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Complementing approaches to demonstrate chlorinated solvent biodegradation in a complex pollution plume: Mass balance, PCR and compound-specific stable isotope analysis

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ABSTRACT

This work describes the use of different complementing methods (mass balance, polymerase chain reaction assays and compound-specific stable isotope analysis) to demonstrate the existence and effectiveness of biodegradation of chlorinated solvents in an alluvial aquifer. The solvent-contaminated site is an old chemical factory located in an alluvial plain in France. As most of the chlorinated contaminants currently found in the groundwater at this site were produced by local industries at various times in the past, it is not enough to analyze chlorinated solvent concentrations along a flow path to convincingly demonstrate biodegradation. Moreover, only a few data were initially available to characterize the geochemical conditions at this site, which were apparently complex at the source zone due to (i) the presence of a steady oxygen supply to the groundwater by irrigation canal losses and river infiltration and (ii) an alkaline pH higher than 10 due to former underground lime disposal. A demonstration of the existence of biodegradation processes was however required by the regulatory authority within a timeframe that did not allow a full geochemical characterization of such a complex site. Thus a combination of different fast methods was used to obtain a proof of the biodegradation occurrence. First, a mass balance analysis was performed which revealed the existence of a strong natural attenuation process (biodegradation, volatilization or dilution), despite the huge uncertainty on these calculations. Second, a good agreement was found between carbon isotopic measurements and PCR assays (based on 16S RNA gene sequences and functional genes), which clearly indicated reductive dechlorination of different hydrocarbons (Tetrachloroethene—PCE-, Trichloroethene—TCE-, 1,2-*cis*Dichloroethene—*cis*-1,2-DCE-, 1,2-*trans*Dichloroethene—*trans*-1,2-DCE-, 1,1-Dichloroethene—1,1-DCE-, and Vinyl Chloride—VC) to ethene. According to these carbon isotope measurements, although TCE biodegradation seems to occur only in the upgradient part of the studied zone, DCE and VC dechlorination (originating from the initial TCE dechlorination) occurs along the entire flowpath. TCE reductase was not detected among the *Dehalococcoides* bacteria identified by quantitative PCR (qPCR), while DCE and VC reductases were present in the majority of the population. Reverse transcriptase PCR assays (rt-PCR) also indicated that bacteria and their DCE and VC reductases were active. Mass balance calculations showed moreover that 1,1-DCE was the predominant DCE isomer produced by TCE dechlorination in the upgradient part of the site. Consequently, coupling rt-PCR assays with isotope measurements removes the uncertainties inherent in a simple mass balance approach, so that when the three methods are used jointly, they allow the identification and quantification of natural biodegradation, even under apparently complex geochemical and hydraulic conditions.

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1. Introduction

Monitored Natural Attenuation (MNA) is currently one of the most cost-effective techniques available to decontaminate groundwater impacted by chlorinated solvents (Ellis, 1997; US EPA, 1998). In Europe, MNA has become of major interest because the European water framework directive (WFD) of 2000 fixes stringent environmental quality standards for surface and ground waters, which must be reached by 2015. Temporary environmental quality standards for several chlorinated compounds, which are currently effective in France in accordance with WFD recommendations, are presented in Table 1. At the same time in France, MNA can be considered an acceptable remedial action by regulatory authorities, only if natural attenuation includes an efficient destructive process of contaminants, such as biodegradation, and not just dilution (ADEME, 2006). In this context, being able to prove in situ biodegradation by a convincing and cost-effective approach is crucial.

Recent research has produced new tools to demonstrate the occurrence of in situ chlorinated hydrocarbon biotransformation. These tools are based on (i) isotope fractionation that occurs during this biotransformation (Compound Specific Isotope Analysis) and (ii) biomolecular characteristics of microbial communities, which catalyze this reaction (polymerase chain reaction assays). These innovative tools have already proved useful in different field studies. Compound Specific Isotope Analysis (CSIA) can differentiate between volatilization and bacteria-mediated attenuation processes (Meckenstock et al., 2004; Slater et al., 2001), as well as characterization of the extent of biodegradation (Sherwood-Lollar et al., 2001; Vieth et al., 2003) and identification of biodegradation pathways (Hunkeler et al., 2005). Quantitative Polymerase Chain Reaction (qPCR) assays based on 16S-RNA genes can inform on the amount of bacteria potentially able to catalyze biotransformation (Fennell et al., 2001; Hendrickson et al., 2002; Loffler et al., 2000) while PCR based on functional genes determines whether bacteria have the specific enzyme to dechlorinate a specific compound (Muller et al., 2004; Rahm et al., 2006; Ritalahti et al., 2006).

Moreover Reverse Transcriptase PCR (rt-PCR) clearly determines if these bacteria, or their specific enzymes, are active in the medium when samples are collected. All these tools have already been used simultaneously by e.g. Carreon-Diazconti et al. (2009) on a polluted site where, reportedly, Tetrachloroethene (PCE) was the only solvent employed. Imfeld et al. (2008) showed the relevance of isotopic and biomolecular tools to study the spatial and temporal evolution of chlorinated solvent biotransformation. More recently, Abe et al. (2009) demonstrated the relationship between geochemical conditions, groundwater discharge rates and the degree of biotransformation in a streambed by means of isotopic tools and PCR assays. But, so far, these techniques have never been used, to our knowledge, in a complex field situation where more than 17 chlorinated solvents had been released into the aquifer, and with apparently dubious geochemical conditions.

This paper describes how the different methods available today were combined to demonstrate the occurrence of biodegradation on a complex alluvial site where the existence of biodegradation cannot be easily proved. We report on a chemical industrial site which has been producing chlorinated solvents and PVC since around 1915. All of the chlorinated contaminants found in the groundwater at this site, notably Tetrachloroethene (PCE), Trichloroethene (TCE), 1,2-*cis*Dichloroethene (*cis*-1,2-DCE) and Vinyl Chloride (VC), may have been directly released by the plant. In this context, using concentration data and parent–daughter relationships to evaluate MNA can lead to erroneous results (Hunkeler et al., 2005) and cannot be considered a biodegradation evidence. This site also features a complex series of river and groundwater exchanges which require proper tools to discriminate between contaminant dilution and biodegradation. The concentrations measured in both ground water and the river did not meet all the temporary environmental quality standards effective in France at the time of this study as a result of the application of the WFD

Table 1
Chlorinated compounds detected in the groundwater (wells P2, P4, P14, P15 in mg/l) and in the river downstream from the site (μg/l) in September 2007 and corresponding Environmental Quality Standard (EQS in μg/l) temporarily defined in France for groundwater bodies (French Ministerial Circular of December 21, 2006) and inland surface water bodies (French Ministerial Circular of May 7, 2007).

Chlorinated compound	P2 (mg/l)	P4 (mg/l)	P14 (mg/l)	P15 (mg/l)	French EQS for ground waters (μg/l)	River (μg/l)	French EQS for inland surface waters (μg/l)
TCE	55.78	32.87	11.43	0.01	10	3	10
PCE	10.98	4.36	1.90	0.04	10	1	10
1,1-DCE	6.81	3.50	6.91	0.35	No EQS	<0.5	11.6
1,1,1,2-Tetrachloroethane	3.95	0.90	0.39	0.15	No EQS	<0.5	No EQS
1,1,2,2-Tetrachloroethane	1.29	<0.01	0.11	0.04	No EQS	<0.5	140
cis-1,2-DCE	1.19	13.98	10.45	3.11	No EQS	5	1100
trans-1,2-DCE	0.51	0.30	0.72	0.22	No EQS	<0.5	1100
VC	0.17	1.90	3.29	2.66	No EQS	7	0.5
Carbon tetrachloride	0.15	<0.01	0.12	0.04	No EQS	<0.5	12
Trichloromethane	0.13	<0.01	0.07	<0.01	No EQS	<0.5	12
1,2-dichloroethane	0.09	0.46	0.29	0.09	No EQS	10	10
1,1,1-trichloroethane	0.09	0.97	0.09	0.02	No EQS	4	26
chloroethane	<0.01	<0.01	<0.01	<0.01	No EQS	<0.5	No EQS
1,1-dichloroethane	<0.01	<0.01	<0.01	<0.01	No EQS	<0.5	92
1,2-dichloropropane	<0.01	<0.01	<0.01	<0.01	No EQS	<0.5	No EQS
1,1,2-trichloroethane	<0.01	<0.01	<0.01	<0.01	No EQS	6	300
pentachloroethane	<0.01	<0.01	<0.01	<0.01	No EQS	<0.5	No EQS

(Table 1). These complex conditions had led the regulatory authority to initially prescribe the deployment of a pump and treat system around the whole site that would have required the treatment of a huge amount of water. A time to think was however granted and this study was performed to explore within a short timeframe if natural attenuation could be a potential alternative solution.

