Carbon and Chlorine Isotope Fractionation During Microbial Degradation of Tetra- and Trichloroethene

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · MAY 2013

Impact Factor: 5.33 · DOI: 10.1021/es305236y · Source: PubMed

10 AUTHORS, INCLUDING:

Manolis Mandalakis
Hellenic Centre for Marine Research
53 PUBLICATIONS **1,281** CITATIONS
SEE PROFILE

Paraskevi Polymenakou
Hellenic Centre of Marine Research Crete
51 PUBLICATIONS **802** CITATIONS
SEE PROFILE

Christoph Aeppli
Bigelow Laboratory for Ocean Sciences
24 PUBLICATIONS **358** CITATIONS
SEE PROFILE

Jirina Machackova
Technical University of Liberec
13 PUBLICATIONS **144** CITATIONS
SEE PROFILE

Carbon and Chlorine Isotope Fractionation During Microbial Degradation of Tetra- and Trichloroethene

Charline Wiegert,[1] Manolis Mandalakis,[2] Tim Knowles,[3+] Paraskevi N. Polymenakou,[2] Christoph Aeppli,[1°] Jiřina Macháčková,[4] Henry Holmstrand,[1] Richard P. Evershed,[3] Richard D. Pancost[3] and Örjan Gustafsson[1]*

[1]Department of Applied Environmental Science (ITM), Stockholm University, 106 91 Stockholm, Sweden

[2]Hellenic Centre for Marine Research (HCMR), 71003, Heraklion, Crete, Greece

[3]School of Chemistry, University of Bristol, Bristol BS8 1TS, United Kingdom

[4]AECOM CZ s.r.o., Liberec 460 11, Czech Republic

[+] current address: Mass Spec Analytical Ltd., Building 20F, Golf Course Lane, Bristol, BS34 7RP

[°]current address: Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

[*] CORRESPONDING AUTHOR. Department of Applied Environmental Science (ITM), Stockholm University, Svante Arrhenius väg 8c, SE-106 91 Stockholm, Sweden. Tel.: +46 (0)8 674 7759. E-mail: charline.wiegert@itm.su.se

Abstract

Two-dimensional Compound Specific Isotope Analysis (2D-CSIA), combining stable carbon and chlorine isotopes, holds potential for monitoring of natural attenuation of chlorinated ethenes (CEs) in contaminated soil and groundwater. However, interpretation of 2D-CSIA data sets is challenged by a shortage of experimental Cl isotope enrichment factors. Here, isotope enrichments factors for C and Cl (i.e. ε_C and ε_{Cl}) were determined for biodegradation of tetrachloroethene (PCE) and trichloroethene (TCE), using microbial enrichment cultures from a heavily CEs contaminated aquifer. The obtained values were $\varepsilon_C = -5.6\pm0.7$‰ (95% CI) and $\varepsilon_{Cl} = -2.0\pm0.5$‰ for PCE degradation, and $\varepsilon_C = -8.8\pm0.2$‰ and $\varepsilon_{Cl} = -3.5\pm0.5$‰ for TCE degradation. Combining the values for both ε_C and ε_{Cl} yielded mechanism-diagnostic $\varepsilon_{Cl}/\varepsilon_C$ ratios of 0.35±0.11 and 0.37±0.11 for the degradation of PCE and TCE, respectively. Application of the obtained ε_C and ε_{Cl} values to a previously investigated field site gave similar estimates for the fraction of degraded contaminant as in the previous study, but with a reduced uncertainty in assessment of the natural attenuation. Furthermore, 16S rRNA gene clone library analyses were performed on three samples from the PCE degradation experiments. A species closely related to *Desulfitobacterium aromaticivorans* UKTL dominated the reductive dechlorination process. This study contributes to the development of 2D-CSIA as a tool for evaluating remediation strategies of CEs at contaminated sites.

Introduction

Chlorinated ethenes (CEs) are frequent soil and groundwater contaminants due to the extensive use of tetrachloroethene (PCE) and trichloroethene (TCE) as industrial solvents.[1] The CEs are sequentially biodegraded under anoxic conditions to the less chlorinated, but more toxic compounds dichloroethenes (DCEs) and vinyl chloride (VC), and eventually to harmless ethene.[2] This sequential reductive dechlorination involves bacteria, indigenous to the environment, which use the CEs as electron acceptors and H_2 or other compounds as the electron donor, to support their growth.[2] Several dehalorespiring bacteria from various genera have been shown to reduce PCE and TCE in pure, mixed and enriched cultures.[3,4]

Monitored Natural Attenuation (MNA) is a cost-effective alternative to traditional remediation methods, as it builds on the indigenous degradation activity in the soil and groundwater.[1,5] However, such in situ degradation requires accurate methods for site characterization, assessment and monitoring, which may not be possible using traditional concentration-based methods alone.[6] However, recent technical advances in compound-specific isotope analysis (CSIA) for chlorine isotopes ($\delta^{37}Cl$) now facilitate dual-isotope analysis (2D-CSIA), which allows for the simultaneous elucidation of the extent and mechanism of biodegradation.[7,8]

Degradation monitoring of CEs by CSIA relies on the kinetic isotope effect (KIE) resulting from sequential scission of chlorine atoms from the alkene carbon backbone during reductive dechlorination. The lighter stable isotope of carbon or chlorine (^{12}C and ^{35}Cl, respectively) forms weaker bonds than the heavier isotopes (^{13}C and ^{37}Cl) due to their higher zero-point energies, and thus react at faster rates.[9] Consequently, the residual reaction substrate typically becomes isotopically enriched, while the products become depleted in the heavier isotopes. The KIE is defined as the position-specific ratio of the reaction rates for a chemical element (e.g., for chlorine written as $KIE_{Cl} = {}^{35}k/{}^{37}k$) and is synonymous with the inverse of a position-specific isotope fractionation factor ($\alpha = KIE^{-1}$).[10] However, unlike α, the KIE is seldom used to report bulk (molecularly averaged) isotope fractionation. The basic

parameters used in CSIA degradation studies are summarized in the Rayleigh equation which describes the relationship between the observed isotope composition (R, the heavy-to-light isotope abundance ratio) and the remaining fraction (f) of the substrate compound, using the initial substrate isotope composition (R_0) and the reaction specific isotope fractionation factor α, often reported in the form of the isotope enrichment factor ε (see Elsner (2010)[7] and Hofstetter and Berg (2010)[11] for review papers):

$$R = R_0 \cdot f^{\alpha-1} = R_0 \cdot f^{\frac{\varepsilon}{1000}} \quad (1)$$

We recently reported the first field application of 2D-CSIA for PCE and TCE to demonstrate its potential to assess ambient biodegradation in a heavily contaminated aquifer.[12] The extent of in situ PCE degradation was estimated using C isotope enrichment factors (ε_C) from the literature, while a Cl isotope enrichment factor (ε_{Cl}) was inferred from the field derived $\varepsilon_{Cl}/\varepsilon_C$ ratios. While demonstrating the applicability of 2D-CSIA for the assessment of in situ degradation of CEs, this work also highlighted the lack of well-constrained ε_{Cl} values derived from laboratory experiments, which limited further interpretation of the 2D-CSIA data sets for PCE and TCE. Abe et al. reported laboratory-derived ε_C and ε_{Cl} for oxidation and reductive dechlorination of cDCE and VC.[13] The 2D-CSIA approach was then applied for cDCE degradation at a field site,[14] which underscored the need for ε_{Cl} values and better understanding of the microbial communities leading the degradation. Recent works described the first dual C and Cl data set for TCE and cDCE abiotic degradation by zero valent iron (ZVI) at a field site[15] and in laboratory experiments.[16] However, no laboratory-derived ε_{Cl} values for microbial PCE and TCE hydrogenolysis are available so far.

