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Response of 1,2-dichloroethane-adapted microbial communities to *ex-situ* biostimulation of polluted groundwater

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Abstract

The microbial community of a groundwater system contaminated by 1,2-dichloroethane (1,2-DCA), a toxic and persistent chlorinated hydrocarbon, has been investigated for its response to biostimulation finalized to 1,2-DCA removal by reductive dehalogenation. The microbial population profile of samples from different wells in the aquifer and from microcosms enriched in the laboratory with different organic electron donors was analyzed by ARISA (Amplified Ribosomal Intergenic Spacer Analysis) and DGGE (Denaturing Gradient Gel Electrophoresis) of 16S rRNA genes. 1,2-DCA was completely removed with release of ethene from most of the microcosms supplemented with lactate, acetate plus formate, while cheese whey supported 1,2-DCA dehalogenation only after a lag period. Microbial species richness deduced from ARISA profiles of the microbial community before and after electron donor amendments indicated that the response of the community to biostimulation was heterogeneous and depended on the well from which groundwater was sampled. Sequencing of 16S rRNA genes separated by DGGE indicated the presence of bacteria previously associated with soils and groundwater polluted by halogenated hydrocarbons or present in consortia active in the removal of these compounds. A PCR assay specific for *Desulfitobacterium* sp. showed the enrichment of this genus in some of the microcosms. The dehalogenation potential of the microbial community was confirmed by the amplification of dehalogenase-related sequences from the most active microcosms. Cloning and sequencing of PCR products indicated the presence in the metagenome of the bacterial community of a new dehalogenase potentially involved in 1,2-DCA reductive dechlorination.

Introduction

Chlorinated alkanes are widely used in industrial applications as solvents, chemical intermediates, detergents, lead scavengers in gasoline, etc. Among these, 1,2-dichloroethane (1,2-DCA) is the most important quantitatively, being used as an intermediate for polyvinyl chloride production (De Wildeman & Verstraete 2003).

The widespread use of 1,2-DCA has caused serious environmental contaminations (Hughes et al. 1994) and for this reason it has been included in the EU and US priority lists of pollutants. The environmental risk connected to 1,2-DCA is due to the acute and long-term toxic effects, including carcinogenesis. 1,2-DCA presents high water solubility (8000 mg l^{-1}) and very long persistence in anoxic groundwater, with an estimated half-life of

months to decades depending on environmental conditions (Barbee 1994; Vogel et al. 1987). Moreover, 1,2-DCA is not chemically dechlorinated by treatments with metals unlike other halogenated aliphatics such as substituted ethenes (Ferrey et al. 2004; Zhang et al. 1998).

However, recent experiments have led to the successful isolation of microorganisms that can dechlorinate 1,2-DCA (De Wildeman et al. 2003; Maymo-Gatell et al. 1999). This has shed new light on bioremediation of polluted sites by biostimulation and bioaugmentation. Biostimulation of 1,2-DCA-contaminated environments could improve the reductive dehalogenation of chlorinated compounds (De Wildeman et al. 2004; Klecka et al. 1998; Nobre and Nobre, 2004; Wenderoth et al. 2003). Biostimulation is typically attained by supplying nutrients and electron donors to the above environment in order to stimulate the electron transfer to 1,2-DCA, which acts as the main terminal electron acceptor. This process results in an energy-conserving net dehalogenation (ATP-producing reaction) (De Wildeman & Verstraete 2003; Holliger et al. 1999), where two halogen atoms are removed requiring only one H_2 mol (Smidt & de Vos 2004).

Exposure of a complex microbial community to externally supplied organic carbon generally exerts a deep effect on its structure and diversity, due to the response of a part of the community to the changed nutritional conditions. The effectiveness of a remediation treatment largely depends on these changes. In the context of this investigation, biostimulation should favor dehalorespiring bacteria supporting 1,2-DCA degradation in anoxic groundwater. Monitoring these changes plays a key role in the prediction of the self-remediating potential of a polluted site in response to biostimulation treatments. It could also guide the selection of the most suitable substrates to be amended and the choice of the most effective operative conditions for a successful *in situ* dehalogenation. However, few data on the molecular structure of the microbial community of 1,2-DCA contaminated sites are currently available.

Therefore, our work set out to evaluate changes in a bacterial community in groundwater strongly contaminated with 1,2-DCA in response to biostimulation with several electron donors. Population structure was followed by ARISA (Automated Ribosomal Intergenic Spacer Analysis) (Fisher & Triplett 1999) and bacterial diversity was analyzed by 16S rRNA gene PCR-DGGE (Denaturing Gradient Gel Electrophoresis; Iwamoto et al. 2000; Muyzer et al. 1998) and sequencing of most prominent bands. Sequence-specific PCR tests were also used to search for specific bacterial species correlated to 1,2-DCA dechlorination, as well as to identify sequences of catabolic genes (dehalogenases) involved in reductive dehalogenation of 1,2-DCA.

Materials and methods

Groundwater sampling, microcosms preparation and analysis

The contaminated area of $\approx$1100 m^2 has been analyzed using several couples of sampling wells; the average distance of each couple (C, E, D) from another is about 10 m except for two control couples (A, B) at more than 25 m from the likely source of contamination (E) (Figure 1). 1,2-DCA biodegradation assays were carried out in 20 ml microcosms in anaerobic vials containing subsurface water collected by pumping from sampling wells A, B, C, D and E at two depths corresponding to the upper (from 4 m to 10 m deep) and lower (from 14 m to 40 m deep) aquifers (Table 1). Water samples were collected in bottles with no head-space in order to minimize oxygen contamination and either processed immediately or stored at room temperature in the dark for up to a few days before setting up the microcosms.

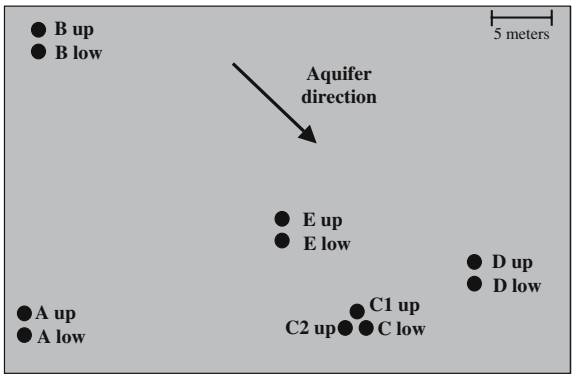

Figure 1. Horizontal layout of the contaminated area, showing location of the sampling wells (black round). Light gray: ground of the contaminated area.

