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Assessment of natural or enhanced in situ bioremediation at a chlorinated solvent-contaminated aquifer in Italy: a microcosm study

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Abstract

A microcosm study was used to assess the potential for in situ natural or enhanced bioremediation at a chloroethane- (i.e., tetrachloroethane, TeCA) and chloroethene-contaminated (i.e., tetrachloroethene, PCE; trichloroethene, TCE) groundwater in Northern Italy. All the live microcosms were positive for dechlorination, indicating the presence of an active native dechlorinating population in the subsurface. All the tested electron donors (i.e., yeast extract, lactate, butyrate, hydrogen) promoted enhanced dechlorination of chlorinated contaminants. Lactate- and butyrate-amended microcosms performed the best, and also dechlorinated the solvents past *cis*-dichloroethene (*cis*-DCE). The microcosm bioaugmented with a PCE-dechlorinating mixed culture containing *Dehalococcoides* spp. dechlorinated groundwater contaminants to DCE, vinyl chloride (VC), and ethene (ETH). In conclusion, results from this microcosm study indicate the potential for enhancing full dechlorination at the contaminated site, through a proper addition of a suitable electron donor (e.g., lactate or butyrate) and/or through bioaugmentation with a *Dehalococcoides*-containing culture.

Keywords: Bioremediation; Chlorinated solvents; *Dehalococcoides*; Microcosm study; Reductive dechlorination

1. Introduction

Chlorinated aliphatic hydrocarbons, such as tetrachloroethene (PCE), trichloroethene (TCE), and tetrachloroethane (TeCA), are ubiquitous groundwater pollutants due to their widespread use as solvents, degreasing agents, and chemical feedstock. These contaminants are of special concern because they are known or suspected carcinogens leading to the setting of maximum concentration levels in groundwater.

For the clean up of groundwater contaminated by chlorinated solvents, the potential of microorganisms to transform toxic chlorinated compounds into harmless products is a major advantage of bioremediation techniques in comparison to chemical–physical techniques where pollutants are often only transferred into another phase.

In general, polychlorinated compounds, such as TeCA and PCE, tend to be resistant to aerobic biodegradation. These compounds are more oxidized than their non-halogenated counterparts due to the presence of the highly electronegative halogen substituents, which provide stability to the molecule (Vogel et al., 1987; Pavlostathis et al., 2003). As a result, reduction of these compounds is more likely to occur than oxidation as the degree of halogenation increases. Generally, reduction entails the replacement of halogen substituents by hydrogen. Several different anaerobic microorganisms can sequentially remove chlorine substituents through the process of reductive dechlorination (RD) (Middeldorp et al., 1999; Pavlostathis et al., 2003). Within the last 15 years, basic research on anaerobic dechlorination has suggested that the transformation of chlorinated contaminants into harmless nonchlorinated end-products can be practically achieved by enhancing of bacterial RD in the field. Enhanced in situ RD has been successfully applied for

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remediation of chlorinated solvent-contaminated sites (Ellis et al., 2000). However, relatively few studies of TeCA degradation in the environment have been conducted compared to the numerous field and laboratory studies of PCE or TCE degradation. Enhanced in situ RD can be accomplished by either enhancing halorespiratory activity of native microbial dechlorinating population (e.g., through the addition of electron donors and/or nutrients to produce favorable reducing conditions) or by inoculating the aquifer with microorganisms that are capable of degrading the target pollutants (bioaugmentation). Prior to the initiation of a full scale RD system, it is important to determine, by means of microcosm studies, whether native dechlorinating bacteria are present that can transform the chlorinated solvents to harmless nonchlorinated products (e.g., ethene, ETH), or whether dechlorination will be carried out only to less-chlorinated products (e.g., dichloroethene, DCE). Moreover, it is important to know if added electron donors are used by the indigenous population, if metabolic processes are in competition with RD for added electron donors (e.g., nitrate reduction, sulfate reduction, methanogenesis), and/or if other unfavorable conditions (e.g., inhibitors) are present.