The purpose of this study was to: (i) determine the C and Cl isotopic enrichment factors during biodegradation of PCE and TCE, using a mixed bacterial culture from a previously investigated contaminated field site, and (ii) explore the changes in the microbial community over the course of PCE degradation. To our knowledge, this work provides the first combined C and Cl isotopes data set for biotic reductive dechlorination of PCE and TCE at the laboratory scale.

Materials and Methods

Site Description

The North Bohemian Carcass Disposal Plant (SAP; Mimoň, Czech Republic) was chosen for this study.[12] The factory used PCE for fat extraction from 1963 to 1988, when drinking water contamination was discovered at a water treatment plant 400 m downstream from the putative source area. The PCE consumption was estimated at 4250 tons, with a net contamination of 149−246 tons. Our recent field study focused on a newly discovered area of the plume that had not been subject to cleanup activities.[12] The 2D-CSIA investigation, combining $\delta^{13}C$ and $\delta^{37}Cl$, revealed ongoing PCE degradation in anoxic areas, although not significant enough to allow MNA as an efficient remediation strategy. Furthermore, concentration data also suggested cDCE accumulation.

Soil Sampling

Three soil cores (Z−32, Z−53, Z−54) were obtained from the contaminated zone of the site, each exhibiting different levels of PCE contamination, while a fourth core (Z−65) was collected at a non-contaminated background location (Table S1). Two soil cores of 0.7 m length each were obtained and subsequently pooled, from each location. Two different depths below the groundwater table were sampled, using a percussion drilling set (Eijkelkamp, Giesbeek, the Netherlands). The soil was covered by a plastic sleeve during drilling to avoid any exposure to the atmosphere after extraction from the subsurface. An additional cover was applied on the sample immediately after retrieval.

Degradation Experiments

All four soil samples were initially screened for their capacity to degrade chlorinated ethenes. For this purpose, separate cultures were established using a reduced anaerobic mineral medium, which was prepared and sterilized according to Cole et al.[17] In brief, each soil (about 5 g) and Cole's basal medium (100 mL) were placed in a 120-mL glass serum bottle, while its headspace was continuously flushed with a stream of N_2. Immediately after transfer, the bottles were tightly sealed with Viton rubber stoppers and each culture was spiked with PCE stock solution (100 µL; ~500 $mmol \cdot L^{-1}$ in methanol) using a gas-tight syringe to achieve an initial concentration of 500 $\mu mol \cdot L^{-1}$. Preliminary experiments

indicated that PCE dissolution in the culture medium was accomplished within a few hours. The maintenance of anaerobic conditions during the preparation of cultures and throughout the biodegradation experiments was verified by using resazurin in the nutrients medium as a redox color indicator. A mixture of butyric acid, propionic acid and ethanol was also added as electron donor and carbon source (final concentration 200 $\mu mol \cdot L^{-1}$ each) and the bottles were incubated at 30 °C without shaking. Note that in heterogeneous systems mass transfer limitations might lead to masking of isotopic effects.[18] However, in our homogeneous system, we did not observe indication of such masking effect, even in the absence of shaking. By using a gas-tight syringe, small aliquots (50 µL) were collected at regular time intervals and the progress of the biodegradation process in each culture was assessed by analyzing the concentration of PCE using an in-vial microscale liquid-liquid extraction method, followed by gas chromatography mass spectrometry (GC-MS). A detailed description of the concentration analysis is provided in the Supporting Information (SI).

During the initial screening, biodegradation activity was evident in the soil samples Z-32, Z-53 and Z-54, as the concentration of PCE substantially decreased after 15 to 20 days of incubation. In contrast, the soil from the non-contaminated background site Z-65 showed no tendency of biodegradation (data not shown). The soil enrichment culture from Z-32, which exhibited the fastest biodegradation rate among all samples, was selected for the inoculation of secondary cultures and the implementation of the final series of biodegradation experiments for PCE and TCE. By following the same procedure as described above, up to twelve identical cultures were prepared for each series of experiments by mixing 100 mL of fresh Cole's medium with 10 mL of cell suspension from the primary culture Z-32. After sealing with Viton stoppers, the bottles from each one of the two series were spiked with the stock solution of PCE or TCE in methanol to reach an initial concentration of 400 or 500 $\mu mol \cdot L^{-1}$, respectively. The progress of biodegradation was monitored in all replicate cultures by analyzing PCE or TCE at regular time intervals. See section S2 in SI for a detailed description of the concentration analyses.

The cultures were sacrificed at a PCE or TCE remaining fraction f ranging from 100 to 5%, by adding 400 µL of concentrated HCl to stop bacterial activity (pH adjusted to 2). Two control samples were

134 taken for each series of biodegradation experiments, by spiking with HCl immediately after their
135 preparation, i.e. samples with f = 100%.
136 Samples for $\delta^{37}Cl$ analyses were shipped to Stockholm University (SU), and an aliquot from each
137 sample was forwarded to the University of Bristol (UB) for $\delta^{13}C$ analyses. The samples were stored in
138 the dark at 4°C until further analysis.
139 *DNA extraction*
140 A total of three samples, corresponding to 100%, 53% and 7.6% remaining fraction of PCE were
141 chosen to characterize the microbial community evolution concomitant to the degradation reaction,
142 using 16S rRNA gene clone library analysis. DNA extraction and PCR amplification was not successful
143 for the TCE experiments and is not reported in the following.
144 Total DNA was extracted from the PCE degrading cultures using the FastDNA-Spin Kit for Soil (Q-
145 BIOgene, Carlsbad, CA). Aliquots of 1 mL were distributed to individual Lysing Matrix tubes included
146 in the extraction kit. DNA extraction was performed according to the manufacturer's protocol and the
147 cell lysis was achieved using a Qiagen TissueLyser II (Retsch GmbH, Haan, Germany). DNA extracts
148 were stored at -80°C until analysis. Nucleic acid extracts from each sample were analyzed
149 spectrophotometrically at 260 and 280 nm using a Nanodrop ND-1000 3.3 spectrophotometer (Coleman
150 Technologies Inc.).
151 *Clone Library Construction and Sequence Analysis of the 16S rRNA genes*
152 Bacterial 16S rRNA genes were amplified from mixed genomic samples by using PCR with the
153 universal bacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-
154 GGYTACCTTGTTACGACTT-3') for the PCE degradation experiments. The detailed protocol is
155 described in SI (section S3).
156 Operational taxonomic units (OTUs) were defined at a minimum sequence similarity of 98%. A total
157 of 44 different OTUs were identified. All 44 partial 16S rRNA gene sequences generated in the present
158 study were deposited in GenBank under accession numbers KC109145-KC109188.
159 *Stable Chlorine Isotope Analysis*

160 The selection and use of PCE and TCE standards as well as the instrumentation and procedure used
161 for $\delta^{37}Cl$ determination followed previously established methods.[12,19] Briefly, sample volumes of 50 µL
162 to 1 mL were extracted with 0.5−2 mL cyclopentane in 4-mL glass vials. These volumes were selected
163 to achieve consistent PCE and TCE concentrations of at least 1 $\mu mol \cdot L^{-1}$ in the solvent. The extracts
164 were shaken for 2 min on a vortex shaker and dried over sodium sulfate.