First, a preliminary evaluation of the contaminant mass flux transported in the adjacent river and in the aquifer is made and discussed, considering (i) the uncertainties which weigh heavily on the flux estimates and (ii) the possible role of volatilization from groundwater to the vadose zone (which could be responsible for a large part of contaminant losses). This mass balance is then compared with a few geochemical data to assess whether geochemical conditions are adequate for reductive dechlorination of pollutants.

Second, molecular biology tools (qPCR and rt-qPCR) and a compound-specific stable isotope analysis (CSIA) are used simultaneously to explore the biodegradation ability and occurrence of the solvents in groundwater.

Third, we discuss the advantages of each approach and suggest a methodology of joining together isotope analyses, rt-PCR assays and mass balance to demonstrate the existence of biodegradation and to provide an estimate of its efficiency.

2. Material and method

2.1. Site description

We focus on an industrial site where chlorinated solvents have been produced sporadically since the 1910s, and continuously since the 1960s. The plant is located on alluvial deposits, composed of calcareous pebble, gravel and sand, close to a river (Fig. 1). Below the chemical plant, the alluvial deposits are organized in embedded terraces. However, downstream from the plant, in the studied site, only recent river deposits

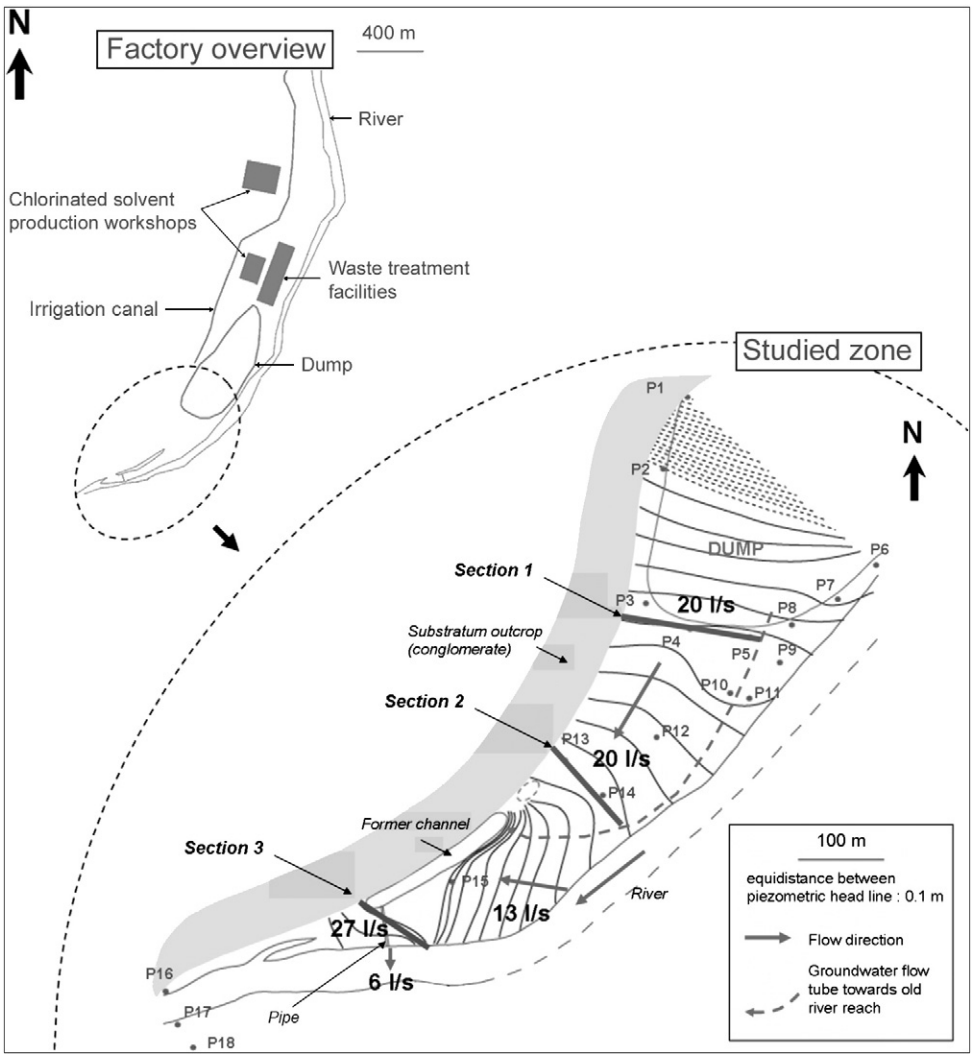

Fig. 1. Overview of the plant site and of the studied zone. On the water-table map, estimates of water fluxes in liters per second through different groundwater sections are reported.

are present and were found to be quite homogenous during the drilling of piezometers. No clay deposits were identified in this part of the site which is dominated by gravel.

The alluvial aquifer lies on an impervious bedrock made of a conglomerate with calcareous cement. The water-table is located 1–2 m below ground surface. In the studied part of the site, downstream from the plant, the aquifer has a hydraulic conductivity ranging from $6.0 \cdot 10^{-3}$ to $1.5 \cdot 10^{-2}$ m/s and a thickness of 5 to 20 m. The lateral extent of the aquifer is limited in this zone by a bedrock outcrop on the western border and by the river on the eastern one.

17 chlorinated compounds produced by the plant contaminate the alluvial aquifer (Table 1). These compounds are mostly chlorinated solvents and this paper will mainly focus on Tetrachloroethene (PCE), Trichloroethene (TCE), 1,2-*cis*Dichloroethene (*cis*-1,2-DCE), 1,2-*trans*Dichloroethene (*trans*-1,2-DCE), 1,1-Dichloroethene (1,1-DCE) and Vinyl Chloride (VC). The possible secondary role of other chlorinated compounds in the biodegradation processes will however be discussed in the mass balance approach. The chlorine necessary to produce these solvents was made by electrolysis, which led to the release in the soil and groundwater of mercury and sodium chloride. A waste dump, which contains unknown by-products of different industrial activities, is also located downstream from the plant. The work concentrates on the last down-gradient portion of the alluvial deposit located downstream from the waste dump (Fig. 1) and downstream from any potential point sources of chlorinated compounds (solvent production units or leaking pipes or ditches).

As the alluvial formation narrows along the studied flow-path, this bottleneck forces all the polluted groundwater to discharge into the river or into a former channel (which also discharges into the river through a pipe, cf. Fig. 1). The maximum measured chlorinated hydrocarbon concentration in groundwater in this zone reached 80 mg/l in September 2007 (Table 1) at the P2 well (location reported in Fig. 1). The chlorinated ethenes TCE, PCE, 1,1-DCE, *cis*-1,2-DCE and *trans*-1,2-DCE represent in weight 94% of the chlorinated compounds detected in this part of the site. Dense non aqueous-phase liquids (DNAPL) were not detected with an interface probe on this part of the site. However, DNAPLs were suspected to be present near the P2 well, since droplets of a free oil phase were visually detected during a sampling campaign in September 2007. The very small amount of DNAPL droplets did not allow them to be analyzed however.

2.2. Sampling

Groundwater was collected for chemical analyses in September, October 2007 and March 2008 and for isotope analysis in March 2008. By pumping for 15 min with a submerged pump at the bottom of each well, 3–4 well-casing volumes were evacuated before sampling. Wells are slotted along the whole alluvial formation thickness and reach the bedrock. In the river and in the former channel, water was sampled by collecting it along different vertical lines across a chosen section. Surface water velocity was also measured at each sampling point. The flux of pollutants transported across the river section was then calculated from (i) the pollutant concentrations measured on each sample and (ii) the water velocity measured with a flow meter at each elementary sampled location. Water samples for chemical analyses were collected in 250 ml glass bottles (or 60 ml for isotope analysis) closed by a Teflon cap without any headspace. For cation and isotope analyses, water samples were stabilized by adding a few drops of concentrated nitric acid and filtrated on a 0.2 μm acetate filter for dissolved ion analysis. This filtration was also performed for Total Organic Carbon (TOC) analysis in order to quantify Dissolved Organic Carbon (DOC) only. Samples for Chemical Oxygen Demand (COD) and 5-day Biological Oxygen Demand (BOD5) were collected in 100 ml brown glass-stoppered bottles without headspace.

