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ASSESSMENT OF NATURAL MICROBIAL DECHLORINATION

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INTRODUCTION

Natural attenuation (NA) is defined as the sum of processes which cause a decrease in contamination without human intervention. Using these natural processes for site remediation is increasingly considered as a sound alternative to active technical measures. Contrary to abiotic attenuation processes such as dispersion and sorption, biodegradation results in a net loss of contaminant mass within a plume. Understanding the site-specific microbial degradation processes is a pre-requisite for applying NA concepts (SUAREZ & RIFAI, 1999; RÖLING & VAN VERSEVELD, 2002; TIEHM & SCHULZE, 2003; SCHULZE & TIEHM, 2004).

Complete dehalogenation of chloroethenes (Figure 1) can be achieved by reductive dechlorination to ethene or by sequential reductive/oxidative degradation (TIEHM *et al.*, 2002; BRADLEY, 2003). During complete reductive dechlorination, tetrachloroethene (PCE) and trichloroethene (TCE) are anaerobically dechlorinated via cis-1,2-dichloroethene (cDCE) and vinyl chloride (VC) to the dehalogenated degradation end-product ethene (EL FANTROUSSI *et al.*, 1998). In the 1980s, only cometabolic processes were considered for reductive microbial dechlorination. However, in recent years halorespiration, i.e. the use of halogenated compounds as terminal electron acceptors, was demonstrated to be the most important reductive degradation process at many sites contaminated with PCE or TCE. Dehalococcoides are the only cluster of bacteria known at present to be capable of complete reductive dechlorination by halorespiration (MAYMÓ-GATELL *et al.*, 1997; FETZNER, 1998; MIDDELDORP *et al.*, 1999; BRADLEY, 2003). Reductive dechlorination of the higher chlorinated ethenes PCE and TCE is often faster than reductive dechlorination of cDCE and VC. Therefore, an accumulation of the lower chlorinated ethenes is frequently observed at contaminated sites (MIDDELDORP *et al.*, 1999), which is of concern due to their toxic (cDCE and VC) and carcinogenic (VC) properties (VERCE *et al.*, 2002). At many sites, the metabolites of reductive dechlorination cDCE and VC form long plumes, often reaching oxidative, aerobic zones of groundwater.

The lower chlorinated compounds cDCE and VC as well as TCE are cometabolically degradable under aerobic conditions with substrates such as ammonia, methane, or ethene (BIELEFELDT & STENSEL, 1999; BRAR & GUPTA, 2000; HOURBRON *et al.*, 2000; TIEHM *et al.*, 2002; TAKEUCHI *et al.*, 2005). Recent findings also proved metabolic degradation of VC (COLEMAN *et al.*, 2002b; DANKO *et al.*, 2004; SINGH *et al.*, 2004; MATTES *et al.*, 2005; TIEHM *et al.*, 2006) as well as cometabolic dechlorination of cDCE with VC as substrate (VERCE *et al.*, 2002, SINGH *et al.*, 2004; FATHEPURE *et al.*, 2005). Furthermore, the use of cDCE as sole carbon source was reported by COLEMAN *et al.* (2002a).

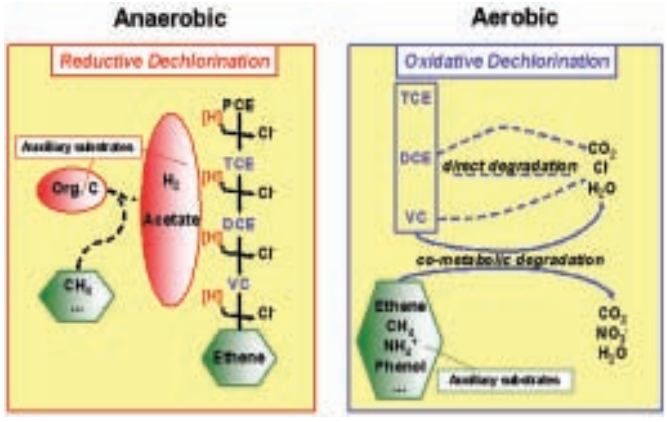

Fig. 1 - Microbial degradation of chloroethenes

The assessment, identification, and quantification of natural biodegradation processes in the field is a pre-requisite for the acceptance of monitored or enhanced NA processes as an alternative to more costly technical remediation measures. This paper represents an overview of microbiological assessment methods, and highlights some recent results at two German field sites.