165 Analyses of $\delta^{37}Cl$ of PCE and TCE were performed according to a previously published method, 12,[19]
166 using the same instrument (GCqMS; GC 8000 gas chromatograph with MD-800 mass analyzer, Fisons,
167 Manchester, UK) and authentic isotopic standards, i.e. the same compounds as the analytes. PCE and
168 TCE were measured on masses of two molecular ions containing zero and one ^{37}Cl, respectively, i.e. *m/z*
169 130 and 132 for TCE, 164 and 166 for PCE.

170 All $\delta^{37}Cl$ values are reported relative to the international Standard Mean Ocean Chlorine (SMOC). To
171 this end, the $\delta^{37}Cl$ values of the PCE and TCE isotopic standards were determined vs SMOC using
172 thermal ionization mass spectrometry (TIMS) according to published procedures.[19,20] The trueness of
173 the instrument was tested with PCE and TCE standards spanning −2.5 to +2.9‰ vs SMOC (Figure S1).
174 This test demonstrated that one isotopic standard with known $\delta^{37}Cl$ was sufficient for determining $\delta^{37}Cl$
175 values, rather than two isotopic standards as were necessary for certain instrumental setups.[21] Note, that
176 due to limited availability of $\delta^{37}Cl$ isotopic standards, we were not able to cover the full range of the
177 samples' $\delta^{37}Cl$ values, which were between −0.9±0.7‰ to 7.5±0.8‰ for PCE, and between 3.0±0.5‰
178 and 16.6±0.5‰ for TCE (Table S3). Although it is in principle possible that such values outside the
179 range of available isotopic standards could suffer from additional uncertainties, it is reasonable to
180 assume that the determined range of $\delta^{37}Cl$ trueness extends in a linear way.[22,23] In future studies, cross-
181 calibration with other laboratories could be integrated as well as standardization through availability of
182 $\delta^{37}Cl$ authentic material. See Table S3 for a list of used isotopic standards. All standards were stored in
183 the dark at 4°C.

184 The obtained average analytical precision of the $\delta^{37}Cl$ analysis was ±0.6‰ vs SMOC. This includes
185 the standard deviation from the GCqMS measurements (n = 5 sample/standard pairs) and the propagated
186 standard deviation from the TIMS measurements of the authentic standards.

187 *Stable Carbon Isotope Analysis*

188 Liquid-liquid extractions were performed in the same manner as for $\delta^{37}Cl$ analysis. The $\delta^{13}C$ analyses
189 were performed using previously described protocols and instrumentation (GC combustion
190 isotope−ratio mass spectrometry GC−C−IRMS).[12] The measurements were performed on a HP 6890 GC
191 with split/splitless injector, hyphenated to a Thermo DeltaPlusXL spectrometer via a Thermo GC/C-III
192 interface (HP, Palo Alto, California, United States; Thermo Finnigan, Bremen, Germany). The average
193 Standard Deviation (SD) of the $\delta^{13}C$ analysis was ±0.4‰, and was determined by replicate injections of
194 target compound (n = 3)

Results and Discussion

Degradation of PCE and TCE by enriched soil bacteria cultures

The microbial consortium dechlorinated PCE via TCE to cDCE, and TCE to cDCE, after a lag phase of 9 and 17 days, respectively (illustrated in Figure 1). No further degradation of cDCE was observed. This is in agreement with the previous observations from the field site, showing cDCE accumulation and no production of vinyl chloride or ethene.[12]

Degradation rates were evaluated from the incubation experiments showing $f < 50\%$ of the initial PCE or TCE. Due to their relatively long incubation time (compared with e.g. experiments with $f > 50\%$), these were the samples with the most data for concentrations over time and the most suitable data sets for fitting of pseudo first-order kinetics. The best fits of the pseudo first order kinetic equation to the obtained data yielded PCE degradation rates ranging from 0.3 to 0.5 day^{-1} in cultures with a specific growth rate (SGR) of 0.3−0.74 $g \cdot g^{-1} \cdot h^{-1}$. The same approach applied to TCE degradation experiments led to a range of 0.4−1.9 day^{-1} for SGR 0.4−2 $g \cdot g^{-1} \cdot h^{-1}$. These ranges fall in the spectrum of previously reported values of 0−0.410 day^{-1} and 0−3.130 day^{-1} for reductive dechlorination of PCE and TCE respectively, at both the laboratory and the field scale.[24]

Microbial Community Changes during PCE Reductive Dechlorination

Clone library analysis of the 16S rRNA genes was used to determine potential changes in the microbial community composition during PCE reductive dechlorination and to identify major microorganisms mediating this process. Before the initiation of PCE degradation, i.e. at 100% remaining fraction, the microbial culture is dominated by an OTU closely related to *Clostridium* sp. strain DR7 (Table S2). In this culture, a total of 55 out of 72 analyzed sequences corresponded to the specific strain, while the other 17 sequences were assigned to 13 different OTUs that were classified into five phyla.

At the intermediate stage of PCE reduction, i.e. at 53% remaining fraction, the microbial community composition was also dominated by the OTU closely related to *Clostridium* sp. strain DR7. In addition, a second OTU closely related to *Desulfitobacterium aromaticivorans* UKTL was found with a high

numbers of clones. More specifically, 51 and 10 out of 80 analyzed sequences corresponded to *Clostridium* sp. strain DR7 and *Desulfitobacterium aromaticivorans* UKTL, respectively, while the other 19 sequences were assigned to 14 different OTUs that were also classified into five phyla (Table S2).

The microbial community at the final stage of PCE reduction, i.e., at 7.6% remaining fraction, was still dominated by close relatives of *Clostridium* sp. strain DR7 and *Desulfitobacterium aromaticivorans* UKTL. However, these two organisms were present at substantially different proportions compared to the intermediate stage. In particular, *Desulfitobacterium aromaticivorans* UKTL became more abundant than *Clostridium* sp. strain DR7, with 34 and 22 out of 74 sequences respectively.

The results show that these two species, which belongs to the taxonomic order of Clostridiales, are involved in PCE reductive dechlorination at the study field site. *Desulfitobacterium aromaticivorans* UKTL is closely related to *Desulfitobacterium chlororespirans* and *Desulfitobacterium dehalogenans*, which are known dechlorination bacteria.[25,26] Generally, the members of the genus *Desulfitobacterium*, are widely known for reductive dehalogenation activity.[27] The second prominent species is closely related to *Clostridium* sp. strain DR7. This species is known for fermentation processes. *Clostridium* spp. are often detected in dechlorinating communities,[28,29] and associated with anaerobic processes other than PCE dechlorination, such as acetogenesis, or fermentation. The later process lead to the production of H_2 that might be used by dechlorinating bacteria as electron donor. [30,31] This might explain the observed increase in *Desulfitobacterium* spp. that occurs only after a lag phase; these organisms only start to dechlorinate (and grow) once a sufficient level of H_2 is present.

Carbon and Chlorine Isotope Fractionation during PCE Reductive Dechlorination

PCE isotopic signatures were measured at different stages of degradation, to determine the C and Cl enrichment factors for the enriched microbial culture, by applying the Rayleigh equation (eq. 3) to the concentration and 2D-CSIA data (Figure 2A).[32] The $\delta^{13}C$ signatures exhibited values from −25.8±0.1‰ to −7.0±0.7‰ at 100% and 3.5% remaining fraction, respectively (Table S4). For PCE, an ε_C value of

246 −5.6±0.7‰ (95% confidence interval, CI; n = 11, R^2 = 0.96, the standard error, SE, was 0.3‰) was
247 determined.