Table 1. Groundwater characteristics and 1,2-DCA concentration in the different wells across the contamination plume

Well	Aquifer	pH	Conductivity (mS)	DO (mg l^{-1})	Eh (mV)	Temperature (°C)	1,2-DCA (mg l^{-1})
A_{up}	Upper	7.38	0.91	1.83	−217	14.3	4.6
A_{low}	Lower	6.91	0.82	2.09	−220	13.6	0.1
B_{up}	Upper	7.39	1.99	1.91	+24	13.5	0
B_{low}	Lower	6.81	0.86	1.77	−82	13.1	0
$C1_{up}$	Upper	n.d.	n.d.	n.d.	n.d.	n.d.	740
$C2_{up}$	Upper	n.d.	n.d.	n.d.	n.d.	n.d.	187
C_{low}	Lower	6.90	0.60	1.65	−99	13.3	0
D_{up}	Upper	7.28	1.48	2.09	−164	15.0	0
D_{low}	Lower	7.13	0.60	1.80	−137	13.6	6.5
E_{up}	Upper	6.89	0.91	1.77	−13	12.7	5932
E_{low}	Lower	6.48	1.97	1.90	−53	12.9	173.7

n.d., Not determined.

Water collected from the sampling wells was known to be contaminated by different concentrations of 1,2-DCA, hence the initial concentration of 1,2-DCA varied among the different series of microcosms (Tables 1 and 2).

In all the microcosms 10 or 50 ppm of 1,2-DCA were added after purging the water with sterile oxygen-free nitrogen. All the microcosms were amended with 1 mM cysteine (to help maintaining the anaerobic condition), 50 mg l^{-1} vitamin B12 (a basic cofactor for some dehalogenating microorganisms), 0.5 mM Hepes/NaOH pH7 [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution] (to keep the pH in a range of 6.8–8.2), yeast extract 0.05% (w/v), a 1:200 dilution of a trace elements stock solution containing: 12.8 g l^{-1} nitrilotriacetic acid, 1.35 g l^{-1} $FeCl_3{\cdot}6H_2O$, 0.1 g l^{-1} $MnCl_2{\cdot}4H_2O$, 0.024 g l^{-1} $CoCl_2{\cdot}6H_2O$, 0.1 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.1 g l^{-1} $ZnCl_2$, 0.025 g l^{-1} $CuCl_2{\cdot}2H_2O$, 0.01 g l^{-1} H_3BO_3, 0.024 g l^{-1} $Na_2{\cdot}MoO_4{\cdot}2H_2O$, 1 g l^{-1} NaCl, 0.12 g l^{-1} $NiCl_2{\cdot}6H_2O$, 0.026 g l^{-1} $Na_2SeO_3{\cdot}5H_2O$ and a supplementary salt solution containing final concentrations of 43 mg l^{-1} NH_4Cl, 0.5 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.01 g l^{-1} $CaCl_2$ $2H_2O$. Microcosms received Na-lactate, Na-acetate (5 mM both), Na-formate (40 mM) or cheese whey (0.2% w/v) as carbon source/electron donors. Microcosms amended with cheese whey did not receive the supplementary salt solution. Individual microcosms were sacrificed at regular time intervals. Control microcosms were prepared by incubating parallel vials containing all amendments with filter-sterilized groundwater samples or additional 0.1% (w/v) benzalconium chloride ethanol as bacteriostat agent. All microcosms were immediately sealed after addition of 1,2-DCA with teflon-faced septa and aluminum crimp seals, and incubated in the dark at 23 °C (Hendrickson et al., 2002).

Analytical methods

The concentrations of 1,2-DCA, ethene, vinyl chloride and other possible degradation products were analyzed by head-space gas chromatography on a 7694 Agilent gas chromatograph equipped with FID set at 200 °C on a DB624 column (J&W Scientific, Folsom, CA) at constant oven temperature at 80 °C. 1,2-DCA limit of detection was about 1–2 μg/l.

ARISA, 16S rRNA gene PCR-DGGE and sequencing

Groundwater (30 ml) and microcosm water (1.5 ml) samples were filtered using Sterivex filters (Millipore, Milan, Italy). DNA was extracted from the filtered bacterial cells by incubating the filter with 2 ml of a lysis solution containing 1 mg ml^{-1} lysozime, 1% (w/v) sodium dodecyl sulfate and 0.5 mg ml^{-1} proteinase K, and purified as previously described by Murray et al. (1998). DNA from each microcosm was extracted, starting from 1.5 ml of culture, as previously described (Ausubel et al. 1994).

An automated ribosomal intergenic spacer analysis was performed by amplifying the 16S-23S rRNA intergenic spacers using one of the primers labeled with a fluorescent dye, and by separating the amplified products in an

Table 2. Microcosm characteristics including sampling well, added electron donor, added and final 1,2-DCA concentrations

Microcosm ID	Sampling well	Aquifer	Added electron donor	Added 1,2-DCA (mg l^{-1})	Total 1,2-DCA (mg l^{-1})	Incubation time (d)	1,2-DCA removal (%)	Ethene
AU0	A_{up}	Upper	n.a.	10	14.6	15	100	+ *
AU1	A_{up}	Upper	Acetate–Formate	10	14.6	15	100	+ *
AU2	A_{up}	Upper	Lactate	10	14.6	15	100	+ *
AU3	A_{up}	Upper	Lactate	50	54.6	45	100	+ *
AU4	A_{up}	Upper	Cheese whey	10	14.6	45	88	+
AL0	A_{low}	Lower	n.a.	10	10.1	45	100	+
AL1	A_{low}	Lower	Acetate–Formate	10	10.1	45	79	–
AL2	A_{low}	Lower	Lactate	10	10.1	15	100	+
AL3	A_{low}	Lower	Lactate	50	50.1	45	100	+
AL4	A_{low}	Lower	Cheese whey	10	10.1	45	72	+
C1U0	$C1_{up}$	Upper	n.a.	10	750	45	100	+
C1U1	$C1_{up}$	Upper	Acetate–Formate	10	750	15	100	+
C1U2	$C1_{up}$	Upper	Lactate	10	750	15	100	+
C1U3	$C1_{up}$	Upper	Lactate	50	790	15	100	+
C1U4	$C1_{up}$	Upper	Cheese whey	10	750	45	61	+
C2U0	$C2_{up}$	Upper	n.a.	10	197	15	100	+
C2U1	$C2_{up}$	Upper	Acetate–Formate	10	197	15	100	+
C2U2	$C2_{up}$	Upper	Lactate	10	197	15	100	+
C2U3	$C2_{up}$	Upper	Lactate	50	237	15	100	+
C2U4	$C2_{up}$	Upper	Cheese whey	10	197	45	32	+
CL0	C_{low}	Lower	n.a.	10	10.6	45	61	–
CL1	C_{low}	Lower	Acetate–Formate	10	10.6	45	100	+
CL2	C_{low}	Lower	Lactate	10	10.6	45	100	+
CL3	C_{low}	Lower	Lactate	50	50.6	45	81	+
CL4	C_{low}	Lower	Cheese whey	10	10.6	45	63	–
DU1	D_{up}	Upper	n.a.	10	10	15	100	+
DU2	D_{up}	Upper	Acetate-Formate	10	10	15	100	+
DU3	D_{up}	Upper	Lactate	10	10	15	100	+
DU4	D_{up}	Upper	Lactate	50	50	15	100	+
DU5	D_{up}	Upper	Cheese whey	10	10	15	100	+
DL0	D_{low}	Lower	n.a.	10	16.5	45	100	–
DL1	D_{low}	Lower	Acetate–Formate	10	16.5	45	100	+
DL2	D_{low}	Lower	Lactate	10	16.5	45	100	+
DL3	D_{low}	Lower	Lactate	50	56.5	45	100	+
DL4	D_{low}	Lower	Cheese whey	10	16.5	45	90	–
EL0	E_{low}	Lower	n.a.	10	183.7	15	100	+
EL1	E_{low}	Lower	Acetate–Formate	10	183.7	15	100	+
EL2	E_{low}	Lower	Lactate	10	183.7	15	100	+
EL3	E_{low}	Lower	Lactate	50	223.7	15	100	+
EL4	E_{low}	Lower	Cheese whey	10	183.7	45	100	+ *

