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Anaerobic bioremediation of groundwater containing a mixture of 1,1,2,2-tetrachloroethane and chloroethenes

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Abstract

This study investigated the biotransformation pathways of 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) in the presence of chloroethenes (i.e. tetrachloroethene, PCE; trichloroethene, TCE) in anaerobic microcosms constructed with subsurface soil and groundwater from a contaminated site. When amended with yeast extract, lactate, butyrate, or H_2 and acetate, 1,1,2,2-TeCA was initially dechlorinated via both hydrogenolysis to 1,1,2-trichloroethane (1,1,2-TCA) (major pathway) and dichloroelimination to dichloroethenes (DCEs) (minor pathway), with both reactions occurring under sulfidogenic conditions. In the presence of only H_2, the hydrogenolysis of 1,1,2,2-TeCA to 1,1,2-TCA apparently required the presence of acetate to occur. Once formed, 1,1,2-TCA was degraded predominantly via dichloroelimination to vinyl chloride (VC). Ultimately, chloroethanes were converted to chloroethenes (mainly VC and DCEs) which persisted in the microcosms for very long periods along with PCE and TCE originally present in the groundwater. Hydrogenolysis of chloroethenes occurred only after highly reducing methanogenic conditions were established. However, substantial conversion to ethene (ETH) was observed only in microcosms amended with yeast extract (200 mg/l), suggesting that groundwater lacked some nutritional factors which were likely provided to dechlorinating microorganisms by this complex organic substrate. Bioaugmentation with an H_2-utilizing PCE-dechlorinating *Dehalococcoides* spp. -containing culture resulted in the conversion of 1,1,2,2-TeCA, PCE and TCE to ETH and VC. No chloroethanes accumulated during degradation suggesting that 1,1,2,2-TeCA was degraded through initial dichloroelimination into DCEs and then typical hydrogenolysis into ETH and VC.

Introduction

Chloroethenes (such as tetrachloroethene, PCE, and trichloroethene, TCE) and chloroethanes (such as 1,1,2,2-tetrachloroethane, 1,1,2,2-TeCA, and 1,1,2-trichloroethane, 1,1,2-TCA) are often found as contaminants in groundwater 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 very stringent maximum allowable concentration levels in groundwater.

Microbial anaerobic reductive dechlorination (RD) is an important mechanism of *in situ* natural attenuation of groundwater contaminated with chlorinated solvents. RD can be enhanced *in situ* by stimulating the activity of native dechlorinating populations through the addition of electron donors and or nutrients to the subsurface, or through

the injection of specialized microorganisms capable to degrade the target contaminants (Ellis et al. 2000).

RD is known to proceed by either cometabolic (fortuitous, non growth-linked) or metabolic (energy providing, growth-linked) mechanisms (Middeldorp et al. 1999). In the growth-linked metabolic RD, also termed dehalorespiration (Holliger et al. 1998), bacteria use the chlorinated compounds as terminal electron acceptors in the presence of suitable electron donors. As for chloroethenes, several bacteria capable of metabolic RD have been isolated. The majority of PCE-respiring bacteria, including several *Desulfitobacterium* species, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, and *Dehalococcoides* species use hydrogen as the actual electron donor for PCE dechlorination, consequently, current efforts to stimulate dechlorination at chloroethene-contaminated sites focus on addition of fermentable substrates to the subsurface or direct hydrogen supply. However, the organisms that carry out the complete dechlorination of PCE to ethene (ETH) are, so far, restricted to the *Dehalococcoides* group of bacteria (Hendrickson et al. 2002).

While extensive research has been conducted within the last 15 years on the RD of PCE and TCE, delineating the metabolic pathways, the pivotal role of hydrogen, and the key factors of microbial competition for the fermentable substrates and for H_2, relatively few studies on the biodegradation of chloroethanes (and 1,1,2,2-TeCA in particular) have been conducted.

Early research indicated that chloroethanes were only partially dechlorinated through cometabolism under methanogenic (Bouwer & McCarty 1983; De Best et al. 1999; Van Eekert et al. 1999), sulfate reducing (Egli et al. 1987), or acetogenic conditions (Wild et al. 1995; De Wildeman et al. 2003a).

Only recently bacteria capable of growth-linked degradation of 1,1,1-trichloroethane (1,1,1-TCA) (Sun et al. 2003) and 1,2-dichloroethane (1,2-DCA) (Maymó-Gatell et al. 1999; De Wildeman et al. 2003b) have been isolated. However, to-date there is no published information on growth-linked 1,1,2,2-TeCA biodegradation.

1,1,2,2-TeCA can be anaerobically converted to non- or less-chlorinated ethanes and ethenes via three mechanisms (Figure 1) (Chen et al. 1996; Lorah & Olsen 1999). The first two mechanisms, hydrogenolysis and dichloroelimination are two different RD mechanisms, in that they require the input of two electrons resulting in the release of one or two chlorine atoms, respectively. The third mechanism, dehydrochlorination, is a non-redox reaction by which HCl is released and a double bond is formed between two neighboring carbon atoms. On the other hand, hydrogenolysis is the primary biological transformation pathway for PCE and TCE (Figure 1).

Elucidation of factors controlling the occurrence of these different reaction pathways 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 (Lorah & Olsen 1999) and in the presence of inocula coming from anaerobic digestion of activated sludge (Chen et al. 1996). In the above mentioned studies only minor formation of ETH or ethane (ETA), which are the desired end products of 1,1,2,2-TeCA dechlorination, was observed.

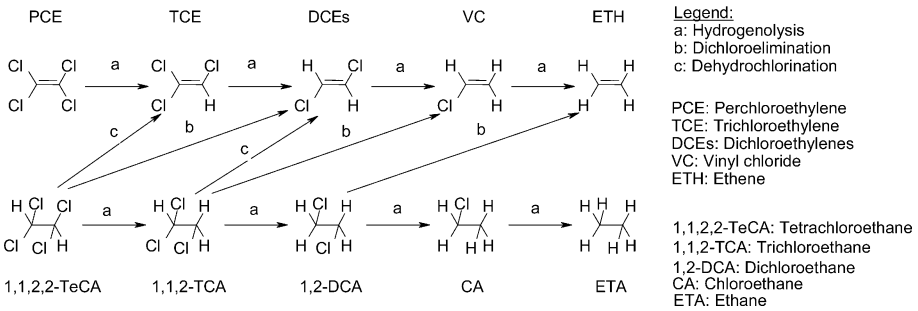

Figure 1. Possible anaerobic degradation pathways for chloroethanes and chloroethenes (modified from Lorah & Olsen 1999). Hydrogenolysis and dichloroelimination are biotic transformations whereas dehydrochlorination is an abiotic transformation.