248 The herein obtained ε_C value is comparable to those obtained for other experiments with mixed
249 consortia, e.g. enriched mixed cultures from contaminated aquifers, yielding ε_C in the range −2‰[33] to
250 −7‰,[34] whereas further comparisons with results from, e.g. pure strains are more difficult to the
251 inherent complexity of our enrichment culture. However we note the difference from abiotic processes,
252 that yielded much higher fractionation behavior with ε_C up to −16.5‰ for reduction by vitamin B12[35]
253 and −25.3‰ for degradation on zero-valent iron.[36]

254 As for the Cl isotopes, an enrichment was observed with δ^{37}Cl values increasing from 1.01±0.6‰ to
255 7.5±0.8‰ at 100% and 3.5% remaining fraction, respectively (Table S4). The ε_{Cl} value was −2.0±0.5‰
256 (95% CI, n = 10, R^2 = 0.91, with 0.2‰ SE; Figure 2B). Numata et al. (2002)[37] first reported Cl isotopes
257 fractionation factors for the reductive dechlorination of PCE to cDCE, with significantly more negative
258 ε_{Cl} values of −9‰, −10‰ and −13‰ for three different anaerobic consortia. However, it is worth noting
259 that these authors used non-compound specific isotopic measurements and therefore relied on a complex
260 Rayleigh-model evaluation scheme, which might be associated with larger uncertainties due to the lack
261 of compound-specific information. Furthermore, the mixed consortia cannot be compared with the
262 diversity of field-derived enrichment cultures, which represent the microbial response to a mix of many
263 controls imposed by a heterogeneous system. The closest comparable ε_{Cl} is our previous field-derived
264 estimate of the in situ $\varepsilon_{Cl,}$ which spanned −0.8 to −7.8‰.[12] Although not directly comparable due to
265 influences from the microbial enrichment process, our laboratory ε_{Cl} likely represents a reduction in the
266 uncertainty at this field site.

267 We calculated the process diagnostic $\varepsilon_{Cl}/\varepsilon_C$ ratio by combining the 2D-CSIA data for C and Cl
268 isotopes. The $\varepsilon_{Cl}/\varepsilon_C$ ratio for the herein reported PCE reductive dechlorination is 0.35±0.11 (95% CI, n =
269 10, R^2 = 0.87, with 0.05‰ SE; Figure 2C), which is at the lower end of the range of our previous field-
270 derived values from 0.42 to 1.12.[12] We also calculated the ratio of the apparent kinetic isotope effects

(AKIE; see Elsner and Hunkeler, 2008)[32] to obtain a diagnostic measure of the mechanism at hand, by removing the influence of non-reactive positions and intramolecular competition of isotopes. Subtraction of one (1) from the AKIEs has been recommended to cancel out any influence of commitment to catalysis.[13] The $(AKIE_{Cl}-1)/(AKIE_{C}-1)$ ratio for the PCE degradation experiment was 0.71 (Figure 3; Table S5).

Carbon and Chlorine Isotope Fractionation during TCE Reductive Dechlorination

The enrichment in $\delta^{13}C$ signatures for the TCE degradation ranged from −26.9±0.01‰ to −2.1±0.2‰ at 100% and 3.5% remaining fraction, respectively (Table S3). The corresponding ε_C value was −8.8±2.0‰ (95% CI, n = 10, R^2 = 0.92, with 0.9‰ SE) (Figure 2D). Our ε_C value falls within the range of literature values for enriched mixed cultures from contaminated aquifers, i.e. −2.5‰[38] to −16.0‰.[39] Values obtained using pure cultures span a range of −3 to −18.8‰.[40] The herein reported value is also similar to a literature value of −7.9‰ for the specific abiotic degradation of TCE to cDCE by zero valent iron (ZVI).[16] However, most studies of abiotic transformation of TCE report larger carbon isotope fractionation with ε_C values in the range −17.2‰ (with vitamin B12),[35] to −26.5±1.5‰ (with nanoparticulate Fe^{2+}).[41]

For Cl isotopes, $\delta^{37}Cl$ values increased from 5.2±0.54‰ to 16.6±0.51‰ at 100% and 3.5% remaining fraction, respectively (Table S4). The determined ε_{Cl} value was −3.5±0.5‰ (95% CI, n = 10, r^2 = 0.97, with 0.2‰ SE; Figure 2E). The obtained ε_{Cl} value is similar to the values of −2.6±0.2‰ and −2.98‰ reported for abiotic degradation of TCE by ZVI in two recent studies.[15,16]

The process diagnostic $\varepsilon_{Cl}/\varepsilon_C$ ratio for microbial TCE hydrogenolysis was 0.37±0.11 (95% CI, n = 10, r^2 = 0.88, with 0.04 SE; Figure 2F), with a $(AKIE_{Cl}-1)/(AKIE_{C}-1)$ ratio of 0.59. The observed differences in $(AKIE_{Cl}-1)/(AKIE_{C}-1)$ ratios between PCE (0.71) and TCE (0.59) could potentially be due to rate limiting but non-fractionating pre-equilibrium steps, e.g. isotopic masking through commitment to catalysis or differences between the PCE and TCE enrichment cultures, if the same reaction mechanism is assumed in both cases.[10] A common dissociative electron transfer has indeed

been suggested for the PCE and TCE degradation using cobalamin as model for the catalytic center of dehalogenation enzymes.[42] The large difference between the herein obtained $(AKIE_{Cl}-1)/(AKIE_{C}-1)$ ratios and that of the biotic transformation of cDCE to VC, i.e. 0.08 (Figure 3; Table S5),[43] suggest that another mechanism is involved in the degradation of these two compounds. This hypothesis is supported by mechanistic investigations. For cobalamin, it has been found that these compounds indeed follow a different reaction mechanism than PCE and TCE. cDCE and VC form a carbon-cobalt bond as initial reaction step rather than a dissociative electron transfer reaction.

Application of incubation derived ε_C and ε_{Cl} to the SAP Field Site Data Set

The remaining fraction (f) of PCE at the SAP wells (n=11) was re-calculated by applying the herein obtained ε_C and ε_{Cl} values to the SAP $\delta^{13}C$ and $\delta^{37}Cl$ data.[12] Three wells gave negative estimates of f, presumably due to seasonally fluctuating anaerobic/aerobic conditions. Thus, they were depleted in ^{37}Cl relative to the designated contaminant source zone, and where therefore excluded from further analysis. The use of chlorine isotope data led to a seemingly slightly higher average estimate (f = 32±21%; 1 SD) than that obtained with the carbon isotope data (f = 16±10%; 1 SD). However, the average residual of f_{Cl}-f_C was 10%, ranging from 4-17% when the two-sided 95% confidence interval for ε_C and ε_{Cl} was taken into consideration. Hence, there is reasonable agreement between these two independent estimates considering the inherent variability of the natural system. It is further possible that any apparent difference in the estimates between the two isotope systems simply reflect instrument bias (e.g. combustion efficiency in the GC-IRMS interface during conversion of organochlorine molecules to CO_2, or small non-linearity effects in GCqMS) for one or both of the used isotope instruments, or small offsets in the calibrations used for isotope analysis. To illustrate, a single sided off-set in the $\delta^{37}Cl$ values by 0.4‰, corresponding to a typical analytical uncertainty, reduces the residual average to zero due to the small value of ε_{Cl}. This could, for instance, be induced by the marginal deviation, which is not statistically significant, from the 1:1 line of the regression line in the linearity plot (Figure S1). In conclusion, future studies are warranted to perform cross-calibrations for two or more isotope systems to obtain the highest possible accuracy in multi-dimensional CSIA field-site investigations. Furthermore

322 the availability of authentic standard with a large $\delta^{37}Cl$ range would improve the confidence in studies
323 of this type.