2.3. Chemical analyses

Concentration of chlorinated hydrocarbons was measured by gas-chromatography (HS/GC/MS) using the NF EN ISO 10301 method. Dissolved ethylene could however not be analyzed. The reason is that the main gas chromatography analyses were done at the industrial plant laboratory, samples were not allowed to be distributed to other labs, and ethylene was not among the statutory analyses made at that lab. Major ions were analyzed by ionic chromatography, alkalinity by titration with sulfuric acid, Total Organic Carbon (TOC) with the EN 1484 (H3) method, dissolved iron by the ISO 11885 method, ferrous iron by the DIN 38406 E1 method, dissolved sulfides by the DIN 38405 D26 method, and BTEX (Benzene, Toluene, Ethyl-benzene, Xylene) by the EN ISO 11423-1 method. Methane was analyzed using an in-house method developed by Wessling laboratory. Chemical Oxygen Demand (COD) analyses were performed by the CRECEP laboratory of Paris (accredited by the French health authorities for water analysis) using the NF T 90-101 method and also by Wessling laboratory using the DIN 38409 H41 method. 5-Day Biological Demand (DBO5) was analyzed by CRECEP (NF EN 1899-2) and by Wessling using the standard EN 1899-1 H51 protocol. Vertical logging was done in each well for O_2 concentration measurements with an oxymeter probe, and for Redox potential (Eh) with a redox probe using an Ag/AgCl reference. Corrections (given by the manufacturer and depending on the water temperature) were then made to determine the Redox with the standard hydrogen reference.

2.4. PCR assays

PCR assays were made on groundwater samples, collected in March 2008 with a surface pump after evacuating 10–30 well-casing volumes. 40 ml of water were collected and mixed with 60 ml of a fixative fluid provided by Bioclear B.V, a commercial laboratory in the Netherlands, where the fixed samples were directly sent by regular post without cooling. DNA and RNA extraction was performed by Bioclear B.V. within 48 h after filtration over 0.2 μm polycarbonate membranes to concentrate the cells prior to DNA extraction. DNA was extracted from the total volume of 50 ml per groundwater sample. The samples were subjected to DNA extraction by bead beating. The DNA was purified using a modified protocol with a silica-based column and eluted in 100 μl TE. RNA was extracted using a similar protocol adapted specifically for RNA isolation. Detailed protocols of DNA and RNA extractions are available, on request, at Bioclear B.V.

First, Quantitative Polymerase Chain Reaction (qPCR) was used to amplify 16S rRNA gene sequences of *Dehalococcoides* spp., genus known to carry out organohalide respiration, with modified primers (M0066P0472). DNA amplification was performed by initial denaturation for 3 min at 94 °C, followed by 35 cycles of amplification (30 s denaturation at 94 °C; 30 s annealing at 58 °C; 30 s elongation at 72 °C), and 5 min at 72 °C to complete elongation. Detailed protocols and primer sequences for q-PCR analysis are available, on request, at Bioclear B.V.

In a second step, new PCR assays were made on the same samples targeting two vinyl chloride reductase (*vcrA* and *bvcA* genes) and one TCE reductase (*tceA* gene). PCR-amplification of vinyl chloride reductase and trichloroethene reductase was performed using modified primers (*vcrA* primers: P0428P0429, *bvcA* primers: P0430P0431 and *tceA* primers: P0590P0591). TCE reductase, encoded by the *tceA* gene, can be synthesized by the *Dehalococcoides ethenogens* strain 195 (Magnuson et al., 2000) and the FL2 strain (He et al., 2005) and converts TCE to DCE and VC. Vinyl chloride reductase encoded by the *bvcA* gene can be synthesized by *Dehalococcoides* BAV1 (Krajmalnik-Brown et al., 2004) and converts DCE isomers and VC to ethene. Vinyl chloride reductase, encoded by the *vcrA* gene, can be synthesized by (i) *Dehalococcoides* VS (Muller et al., 2004) which converts DCE isomers and VC to ethene, and by (ii) the GT strain (Sung et al., 2006) which converts TCE, DCE isomers and VC to ethene.

In a third step, Reverse Transcriptase PCR (rt-qPCR) was performed targeting messenger RNA (mRNA) which transcripts TCE and VC reductases. As rRNA is generally abundant and more stable than mRNA, rt-qPCR was also done targeting 16S rRNA transcripts. Prior to the analysis, RNA samples were converted to cDNA using specific primers and according to the protocol provided by the supplier (Bio-Rad, iScript advanced cDNA synthesis kit for rt-qPCR). Details about rt-qPCR protocols are also available on request at Bioclear B.V.

The detection limit of DNA was $1.3 \cdot 10^2$ copies per ml. The detection limit of RNA was different for each sample and is given in Table 3 when it was not reached.

2.5. Isotope analysis

Stable carbon isotope ratios were measured using a gas chromatograph connected to a mass spectrometer via a combustion interface as described in Hunkeler and Aravena (2000). Different dilutions were used to insure that each chlorinated hydrocarbon concentration lies in the spectrometer range. Carbon isotope ratios ($^{13}C/^{12}C$) are given with the usual delta notation ($\delta^{13}C$) relative to the Vienna Peedee Belemnite Standard. To ascertain that each measurement was steady and valid, the carbon isotope ratio was measured on four different samples for each well. Considering the results obtained on each analyzed sample, the precision and accuracy of these $\delta^{13}C$ values are estimated at 0.3‰.

3. Results and discussion

3.1. Mass balance estimate of contaminant fluxes along the flowpath

In the studied zone, we conducted a mass balance estimate of pollutant fluxes along the flowpath. To this end, we first measured in April 2007 the total flux of chlorine contained in

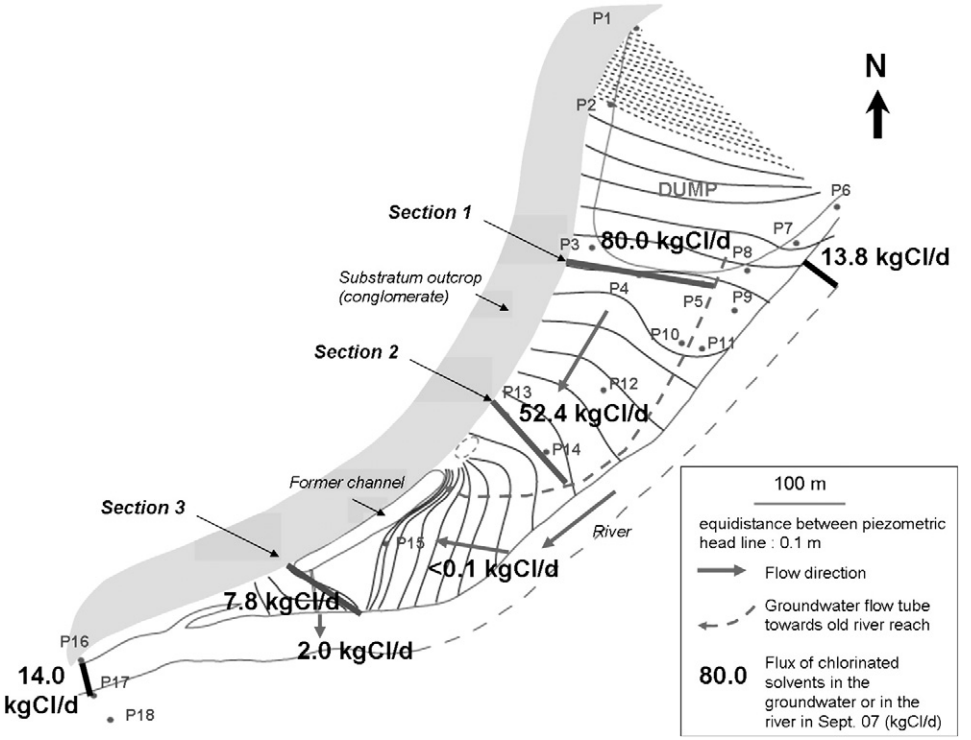

Fig. 2. Assessments of chlorinated solvent fluxes (in kg Chlorine per day) in different groundwater and river sections in the studied zone in September 2007.

the chlorinated solvent transported by the river between two river sections, 900 m apart, bordering the studied site (see Fig. 2). These results showed a very low flux of pollutants discharged into the river in this zone, as the measured chlorine flux transported by the river remained constant (around 14 kg of chlorine per day) between the two river sections. This means that the amount of pollutants discharged by groundwater flow to the river, between the two chosen sections, made up for the losses of dissolved solvents by volatilization from surface waters and was negligible (on the order of a few kg/day).

Moreover river concentration analysis revealed that the only compounds whose concentration increased between the two river sections were VC and *cis*-1,2-DCE while the major component in the groundwater at the highest concentration (directly downstream from the dump at the P2 well) was TCE (Table 1). To try to explain these observations, we computed the mass balance of chlorinated solvents transported by groundwater in September 2007 in this zone, on different aquifer sections.