KEY-WORDS: *natural attenuation, chloroethenes, 16S-PCR, halorespiration, microcosms, isotope fractionation*

SITE DESCRIPTIONS (POLLUTANT PROFILES, HYDROCHEMICAL DATA)

Killisfeld site

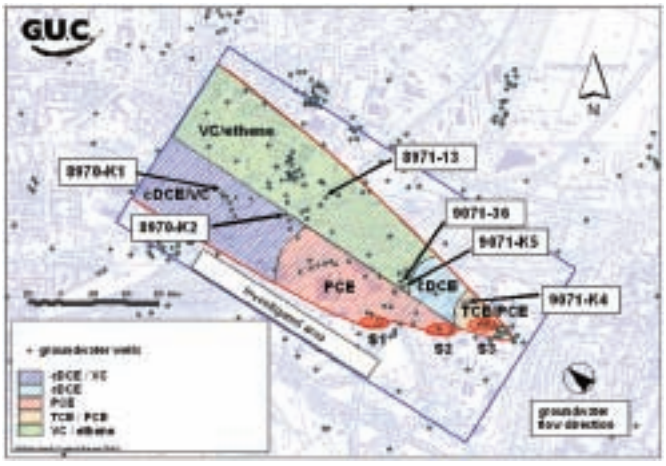

Fig. 2 - Killisfeld site

The Killisfeld site (Figure 2) consists of an industrial area located in the Rhine river valley in Karlsruhe, Germany. The plume has developed in a highly permeable sand and gravel aquifer (hydraulic conductivity: kf = 1.5-3.5 x 10-3 m/s; groundwater velocity: 0.4-0.9 m/d; water table: 2-3 m below surface; aquifer thickness: 12-16 m) interspersed with less permeable areas rich in humic acids. The chloroethene plume is originating from at least three different source areas, where a maximum concentration of 10 mg/L chloroethenes was observed. Downgradient the chloroethene source areas, disposal sites are located containing domestic wastes, which represent possible auxiliary substrates for reductive dechlorination.

Dechlorination of PCE and TCE to cDCE was observed within the first 350 m downgradient the northern source area S3. After passage of the disposal sites, the higher chlorinated pollutants disappeared and VC, ethene and ethane were detected. Along with the occurrence of VC, ethene and ethane the redox potential in the plume dropped from 300 mV to 100 mV and up to 10 mg/L of ferrous iron and 2 mg/L of methane were detected in the groundwater. Obviously, conditions were favorable for reductive dechlorination downgradient the disposal sites.

Frankenthal site

At the Frankenthal site (Figure 3) the groundwater contamination is located in the city center of Frankenthal, Germany. The polluted upper aquifer consists of sand and gravel (hydraulic conductivity: kf = 1 - 7 x 10^{-4} m/s; groundwater velocity: 0.5-1 m/d; water table: 5-9 m below surface; aquifer thickness: 10-20 m) and is delimited downwards by an impermeable clay-layer. There are at least two different source areas clearly separated in space. At the west end of the city center a metal-working industry polluted the groundwater with TCE. In