324 *Implications*

325 We performed a 2D CSIA investigation to address two aspects of PCE and TCE dechlorination. First,
326 this study shows that 2D CSIA assessment has the potential to distinguish reaction mechanisms, based
327 on the characteristic $\varepsilon_{Cl}/\varepsilon_C$ or $(AKIE_{Cl}\text{-}1)/(AKIE_C\text{-}1)$ ratios. Our empirical results support earlier
328 hypotheses that microbial PCE and TCE hydrogenolysis follows a dissociative electron transfer as first
329 reaction step, while cDCE and VC hydrogenolysis follows a different reaction pathway (i.e., formation
330 of a Co-C bond).

331 Second, we aimed to improve confidence in CSIA-based assessment of in situ natural attenuation by
332 introducing an additional line of evidence. This is based on the idea that appropriate ε values can be
333 chosen based on the characteristic $\varepsilon_{Cl}/\varepsilon_C$ or $(AKIE_{Cl}\text{-}1)/(AKIE_C\text{-}1)$ ratios that different dehalogenation
334 bacteria exhibit. To this end, our study is a first step towards creating a library of such ratios.
335 Furthermore, our values determined in the laboratory were in the range of values determined at the field
336 site where the used microbial enrichment culture originated. Although this result generally supports the
337 validity of the concept of using laboratory-enrichment cultures to investigate PCE/TCE dechlorination
338 processes occurring at field sites, we saw more variability in the field data than in the laboratory
339 experiment. This was in spite of the fact that the 2D approach is expected to correct for physical
340 processes influencing isotopic fractionation such as sorption, dilution and isotopic masking due to pre-
341 equilibrium steps. The reason for the observed variability in $\varepsilon_{Cl}/\varepsilon_C$ in the field data is most likely the
342 variability in microbial community at the field site. It is worth noting that by the choice of the medium,
343 electron donor and carbon source, selective pressure is introduced during the microbial cultivation
344 process, thereby reducing the microbial diversity.

345 This study suggests that the presented diagnostic $(AKIE_{Cl}\text{-}1)/(AKIE_C\text{-}1)$ ratio is indicative for
346 *Desulfitobacterium* spp. that are able to reduce PCE and TCE to cDCE. Other known dehalogenating
347 bacteria, especially *Sulfurospirillum* spp. (known for PCE-to-cDCE dehalogenation) and

348 *Dehalococcoides* spp. (PCE-to-ethene dehalogenation) may exhibit different $\varepsilon_{Cl}/\varepsilon_{C}$ ratios, and need to be
349 investigated as a next step to facilitate 2D-CSIA-based assessment of PCE/TCE-contaminated field
350 sites.
351

352 **ACKNOWLEDGMENT**

353 This study received funding from the European Community´s Seventh Framework Programme (FP7
354 2009-2012) isoSoil project, under grant agreement No. 212781. The work at SU was further supported
355 by the Delta Facility of the Faculty of Science. CA acknowledges a postdoctoral fellowship from the
356 Swiss National Science Foundation. RPE and RDP acknowledge the Royal Society Wolfson Research
357 Merit Awards. ÖG acknowledges support as an Academy Research Fellow at the Swedish Royal
358 Academy of Sciences through a grant from the Knut and Alice Wallenberg Foundation. Petr Dostal,
359 Monika Kralova and Monika Stavelova (Aecom) are gratefully acknowledged for the soil sampling. The
360 authors thank Yngve Zebühr (SU) for his helpful technical support concerning the GCqMS
361 measurements and Heike Siegmund at SIL (SU) for $\delta^{13}C$ determination of the PCE and TCE standards.
362 We thoroughly thank three anonymous reviewers for their in-depth comments on the manuscript.

363 **Supporting Information Available**

364 The groundwater data, a detailed description of the CEs concentration analyses run during the
365 degradation experiments, the protocol used for the clone library construction, details about the
366 calibration approach used for $\delta^{37}Cl$ measurements, a table with the CEs concentrations and stable C and
367 Cl isotopic data set, as well as a table comparing the ε and AKIE values for C and Cl isotopes in
368 different studies on CEs degradation.

369 This material is available free of charge via the Internet at http://pubs.acs.org.

370 The authors declare no competing financial interest.

371 **References**

372 (1) Committee on Intrinsic Remediation, Water Science and Technology Board, Board on
373 Radioactive Waste Management, National Research Council (NRC). *Natural Attenuation for*
374 *Groundwater Remediation*; National Academy Press: Washington, D.C., 2000.

375 (2) Wiedemeier, T. H.; Newell, C. J.; Rifai, H. S.; Wilson, J. T. *Natural attenuation of fuels and*
376 *chlorinated solvents*; John Wiley & Sons Inc.: New York, USA, 1999.

377 (3) Löffler, F. E.*;* Cole, J. R.*;* Ritalahti, K. M.*;* Tiedje. J. M. Diversity of Dechlorinating Bacteria. In
378 Häggblom M.M., and Bossert I.D. (Eds)*, Dehalogenation: Microbial Processes and Environmental*
379 *Applications;* Kluwer Academic Publishers: Boston/Dordrecht/London, 2003.

380 (4) Futagami, T.; Goto, M.; Furukawa, K. Biochemical and genetic bases of dehalorespiration.
381 *Chem Record* **2008**, *8*, 1–12.

382 (5) Mulligan, C. N.; Yong, R. N. Natural attenuation of contaminated soils. *Environment*
383 *International* **2004**, *30*, 587–601.

384 (6) Bombach, P.; Richnow, H. H.; Kästner, M.; Fischer, A. Current approaches for the assessment
385 of in situ biodegradation. *Applied Microbiology and Biotechnology* **2010**, *86*, 839–852.

386 (7) Elsner, M. Stable isotope fractionation to investigate natural transformation mechanisms of
387 organic contaminants: principles, prospects and limitations. *J. Environ. Monit.* **2010**, *12*, 2005.

388 (8) Cincinelli, A.; Pieri, F.; Zhang, Y.; Seed, M.; Jones, K. C. Compound Specific Isotope Analysis
389 (CSIA) for chlorine and bromine: A review of techniques and applications to elucidate environmental
390 sources and processes. *Environmental Pollution* **2012**, *169*, 112–127.

391 (9) Criss, R. E. *Principles of Stable Isotope Distribution;* Oxford University Press, Inc.: New York,
392 USA, 1999.

(10) Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P. A New Concept Linking Observable Stable Isotope Fractionation to Transformation Pathways of Organic Pollutants. *Environ. Sci. Technol.* **2005**, *39*, 6896–6916.

(11) Hofstetter, T. B.; Berg, M. Assessing transformation processes of organic contaminants by compound-specific stable isotope analysis. *TrAC Trends in Analytical Chemistry* **2010**.

(12) Wiegert, C.; Aeppli, C.; Knowles, T.; Holmstrand, H.; Evershed, R. P.; Pancost, R.; Macháčková, J.; Gustafsson, O. Dual carbon-chlorine stable isotope investigation of sources and fate of chlorinated ethenes in contaminated groundwater. *Environ. Sci. Technol.* **2012**.

(13) Abe, Y.; Aravena, R.; Zopfi, J.; Shouakar-Stash, O.; Cox, E.; Roberts, J. D.; Hunkeler, D. Carbon and Chlorine Isotope Fractionation during Aerobic Oxidation and Reductive Dechlorination of Vinyl Chloride and cis-1,2-Dichloroethene. *Environmental Science & Technology* **2009**, *43*, 101–107.