The groundwater flow (in l/s) through three different sections (see Fig. 1 for their location) was first evaluated by Darcy's law. The flow tube for these calculations was chosen so that all flow lines in the tube converged at the former river channel. The results of our calculations are reported in Fig. 1. All the hydraulic parameters needed to compute these fluxes were measured beforehand: hydraulic gradient (i) by leveling, groundwater permeability (K) on each section with pumping tests, thickness (L) of the aquifer from piezometer drilling and width (D) of groundwater sections considering the groundwater flow tube hand-drawn on the piezometric map.

Chlorinated solvent concentrations were measured on different wells in September and October 2007 (using the sampling method previously described). Considering that the effective porosity of the alluvial medium probably ranges from 10 to 20% and assuming moreover that the dissolved chlorinated solvents are not retarded along the flowpath, Darcy's law indicates that these compounds are transported by groundwater within a range between only 30 and 60 days from the P4 piezometer to the former channel, 300 m downstream. Since the concentrations were steady between September and October 2007 on the piezometers for which data were available, we used the concentrations (C) measured at the same time on each piezometer in September 2007 (the most complete available data set) to assess the chlorinated solvent fluxes (F) across each groundwater section (Fig. 2 and Eq. (1)). We assumed that these fluxes represented steady-state conditions. This hypothesis is supported by the spatial evolution of the total carbon concentration contained in the sum of chlorinated ethenes in September 2007 (Fig. 3). If dispersion and volatilization to the vadose zone are low and VC biodegradation is limited in the upgradient part of the site, as shown below, the mass of carbon contained in the sum from PCE to VC should be constant in the groundwater flow tube, whether there is biodegradation or not. This was indeed observed in September 2007 (Fig. 3). The carbon concentration of the sum from PCE to VC was 11.1 mg C/l at the P4 piezometer

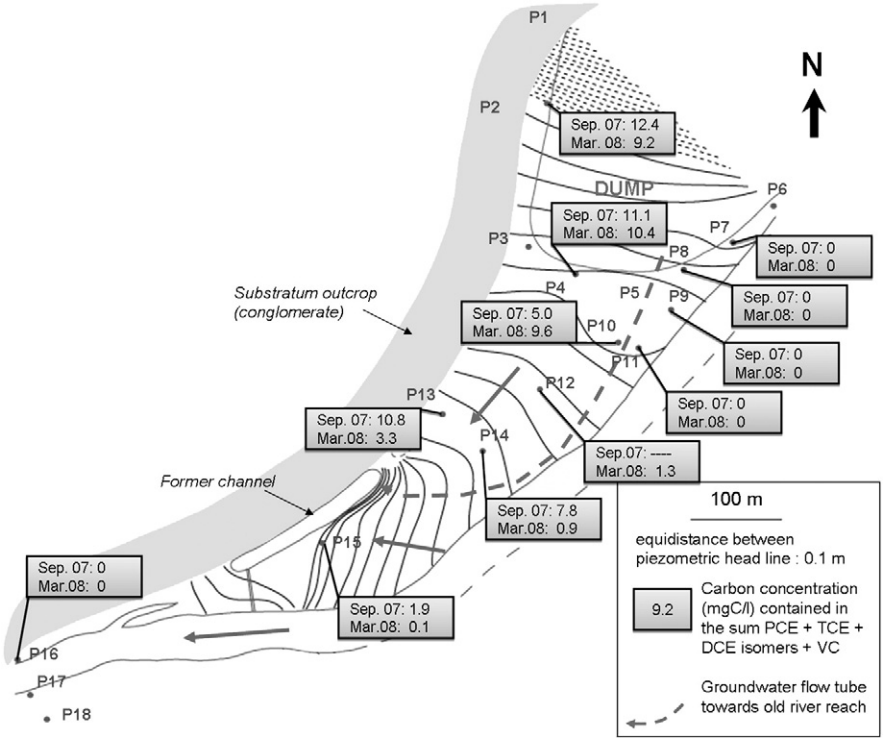

Fig. 3. Carbon concentration (mgC/l) contained in the sum PCE + TCE + DCE isomers + VC measured in September 2007 and March 2008 in the studied zone.

and 10.8 mg C/l at the P13 piezometer, leading to a carbon flux of 222 and 216 mg C/s, respectively, in the flow tube. The concentration decreased sideways toward the eastern limit of the selected flow tube at the P14 piezometer (7.8 mg C/l) most likely because of lateral dispersion.

$$F = KiDLC \tag{1}$$

$$\frac{dF}{F} = \frac{dK}{K} + \frac{di}{i} + \frac{dD}{D} + \frac{dL}{L} + \frac{dC}{C} \tag{2}$$

The uncertainty on chlorinated solvent flux (dF/F) is given by Eq. (2). Considering a 4% uncertainty on the hydraulic gradient (due to the leveling method) and distances (section width and aquifer thickness) and considering that concentrations are given at ± 1 mg/l (2% on the first section) and permeability at $\pm 1.10^{-3}$ m/s (10%), uncertainty on the flux (dF/F) did not exceed 25% on each groundwater section.

Contrary to the mass flux of carbon contained in the sum of solvent, the mass flux of chlorine contained in the sum chlorinated solvents, decreases considerably along the groundwater flowpath. In fact, it decreases by 34% between sections 1 and 2 of the flow tube (results reported in Fig. 2), and by more than 80% between sections 2 and 3. This decrease seems significant in comparison with the 25% estimate of accuracy of these fluxes.

As the only known outlet of the groundwater in the studied zone is the river in the downgradient part, a decreasing flux of chlorine in the groundwater along the flow tube cannot be explained by groundwater discharge outside the flow tube (e.g. by dispersion), as the measurements of the chlorine flux transported by the river showed that groundwater did not contribute significantly to the river load in this zone.

To explain the decrease in the flux of chlorine transported by groundwater, different natural attenuation processes can be considered. First, the decrease could result from pollutant dispersion outside the groundwater tube chosen to compute these fluxes. However, outside this tube, chlorinated solvent concentrations were below the detection threshold of a few µg/l in September 2007. So, this process could not explain such a huge decrease of the chlorine flux along the groundwater flowpath (Fig. 3). Second, dilution by river water infiltration in the groundwater near the former channel could lead to reduced groundwater concentrations. However, in our mass balance, we took into account this increase in groundwater flow and even if concentrations were diminishing because of this process, solvent fluxes were also diminishing. This reasoning leads to the conclusion that natural destructive processes of contaminants such as volatilization or/and biodegradation are needed to explain the observations.

To estimate the contribution of volatilization, a preliminary rough estimate of the TCE volatilization flux between sections 1 and 2 can be conducted, assuming that the main chlorine loss by volatilization between sections 1 and 2 is due to TCE volatilization as this compound represents 58% of the total chlorine flux transported by the groundwater across the first section.

We base our TCE volatilization calculation on Bohy et al. (2006) who compared measurements of TCE and PCE volatilization rates from the soil surface with predictions obtained with a quasi-analytical approach based on Fick's first law. They found, in an experiment with a sand-filled tank (0.4 mm uniform grain diameter, 40% porosity, 8.10^{-4} m/s saturated permeability, 2 m-depth to groundwater table), a TCE volatilization flux varying between 3 and 0.1 $g.m^{-2}.day^{-1}$, as a function of time and distance from the TCE injection point. Assuming that volatilization was governed by diffusion in the unsaturated zone, Bohy et al. (2006) also found that their flux measurements at the ground surface verified a simple 1-D expression of Fick's law, given by Eq. (3), where φ is the diffusive flux ($g.m^{-2}.s^{-1}$), D_a is the molecular diffusion coefficient of TCE in the air ($m^2.s^{-1}$), τ_a is the tortuosity of the porous medium toward air (−), ω is the total porosity (−) and dC/dz the concentration gradient in the gaseous phase ($g.m^{-4}$). The tortuosity of the porous medium toward air (τ_a) can be assessed using the empirical expression of Millington and Quirk (1961), given by Eq. (4) where S_a is the air saturation (−). To assess the concentration gradient in the gaseous phase (dC/dz) at the first groundwater section, we assume that TCE gas concentration linearly increases with depth from zero at the ground surface to the bottom of the unsaturated zone where the gas concentration (C_a, $g.m^{-3}$) is supposed to be at equilibrium with the groundwater and then given by Eq. (5), where C_w is the aqueous TCE concentration in the aquifer ($g.m^{-3}$) at the first section and H is the dimensionless Henry's law constant for TCE (−).

$$\varphi = -D_a \tau_a \varpi \frac{dC}{dz} \tag{3}$$

$$\tau_a = S_a^{7/3} \varpi^{1/3} \tag{4}$$

$$C_a = HC_w \tag{5}$$

Considering the molecular diffusion coefficient of TCE in the air given by Bohy et al. (2006) ($7.6 \cdot 10^{-6}$ $m^2.s^{-1}$), a total porosity of 40%, an air saturation of 80%, the TCE dimensionless Henry's law constant at 17.5 °C given by Gossett (1987) (0.265), a 2 m-thick unsaturated zone and an aqueous TCE concentration of 33 $g.m^{-3}$ at the first section in September 2007, the TCE volatilization flux can be roughly estimated on the first section at $5.8 \cdot 10^{-6}$ $g.m^{-2}.s^{-1}$, i.e. 0.5 $g.m^{-2}.d^{-1}$. Considering that the area involved between sections 1 and 2 is 36,500 m², the flux of TCE lost by volatilization between these sections is on the order of 18 $kg.d^{-1}$, i.e. 15 kg of chlorine per day.