Thus, also due to the lack of basic knowledge, *in situ* bioremediation of chloroethanes is largely unexplored, and particularly when groundwater contains chloroethanes and chloroethenes, as well as other competing electron acceptors (e.g. nitrate and sulfate).

This paper describes a microcosm study designed to assess the potential for microbial *in situ* RD at a chlorinated solvent-contaminated aquifer, located in the Northern Italy where chloroethanes (i.e. 1,1,2,2-TeCA), and chloroethenes (i.e. PCE, TCE) were simultaneously present as parent contaminants. In particular, this study was aimed at evaluating the pathways of contaminant transformation by both native soil populations and a dechlorinating inoculum containing *Dehalococcoides* spp., under different amendment conditions relevant to enhanced *in situ* remediation. Main attention was paid at distinguishing the factors and the conditions which control the distribution and the persistence of the daughter products of chloroethanes and chloroethenes dechlorination.

Materials and methods

Site description

A former chemical facility located in Rho (Milano, Italy), mainly involved in the production of synthetic dyes, is the source of a chlorinated solvent plume that extends over approximately 0.4 km^2 (Petrangeli Papini et al. 2003). The plume possibly originated from the leakage of an underground (2–6 m below ground surface, bgs) storage tank located approximately 20 m outside the industrial area. In 1982, local authorities undertook an emergency containment action, which consisted in the lateral and superficial isolation of the storage tank. Despite this action, groundwater-monitoring downgradient the source area during the last 15 years, indicated the almost stable presence of high concentrations of chlorinated solvents (e.g. up to 180 mg/l TCE, and 50 mg/l 1,1,2,2-TeCA). Both a shallow (from 5 to 12 m bgs) and a deep (from 15 to 40 m bgs) aquifer underlying the industrial site are impacted by the contamination. Groundwater and site characterization data are consistent with a scenario where spilled contaminants, migrated from the underground tank downward through the aquifers where they formed dense non-aqueous phase liquid (DNAPL) pools acting as long-term sources of contamination.

Aquifer material collection

Microcosms were prepared by using soil and groundwater samples taken from the contaminated site, in the proximity of the contamination source. The location for obtaining the sediment core was chosen based on the outcome of preliminary chemical analyses which indicated the presence in the groundwater of biodegradation products (i.e. VC and *cis*-DCE), hence providing preliminary evidence of the presence of native dechlorinating populations. Soil samples were collected by following the procedures described by Fennell et al. (2001), and by Morse et al. (1997). A soil core was obtained from 9.5 to 10.5 m bgs using a hollow-stem auger. In order to transport the (unconsolidated) soil core, upon removal it was transferred into a sterile glass jar which had been first filled with groundwater from the site. Groundwater was obtained from a well located less than 2 m from the coring location. Twenty liters of groundwater were collected in sterile glass jars and capped with a positive meniscus to exclude air. Soil and groundwater samples were maintained in coolers at 4 °C until use. Groundwater used for microcosms contained 1,1,2,2-TeCA (80.2 μmol/l), PCE (7.8 μmol/l), TCE (15.0 μmol/l), *cis*-DCE (3.58 μmol/l), *trans*-DCE (0.52 μmol/l), chloride (40 mg/l), nitrate (37 mg/l), and sulfate (500 mg/l). The pH of groundwater was 6.5.

Microcosm protocol

For microcosm preparation all materials (including soil samples, groundwater, autoclaved 250-ml serum bottles, gray butyl Teflon-faced stoppers, spatulas, and other minor materials) were placed inside an anaerobic glovebox under nitrogen (H_2-free) atmosphere. Twelve microcosm treatments, based on a modification of the RABITT protocol (Morse et al. 1997), were set up and triplicate bottles were prepared for each treatment. The experimental conditions for the microcosm treatments are shown in Table 1.

Treatment 1 (abiotic control) was prepared as follows: 60 g (dry weight) of soil were dispensed in a 250 ml serum bottle and diluted with 150 ml of

Table 1. Microcosms experimental conditions (each treatment in triplicate)

Treat.	Microcosm composition	Electron donor	Growth factors[a]	Added electron equivalents (meq/l)[b]
1 (abiotic control)	Soil + RAMM[c] (spiked with 1,1,2,2-TeCA)	None	–	–
2 (biotic control)	Soil + GW[d]	None	–	–
3 (biotic control)	Soil + GW	None	+	3.6
4	Soil + GW	Yeast extract (180 mg/l)	+	36
5	Soil + GW	Lactate (3 mmol/l)	–	36
6	Soil + GW	Lactate (3 mmol/l)	+	39.6
7	Soil + GW	Butyrate (3 mmol/l)	–	60
8	Soil + GW	Butyrate (3 mmol/l)	+	63.6
9	Soil + GW	Hydrogen (3 mmol/l)[e]	–	6
10	Soil + GW	Hydrogen (3 mmol/l)	+	9.6
11	GW + Inoculum[f]	Hydrogen (3 mmol/l)	+	9.6
12	Soil + RAMM (spiked with TCE)	Butyrate (3 mmol/l)	+	63.6

[a]Growth factors: yeast extract (20 mg/l) and vitamin B_{12} (0.05 mg/l).
[b]equivalent conversion factors for the electron donors (eq/mol) are based on their complete oxidation. The reducing equivalents available from yeast extract were estimated as described by Yang & McCarty (1998).
[c]RAMM: reduced anaerobic mineral medium prepared according to Tandoi et al (1994).
[d]GW: groundwater.
[e]nominal concentration, i.e. total moles divided by the volume of liquid phase; At days 120 and 250 treatment 9 and 10 were also amended with acetate 3 mmol/l.
[f]the inoculum is a H_2-utilizing PCE-dechlorinating culture containing *Dehalococcoides* spp. (Aulenta et al 2002, 2004).

reduced anaerobic mineral medium (RAMM). RAMM composition and preparation is that reported by Tandoi et al. (1994). After preparation, the bottle was sealed with Teflon-faced butyl rubber stoppers and autoclaved at 121 °C for 1 h. Thereafter, the bottle was spiked with neat 1,1,2,2-TeCA (to a final nominal concentration of 60 μmol/l) using aseptic conditions.

