This is the accepted version of the following article: Natàlia Blázquez-Pallí, Mònica Rosell, Joan Varias, Marçal Bosch, Albert Soler, Teresa Vicent, et al. Multi-method assessment of the intrinsic biodegradation potential of an aquifer contaminated with chlorinated ethenes at an industrial area in Barcelona (Spain). *Environmental Pollution*, 244:2019, p.165-173, which has been published in final form at https://doi.org/10.1016/j.envpol.2018.10.013.

Multi-method assessment of the intrinsic biodegradation potential of an aquifer contaminated with chlorinated ethenes at an industrial area in Barcelona (Spain)

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Environmental Pollution

Total number of pages (including cover): 32

Figures: 3

Tables: 1

Abstract

The bioremediation potential of an aquifer contaminated with tetrachloroethene (PCE) was assessed by combining hydrogeochemical data of the site, microcosm studies, metabolites concentrations, compound specific-stable carbon isotope analysis and the identification of selected reductive dechlorination biomarker genes. The characterization of the site through 10 monitoring wells evidenced that leaked PCE was transformed to TCE and *cis*-DCE via hydrogenolysis. Carbon isotopic mass balance of chlorinated ethenes pointed to two distinct sources of contamination and discarded relevant alternate degradation pathways in the aquifer. Application of specific-genus primers targeting *Dehalococcoides mccartyi* species and the vinyl chloride-to-ethene reductive dehalogenase *vcrA* indicated the presence of autochthonous bacteria capable of the complete dechlorination of PCE. The observed *cis*-DCE stall was consistent with the aquifer geochemistry (positive redox potentials; presence of dissolved oxygen, nitrate, and sulphate; absence of ferrous iron), which was thermodynamically favourable to dechlorinate highly chlorinated ethenes but required lower redox potentials to evolve beyond *cis*-DCE to the innocuous end product ethene. Accordingly, the addition of lactate or a mixture of ethanol plus methanol as electron donor sources in parallel field-derived anoxic microcosms accelerated dechlorination of PCE and passed *cis*-DCE up to ethene, unlike the controls (without amendments, representative of field natural attenuation). Lactate fermentation produced acetate at near-stoichiometric amounts. The array of techniques used in this study provided complementary lines of evidence to suggest that enhanced anaerobic bioremediation using lactate as electron donor source is a feasible strategy to successfully decontaminate this site.

Capsule

The combination of complementary diagnostic techniques provides different lines of evidence for *in situ* bioremediation potential of a tetrachloroethene-contaminated aquifer.

Keywords

Stable isotope; *Dehalococcoides mccartyi*; biostimulation; DCE stall; reductive dehalogenase.

1. Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are widely used as degreasing agents in industry and are frequently detected in subsurface waters due to improper disposal or accidental spills. The lesser chlorinated ethenes, dichloroethene (DCE) and vinyl chloride (VC), are usually detected in groundwater because of incomplete reductive dechlorination reactions of PCE and TCE, in a common phenomenon referred to as "DCE or VC stall" (Bradley, 2000; Stroo and Ward, 2010). In this situation, PCE, TCE and their daughter products coexist in groundwater forming a hazardous chemical mixture. These compounds are all regulated and considered priority substances by the Agency for Toxic Substances and Disease Registry of the United States (ATSDR, 2016) and by the 2008/105/EC European Directive (European Commission, 2008), which set maximum contaminant levels (MCL) for all of them.

Remediation strategies for contaminated aquifers have traditionally included physicochemical treatments such as pump and treat, thermal desorption, *in situ* chemical oxidation and soil vapor extraction, among others. However, in the last decade, *in situ* bioremediation, which uses the metabolism of microorganisms to degrade pollutants, has emerged as one of the most preferable technologies used to clean up the subsoil (Pandey et al., 2009). This shift towards *in situ* biological treatments is likely due to their cost-effectiveness and less invasive features compared with the conventional physicochemical methods (Lemming et al., 2010; Lyon and Vogel, 2013).

Organohalide-respiring bacteria (OHRB) are the key microorganisms in the bioremediation of chlorinated ethenes because they can use them as terminal electron acceptors during microbial respiration, resulting in the formation of lesser-chlorinated compounds (Leys et al., 2013). OHRB tend to harbour several distinct reductive

dehalogenases (RDase), which are the enzymes driving the organohalide respiration process. The identification of their function allows for the exploitation of the genes encoding the catalytic subunit, reductive dehalogenase homologous A subunit (*rdhA*), as biomarkers to assess the intrinsic bioremediation potential of contaminated sites (Cupples, 2008; Hug et al., 2013; Hug and Edwards, 2013; Pöritz et al., 2013). OHRB are strictly anaerobic and when redox potential or nutritional requirements (i.e. acetate and hydrogen as carbon source and electron donor, respectively) are not adequate, the aquifer can be conditioned by supplying organic fermentable substrates such as lactate, butyrate or benzoate in a process referred to as biostimulation (Adrian and Löffler, 2016; Leeson et al., 2004). Some OHRB can partially dechlorinate PCE to TCE or *cis*-DCE, such as *Clostridium* sp., *Dehalobacter* sp., *Desulfitobacterium* sp., *Desulfuromonas* sp., *Geobacter* sp., or *Sulfurospirillum* sp. (Löffler et al., 2013), but *Dehalococcoides mccartyi* sp. (*Dhc*) has been considered, for a long time, the unique genus capable of fully dechlorinating PCE to innocuous ethene (Cupples et al., 2003; He et al., 2005; Sung et al., 2006). However, it may be possible that other bacteria, still unknown and not described yet, are able to perform such reaction, as recent studies have shown that a *Dehalogenimonas* species can dechlorinate TCE to ethene (Yang et al., 2017b), and that a bacterial consortium not containing *Dhc* was able to completely dechlorinate PCE as well (Yu et al., 2016). Likewise, Lu et al. (2006) observed that VC disappeared at contaminated sites where no *Dhc* markers were present, and Da Silva and Alvarez (2008) and He et al. (2015) demonstrated that dechlorination activity and formation of ethene is not always correlated with the presence of *Dhc* genes.