The highest 1,2-DCA removal (as percentage) and the incubation time when it was firstly recorded are reported. At the indicated incubation times the presence of ethene and ethane is indicated by + and *, respectively. Ethane was never detected in the microcosms at T0. Acetate and formate were supplied together.

n.a., No organic electron donors/carbon sources were added. These microcosms were supplemented with solutions containing inorganic salts, trace element and vitamins as reported in the section Materials and Methods.

automated capillary sequencer working in Genescan mode. Reaction mixture contained 1× PCR buffer (Amersham, Milan, Italy), 2.5 mM $MgCl_2$, 0.10 mM dNTPs, 0.3 mM of each primer, 1 U of *Taq* polymerase and 50 ng of environmental DNA in a final volume of 50 μl. The bacterial universal

primers used were the S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 previously described (Daffonchio et al. 1998) except that primer S-D-Bact-1494-a-S-20 was 5′ end labeled with the phosphoramidite dye 5-FAM. The amounts of PCR products were estimated on agarose gel, and 3 μl of the product was added to 0.8 μl of 1000 ROX-labeled internal size standard (Applied Biosystems, Monza, Italy) and 15 μl of deionized formamide. Samples were denatured at 95 °C for 8 min and rapidly put into ice for 5 min. ARISA fragments were loaded on an Abi Prism 310 capillary electrophoresis system in denaturing conditions using POP-4 running polymer. Samples were run for 50 min at 15 kV. The injection time of each sample was 5 s at 15 kV. The ARISA data were analyzed with Genescan 3.1.2 software (Applied Biosystems), and a threshold of 50 fluorescent units was used.

For DGGE analysis primers GC-357f and 907r were used to amplify 16S rRNA genes as previously described (Sass et al. 2001). Polyacrylamide gels (7% of a 37:1 acrylamide–bisacrylamide mixture in 1× TAE buffer) were prepared with a gradient maker (BioRad, Milan, Italy) according to the manufacturer's guidelines, with a gradient of 40% (top)–60% (bottom) denaturant where 100% denaturation is considered urea 7 M and formamide 40% according to Muyzer et al. (1993). Gels were run for 15 h at 110 V in 1× TAE buffer at a constant temperature of 60 °C in a D-Code electrophoresis system (BioRad). The gels were stained for 30 min in 1× TAE buffer containing 1.2 μl ml^{-1} of SYBR Green stain (Molecular Probes, Leiden, The Netherlands). Visualization and digital image recording was performed with GelDoc 2000 apparatus (BioRad) using the Diversity Database software (BioRad).

DGGE bands were excised from the gel using a sterile blade, transferred to 50 μl of MilliQ water and frozen at −20 °C until reamplification. Before amplification the DNA was eluted at 37 °C for 6 h. Reamplification was performed using primers 357f (without GC clamp) and 907r, 7 μl of the eluted DNA fragments as template, adding 1 mM $MgCl_2$ to the reaction mixture and following this protocol: an initial DNA denaturation at 94 °C for 4 min followed by 30 cycles at 94 °C for 30 s, 64 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 7 min. PCR products were purified by the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Purified products were hence sequenced with 357f primer using ABI Prism BigDye terminator cycle sequencing (Applied Biosystems) and ABI 310 automated sequencer (Applied Biosystems). The resulting sequences were compared with the sequence database at the National Center for Biotechnology Information (NCBI) using BLAST (Altschul et al. 1990).

PCR primers specific for the 16S rRNA gene of *Desulfitobacterium* sp. were designed by aligning known *Desulfitobacterium* sequences and selecting species-specific regions. Primers Desulfo16sF3 (5′-TTA(AG)TAGATGGATCCGCGTCTG-3′) and Desulfo16sR5 (5′-TTTCCGATGCAGTCCCAGG -3′) were designed and utilized at an annealing temperature of 51 °C. The 500-bp long product of the amplification reactions was sequenced to confirm the specificity. In a few cases the sequenced product of amplification showed the highest degree of homology with *Desulfosporosinus* sp.

Community fingerprinting statistical analysis

The peak matrices corresponding to the ARISA profiles were subjected to principal component analysis (PC analysis – Ranjard et al. 2001) by using the Jaccard coefficient in the NTSYS pc 2.01 software (Applied Biostatistics Inc., USA).

The species richness in the microcosms was estimated on the basis of the ARISA peak patterns according to the assumption of Yin et al. (2000). The number of peaks in each profile was taken to represent the number of different organisms (species richness) present in the groundwater microcosms during the treatment. This represents an approximation as many bacteria harbor several rRNA operons per cell that can be different, thus giving a pattern of bands.

PCR amplification, cloning and sequencing of reductive dehalogenases

To identify potential catabolic genes involved in reductive dehalogenation, PCR amplification with degenerated primers (ceRD2Sf, ceRD2Lf, RD7r) has been performed on DNA extracted from groundwater microcosms according to Regeard et al. (2004). Positive PCR products have been cloned using pMOS Blue cloning kit (Amersham) according to the manufacturer's instructions.

A direct PCR assay has been performed on white colonies to amplify the insert using primers T7 and U19. Amplification reactions were performed in a 25 μl total volume containing, 10× reaction buffer, 0.12 mM of each dNTP, 0.7 U of *Taq* DNA polymerase, 0.3 μM of each primer. Reactions were performed with the following protocol: an initial melting at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1.5 min. A final extension step at 72 °C for 5 min. PCR products were purified by the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sequenced as previously described.

Results

Groundwater 1,2-DCA contamination and dechlorination in groundwater microcosms

Groundwater samples from a 1,2-DCA contaminated aquifer were collected from a confined area internal to an industrial site. The entire area was monitored through 11 wells to detect the pollutant levels in the plume both in the upper and in the lower aquifer. Different levels of DCA contamination characterized the two aquifers. Monitoring of the contamination plume showed that the 1,2-DCA concentrations over 200 mg l^{-1} were restricted to the upper aquifer in a relative narrow area that included the E wells (Table 1). The chemical–physical conditions of the aquifers were suitable for supporting anaerobic microbial life with pH ranging between 6.4 and 7.4 and negative Eh values down to −220 mV. The 1,2-DCA contamination level was highest in the upper central sector of the plume.