Note that the assumption of a linear gradient from water table to atmosphere leads to an estimate of the maximal TCE volatilization flux as it does not take into account the effect of a lower diffusion through the capillary fringe.

Even if the same calculation cannot be conducted between sections 2 and 3 as the presence of the former river channel probably increases volatilization effects (direct exchange from surface water to the atmosphere), it shows however that volatilization represents at most 50% of the lost flux of chlorine (27.6 kg Cl/d) between sections 1 and 2 (Fig. 2).

Consequently, volatilization alone does not explain the loss of chlorinated solvents along the flowpath.

The same mass balance (in the river and in the aquifer) was done for other time periods and, at no time, was any

significant increase of the chlorine flux observed between the two river sections. The contribution of groundwater discharge to contaminant river fluxes seemed to stay low at any time. However, the mass of carbon contained in the sum from PCE to VC in the groundwater (Fig. 3) did not seem to be a conservative parameter over time in the chosen flow tube. In March 2008, an increase of chlorinated solvent concentration was observed at the P10 piezometer without any impact downstream on P12 and P14 (where concentrations were lower than in September 2007). This could reflect unsteady transport conditions of the contaminants in the groundwater. The same mass balance in March 2008 showed a decrease of 87% of the total groundwater chlorine flux between sections 1 and 2, instead of 34%. This could cast doubts on the applicability of the mass balance method, and thus on its capacity to demonstrate the existence of strong biodegradation.

In this context, to investigate if the decrease of chlorine fluxes observed in a simple mass-balance approach could be due to biodegradation, to volatilization or be an artifact of unsteady transport conditions, tools capable of making this differentiation are required.

3.2. Overview of geochemical conditions

In order to explore if biodegradation is a relevant destructive process in this zone, we performed a basic geochemical characterization of the groundwater (Table 2) in March 2008.

Eh and O_2 measurements revealed that redox conditions are quite heterogeneous in this zone. Reducing conditions were revealed by a negative Eh at the P10 (−192 mV) and P12 wells (−61 mV) and confirmed by the absence of oxygen and nitrates (Table 2). These reducing conditions did not seem to reach sulfate reduction as (i) no significant decrease of sulfate concentration was observed in these zones and (ii) sulfides were not detectable. It has to be noted that the sulfate concentrations in this part of the site are quite high (>200 mg/l) but are not necessarily unfavorable to biodegradation even if they can raise competition for hydrogen in reducing zones (Heimann et al., 2005).

Oxygen was found in groundwater upstream from these reducing zones (at P2 and P3) and at shallow depths in most wells. Oxygen may come from (i) irrigation-canal losses near the zone where the canal flows and (ii) river infiltration toward the former channel. It has to be noted that the few chemical analyses performed in the river (Table 2) did not allow us to identify canal losses or river infiltration as the chemistry of these surface waters (at least for the major ion concentrations) was close to that of groundwater.

The existence of reducing conditions, directly downstream from the waste dump at the P4 well, could be explained by the presence of a carbon source revealed by Dissolved Organic Carbon (DOC) measurements. More than 30 mg C/l were indeed found below the dump at the P2 well, upstream from the reducing zone (P4). The dump was known to contain municipal wastes that could generate this DOC but no data about the organic content of the soils through which the groundwater was moving was available. Moreover no historical information was available as DOC measurements in groundwater were not part of the statutory analyses required by the regulatory authority. Consequently, the origin of this high DOC level below the dump is still currently unknown. In March 2008, the DOC concentration decreased along the flowpath, which might signal its consumption. In order to roughly evaluate if these high DOC levels below the dump correspond to organic compounds that can be easily oxidized, the Chemical Oxygen Demand (COD) was measured in March 2008 and compared to the 5-day Biological Oxygen Demand (BOD5). These measurements were complicated by the high chloride background in the ground waters which is well known to interfere with the determination of COD. Protocols which include the addition of mercuric sulfate to minimize chloride interference in COD determination were thus used (DIN 38409 H41, NF T 90-101). The points which showed the highest COD and BOD5, regardless of the considered protocol, were also the points which showed the highest DOC concentrations (P2 and P4). However a quite high COD/BOD5 ratio was found for these points (which ranges from 3.7 at P2 to 4.5 at P4), whereas dissolved organic wastes in waters usually show a COD/BOD5 ratio around 2 which indicates a good biodegradability (Wentzel et al., 2003). The BOD5 was always lower than the detection limit or quite low (<5 mg O_2/l), regardless of the protocol, for the rest of the wells. These complementary measurements show the need of further analyses to identify the origin of the high DOC level below the dump which may not correspond to an easily oxidized (or fermentable) source of non-chlorinated organic carbon whereas such a source is required for sustainable biodegradation to occur.

Additionally, BTEX were analyzed to identify if volatile organic compounds could represent a relevant source of non-chlorinated carbon. The main BTEX found in groundwater was however benzene (Table 2) which is known to be hardly biodegraded even under extreme anaerobic conditions (Grbic-Galic and Vogel, 1987; Lovley, 2000). The highest concentration was found at the P4 well (280 μg/l) where anoxic conditions were also identified. Further studies are required to evaluate the role of benzene in the geochemistry of this site.

Moreover, the pH was very high (alkaline) directly downstream from the waste dump (around 10 to 12) because of lime disposal in this dump. A pH greater than 9 is usually recognized as less favorable to biodegradation than a pH range of 6 to 8 (US EPA, 1998). However, a more favorable pH range of 7 to 7.5 was quickly restored downstream from the high alkaline zones (P10 and P11). Furthermore, in the presence of a calcareous medium, the high alkalinity measurements prevented us from identifying an alkalinity increase attributable to reductive dechlorination.

Consequently, a simple geochemical characterization tends to show that reducing conditions downstream from the dump could be suitable for reductive dechlorination even if at the source zone, the presence of an oxygen input into the groundwater due to losses from an irrigation canal leads to less favorable conditions. The consumption of oxygen may be favored by the oxidation of the high DOC level below the dump but our limited measurements did not provide a basis for identifying its source neither its availability over time.

We must emphasize that chloride ion concentrations could not be used to identify dechlorination, as the chloride concentration on this site is very high and heterogeneous because of the storage and release of sodium chloride used for electrolysis (concentrations up to 700 mg/l, cf. Table 2).

Table 2
Geochemical data acquired between December 2007 and March 2008 on different wells (oxygen measurements are given at the deepest point of each well; the other data were analyzed on an average groundwater sample), the irrigation canal near the dump, the river downstream from the site and the former channel.

