPL) chlorinated compounds while acting as electron donors for microbial dechlorination reactions.

RESEARCH NEEDS

The presence in an aquifer of multiple, and in many cases unknown, co-contaminants may drastically reduce our ability to predict the outcome of a bioremedial action. As an example, in a PCE-enriched culture containing *Dehalococcoides* spp. as the putative dechlorinating organism,[104] we observed that a 1,1,2,2-TeCA spike had a negative effect on the last step of dechlorination (i.e. from VC to ETH). Adamson and Parkin[105–107] observed that a PCE-degrading, methanogenic, lactate-enriched culture was able to degrade cometabolically both carbon tetrachloride (CT) and 1,1,1-TCA despite no previous exposure to these compounds. However, while the presence of $<20\,\mu mol\,L^{-1}$ 1,1,1-TCA had little effect on PCE removal, the addition of

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$10-15\,\mu mol\,L^{-1}$ CT negatively impacted both the PCE and VC dechlorination steps. In some other cases the presence of co-contaminants may even be beneficial to dechlorinating micro-organisms. For instance, organic co-contaminants may act as electron donors for dechlorination reactions or may lead to the establishment of complex mixed cultures in which organisms growing on co-contaminants provide dechlorinators with nutritional factors through lysis or excretion.[108] To address the problem of complex contaminated aquifers, bioremediation studies will preferably have to be carried out using real groundwater.

Furthermore, most knowledge regarding the kinetics of chlorinated solvent dechlorination, the physiology of dechlorinating micro-organisms and the microbial competitive aspects of the process has been obtained from suspended growth systems, whereas only limited information is available for dechlorinating biofilms, even though these are present in most systems, either naturally (e.g. contaminated soils and aquifers) or engineered (e.g. permeable barriers). In biofilm systems the transport of substrates contributes to determining overall process performance along with intrinsic reaction kinetics. In particular, for dechlorinating biofilms the presence of mass transport resistances for the primary substrate (e.g. fermentable carbon sources), the electron donor (i.e. H_2) or the electron acceptors (i.e. chloroethenes) may control both the kinetics of the RD steps and the competition for the primary electron donor as well as for H_2 among the different trophic groups.[109] Indeed, the presence of concentration gradients for the electron donors and/or the electron acceptors within the biofilm depth may result in the stratification of microbial processes inside the microbial aggregates. Although organisms in biofilms are largely responsible for most degradation that occurs naturally, little is known about the physiology of organisms in biofilms and how the interaction among organisms in biofilms leads to degradation of toxic compounds. As an example, there is some evidence[29,110] suggesting that mixed dechlorinating cultures perform better than pure cultures and that dechlorinating micro-organisms live in close association with other micro-organisms in microbial aggregates. The identification and quantification of members of particular microbial communities, as well as a clear understanding of the functional relationships between members, are required in order to fully appreciate and possibly manage the complex processes that these communities perform.[111] Until recently, however, the lack of methods for exploring these complex microbial communities *in situ* has hampered detailed understanding. Fortunately, recent advances in microscopy and molecular techniques have made it possible to examine such communities *in situ* in great detail. More studies, preferably carried out under conditions more representative of contaminated aquifers, are needed, however, to fully understand and exploit microbial communities performing *in situ* bioremediation of chlorinated solvents.

At present, microcosm studies, in which soil and groundwater samples are incubated in serum bottles in the presence of various amendments, represent the most common way to test the response of native microbial populations to amendments aimed at stimulating RD. In our experience the choice of optimal electron donor is not straightforward. In a recent microcosm study,[12] not only were different rates and end-products obtained from different substrates, but also accumulation of fermentation products, Fe and Mn release and residual groundwater ecotoxicity were affected (Aulenta F *et al.*, unpublished). Moreover, comparison between microcosms performed in the presence of groundwater and defined mineral medium could provide additional information on whether other groundwater constituents may have any further effects on reductive dechlorination reactions.[12,104]

