Hydrogen isotope fractionation during biodegradation of 1,2-dichloroethane: potential for pathway identification using a multi-element (C, Cl and H) isotope approach

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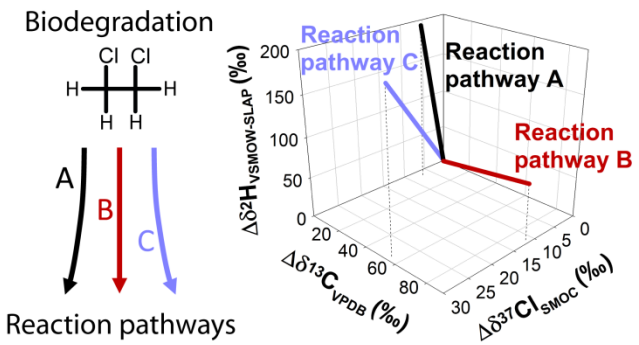
ABSTRACT

Even though multi-element isotope fractionation patterns provide crucial information to identify contaminant degradation pathways in the field, those involving hydrogen are still lacking for many halogenated groundwater contaminants and degradation pathways. This study investigates for the first time hydrogen isotope fractionation during both aerobic and anaerobic biodegradation of 1,2-dichloroethane (1,2-DCA) using five microbial cultures. Transformation-associated isotope fractionation values (ε^{H}_{bulk}) were: -115 ± 18‰ (aerobic C-H bond oxidation), -34 ± 4‰ and -38 ± 4‰ (aerobic C-Cl bond cleavage via hydrolytic dehalogenation), -57 ± 3‰ and -77 ± 9‰ (anaerobic C-Cl bond cleavage via reductive dihaloelimination). The dual element C-H isotope approach ($\Lambda_{C\text{-}H} = \Delta\delta^{2}H/\Delta\delta^{13}C \approx \varepsilon^{H}_{bulk}/\varepsilon^{C}_{bulk}$, where $\Delta\delta^{2}H$ and $\Delta\delta^{13}C$ are changes in isotope ratios during degradation) resulted in clearly different $\Lambda_{C\text{-}H}$ values: 28 ± 4 (oxidation), 0.7 ± 0.1 and 0.9 ± 0.1 (hydrolytic dehalogenation), 1.76 ± 0.05 and 3.5 ± 0.1 (dihaloelimination). This result highlights the potential of this approach to identify 1,2-DCA degradation pathways in the field. In addition, distinct trends were also observed in a multi (i.e., $\Delta\delta^{2}H$ vs $\Delta\delta^{37}Cl$ vs $\Delta\delta^{13}C$) isotope plot, which opens further possibilities for pathway identification in future field studies. This is crucial information to understand the mechanisms controlling natural attenuation of 1,2-DCA and to design appropriate strategies to enhance biodegradation.



TOC Art

60 **INTRODUCTION**

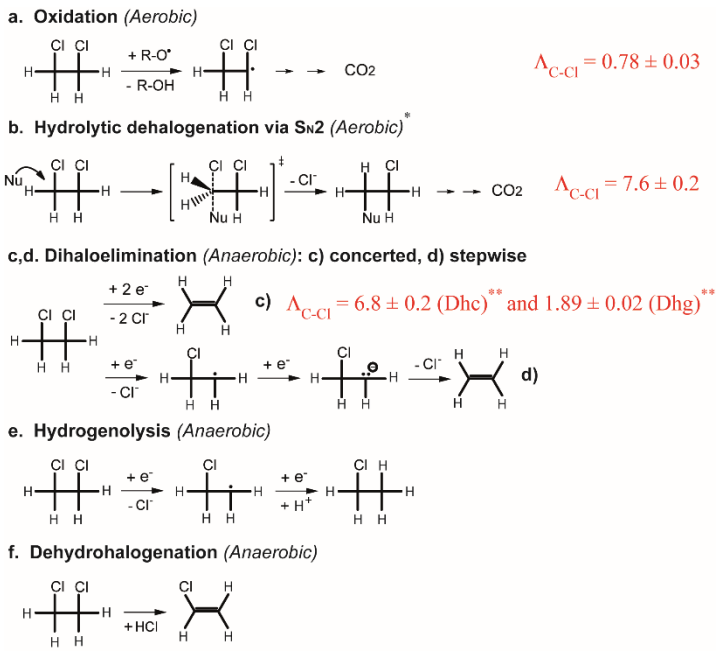
61 1,2-dichloroethane (1,2-DCA) is widely used as a chemical intermediate in the industrial
62 production of polyvinyl chloride, as a solvent and also as a lead scavenger in leaded
63 gasoline.[1] Due to its high production, accidental leakage and improper disposal, 1,2-DCA
64 has become a prevalent groundwater contaminant. For instance, in 2015 a total of 186 tons
65 of 1,2-DCA (not including on-site land disposal) were released to the environment in the
66 U.S.,[2] which poses a threat to human and wildlife health due to its high toxicity.[3]

67 1,2-DCA can undergo biodegradation via distinct degradation pathways under oxic[4-6]
68 and anoxic conditions[7-11] (Scheme 1). Under oxic conditions, 1,2-DCA can be biodegraded
69 by oxidation via a monooxygenase[4] (Scheme 1a) and hydrolytic dehalogenation[5, 6]
70 (Scheme 1b). Initial products of both reactions are further degraded to innocuous end
71 products. Under reducing conditions, 1,2-DCA is usually transformed by dihaloelimination
72 to ethene[7, 8] (either concerted or stepwise β-elimination, Scheme 1c, d) or hydrogenolysis
73 to chloroethane (CA)[11] (Scheme 1e). In addition, 1,2-DCA can be transformed to vinyl
74 chloride (VC) via dehydrohalogenation (Scheme 1f) by pure *Dehalococcoides* strains[12, 13]
75 and *Dehalococcoides*-containing cultures,[10, 14] however, VC is typically detected at much
76 lower concentrations compared to ethene. Therefore, chlorinated products such as CA and
77 VC can accumulate under anoxic conditions. Like 1,2-DCA, both CA and VC are
78 groundwater contaminants and are considered as priority pollutants by the U.S.
79 Environmental Protection Agency (USEPA),[3] emphasizing the need for elucidation of
80 active biodegradation pathways in the field.