Different approaches allow for the monitoring and characterization of *in situ* biodegradation of organic contaminants and organohalides in particular (Bombach et al., 2010; Nijenhuis and Kuntze, 2016). Monitoring of contaminants' concentrations can

provide information on the removal of certain contaminants in the polluted site, but the main drawback is that physical attenuation processes such as dilution, sorption or dispersion cannot be distinguished from biological processes and, therefore, these measurements can sometimes be misinterpreted. Likewise, co-contamination and off-site sources of the contaminants make difficult site degradation assessments. To overcome this bottleneck, a more reliable method that is increasingly used to monitor *in situ* chlorinated ethenes transformation is the compound specific isotope analysis (CSIA) (Hunkeler et al., 1999; Nijenhuis et al., 2007; Palau et al., 2014; Wanner et al., 2016). This technique measures the abundance ratio of specific stable isotopes (i.e. $^{13}C/^{12}C$) in targeted molecules relative to an international standard. In principle, lighter isotope molecules react at a higher rate than heavier isotope molecules during biochemical transformations. This results in a progressive enrichment of the heavier isotope that can be used to confirm and quantify *in situ* biodegradation and elucidate degradation pathways (Elsner et al., 2010). CSIA can also give information regarding source apportionment of the involved pollutants in field studies (Filippini et al., 2018; Hunkeler et al., 2008; Nijenhuis et al., 2013).

The detection of *Dhc* and specific *rdhA* genes based on polymerase chain reaction (PCR) is a qualitative indication of the reductive dechlorination potential at contaminated sites, but it does not provide information about the physiological activity of *Dhc*. Likewise, stable carbon isotope fractionation of PCE or TCE can serve as an indicator of *in situ* reductive dechlorination of these contaminants, but it does not confirm that biodegradation beyond *cis*-DCE is feasible. Therefore, it is necessary an integrated approach to provide different and complementary lines of evidence for the assessment of the potential of contaminated sites to fully dechlorinate chlorinated solvents (Badin et al., 2016; Courbet et al., 2011; Stelzer et al., 2009).

Consequently, the aim of this work was to assess the bioremediation potential of a chlorinated ethenes contaminated site from an industrial area in Barcelona (Spain) using several techniques, including (1) the assessment of the hydrogeochemical conditions of the aquifer, (2) the analysis of the carbon isotopic composition of chlorinated ethenes, (3) the establishment of microcosms to evaluate the effect of different biostimulants to detoxify groundwater samples, and (4) the use of PCR primers targeting specific functional genes.

2. Materials and methods

2.1. Chemicals

PCE (99.9% purity) and TCE (≥98% purity) were purchased from Panreac, and ethene (≥99.95% purity) and sodium lactate (≥98% purity) from Sigma-Aldrich. Methanol and ethanol were purchased from Scharlab at the highest purity available. Other chemicals and reagents used for the present study were purchased from Sigma-Aldrich, Thermofisher and Bio-Rad at scientific grade or higher.

2.2. Hydrogeochemical description of the aquifer

The studied aquifer is located in the province of Barcelona (Spain). This site was significantly contaminated with PCE due to improper disposal practices after its former use as degreasing agent at an industrial plant. The aquifer is an unconfined bedrock mainly consisting of Tertiary sediments.

For the geological and hydrogeological characterization of the studied area, 55 rotational probes between 10 to 20 m depth with continuous sample extraction were carried out and habilitated as piezometers. According to the probes, from the bottom to

top, mainly three main lithological facies were differentiated: i) a lower layer of red marl located at 10 to 3 m depth, ii) an intermediated brown silty mudstone, and iii) a higher level represented by ochre silty mudstone that are located up to 3 to 6 m depth. An intermediated layer of silty mudstone with sandstone beds is developed in the south part of the site, and it locally evolves to a sandstone – microconglomerate strata that probably corresponds to an old Tertiary sedimentary paleochannel. This most transmissive layer is intersected at the piezometer MW-7. Such Tertiary formation is locally covered by Quaternary deposits including sands, silts and clays with a variable vertical extension and anthropogenic materials (concrete, etc.).

For site characterization and monitoring, 55 wells completely screened were installed. The water table is located at depths ranging between 2.5 and 7.5 m below ground surface (266 to 272 m.a.s.l.). Groundwater is mainly concentrated in the sandstone or microconglomerate intervals that intercalate with the mudstone matrix forming a multi-layered aquifer. Due to the limited continuity of the permeable stratum, the potential hydrological exploitation of the area is low. Locally, when monitoring wells intersect layers of sandstone, silty sandstones, silty mudstones and microconglomerates, the hydraulic conductivity increases to medium–low. Pumping tests determined that the hydraulic parameters of the aquifer are from 0.1 to 0.8 m^2/day for transmissivity, and from 0.02 to 0.32 m/day for hydraulic conductivity .

2.3. *Collection of aquifer samples*

The plume characterization monitoring campaign was carried out in May 2016 on 10 monitoring wells identified by numerical codes (Figure 1) using a peristaltic pump. First, the following parameters were measured *in situ*: (1) the piezometric level; (2) the worker short-term exposure to volatile organic compounds (VOCs) and other gases, with a

MiniRAE Lite direct-reading photoionization detector (RAE Systems, Spain), and (3) the hydrogeochemical parameters of groundwater once they were stabilized using a flow-through cell to avoid contact with the atmosphere (temperature, pH, electric conductivity, and redox potential (Eh)), with a multiparameter probe 3430 WTW (Weilheim). For Eh the redox sensor was a SenTix ORP 900 and the measurements were corrected to the standard hydrogen electrode system (UH) by adding the reference electrode potential at the groundwater temperature to the measured potential. Concentration of dissolved oxygen was measured with a Dissolved Oxygen Meter HI 9147 (Hanna Instruments). Then, samples from the aquifer were obtained.

Groundwater for stable carbon isotope analysis and chemical characterization was collected at 1 m above the bottom of the 10 wells, to avoid sediments. The ones for CSIA were killed with NaOH (pH>10) to prevent further biodegradation reactions.

For the establishment of microcosms, groundwater with fine sediments was collected from the bottom of the monitoring well MW-2 in transparent autoclaved glass bottles, which were previously filled with N_2 gas to minimise bacterial contact with oxygen, and sealed with PTFE caps to minimise VOCs' adsorption. All groundwater samples were kept in the dark at 4ºC until analysis.