On the other hand, microcosms are usually performed for very long residence times (which can be quite different from field conditions) and there is some evidence that accumulation of metabolic intermediates and/or daughter products can reduce the rate and extent of RD.[45] Also, increasing evidence indicates that aquifers are extremely heterogeneous systems, even at very small scale, in terms of both geochemical composition and microbial and contaminant distribution.[112] Such complexity may not be captured by a single microcosm study, especially if DNAPL is present. Although more expensive and time-consuming, small-scale field tests should also be conducted along with microcosm studies to assess the potential for *in situ* bioremediation of chlorinated solvents at specific sites. On the other hand, even at field scale, these tests need a research-quality approach including careful design, operation and monitoring. Particularly, dealing with the addition of soluble electron donors, the design of research-quality field tests should be aimed at creating a hydraulically controlled reaction volume in the aquifer, controlling the residence time in the reaction volume, obtaining good mixing of contaminated groundwater with substrates and carefully monitoring inside and outside the test area. Good example applications of field tests are given in the RABITT protocol.[75]

REFERENCES

1 Vogel TM, Criddle CS and McCarty PL, Transformation of halogenated aliphatic compounds. *Environ Sci Technol* **21**:722–736 (1987).
2 Field JA and Sierra-Alvarez R, Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds. *Rev Environ Sci Biotechnol* **3**:184–254 (2004).
3 Middeldorp PJM, Luijten MLGC, van de Pas BA, van Eekert MHA, Kengen SWM, Schraa G, *et al*, Anaerobic microbial reductive dechlorination of chlorinated ethenes. *Bioremed J* **3**:151–169 (1999).
4 Pavlostathis SG, Prytula MT and Yeh DH, Potential and limitations of microbial reductive dechlorination for bioremediation applications. *Water Air Soil Pollut Focus* **3**:117–129 (2003).

5 Lee MD, Odom JM and Buchanan Jr RJ, New perspectives on microbial dehalogenation of chlorinated solvents: insights from the field. *Annu Rev Microbiol* **52**:423–452 (1998).

6 Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, *et al*, Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environ Sci Technol* **36**:5106–5116 (2002).

7 ESTCP, *Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents*. [Online]. (2004). Available: www.afcee.brooks.af.mil.

8 De Wildeman S and Verstraete W, The quest for microbial reductive dechlorination of C(2) to C(4) chloroalkanes is warranted. *Appl Microbiol Biotechnol* **61**:94–102 (2003).

9 Lorah MM and Voytek MA, Degradation of 1,1,2,2-tetrachloroethane and accumulation of vinyl chloride in wetland sediment microcosms and *in situ* porewater: biogeochemical controls and associations with microbial communities. *J Contam Hydrol* **70**:117–145 (2004).

10 Chen C, Puhakka JA and Ferguson JF, Transformations of 1,1,2,2-tetrachloroethane under methanogenic conditions. *Environ Sci Technol* **30**:542–547 (1996).

11 Chen C, Ballapragada BS, Puhakka JA, Strand SE and Ferguson JF, Anaerobic transformation of 1,1,1-trichloroethane by municipal digester sludge. *Biodegradation* **10**:297–305 (1999).

12 Aulenta F, Potalivo M, Majone M, Petrangeli Papini M and Tandoi V, Anaerobic bioremediation of groundwater containing a mixture of 1,1,2,2-tetrachloroethane and chloroethenes. *Biodegradation* **17**:193–206 (2006).

13 Griffin BM, Tiedje JM and Loffler FE, Anaerobic microbial reductive dechlorination of tetrachloroethene to predominately *trans*-1,2-dichloroethene. *Environ Sci Technol* **38**:4300–4303 (2004).

14 Häggblom MM and Bossert ID, *Dehalogenation: Microbial Processes and Environmental Applications*. Kluwer Academic, Boston, MA (2003).

15 Loffler FE, Ritalahti KM and Tiedje JM, Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. *Appl Environ Microbiol* **63**:4982–4985 (1997).

16 Gerritse J, Renard V, Pedro Gomes TM, Lawson PA, Collins MD and Gottschal JC, *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols. *Arch Microbiol* **165**:132–140 (1996).