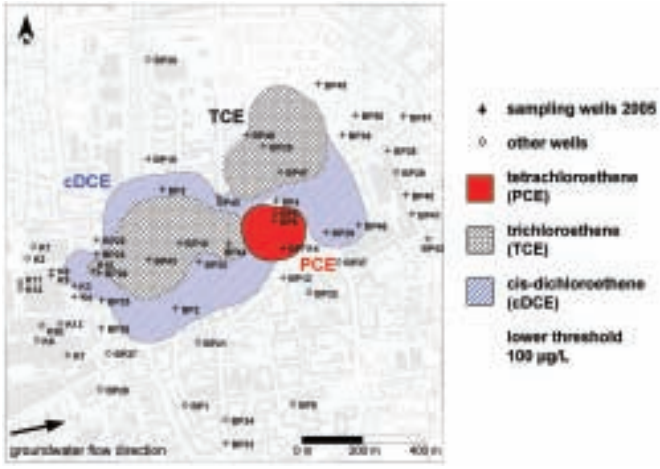

Fig. 3 - Frankenthal site

the city center a PCE contamination was caused by a dry-cleaner.

The primary contaminants PCE and TCE are degraded to cDCE, which is detected at concentrations > 1 mg/L. The intermediary reductive degradation product VC could only be found in lower amounts and ethene - end-product of reductive dechlorination - was not detected at all (detection limit: 5 µg/L). Redox-conditions in Frankenthal are more oxidizing than in Killisfeld, with higher oxygen, sulfate and nitrate concentrations, higher redox potential and lower contents of ferrous iron and methane (Table 1). Thus, the Frankenthal site conditions do not seem to be suitable for complete reductive dechlorination.

	Unit	Killisfeld	Frankenthal
Redox Potential	[mV]	47-469	140-555
Oxygen	[mg/L]	0-2.6	0-5.5
Nitrate	[mg/L]	<0.5-46	<0.5-100
Nitrite	[mg/L]	<0.01-0.14	<0.01-0.85
Sulfate	[mg/L]	26-262	64-427
Iron	[mg/L]	<0.01-8.5	<0.01-5.9
Manganese	[mg/L]	0.01-1.69	0.02-1.56
Methane	[µg/L]	<10-2000	<10-530
Total Organic Carbon	[mg/L]	1.1-15	1.6-7.6

Table 1 - Comparison of hydrochemical site data

MICROBIOLOGICAL METHODS

MICROBIAL SURVEY WITH MPN-TESTS

Most probable number (MPN) techniques are used to enumerate metabolically active microorganisms in groundwater samples or soil eluates. Samples are diluted in microplates (Figure 4, left side). After incubation the turbid wells, i.e. wells in which microorganisms did grow, are counted (Figure 4, right side). Based on a Poisson-distribution the most probable numbers are determined using appropriate tables or software.

Microorganisms with different metabolic physiologies can be detected with selective media, making it possible to distinguish

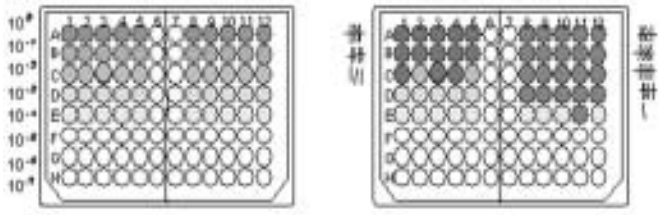

Fig. 4 - Most probable number enumeration. Left: dilution series, right: counting of the turbid or coloured wells

between active organisms of different redox zones. Total aerobic viable numbers, methanotrophs, ammonia-oxidizing and nitrite-oxidizing bacteria can be detected separately. Anaerobic organisms can be differentiated by denitrifying, nitrate-ammonifying, iron-reducing, sulphate-reducing and methanogenic microorganisms. Specific contaminant degrading bacteria can be assessed by adding the relevant pollutants as sole carbon source (STIEBER *et al.*, 1994). This method can easily be adopted to new compounds, even if the microorganisms capable of biodegradation have not been characterized.

The MPN-technique is applied to analyse microbial community composition in terms of metabolically active pollutant degraders or microorganisms using different electron acceptors. The survey of the site-specific microbial community in combination with geochemical investigations enables identification of redox zones relevant for degradation processes. In addition, changes in the microbial community composition during NA processes can be monitored.