(14) Hunkeler, D.; Abe, Y.; Broholm, M. M.; Jeannottat, S.; Westergaard, C.; Jacobsen, C. S.; Aravena, R.; Bjerg, P. L. Assessing chlorinated ethene degradation in a large scale contaminant plume by dual carbon–chlorine isotope analysis and quantitative PCR. *Journal of Contaminant Hydrology* **2011**, *119*, 69–79.

(15) Lojkasek-Lima, P.; Aravena, R.; Shouakar-Stash, O.; Frape, S. K.; Marchesi, M.; Fiorenza, S.; Vogan, J. Evaluating TCE Abiotic and Biotic Degradation Pathways in a Permeable Reactive Barrier Using Compound Specific Isotope Analysis. *Groundwater Monit R* **2012**, *32*, 53–62.

(16) Audí-Miró, C.; Cretnik, S.; Otero, N.; Palau, J.; Shouakar-Stash, O.; Soler, A.; Elsner, M. Cl and C isotope analysis to assess the effectiveness of chlorinated ethene degradation by zero-valent iron: Evidence from dual element and product isotope values. *Applied Geochemistry* **2013**, *32*, 175–183.

414 (17) Cole, J. R.; Cascarelli, A. L.; Mohn, W. W.; Tiedje, J. M. Isolation and characterization of a
415 novel bacterium growing via reductive dehalogenation of 2-chlorophenol. *Applied and Environmental*
416 *Microbiology* **1994**, *60*, 3536–3542.

417 (18) Aeppli, C.; Berg, M.; Cirpka, O. A.; Holliger, C.; Schwarzenbach, R. P.; Hofstetter, T. B.
418 Influence of Mass-Transfer Limitations on Carbon Isotope Fractionation during Microbial
419 Dechlorination of Trichloroethene. *Environ. Sci. Technol.* **2009**, *43*, 8813–8820.

420 (19) Aeppli, C.; Holmstrand, H.; Andersson, P.; Gustafsson, O. Direct Compound-Specific Stable
421 Chlorine Isotope Analysis of Organic Compounds with Quadrupole GC/MS Using Standard Isotope
422 Bracketing. *Analytical Chemistry* **2010**, *82*, 420–426.

423 (20) Holmstrand, H.; Andersson, P.; Gustafsson, Ö. Chlorine Isotope Analysis of Submicromole
424 Organochlorine Samples by Sealed Tube Combustion and Thermal Ionization Mass Spectrometry. *Anal.*
425 *Chem.* **2004**, *76*, 2336–2342.

426 (21) Bernstein, A.; Shouakar-Stash, O.; Ebert, K.; Laskov, C.; Hunkeler, D.; Jeannottat, S.;
427 Sakaguchi-Söder, K.; Laaks, J.; Jochmann, M. A.; Cretnik, S.; Jager, J.; Haderlein, S. B.; Schmidt, T.
428 C.; Aravena, R.; Elsner, M. Compound-Specific Chlorine Isotope Analysis: A Comparison of Gas
429 Chromatography/Isotope Ratio Mass Spectrometry and Gas Chromatography/Quadrupole Mass
430 Spectrometry Methods in an Interlaboratory Study. *Anal. Chem.* **2011**, *83*, 7624–7634.

431 (22) Aeppli, C.; Bastviken, D.; Andersson, P.; Gustafsson, Ö. Chlorine Isotope Effects and
432 Composition of Naturally Produced Organochlorines from Chloroperoxidases, Flavin-Dependent
433 Halogenases, and in Forest Soil. *Environmental Science & Technology* **2013**, 130131073931007.

434 (23) Laube, J. C.; Kaiser, J.; Sturges, W. T.; Bönisch, H.; Engel, A. Chlorine Isotope Fractionation in
435 the Stratosphere. *Science* **2010**, *329*, 1167–1167.

436 (24) Suarez, M. P.; Rifai, H. S. Biodegradation Rates for Fuel Hydrocarbons and Chlorinated
437 Solvents in Groundwater. *Bioremediation Journal* **1999**, *3*, 337–362.

438 (25) Dennie, D.; Gladu, I.; Lépine, F.; Villemur, R.; Bisaillon, J.-G.; Beaudet, R. Spectrum of the
439 Reductive Dehalogenation Activity of Desulfitobacterium frappieri PCP-1. *Applied and Environmental*
440 *Microbiology* **1998**, *64*, 4603–4606.

441 (26) Kunapuli, U.; Jahn, M. K.; Lueders, T.; Geyer, R.; Heipieper, H. J.; Meckenstock, R. U.
442 Desulfitobacterium aromaticivorans sp. nov. and Geobacter toluenoxydans sp. nov., iron-reducing
443 bacteria capable of anaerobic degradation of monoaromatic hydrocarbons. *International Journal of*
444 *Systematic and Evolutionary Microbiology* **2010**, *60*, 686–695.

445 (27) Villemur, R.; Lanthier, M.; Beaudet, R.; Lépine, F. The Desulfitobacterium genus. *FEMS*
446 *Microbiology Reviews* **2006**, *30*, 706–733.

447 (28) Smidt, H.; de Vos, W. M. Anaerobic Microbial Dehalogenation. *Annu. Rev. Microbiol.* **2004**, *58*,
448 43–73.

449 (29) Chang, Y. C.; Hatsu, M.; Jung, K.; Yoo, Y. S.; Takamizawa, K. Isolation and characterization of
450 a tetrachloroethylene dechlorinating bacterium, Clostridium bifermentans DPH-1. *Journal of Bioscience*
451 *and Bioengineering* **2000**, *89*, 489–491.

452 (30) Flynn, S. J.; Löffler, F. E.; Tiedje, J. M. Microbial Community Changes Associated with a Shift
453 from Reductive Dechlorination of PCE to Reductive Dechlorination of cis-DCE and VC. *Environmental*
454 *Science & Technology* **2000**, *34*, 1056–1061.

455 (31) Lee, P. K. H.; Warnecke, F.; Brodie, E. L.; Macbeth, T. W.; Conrad, M. E.; Andersen, G. L.;
456 Alvarez-Cohen, L. Phylogenetic Microarray Analysis of a Microbial Community Performing Reductive
457 Dechlorination at a TCE-Contaminated Site. *Environ. Sci. Technol.* **2011**, *46*, 1044–1054.

458 (32) Elsner, M.; Hunkeler, D. Evaluating Chlorine Isotope Effects from Isotope Ratios and Mass
459 Spectra of Polychlorinated Molecules. *Anal. Chem.* **2008**, *80*, 4731–4740.

460 (33) Hunkeler, D.; Aravena, R.; Butler, B. J. Monitoring Microbial Dechlorination of
461 Tetrachloroethene (PCE) in Groundwater Using Compound-Specific Stable Carbon Isotope Ratios:
462 Microcosm and Field Studies. *Environmental Science & Technology* **1999**, *33*, 2733–2738.

463 (34) Liang, X.; Dong, Y.; Kuder, T.; Krumholz, L. R.; Philp, R. P.; Butler, E. C. Distinguishing
464 Abiotic and Biotic Transformation of Tetrachloroethylene and Trichloroethylene by Stable Carbon
465 Isotope Fractionation. *Environmental Science & Technology* **2007**, *41*, 7094–7100.