17 Gerritse J, Drzyzga O, Kloetstra G, Keijmel M, Wiersum LP, Hutson R, *et al*, Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene by *Desulfitobacterium frappieri* TCE1. *Appl Environ Microbiol* **65**:5212–5221 (1999).

18 Miller E, Wohlfarth G and Diekert G, Comparative studies on tetrachloroethene reductive dechlorination mediated by *Desulfitobacterium* sp. strain PCE-S. *Arch Microbiol* **168**:513–519 (1997).

19 Suyama A, Iwakiri R, Kai K, Tokunaga T, Sera N and Furukawa K, Isolation and characterization of *Desulfitobacterium* sp. strain Y51 capable of efficient dehalogenation of tetrachloroethene and polychloroethanes. *Biosci Biotechnol Biochem* **65**:1474–1481 (2001).

20 Chang YC, Hatsu M, Jung K, Yoo YS and Takamizawa K, Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1. *J Biosci Bioeng* **89**:489–491 (2000).

21 Holliger C, Schraa G, Stams AJ and Zehnder AJ, A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol* **59**:2991–2997 (1993).

22 Holliger C, Hahn D, Harmsen H, Ludwig W, Schumacher W, Tindall B, *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* **169**:313–321 (1998).

23 Wild A, Hermann R and Leisinger T, Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene. *Biodegradation* **7**:507–511 (1996).

24 Sharma PK and McCarty PL, Isolation and characterization of a facultative bacterium that reductively dehalogenates tetrachloroethene to *cis*-dichloroethene. *Appl Environ Microbiol* **62**:762–765 (1996).

25 Krumholz LR, Sharp R and Fishbain S, A freshwater anaerobe coupling acetate oxidation to tetrachloroethene dehalogenation. *Appl Environ Microbiol* **62**:4108–4113 (1996).

26 Sung Y, Ritalathi KM, Sanford RA, Urbance JW, Flynn SJ, Tiedje JM, *et al*, Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. nov. *Appl Environ Microbiol* **69**:2964–2974 (2003).

27 Scholz-Muramatsu H, Neumann A, Messmer M, Moore E and Diekert G, Isolation and characterization of *Dehalospirillum multivorans* gen. Nov., sp. Nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch Microbiol* **163**:48–56 (1995).

28 Luijten ML, de Weert J, Smidt H, Boschker HT, de Vos WM, Schraa G, *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* **53**:787–793 (2003).

29 Maymò-Gatell X, Chien YT, Gossett JM and Zinder SH, Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* **276**:1568–1571 (1997).

30 Maymo-Gatell X, Anguish T and Zinder SH, Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by '*Dehalococcoides ethenogenes*' 195. *Appl Environ Microbiol* **65**:3108–3113 (1999).

31 He J, Sung Y, Krajmalnik-Brown R, Ritalahti KM and Loffler FE, Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe. *Environ Microbiol* **7**:1442–1450 (2005).

32 He J, Ritalahti KM, Yang KL, Koenigsberg SS and Loffler FE, Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature* **424**:62–65 (2003).

33 Cupples AM, Spormann AM and McCarty PL, Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. *Appl Environ Microbiol* **69**:953–959 (2003).

34 El Fantroussi S, Naveau H and Agathos SN, Anaerobic dechlorinating bacteria. *Biotechnol Prog* **14**:167–188 (1998).

35 Loeffler FE, Cole JR, Ritalathi KM and Tiedje JM, Diversity of dechlorinating bacteria, in *Dehalogenation: Microbial Processes and Environmental Applications*, ed. by Häggblom MM and Bossert ID. Kluwer Academic, Boston, MA, pp. 53–87 (2003).

36 Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, *et al*, Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. *Appl Environ Microbiol* **68**:485–495 (2002).

37 Kube M, Beck A, Zinder SH, Kuhl H, Reinhardt R and Adrian L, Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat Biotechnol* **23**:1269–1273 (2005).