To evaluate the potential of 1,2-DCA removal in the aquifer, sets of microcosms made of groundwater samples collected from 11 wells across the contaminated area and supplemented with different electron donors were incubated in anaerobic conditions (Table 2). Samples from the most contaminated well (E_{up}) were not used. The effectiveness of these electron donors/carbon sources to feed 1,2-DCA dehalogenation was measured in the microcosms at different times of incubation (Table 2). In most of the microcosms 1,2-DCA was completely removed. Efficient dechlorinating microcosms were those prepared with groundwater from wells $C1_{up}$ (750 mg l^{-1} of 1,2-DCA eliminated in less than 15 days), $C2_{up}$, E_{low} (around 200 mg l^{-1} of 1,2-DCA eliminated in less than 15 days) and D_{up} (up to 50 mg l^{-1} of 1,2-DCA eliminated in less than 15 days). The microcosms prepared with groundwater from the other wells were contaminated with lower amounts of 1,2-DCA (between 10 and 60 mg l^{-1}). In most of these microcosms 1,2-DCA was completely removed in 15 or 45 days.

In general, both lactate and acetate plus formate were equally effective as electron donor in supporting 1,2-DCA removal, with the exception of microcosms prepared from A_{low}, where lactate was the most efficient. For all water samples, effective removal of 1,2-DCA was supported also in microcosms amended with inorganic salts even though, in case of microcosm CL0, 1,2-DCA removal was partially supported (61%) and in microcosms AL0 and C1U0 100% removal occurred after 45 days. 1,2-DCA was removed also in microcosms amended with cheese whey but more slowly with respect to microcosms amended with the other organic electron donors or inorganic salts, reaching 100% removal only in microcosms DU5 and EL4 after 15 and 45 days of incubation, respectively. However, for all microcosms amended with cheese whey 1,2-DCA reached concentrations below the limit of detection after more than 45 days of incubation (data not shown). In Figure 2 the time course of 1,2-DCA degradation is reported for microcosms prepared with groundwater from well $C1_{up}$ completely degrading 750 mg l^{-1} of the contaminant in 2 weeks.

In most of the microcosms ethene was detected as dechlorination product of 1,2-DCA (Table 2). Vinyl chloride (which is constantly present in aquifer groundwater at concentrations of about 0.02% of the 1,2-DCA level) was below detection levels in the microcosms and did not accumulate. In a few microcosms ethane was found together with ethene, while in one sample only ethane was present after 45 days of incubation. A relative decrease in ethene was observed in some samples at the longest incubation times.

ARISA, 16S rRNA gene PCR-DGGE and sequencing

To evaluate the response of the aquifer microbial community to the biostimulation treatments, microcosms prepared from a subset of the

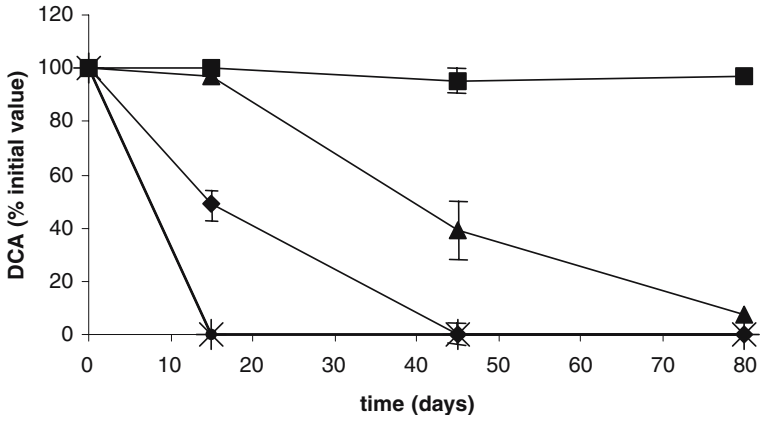

Figure 2. Time course of 1,2-DCA dehalogenation for the 1,2-DCA-adapted microbial community selected from well C1 upper after addition of: Salts-vitamins and no additional C source (♦), lactate (●), lactate and additional 1,2-DCA (+), formate plus acetate (×), cheese whey (▲), control (■). Curves of lactate (●), lactate and additional 1,2-DCA (+) and formate plus acetate (×) are overlapped in the graph.

sampling points showing the fastest 1,2-DCA removals were analyzed for the structure of the bacterial population.

First, we wished to evaluate whether in the microcosms most efficient in 1,2-DCA degradation, i.e. microcosms C1U and C2U amended with lactate, bacterial populations with a different structure respect to the other microcosms were selected. The community structure of microcosms C1U2, C1U3, C2U2 and C2U3, which degraded the highest 1,2-DCA concentrations, was analyzed by ARISA after sampling at two different times of incubation. The profiles were compared to those of microcosms AL2, AL3, CL0, CL2, CL3, DL2 and DL3 (Table 2). The selected microcosms were from different wells, all amended with lactate except CL0 that received only salt and vitamin solutions. The ARISA peak data were used for PC analysis. The scatter plot resulting from the analysis of the first three principal components contributing 29% of the total variance is shown in Figure 3a. The PCA scatter plot of the ARISA patterns tended to group the microcosms according to the well from which they originated by dividing the analyzed microcosms in three main clusters, the first including ARISA profiles obtained from microcosms CL and AL (wells C_{low} and A_{low}, lower aquifer), the second from microcosms DL (well D_{low}, lower aquifer) and the third from microcosms C1U and C2U (wells $C1_{up}$ and $C2_{up}$, upper aquifer).

We evaluated the effect of biostimulation treatment on species richness, since it has been shown (De Wildeman et al. 2003; Kassenga et al. 2004) that the primary electron donor in dechlorination reaction is hydrogen and substrates like lactate, acetate etc, may be used by bacteria other than dechlorinators, leading to hydrogen generation and hence indirectly to dechlorination. To evaluate how the biostimulation treatments affected the species richness of the contaminated groundwater, the average and standard deviation of the species richness in the microcosms derived from different wells was calculated from the ARISA profiles for: (i) microcosms amended with inorganic salts, (ii) microcosms amended with different organic electron donors (lactate, acetate plus formate and cheese whey) at two incubation times (15 and 45 days). These values were then compared to the species richness in the original groundwater (Figure 3b). For microcosms derived from wells A_{low}, C_{low} and D_{low}, amendments clearly affected the species richness by remarkably increasing the number of peaks in the ARISA profiles compared to the species richness in the microbial community of the original groundwater. For microcosms derived from wells $C1_{up}$ and $C2_{up}$ the peaks increase was much less evident due to an already richer species complexity in the original groundwater.