For treatment 2 to 10, 60 g (dry weight) of soil were dispensed in a 250 ml serum bottle, and added with 150 ml of groundwater. Groundwater was previously added with 1 mg/l resazurin (as a redox indicator). After preparation, the bottles were sealed with Teflon-faced butyl rubber stoppers, maintained in a shaker overnight to equilibrate and their gas phase was analyzed the following day to determine the initial 1,1,2,2-TeCA, PCE, TCE, and DCEs level. Thereafter the microcosms were spiked with the selected electron donor (yeast extract, lactate, butyrate, hydrogen, or none) as illustrated in Table 1. 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 anoxic stock solutions. Hydrogen gas was added in the headspace of the serum bottles, by using gas-tight syringes. For treatment 11 (bioaugmentation microcosm), 35 ml of an H_2-utilizing, PCE-dechlorinating culture containing large amount of *Dehalococccoides* spp. (i.e. 59.0 ± 10.4 mgVSS/l) (Aulenta et al. 2002, 2004) were dispensed in a 250-ml serum bottle and added with 150 ml of groundwater (in the absence of soil). Thereafter, the bottle was sealed and spiked with H_2 and growth factors. The *Dehalococcoides* spp. used as inoculum for microcosm tests has been previously enriched for about 1 year on PCE and methanol or H_2. It had been also verified that hydrogen was the ultimate electron donor for PCE dechlorination in that culture (Aulenta et al. 2004). During the 1-year enrichment period, the culture was not previously exposed to 1,1,2,2-TeCA or any other chloroethanes.

For treatment 12, 60 g (dry weight) of soil were dispensed in a 250 ml serum bottle, and diluted with 150 ml RAMM. Thereafter the bottle was sealed and spiked with TCE and butyrate. All the microcosms were incubated statically in the dark at room temperature (i.e. 18–22 °C).

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, except the biotic control (treatment 2), turned clear indicating establishment of reduced conditions.

Analytical procedures and monitoring protocol

Every 14 days, the microcosms were analyzed for chlorinated solvents and electron donors. Electron donors were re-added every time analyses indicated they were completely exhausted. Growth factors, i.e. yeast extract (20 mg/l) and vitamin B_{12} (0.05 mg/l), were re-added to microcosms every 100 days. At days 120 and 250, H_2-amended microcosms (treatments 9–10) were spiked with acetate 3 mmol/l. Treatment 4 was re-spiked with yeast extract (200 mg/l) every 100 days. Chloroethenes, ETH, ETA, 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, 2004). 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, I.D. 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). Besides chloroethanes, this chromatographic method was also used to quantify the different DCE isomers (i.e. *cis*-DCE, *trans*-DCE, 1,1-DCE) which are not separated within the packed column used for chloroethene analysis. Hydrogen was analyzed in 500 μl headspace samples, by using GC with thermal-conductivity detector (TCD) as described elsewhere (Aulenta et al. 2002). Headspace samples were taken using gastight glass syringes dedicated to each single microcosm. Standards for chlorinated compounds, ETH, ETA, CH_4, and H_2 were prepared by adding, with gastight syringe, a known amount of each compound to a serum bottle with the same headspace to liquid ratio as the microcosm bottles (Gossett et al. 1987). Concentrations for volatile compounds are expressed as total moles in the bottle divided by the liquid phase (i.e. nominal concentrations). Liquid samples (1 ml) were taken using sterile disposable plastic syringes, filtered (0.22 μm), and analyzed for lactate and volatile fatty acids by using GC-FID (1 μl sample, GC Perkin-Elmer 8400, 2 m × 2 mm glass column packed with Carbograph 1 AL 80/120, flame-ionization detector), and for nitrate, nitrite, sulfate, and chloride, by using ion chromatography (0.5 ml sample, Dionex DX-100, Ionpac As9-Sc column, conductivity detector).

All the chemicals used as microcosm amendments or used to prepare analytical standards were analytical grade.

Data interpretation

Since chloroethanes and chloroethenes can share common reaction intermediates (i.e. TCE, DCEs, VC), depending on chloroethanes degradation pathways (for instance DCEs formation could result from both 1,1,2,2-TeCA dichloroelimination and TCE hydrogenolysis), an integrated parameter, the 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 dechlorination intermediates (Aulenta et al. 2004). Because TCE and DCEs could be either native substrates or products of dechlorination, their contribution to the chloride cumulative release was considered positive or negative depending on their increase or decrease with respect to the background value.

The overall dechlorinating activity could not be measured by following the actual concentration of Cl^- (as measured by ion chromatography) because of its high background concentration (> 1 mM) in the groundwater.

Results

Summary of enhanced reductive dechlorination by native soil dechlorinating populations

Table 2 summarizes the results of the microcosm study. The distribution (percentage of initial contaminant concentration, on a molar basis) of chlorinated contaminants and transformation products at the end of the 384-day incubation period is shown (mean value of three replicates). Good mass balances could be maintained through the study, with recoveries of initial chlorinated compounds into transformation products ranging between 74.1% and 106.3%, thus providing evidence that the decrease in parent compounds over

Table 2. Distribution (%) of dechlorination products at the end of the 384-day incubation period (error based on 90% confidence interval)