81 Owing to the high susceptibility for 1,2-DCA to be transformed under different redox
82 conditions, the assessment of its fate in the subsurface is not an easy task. On the one hand,
83 aerobic biodegradation of 1,2-DCA might be sustained at very low dissolved oxygen
84 concentrations as shown for VC oxidation.[15] Furthermore, anaerobic oxidation of 1,2-DCA

was demonstrated under nitrate-reducing conditions,[16, 17] likely via hydrolytic dehalogenation (Scheme 1b).[18] On the other hand, contaminated sites are usually impacted by mixtures of compounds, which complicates pathway identification from analysis of degradation products because the same products can be formed from different precursors. For instance, CA can also be formed from 1,1,1-trichloroethane (1,1,1-TCA)[19] and VC and ethene from trichloroethene (TCE)[12] by reductive dechlorination. In addition, end products of the aerobic degradation pathways of 1,2-DCA, i.e., inorganic carbon and Cl^-, are ubiquitous and often occur at high background concentrations in groundwater. Hence, additional tools are necessary for better characterization of 1,2-DCA biodegradation in the field. This information is essential for i) evaluating the natural attenuation of 1,2-DCA at contaminated sites; and ii) predicting potential accumulation of toxic products.

Scheme 1. Biodegradation pathways of 1,2-DCA in aqueous systems and reported $\Lambda_{C\text{-}Cl}$ values.[14] (*) Transformation via hydrolytic dehalogenation was also proposed under nitrate-reducing conditions.[18] (**) Values obtained from experiments with *Dehalococcoides*- (Dhc) and *Dehalogenimonas*- (Dhg) containing enrichment cultures.[14]



102 Dual carbon and chlorine isotope analysis during substrate transformation is
103 increasingly used to elucidate biodegradation pathways and obtain insight into enzymatic
104 reaction mechanisms for chlorinated ethenes[20-23] and ethanes.[14, 24] However, until recently,
105 a multi-element isotope approach including hydrogen isotope data was not feasible because
106 on-line hydrogen isotope analysis of chlorinated compounds was hampered by the
107 formation of HCl during the high-temperature conversion of chlorinated analytes to H_2.[25]
108 New analytical methods for on-line compound-specific hydrogen isotope analysis (H-
109 CSIA) by gas chromatography - isotope ratio mass spectrometry (GC-IRMS) were
110 developed which largely circumvent the formation of HCl by the use of a chromium metal
111 reactor interface to form H_2. These new methods were validated using different compounds
112 such as chlorinated ethenes and hexachlorocyclohexane.[26-29] For instance, δ^2H values with
113 a precision better than ± 7‰ were obtained for both trichloroethene and *cis*-1,2-
114 dichloroethene (*cis*-DCE) by Shouakar-Stash and Drimmie.[26] H-CSIA studies have so far
115 been applied to only a few chlorinated compounds[30-32] and, to the best of our knowledge,
116 multi-element isotope studies including hydrogen isotope data are currently nonexistent for
117 chlorinated ethanes.

118 Combined shifts in isotope ratios of two elements (e.g., $\Delta\delta^2H$ vs. $\Delta\delta^{13}C$) generally
119 exhibit a linear relationship with a slope ($\Lambda_{C\text{-}H} = \Delta\delta^2H/\Delta\delta^{13}C$) reflecting the extent of H
120 and C isotope effects, which are controlled by chemical bond breakage or formation.
121 Therefore, different $\Lambda_{C\text{-}H}$ values may be expected for distinct transformation mechanisms
122 involving different elements.[33] For 1,2-DCA, dual C-Cl isotope data were recently reported
123 and different $\Lambda_{C\text{-}Cl}$ values (Scheme 1) were observed during both aerobic[24] and anaerobic[14]
124 biodegradation of 1,2-DCA. However, relatively similar $\Lambda_{C\text{-}Cl}$ values were observed for
125 aerobic hydrolytic dehalogenation by *Xanthobacter autotrophicus* GJ10 and *Ancylobacter*
126 *aquaticus* AD20 (7.6 ± 0.2) and anaerobic dihaloelimination by a *Dehalococcoides*-

127 containing enrichment culture (6.8 ± 0.2). Taking into account the uncertainty of
128 measurements at contaminated sites, it may be difficult to distinguish the two pathways
129 (i.e., hydrolytic dehalogenation vs. dihaloelimination) in field studies solely based on dual
130 C-Cl isotope data. In addition to C and Cl, analysis of H isotope ratios for 1,2-DCA may
131 increase the possibilities for a dual- (C vs. H or Cl vs. H) or multi-element (C vs. Cl vs. H)
132 isotope approach for differentiating between hydrolytic dehalogenation and
133 dihaloelimination pathways.

134 For dihaloelimination of 1,2-DCA, distinct $\Lambda_{C\text{-}Cl}$ values were reported from microcosm
135 experiments with *Dehalococcoides* (6.8 ± 0.2) and *Dehalogenimonas* (1.89 ± 0.02)
136 containing enrichment cultures and a different mode of *concerted* bond cleavage rather
137 than two different reaction mechanisms (i.e., *stepwise* vs *concerted*) was proposed to
138 explain this difference.[14] However, further insight into the reductive dehalogenation
139 mechanisms of 1,2-DCA may be obtained from hydrogen isotope data. Owing to the
140 usually large hydrogen isotope fractionation (ε^{H}_{bulk}), secondary hydrogen isotope effects
141 during C-Cl bond breakage might be detected and measured.[34] In this case, the magnitude
142 of secondary ε^{H}_{bulk} values may vary depending on the reaction mechanism (i.e., *stepwise* vs
143 *concerted* dehalogenation).

144 In this study, hydrogen isotope fractionation during biodegradation of 1,2-DCA via
145 aerobic (oxidation and hydrolytic dehalogenation) and anaerobic (dihaloelimination)
146 degradation pathways was determined for the first time using different pure microbial
147 strains and enrichment cultures in laboratory experiments. 2D (H vs. C and H vs. Cl) and
148 3D (H vs. C vs. Cl) multi-element isotope approaches were used i) to characterize the $\Lambda_{C\text{-}H}$
149 and $\Lambda_{Cl\text{-}H}$ values during biodegradation of 1,2-DCA under different redox conditions; ii) to
150 determine whether the resultant multi-element isotope patterns are sufficiently different to
151 distinguish between different pathways, particularly between hydrolytic dehalogenation

152 and dihaloelimination pathways; and iii) to obtain further insight into underlying reaction
153 mechanisms. In addition, for the 3D isotope approach, new procedures were proposed to
154 characterize pathway-specific multi-element (Cl, C, H) isotope trends, which can be
155 applicable to other multi-element isotope studies with three elements.
156