The species diversity in the groundwater from all the wells except B_{up}, B_{low} and E_{up}, and in microcosms derived from wells $C1_{up}$ and $C2_{up}$, sampled after 15 and 45 days of incubation, was examined by DGGE analysis of amplified bacterial 16S rRNA gene and further band sequencing.

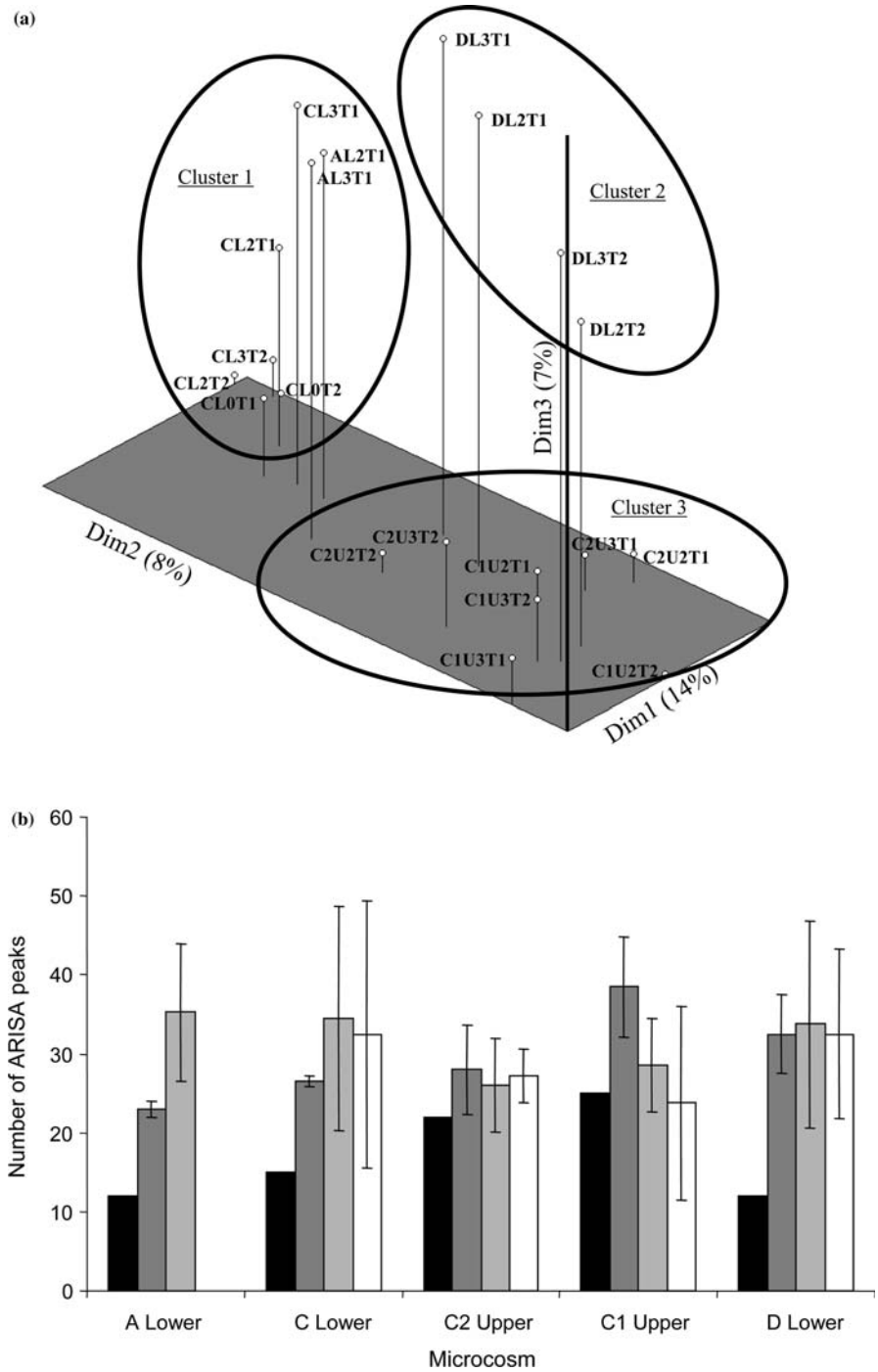

Figure 3. (a) Three dimensional scatter plots of the principal component analysis of ARISA fingerprinting: the axes represent the first three variability components explaining together the 29% of the variance among the samples analyzed. The analysis groups the microcosms in three clusters according to the well from which they originated. T1: 15 days of incubation; T2: 45 days of incubation. (b) Graphical representation of richness index: black column for the Time 0 samples, dark gray for microcosm amended only with salts (analyzed after 15 days of incubation), light gray for average of lactate–formate plus acetate–cheese whey amended samples analyzed after 15 days and white columns for average of lactate–formate plus acetate–cheese whey amended samples analyzed after 45 days.

DGGE analysis did not show conserved band profiles in response to the presence of different 1,2-DCA concentrations or of selected electron donors (Figure 4). Band sequencing identified the presence of some microorganisms in the groundwater belonging to species potentially involved in dehalogenation and band enrichment in the microcosms in response to the different amendments (Table 3). In the DGGE analysis of the original groundwaters considered as the 'time zero' of the biostimulation experiment (Figure 4a), bands 4, 6, 7 and 10 from groundwater derived from A_{up}, C_{low} and E_{low} wells, showed sequence similarity to microorganisms recently identified in sites polluted by trichloroethene (an Alphaproteobacterium), chlorinated solvents (*Spirochaetales*), monochlorobenzene (Comamonadaceae) and polychlorinated biphenyls (*Variovorax*), respectively (Alfreider et al. 2002; Dojka et al. 1998; Lowe et al. 2002; Nogales et al. 2001). Additional bands with sequence homology to 1,2-dichloropropane (1,2-DCP) dehalogenating *Trichlorobacter* (De Wever et al. 2000) and to a *Acetobacterium* involved in 1,2-DCP dehalogenating community (Acc.n. AY185330) were detected in subsequent DGGE analysis of samples E_{low} and $C2_{up}$ (data not