N°	Treatment	TeCA[a]	TCA[a]	DCA[a]	PCE	TCE	cDCE	tDCE	VC	ETH	Recovery (%)
1	Day 0 →	100	–[b]	–	–	–	–	–	–	–	100
	Day 384 ↓										
1	Abiotic control	40.1 ± 4.8	–	–	–	56.3 ± 7.3	–	–	–	–	96.4 ± 12.1
2–11	Day 0 →	76.4	–	–	5.8	13.6	3.6	0.5	–	–	100
	Day 384 ↓										
2	Biotic control	58.4 ± 20.2	–	–	2.2 ± 0.6	40.0 ± 5.0	4.1 ± 0.2	1.4 ± 0.2	–	0.2 ± 0.1	106.3 ± 26.6a
3	Biotic control + g.f.[c]	41.0 ± 10.0	–	–	1.6 ± 0.9	37.9 ± 4.0	10.0 ± 4.6	4.3 ± 0.9	–	0.2 ± 0.1	95.0 ± 20.5
4	YE[d](180 mg/L) + g.f.	–	–	–	–	0.2 ± 0.7	–	11.3 ± 9.2	37.6 ± 8.5	45.9 ± 20.7	95.0 ± 39.1
5	Lactate	–	31.9 ± 10.9	17.1 ± 15.4	1.5 ± 0.3	12.4 ± 0.7	7.3 ± 2.4	2.3 ± 0.8	19.1 ± 3.9	0.3 ± 0.1	91.9 ± 34.5
6	Lactate + g.f.	–	23.2 ± 9.5	28.3 ± 5.0	0.8 ± 0.2	6.0 ± 2.7	7.8 ± 0.7	2.7 ± 0.4	24.3 ± 12.8	0.5 ± 0.3	93.7 ± 31.6
7	Butyrate	17.3 ± 4.5	–	–	1.1 ± 0.2	35.6 ± 22.4	14.4 ± 2.3	5.6 ± 2.2	0.0 ± 0.5	0.1 ± 0.1	74.1 ± 32.2
8	Butyrate + g.f.	–	–	–	–	4.9 ± 0.3	6.5 ± 1.0	2.5 ± 1.2	60.2 ± 15.2	16.0 ± 14.3	90.1 ± 32.0
9	H_2	–	–	–	1.1 ± 0.2	24.3 ± 2.5	4.7 ± 2.3	1.6 ± 0.4	40.8 ± 1.1	7.3 ± 5.5	79.9 ± 12.0
10	H_2 + g.f.	–	–	–	1.4 ± 0.3	23.9 ± 2.1	11.9 ± 1.0	6.8 ± 2.3	32.0 ± 1.1	5.2 ± 3.0	81.2 ± 9.8
11	Inoculum + H_2 + g.f.	–	–	–	–	–	–	–	20.6 ± 7.5	58.6 ± 20.7	79.2 ± 28.2

[a]1,1,2,2-TeCA was abbreviated to TeCA; 1,1,2-TCA was abbreviated to TCA; 1,2-DCA was abbreviated to DCA.
[b]Hyphens indicate that measured concentration was below detection limit.
[c]g.f.: growth factors.
[d]YE: yeast extract.

time was due largely to degradation rather than to sorption or volatilization.

In the abiotic (autoclaved) control (treatment 1) 1,1,2,2-TeCA was partially transformed into TCE via dehydrochlorination, a process that does not require the input of electrons. Biotic controls (treatments 2 and 3) also showed an increase of TCE concentration with respect to the initial value (Table 2), so indicating that abiotic dehydrochlorination was occurring but no further biotic dechlorination.

For all other soil microcosms, the electron donors (i.e. yeast extract, lactate, butyrate, hydrogen, treatments 4–10) enhanced dechlorination with respect to the biotic controls, indicating the presence in the soil of native dechlorinating populations, whose dechlorinating activity was probably limited by the lack of appropriate electron donors in the soil and/or in the groundwater. 1,1,2,2-TeCA was fully converted into less chlorinated ethenes or ethanes in all amended microcosms (treatment 4–10), with the only exception for a butyrate-amended microcosm (treatment 7). PCE and TCE were not exhausted in most microcosms, but for treatments 4 (i.e. yeast extract-amended microcosm) and 11 (i.e. *Dehalococcoides* spp. bioaugmented microcosm). It is noteworthy that TCE increased during time in some cases (treatments 2, 3, 7, 9, and 10) due to its formation from 1,1,2,2-TeCA dehydrochlorination. ETH was the main end product in treatments 4 and 11, while VC was the main product in treatments 6, 8, 9, and 10.

Based on Table 2, the addition of growth factors (i.e. vitamin B_{12} 0.05 mg/l, and yeast extract 20 mg/l) had a clear beneficial effect only in butyrate-amended microcosm (treatment 8) which performed better than corresponding treatment with no growth factors (treatment 7).

Dechlorination pathways

In order to describe in a greater detail the dechlorination pathways, Figure 2a and b show the results of a typical microcosm amended with butyrate and growth factors (treatment 8). For clarity, the daughter products are displayed whereas native contaminants PCE and 1,1,2,2-TeCA are not (even though TCE and DCEs were also native contaminants, their profiles were included in the graph since these compounds were also produced during 1,1,2,2-TeCA and PCE dechlorination).

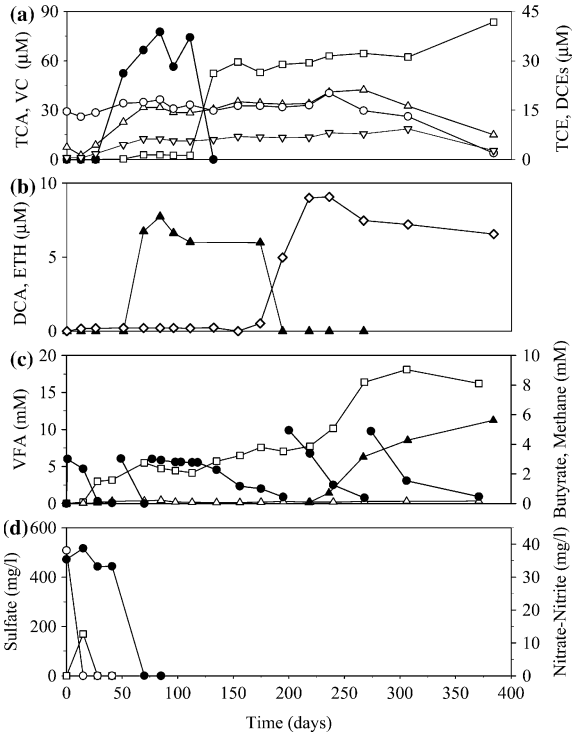

Figure 2. Results from a typical (one of the three replicates) butyrate-amended microcosm (in the presence of growth factors). (a) Time course of dechlorination. Symbols: ●, 1,1,2-TCA; ○, TCE; △, *cis*-DCE; ▽, *trans*-DCE; □, VC; (b) Symbols: ▲, 1,2-DCA; ◇, ETH. (c) Electron donor utilization. Symbols: ●, butyrate; □, acetate; △, propionate; ▲, methane. (D) Inorganic electron acceptors consumption. Symbols: ○, nitrate; □, nitrite; ●, sulfate.