38 Magnuson JK, Romine MF, Burris DR and Kingsley MT, Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of tceA and substrate range characterization. *Appl Environ Microbiol* **66**:5141–5147 (2000).

39 Holscher T, Krajmalnik-Brown R, Ritalahti KM, Von Wintzingerode F, Gorisch H, Loffler FE, *et al*, Multiple nonidentical reductive-dehalogenase-homologous genes are common in *Dehalococcoides*. *Appl Environ Microbiol* **70**:5290–5297 (2004).

40 Regeard C, Maillard J and Holliger C, Development of degenerate and specific PCR primers for the detection

and isolation of known and putative chloroethene reductive dehalogenase genes. *J Microbiol Meth* **56**:107–118 (2004).
41 Ballapragada BS, Stensel DH, Puhakka JA and Ferguson JF, Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ Sci Technol* **31**:1728–1734 (1997).
42 Cupples AM, Spormann AM and McCarty PL, Vinyl chloride and *cis*-dichloroethene dechlorination kinetics and microorganism growth under substrate limiting conditions. *Environ Sci Technol* **38**:1102–1107 (2004).
43 Smatlak CR, Gossett JM and Zinder SH, Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ Sci Technol* **30**:2850–2858 (1996).
44 Schink B, Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* **61**:262–280 (1997).
45 Aulenta F, Gossett JM, Petrangeli Papini MP, Rossetti S and Majone M, Comparative study of methanol, butyrate, and hydrogen as electron donors for long-term dechlorination of tetrachloroethene in mixed anerobic cultures. *Biotechnol Bioeng* **91**:743–753 (2005).
46 Fennell DE, Gossett JM and Zinder SH, Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ Sci Technol* **31**:918–926 (1997).
47 Aulenta F, Majone M, Verbo P and Tandoi V, Complete dechlorination of tetrachloroethene to ethene in presence of methanogenesis and acetogenesis by an anaerobic sediment microcosm. *Biodegradation* **13**:411–424 (2002).
48 Yang Y and McCarty PL, Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environ Sci Technol* **32**:3591–3597 (1998).
49 Fennell DE and Gossett JM, Modeling the production of and competition for hydrogen in a dechlorinating culture. *Environ Sci Technol* **32**:2450–2460 (1998).
50 Lovley DR, Chapelle FH and Woodward JC, Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. *Environ Sci Technol* **28**:1205–1210 (1994).
51 Loffler FE, Tiedje JM and Sanford RA, Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Appl Environ Microbiol* **65**:4049–4056 (1999).
52 Luijten ML, Roelofsen W, Langenhoff AA, Schraa G and Stams AJ, Hydrogen threshold concentrations in pure cultures of halorespiring bacteria and at a site polluted with chlorinated ethenes. *Environ Microbiol* **6**:646–650 (2004).
53 Kassenga G, Pardue JH, Moe WM and Bowman KS, Hydrogen thresholds as indicators of dehalorespiration in constructed treatment wetlands. *Environ Sci Technol* **38**:1024–1030 (2004).
54 Lovley DR and Goodwin S, Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochim Cosmochim Acta* **52**:2993–3003 (1988).
55 Lu XX, Tao S, Bosma T and Gerritse J, Characteristic hydrogen concentrations for various redox processes in batch study. *J Environ Sci Health A* **36**:1725–1734 (2001).
56 Wu W-M, Nye J, Jain MK and Hickey RF, Anaerobic dechlorination of trichloroethylene (TCE) to ethylene using complex organic materials. *Water Res* **32**:1445–1454 (1998).
57 Yang Y and McCarty PL, Biomass, oleate, and other possible substrates for chloroethene reductive dechlorination. *Bioremed J* **4**:125–133 (2000).
58 Yang Y and McCarty PL, Comparison between donor substrates for biologically enhanced tetrachloroethene DNAPL dissolution. *Environ Sci Technol* **36**:3400–3404 (2002).
59 DiStefano TD, Bral R, Duran M and Speece RE, A comparison of complex electron donors for anaerobic dechlorination of PCE. *Bioremed J* **5**:131–143 (2001).