157 **MATERIALS AND METHODS**

158 **Pure and enrichment cultures**

159 Three pure strains with known initial biotransformation mechanisms were used for the
160 aerobic experiments: *Pseudomonas* sp. strain DCA1 (oxidation)[4] and *Xanthobacter*
161 *autotrophicus* GJ10 and *Ancylobacter aquaticus* AD20 (hydrolytic dehalogenation)[5, 6].
162 *Pseudomonas* sp. strain DCA1 was kindly provided by E. Edwards (Department of
163 Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON) and *X.*
164 *autotrophicus* GJ10 (DSMZ 3874) and *A. aquaticus* AD20 (DSMZ 9000) were purchased
165 (DSMZ, Braunschweig, Germany). The growth medium was prepared as described by
166 Hunkeler and Aravena[35] and further cultivation details are available in Palau *et al.*[24]

167 Anaerobic cultures for reductive dihaloelimination experiments were prepared using
168 two enrichment cultures with different bacterial populations, which were characterized in
169 previous studies to determine organohalide-respiring bacteria (ORB) capable of 1,2-DCA
170 degradation.[10, 14, 36] The growth media used and cultivation details for *Dehalococcoides* and
171 *Dehalogenimonas*-containing cultures are available in Palau *et al.*[14]

172 **Batch experiments preparation and sampling**

173 Aerobic biodegradation experiments were performed at the University of Neuchâtel (UN),
174 Switzerland. Microcosm batch tests were prepared in 250 mL glass bottles, which
175 contained 185 mL of medium and were capped with Mininert™ valves (VICI Precision
176 Sampling, Baton Rouge, LA). Experiments and controls were amended with 22.5 μL of

177 pure 1,2-DCA to produce an initial aqueous concentration of 1.5 mM (when taking into
178 account partitioning between the headspace and liquid using Henry's Law). All
179 experiments were conducted in triplicate. Bottles were shaken upside down to prevent
180 leakage of the gas phase through the valve. For concentration and isotopic analysis,
181 aqueous samples (1.5 mL) were taken from the 250 mL bottles at selected time points and
182 preserved frozen[37] in 2 mL vials with NaN_3 (1 g/L). Abiotic control bottles were prepared
183 with 185 mL of autoclaved mineral medium and samples were collected and preserved as
184 described for the experimental bottles.

185 Anaerobic biodegradation experiments with *Dehalococcoides* and *Dehalogenimonas*-
186 containing cultures were performed at Clemson University (CU), US, and at the
187 Universitat Autònoma de Barcelona (UAB), Spain, respectively. Microcosms were
188 prepared in anoxic chambers and the bottles (120 mL total volume) were sealed with
189 Teflon-faced rubber septa and aluminum crimp caps to maintain anoxic conditions.

190 For the batch tests with *Dehalococcoides*-containing culture, a total of 30 serum bottles
191 were prepared by dispensing 75 mL of the enrichment culture. 1,2-DCA was added as a
192 water saturated solution (225 μL per bottle) to produce an initial aqueous phase
193 concentration of ~0.25 mM. Sodium lactate was added to ensure an excess of electron
194 equivalents for dechlorination (150 μL of a sodium lactate stock solution containing 456.2
195 g/L of 60% sodium lactate syrup).[10] Killed controls were prepared by adding phosphoric
196 acid to the bottles, followed by the 1,2-DCA.

197 For the experiments with *Dehalogenimonas*-containing culture, a total of 16 serum
198 bottles were prepared by dispensing 65 mL of a sterilized anoxic medium described
199 elsewhere;[36] however, in half of the bottles pyruvate (5 mM) was replaced by acetate (5
200 mM) as carbon source. The microcosms were inoculated with 3 mL of the
201 *Dehalogenimonas*-containing culture and 1,2-DCA was added from a stock solution in

202 acetone to give an initial aqueous phase concentration of ~0.1 mM. Abiotic control bottles
203 containing the growth medium with 1,2-DCA but without inoculum were prepared as
204 described for the experimental bottles. In addition, live controls without 1,2-DCA were
205 prepared to account for the transfer of compounds from previous degradation experiments
206 with the inoculum.

207 **Isotopic and concentration analysis**

208 A detailed description of analytical methods and equipment used for the isotopic and
209 concentration analysis is available in supporting information (SI). On-line H-CSIA of 1,2-
210 DCA was performed at Isotope Tracer Technologies Inc., Canada, according to Shouakar-
211 Stash and Drimmie.[26] Briefly, measurements of hydrogen isotope ratios for 1,2-DCA at
212 natural abundance were determined by GC-IRMS equipped with a chromium reduction
213 system. The instrument was tuned and the H_3^+ factor was determined every day before
214 analysis of standards and samples. The H_3^+ factor usually ranged between 4.5 and 5.5.
215 Isotope ratios were reported using the δ-notation (eq. 1):

216

217 $$\delta^2\mathrm{H}_{\mathrm{sample}} = \frac{\mathrm{R}_{\mathrm{sample}}}{\mathrm{R}_{\mathrm{standard}}} - 1 \qquad (1)$$

218

219 where R = $^2H/^1H$, corresponding to the ratio of *m/z* 3 ($^2H^1H$) to *m/z* 2 ($^1H^1H$) measured in
220 separate Faraday caps. The δ-values were expressed in per mil (1 ‰ = 1 mUr) and the
221 notations $\delta^2H_{VSMOW\text{-}SLAP}$ and $\Delta\delta^2H_{VSMOW\text{-}SLAP}$ were used to indicate calibrated δ-values and
222 changes in calibrated δ-values during degradation (i.e., $\Delta\delta^2H = \delta^2H_t - \delta^2H_0$), respectively.
223 Hydrogen isotope ratios were calibrated using 1,2-DCA and TCE as reference compounds
224 in the $\delta^2H_{VSMOW\text{-}SLAP}$-range between -50 ± 1‰ to +565 ± 4‰.[27, 38] External laboratory
225 standards of 1,2-DCA and TCE were dissolved in water and measured similarly to the
226 samples. Further details about δ^2H values (two-point) calibration to the VSMOW-SLAP

227 scale are available in the SI. Additional aqueous standards of 1,2-DCA were interspersed in
228 each sample sequence to ensure stability of the measurements during the course of sample
229 analyses. Samples and standards were diluted to a similar concentration and measured in
230 duplicate. Precision (1σ) of the δ^2H values for 1,2-DCA on the analysis of the standards
231 was < 5‰ (n=54) (see further details in SI).