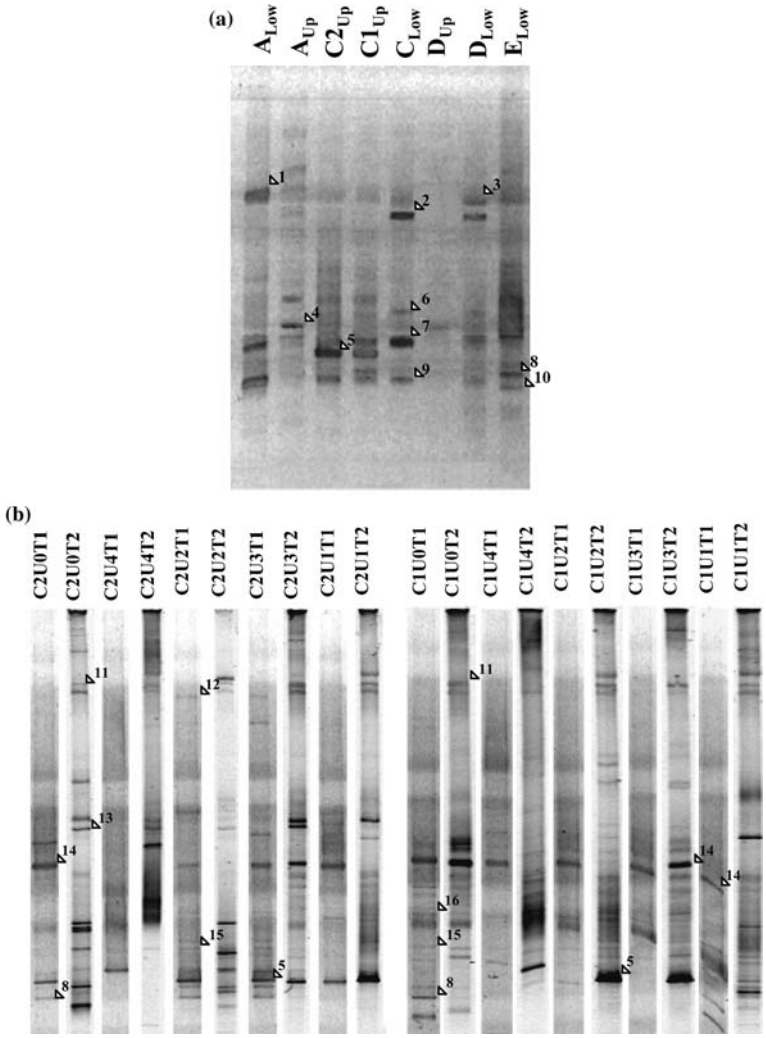

Figure 4. DGGE patterns on a 7% polyacrylamide denaturing gel (30–60% urea and formamide); (a) DGGE analysis on DNA extracted from aquifers (T0); (b) DGGE pattern from analysis of microcosms C1U and C2U after 15 (T1) and 45 (T2) days. Bands cut for sequencing have been marked with an arrow and numbered. Individual lanes from different gels were graphically assembled in the figure after alignment of reference bands.

Table 3. Summary of the sequencing results with band number, homology and reference code in NCBI, most correlated species and groundwater-microcosms showing the band

Band	Homology and reference	Most related species	Characteristics – Habitat	Groundwaters and microcosms showing band
1	95 (AY145619)	Uncult. clone DC11	Weser estuary β Proteobacteria	A_{low}
2	95 (AY424830)	Uncult. β clone mv13	Analysis of methanotrophic bacteria	C_{low}; D_{low}
3	94 (AJ532690)	Uncult. β Proteobacteria	Methylophilus interaction with heavy metals	D_{low}
4	96 (AF422583)	Uncult. clone T008	TCE dechlorinating α Proteobacteria	A_{up}
5	99 (AY032610)	*Dechloromonas aromatica*	Anaerobic benzene oxidation	C1U1T1; C1U2T1; C1U2T2; C1U3T2; $C1_{up}$; C2U0T1; C2U1T1; C2U1T2; C2U2T1; C2U3T1; C2U3T2; $C2_{up}$
6	96 (AF050550)	Uncult. *Spirochaetales*	Chlorinated-solvent-contaminated aquifer bioremediation	$C1_{up}$; C_{low}
7	95 (AF407413)	Uncult. *Comamonadaceae*	Monochlorobenzene reactor	C1U0T1; C2U2T1; C2U3T1; $C1_{up}$; C_{low}; D_{low}; E_{low}
8	96 (AY168754)	Hydrogenophaga	Arsenite oxidizing biofilms	C1U0T1; C2U0T1; C2U2T1; C2U3T1; E_{low}
9	96 (AY032611)	*Dechloromonas* sp. JJ	Anaerobic benzene oxidation	A_{low}; $C1_{up}$; $C2_{up}$; C_{low}
10	96 (AJ292659)	Uncult. *Variovorax*	Polychlorinated biphenyl-polluted soil	E_{low}
11	97 (AY133091)	Uncult. Low GC Bacteria	TCE-contaminated site	C1U0T2; C1U1T2; C1U2T2; C1U3T2; C1U4T2; C2U0T2; C2U1T2; C2U2T1; C2U2T2; C2U3T2; C2U4T1; C2U4T2
12	95 (AF233880)	*Comamonas denitrificans*	Activated sludge	C1U1T2; C1U2T2; C1U3T2; C2U1T2; C2U2T1; C2U3T1
13	96 (AY530552)	Uncult. β Proteobacteria	Perchlorate reducing bacteria	C2U0T1; C2U0T2; C2U1T1; C2U3T2 ; C2U4T2
14	93 (AF255644)	Uncult. *Clostridium*	Biological phosphorus removal (dichloropropane)	C1U0T1; C1U0T2; C1U1T1; C1U2T1; C1U3T1; C1U3T2; C1U4T1; C2U0T1; C2U1T1; C2U2T1; C2U3T1; C2U3T2
15	97(UBA306754)	Uncult. Bacterium	*Acidaminobacter hydrogenoformans*	C1U0T1; C1U0T2; C2U0T2; C2U2T1; C2U3T1; C2U4T2
16	92 (AJ306754)	Uncult. Clostridiaceae	Anaerobic treatment of sulfurous effluents	C1U0T1; C1U0T2; C1U4T1; C1U4T2

For the groundwater samples, please refer to the sampling well (A_{up}; A_{low}; $C1_{up}$; $C2_{up}$; C_{low}; D_{low}; E_{low}) ID in Table 2. The last two digits of each microcosm name represent the time at which the microcosm was sampled and analyzed (T1 = 15 days; T2 = 45 days), while the first four digits represent the microcosm ID according to Table 2.

shown). In the DGGE profiles of the bacterial community in anaerobic microcosms (Figure 4b), band 11 appeared after 15 days in microcosms derived from groundwater of $C1_{up}$ and $C2_{up}$ wells, while band 13 has been identified only in $C2_{up}$ well-derived microcosms. These bands correspond to sequences with high homology to 16S rRNA of microorganisms adapted to soils and waters contaminated with TCE and perchlorate. The only prominent band conserved in all DGGE profiles both of the original groundwaters and in the microcosms was band number 5 which showed high homology with *Dechloromonas aromatica* (Coates et al. 2001). No other band identified in original groundwater and potentially correlated to microrganisms able to dechlorinate chlorinated aliphatics was recovered in the following sampling times. Band 14, found in several microcosms derived from both $C1_{up}$ and $C2_{up}$ wells, showed homology with an uncultured *Clostridium* involved in a biological phosphorous removal process (Liu et al. 2001) but also with another uncultured *Clostridium* identified in a consortium able to degrade dichloropropane (Schloetelburg 2001).