60 Kao CM, Chen YL, Chen SC, Yeh TY and Wu WS, Enhanced PCE dechlorination by biobarrier systems under different redox conditions. *Water Res* **37**:4885–4894 (2003).
61 Yu S and Semprini L, Comparison of trichloroethylene reductive dehalogenation by microbial communities stimulated on silicon-based organic compounds as slow-release anaerobic substrates. *Water Res* **36**:4985–4996 (2002).
62 Novak PJ, Daniels L and Parkin GF, Enhanced dechlorination of carbon tetrachloride and chloroform in the presence of elemental iron and *Methanosarina barkeri, Methanosarina thermophila*, or *Mmethanosaeta concillii*. *Environ Sci Technol* **32**:1438–1443 (1998).
63 Parkin GF, Weathers LJ and Novak PJ, Enhanced anaerobic bioremediation using elemental iron. *Proc Water Resources and Urban Environment*, Chicago, IL, pp. 128–133 (1998).
64 Karri S, Sierra-Alvarez R and Field JA, Zero-valent iron as an electron donor for methanogenesis and sulfate reduction in anaerobic sludge. *Biotechnol Bioeng* **92**:810–819 (2005).
65 Daniels L, Belay N, Rajagopal BS and Weimer PJ, Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons. *Science* **237**:509–511 (1987).
66 Shrout JD, Williams AG, Scherer MM and Parkin GF, Inhibition of bacterial perchlorate reduction by zero-valent iron. *Biodegradation* **16**:23–32 (2005).
67 El Mamouni R, Jacquet R, Gerin P and Agathos SN, Influence of electron donors and acceptors on the bioremediation of soil contaminated with trichloroethene and nickel: laboratory- and pilot-scale study. *Water Sci Technol* **45**:49–54 (2002).
68 Deweerd KA, Concannon F and Suflita JM, Relationship between hydrogen consumption, dehalogenation, and the reduction of sulfur oxyanions by *Desulfomonile tiedje*. *Appl Environ Microbiol* **57**:1929–1934 (1991).
69 Hoelen TP and Reinhard M, Complete biological dehalogenation of chlorinated ethylenes in sulfate containing groundwater. *Biodegradation* **15**:395–403 (2004).
70 Cabirol N, Jacob F, Perrier J, Fouillet B and Chambon P, Interaction between methanogenic and sulfate-reducing microorganisms during dechlorination of a high concentration of tetrachloroethylene. *J Gen Appl Microbiol* **44**:297–301 (1998).
71 Townsend GT and Suflita JM, Influence of sulfur oxyanions on reductive dehalogenation activities in *Desulfomonile tiedjei*. *Appl Environ Microbiol* **63**:3594–3599 (1997).
72 Nelson DK, Hozalski RM, Clapp LW, Semmens MJ and Novak PJ, Effect of nitrate and sulfate on dechlorination by a mixed hydrogen-fed culture. *Bioremed J* **6**:225–236 (2002).
73 Warner KA, Gilmour CC and Capone DG, Reductive dechlorination of 2,4-dichlorophenol and related microbial processes under limiting and non-limiting sulfate concentration in anaerobic mid-Chesapeake Bay sediments. *FEMS Microbiol Ecol* **40**:159–165 (2002).
74 Fennell DE and Gossett JM, Microcosms for site-specific evaluation of enhanced biological reductive dehalogenation, in *Dehalogenation: Microbial Processes and Environmental Applications*, ed. by Häggblom MM and Bossert ID. Kluwer Academic, Boston, MA, pp. 385–420 (2003).
75 Morse JJ, Alleman BC, Gossett JM, Zinder SH, Fennell DE, Sewell GW, *et al*, *A Treatability Test for Evaluating the Potential Applicability of the Reductive Anaerobic Biological In Situ Treatment Technology (RABITT) to Remediate Chloroethenes. A Draft Technical Protocol Developed for the Environmental Security Technology Certification Program, Department of Defense*. US Government Printing Office, Washington, DC (1997).
76 Clapp LW, Semmens MJ, Novak PJ and Hozalski RM, Model for *in situ* perchloroethene dechlorination via membrane-delivered hydrogen. *J Environ Eng* **130**:1367–1381 (2004).
77 Fang Y, Hozalski RM, Clapp LW, Novak PJ and Semmens MJ, Passive dissolution of hydrogen gas into groundwater using hollow-fiber membranes. *Water Res* **36**:3533–3542 (2002).