232 The concentrations of 1,2-DCA were measured by headspace analysis using a GC -
233 mass spectrometer (GC-MS) at UN[24] laboratory (aerobic experiments) and a GC - flame
234 ionization detector (GC-FID) at the CU[10] and UAB[36] laboratories (anaerobic experiments).
235 The concentration of 1,2-DCA in the abiotic controls for the aerobic (1.55 ± 0.03 mM, n =
236 12) and anaerobic experiments (0.257 ± 0.004 mM, n = 5 and 0.094 ± 0.009 mM, n = 6)
237 remained at the initial concentration during the experiments, indicating that compound
238 losses through the caps and abiotic degradation during incubation were insignificant.

239 **Evaluation of isotope fractionation**

240 For a given substrate, the relationship between observable compound-average isotope
241 fractionation (ε_{bulk}) and the extent of biotransformation can be described by a modified
242 form of the Rayleigh distillation equation (2) in laboratory experiments:

243

244 $$\ln\frac{R_t}{R_0} = \ln\left(\frac{1+\delta^2H_t}{1+\delta^2H_0}\right) = \varepsilon^{H}_{bulk} \cdot \ln f \qquad (2)$$

245

246 where the subscripts "t" and "0" refer to the current and initial bulk isotope ratios,
247 respectively, and f is the remaining fraction of the substrate.

248 For the aerobic experiments, f was corrected for substrate removal by repetitive liquid
249 samples withdrawn from the same batch reactor according to Buchner *et al*. (see SI, eq. S1
250 and eq. S2),[39] which also takes into account volatilization of the substrate to the bottle
251 headspace. An aqueous phase 1,2-DCA concentration decrease of < 5% was estimated as a

result of the change in the headspace to solution ratio during the aerobic experiments. The correction of f due to mass removal during sampling was not necessary for the anaerobic experiments because these were prepared with numerous parallel replicates, which were sequentially sacrificed for sampling.

ε_{bulk} values were quantified by least squares linear regression of eq. 2 without forcing the regression through the origin (see SI, Figure S1).[40] Uncertainties are represented by 95% confidence intervals (C.I.). Calculation of position-specific apparent kinetic isotope effects (AKIEs) is indicated in the SI.

Dual-element isotope fractionation patterns for different degradation pathways were characterized by the slope of the linear regressions in a 2D isotope plot, i.e., $\Lambda_{C\text{-}H} = \Delta\delta^2H/\Delta\delta^{13}C$ and $\Lambda_{Cl\text{-}H} = \Delta\delta^2H/\Delta\delta^{37}Cl$, ± 95% C.I. For each degradation experiment, the observed multi-element (Cl, C, H) isotope fractionation trend in a 3D isotope plot was characterized by principal component analysis in SigmaPlot v.13.0. As a result, a characteristic unit vector ($\vec{P}$) was determined for each degradation pathway (see SI and Table S1). For a given degradation pathway, the unit vector $\vec{P}$ can also be calculated from ε_{bulk} values determined in laboratory experiments according to the expression

$$\vec{P} = \frac{1}{\sqrt{(\varepsilon_{bulk}^{Cl})^2 + (\varepsilon_{bulk}^{C})^2 + (\varepsilon_{bulk}^{H})^2}} \cdot \overrightarrow{\left(\varepsilon_{bulk}^{Cl},\ \varepsilon_{bulk}^{C},\ \varepsilon_{bulk}^{H}\right)} \qquad (3)$$

provided that absolute ε_{bulk} values are used (see SI). A comparison of $\vec{P}$-vectors determined by principal component analysis with those calculated from eq. 3 is available in the SI (Table S2).

RESULTS AND DISCUSSION

Hydrogen isotopes fractionation

277 Aerobic biodegradation experiments lasted between 12 and 21 hours (half-life from ~3.5 to
278 ~6.3 hours, see SI) and 1,2-DCA transformation above 90% was reached for all replicates.
279 δ^2H values for 1,2-DCA showed a trend towards more positive values during its
280 transformation by C-H bond oxidation (Figure 1a) or hydrolytic dehalogenation (Figure
281 1b), reflecting an enrichment of 1,2-DCA in the heavy isotope (^{2}H). This is indicative of a
282 normal isotope effect. The δ^2H values for 1,2-DCA in the controls remained constant
283 throughout the experiments ($\delta^2H_{VSMOW\text{-}SLAP}$ = -53 ± 3‰, ±1σ, n = 6). For *Pseudomonas* sp.
284 strain DCA1 (C-H bond oxidation, Scheme 1a) a very large shift in δ^2H values was
285 observed, with up to $\Delta\delta^2H_{VSMOW\text{-}SLAP}$ = +188 ± 10‰ after ~80% degradation (Figure 1a).
286 This resulted in a large ε^H_{bulk} value of -115 ± 18‰ ($AKIE_H$ = 1.6 ± 0.2). Large ε^H_{bulk} values
287 were also observed, for instance, during oxidation of ethylbenzene by *Aromatoleum*
288 *aromaticum* (ε^H_{bulk} = -111 ± 7‰, $AKIE_H$ = 6.0, for variations of δ^2H within the range $\Delta\delta^2$H
289 < 100)[41] or methyl *tert*-butyl ether (MTBE) by *Pseudonocardia tetrahydrofuranoxydans*
290 K1 (ε^H_{bulk} = -100 ± 10‰, $AKIE_H$ = 14.2)[42]. In comparison to the large ε^H_{bulk} value
291 determined for oxidation of 1,2-DCA, for hydrolytic dehalogenation of 1,2-DCA (C-Cl
292 bond cleavage via S_N2, Scheme 1b) a much lower enrichment in ^{2}H was measured in
293 experiments with *A. aquaticus* and *X. autotrophicus* (ε^H_{bulk} values of -34 ± 4‰ and -38 ±
294 4‰, respectively, Figure 1b).
295 Anaerobic biodegradation experiments lasted approximately 9 and 40 days (half-life of ~30
296 and ~289 hours, see SI) for the microcosms with *Dehalococcoides* and *Dehalogenimonas*-
297 containing cultures, respectively, at which point most all of the initial 1,2-DCA was
298 transformed to ethene via dihaloelimination. VC was detected only in the
299 *Dehalococcoides*-containing microcosms, at concentrations much lower than those of
300 ethene. The maximum accumulation of VC represented less than 6% of the initial 1,2-DCA
301 added. Further information on concentrations of ethene and VC during degradation of 1,2-