2.4. Laboratory microcosms

To study whether organic fermentable substrates could enhance the biodegradation of chlorinated ethenes and ethene formation, three different treatments were prepared in triplicate: (1) control containing only groundwater, (2) groundwater with a mixture of methanol plus ethanol (3 mM each), and (3) groundwater with sodium lactate (3 mM). Each microcosm consisted of 100 mL glass serum sterile bottles sealed with Teflon-coated butyl rubber septa and aluminium crimp caps, and contained 65 mL of sampled

198 groundwater and fine sediments. All microcosms were prepared in an anaerobic glovebox
199 and incubated at 25 °C in the dark under static conditions. After setting up the
200 microcosms, the initial concentration of chlorinated ethenes was analysed by headspace
201 gas chromatography as described in section 2.5 (Table S1) and afterwards it was
202 periodically monitored.

203 To determine the carbon isotopic fractionation (ε_C) during anaerobic reductive
204 dechlorination of PCE, six parallel cultures were simultaneously prepared as described
205 above, but using anaerobic defined media as reported elsewhere (Martín-González et al.,
206 2015) and groundwater from MW-2 (1.5% v/v) as inoculum. Each microcosm was spiked
207 with PCE (160 µM), and sacrificed with NaOH (10 M) at different time points of PCE
208 degradation. In this experiment, three different controls were included, at least in
209 duplicate: (1) NaOH-killed controls containing inoculum and PCE; (2) NaOH-killed
210 controls with inoculum but without PCE, and (3) abiotic controls with PCE but without
211 inoculum, to account for abiotic transformations and control potential impurities from the
212 PCE stock solution.

213 *2.5. Analytical methods*

214 Chemical characterization of groundwater was determined through the analysis of major
215 anions and cations. Aliquots of samples were preserved with nitric acid to measure total
216 concentrations of Fe, Ca and Na by inductively coupled plasma-optic emission
217 spectrometry (ICP-OES, Optima 3200 RL) and by inductively coupled plasma mass
218 spectrometry (ICP-MS, Elan 6000) at the *Centres Científics i Tecnològics de la*
219 *Universitat de Barcelona* (CCiT-UB). HCO_3^- was determined by titration (METROHM
220 702SM Titrino). NO_3^-, Cl^- and SO_4^{-2} concentrations were analyzed by high-performance
221 liquid chromatography (HPLC) using a WATERS 515 HPLC pump with an IC-PAC

anion column and a WATERS detector (mod 432) at the CCiT-UB.

Headspace samples (500 µL) were collected from microcosms to quantify chlorinated ethenes and ethene by gas chromatography (GC) coupled to a flame ionization detector (FID) as described elsewhere (Martín-González et al., 2015). Lactate, pyruvate, acetate, and formate were analysed from 1 mL filtered liquid samples (0.22 µm, Millex) by HPLC as previously described (Mortan et al., 2017).

Stable carbon isotope analyses were performed with an Agilent 6890 GC equipped with a split/splitless injector, coupled to a Delta Plus isotope ratio mass spectrometer (IRMS) through a GC-Combustion III interface (Thermo Finnigan). The analyses were carried out by headspace solid-phase microextraction (HS-SPME) following the same methodology used in Martín-González et al. (2015), but the injector temperature was 250ºC and the initial oven temperature of 60ºC was kept for 5 min. Carbon isotopic compositions of the contaminants are reported in delta notation ($\delta^{13}C$), relative to the international standard VPDB (Vienna Pee Dee Belemnite), following

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{std}} - 1\right) \qquad \text{(Eq.1)}$$

where R_{sample} and R_{std} refer to the isotope ratios ($^{13}C/^{12}C$) of the sample and the standard, respectively (Elsner, 2010). $\delta^{13}C$ is usually expressed in parts per mil (‰). All samples were measured at least in duplicate and corrected for slight carbon isotopic fractionation induced by the HS-SPME with respect to daily aqueous control standards of chlorinated ethenes with known carbon isotope ratios. The $\delta^{13}C$ of these pure in-house standards was determined previously using a Flash EA1112 (Carlo-Erba) elemental analyzer (EA) coupled to a Delta C Finnigan MAT IRMS (Thermo Finnigan) through a Conflo III interface (Thermo Finnigan) using six international reference materials (NBS 19, IAEA-

CH-6, USGS40, IAEA-600, IAEACH-7, L-SVEC) with respect to the VPDB standard according to (Coplen et al., 2006). All the chlorinated ethenes aqueous control standards that were injected together with the experimental samples had a one standard deviation (1σ) lower than 0.5‰ and their mean values were used to normalise the $\delta^{13}C$ of the samples, the uncertainties of which were calculated by error propagation.

Since PCE was the unique chlorinated precursor released in this aquifer and it is transformed sequentially via reductive dechlorination to TCE, *cis*-DCE and minor amounts of VC, the weighted average of the isotope signature of the chlorinated solvents must remain constant if VC is not further degraded and PCE released over the years has identical isotopic composition (Aeppli et al., 2010; Hunkeler et al., 1999; Palau et al., 2014) . Carbon isotopic mass balance for chlorinated ethenes at each well of the site was calculated for the sequential reductive dechlorination, as follows

$$\delta^{13}C_{sum} = x_{PCE} \cdot \delta^{13}C_{PCE} + x_{TCE} \cdot \delta^{13}C_{TCE} + x_{DCE} \cdot \delta^{13}C_{DCE} + x_{VC} \cdot \delta^{13}C_{VC} \quad \text{(Eq.2)}$$

where *x* refers to the molar fraction of each compound with respect to the total molar mass (sum of chlorinated ethenes) at the sampling event. Calculated $\delta^{13}C_{sum}$ from wells at the field site were compared to assess potential point sources of PCE in the aquifer.

A simplified version of the Rayleigh equation allows to quantify the carbon isotopic fractionation, ε_C (Elsner, 2010), which defines the relationship between changes in carbon isotopic composition (R_t/R_0) and concentrations ($f = C_t/C_0$) with time, as follows

$$\ln\left(\frac{R_t}{R_0}\right) = \varepsilon_C \cdot \ln(f) \quad \text{(Eq.3)}$$

where R_t/R_0 can be described as $(\delta^{13}C_t + 1) / (\delta^{13}C_0 + 1)$ according to $\delta^{13}C$ definition. ε_C can be used to quantify the extent of biodegradation of a target contaminant if a site-specific ε_C-value can be obtained (Elsner, 2010). In addition, the ε_C value is characteristic

for a given degradation pathway and can provide information into the reactions taking place in the field (Elsner, 2010).