At present, *Dehalococcoides ethenogenes* strain 195 (Maymò-Gatell et al., 1997), is the only isolated bacterium capable of dechlorination of chloroethenes (and dichloroethanes) past *cis*-DCE and therefore, bioaugmentation with inocula containing this bacterium can play an important role in full remediation of chloroethene- and chloroethane-contaminated sites.

This paper describes a microcosm study designed to assess the potential for microbial in situ RD at a chlorinated solvent-contaminated (i.e., tetrachloroethane (TeCA), trichloroethene (TCE), and tetrachloroethene (PCE)) aquifer (Petrangeli Papini et al., 2003), located in Northern Italy. In particular, this study was aimed at evaluating if RD by native population could be enhanced by addition of substrates (i.e., yeast extract, lactate, butyrate, hydrogen) or trace elements (i.e., yeast extract, vitamin B_{12}) and could proceed past *cis*-DCE. Moreover, the influence on RD of competitive metabolic processes (i.e., nitrate reduction and sulfate reduction) and the application of a *Dehalococcoides* spp.-containing inoculum for the treatment of the TeCA-, PCE-, and TCE-contaminated groundwater were also evaluated by performance of appropriate microcosm tests.

2. Materials and methods

2.1. Microcosm setup

Microcosms were prepared by using soil and groundwater samples taken from the contaminated site, near the contamination source (Petrangeli Papini et al., 2003). Groundwater used for microcosm studies contained TeCA (80.2 μmol/l), PCE (7.8 μmol/l), TCE (15.0 μmol/l), nitrate (0.6 mmol/l), and sulfate (5.1 mmol/l) along with trace levels of other chlorinated aliphatic and aromatic compounds (overall COD approx. 30 mg/l). Soil samples were collected by following the procedures described by Fennell et al. (2001) and by Morse et al. (1997). After collection, soil and groundwater were stored in glass jars and maintained in coolers at 4 °C until use. Soil total organic carbon content was 1.5 g/kg. For microcosm preparation, the soil samples, groundwater, autoclaved 250-ml serum bottles, gray butyl Teflon-faced stoppers, spatulas, and other material were placed inside an anaerobic glove box, under nitrogen atmosphere. Thirteen microcosm treatments were set-up and triplicate bottles were prepared for each treatment. The experimental conditions for the bottle microcosms are shown in Table 1. For treatments 1 to 10 (Table 1), 60 g (dry weight) of soil was dispensed in a 250-ml serum bottle, and amended with 150 ml of groundwater. Groundwater was previously spiked with resazurin (final concentration 1 mg/l), as a redox indicator. After preparation, the bottles were sealed with Teflon-faced butyl rubber stoppers and spiked with the selected electron donor (yeast extract, lactate, butyrate, hydrogen, or none). Each electron donor was added either along with growth factors (i.e., yeast extract at 20 mg/l and vitamin B_{12} at 0.05 mg/l) or without. Yeast extract, lactate, butyrate, and growth factors were added from a stock solution using syringes. Hydrogen gas was added in the headspace of the serum bottles at 3 mM (nominal concentration: total moles divided by the volume of liquid phase), by using gas-tight syringe. After preparation, treatment 1 was autoclaved at 121 °C for 1 h (abiotic control). For treatment 11 (bioaugmentation microcosm), 35 ml (~5 mg VSS) of a H_2-utilizing, PCE-dechlorinating culture containing *Dehalococccoides* spp. (Aulenta et al., 2002; Aulenta et al., 2004) was dispensed in a 250-ml serum bottle and amended with 150 ml of groundwater (in the absence of soil). Thereafter, the bottle was sealed and spiked with H_2 and growth factors. For treatments 12 and 13, 60 g (dry weight) of soil was dispensed in a 250-ml serum bottle, and diluted with 150 ml reduced anaerobic mineral medium (RAMM). RAMM composition and preparation is that reported by Tandoi et al. (1994). Thereafter, the bottles were sealed and spiked with TCE and H_2 (treatment no. 12) or TCE and butyrate (treatment no. 13). All the microcosms were incubated statically in the dark at room temperature (18–22 °C).