Dechlorination commenced after about a 25-day lag and resulted in the rapid accumulation of 1,1,2-TCA (through the hydrogenolysis of 1,1,2,2-TeCA). 1,1,2-TCA reached a peak concentration of 75 μM (amounting to about 85% of initial 1,1,2,2-TeCA) then it was rapidly transformed to VC via dichloroelimination (Figure 2a). Between day 50 and 200, a little accumulation of 1,2-DCA was also observed (hydrogenolysis of 1,1,2-TCA), then its disappearance was coupled to ETH formation (via dichloroelimination) (Figure 2b). Interestingly, no further increase of ETH (e.g. from hydrogenolysis of VC) was noticed during the remaining incubation period.

During the initial 200 days of incubation, little formation of DCEs was observed, with the *cis*-isomer accounting for by 66% of the produced

DCE, and the *trans*-isomer accounting for by the remaining 33% (Figure 2a). Although there is recent evidence that *trans*-DCE can be formed from hydrogenolysis of TCE (Griffin et al. 2004), a mass balance indicated that both DCE isomers were formed from dichloroelimination of 1,1,2,2-TeCA and not from the hydrogenolysis of TCE.

Thus, 1,1,2,2-TeCA (data not shown) and the daughter products resulting from its hydrogenolysis (i.e. 1,1,2-TCA and 1,2-DCA) were fully consumed in about 200 day, mostly by dichloroelimination into less-chlorinated ethenes. Once formed, the produced chloroethenes (mainly DCEs) and the background TCE persisted in the microcosm until day 220. Also PCE persisted in the microcosm for very long time (being completely consumed only after 384 days, data not shown).

The hydrogenolysis of chloroethenes started at day 220 and resulted in a significant decrease of TCE, and DCEs and correspondent increase of VC (Figure 2a).

Figure 2c shows the fate of amended butyrate in the microcosm: butyrate was initially used for nitrate reduction (Figure 2d); then it was fermented to acetate (and trace amounts of propionate) during the following sulfate reduction and dechlorination. It is noteworthy that sulfate reduction and the first steps of chloroethanes dechlorination occurred simultaneously; hence both hydrogenolysis and dichloroelimination of chlorinated ethanes were possible under sulfate-reducing conditions. Methane formation commenced only after 220 day of incubation that also corresponded to the period when the hydrogenolysis of TCE and chloroethenes was acting. Thus, the hydrogenolysis of TCE and accumulated chloroethenes and methanogenesis required the same reducing and environmental conditions to occur.

In the microcosms amended with lactate (both in the presence and in absence of growth factors) initial hydrogenolysis of 1,1,2,2-TeCA was faster than with butyrate, however at the time of 1,1,2,2-TeCA depletion, product distribution in lactate- and butyrate- amended microcosms was similar (data not shown). Differently, the following steps of dechlorination (i.e. dichloroelimnation of 1,1,2-TCA and 1,2-DCA) were more rapid with butyrate than with lactate. This resulted in the persistence of 1,1,2-TCA and 1,2-DCA in lactate-amended microcosms and in the formation of less VC and ETH compared to butyrate-amended microcosms (Table 2).

Differently, in the H_2-amended microcosms (treatment 10, Figure 3a) 1,1,2-TCA (from hydrogenolysis of 1,1,2,2-TeCA) was not formed initially and only little amounts of DCEs accumulated (from dichloroelimination of 1,1,2,2-TeCA). Since the microcosm showed little acetate formation through homoacetogenesis (Figure 3b), at day 120, acetate (3 mM) was added. The acetate spike resulted in the rapid formation of 1,1,2-TCA (up to 50 μM), and little amounts of 1,2-DCA. Moreover, the acetate spike corresponded to an increase of the sulfate reduction rate. Thereafter, 1,1,2-TCA and 1,2-DCA were transformed via dichloroelimination reactions to VC and ETH, respectively (Figure 3a), so following the same pattern observed with butyrate and lactate. Once 1,2-DCA was depleted, ETH did not further increase even though VC (which is the possible precursor of ETH via hydrogenolysis) was present.

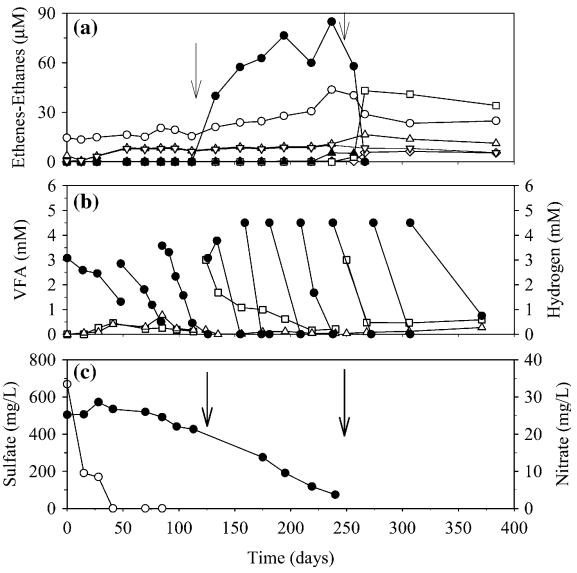

Figure 3. Results from a typical (one of the three replicates) H_2- and growth factors- amended microcosm. (a) Time course of dechlorination. Symbols: ●, 1,1,2-TCA; ▲, 1,2-DCA; ○, TCE; △, *cis*-DCE; ▽, *trans*-DCE; □, VC; ◇, ETH. (b) Electron donor utilization. Symbols: ●, H_2; □, acetate; △, propionate. No methane was detected throughout the incubation period. (c) Inorganic electron acceptors consumption. Symbols: ○, nitrate; ●, sulfate. Arrows indicate additions of acetate 3 mM.