2.6. DNA extraction and PCR

DNA was extracted from enriched cultures inoculated with aquifer samples from MW-2. Cell harvesting was carried out via centrifugation of 65 mL-samples. Genomic DNA was isolated using NucleoSpin Tissue DNA extraction kit following the instructions provided by the manufacturer (Macherey-Nagel). Primer sets used to detect *Dhc* 16S rRNA gene and *Dhc* reductive dehalogenase gene *vcrA* were previously described (Manchester et al., 2012; Ritalahti et al., 2006) (Table S2). *Dehalococcoides mccartyi* strain BTF08 (Pöritz et al., 2013) was used as positive control. Each 10 µL reaction mixture contained 5 µL of iQ Supermix (2x) (Bio-Rad), 250 nM of each primer (1 µL volume each) (Thermofisher) and a concentration of template DNA ranging between 5-50 ng/µl (3 µL volume). The thermal program used for PCR amplification of *Dhc* and *vcrA* genes was described elsewhere (Martín-González et al., 2015; Ritalahti et al., 2006).

3. Results

3.1.Physicochemical characterization of the site

The hydrogeochemical data collected from the 10 monitoring wells of the aquifer is summarized in Table 1. MW-4 was never impacted by chlorinated ethenes contamination and therefore is considered as the natural background of the area, which belongs to magnesium-calcium-bicarbonate facies. The distribution of native potential electron acceptors in the aquifer varied among different monitoring wells. Dissolved oxygen concentration ranged from 0.2 to 2.0 mg/L, total Fe was insignificant (<0.1 mg/L), nitrate

concentration exceeded 25 mg/L in all piezometers except in PZ-1 and Prof A, and sulphate was detected at elevated levels in most of the wells (values ranging from 94 to 1435 mg/L). Elevated levels of chloride were detected in most of the wells (up to 1053 mg/L at the most contaminated well PZ-5), whereas MW-4 showed the lowest value (30.5 mg/L), considered the natural background. The averaged concentration of bicarbonate, which serves as an indicator of the natural buffering capacity of the aquifer, was 459 ± 146 mg/L. The pH and temperature values were on average 7.4 ± 0.2 and 19 ± 2 ºC, respectively.

The concentration analyses of chlorinated ethenes across the site showed that PCE was the main VOC in the aquifer (concentrations ranging from 2.1 to 77 µM), but it was always accompanied by minor amounts of TCE and *cis*-DCE (Figure 2, Table S1). Traces of *trans*-DCE and VC were detected in MW-2 and, as expected, no chlorinated ethenes were detected in MW-4 (Table S1).

3.2. Carbon stable isotope analysis of chlorinated ethenes

The carbon isotopic signatures ($\delta^{13}C$) of PCE and its dechlorination products were analysed at the different monitoring wells to investigate the relevance of biodegradation processes at the contaminated site. They were found from -32.6 to -26.4‰, -37.7 to -29.7‰, and -33.0 to -26.0‰ for PCE, TCE, and *cis*-DCE, respectively (Figure 2, Table S1 for more details). In the case of PCE, which is the source of the contamination plume, all values were within the range of commercial solvents (-37.2 to -23.2‰) (Jendrzejewski et al., 2001; van Warmerdam et al., 1995). The isotopic mass balance based on the concentration-weighted $\delta^{13}C$ signatures of the chlorinated ethenes and assuming hydrogenolysis ($\delta^{13}C_{sum}$, Eq. 2) was established for each well (Figure 2, Table S1). Except for wells MW-7 and Prof A, which had similar isotopic balances of -26.4 ± 0.6‰ and -

315 26.0 ± 0.7‰, respectively, the $\delta^{13}C_{sum}$-values of the rest of the wells were within the same
316 average value of -31 ± 1‰.

317 In an attempt to determine the site-specific ε_C for PCE dechlorination in this aquifer,
318 six parallel cultures inoculated with 1.5% v/v MW-2 groundwater were killed after
319 approximately 0, 7, 21, 46, 79, 81 and 82% of PCE degradation and the $\delta^{13}C$ of
320 chlorinated ethenes was analysed. Abiotic and NaOH-killed controls showed no PCE
321 degradation as its concentration remained constant throughout the whole experiment (163
322 ± 5 µM, n=7, Figure S1A). In the active microcosms, although the $\delta^{13}C$ of PCE shifted
323 1.6‰ after 82% degradation (see Figure S1B) and the one standard deviation (1σ) for
324 duplicate measurements were, for all samples, below total instrumental uncertainty of
325 0.5‰ (Sherwood Lollar et al., 2007), PCE dechlorination to TCE did not fit the Rayleigh
326 model. The data exhibited poor linearity ($R^2 = 0.46$) when plotting according to Eq.3 and
327 the calculated εC would be -0.6 ± 0.8‰, showing a 95% confidence interval bigger than
328 the value itself and, therefore, expressed as not significant. In contrast, TCE showed a
329 stronger enrichment when degrading to *cis*-DCE.

330 *3.3. Laboratory microcosms amended with different biostimulants*

331 Three different microcosm treatments were prepared with groundwater material from
332 well MW-2 to test whether different fermentable organic compounds could enhance the
333 complete reductive dechlorination of PCE to ethene. This well was chosen for the
334 microcosms experiment because it was a candidate for the injection of amendments in a
335 foreseeable future *in situ* biostimulation pilot test.

336 The unamended microcosms used as natural biodegradation controls fully converted
337 PCE to *cis*-DCE by day 150, but the reaction remained stalled at this stage without ethene
338 formation (Figure S2A).

In the microcosms amended with a mixture of methanol plus ethanol, PCE was degraded within the first week to *cis*-DCE and it accumulated in the medium for 85 d (Figure S3A). At day 125, *cis*-DCE and VC disappeared from the medium. At day 139, the microcosm was spiked with 75 µM of TCE and, after 60 days, TCE, *cis*-DCE and VC were sequentially transformed, and ethene was detected (Figure S3A).

Microcosms amended with lactate also transformed PCE to *cis*-DCE within the first week and the latter accumulated in the medium. At day 63, VC was detected and at day 75 ethene was observed for the first time. After 125 d, ethene was the only remaining compound in the microcosms (Figure 3A).