78 Ma X, Novak PJ, Clapp LW, Semmens MJ and Hozalski RM, Evaluation of polyethylene hollow-fiber membranes for hydrogen delivery to support reductive dechlorination in a soil column. *Water Res* **37**:2905–2918 (2003).
79 Newell CJ, Fisher RT and Hughes J, Direct hydrogen addition for the in-situ biodegradation of chlorinated solvents. *NGWA Petroleum Hydrocarbons Conf*, Houston, TX, pp. 791–800 (1997).
80 Zhang XH, Sewell GW and Cui SY, An improved method of hydrogen production as electron donor for anaerobic bioremediation. *J Environ Sci Health A* **36**:1661–1670 (2001).
81 DiStefano TD, Gossett JM and Zinder SH, Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl Environ Microbiol* **58**:3622–3629 (1992).
82 Krumholz LR, *Desulfuromonas chloroethenica* sp. nov. uses tetrachloroethylene and trichloroethylene as electron acceptors. *Int J Syst Bacteriol* **47**:1262–1263 (1997).
83 He Q and Sanford RA, Acetate threshold concentrations suggest varying energy requirements during anaerobic respiration by *Anaeromyxobacter* 4. *Appl Environ Microbiol* **70**:6940–6943 (2004).
84 Oude Elferink SJWH, Luppens SBI, Maecelis CLM and Stams AJM, Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge. *Appl Environ Microbiol* **64**:2301–2303 (1998).
85 He J, Sung Y, Dollhopf ME, Fathepure BZ, Tiedje JM and Loffler FE, Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environ Sci Technol* **36**:3945–3952 (2002).
86 Mackay DM and Cherry JA, Groundwater contamination: pump-and-treat remediation. *Environ Sci Technol* **23**:630–636 (1989).
87 Oolman T, Godard ST, Pope GA, Jin M and Kirchner K, DNAPL flow behavior in a contaminated aquifer: evaluation of field data. *Ground Water Monit Remed* **15**:125–137 (1995).
88 Christ JA, Ramsburg CA, Abriola LM, Pennell KD and Loffler FE, Coupling aggressive mass removal with microbial reductive dechlorination for remediation of DNAPL source zones: a review and assessment. *Environ Health Perspect* **113**:465–477 (2005).
89 Londergan JT, Meinardus HW, Mariner PE, Jackson RE, Brown CL, Dwarakanath V, *et al*, DNAPL removal from a heterogeneous alluvial aquifer by surfactant-enhanced aquifer remediation. *Ground Water Monit Remed* **21**:57–67 (2001).
90 Rao PSC, Annable MD, Sillan RK, Dai D, Hatfield K, Graham WD, *et al*, Field-scale evaluation of *in situ* cosolvent flushing for enhanced aquifer remediation. *Water Resources Res* **33**:2673–2686 (1997).
91 Ramsburg CA, Abriola LM, Pennell KD, Loffler FE, Gamache M, Amos BK, *et al*, Stimulated microbial reductive dechlorination following surfactant treatment at the Bachman Road site. *Environ Sci Technol* **38**:5902–5914 (2004).
92 Yu S and Semprini L, Kinetics and modeling of reductive dechlorination at high PCE and TCE concentrations. *Biotechnol Bioeng* **88**:451–464 (2004).
93 Nielsen RB and Keasling JD, Reductive dechlorination of chlorinated ethene DNAPLs by culture enriched from contaminated groundwater. *Biotechnol Bioeng* **62**:160–165 (1999).
94 Yang Y and McCarty PL, Biologically enhanced dissolution of tetrachloroethene DNAPL. *Environ Sci Technol* **34**:2979–2984 (2000).
95 Adamson DT, McDade JM and Hughes JB, Inoculation of a DNAPL source zone to initiate reductive dechlorination of PCE. *Environ Sci Technol* **37**:2525–2533 (2003).