No methane production was observed in the H_2-amended microcosms, regardless of acetate addition. This finding confirms that methanogenic conditions were not required for the initial hydrogenolysis and dichloroelimination of 1,1,2,2-TeCA and its daughter products (which apparently required acetate or sulfidogenic conditions). The lack of methane also corresponded to the absence of hydrogenolysis of TCE, DCEs, and VC, so confirming that methanogenesis and hydrogenolysis of TCE, DCE, and VC required similar conditions.

One more different pattern of contaminant transformation was observed for the yeast extract-amended microcosms (treatment 4, Figure 4). Indeed, 1,1,2-TCA appeared later (day 75) than in butyrate-, H_2-, or lactate- amended microcosms and accumulated at lower concentration. This late appearance of 1,1,2-TCA could be again due to that little acetate was available through the slow fermentation of yeast extract (Figure 4b). On the other hand, in this case an intense hydrogenolysis of chloroethenes was observed and, more important, clear evidence of VC conversion into ETH was observed. At the end of the incubation period, the yeast extract-amended microcosms exhibited the almost full conversion of chlorinated ethenes to ETH (but for some residual *trans*-DCE). It is noteworthy that the *trans*-isomer of DCE, produced from 1,1,2,2-TeCA, was far more recalcitrant to biodegradation than the *cis*-isomer and other chloroethenes and persisted in the bottle even when higher chlorinated ethenes were completely transformed to ethene. Similarly to other microcosms, methanogenic activity only established after 275 days of incubation (Figure 4b) in the period when hydrogenolysis of chloroethenes was also occurring.

Figure 5 shows the effect of different amendments (in the presence of growth factors) on the time-course of overall dechlorinating activity (in terms of chloride cumulative release). It is clear from Figure 5 that the observed initial maximum dechlorination rate followed the order lactate > butyrate > hydrogen > yeast extract > biotic control. This order is in agreement with the rate of fermentation of these organic compounds previously reported by other researchers (Fennell et al. 1997; He et al. 2002) which observed that lactate is fermented quicker and produces larger amounts of acetate and H_2 than butyrate and yeast extract. This is also in agreement with a scenario where initial dechlorination is mainly due to acetate-dependent 1,1,2,2-TeCA dechlorination to 1,1,2-TCA.

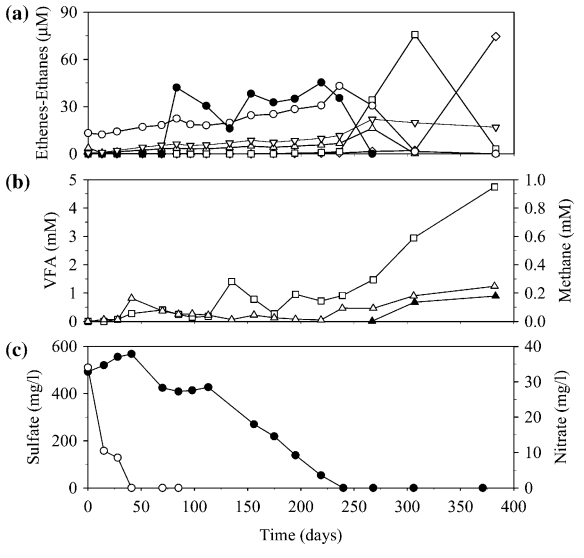

Figure 4. Results from a typical (one of the three replicates) yeast extract- and growth factors- amended microcosm. (a) Time course of dechlorination. Symbols: ●, 1,1,2-TCA; ▲, 1,2-DCA; ○, TCE; △, *cis*-DCE; ▽, *trans*-DCE; □, VC; ◇, ETH. (b) Electron donor utilization. Symbols: □, acetate; △, propionate; ▲, methane. (c) Inorganic electron acceptors consumption. Symbols: ○, nitrate; ●, sulfate.

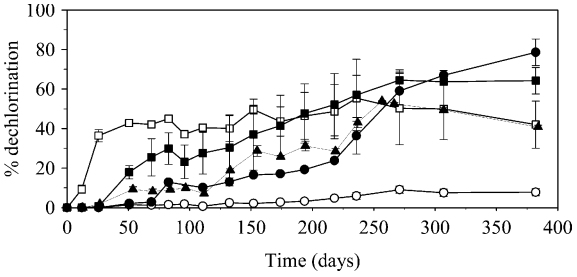

Figure 5. Effect of different electron donors (in the presence of growth factors) on the time course of dechlorination. % dechlorination is calculated, at any time, as the ratio between the chloride cumulative release and the initial level of chloride in groundwater contaminants. Error bars (when larger than symbols) are standard deviation of three replicates. Symbols: ○, biotic control; ●, yeast extract; □, lactate; ■, butyrate; ▲, hydrogen.

Effect of groundwater composition on RD

In all the amended treatments, chloroethanes were ultimately converted via dichloroelimination pathways to chloroethenes. The produced chloroethenes (i.e. mainly DCEs and VC) and the background TCE and PCE, persisted in the microcosms for very long periods. Apparently, further, even though slow, hydrogenolysis of the produced chloroethenes was observed only after sulfate had been depleted and more reducing conditions were established. Moreover, only in the yeast extract-amended microcosms there was clear evidence of hydrogenolysis of VC to ETH. Indeed, the little ETH, which was produced in butyrate-, lactate-, or H_2-amended microcosms, resulted only from dichloroelimination of 1,2-DCA.

A different scenario was observed when the soil was diluted with RAMM (in the presence of butyrate as the electron donor), and spiked with TCE (no chloroethanes present) (treatment 12). In that case, the complete hydrogenolysis of TCE to ETH was rapidly achieved (Figure 6), indicating that a high population of dechlorinating microorganisms was already present in the soil added to the microcosms. Similarly a more rapid methanogenic activity was also observed.