To get an insight into the predominant fermentation pathways used by native microbial populations for the organic substrates selected, low-molecular-weight fatty acids (lactate, pyruvate, acetate, and formate) were monitored during the time-course experiments. Organic acids were not detected in the unamended microcosms (Figure S2B). In the microcosms amended with ethanol and methanol, approximately 3 mM of acetate was produced after 15 d and it remained in the medium without a significant decrease for approximately two months (Figure S3B). In the case of lactate-amended microcosms, lactate was fermented to near-stoichiometric amounts of acetate, which was slowly consumed in the microcosms and after 75 d it was completely depleted (Figure 3B).

A remarkable difference observed between the microcosms amended with lactate and the mixture of ethanol plus methanol was the vigorous generation of methane in the latter treatment. As depicted in Figure S4, methane was not detected in the control and it was barely produced in the lactate-amended microcosms but, in the treatment with alcohols, methane concentration remarkably increased after approximately 50 d without reaching a plateau in the monitored period.

3.4. Identification of native OHRB

Dhc are keystone bacteria for the detoxification of chlorinated ethenes to nontoxic ethene. PCR amplifications with *Dhc* 16S rRNA and the *vcrA* gene-targeted primers yielded diagnostic amplicons indicating that the enrichment culture inoculated with groundwater from MW-2 contained *Dhc* species implicated in the VC-to-ethene dechlorination (Figure S5).

4. Discussion

The presence of the degradation products of the hydrogenolysis pathway of PCE in the monitoring wells is a qualitative evidence for intrinsic anaerobic reductive dechlorination because PCE was the only degreasing solvent used on site (Table S1). The values of $\delta^{13}C_{sum}$ in 7 of 9 wells (Table S1) were within the same value (-31 ± 1‰) indicating that they all share the same source of contamination and that PCE biodegradation beyond *cis*-DCE was not significant in these seven wells (the balance would have become heavier as the production of lighter VC was not taken into account). In the same way, it discarded the existence of important alternate degradation pathways or production of unidentified by-products. On the other hand, the $\delta^{13}C_{sum}$ observed in the other two distal wells (MW-7 and Prof A), with an average value of $\delta^{13}C_{sum}$ of -26.2 ± 0.5‰ obtained from different chlorinated ethenes in each well, suggest that two different sources of PCE could have been leaked in this industrial area and they are statistically different from the rest (ANOVA, $p < 0.0009$). An additional indicator of *in situ* PCE biodegradation is provided by the concentration of chloride ions detected in the aquifer, which is between 3 and 30 times higher in the monitoring wells of the impacted aquifer compared to a non-impacted well (MW-4), which served as a background control (Table 1). Furthermore, measured

387 temperature and pH showed neutral and temperate shallow groundwater conditions which
388 are quite optimal for bioremediation purposes.

389 The extent of *in situ* PCE biodegradation could not be quantified using the site-specific
390 ε_C of PCE because the variation of $\delta^{13}C$ values obtained from the stablished microcosms
391 at different degradation stages of PCE was not linear. This fact leads us to the conclusion
392 that probably the degrading bacterial community was evolving differently in the
393 microcosm bottles which were sacrificed at different PCE degradation points. Several ε_C
394 values have been reported for the biodegradation of PCE under anoxic conditions in the
395 literature. They range from strong isotope fractionation (e.g. -16.7‰ by
396 *Desulfitobacterium* sp.) to very weak (-0.4 to -1.7‰) or even not significant isotope
397 fractionation (e.g. *Sulfurospirillum*, *Desulfuromonas* or *Geobacter* species all belonging
398 to ε-Proteobacteria, Table S3), and the reported ε_C of PCE during anaerobic reductive
399 dechlorination by *Dhc* isolates or *Dhc*-containing cultures ranges from -1.6 to -6.0‰
400 (Table S3). The non-linear low fractionation (<2‰) obtained in our enrichment is likely
401 a combination of the degradation of several bacterial species present in the aquifer with a
402 major contribution of non-fractionating species. These results differ a bit from the higher
403 and significant, according to the EPA guide (Hunkeler et al., 2008), isotopic shift for PCE
404 of 3.6‰ observed on-site among the different monitoring wells with equal isotopic
405 balance (Figure 2, excluding MW-7 and Prof A), suggesting that such shift could be due
406 to a higher extent of degradation than the one measured in the microcosm (>82%) or a
407 major activity of higher fractionating species depending on specific well conditions.
408 Moreover, the remarkable isotopic enrichments observed in the same wells for TCE
409 (8.1‰) and *cis*-DCE (5.3‰) point clearly to biodegradation processes (Figure 2,
410 excluding MW-7 and Prof A).

411 The detection of the biomarker genes implicated in the VC-to-ethene transformation
412 (Figure S5) indicated that the aquifer contained *Dhc* with the potential to detoxify PCE,
413 however, groundwater geochemistry exerted a primary control over anaerobic
414 dechlorination reactions. The presence of low concentrations of dissolved oxygen, the
415 relatively high concentrations of nitrate and sulphate, and the non-detection of iron (Table
416 1) indicate that the aquifer has mainly hypoxic to iron-reducing conditions, which is
417 thermodynamically appropriate to reduce highly chlorinated compounds such as PCE or
418 TCE to *cis*-DCE, but not to fully dechlorinate to harmless ethene (Bouwer, 2017). The
419 optimum redox potential for a complete reductive dechlorination is less than -100 mV
420 (Elsner and Hofstetter, 2011) but, as indicated in Table 1, no negative redox potentials
421 were measured in the monitored wells, although higher reducing microenvironments in
422 the aquifer cannot be discarded. As oxygen, nitrate, and sulphate are consumed, the redox
423 potential is expected to fall, but reduction of these electron acceptors can be hampered by
424 the lack of electron donor in groundwater (Yu et al., 2018). According to this hypothesis,
425 the addition of easily fermentable organic substrates (lactate and the mixture of ethanol
426 plus methanol) to the microcosms enhanced the dechlorination of PCE with respect to the
427 control by shortening the lag phase of PCE dechlorination and overcoming the "DCE
428 stall", which permitted the full dechlorination to ethene (Figure 3A and S3A).