Microbial degradation studies (microcosms)

In microcosms, microbial degradation studies are performed with the autochthonous, site-specific microflora in batchtests or flow-through column systems allowing the assessment of the degradation processes occurring in the field. The idea behind such studies is that understanding the activity of a small portion of aquifer material (groundwater and sediment) gives much information about the aquifer as a whole (TIEHM & SCHULZE, 2003). Redox conditions are maintained through the use of appropriate techniques for sampling, transport and storage of aquifer material. On the other hand it is also possible to intentionally change redox conditions in the laboratory in order to understand the effect of specific electron acceptors or auxiliary substrates. With these degradation studies substantiated information is gathered about degradable pollutants and formed metabolites under different redox conditions.

Detection of specific degraders with 16S-PCR

16S-PCR (Polymerase Chain Reaction, see Figure 5 for a general scheme of a PCR reaction) can be used for the fast detection of microbial groups or single species. The detection of specific pollutant degraders is a promising tool to assess the site-specific degradation potential (FENNELL *et al.*, 2001; HOHNSTOCK-ASHE *et al.*, 2001). The presence of Dehalococcoides bacteria, which are the only bacteria reported so far to catalyze the complete reductive dechlorination of PCE to ethene, is of particular interest for sites contaminated with chloroethenes. Dehalococcoides have been detected at sites, where complete reductive dechlorination occurred (HENDRICKSON *et al.*, 2002; SCHMIDT *et al*, 2006a). During a 16S-PCR reaction Dehalococcoides-characteristic parts of the 16S-rDNA are amplified specifically allowing their detection with agarose-gel-electrophoresis. For an increase in sensitivity, a nested PCR approach is used: in a first PCR reaction universal bacterial 16S-rDNA is amplified augmenting the DNA-material serving as template for the second PCR reaction, the amplification of Dehalococcoides-specific rDNA-regions.

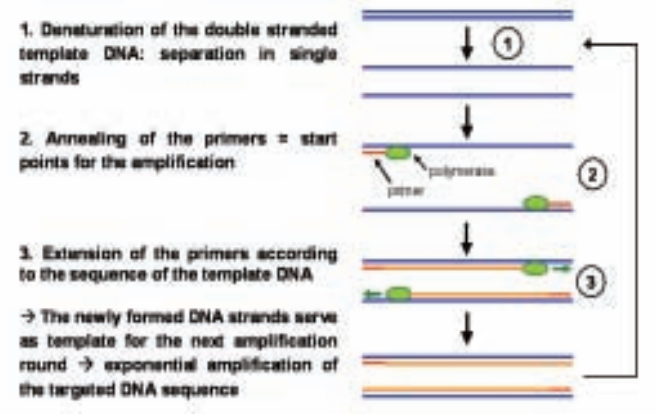

Fig. 5 - Scheme of a PCR reaction

In our study, 16S-PCR complements the microbial community analysis by MPN methods. Since PCR is a fast technique, numerous samples can be analysed for a comprehensive site assessment.

Carbon isotope fractionation

Molecules consisting of light isotopes (e.g. ^{12}C) are more rapidly biologically degraded than molecules containing heavy isotopes (e.g. ^{13}C) (ARAVENA *et al.*, 1999). Thus, microbial degradation causes an isotope fractionation effect, i.e. the enrichment of heavy isotopes in the remaining substrate (CHU *et al.*, 2004; MECKENSTOCK *et al.*, 2004) (Figure 6). The detection of fractionation effects in the field makes it possible to prove microbial degradation and to distinguish degradation from other NA processes such as dilution or sorption (CHARTRAND *et al.*, 2005). The extent of microbial fractionation is expressed with an isotope enrichment factor. These isotope enrichment factors differ for different compounds as well as for different enzymatic reactions and bacterial species (BLOOM *et al.*, 2002). Quantification of microbial degradation processes in the field is possible, if the specific fractionation factors are known. Specific fractionation factors can be determined in laboratory degradation studies (e.g. microcosms) under appropriate conditions.