96 Adamson DT, Lyon DY and Hughes JB, Flux and product distribution during biological treatment of tetrachloroethene dense non-aqueous-phase liquid. *Environ Sci Technol* **38**:2021–2028 (2004).
97 Coleman NV, Mattes TE, Gossett JM and Spain JC, Biodegradation of *cis*-dichloroethene as the sole carbon source by a beta-proteobacterium. *Appl Environ Microbiol* **68**:2726–2730 (2002).
98 Coleman NV, Mattes TE, Gossett JM and Spain JC, Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. *Appl Environ Microbiol* **68**:6162–6171 (2002).
99 Newell CJ, Aziz CE, Haas PE, Hughes J and Khan TA, Two novel methods for enhancing source zone bioremediation: direct hydrogen addition and electron acceptor diversion. *Proc Sixth Int Symp on In Situ and On-Site Bioremediation*, San Diego, CA, Vol. 7, pp. 19–26 (2001).
100 Newell CJ, Aziz CE, Haas PE, Hughes J and Khan TA, Results from two direct hydrogen delivery field tests for enhanced dechlorination. *Proc Second Int Conf on Remediation of Chlorinated and Recalcitrant Compounds*, Monterey, CA, Vol. 4, pp. 31–37 (2000).
101 McCarty PL, Chu MY and Kitanidis PK, Biological degradation and enhanced dissolution of chlorinated solvent DNAPL. *Proc 3rd Eur Bioremediation Conf*, Chania, (2005).
102 Yeh DH and Pavlostathis SG, Development of hexachlorobenzene-dechlorinating mixed cultures using polysorbate surfactants as a carbon source. *Water Sci Technol* **43**:43–50 (2001).
103 Yeh DH and Pavlostathis SG, Anaerobic biodegradability of Tween surfactants used as a carbon source for the microbial reductive dechlorination of hexachlorobenzene. *Water Sci Technol* **52**:343–349 (2005).
104 Aulenta F, Fina A, Potalivo M, Petrangeli Papini M, Rossetti S and Majone M, Anaerobic transformation of tetrachloroethane, perchloroethylene, and their mixtures by mixed-cultures enriched from contaminated soils and sediments. *Water Sci Technol* **52**:357–362 (2005).
105 Adamson DT and Parkin GF, Impact of mixtures of chlorinated aliphatic hydrocarbons on a high-rate, tetrachloroethene-dechlorinating enrichment culture. *Environ Sci Technol* **34**:1959–1965 (2000).
106 Adamson DT and Parkin GF, Product distribution during transformation of multiple contaminants by a high-rate, tetrachloroethene-dechlorinating enrichment culture. *Biodegradation* **12**:337–348 (2001).
107 Adamson DT and Parkin GF, Biotransformation of mixtures of chlorinated aliphatic hydrocarbons by an acetate-grown methanogenic enrichment culture. *Water Res* **33**:1482–1494 (1999).
108 Adamson DT and Pakin GF, Dependence of a high-rate PCE-dechlorinating enrichment culture on methanogenic activity. *Bioremed J* **5**:51–62 (2001).
109 Aulenta F, Di Tomassi C, Cupo C and Majone M, Influence of hydrogen on the reductive dechlorination of tetrachloroethene (PCE) to ethene in a methanogenic biofilm reactor: role of mass transport phenomena. *J Chem Technol Biotechnol* in press.
110 Tandoi V, DiStefano TD, Bowser PA, Gossett JM and Zinder SH, Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high rate anaerobic enrichment culture. *Environ Sci Technol* **28**:973–979 (1994).
111 Davey ME and O'Toole GA, Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* **64**:847–867 (2000).
112 Fennell DE, Carroll AB, Gossett JM and Zinder SH, Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environ Sci Technol* **35**:1830–1839 (2001).