429 Lactate, ethanol, and methanol can be potentially transformed by native bacteria using
430 different pathways (Fennell et al., 1997). Production of acetate and hydrogen from
431 fermentation reactions is preferred to stimulate growth of OHRB because they can serve
432 as carbon source and electron donor, respectively. The production of ~ 3 mM of acetate
433 in the microcosms with a mixture of ethanol and methanol was consistent with the
434 stoichiometric transformation of ethanol (3 mM) to acetate and hydrogen ($C_2H_6O + H_2O$
435 $\rightarrow C_2H_3O_2^- + H^+ + 2H_2$) and the fermentation of methanol to carbon dioxide and hydrogen

436 ($CH_4O + 2H_2O \rightarrow CO_2 + H_2O + 4H_2$) (Figure S3B). Similarly, the near stoichiometric
437 conversion of lactate to acetate observed in Figure 3B agrees with the fermentation
438 reaction $C_3H_5O_3^- + 2H_2O \rightarrow C_2H_3O^{-2} + HCO^- + H^+ + 2H_2$. The absence of short-chain
439 fatty acids in the microcosms used as controls corroborate that acetate was produced from
440 the organic amendments.

441 Acidity generated from fermentation reactions of the organic acids and dechlorination
442 reactions (i.e. HCl) can affect the success of the biodegradation of lesser chlorinated
443 ethenes (Christ et al., 2004). The successful reductive dechlorination of PCE to ethene
444 observed in the amended MW-2 microcosms shows that the aquifer was naturally well-
445 buffered (Table 1) and it can be assumed that pH was maintained within the range of 6-
446 8, which is described as optimal for dechlorinators (Yang et al., 2017a).

447 Similarly to our results, previous studies showed that methane production also
448 developed more slowly in lactate rather than ethanol-amended microcosms (Fennell et
449 al., 1997). Such pattern may be correlated with the amount of hydrogen released per mole
450 of ethanol and methanol, which is larger than that produced from lactate (Fennell et al.,
451 1997). In the present work, since the concentration of fermentable organic substrates was
452 higher in the microcosms with ethanol and methanol (6 mM) than in the lactate ones (3
453 mM), the high amount of hydrogen released in the microcosms amended with the alcohols
454 could have caused a rapid shift to methanogenic conditions and stimulate the activity of
455 hydrogenotrophic methanogens with the subsequent methane production.

456

457 ## 5. Conclusions

458 The use of an integrated approach that combined different complementary techniques
459 provided insights into the intrinsic biodegradation potential of a site contaminated with

chlorinated ethenes. The application of carbon stable isotopic balances and a statistical analysis of the results suggested that two sources of PCE were responsible for the contamination plume in this industrial area, but they have not been mixed, and that PCE has been transformed, in any case, via the hydrogenolysis pathway to *cis*-DCE. The identification of *Dhc* 16S rRNA and *vcrA* genes provided evidence of the aquifer potential to detoxify PCE to ethene. The geochemistry of the aquifer suggested that activity of *Dhc* in the *cis*-DCE stalled aquifer was impeded by the lack of sufficient electron donors to lower the redox potential, and it was further corroborated with the establishment of microcosms amended with fermentable substrates. The results obtained in this study discourage natural attenuation as a remediation strategy in this contaminated site due to the *cis*-DCE stall observed in microcosms miming the natural conditions of the aquifer (unamended controls). The two treatments with organic amendments (lactate and the mixture of ethanol plus methanol) accelerated the dechlorination of PCE and produced ethene, but methane was vigorously produced in the microcosm containing methanol and ethanol. In light of these results, an enhanced anaerobic bioremediation injecting lactate as electron donor is recommended to detoxify this particular contaminated site.

6. Acknowledgements

This research has been supported by the Spanish Ministry of Economy and Competitiveness, MINECO (CTM2013-48545-C2-1-R, CTM2016-75587-C2-1-R, CGL2014-57215-C4-1-R and CGL2017-87216-C4-1-R projects) co-financed by the European Union through the European Regional Development Fund (ERDF). This work was also partly supported by the *Generalitat de Catalunya* through the consolidate research groups (2017-SGR-14 and 2017SGR-1733) and N. Blázquez-Pallí's Industrial

Doctorate grant (2015-DI-064). M. Rosell acknowledges a Ramón y Cajal contract (RYC-2012-11920) from MINECO. The *Departament d'Enginyeria Química, Biològica i Ambiental* of the *Universitat Autònoma de Barcelona* is a member of the *Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya*. We thank Dr. Ivonne Nijenhuis for providing *Dehalococcoides mccartyi* BTF08 and CCiT-UB and Dr. Roger Puig for excellent technical assistance.

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686

687

688 **Table 1**. Hydrogeological and physicochemical parameters of the studied fully screened boreholes and groundwater samples. Numerical codes correspond
689 to monitoring wells depicted in Figure 1.

	MW-2	**MW-3**	**MW-4**	**MW-6**	**MW-7**	**PZ-1**	**PZ-3**	**PZ-5**	**PZ-13**	**Prof A**
Borehole depth (m)	7.0	6.0	8.0	7.2	8.0	6.8	6.0	11.2	8.0	15.0
WT (m.a.s.l.)	268.2	269.3	270.4	269.1	265.5	272.2	271.8	n.a.	268.1	271.1
T (ºC)	17.6	24.5	16.5	19.1	18.8	20.7	20.3	18.3	18.4	18.9
pH	7.4	7.8	7.5	7.2	7.7	7.2	7.5	7.3	7.4	7.3
EC (mS/cm)	2.8	1.5	1.1	1.5	2.0	3.4	1.4	4.3	2.2	3.4
DO (mg/L)	0.2	n.m.	n.m.	1.8	n.m.	1.2	1.0	2.0	1.5	n.m.
Eh (mV)	247	337	392	288	266	469	401	361	273	94
SO_4^{-2} (mg/L)	301.9	199.2	105.0	94.2	159.6	1435.1	182.4	179.2	181.7	144.3
NO_3^- (mg/L)	49.3	60.8	24.9	31.2	72.7	<20	130.5	41.3	116.8	<20
Fe (mg/L)	<0.1	<0.02	<0.02	<0.02	<0.02	<0.1	<0.02	<0.1	<0.02	<0.1
Cl^- (mg/L)	382.2	205.8	30.5	125.0	372.9	96.0	114.3	1053.0	368.6	711.3
HCO_3^- (mg/L)	605.3	251.9	585.3	652.3	260.1	543.4	351.3	369.1	364.5	605.3
Na (mg/L)	412.7	149.2	32.9	58.2	167.4	336.7	57.8	383.2	186.1	266.0
K (mg/L)	0.7	1.3	0.6	1.8	1.9	0.5	0.3	0.5	1.8	1.9
Ca (mg/L)	121.1	125.0	77.9	122.0	166.9	287.0	118.6	275.3	173.3	135.6
Mg (mg/L)	27.3	29.0	74.9	88.4	58.2	138.9	69.6	110.9	61.6	166.5