By comparison with the corresponding treatment in the presence of groundwater (treatment 8) where no ETH formation from hydrogenolysis of TCE was observed and methanogenic activity established only after 220 days, the results of this microcosms could indicate that hydrogenolysis and methanogenesis were both inhibited by higher redox potential of the groundwater (such as in the presence of sulfate or nitrate) and/or by the presence of chloroethanes or of other contaminants present in the groundwater.

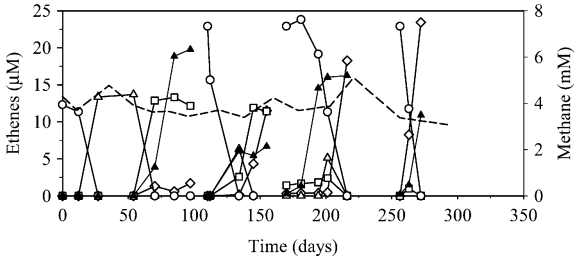

Figure 6. Reductive dechlorination of TCE with butyrate and growth factors by the soil suspended in reduced anaerobic mineral medium (RAMM). Before each TCE spike the headspace of the bottle was purged with N_2–CO_2 mixture to remove volatile compounds. Symbols: ○, TCE; △, *cis*-DCE; □, VC; ◇, ETH; ▲, methane. TCE in abiotic control — .

Effect of bioaugmentation with Dehalococcoides spp.

In the microcosm (treatment 11) where groundwater was bioaugmented with an H_2-utilizing PCE-dechlorinating mixed culture containing large amounts of *Dehalococcoides* spp. (Aulenta et al. 2004), the chlorinated contaminants (1,1,2,2-TeCA, PCE, and TCE) were degraded at higher rate than in the parallel soil microcosm (treatment 10), and converted to ETH and VC (Figure 7a).

A mass balance indicated that VC and ETH were also produced from 1,1,2,2-TeCA conversion. It is noteworthy that only chloroethenes accumulated during chlorinated contaminants biotransformation, which indicates that 1,1,2,2-TeCA was mostly converted into chloroethenes, probably through dichloroelimination to DCEs,

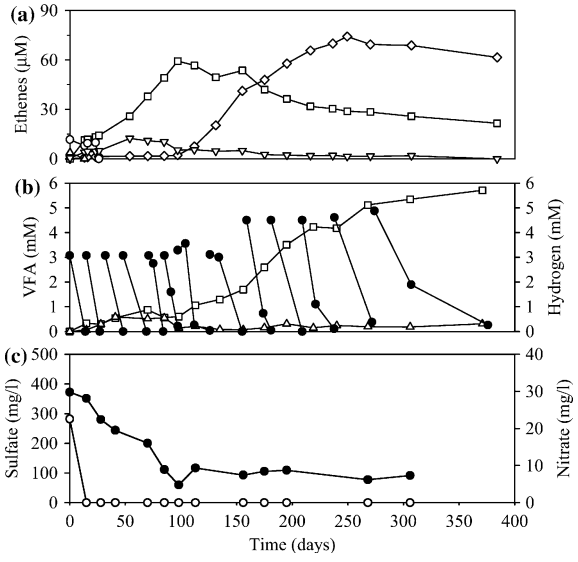

Figure 7. Results from a typical (one of the three replicates) microcosm bioaugmented with *Dehalococccoides* spp. culture, in the presence of H_2 and growth factors. (a) time-course of dechlorination. Symbols: ○, TCE; △, *cis*-DCE; ▽, *trans*-DCE; □, VC; ◇, ETH. No chloroethanes were detected throughout the incubation period. (b) Electron donor utilization. Symbols: ●, H_2; □, acetate; △, propionate. No methane was detected throughout the incubation period. (c) Inorganic electron acceptors consumption. Symbols: ○, nitrate; ●, sulfate.

whereas 1,1,2,2-TeCA hydrogenolysis (to 1,1,2-TCA) was not active.

VC was the main chlorinated intermediate which accumulated during 1,1,2,2-TeCA dechlorination and reached a peak concentration of 65 μM (amounting to about 75% of initial 1,1,2,2-TeCA) at the time of 1,1,2,2-TeCA depletion (data not shown). Different from other treatments, VC formation was observed under sulfate reducing conditions (Figure 7c). Delayed and slow ETH formation occurred only after 1,1,2,2-TeCA was completely depleted. In our previous studies with the same dechlorinating culture (Aulenta et al. 2004), ETH formation did not require the depletion of higher chlorinated ethenes (i.e. TCE, cDCE) to begin. Hence, by comparison with our previous findings, the results of the bioaugmentation microcosms indicate that 1,1,2,2,-TeCA was inhibitory to ETH formation.

As shown in Figure 7b, acetate formation was observed due to intense H_2/CO_2 homoacetogenic activity. At day 100 sulfate reduction stopped even if H_2 and acetate were still present in the microcosms (Figure 7c). The reason for this observed behavior is still unclear.

Discussion

This study investigated the degradation pathways of 1,1,2,2-TeCA, PCE and TCE in anaerobic microcosms constructed with soil and groundwater from a contaminated site in Northern Italy.

1,1,2,2-TeCA, the main groundwater contaminant, was degraded by soil dechlorinating populations via multiple reaction pathways including hydrogenolysis, dichloroelimination and abiotic dehydrochlorination. However, 1,1,2,2-TeCA degradation occurred mostly through hydrogenolysis, which resulted in peak concentrations of 1,1,2-TCA comprising up to 85% of initial 1,1,2,2-TeCA, and to a minor extent through dichloroelimination to DCEs (both the *cis*- and *trans*-isomers).

1,1,2-TCA formation occurred in microcosms under sulfate-reducing conditions and required the presence of acetate (either produced during substrate fermentation or spiked to the microcosms).

The finding that highly reducing conditions were not required for initial dechlorination of 1,1,2,2-TeCA has been also observed by Lorah & Olsen (1999) who reported 1,1,2-TCA and DCE formation in a freshwater tidal wetland under iron-reducing conditions. Moreover, Lorah & Olsen observed that continued anaerobic degradation of these daughter products was dependent on the more reducing conditions of methanogenesis.