All the chemicals used as microcosm amendments or used to prepare analytical standards were of analytical grade. Liquid-chlorinated solvents used to prepare analytical standards were purchased from Aldrich (Milwaukee, WI).

2.2. Analytical procedures and monitoring protocol

Upon setup, the microcosms had a purple tint (given by the resazurin added to the groundwater) indicating unreduced conditions. After few days, all the microcosms,

Table 1
Bottle microcosm experimental conditions and dechlorinating activity observed after 98 days of incubation

Treatment no.	Soil and groundwater amended with	Chloride cumulative release (μmol/l)[a]	Dechlorination (%)[b]	Dechlorination past *cis*-DCE
1	None (abiotic control)	0.0 (0.7)	0.0 (0.2)	No
2	None (biotic control)	12.6 (1.9)	3.2 (0.48)	No
3	None+g.f.[c]	32.1 (1.6)	8.2 (0.4)	No
4	YE (180 mg/l)+g.f.	54.2 (3.0)	13.8 (0.8)	No
5	Lactate (3 mmol/l)	110.0 (11.7)	27.9 (3.0)	Yes (mainly VC)
6	Lactate (3 mmol/l)+g.f.	157.8 (9.8)	40.0 (2.5)	Yes (mainly VC)
7	Butyrate (3 mmol/l)	48.7 (20.8)	12.3 (5.3)	Yes (mainly VC)
8	Butyrate (3 mmol/l)+g.f.	106.7 (20.2)	27.1 (5.1)	Yes (mainly VC)
9	Hydrogen (3 mmol/l)	12.9 (0.5)	3.3 (0.1)	No
10	Hydrogen (3 mmol/l)+g.f.	60.3 (6.2)	15.3 (1.6)	No
	Groundwater amended with			
11	Hydrogen (3 mmol/l)+Inoculum[d]+g.f.	167.8 (14.5)	42.6 (3.7)	Yes (VC, ETH)
	Soil amended with			
12	RAMM[e]+TCE+Hydrogen (3 mmol/l)+g.f.	8.5 (3.9)	22.5 (10.3)	No
13	RAMM[e]+TCE+Butyrate (3 mmol/l)+g.f.	35.4 (3.1)	93.7 (8.2)	Yes (ETH)

[a] Mean value and standard deviation (in parentheses) of three replicates.
[b] Dechlorination (%) is calculated as the ratio between the chloride cumulative release at day 98 and the initial chloride associated with groundwater contaminants. For treatments 12 and 13, dechlorination (%) refers to the spiked TCE. 100% dechlorination corresponds to the complete transformation of chlorinated compounds into nonchlorinated end-products.
[c] g.f. (growth factors): yeast extract (20 mg/l) and vitamin B_{12} (0.05 mg/l).
[d] The inoculum is a H_2-utilizing PCE dechlorinating culture containing *Dehalococcoides* spp. (Aulenta et al., 2004).
[e] RAMM: reduced anaerobic mineral medium.

except for the autoclaved, abiotic control (treatment no. 1), and the biotic control (treatment no. 2), turned clear indicating establishment of reduced conditions.