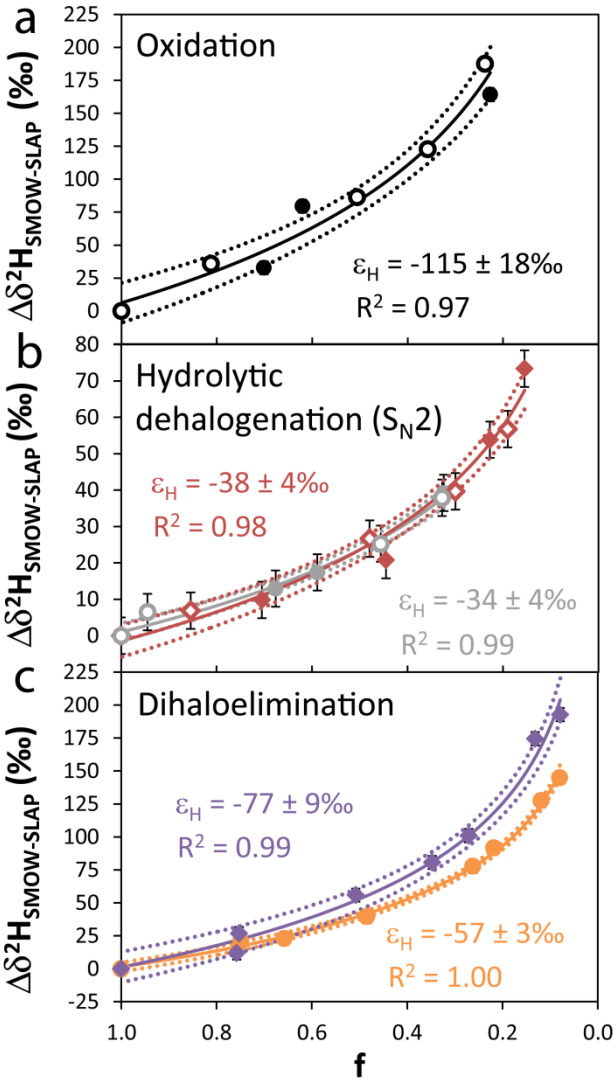
302 DCA is available in a previous study.[14] The concentration pattern of products observed in
303 this previous study[14] indicated that ethene and VC were formed in parallel reaction
304 pathways via dihaloelimination and dehydrohalogenation (Scheme 1), respectively. Hence,
305 products concentrations indicated that only a small fraction of 1,2-DCA was transformed
306 via dehydrohalogenation in the *Dehalococcoides*-containing microcosms.

307 As observed for the aerobic experiments, enrichment in ^{2}H was obtained during
308 dihaloelimination of 1,2-DCA by both anaerobic enrichment cultures (ε^{H}_{bulk} values of -57 ±
309 3‰ and -77 ± 9‰, Figure 1c). However, in contrast to the similar ε^{H}_{bulk} values obtained for
310 hydrolytic dehalogenation by *A. aquaticus* and *X. autotrophicus*, a significantly higher
311 value was determined for dihaloelimination by *Dehalogenimonas* (ε^{H}_{bulk} = -77 ± 9‰)
312 compared to that of *Dehalococcoides* (ε^{H}_{bulk} = -57 ± 3‰) containing cultures. The $\delta^{2}H$
313 values for 1,2-DCA in the controls did not change significantly during both experiments
314 (i.e., $\delta^{2}H_{VSMOW\text{-}SLAP}$ of -44 ± 1‰ and -50 ± 2‰, ±1σ, n = 4, respectively). It is also
315 interesting to note that, although 1,2-DCA was purchased from different suppliers in each
316 laboratory (see SI), their hydrogen isotopic signatures were relatively similar, varying
317 between $\delta^{2}H_{VSMOW\text{-}SLAP}$ values of -44 ± 1‰ and -53 ± 3‰.

318 Even though clearly distinct ε^{H}_{bulk} values were determined for different aerobic and
319 anaerobic biodegradation pathways for 1,2-DCA during experiments performed in the
320 laboratory, pathway distinction based on isotope fractionation of one element alone is not
321 possible under field conditions. The reason is that changes in substrate concentrations are
322 also related to processes other than transformation (e.g., hydrodynamic dispersion). This
323 prevents accurate calculation of ε^{H}_{bulk} values and, hence, precludes mechanistic information
324 based on isotope effects. The situation is different if isotope analysis is conducted on two
325 or more elements. The proportion of changes in δ-values of both elements relative to each
326 other (e.g., $\Delta\delta^{2}H$ / $\Delta\delta^{13}C$) is largely unaffected by non-degradative processes.[43, 44]

327 Therefore, measurements of isotope fractionation of two or more elements are crucial for

328 investigating contaminant biodegradation pathways in the field.



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330 **Figure 1**. Hydrogen isotopic fractionation of 1,2-DCA during biodegradation by

331 *Pseudomonas* sp. (a), *A. aquaticus* (b, grey circles), *X. autotrophicus* (b, red diamonds) and

332 *Dehalococcoides*- (c, orange circles) and *Dehalogenimonas*-containing cultures (c, violet

333 diamonds); f is the fraction of 1,2-DCA remaining. For the aerobic experiments (a, b),

334 solid and empty symbols represent data from two replicate bottles. The error bars for

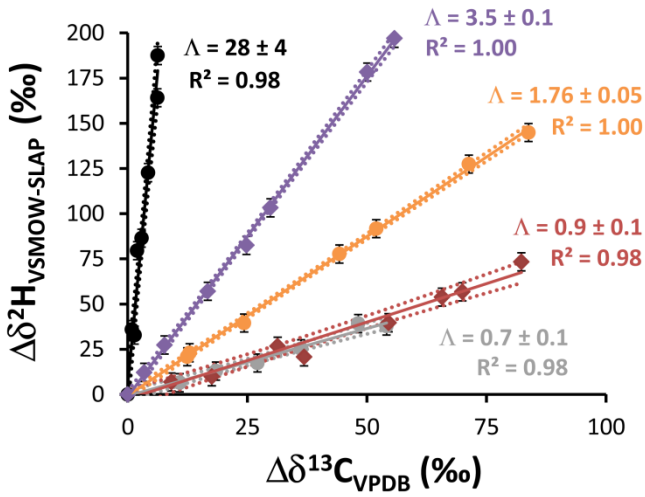
335 isotope values in panels (a) and (c) are smaller than the symbols. The solid lines are fits in

336 SigmaPlot® according to eq. 2 and dotted lines the 95% confidence intervals of the

337 nonlinear regressions.