The key role of acetate as a carbon source/electron donor for the hydrogenolysis of 1,1,2,2-TeCA has not been previously described in the literature and may suggests the direct involvement of an acetotrophic dechlorinator in the formation of 1,1,2-TCA from 1,1,2,2-TeCA. At present, information on the effect of different electron donors on the reductive transformation pathways of 1,1,2,2-TeCA is scarce as well as the knowledge on the identity and physiology of 1,1,2,2-TeCA-dechlorinating microorganisms. However, recent finding suggested that acetate, rather than hydrogen, is a relevant direct electron donor to stimulate the reductive dechlorination of some chlorinated pollutants (Cutter et al. 2001; He et al. 2002).

Different from 1,1,2-TCA, formation of DCEs (through 1,1,2,2-TeCA dichloroelimination) seemed to be independent on the presence of acetate.

The 1,1,2-TCA produced from hydrogenolysis of 1,1,2,2-TeCA was degraded through a different pathway compared to its parent compound; i.e. through minor hydrogenolysis to 1,2-DCA and major dichloroelimination to VC. 1,2-DCA was fully converted to ETH, through dichloroelimination. Apparently, the relative importance of dichloroelimination with respect to hydrogenolysis increased as the chlorination degree decreased.

Ultimately, TCE (from abiotic dehydrochlorination of 1,1,2,2-TeCA), DCE (both *cis*- and *trans*-isomers, from dichloroelimination of 1,1,2,2-TeCA) and VC (from dichloroelimination of 1,1,2-TCA), accumulated in most microcosms, where persisted for long periods with no or slow hydrogenolysis to ETH. Hydrogenolysis of parent or produced (from 1,1,2,2-TeCA) chloroethenes was observed only after highly reducing conditions were established, which also corresponded to methanogenic conditions.

The relation between hydrogenolysis of produced chloroethenes and the presence of the extremely reducing conditions of methanogenesis agrees with the study of Lorah & Olsen (1999). The long time to begin either hydrogenolysis or

methanogenesis support the hypotesis that groundwater contained inhibitory compounds which had a significant toxic effect on both dechlorinators and methanogens. Nitrate, sulfate and chlorinated ethanes have all been previously reported to inhibit both hydrogenolysis of chloroethenes and methanogenesis (Gerritse et al. 1997; Adamson & Parkin 2000; Nelson et al. 2002; Hoelen & Reinard 2004). However, the only amendment which stimulated the almost complete transformation to ETH (but for some residual *trans*-DCE) was the yeast extract, suggesting that one more possible factor was that groundwater lacked some nutritional factors which were likely provided to dechlorinating microorganisms by this complex organic substrate or one of its fermentation products (Fennell et al. 1997).

Faster dechlorination of chloroethanes and chloroethenes to ETH and VC was also achieved with a *Dehalococcoides* spp. – containing culture. The culture, even though not previously exposed to 1,1,2,2-TeCA was able to degrade it without any initial lag: 1,1,2,2-TeCA biotransformation produced only DCEs, whereas no transient accumulation of chloroethanes was observed. This degradation pathway could not be ascribed to any isolated dechlorinating microorganism, including *Dehalococcoides* spp. On the other hand, even though a large percentage (up to 40% of total VSS) of the biomass used as the inoculum for this microcosms was composed of *Dehalococcoides* spp., the direct involvement of this microorganism in the observed dechlorination could not be verified. Moreover, the PCE-dechlorinating culture bioaugmented microcosm (treatment 11) confirmed that conversion to ETH is possible also in the presence of groundwater, as already observed in the presence of excess yeast extract (treatment 4) for soil microcosms.

Conclusions

This study investigated the anaerobic transformation pathways of a mixture of 1,1,2,2-TeCA and chloroethenes by soil microbial populations and a dechlorinating inoculum containing *Dehalococccoides* spp., under different amendment conditions relevant to enhanced *in situ* remediation. The following conclusions can be drawn from this study:

- In the presence of soil populations and organic electron donors, 1,1,2,2-TeCA was dechlorinated via different reaction pathways including hydrogenolysis (major pathway), dichloroelimination (minor pathway), and abiotic dehydrochlorination (minor pathway). In particular, hydrogenolyis of 1,1,2,2-TeCA to 1,1,2-TCA occurred under sulfate reducing conditions and required the presence of acetate (either produced during substrate fermentation or spiked to the microcosms). The key role of acetate as a carbon source/electron donor for the hydrogenolysis of 1,1,2,2-TeCA has not been previously described in the literature and may suggests the direct involvement of an acetotrophic dechlorinator in the formation of 1,1,2-TCA from 1,1,2,2-TeCA;
- Once produced 1,1,2-TCA was degraded to VC via dichloroelimination (major pathway) and hydrogenolysis to 1,2-DCA (minor pathway). 1,2-DCA was then converted to ETH via dichloroelimination. Apparently, the relative importance of dichloroelimination with respect to hydrogenolysis increased as the chlorination degree decreased. 1,1,2,2-TeCA and chloroethenes were ultimately converted into chloroethenes.
- The hydrogenolysis of chloroethenes started only after highly reducing methanogenic conditions were established. The long time to begin either hydrogenolysis or methanogenesis was likely due to the presence in the groundwater of 1,1,2,2-TeCA and or sulfate. However, even when 1,1,2,2-TeCA and sulfate had been depleted, the only amendment which stimulated the almost complete transformation to ETH (but for some residual *trans*-DCE) was the yeast extract, suggesting that groundwater lacked some nutritional factors which were likely provided to dechlorinating microorganisms by this complex organic substrate or one of its fermentation products.
- From a practical point of view, the results of this microcosm study indicate that the best options for site remediation could be the addition of yeast extract or of a *Dehalococcoides*-containing culture to the subsurface. To our knowledge, this is the first time that the possibility of full conversion of a mixture of chlorinated ethanes (1,1,2,2-TeCA) and eth-

enes (PCE and TCE) into ETH is reported. Since the most rapid dechlorination of 1,1,2,2-TeCA was stimulated by lactate (or acetate) while the further conversion to ETH was possible only with yeast extract, a possible strategy for site remediation could involve the addition of lactate close to the contamination source, to stimulate the rapid reduction of 1,1,2,2-TeCA to mainly DCEs and VC, and the downgradient addition of yeast extract to promote their further and full transformation to ETH.

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