Every 14 days, microcosms were analyzed for chlorinated solvents and electron donors. The electron donors were re-added every time analyses indicated they were completely exhausted. Chloroethenes, ETH, and CH_4 were quantified in 100 μl headspace samples by using gas-chromatography (GC) with flame-ionization detector (FID) as previously described (Aulenta et al., 2002). Chloroethanes were quantified by injecting 50 μl of serum bottle headspace into a Carlo Erba 5300 Mega Series gas-chromatograph (capillary column HP-5, length 30 m, ID 0.53 mm, film 5 μm; helium carrier gas 3 ml/min; oven temperature 50 °C for 2 min then raised to 210 °C at 10 °C/min; flame ionization detector at 260 °C). Hydrogen was analyzed in 500 μl headspace samples by using GC with thermal-conductivity detector (TCD) as described previously (Aulenta et al., 2002). Standards for chlorinated compounds, ETH, CH_4, and H_2 were prepared by adding a known amount of each compound to a serum bottle with the same headspace to liquid ratio as the microcosm bottles (Gossett, 1987). Concentrations of volatile compounds are expressed as total moles in the bottle divided by the liquid phase (i.e., nominal concentrations). Filtered (0.22 μm) liquid samples were analyzed for lactate and volatile fatty acids by using GC-FID (GC Perkin-Elmer 8400, 2 m×2 mm glass column packed with Carbograph 1 AL 80/120), and for nitrate, sulfate, and chloride, by using ion chromatography (Dionex DX-100, Ionpac As9-Sc column).

2.3. Data interpretation

TeCA can be anaerobically converted to non- or less-chlorinated ethanes and ethenes via three mechanisms

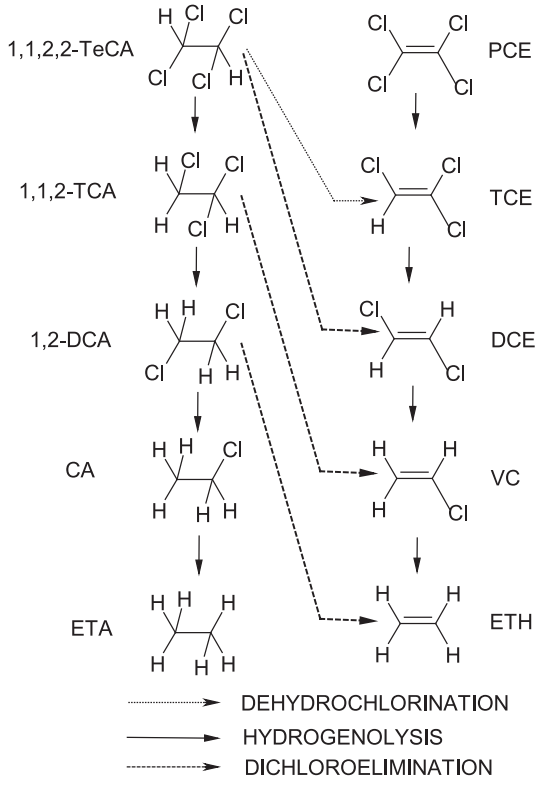

Fig. 1. Anaerobic degradation pathways for chloroethanes and chloroethenes. Modified from Lorah and Olsen (1999).

(Fig. 1; Chen et al., 1996; Lorah and Olsen, 1999). Two mechanisms, hydrogenolysis and dichloroelimination, require the input of two electrons resulting in the release of one or two chlorine atoms, respectively. The third mechanism, dehydrochlorination, is a nonredox reaction by which HCl is released and a double bond is formed between two neighboring carbon atoms. On the other hand, PCE and TCE are generally dechlorinated via hydrogenolysis (Fig. 1). Hence, chloroethanes and chloroethenes can share common reaction intermediates (i.e., TCE, DCE, and vinyl chloride (VC)), depending on chloroethane degradation pathways. For instance, DCE formation could result from both TeCA dichloroelimination and TCE hydrogenolysis. In this paper, an integrated parameter (i.e., chloride cumulative release) was introduced to assess the overall dechlorinating activity in each microcosm. The chloride cumulative release is the amount of chloride released by the dechlorination processes as calculated (at any time) from the sum of all the measured (by gas-chromatography) dechlorination intermediates, by taking into account their initial and residual chlorination degree. Because TCE could be either a native substrate or a product of TeCA dechlorination, its contribution to the chloride cumulative release was considered as positive or negative dependent on its increase or decrease from the background value. This approach has been previously described by Aulenta et al. (2004).