338 **Multi-element isotope approach**

339 Hydrogen δ-values of 1,2-DCA were combined with previously determined carbon and
340 chlorine isotopic data for these experiments[14, 24] in dual- (δ^2H vs. $\delta^{13}C$ and δ^2H vs. $\delta^{37}Cl$)
341 and multi-element (δ^2H vs. $\delta^{13}C$ vs. $\delta^{37}Cl$) isotope plots. The dual C-H isotope approach
342 resulted in very good linear correlations ($r^2 \geq 0.98$, Figure 2), which is consistent with
343 Dorer *et al.*[41] These authors showed that for variations of δ^2H within the range $\Delta\delta^2H < 100$
344 – 200‰, the ε^H_{bulk} and $\Lambda_{C\text{-}H}$ values (i.e., $\Lambda_{C\text{-}H} = \Delta\delta^2H / \Delta\delta^{13}C \approx \varepsilon^H_{bulk} / \varepsilon^C_{bulk}$) can be
345 evaluated using eq. 2 (Figure S1) and $\Delta\delta^2H$ against $\Delta\delta^{13}C$ data in a dual element isotope
346 plot (Figure 2), respectively. A different procedure may be necessary for evaluating
347 stronger H isotope fractionation that lead to larger shifts in δ^2H, since previous studies
348 observed a nonlinear behavior in H isotope ratios in Rayleigh and dual element isotope
349 plots at a late stage of reaction.[38, 41]



350

351 **Figure 2**. Dual C-H isotope trends during biodegradation of 1,2-DCA via oxidation by
352 *Pseudomonas* sp. (black circles), hydrolytic dehalogenation by *A. aquaticus* (grey circles)
353 and *X. autotrophicus* (red diamonds), and dihaloelimination by *Dehalococcoides*- (orange
354 circles) and *Dehalogenimonas*-containing cultures (violet diamonds). Dotted lines indicate
355 the 95% confidence intervals of the linear regression. Error bars of $\Delta\delta^{13}C$ values are

356 smaller than the symbols. Λ values (±95% C.I.) are given by the slope of the linear
357 regressions.

358 The clearly distinct isotope patterns observed in Figure 2 for all the investigated
359 degradation pathways, with $\Lambda_{C\text{-}H}$ values ranging between 28 ± 4 (oxidation) and 0.8 ± 0.1
360 (average value for hydrolytic dehalogenation), opens the possibility of a dual C-H isotope
361 approach to identify the different aerobic and anaerobic degradation pathways for 1,2-DCA
362 in the field. Particularly, for aerobic hydrolytic dehalogenation by *X. autotrophicus* and *A.*
363 *aquaticus* (average $\Lambda_{C\text{-}H}$ value of 0.8 ± 0.1) and reductive dihaloelimination by a
364 *Dehalococcoides*-containing culture ($\Lambda_{C\text{-}H}$ = 1.76 ± 0.05), a larger relative difference in
365 $\Lambda_{C\text{-}H}$ values was obtained (around 50% relative to the higher value) compared to that of
366 their respective $\Lambda_{C\text{-}Cl}$ values (around 10%, Scheme 1). Therefore, the use of a C-H isotope
367 approach enables improved identification of the two pathways (i.e., hydrolytic
368 dehalogenation and dihaloelimination).

369 A dual Cl-H isotope approach was also investigated and good linear correlations ($r^2 \geq$
370 0.97, Figure S2) were observed for all the degradation experiments in a dual isotope plot
371 (see SI). However, similar $\Lambda_{Cl\text{-}H}$ values were obtained for aerobic hydrolytic
372 dehalogenation by *X. autotrophicus* and *A. aquaticus* (6.5 ± 1.0 and 5.7 ± 0.9, respectively)
373 and dihaloelimination by a *Dehalogenimonas*-containing culture (6.7 ± 0.3), which
374 hampers differentiation of these two pathways (i.e., hydrolytic dehalogenation and
375 dihaloelimination) using a dual Cl-H isotope approach.

376 The large differences among all trends obtained for the dual C-H isotope approach
377 (Figure 2) also enables estimation of the proportion of two competing pathways (e.g.,
378 oxidation and hydrolytic dehalogenation reactions under aerobic conditions or
379 dihaloelimination by *Dehalococcoides* and *Dehalogenimonas* populations under anaerobic
380 conditions) based on the resultant slope,[45, 46] assuming simultaneous activity with a

constant ratio between both pathway rates. However, for three or more pathways unique solutions are not possible. An improved evaluation of up to three different degradation pathways might be possible by combining isotope data of three elements.

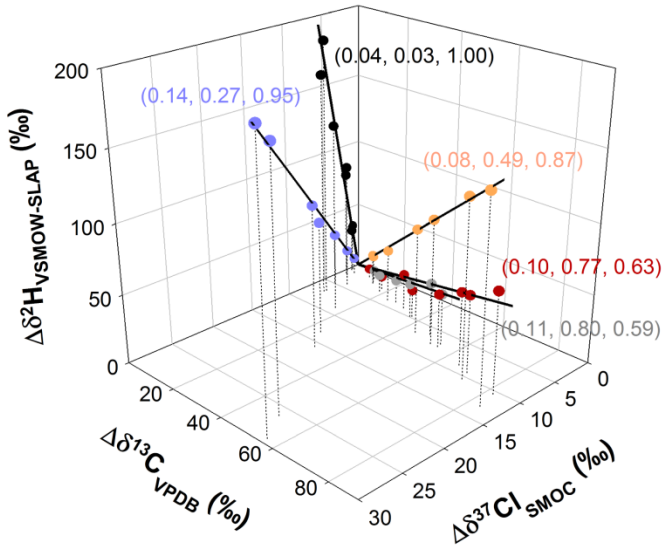


Figure 3. Multi-element isotope patterns during biodegradation of 1,2-DCA via different reaction pathways: oxidation by *Pseudomonas* sp. (black), hydrolytic dehalogenation by *A. aquaticus* (grey) and *X. autotrophicus* (red), and dihaloelimination by *Dehalococcoides*- (orange) and *Dehalogenimonas*-containing cultures (violet). Solid lines are defined by the unit $\vec{P}$-vectors indicated in brackets (Table S1).