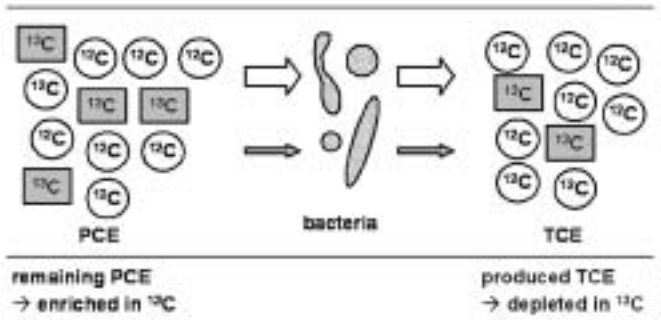

Fig. 6 - Scheme of isotope fractionation during transformation of PCE to TCE

RESULTS

Microbial survey with MPN-tests

At the Killisfeld site a microbial survey was conducted using different MPN-tests on sediment samples taken at different depths during the construction of four groundwater wells (see Figure 2 for location of the wells):

- 9071-K4: downgradient close to the chloroethene source; moderately reducing conditions; detection of PCE, TCE and cDCE
- 9071-K5: downgradient close to a disposal site; strongly reducing conditions; detection of VC and ethene
- 8970-K2: further downgradient; reducing conditions; cDCE and VC predominating
- 8970-K1: further downgradient; reducing conditions; cDCE and VC predominating

Under aerobic conditions the microbial numbers of total heterotrophs, BTEX-degraders, ammonia and nitrite oxidising bacteria and methanotrophs were determined. As anaerobes denitrifying bacteria, methanogenic bacteria, Fe(III) reducers and sulphate reducers were investigated (see Figure 7 for exemplary results of two wells).

All four wells investigated showed highest microbial numbers in regions were silt/clay/peat sediments predominate. High numbers of aerobic, BTEX-degrading, nitrifying and methanotrophic bacteria could be detected. Microbial numbers are decreasing with increasing depth. Furthermore, the microbial community composition shows changes depending on well location within the plume. In well 9071-K5 located in a strongly reducing region of the aquifer downgradient the disposal sites, methanogenic but no nitrifying bacteria and only a few methanotrophs were detected. Higher numbers of aerobic organisms were detected further downgradient in well 8970-K2, were changing, less reducing conditions occurred.

Microbial degradation studies (microcosms)

The results of degradation studies are shown for the Killisfeld site. Aerobic and anaerobic dechlorination was observed with groundwater from different parts of the plume. Results are exemplarily shown for the following two wells:

- 9071-36: downgradient close to a disposal site; strongly reducing conditions; detection of VC and ethene;