Parameter	P1	P2	P3	P4	P10	P11	P12	P13	P14	P15	P17	Canal	Former Channel	River
pH	7.1	12.1	10.0	11.4	7.0	7.0	9.0	7.4	7.1	7.5		–	—	–
O_2	–	5.42	5.59	<0.01	–	–	–	0.10	<0.01	<0.01	6.00	9.90	1.72	9.91
Eh (mV)	−55	–	−84	−240	−192	–	−61	19	22	20	326	–	−195 (bottom)	–
Na^+ (mg/l)	61.45	51.94	52.56	239.26	35.33	139.88	112.37	329.25	149.13	32.43	129.96	14.81	110.07	16.66
K^+ (mg/l)	0.60	28.46	9.65	13.03	0.18	2.62	3.26	16.81	16.47	9.47	0.03	1.79	4.58	2.32
Mg^{2+} (mg/l)	3.47	23.21	0.15	8.18	3.00	13.83	53.89	3.57	10.56	8.27	1.62	14.82	11.50	15.52
Ca^{2+} (mg/l)	16.59	<0.01	62.51	255.09	21.66	84.82	0.57	284.39	240.25	94.93	17.80	73.85	114.18	81.15
$NH4^+$ (mg/l)	126.92	184.09	5.95	9.72	86.92	1.13	181.93	10.31	4.10	2.87	80.22	0.00	1.28	0.00
Cl^- (mg/l)	102.37	177.56	111.73	526.48	105.25	172.88	614.15	733.88	417.05	187.06	122.16	18.11	178.64	17.78
NO_2^- (mg/l)	<0.01	2.14	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
NO_3^- (mg/l)	16.94	<0.01	<0.01	<0.01	6.51	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	2.16	0.88	2.76
SO_4^{2-} (mg/l)	80.11	51.42	115.30	102.36	78.03	118.02	96.27	117.19	88.39	101.86	113.04	118.81	103.20	109.27
HCO_3^- (mg/l)	315.70	531.02	58.88	210.40	285.99	190.80	57.00	132.48	166.73	187.64	319.37	139.40	164.19	187.10
S^{2-} (mg/l)	–	–	–	0.1	<0.1	–	–	<0.1	<0.1	<0.1	–	–	–	–
Fe (mg/l)	–	–	–	0.032	–	–	–	0.16	0.024	0.55	–	–	–	–
Fe^{2+} (mg/l)	–	–	–	<0.1	–	–	–	<0.1	–	<0.1	–	–	–	–
CH_4 (mg/l)	–	–	–	0.016	<0.01	–	–	0.011	0.023	0.019	–	–	–	–
DOC (mgC/l)	2.15	32.42	–	15.69	0.94	–	3.91	2.54	0.97	6.84	3.3	–	1.97	–
BTEX (Benzene) (μg/l)	–	–	45 (30)	283 (280)	15 (12)	–	–	9.7 (8.5)	4.3 (4)		–	–	–	–
COD (mgO_2/l) NF T 90-101	–	51	<30	79	–	–	–	63	30	53	–	–	45	<30
COD (mgO_2/l) DIN 38409 H41	–	99	–	63	38	–	–	36	24	12	22	–	–	–
BOD5 (mgO_2/l) NF EN 1899-2	–	5.1	<1	3.5	–	–	–	5.2	<3	<3	–	–	<3	3.7
BOD5 (mgO_2/l) EN 1899-1 H51	–	27	–	14	<3	–	–	<3	<3	<3	<3	–	–	–

Table 3
qPCR results on groundwater samples collected in March 2008, targeting (i) DNA of *Dehalococcoides* spp., *tceA*, *vcrA* and *bvcA* genes and (ii) the corresponding RNA by rt-qPCR.

Point	*Dehalococcoides* spp. cells/ml	*Dehalococcoides* spp. RNA copy/ml	*tceA* DNA copy/ml	*tceA* mRNA copy/ml	*vcrA* and *bvcA* DNA copy/ml	*vcrA* and *bvcA* mRNA copy/ml
P4	$3.8 \cdot 10^2$	$<4.2 \cdot 10^4$	$<1.3 \cdot 10^2$	$<4.2 \cdot 10^4$	$2.2 \cdot 10^2$	$<4.2 \cdot 10^4$
P11	$2.2 \cdot 10^5$	$2.7 \cdot 10^5$	$2.3 \cdot 10^2$	$<1.9 \cdot 10^5$	$7.8 \cdot 10^3$	$9.0 \cdot 10^5$
P13	$3.2 \cdot 10^3$	$1.5 \cdot 10^5$	$<1.3 \cdot 10^2$	$<5.3 \cdot 10^3$	$4.5 \cdot 10^3$	$4.8 \cdot 10^5$
P14	$5.0 \cdot 10^3$	$3.2 \cdot 10^5$	$<1.3 \cdot 10^2$	$3.2 \cdot 10^3$	$6.8 \cdot 10^3$	$4.9 \cdot 10^5$
P15	$2.1 \cdot 10^3$	$2.2 \cdot 10^4$	$<1.3 \cdot 10^2$	$<4.3 \cdot 10^3$	$4.1 \cdot 10^3$	$2.7 \cdot 10^4$

3.3. Polymerase chain reaction assays results

3.3.1. PCR based on 16S rRNA

Quantitative PCR assays to target 16S rRNA showed that *Dehalococcoides* spp. was naturally present in the groundwater of the five sampled wells (Table 3). Moreover, rt-qPCR assays made clear that this group was active at least in four wells when the samples were collected.

These results support the existence of a biodegradation process in the groundwater, mediated by the *Dehalococcoides* group.

The most bacteria-concentrated well was P11, which is located outside the groundwater flow tube previously chosen to compute mass balance. At this point, the concentration of $2.7 \cdot 10^8$ cells per liter was one order of magnitude higher than the threshold of 10^7 cells per liter necessary to produce a "useful rate" of natural attenuation of 1,2-*cis*-DCE and VC (defined as greater than or equal to 0.3 per year) according to Lu et al. (2006). This high bacteria concentration at this point therefore confirms the existence of high biodegradation ability which can explain why no chlorinated solvents were found outside the flow tube in this zone.

Moreover, it has to be noted that this order of magnitude of *Dehalococcoides* cell concentration (10^8 cells per liter) is equal to the one detected by Lee et al. (2008) at their studied site a few months after implementing bioaugmentation using enriched cultures and biostimulation with whey. This tends to support that *Dehalococcoides* spp. is naturally well established at our studied site under conditions sufficiently favorable to yield bacteria concentration close to what can be expected on a biostimulated and bioaugmented site. However these conditions do not seem to be so good near the source zone at the P4 well (characterized by an alkaline pH of 11) where (i) bacteria were two orders of magnitude less concentrated than at P11 (characterized by a neutral pH) and (ii) expressions of the 16S rRNA genes were not detected ($<4.2 \cdot 10^4$ transcripts per ml).

3.3.2. PCR based on functional genes

Quantitative PCR assays to target TCE reductase showed that this enzyme is not well represented in the previously identified *Dehalococcoides* population. All the results were below detection threshold, except rt-qPCR results on P14, which tends to show the possible *tceA* gene implication on TCE reduction at this point when the sample was collected. Sung et al. (2006) reported however that the *Dehalococcoides* strain GT is able grow on TCE without expressing *tceA*. Consequently, the lack of expression of the gene *tceA* in our study does not imply that TCE dechlorinators were not active at this site.

Unlike PCR assays targeting TCE reductase, qPCR assays targeting VC reductase gave positive results on the five sampled wells. The number of VC reductase DNA copies had the same order of magnitude as the number of *Dehalococcoides* cells identified by targeting 16S rRNA. This result tends to show that most of the *Dehalococcoides* bacteria targeted by these analyses are able to grow on VC. Moreover, rt-qPCR made clear that VC reduction was occurring when groundwater samples were collected on P11, P13, P14 and P15.

It has been shown that different bacteria communities are responsible for each sequential dechlorination step of chloroethene biodegradation (Flynn et al., 2000). Commonly, bacteria which mediate PCE and TCE reduction are more ubiquitous than bacteria able to grow on 1,2-DCE and VC. In our case study, most of the identified *Dehalococcoides* bacteria were equipped with a VC reduction enzyme while the targeted TCE reductase was not detectable in this population. This apparent lack of *tceA* expression could be due to a variant *tceA* gene among the *Dehalococcoides* population present at this site which was not amplified with the primers used in this study, as concluded by Sung et al. (2006) for strain GT.

Furthermore, it can be noted that the *vcrA* and *bvcA* genes were highly expressed in the different samples except at P4 (the most alkaline point). The number of transcripts per copy of gene ranged from 6 at P15 to 115 at P11. This level of expression at P11 (115 transcripts per copy of gene) and P13 (106 transcripts per copy of gene) is for instance one order of magnitude higher than the expression level detected by Lee et al. (2008) for *vcrA* and *bvcA* on their bioaugmented and biostimulated site. Consequently, this site seems to be naturally more favorable to the development of VC dechlorinators, than a bioaugmented and biostimulated site.