3. Results and discussion

Table 1 shows the chloride cumulative release in the different treatments after 98 days of incubation (mean value and standard deviation of three replicates are reported). All the microcosms, with the exception of the abiotic control, showed dechlorinating activity, suggesting the presence of native dechlorinating populations in soil.

Little dechlorination (12.6 μmol Cl^- released/l) was observed in the biotic control (treatment no. 2) and resulted in slow but steady increase of TCE concentration, probably due to TeCA dehydrochlorination, a nonredox reaction that does not require the input of electrons.

All the electron donors (i.e., yeast extract, lactate, butyrate, hydrogen, treatments 4 to 10) enhanced dechlorination with respect to the biotic control, with lactate- and butyrate-amended microcosms performing the best (Fig. 2A). The lactate-amended microcosms showed the shortest lag-phase (i.e., time prior to the onset of dechlorination) and the highest initial dechlorination rate (Fig. 2A). Dechlorination of *cis*-DCE was observed only in lactate- and butyrate-amended microcosms where VC was formed. After 98 days of incubation, the main daughter products formed in the lactate-amended microcosms were TCA (77.5 μmol/l), DCA (20.2 μmol/l), DCE (20.9 μmol/l), and VC (2.3 μmol/l), along with trace levels of ETH (0.22 μmol/l). The TCA produced, accounted for most of initial TeCA (on a molar basis), clearly indicating that hydrogenolysis was the main

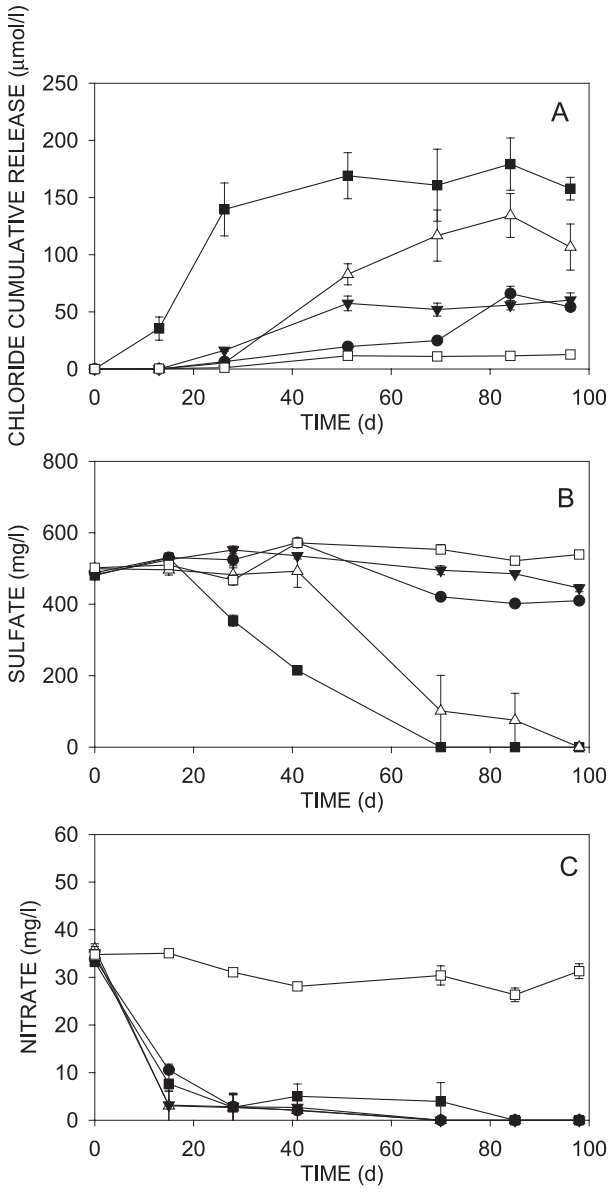

Fig. 2. Effect of the different amendments on: chloride cumulative release (A), sulfate reduction (B), and nitrate reduction (C). (Mean value and standard deviation of three replicates). Lactate (treatment No. 6; ■); butyrate (treatment No. 8; △); hydrogen (treatment No. 10; ▼); yeast extract (treatment No. 4; ●); and biotic control (treatment No. 2; □).