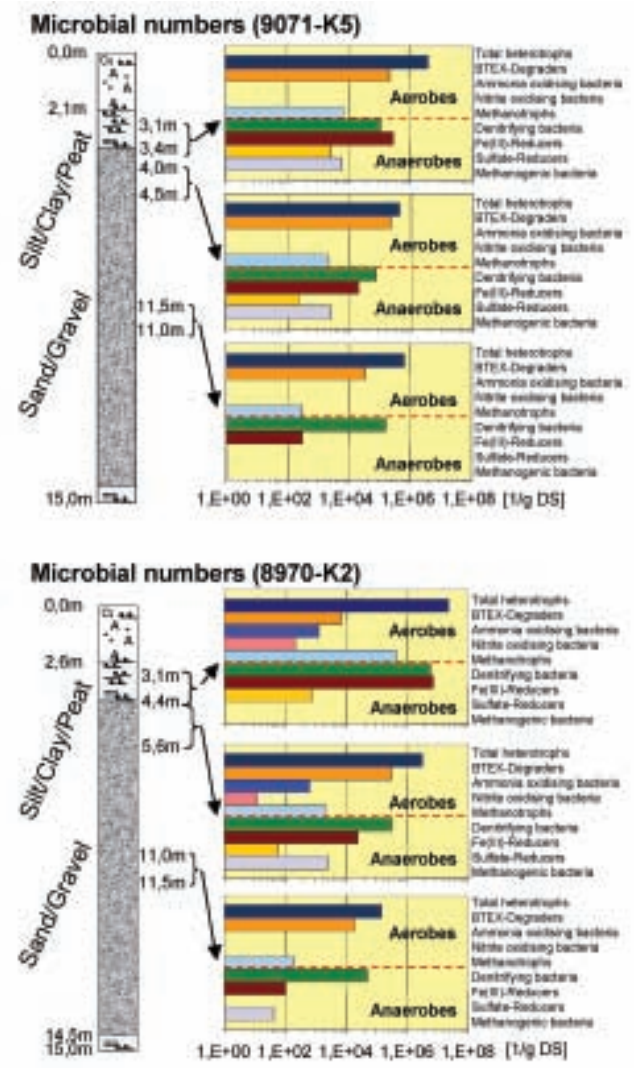

Fig. 7 - Killisfeld site: aerobic and anaerobic microbial numbers of two wells in three different depths (n.d. = not detected, DS = dry substance)

- 8971-13: further downgradient; reducing conditions; high concentrations of VC.

PCE (9 µM) was completely dechlorinated during anaerobic incubation with acetate and hydrogen as auxiliary substrates (Figure 8). The intermediary metabolites TCE and cDCE were detected, but not VC and ethene. It is assumed that VC and ethene were formed in concentrations below the detection limit (306 µg/L / 4.9 µM for VC and 133 µg/L / 4.7 µM for ethene) due to the relatively low initial concentration of PCE. In other degradation studies with repeated additions of PCE (cumulative addition of approx. 40 µM), the formation of VC and ethene was demonstrated (Figure 9).

Groundwater from well 9071-36 directly downgradient a disposal site in a strongly reducing zone of the plume showed faster dechlorination than groundwater from well 8971-13 further downgradient.

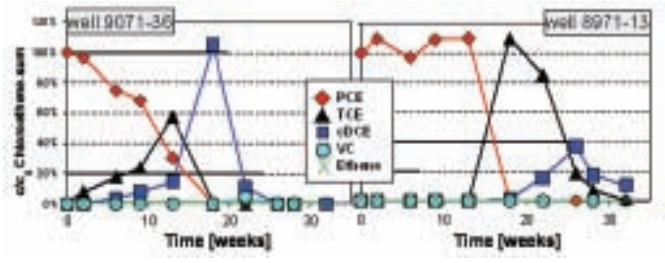

Fig. 8 - Killisfeld site: reductive dechlorination in groundwater from different zones of the plume

A lag-phase of about 12 weeks occurred with groundwater from well 8971-13 before the onset of dechlorination (Figure 8). Addition of yeast extract, trace elements and vitamins did not result in an increase of dechlorination rate (data not shown). Hence, groundwater from the investigated wells contained all nutrients required by dechlorinating microorganisms.

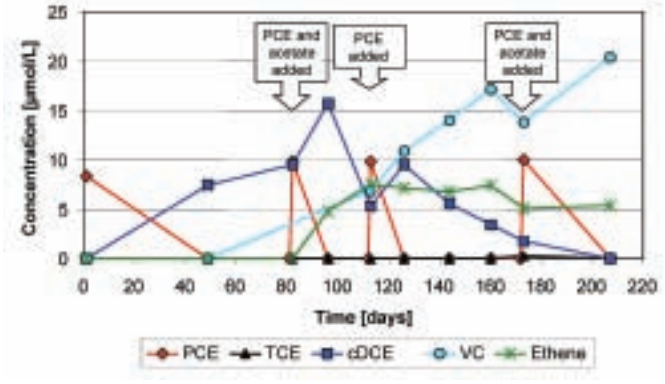

Fig. 9 - Killisfeld site: formation of VC and ethene during reductive dechlorination of PCE

The lower chlorinated metabolites from reductive dechlorination were also degradable under aerobic conditions (Figure 10). Again, degradation of cDCE (4.1 µM) and VC (19.2 µM) was faster with groundwater from well 9071-36 close to the source zones. The increasing degradation rate of VC after repeated spiking with VC indicates metabolic degradation of this compound. cDCE was degraded as long as VC was available in the microcosms, indicating cometabolic degradation of cDCE with VC as auxiliary substrate.