3.4. Compound-specific carbon isotope analysis

Results of carbon isotope ratio measurements are reported in Fig. 4. They revealed, directly at the bottom of the waste dump at the P4 well, that the $\delta^{13}C$ TCE ratio is higher ($-21.7‰$) than industrial TCE ratios which usually range between -24.3 and $-33.5‰$ according to Auer and Schulz-Bull (2006) and Jendrzejewski et al. (2001). This leads to the conclusion that TCE had probably already undergone a biodegradation process along the flowpath when entering the studied site. Even if PCE concentration is of secondary importance in comparison to TCE, these measurements also showed that TCE could come from PCE biodegradation. The PCE $\delta^{13}C$ ratio

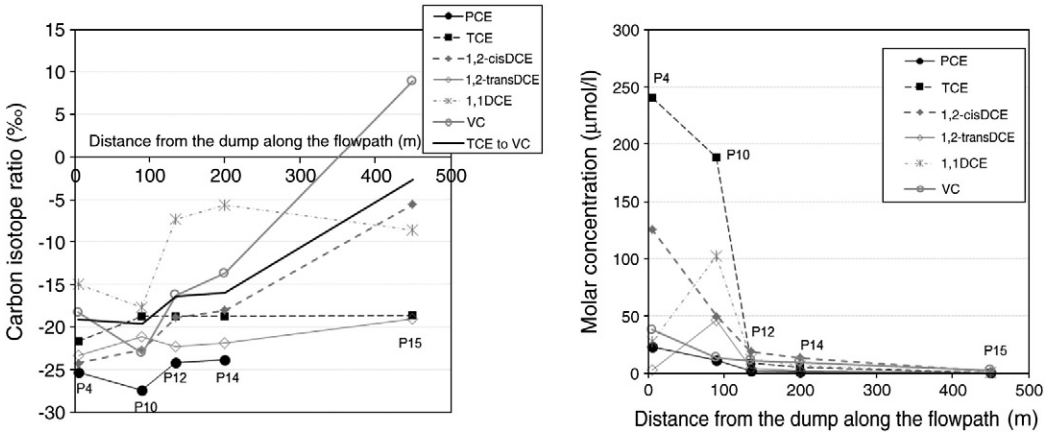

Fig. 4. Evolution of carbon isotope ratios (on the left) and molar concentrations (on the right) of different chlorinated compounds along the flowpath in March 2008.

at the P4 well (−22.3‰) is indeed lower than the TCE $\delta^{13}C$ ratio (−21.7‰).

Then, the TCE $\delta^{13}C$ ratio increased along the flowpath from P4 (−21.7‰) to P10 well (−18.8‰), which indicated a biodegradation of TCE.

From the P10 to the P15 well, isotope ratios of TCE remained steady along the flowpath which tends to show that this compound was no longer biodegraded, as implied by the PCR results (absence of TCE reductase in this zone). We can assume that the decrease of TCE concentration between these two points (Fig. 4) was mainly due to dispersion, volatilization and river infiltration (which leads to dilute concentration). The same evolution was observed on PCE isotope ratios which remained roughly steady from P12 to P14.

Note (Fig. 4) that the main decrease of TCE concentration was observed between P10 and P12 where the isotope ratio was stable around −18.8‰ (within the accuracy range of isotope measurements). Contrary to the situation observed in September 2007, carbon concentration contained in the sum from PCE to VC was not stable along the flowpath in March 2008, when the samples for isotope analyses were collected (Fig. 3). The carbon concentration decreased strongly between P10 and P12, which could be attributed to high lateral dispersion in this zone or might be an expression of locally unsteady conditions between these points, which, in turn, could explain the high TCE concentration drop without any significant increase of TCE isotope ratio.

Unlike PCE and TCE, the isotope ratio of *cis*-1,2-DCE highly increased between P4 (−22.7‰) and P15 (−5.6‰). As the ratio of *cis*-1,2-DCE (−22.7‰) was initially lower than that of TCE (−21.7‰) at the P4 well, we could assume that *cis*-1,2-DCE could come partly from TCE dechlorination and then be biodegraded along the flowpath. In addition, at the same time as TCE biodegradation between P4 and P10 occurs, a production of *trans*-1,2-DCE and 1,1-DCE was observed as their concentrations increased. This production was associated with a decrease of the 1,1-DCE isotope ratio (from −15.0 to −17.7‰) but stayed above that of TCE (−18.8‰) at the P10. In these conditions, two hypotheses to explain this observation can be considered. Either the 1,1-DCE came from another chlorinated product which had a higher initial isotope ratio than TCE, or the 1,1-DCE isotope ratio observed at the P10 well resulted from a mixing between an impoverished 1,1-DCE in ^{13}C which came from TCE dechlorination and an initial enriched 1,1-DCE. The selection of one of these two hypotheses will be made through a mass balance approach in the following section.

Unlike 1,1-DCE, the isotope ratio of *trans*-1,2-DCE increased (from −23.3 to −21.2‰) between P4 and P10 as its concentration increased but stayed isotopically lower than that of TCE (−18.8‰). Consequently, we could assume that *trans*-1,2-DCE might come from TCE dechlorination. The *trans*-1,2-DCE isotope ratio increase may be due either to *trans*-1,2-DCE biodegradation at the same time as it was produced, or to unsteady transport conditions (a local load of enriched *trans*-1,2-DCE for instance).

Isotope ratios of VC decreased from P4 (−18.3) to P10 (−23.1‰) by so much that they became lower than that of *cis*-1,2-DCE (−22.6‰) and *trans*-1,2-DCE (−21.2‰) at this point. Because of this shift, we could conclude that VC is produced by 1,2-DCE isomer dechlorination between these points. The decrease of VC concentration in this zone could therefore be explained by other attenuation processes (volatilization, dispersion).

From P10 (−23.1‰) to P15 (8.9‰), the isotope ratios of VC continued to reach positive values. This huge positive shift along the flowpath requires a very fractionating process. It has indeed been demonstrated that reductive dechlorination of VC (enrichment factor of about −21.5 to −31.1‰ according to Bloom et al. (2000) and Hunkeler et al. (2002)) is a stronger fractionating process than aerobic biodegradation of VC (enrichment factor about −3.2 to −8.2‰ according to Chartrand et al. (2005) and Chu et al. (2004)). Therefore we can conclude that the VC biodegradation between P10 to P15 resulted from reductive dechlorination.

Finally the carbon isotope ratio of the molar sum from TCE to VC showed a small decrease between P4 and P10 which tends to signal unsteady transport conditions, as a differential sorption between parent compounds and their by-products along the flowpath could lead to a lower isotope ratio of the molar sum of these compounds (Van Breukelen et al., 2005). Then an increase of the TCE to VC isotope ratio was observed from P10 to P15. This second evolution signals a loss of ^{12}C and confirms the biotransformation of the last

chlorinated ethene of this biodegradation chain (VC) in the downgradient part of the site. So, even if it were not possible to measure ethene concentrations, isotope measurements indicate the existence of TCE and PCE dehalogenation to ethene on the studied site.

3.5. Final mass balance estimate of the MNA

We can now conclude that biodegradation of chlorinated solvents is demonstrated to occur naturally at the studied site. All three methods used to confirm the existence of this process give positive answers (mass balance of chlorine summed from PCE to VC and compared to the mass balance of carbon, PCR analyses, and carbon isotope ratios). All alternative natural attenuation processes such as dilution, dispersion or volatilization may participate in the attenuation, but at a rate which is much lower than that of biodegradation.

For the September 2007 quasi-steady situation, we can estimate by mass balance the biodegradation rate for each solvent (Table 4).

As we have previously estimated at around 15 kg Cl/d the volatilization flux of TCE between sections 1 and 2, we can deduce from the loss of TCE (28 kg Cl/d) reported in Table 4 that biodegradation represents a loss of TCE of around 13 kg Cl/d. As volatilization is probably greater between sections 1 and 2 because of the presence of the former river channel, the decrease of the loss flux of TCE (from 28 to 18 kg Cl/d) along the flowpath shows that biodegradation is not uniform in space, and acts preferentially for TCE between sections 1 and 2 and decreases between sections 2 and 3. As also shown by PCR and carbon isotope ratios, on the contrary, biodegradation of DCE isomers and VC is more active between sections 2 and 3 than between sections 1 and 2 as it counterbalances the production of these compounds observed between sections 1 and 2. Further work is needed to identify the causes of this behavior and to precise the role of the old river channel (volatilization, biodegradation) in this evolution.

Moreover, these calculations highlight the fate of the biodegraded TCE. Indeed, as 1 mol of TCE produces 1 mol of DCE and then 1 mol of VC:

- the production of 0.5 kg Cl/d of *trans*-1,2-DCE between sections 1 and 2 corresponds stoichiometrically to a TCE biodegradation of 0.7 kg Cl/day,
- the production of 3.8 kg Cl/d of 1,1-DCE between sections 1 and 2 corresponds stoichiometrically to a TCE biodegradation of 5.1 kg Cl/day,
- the production of 1.3 kg Cl/d of VC between sections 1 and 2 corresponds stoichiometrically to a TCE biodegradation of 2.2 kg Cl/day.

