Supporting Information

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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CHEMICALS

The purity and suppliers of 1,2-DCA used in the laboratories of Clemson University (CU), University of Neuchâtel (UN) and Universitat Autònoma de Barcelona (UAB) to prepare the biodegradation experiments are: Mallinckrod (99%), Fluka ($\geq$ 99.5%) and Sigma-Aldrich (> 99%), respectively.

ANALYTICAL METHODS

Concentration analysis

Concentration measurements in each laboratory were performed as described previously.[1-3] Briefly, concentrations of 1,2-DCA for the aerobic experiments were measured at the UN laboratory by headspace (HS) analysis using a TRACE GC-DSQII MS instrument (Thermo Fisher Scientific, Waltham, MA, USA) in single-ion mode (*m/z* 51, 62, 64, 98, and 100). The samples were prepared in 20 mL HS vials filled with 10 mL of solution. HS injections were performed using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) and HS volumes of 500 µL were injected through a split / splitless injector (split ratio 1:10) set to 200 °C. The GC was equipped with a RTX-VMS column (30 m × 0.25 mm ID × 1.4 µm; Restek, Bellefonte, PA, USA). Obtained aqueous-phase concentrations were corrected for gas-water partitioning and repetitive liquid sample removal during the experiments according to Buchner *et al.*[4] (see below).

The amounts of 1,2-DCA in the anaerobic *Dehalococcoides*-containing microcosms were measured at CU laboratory using a GC-FID (Hewlett Packard 5890 Series II, Wilmington, DE, USA) equipped with a Supelco 1% SP-1000 on 60/80 Carbopack B column (Supelco, St. Louis, MO, USA). The GC-FID response to a HS sample (0.5 mL) was calibrated to give the total mass of 1,2-DCA in that bottle, which was then converted to an aqueous-phase concentration using Henry's Law constants.

Concentration measurements of 1,2-DCA in the anaerobic *Dehalogenimonas*-containing microcosms were performed at the UAB laboratory. HS samples of 0.5 mL were analyzed using a GC model 6890N (Agilent Technologies; Santa Clara, CA, USA) equipped with a DB-624 column (30 m × 0.32 mm ID × 0.25 µm; Agilent Technologies) and a FID detector. The target compounds were identified using retention times of chemical standards. Calibration was based on aqueous standards with the same liquid and HS volumes as in the experimental bottles.

Hydrogen isotope analysis

The H-CSIA of 1,2-DCA was performed at Isotope Tracer Technologies Inc., Canada, according to Shouakar-Stash and Drimmie.[5] The 1,2-DCA in solution was extracted by solid phase micro extraction (SPME). The sample solution was stirred at room temperature and 1,2-DCA was extracted during 20 min by headspace SPME using a manual sampler holder equipped with a 75 μm Carboxen-PDMS fiber (Supelco, Bellefonte, PA). Hydrogen isotope ratios were measured using a Continuous Flow – Isotope Ratio Mass Spectrometer (CF-IRMS, Deltaplus XL, ThermoFinnigan, Bremen, Germany). The IRMS is coupled with a GC model 6890 (Agilent Technologies) and a reduction system. The GC is equipped with a split/splitless injection inlet set at 250ºC and a DB-5ms capillary column (60 m × 0.32 mm ID × 1 μm; Agilent Technologies, Mississauga, ON, Canada). A high temperature (Max T: 1200°C) tube furnace (Thermcraft, model XST-3-0-12-10, Winston-Salem, NC, USA) is used for the Cr reduction system.

Pure 1,2-DCA and TCE isotopic standards were used for instrument monitoring and external two-point calibration of sample δ^2H values to the international scale. These H isotope standards were characterized beforehand ($\delta^2H_{1,2-DCA}$ = -50 ± 1‰, 1σ, n = 10 and δ^2H_{TCE} = +565 ± 4‰, 1σ, n = 8) by analyzing the pure phase compounds on an elemental analyzer (Heraeus D-6450 Hanau, Type VT, CHN-O-Rapid, Germany) coupled to and IRMS (Deltaplus XL, ThermoFinnigan).[6, 7] The EA was equipped with a Cr reduction tube and a high precision A200S autosampler (CTC Analytics CH-4222, Zwingen).[8] The standards were calibrated with the International Atomic Energy Agency (IAEA) international reference standards for hydrogen isotopes (i.e., VSMOW, GISP and SLAP).

Previous to sample analysis, a mass dependence test was performed using the isotopic standard of 1,2-DCA. Several aqueous standard measurements were performed at different concentrations, within a range of signal amplitude of m/z 2 between 803 mV and 6975 mV. The δ^2H value of the standard was highly reproducible within this range of signal amplitude (± 4 ‰, 1σ, n = 33) and linearity issues were not observed.

KINETICS OF 1,2-DCA BIODEGRADATION

For the aerobic experiments, substrate degradation rates were estimated using a zero-order kinetic equation. Lag times between ~3 hours (for a microcosm with *Ancylobacter*) and ~12 hours (for a microcosm with *Pseudomonas*) were considered for

the experiments. Determined substrate degradation rate constants (k_{obs}) were between 0.23 and 0.27 mM·h^{-1} ($r^2 > 0.97$) for the experiments with *Ancylobacter*, between 0.14 and 0.16 mM·h^{-1} ($r^2 > 0.96$) for the experiments with *Xanthobacter* and between 0.17 and 0.22 mM·h^{-1} ($r^2 > 0.92$) for the experiments with *Pseudomonas*. The results indicated that the substrate decay can be fairly well described by a zero-order kinetic equation (half-lives ($t_{1/2}$) between ~3.5 and 6.3 h for all the aerobic experiments).