WT = water table, m.a.s.l. = meters above sea level, T = temperature, EC = electrical conductivity, DO = dissolved oxygen, Eh = redox potential, n.m. = not measured

690

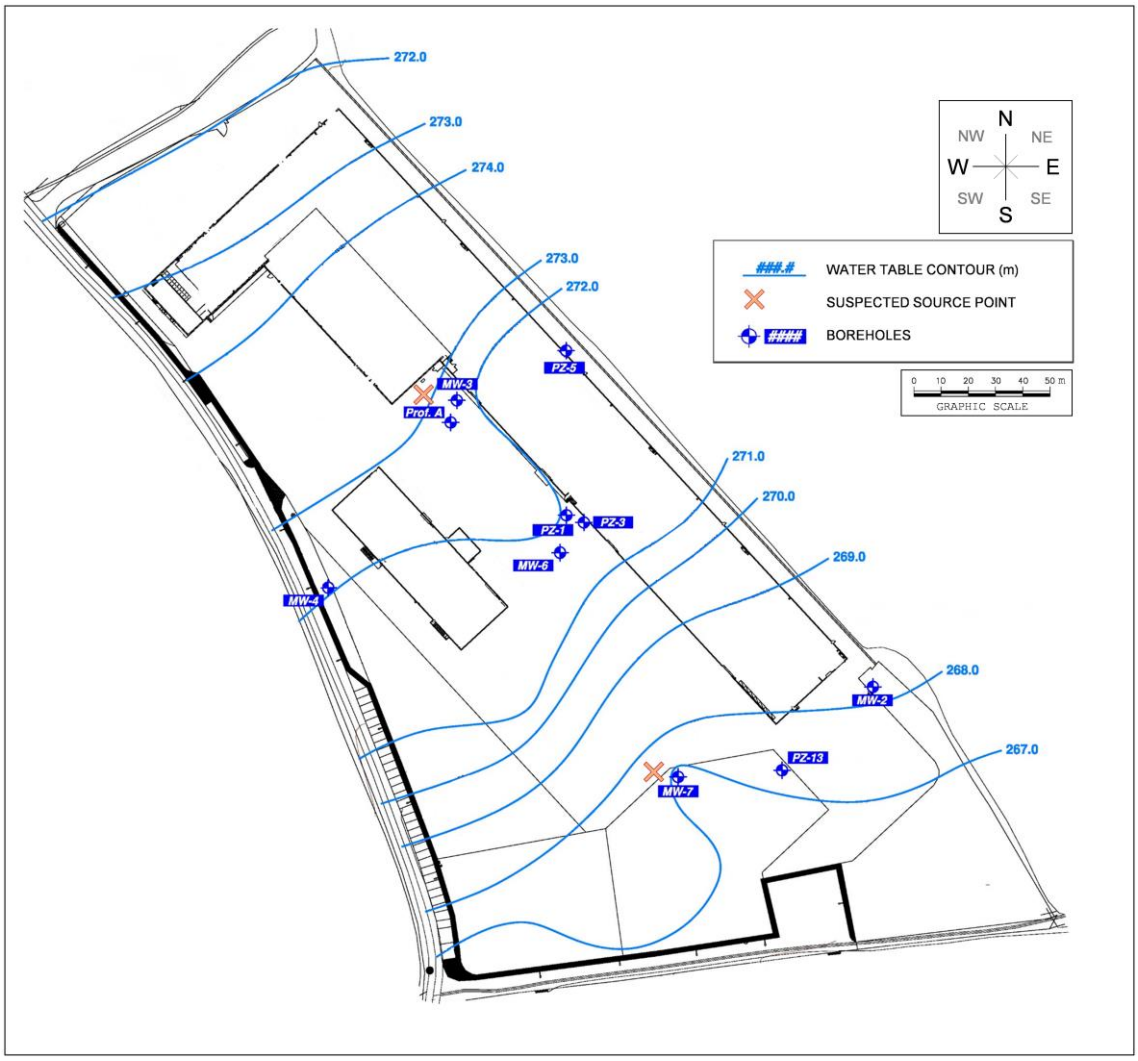


Figure 1. Head contour lines, groundwater flow direction, location of boreholes and suspected source points at the industrial contaminated area.