Consequently, if the TCE biodegradation loss deduced from volatilization calculation is correct (around 28–15 = 13 kg Cl/d), TCE must be at least biodegraded into VC, *trans*-1,2-DCE and 1,1-DCE between sections 1 and 2, to verify the stoichiometry of the biodegradation chain (0.7 + 5.1 + 2.2 = 8 kg Cl/d of biodegraded TCE). This conclusion is mainly supported by the fact that the other high-chlorinated compounds whose biodegradation can also lead to 1,1-DCE production are not sufficiently concentrated in groundwater to explain such a 1,1-DCE production between sections 1 and 2. For instance, 1,1,1-trichloroethane may also be degraded along the flow path into 1,1-DCE by abiotic dehydrochloration (Butler and Hayes, 2000; Vogel and McCarty, 1987) or even by a bacteria-mediated process (Gregory et al., 2000). However, its degradation represents only a loss of 1.3 kg Cl/d between sections 1 and 2 (as its concentration through section 1 is approximately 1 mg/l and is only 0.2 mg/l through section 2). Such a degradation only represents a production of 0.7 kg Cl/d of 1,1-DCE which cannot explain by itself only the observed production of 3.8 kg Cl/d of 1,1-DCE. Similarly, 1,1,1,2-trichloroethane degradation could also produce 1,1-DCE by abiotic dechlorination (Butler and Hayes, 2000) between sections 1 and 2 as a loss of 0.7 kg Cl/d of this compound can be observed. However, such a degradation only represents a production of 0.35 kg Cl/d of 1,1-DCE. Consequently, as a first approximation, it can be considered that the huge production of 1,1-DCE (3.8 kg Cl/d) observed between sections 1 and 2 must be mainly attributed to the observed degradation of TCE and, to a lesser extent, of PCE, the major chlorinated compounds identified in that zone, to verify the stoichiometry of the biodegradation chain. This rough mass balance calculation thus clarify the origin of 1,1-DCE allowing to dismiss one of the two above hypotheses explaining its carbon isotope ratio.

The difference between the TCE biodegradation loss deduced from volatilization calculation (13 kg Cl/d) to which must be added the loss of PCE (3.5 kg Cl/d) which represent a production of 2.6 kg Cl/l TCE (total of 13 + 2.6 = 15.6 kg Cl/l) and the one deduced from by-product production (8 kg Cl/d) could be due to (i) VC biodegradation between sections 1 and 2 at the same time as it is produced, which would increase our estimate of the initial TCE flux, (ii) DCE and VC volatilization between sections 1 and 2 and (iii) PCE volatilization which was here neglected as a first approximation. Moreover, if all the VC produced between sections 1 and 2 comes from *cis*-1,2-DCE dechlorination, these calculations show that TCE

Table 4
Estimated amount of chlorinated solvents in kg Cl/d lost or produced between the three sections of the flow tube of Fig. 2 and% of the incoming flux. The degradation takes place in about 1 month and a travel distance of 300 m between sections 1 and 2.

Chlorinated solvent	Between sections 1 and 2	Between sections 2 and 3
PCE	Loss of 3.5 kg Cl/day, 54% of the flux	Loss of 2.9 kg Cl/day, 98% of the flux
TCE	Loss of 28.0 kg Cl/day, 61% of the flux	Loss of 18.0 kg Cl/day,100% of the flux
cis-1,2-DCE	Loss of 0.2 kg Cl/day, 1% of the flux	Loss of 13.5 kg Cl/day, 77% of the flux
trans-1,2-DCE	Production of 0.5 kg Cl/day, 121% of the flux	Loss of 0.6 kg Cl/day, 66% of the flux
1,1-DCE	Production of 3.8 kg Cl/day, 86% of the flux	Loss of 7.8 kg Cl/day, 95% of the flux
VC	Production of 1.3 kg Cl/day, 75% of the flux	Loss of 0.6 kg Cl/day, 19% of the flux

is biodegraded preferentially into 1,1-DCE (64%), *cis*-1,2-DCE (27%) and *trans*-1,2-DCE (9%). These results are surprising as the *cis*-1,2-DCE is known to be the most common intermediate of TCE biodegradation among the different DCE isomers. However, some studies show that the production of *trans*-1,2-DCE (Cheng et al., 2010; Griffin et al., 2004) and 1,1-DCE (Zhang et al., 2006) was observed in some cases. In addition, we can note that the 1,1-DCE-to-*cis*-1,2-DCE ratio found in our study is very close (2.3) to the one found by Zhang et al. (2006) (2.4 ± 0.3) but in their case, no *trans*-1,2-DCE production was observed. This leads to the conclusion that a complex mixture of several bacteria communities is implied in TCE biodegradation in our case study.

Moreover we have also shown that biodegradation varied with time, between October 2007 and March 2008. The primary cause of the observed variations is the change in the incoming flux of solvents at the upstream side of the studied zone. In an unsteady situation, the simple mass balance calculations of Table 5 are no longer possible, and only a groundwater flow and transport model taking into account biodegradation and parent–daughter relationships could estimate the actual biodegradation rate. Such work is currently in progress. In addition, a microcosm study is also in progress to confirm the observed biodegradation pathways, and to select appropriate biostimulation measures.

4. Conclusion

In this study, we have demonstrated that use of three complementing methods (mass balance, polymerase chain reaction assays and compound-specific stable isotope analysis) is able to demonstrate the existence and effectiveness of biodegradation of chlorinated solvents in an alluvial aquifer. PCR and compound-specific isotope analyses (CSIA) were in good agreement in our case study. As PCR showed a lack of TCE reductase representation among the identified *Dehalococcoides* bacteria, the spatial evolution of the TCE carbon isotope ratio tends to prove that biodegradation occurred only in the upgradient zone of the site. Unlike the scaled-down TCE dechlorination ability, rt-qPCR indicated that bacteria able to grow on VC and DCE were active and widespread in the groundwater. Furthermore, CSIA confirmed the dechlorination of DCE and VC all along the flowpath as soon as they were produced. It indicated moreover that these by-products originated from a sequential biodegradation of the initial TCE and, to a lesser extent, of PCE.

These results, compared to a basic geochemical characterization of the aquifer, indicate that, even in extreme geochemical conditions, characterized by a groundwater pH greater than 10 at the source zone, natural biodegradation can be observed and demonstrated with PCR measurements and CSIA without requiring a full characterization of the geochemical conditions, even if such a characterization would have been preferable from a scientific point of view. Once biodegradation is recognized, simple mass-balance calculations can produce an estimate of the amount of solvents that are degraded between successive sections of the aquifer. In our study, this mass balance allowed us to show that 1,1-DCE was the predominant DCE isomer produced by TCE dechlorination.

These high-performance tools have thus revealed a spatially heterogeneous distribution of dechlorination ability in the studied alluvial medium. Note, moreover that a simple and cost-effective sampling method (one average sample per well) was used in this study. Although this sampling does not give information on the vertical distribution of water quality and redox conditions, it allows a quick answer to the question "Do biodegradation processes occur in the aquifer?"

This answer is an important factor in determining if monitored natural attenuation could be an efficient method to decontaminate groundwater at a lower cost than more active techniques. For instance, we show in this paper that, together with a loss of about 15 kg Cl/d due to volatilization, about 10 kg Cl/d of total dissolved chlorinated solvents can be eliminated from the groundwater along a flowpath of 300 m by biodegradation processes. However, stable TCE isotope ratios also showed that biodegradation was not the only factor contributing to the huge concentration decrease of contaminants observed along the flowpath. In this context, coupling rt-PCR analysis and CSIA appeared to be a powerful method to describe the nature of a biodegradation process occurring under unsteady transport conditions at the same time as other attenuation processes. These results can thus be submitted to the regulatory authority within a short timeframe to discuss the most suitable remedial solution and to demonstrate the importance of better characterizing the geochemical conditions.

The next step of this work will be to quantify the role of bacteria-mediated attenuation compared with dilution, dispersion and volatilization. We will also assess the role of surface water (and the former channel).

More research is particularly needed on this site to better characterize the geochemical conditions in order to answer the three crucial following questions: (i) What source of fermentable non-chlorinated carbon produces the hydrogen and/or acetate required for *Dehalococcoides* to develop at this site? (ii) Is this amount of carbon sufficient to sustain biodegradation or is it a limiting factor? (iii) How can VC reductive dechlorinators, identified by PCR analyses, develop in the last downgradient part of the site where oxygen-rich water coming from the river infiltrates into the aquifer and where TCE and PCE biodegradation does not seem to be effective anymore (confirmed by both isotope measurements and PCR)? Microcosm studies coupled with CSIA and PCR assays could make an efficient tool to appreciate the geochemical factors implied in this process and to confirm the biodegradation pathways deduced from mass balance calculations. Measurement of the dissolved ethylene concentration in groundwater is also required to confirm the biodegradation pathways. New in situ PCR assays targeting the *pceA* gene could also be performed in order to see if this gene, well known to be implied in anaerobic dechlorination of PCE and TCE, as demonstrated by Holliger et al. (1998) and Maillard et al. (2003), is more represented than the *tceA* one in the identified *Dehalococcoides* population.

All of these new pieces of information could then be integrated into a reactive transport model in a more quantitative approach.

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