By using primers specifically designed for the amplification of *Desulfitobacterium*, PCR products of the expected size were obtained from microcosms derived from well $C1_{up}$. The sequence of this fragment showed 97% nucleotide identity with *Desulfitobacterium chlororespirans,* an anaerobic dehalorespiring bacterium, which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate (Sanford et al. 1996) and 96% with *Desulfitobacterium dichloroeliminans* strain DCA1, a species recently shown to efficiently dehalogenate 1,2-DCA (De Wildeman et al. 2003). Fragments with homology to this bacterium were not identified in DGGE profiles of the original groundwater, nor in the microcosms undergoing biostimulation treatments.

PCR amplification, cloning and sequencing of a putative reductive dehalogenase gene

To identify potential catabolic genes involved in reductive dehalogenation, PCR amplification with highly degenerated primer sets (ceRD2Sf/RD7r, ceRD2Lf/RD7r; Regeard et al. 2004) was performed on total DNA extracted from microcosms originating from $C1_{up}$ well which showed the best performance in 1,2-DCA removal by completely degrading the 750 mg l^{-1} of contaminant (Figure 2). By using the same primers on DNA of bacterial isolates Regeard et al. (2004) found a PCR product of 750–800 bp depending on the strain, while in this work PCR products longer than 1000 bp were amplified only from microcosm C1U2 with the ceRD2Lf/RD7r primer set and from microcosms C1U0 and C1U2 with the ceRD2Sf/RD7r primer set. These PCR products were cloned and screening of the cloned insert by vector specific PCR showed all the clones to contain about 1150-bp long inserts, with the exception of one insert 800-bp long. Three purified plasmid inserts were sequenced in both directions: DHLclone89 (ca. 800 bp) and DHLclone84 (ca. 1150 bp) from microcosm C1U0, and DHLclone54 (ca. 1150 bp) from microcosm C1U2. These inserts showed a complete nucleotide identity for the overlapping sequence. Alignment of the longest sequence with public sequence databases showed that the amplified fragments were putative reductive dehalogenase gene fragments with a 94% nucleotide identity (1036 nt positions), and 92% aminoacid similarity (88% identity on 344 aa positions), with *Dehalobacter restrictus pceA* gene coding for tetrachloroethene reductive dehalogenase (Figure 5).