The obtained results are consistent with site data. The groundwater downgradient the disposal sites contains VC, ethene and ethane - the products of microbial reductive dechlorination. Obviously, the emission of organic substances from the disposal sites creates strongly reducing conditions, and delivers auxiliary substrates required for reductive dehalogenation. Aerobic degradation is possible at the plume fringes, i.e. further downgradient and in the groundwater fluctuation zones, where oxygen is available.

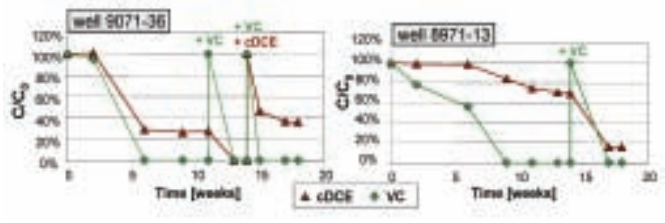

Fig. 10 - Killisfeld site: aerobic dechlorination of cDCE and VC in groundwater from different zones of the plume

Detection of specific degraders with 16S-PCR

Reductively dechlorinating groundwater microcosms from the two sites Killisfeld (6 different microcosms) and Frankenthal (9 different microcosms) were investigated for rDNA-sequences of Dehalococcoides with nested 16S-PCR. All microcosms originating from the Killisfeld site showed complete anaerobic degradation of PCE, TCE and cDCE (see exemplarily Figure 8 and Figure 11, left side). In contrast, the Frankenthal microcosms only transformed PCE via TCE to cDCE, which accumulated (see exemplarily Figure 11, right side). The obtained 16S-PCR results are corresponding to the observed dechlorinating capabilities of the microcosms. Organisms of the Dehalococcoides-cluster were detected only in completely dechlorinating microcosms (Figure 11, bottom). These results confirm previous reports that only Dehalococcoides are capable to degrade PCE completely down to ethene (Maymó-Gatell *et al.*, 1997; Hendrickson *et al.*, 2002).

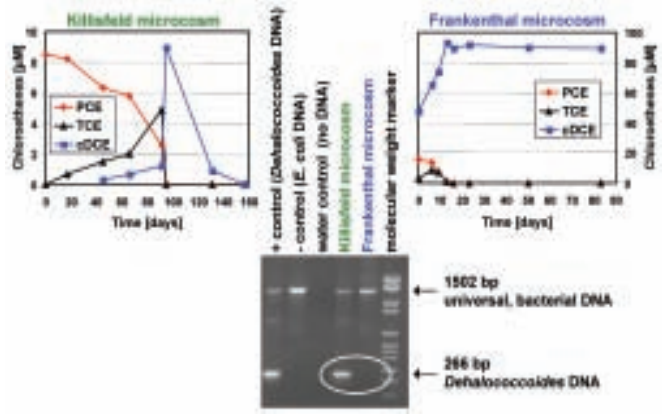

Fig. 11 - Dechlorination activity and 16S-PCR results of groundwater microcosms

In addition, groundwater samples of the two contaminated sites were assayed for Dehalococcoides with nested 16S-PCR. All sampling wells (7 at the Killisfeld site and 11 at the Frankenthal site) are located in the plume downgradient the source areas. Bacterial rDNA could successfully be extracted from all groundwater samples. But only groundwater samples from the Killisfeld site contained Dehalococcoides-rDNA, whereas in none of the Frankenthal groundwater samples such sequences were detected.