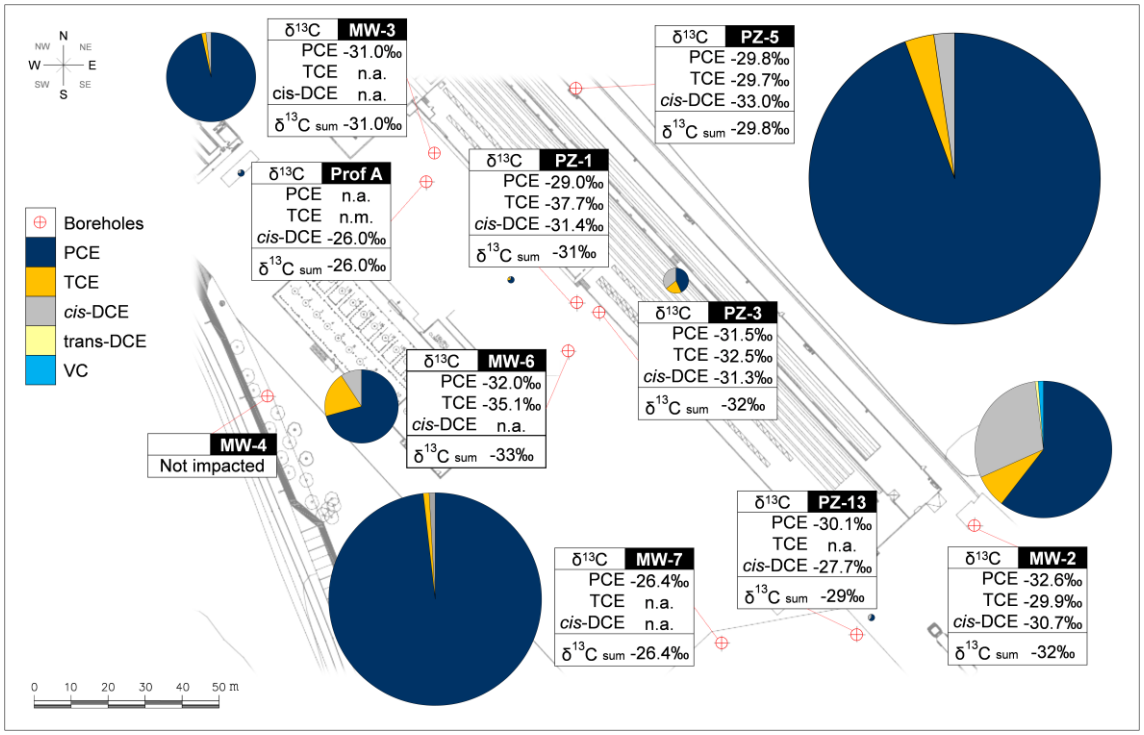
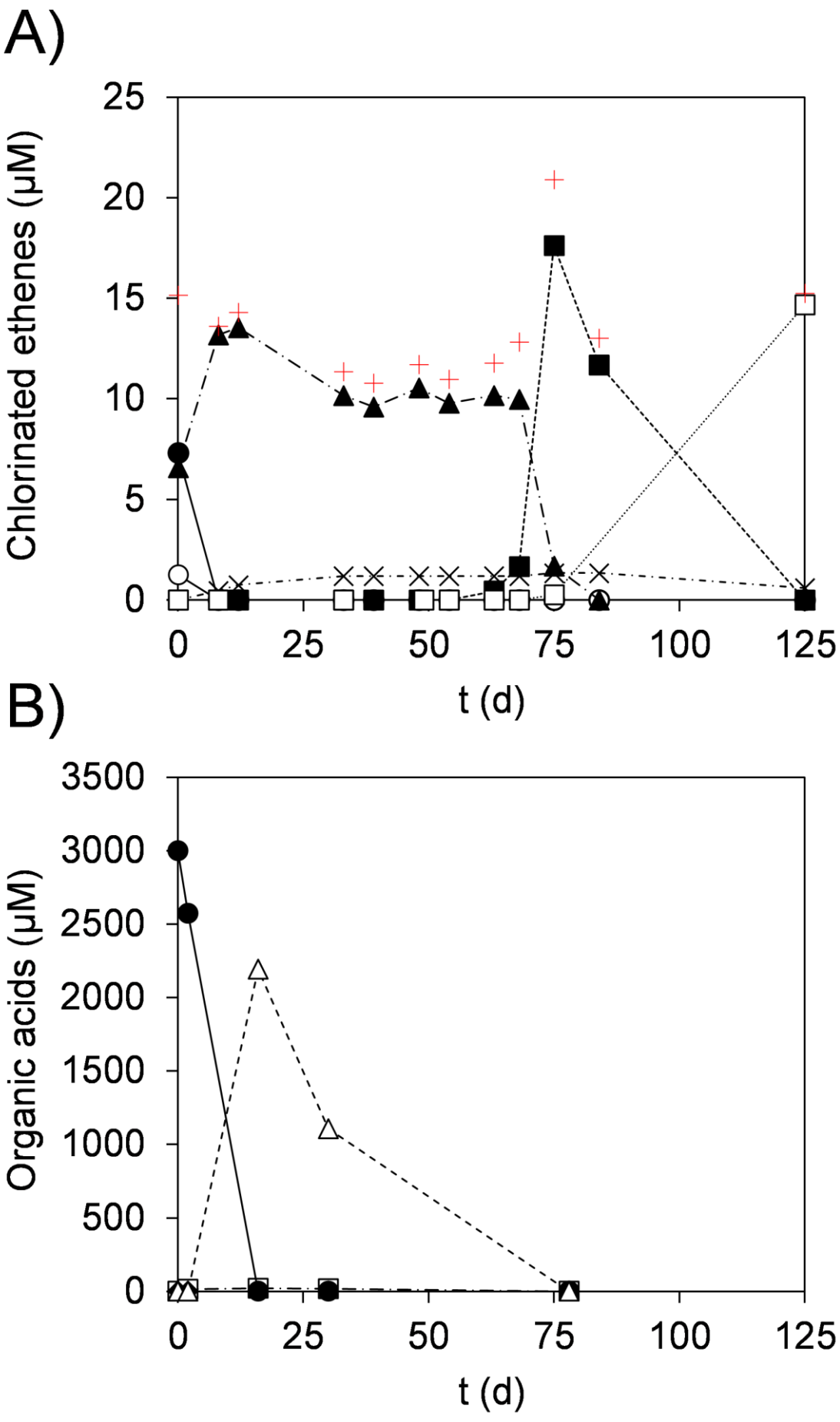


Figure 2. Molar concentrations distribution and carbon isotopic signatures ($\delta^{13}C$) of chlorinated ethenes at the industrial contaminated area. The pie charts are proportionally sized according to the total concentration of chlorinated ethenes in each well (from 2.8 to 82 µM). The carbon isotopic mass balance ($\delta^{13}C_{sum}$) included all chlorinated ethenes detected and it was calculated using Eq. 2. The numerical codes in the black rectangle indicate the name of the well. Detailed information about the concentration and $\delta^{13}C$ of chlorinated ethenes in all monitoring wells can be found in Table S1.



703 **Figure 3.** Time-course of reductive dechlorination of chlorinated ethenes (●: PCE, ○:
704 TCE, ▲: *cis*-DCE, ×: *trans*-DCE, ■: VC, □: ethene and ┼: sum of chlorinated ethenes
705 plus ethene, Panel A) and fermentation of lactate (●: lactate, □: pyruvate, Δ: acetate, ◆:
706 formate, Panel B) in a lactate-amended microcosm constructed with aquifer materials
707 from well MW-2. Concentrations of chlorinated solvents and ethene are presented as
708 nominal concentrations. Data presented is from an individual microcosm, but it is
709 representative of triplicate microcosms.

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