Discussion

The prediction of the success of an *in situ* remediation process in polluted soils and aquifers remains a black box in most cases. It is well known that standardization of such processes is practically impossible and each case requires specific feasibility studies. This is particularly true for remediation processes based on the activity of the autochthonous microbiota of polluted sites. When the main catalyzers of the remediation process are bacteria, the feasibility of the process must be evaluated in order to optimize results. In particular, three factors need to be carefully evaluated to choose the optimal treatment: the presence of suitable microorganisms in the site, an evaluation of the factors that can foster the site reclamation (such as carbon sources, electron donors and acceptors, etc.), and the response to biostimulation in terms of removal of the toxic compounds.

```
DHLclone54          QRSKETGWTSLDSALQAGVWAVEFDFSGFNATDSGPGSVITPYPINPMTNEIANEPVMVP
PceA Dr             QRHNETGWTGLDEALNAGAWAVEFDYSGFNAAGGGPGSVIPLYPINPMTNEIANEPVMVP
                    **  :***** .** .** :** .****** :***** :. ******.  **********

DHLclone54          GLYNWDNIDVESVRQQGQQWKFKSKEEASKMVKKAACFLGADLVGIAPYDERWTYSTWGR
PceA Dr             GLYNWDNIDVESVRQQGQQWKFESKEEASKMVKKATRLLGADLVGIAPYDERWTYSTWGR
                    **********************:***********: :**********************

DHLclone54          KIPKPCKMPNGRTKLMPWDLPKVLSGGGVEVFGHAKFEPDWEKHAGFKPKSVIVFVLEED
PceA Dr             KILKPCKMPNGRTKYLPWDLPKMLSGGGVEVFGHAKFEPDWEKYAGFKPKSVIVFVLEED
                    ** ***********  :****** :********************:****************

DHLclone54          YEALRTSPSVIASAATGKVYSSMGGVSYKIAVFLRKLGYYAAPSGNDTGLNVPMAVQAGL
PceA Dr             YEAIRTSPSVISSATVGKSYSNMAEVAYKIAVFLRKLGYYAAPCGNDTGLSVPMAVQAGL
                    ***:******* :** :.** **.*. *:**************** .****** .*********

DHLclone54          GEAGRNGLLITQKFGPRHRIAKVYTDLELAPDKPRKFGVREFCRLCKKCADACPAQAISH
PceA Dr             GEAGRNGLLITQKFGPRHRIAKVYTDLELAPDKPRKFGVREFCRLCKKCADACPAQAISH
                    ************************************************************

DHLclone54          EKDPKVLQPGDCEESENPYTEKWHVDSNRCGSFWAYNGGLCANC (344aa)
PceA Dr             EKDPKVLQPEDCEVAENPYTEKWHLDSNRCGSFWAYNGSPCANC (344aa)
                    ********* *** :********* :************. ****
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Figure 5. Clustal X alignment of *Pce*A Dr reductive dehalogenase gene by *Dehalobacter restrictus* (AJ439607) with DHLclone54. Underlined letters represent FeS cluster 1. '*' = a conserved position in both the reductive dehalogenases; ':' = a position with high positives; '.' = a position with low positives.

Hence the description of the microflora present in the site and of its putative response to different treatments, including biostimulation or bioaugmentation and even natural attenuation-mediated processes, would contribute to improving the effectiveness of the site reclamation.

In the case of groundwater contamination by 1,2-DCA, one of the most interesting possibilities for detoxification is reductive dehalogenation of the pollutant. This reaction is catalyzed by several bacteria through dehalogenases that mediate electron transfer from donors like organic acids to 1,2-DCA which is converted to ethene as a result (De Wildeman et al. 2004; Kassenga et al. 2004; Smidt and de Vos, 2004). Reductive dehalogenation can be favored by biostimulation with suitable electron donors, when their *in situ* level is low (Nobre & Nobre 2004).

The case considered in this study refers to a site with a relatively complex situation in which an old 1,2-DCA contamination resulted in a plume spanning over two overlaying aquifers. The chemical–physical characterization of the plume showed a heterogeneous level of contamination ranging from μg to g l^{-1} of contaminant. The environmental conditions suggested that a process of reductive dechlorination of 1,2-DCA could already be under way and could be improved by biostimulation treatment. Therefore, we evaluated the potential of such a treatment by assaying the response of the microbial community to several electron donors. We chose formate plus acetate and lactate as our electron donors since they are known to be suitable for supporting dehalogenation of 1,2-DCA in aquifers (Klecka et al. 1998). In addition, cheese whey was also tested as an electron donor/carbon source since it can be an interesting and inexpensive alternative to lactate or acetate plus formate to be used in field application (Camilli et al. 2002). In most of the microcosms prepared with groundwater from different wells across the plume, lactate and acetate plus formate resulted suitable amendment for 1,2-DCA removal. Nevertheless, the simple addition of salts appeared to support 1,2-DCA removal in most cases, albeit at a slower rate (Table 2). On the other hand, cheese whey seemed to be a less effective amendment since it supported 1,2-DCA removal but at a relatively low rate. This could be due to a lack of inorganic salts, since microcosms

with cheese whey did not receive the supplementary salt solution. It should be noted that the cheese whey solution used contains levels of inorganic and organic nitrogen and phosphorous that are high enough not to be limiting for bacterial growth. Another possible explanation is that this raw and complex C-source contained alternative electron acceptors, which can initially compete with 1,2-DCA thus causing a delay in the onset of an efficient 1,2-DCA removal. Moreover, whey lactose fermentation might have caused a pH decrease that was not promptly buffered in the medium, hence determining a delay of the metabolism of those microorganisms sensitive to acidity. However, after the initial lag phase, the microbial metabolism seemed to slowly replace suitable conditions for 1,2-DCA dehalogenation as shown in Figure 2 (triangle line).

The microcosms showing the minimum best degradation rate of 1,2-DCA have been chosen to further investigate the response of the microbial communities to the different biostimulation amendments. ARISA profiles of the bacterial communities showed some heterogeneity as indicated by the relatively low level of variability (29%) accounted for by the first three principal components of ARISA patterns PCA (Figure 3a). This indicated that the bacterial communities selected by the biostimulation treatments of groundwater from different wells were rather dissimilar, suggesting that the environmental conditions in the plume were not homogeneous thus causing the establishment of different bacterial populations. The clustering of the ARISA profiles showed that the microbial community from different wells responding to the same electron donor, lactate, tends to cluster according to the well and the lower or upper aquifer from which they originated. A similar consideration can be hypothesized from the species richness index (Figure 3b). The latter indicates that the most polluted microcosm from the upper aquifer showed minor changes in species richness with respect to microcosms originating from the lower aquifer where 1,2-DCA concentration was lower. We speculate that microrganisms in the most polluted well in the plume were well-adapted to the environmental conditions and already metabolizing 1,2-DCA. Moreover, *in situ* degradation of 1,2-DCA in wells $C1_{up}$ and $C2_{up}$ is supported by the presence in the aquifer of traces of ethene, the catabolic end product of the reductive microbial dichloroelimination of 1,2-DCA. In other less polluted wells the microbial community seemed to be less adapted to 1,2-DCA and could be enriched in dechlorinating species only following the addition of a suitable electron donor/carbon source which fostered dechlorination at a lower rate than microbial communities of wells $C1_{up}$ and $C2_{up}$ mentioned earlier (Table 2). However, all the wells were found to host microbial communities, which properly respond to biostimulation by degrading 1,2-DCA to ethene without the release of highly toxic by-products such as vinyl chloride. Consequently, we can conclude that bacterial populations could already dechlorinate 1,2-DCA *in situ* in the area surrounding wells $C1_{up}$ and $C2_{up}$ but lactate or other amendments might further increase the rate of dehalogenation.

The adaptation of wells $C1_{up}$ and $C2_{up}$ is also partially supported by the bacterial diversity found with DGGE analysis of 16S rRNA gene (Figure 4a and b). Among others, sequences related to *Dechloromonas aromatica* (band 5 in Figure 4a and b), a species that can dehalogenate aromatic pollutants (Coates et al. 2001) were found in the bacterial community associated with the groundwater of these wells as well in the derived microcosms. It would be interesting to evaluate whether strains of this species could mediate 1,2-DCA dehalogenation. Following biostimulation, other sequences related to bacteria that were found in the context of soils and waters polluted with chloroaliphatics were enriched in groundwater microcosms such as several low G+C gram-positive bacteria related to Clostridia. Also, a sequence with a homology of 97% with *Desulfitobacterium chlororespirans* (Sanford et al. 1996) has been identified by a specific PCR assay in microcosm C1U2 after amendment with lactate. Sequences that were found to be homologous to other known dechlorinating bacteria (*Clostridium*, *Trichlorobacter*, *Acetobacterium*) were also enriched in the amended cultures. It is interesting to note that no sequences correlated to *Dehalococcoides ethenogenes*, a microrganism known to be able to degrade 1,2-DCA and identified in several sites polluted by chloroaliphatics (Kassenga et al. 2004; Maymo-Gatell et al. 1999), were identified neither in DGGE analysis nor with taxon-specific PCR assay.

Another important piece of evidence in support of the potential of a given site for a bioremediation process is the presence and/or the enrichment of catabolic genes associated with the key steps of the detoxification process. Dehalogenases are the key enzymes, which mediate reductive dechlorination. By using a PCR assay recently developed for the amplification of unknown dehalogenases (Regeard et al. 2004), we were able to amplify, clone, and sequence a fragment which appeared to be a new putative dehalogenase from the metagenome of the two microcosms that showed the highest 1,2-DCA removal rate. We had previously used many pairs of primers obtained from the literature that were found to be unsuitable for the identification of sequence markers potentially associated to dehalogenation. The low efficiency of these primers is due to the low level of conservation found among known dehalogenases (Suyama et al. 2002). Using the highly degenerated primers (containing inosine) from Regeard et al. (2004), designed to amplify chloroethene and chlorophenol reductive dehalogenase genes, we were able to obtain a 1036-bp fragment that showed an homology of 94% with the *pceA* gene coding for a 60 kDa reductive dehalogenase of *Dehalobacter restrictus* (Maillard et al. 2003). PceA is a dehalogenase with a rather broad substrate spectrum (1,1,1-TCA among others) but it has not been tested on 1,2-DCA (Maillard et al. 2003). The fact that we have found the PceA sequence only in those microcosms showing the highest rates of 1,2-DCA leads us to speculate that this sequence could be involved in the *in situ* dehalogenation process. Further work is necessary to confirm this hypothesis.

In conclusion, we have found that *ex situ* biostimulation with different electron donors may support 1,2-DCA dechlorination with concomitant release of ethene, indicating the presence in the contaminated groundwater of suitable microflora. Lactate was found to be the best electron donor for stimulating dechlorination of 1,2-DCA. Despite the heterogeneous response of the microbial community to the biostimulation in terms of changes of community structure following the different amendments, most of the communities were able to support 1,2-DCA dechlorination, albeit at different rates. Species diversity analysis showed the presence of microbial species, which have previously been correlated with degradation of chlorinated solvents. Among these, we found sequences related to Firmicutes like *Clostridium* and a *Desulfitobacterium* and to Rhodocyclaceae of the beta-proteobacteria class like *Dechloromonas aromatica*. Lastly, from the most efficient 1,2-DCA dechlorinating microcosms, we were able to amplify a sequence of 1036 bp, which we identified as a new putative dehalogenase.

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