Field pollutant profiles, hydrochemical site characteristics (refer to Table 1), dechlorination activity in microcosms and presence of

	Killisfeld	Frankenthal
Primary contaminants on site	PCE and TCE	PCE and TCE
Degradation products detected on site	cDCE, VC, ethene	mainly cDCE
Dechlorination in groundwater microcosms	PCE to ethene	PCE to cDCE
16S-PCR detection of Dehalococcoides in groundwater microcosms	positive	negative
16S-PCR detection of Dehalococcoides in groundwater samples	positive	negative

Tab. 2 - Site characteristics and PCR-results

Dehalococcoides were well correlated at the investigated sites (Table 2). Bacteria belonging to the Dehalococcoides-cluster were only detected (i) in microcosms, where complete reductive dechlorination was observed, and (ii) in groundwater samples from the Killisfeld site favourable for reductive dechlorination. Thus, 16S-PCR detection of Dehalococcoides proved to be a quick and easy means to assess the potential for complete reductive dechlorination of chloroethenes at a given site. Other dechlorinating microorganisms can also be detected via 16S-PCR (Schmidt *et al.*, 2006b). 16S-PCR detection of specific pollutant degrading bacteria may complement analyses of pollutant profiles at contaminated sites and may be used instead of rather long-lasting degradation studies in the laboratory.

Carbon isotope fractionation

Selected microcosms from the Killisfeld and Frankenthal sites were assayed for isotope fractionation during anaerobic dechlorination of PCE and TCE and aerobic degradation of cDCE and VC. Results are shown exemplarily for reductive dechlorination of PCE (Figure 12). Significant fractionation effects could also be observed for anaerobic degradation of TCE and aerobic dechlorination of cDCE and VC (Tiehm *et al.*, 2006). The determined enrichment factors differ for the two sites. Thus, the site-specific enrichment factors have to be determined in order to quantify NA processes in the field.

CONCLUSION

A multiple lines of evidence approach was applied for the assessment of NA processes at two chloroethene contaminated sites in Germany. Hydrochemical site conditions and microbial degradation activities were studied. The following analytical methods proved to be suitable for the assessment of microbiological NA processes:

- analysis of pollutant profiles and hydrochemical site data (availability of auxiliary substrates and electron acceptors);
- microbial survey with MPN techniques;
- detection of specific degraders with 16S-PCR;
- analysis of isotopic signatures in the field and determination of isotope fractionation factors in the laboratory;
- identification of microbial degradation processes and evaluation of specific redox processes in microcosms studies.

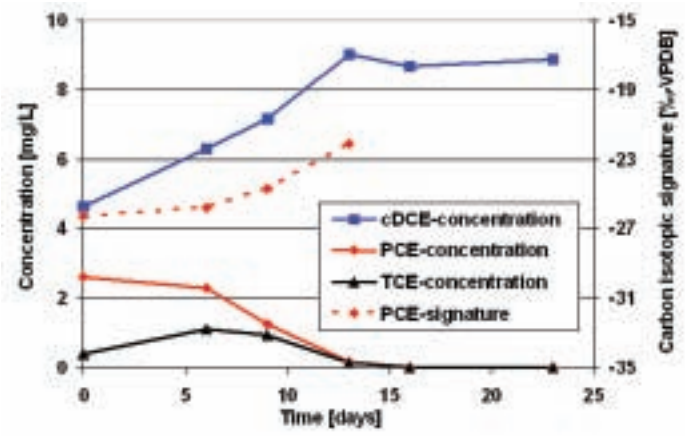

Fig. 12 - Frankenthal site: isotope fractionation during reductive dechlorination of PCE

The results obtained at the Killisfeld and Frankenthal sites demonstrate that the different assessment methods complement each other and allow a profound evaluation of the degradation processes occurring in the field. The different techniques can be combined depending on the specific site conditions. Similar strategies are recommended for other classes of contaminants such as tar oil pollutants (BTEX, PAH, heterocycles) or other halogenated compounds.

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