466 (35) Slater, G. F.; Lollar, B. S.; Lesage, S.; Brown, S. Carbon Isotope Fractionation of PCE and TCE
467 During Dechlorination by Vitamin B12. *Ground Water Monitoring & Remediation* **2003**, *23*, 59–67.

468 (36) Dayan, H.; Abrajano, T.; Sturchio, N. .; Winsor, L. Carbon isotopic fractionation during
469 reductive dehalogenation of chlorinated ethenes by metallic iron. *Organic Geochemistry* **1999**, *30*, 755–
470 763.

471 (37) Numata, M.; Nakamura, N.; Koshikawa, H.; Terashima, Y. Chlorine Isotope Fractionation
472 during Reductive Dechlorination of Chlorinated Ethenes by Anaerobic Bacteria. *Environ. Sci. Technol.*
473 **2002**, *36*, 4389–4394.

474 (38) Bloom, Y.; Aravena, R.; Hunkeler, D.; Edwards, E.; Frape, S. K. Carbon Isotope Fractionation
475 during Microbial Dechlorination of Trichloroethene, cis-1,2-Dichloroethene, and Vinyl Chloride:
476 Implications for Assessment of Natural Attenuation. *Environmental Science & Technology* **2000**, *34*,
477 2768–2772.

478 (39) Lee, P. K. H.; Conrad, M. E.; Alvarez-Cohen, L. Stable Carbon Isotope Fractionation of
479 Chloroethenes by Dehalorespiring Isolates. *Environmental Science & Technology* **2007**, *41*, 4277–4285.

480 (40) Aeppli, C.; Hofstetter, T. B.; Amaral, H. I. F.; Kipfer, R.; Schwarzenbach, R. P.; Berg, M.
481 Quantifying In Situ Transformation Rates of Chlorinated Ethenes by Combining Compound-Specific
482 Stable Isotope Analysis, Groundwater Dating, And Carbon Isotope Mass Balances. *Environmental*
483 *Science & Technology* **2010**, *44*, 3705–3711.

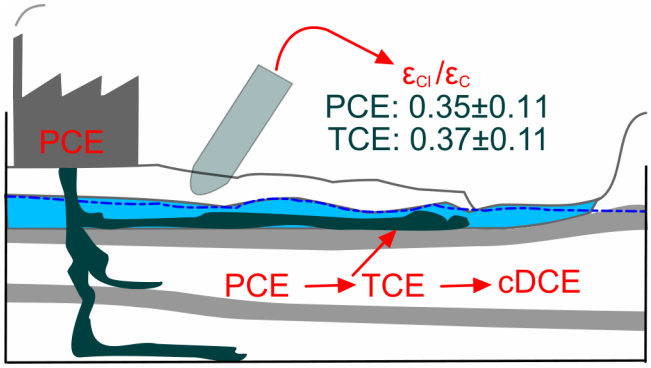
484 (41) Elsner, M.; Chartrand, M.; VanStone, N.; Lacrampe Couloume, G.; Sherwood Lollar, B.
485 Identifying Abiotic Chlorinated Ethene Degradation: Characteristic Isotope Patterns in Reaction
486 Products with Nanoscale Zero-Valent Iron. *Environ. Sci. Technol.* **2008**, *42*, 5963–5970.

487 (42) Glod, G.; Angst, W.; Holliger, C.; Schwarzenbach, R. P. Corrinoid-Mediated Reduction of
488 Tetrachloroethene, Trichloroethene, and Trichlorofluoroethene in Homogeneous Aqueous Solution:
489 Reaction Kinetics and Reaction Mechanisms. *Environmental Science & Technology* **1997**, *31*, 253–260.

490 (43) Abe, Y.; Aravena, R.; Zopfi, J.; Parker, B.; Hunkeler, D. Evaluating the fate of chlorinated
491 ethenes in streambed sediments by combining stable isotope, geochemical and microbial methods.
492 *Journal of Contaminant Hydrology* **2009**, *107*, 10–21.

493

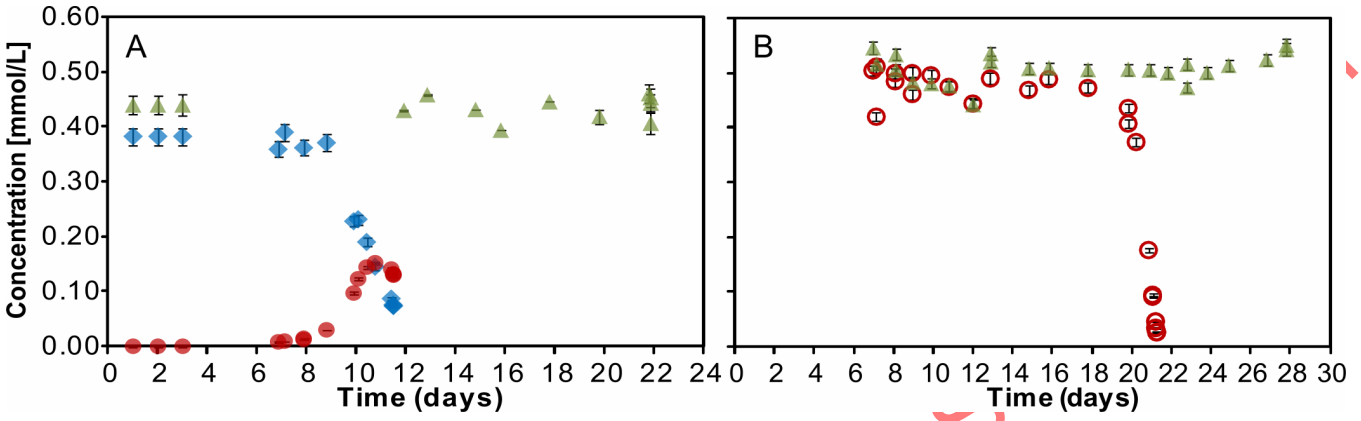
494 **TOC / Abstract Art**



495
496

497 **Figures**

498



499

500 **Figure 1.** Typical results from one replicate of a microbial reductive degradation experiment for **(A)**
501 PCE (blue diamonds) and **(B)** TCE (red circles). For each compound a set of 12 such individual culture
502 experiments were run. The concentration of a control sample, i.e. a culture immediately spiked with HCl
503 to inhibit any bacterial activity (see main text), is indicated for each compound with the green triangles.
504 The PCE degradation product TCE is also depicted with red filled circles in panel A. The error bars
505 show the standard deviation of the concentration measurements and were typically 4% and 2% for PCE
506 and TCE respectively.