Measurement of hydrogen isotope ratios enables the combination of δ^2H with δ^{13}C and δ^{37}Cl data in a multi-element isotope plot (Figure 3). This new approach was investigated to determine whether the multi-element isotope patterns are sufficiently different to potentially distinguish among different aerobic and anaerobic biodegradation pathways in the field. As observed in Figure 3, the strongly different trends further strengthen pathway identification in future biodegradation studies for 1,2-DCA. In order to identify the degradation pathway of 1,2-DCA using a 3D approach, multi-element isotope data from new field (or laboratory) studies can be characterized by principal component analysis (see SI). The obtained $\vec{P}$-vector can then be compared to those reference $\vec{P}$-vectors reported in

399 this study for different reactions (Figure 3) by determining the angle between them (see
400 S.I., eq S8). In complex sites where different biodegradation pathways may be involved,
401 the occurrence of up to three different pathways might be detected using a 3D approach.
402 For instance, in a site where three potential biodegradation pathways may control the fate
403 of 1,2-DCA (e.g., aerobic biodegradation via oxidation and hydrolytic dehalogenation and
404 anaerobic dihaloelimination by *Dehalogenimonas* populations), data points situated
405 between their respective reference $\vec{P}$-vectors would indicate the effect of all of them.
406 However, unequivocal information is hampered if more than three pathways occur at the
407 site. If there are more than three pathways, additional information (e.g., redox data) is
408 required to constrain pathways.

409 The distinctly different $\Lambda_{C\text{-}H}$ values in Figure 2 can be rationalized in terms of the
410 corresponding degradation mechanisms. For oxidation of 1,2-DCA, a highly pronounced H
411 isotope fractionation was observed (ε^{H}_{bulk} = -115 ± 18‰), reflecting a strong primary H
412 isotope effect during oxidative C-H bond cleavage (Scheme 1a). In addition, smaller but
413 non-negligible secondary isotope effects in H atoms located in proximity to the reacting
414 bond (see below) are likely represented in the observable bulk fractionation.[41, 47] In
415 contrast, for hydrolytic dehalogenation and dihaloelimination reactions (Schemes 1b-d),
416 smaller ε^{H}_{bulk} values were determined, ranging from -34 ± 4‰ to -77 ± 9‰, since only
417 secondary H isotope effects are involved during C-Cl bond cleavage. Conversely, a lower
418 ε^{C}_{bulk} value was obtained for carbon during oxidation (-3.5 ± 0.1‰ for C-H bond
419 cleavage),[24] relative to those determined for hydrolytic dehalogenation and
420 dihaloelimination reactions (from -23 ± 2‰ to -33.0 ± 0.4‰ for C-Cl bond cleavage),[14, 24]
421 due to the different mass of its bonding partner.[34] As a result, the largest $\Lambda_{C\text{-}H}$ value was
422 obtained for the C-H oxidation pathway (Figure 2). For the reactions without an initial
423 primary H isotope effect, the different ε^{H}_{bulk} values for hydrolytic dehalogenation (average

424 value of -36 ± 3‰) and dihaloelimination by *Dehalococcoides* (-57 ± 3‰) and
425 *Dehalogenimonas* (-77 ± 9‰) containing cultures compared with their relatively similar
426 ε^{C}_{bulk} values (from -23 ± 2‰ to -33.0 ± 0.4‰)[14, 24] also resulted in distinctly different $\Lambda_{C\text{-}H}$
427 values (Figure 2). The variation between ε^{H}_{bulk} values for hydrolytic dehalogenation and
428 dihaloelimination reactions and also between dihaloelimination by *Dehalococcoides* and
429 *Dehalogenimonas*-containing cultures is discussed below.

430 **Secondary H isotope fractionation and insight into anaerobic dihaloelimination**
431 **mechanism**

432 During transformation of 1,2-DCA by *A. aquaticus* and *X. autotrophicus* by a haloalkane
433 hydrolytic dehalogenase reaction (i.e., nucleophilic substitution S_N2-type,[5, 6] Scheme 1b), a
434 single C-Cl bond cleavage occurs in the first reaction step. Therefore, no primary H isotope
435 effect would be expected for this pathway and the measured ε^{H}_{bulk} values (-34 ± 4‰ and -
436 38 ± 4‰) represent the average secondary H isotope fractionation of all positions, i.e., α-
437 secondary isotope effects in two H atoms located next to the reacting bond and β-
438 secondary isotope effects in two H atoms situated one position away from the reacting
439 bond. The H atoms situated one bond apart from the reacting bond might exhibit a smaller
440 secondary isotope effect (see[33, 34] and references herein) compared to those atoms adjacent
441 to the reaction bond. For instance, α-secondary KIE_H = 1.1-1.2 and β-secondary KIE_H =
442 1.05-1.15 are expected for a nucleophilic substitution S_N1-type involving C-Cl bonds.[34]

443 Similarly to hydrolytic dehalogenation, no primary H isotope effect is expected in the
444 initial transformation of 1,2-DCA by *Dehalococcoides* and *Dehalogenimonas*-containing
445 cultures since no C-H bond is broken during dihaloelimination of 1,2-DCA to ethene
446 (Scheme 1c). However, much higher secondary ε^{H}_{bulk} values (-57 ± 3‰ and -77 ± 9‰)
447 were determined for both cultures compared to those obtained for hydrolytic
448 dehalogenation. The large secondary compound average H isotope fractionation values

449 measured for dihaloelimination are ~ 50 – 70% of that measured for C-H oxidation by
450 *Pseudomonas* (-115 ± 18‰), which is remarkable given that no C-H bond is broken in the
451 dihaloelimination reaction.

452 For enzymatic dihaloelimination of 1,2-DCA, a previous study based on C and Cl
453 isotope fractionation suggested that the difference between ε^{C}_{bulk} and ε^{Cl}_{bulk} values obtained
454 in experiments with *Dehalococcoides* and *Dehalogenimonas*-containing cultures could be
455 associated with a different mode of *concerted* bond cleavage rather than with *stepwise*
456 versus *concerted* reactions (Scheme 1c, d).[14] Assuming *concerted* dihaloelimination of 1,2-
457 DCA (Scheme 1c), α-secondary isotope effects may be anticipated for all H atoms. The
458 location of all H atoms next to simultaneously reacting bonds could explain the higher
459 secondary ε^{H}_{bulk} values measured for dihaloelimination compared to hydrolytic
460 dehalogenation, where β-secondary isotope effects are involved in the latter.

461 α-secondary AKIEs of 1.060 ± 0.003 and 1.08 ± 0.01 were calculated for *concerted*
462 dihaloelimination of 1,2-DCA by *Dehalococcoides* and *Dehalogenimonas*-containing
463 cultures, respectively, assuming simultaneous secondary effects without intramolecular
464 competition (see SI). These values agree well with the α-secondary KIE_H (i.e., from 0.95 to
465 1.2) reported for nucleophilic substitution (S_N1- and S_N2-type).[34] The different magnitude
466 of α-secondary $AKIE_H$ during 1,2-DCA transformation by *Dehalococcoides* and
467 *Dehalogenimonas*-containing cultures might reflect a different interaction mode between
468 reductive dehalogenases and 1,2-DCA (e.g., how leaving groups were stabilized in
469 different enzyme environments) as previously proposed to explain the differences on C and
470 Cl isotope effects (see Palau *et al.*[14] and references therein). Comparison of the hydrogen
471 AKIEs determined in this study with those obtained using quantum mechanical/molecular
472 mechanical modeling (QM/MM) in future studies can help to elucidate the enzymatic
473 reaction mechanisms in more detail.