TeCA degradation pathway. However, the accumulation of DCE indicates that TeCA dichloroelimination was also acting. Conversely, the abiotic production of TCE (dehydrochlorination route) was a minor degradation pathway. Different results were reported by Chen et al. (1996). The authors investigated the transformation of TeCA under methanogenic conditions: dichloroelimination resulting in the formation of DCEs and abiotic dehydrochlorination resulting in the formation of TCE, were the more important routes in the initial degradation of TeCA.

Product distribution in butyrate-amended microcosms was similar to that of lactate-amended microcosms. The presence of VC and ETH in lactate- and butyrate-amended

microcosms indicates that, after 98 days of incubation, the complete dechlorination of contaminants to nonchlorinated end-products is not limited by microbial constrains (i.e., lack in the subsurface of microorganisms capable to metabolize contaminants) and it could be achieved in longer incubation times.

A significantly different chlorinated contaminants transformation pathway was observed in H_2-amended microcosms: TCA (from hydrogenolysis of TeCA) was not formed and only little DCE (29.9 μmol/l) accumulated.

For all the tested donors, the addition of growth factors (i.e., yeast extract and vitamin B_{12}) had a beneficial effect on the dechlorinating activity (Table 1). This suggests that the activity of soil microorganisms was probably limited by the lack of micronutrients. However, vitamin B_{12} and other micronutrients have been previously reported to increase the rate and extent of chlorinated solvents degradation by some anaerobic cultures. For example, the pure culture of *D. ethenogenes* requires vitamin B_{12} and anaerobic digester sludge supernatant or cell extract to dechlorinate PCE (Maymò-Gatell et al., 1997). Corrinoids such as vitamin B_{12} are a factor in biological reductive dechlorination by pure strains of *Dehalospirillum multivorans* (Neumann et al., 1994). Additions of vitamin B_{12} to a dichloromethane-degrading enrichment culture increased the rate and extent of biodegradation of carbon tetrachloride tenfold, and minimized accumulation of dichloromethane and chloromethane compared to culture without the vitamin (Hashsham et al., 1995). Moreover, vitamin B_{12} was reported to catalyze the abiotic dechlorination when an external reductant is added. Additions of vitamin B_{12} in the presence of titanium citrate can support the abiotic dechlorination of PCE (Lesage et al., 1996).

The presence of native microorganisms capable of performing full dechlorination (at least of chloroethenes) in soil is confirmed by the results of treatment 13 where the soil was diluted with RAMM and spiked with butyrate and TCE (approx. 15 μmol/l) as the only contaminant: complete dechlorination to ETH was observed in less than 100 days,

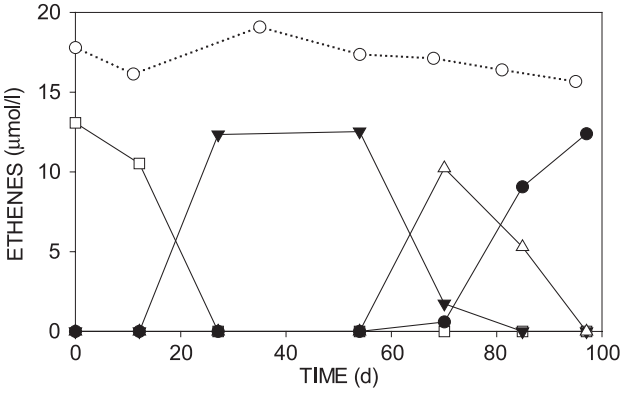

Fig. 3. Reductive dechlorination of TCE by the soil microcosm amended with RAMM+butyrate+yeast extract+vitamin B_{12}+TCE (treatment No. 13). TCE (□); DCE (▼); VC (△); ETH (●); and TCE (abiotic control; ○).

with only transient accumulation of *cis*-DCE and VC (Fig. 3). On the contrary, only partial dechlorination of TCE to *cis*-DCE was observed when the soil, diluted with RAMM, was spiked with H_2 (treatment no. 12). This confirms that organic substrates (i.e., lactate, butyrate) were more effective than H_2, stimulating native dechlorinating populations.