507

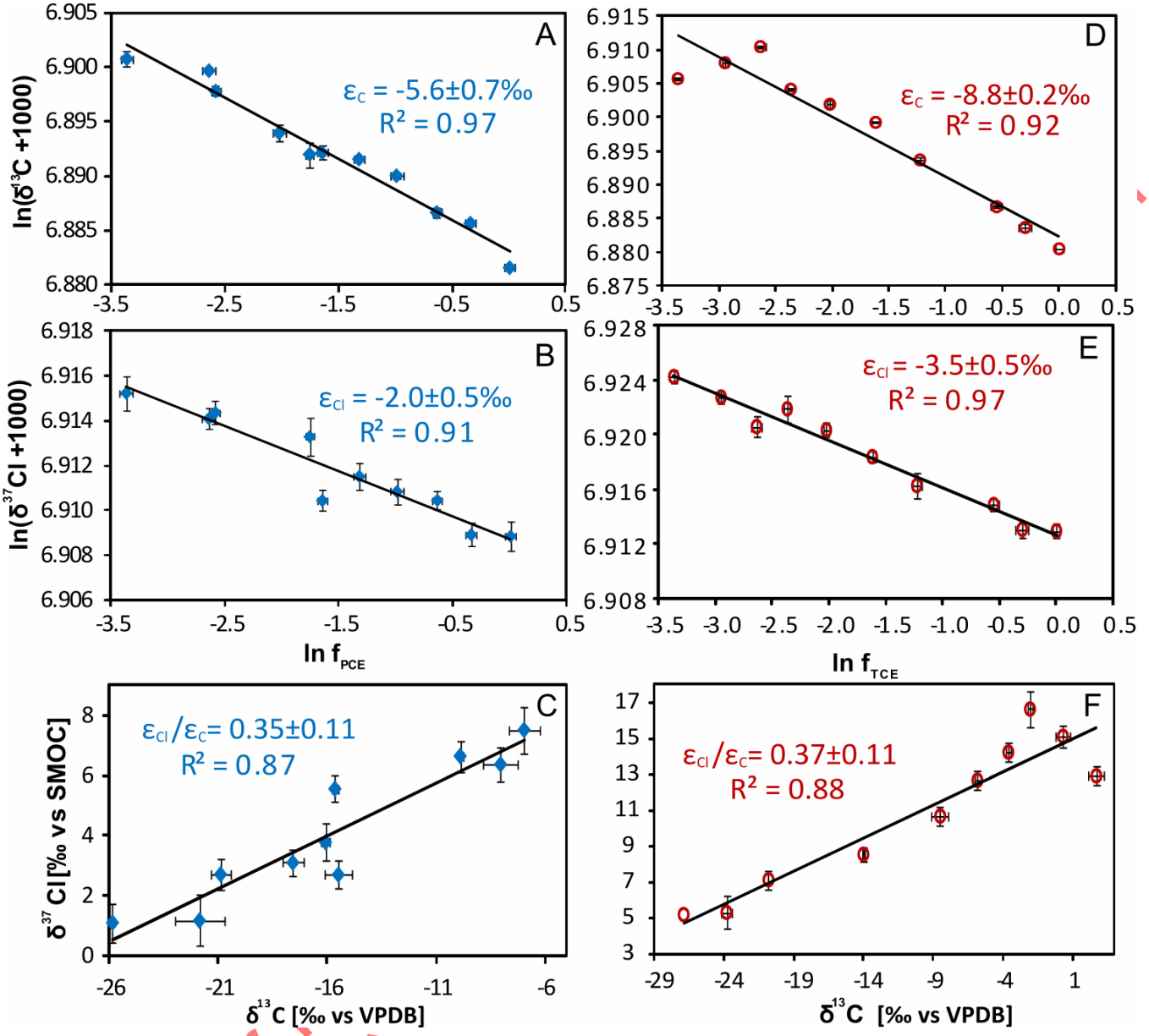
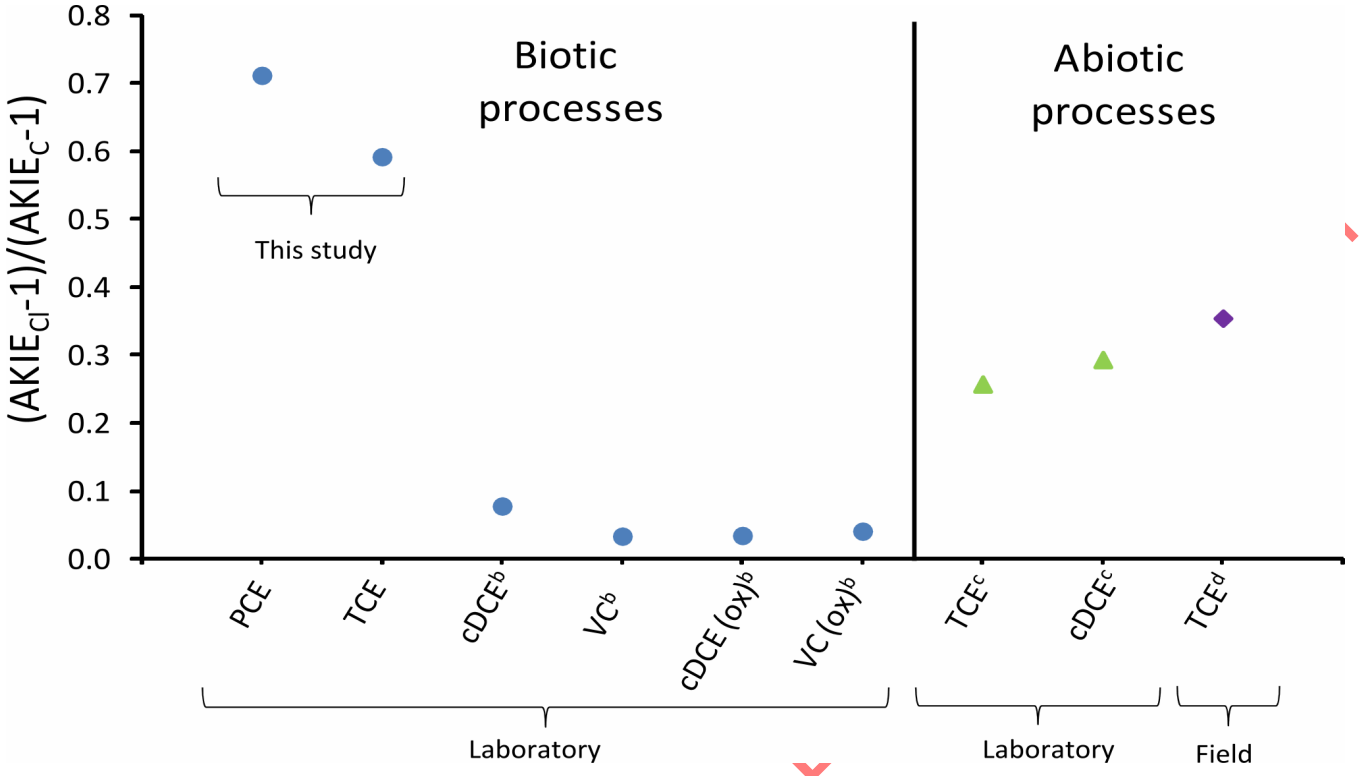


Figure 2. (A and D) Rayleigh plots for carbon isotopes and (**B and E)** chlorine isotopes data from degradation experiments of PCE (left panel) and TCE (right panel). The concentrations and C and Cl isotopic composition of each sacrificed culture bottle were plotted as natural logarithms to allow treatment according to the linearized version of eq. 3. The remaining fraction of PCE is denoted by f. (**C and F)** Dual stable carbon - chlorine isotope plot, yielding the process diagnostic ratio $\varepsilon_{Cl}/\varepsilon_C$. The error bars represent the SD replicate injections of target compound (n = 3) for the $\delta^{13}C$ analysis and the analytical uncertainties vs SMOC for $\delta^{37}Cl$.



517

518 **Figure 3.** Comparison of the apparent kinetic isotope effect ratios (AKIE$_{Cl}$-1)/(AKIE$_{C}$-1) from different

519 field and laboratory studies (according to Abe et al., 2009)[13], calculated from bulk ε values according to

520 Elsner and Hunkeler (2008).[32] The mechanism was reductive dechlorination in all cases, except for the

521 two aerobic oxidation data points cDCE(ox) and VC(ox). Data was obtained from [a]Abe et al. (2009)[13],

522 [c]Audí-Mirò et al.(2012)[16], and [d]Lojkasek-Lima et al.(2012).[15]

523