474 The evidence from H isotope ratios obtained in this study are in agreement with
475 *concerted* dihaloelimination of 1,2-DCA by *Dehalococcoides* and *Dehalogenimonas*-
476 containing cultures, showing that further insight into enzymatic reductive dechlorination of
477 1,2-DCA can be obtained from hydrogen isotope fractionation. Such insight cannot be
478 obtained from end product analysis because the same product (i.e., ethene) is formed
479 during transformation of 1,2-DCA via *concerted* or *stepwise* dihaloelimination (Scheme
480 1c,d). As observed for 1,2-DCA in this study, the different magnitude of secondary H
481 isotope effects might help to differentiate between *concerted* and *stepwise*
482 dihaloelimination mechanisms during biodegradation of other chlorinated ethanes of
483 environmental concern, such 1,1,2-trichloroethane or 1,1,2,2-tetrachloroethane, in future
484 studies.

485 **Environmental Significance**

486 In addition to elucidating natural biodegradation processes for 1,2-DCA, multi-element
487 isotope analysis can also be useful for obtaining insight into enhanced remediation
488 processes such as in-situ bioaugmentation.[48-50] This form of enhanced biodegradation
489 typically reduces the time required to reach target remediation goals at contaminated sites.
490 However, its application and evaluation can be difficult at numerous sites impacted by
491 mixtures of chlorinated compounds such as ethenes and ethanes. A recent study by Mayer-
492 Blackwell *et al.*[51] showed that reductive dihaloelimination of 1,2-DCA by a
493 *Dehalococcoides mccartyi* consortium was strongly inhibited by *cis*-DCE. These authors
494 also suggested that the presence of a significant 1,2-DCA concentration in groundwater
495 that is co-contaminated with chlorinated ethenes may alter the *Dehalococcoides* population
496 structure away from conditions ideal for complete degradation of VC. In this case,
497 degradation of 1,2-DCA by other ORB such as *Dehalogenimonas* could eventually
498 eliminate its inhibitory effect on VC degradation by *Dehalococcoides* populations in the

field. Therefore, knowledge of the fate of 1,2-DCA at sites co-contaminated with chlorinated ethenes is crucial for site remediation. However, the assessment of 1,2-DCA transformation by *Dehalococcoides* or *Dehalogenimonas* populations in groundwater co-contaminated with chlorinated ethenes is particularly challenging because the same products (i.e., ethene and VC) are also obtained during biodegradation of chlorinated ethenes. The results of this study show that dihaloelimination of 1,2-DCA by ORB such *Dehalococcoides* or *Dehalogenimonas* could be identified using a dual- (δ^2H vs. δ^{13}C) and a multi-element (δ^2H vs. δ^{13}C vs. δ^{37}Cl) isotope approach, illustrating the potential of H isotope analysis in combination with C and Cl isotope data to investigate transformation pathways for 1,2-DCA in the field. In addition to the ORBs investigated in this study, other anaerobic bacteria are also able to reductively dechlorinate 1,2-DCA. Therefore, the multi-element isotope patterns determined for the *Dehalococcoides* and *Dehalogenimonas*-containing enrichment cultures should be compared to the patterns obtained using other types of microbes, such *Dehalobacter*[8] or *Desulfitobacterium*,[52] in future studies.

Identification of the 1,2-DCA degradation pathway in the field can help constrain the range of ε_{bulk} values used to estimate contaminant degradation extent using the Rayleigh equation,[53, 54] which is one of the main applications of CSIA to field studies. The new hydrogen isotope fractionation values for 1,2-DCA determined in this study, under both oxic and anoxic conditions, opens the possibility for using H-CSIA to quantify degradation at sites polluted by 1,2-DCA. In addition, the ε^{H}_{bulk} values for the different reaction pathways determined in this study are larger than their respective ε^{C}_{bulk}[13, 14, 24, 35, 55-58] and ε^{Cl}_{bulk}[14, 24] values, indicating that hydrogen isotopic fractionation can be a more sensitive indicator than carbon and chlorine isotopic fractionation. This can be particularly important for obtaining a better assessment of microbial oxidation of 1,2-DCA in the field (ε^{H}_{bulk}= -

523 115 ± 18‰ compared to the ε^{C}_{bulk} average value of -3.8 ± 0.8‰, ± 1σ, n = 6 [24, 57] and the
524 ε^{Cl}_{bulk} value of -3.8 ± 0.2 [24]).

525 Groundwater contaminant plumes are dynamic and highly heterogeneous systems
526 subject to temporal and spatial geochemical variations that control biodegradation
527 processes in an aquifer.[59] At contaminated sites, multi-isotope analysis in combination with
528 appropriately high resolution sampling could be used to investigate the distribution of 1,2-
529 DCA biodegradation processes associated with steep redox gradients, from oxic to anoxic
530 environments (e.g., as often occurs with depth), which has rarely been considered in
531 contaminant biodegradation studies. Understanding active degradation processes in the
532 field is essential for evaluating natural attenuation and predicting how far a groundwater
533 plume of 1,2-DCA might migrate. In this case, it is important to know if aerobic or
534 anaerobic biodegradation occurs, especially since aerobic degradation rates for 1,2-DCA
535 are likely controlled by the availability of oxygen. Hence, the conceptual model, and
536 accordingly the mathematical model, used to predict plume behavior would be quite
537 different. It also has implications for enhancing one remediation approach over another,
538 e.g., adding more oxygen or adding electron donor, depending on the identified
539 biodegradation pathway. Based on the results of this study, multi-element isotope patterns
540 are expected i) to identify the active transformation pathway for 1,2-DCA; and ii) to detect
541 changes in biodegradation conditions (i.e., aerobic vs. anaerobic biodegradation) allowing
542 for better characterization of degradation processes in the field.

543 ASSOCIATED CONTENT

544 **Supporting Information**

545 Further information about chemicals, analytical methods, reaction kinetics, correction of
546 the substrate remaining fraction, Rayleigh isotope plot, calculation of H-AKIEs, dual Cl-H

isotope plot, and characterization of multi-element (Cl, C, H) isotope trends is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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