It is noteworthy that dechlorination of TeCA and TCE was observed also in the presence of nitrate and sulfate (Fig. 2A–C). Previous studies indicated that, in the presence of nitrate or sulfate, the reductive dechlorination was inhibited (Bagley and Gossett, 1990). The inhibitory effect was explained in terms of competition between dechlorinators and nitrate or sulfate reducers for available electron donor; moreover, nitrous oxide and sulfide, produced by nitrate and sulfate reduction, were found to inhibit the reductive dechlorination of chlorinated solvents (Nelson et al., 2002; Hoelen and Reinhard, 2004). On the other hand, the cometabolic dechlorination of 1,1,1-tetrachloroethane, mediated by sulfate reducing bacteria, was also reported (Klecka et al., 1990).

The finding that in the present study dechlorination occurred in the presence of sulfate and nitrate could be due to that sufficient electron donor was added so minimizing the competition between dechlorination and competing metabolic processes.

On the other hand, it could not be excluded that initial dechlorination of TeCA was a fortuitous process mediated by sulfate-reducing bacteria.

All added electron donors promoted the rapid reduction of nitrate, whereas sulfate reduction was promoted only by lactate and butyrate (Fig. 2B, C). Neither sulfate nor nitrate reduction was observed in the nonamended biotic control (treatment no. 2), likely indicating that soil organic carbon was probably not enough to support activity of native microorganisms.

Nevertheless, added substrates were effectively utilized by native soil microorganisms. In particular, lactate and butyrate were rapidly converted to acetate, which accumulated up to 6 mM (and also propionate for lactate), whereas only little acetate was produced from hydrogen (<0.4 mM). Methanogenic activity was absent in all the microcosms probably because of the inhibitory effect of the chlorinated solvents on methanogenic populations (data not shown). However, it is also possible that methanogens were present initially in the soil at such low number (also considering the low soil organic carbon content); hence, the development of significant methanogenic activity would have required longer incubation time.

In the microcosm where groundwater was bioaugmented with a PCE-dechlorinating mixed culture containing *Dehalococcoides* spp. (treatment no. 11), chlorinated contaminants (TeCA, PCE, and TCE) were degraded at higher rates than in the presence of soil consortia, and mainly converted to VC (45.0 μmol/l), *cis*-DCE (22.1 μmol/l), and ETH (5.5 μmol/l). Comparison with treatment no. 10 showed that bioaugmentation was effective in increasing of dechlorination rate (at

least with H_2 as the electron donor). Also in this case, dechlorination occurred in the presence of nitrate and sulfate.

4. Conclusions

From the present microcosm study, the following statements can be concluded:

- All the live microcosms were positive for dechlorination indicating the presence in the site of native dechlorinating populations; however, dechlorinating activity in the nonamended microcosm (biotic control) was very slow and only resulted in accumulation of TCE, from dehydrochlorination of TeCA.
- All the electron donors tested (yeast extract, hydrogen, lactate, butyrate) enhanced dechlorination with respect to the nonamended microcosm (biotic control); moreover, for all the tested donors, the addition of growth factors (i.e., yeast extract and vitamin B_{12}) had a beneficial effect on dechlorination.
- High-rate dechlorinating activity was observed in lactate- and butyrate-amended microcosms and in the microcosm inoculated with a *Dehalococoides* spp.-containing culture.

In conclusion, the potential exists for enhancing full dechlorination at the chlorinated solvent-contaminated site, through a proper addition of a suitable electron donor (e.g., lactate or butyrate) and/or through bioaugmentation with a *Dehalococcoides*-containing culture.

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