For the anaerobic experiments, substrate degradation and product formation rates were estimated using first-order kinetic equations in a previous study.[9] Briefly, substrate degradation rates (k_{obs}) of 0.023 ± 0.002 h^{-1} ($\pm$ 95% C.I., $r^2 = 0.91$) and 0.0024 ± 0.0002 h^{-1} ($\pm$ 95% C.I., $r^2 = 0.92$) were determined for the microcosms containing *Dehalococcoides* and *Dehalogenimonas*, respectively ($t_{1/2}$ of 30 and 289 h for the experiments with *Dehalococcoides* and *Dehalogenimonas*-containing cultures, respectively).

CORRECTION OF THE SUBSTRATE REMAINING FRACTION (f) FOR SUBSTRATE REMOVAL BY REPETITIVE LIQUID SAMPLES WITHDRAWN DURING THE AEROBIC EXPERIMENTS

For the aerobic experiments, a stepwise correction method proposed by Buchner *et al.*[4] was used to account for repetitive liquid mass removal and for volatilization into the bottle headspace. This method applies an iterative correction scheme using eq. S1 and eq. S2. First, for two consecutive sampling times (i.e., times t and t-1) individual substrate fractions are determined ($f_{(t-1)\to t}$):

$$f_{(t-1)\to t} = \frac{c_w(t)\cdot\left[V_w(t) + \frac{V_g(t)}{K_H}\right]}{c_w(t-1)\cdot\left[V_w(t-1) + \frac{V_g(t-1)}{K_H} - V_{rem}(t-1)\right]} \qquad \text{S1}$$

where $c_w(t)$ and $c_w(t-1)$, $V_w(t)$ and $V_w(t-1)$, $V_g(t)$ and $V_g(t-1)$ are measured concentrations, water volumes and the volumes of the gas phase at time t and t-1, respectively; $V_{rem}(t-1)$ is the sample volume taken at time t-1 and K_H is the dimensionless Henry-constant ($K_H = c_w / c_g$) at the appropriate temperature. Second, the overall substrate fraction at time t (i.e., $f(t)$) is calculated from eq. S2 considering the results of eq. S1 for all preceding time steps and $f(0) = 1$:

$$f(t) = f(0) \cdot f_{t0 \to t1} \cdot f_{t1 \to t2} \cdot \ldots \cdot f_{t(n-1) \to tn}$$ S2

RAYLEIGH ISOTOPE PLOT

Bulk hydrogen ε values were obtained from the slopes of Rayleigh plots according to eq. 2 in the in the main document (Figure S1).
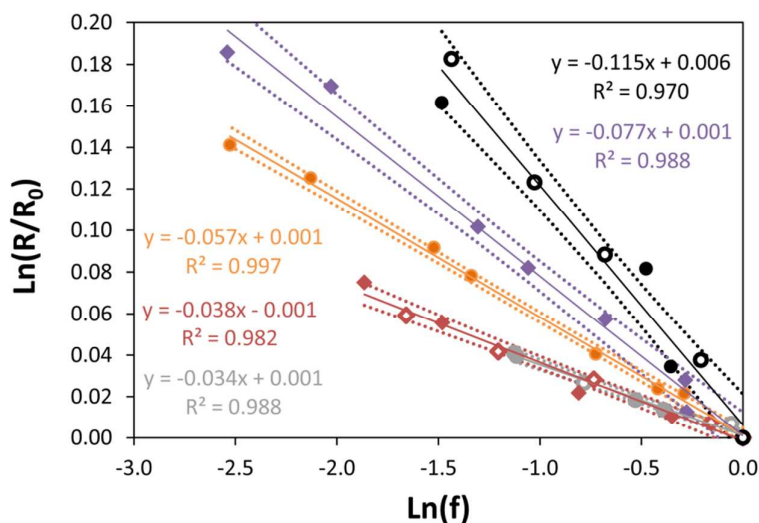

Figure S1. Hydrogen isotope regressions for 1,2-DCA during transformation via oxidation by *Pseudomonas* sp. (black circles), hydrolytic dehalogenation by *A. aquaticus* (grey circles) and *X. autotrophicus* (red diamonds), and dihaloelimination by *Dehalococcoides*- (orange circles) and *Dehalogenimonas*-containing cultures (violet diamonds). For oxidation and hydrolytic dehalogenation experiments, solid and empty symbols represent data from two replicate bottles. Dotted lines indicate the 95% confidence intervals of the linear regression.

ESTIMATION OF ^{2}H-AKIEs FOR OXIDATION AND CONCERTED DIHALOELIMINATION OF 1,2-DCA

Intrinsic KIEs are position specific whereas observable ε_{bulk} values are calculated from compound-average isotope data (eq. 2 in the main text). Therefore, for the calculation of ^{2}H-AKIEs changes in bulk isotope ratios need to be converted into position-specific changes (eq. S3):[10]

$$\ln\left(\frac{R_0 + \frac{n}{x}(R_t - R_0)}{R_0}\right) = \varepsilon_{rp}^H \cdot \ln f \qquad \text{S3}$$

$$AKIE_H = \frac{1}{\varepsilon_{rp}^H + 1} \qquad \text{S4}$$

where f is the substrate remaining fraction, R_t and R_0 are the current and initial isotope ratios, ε_{rp}^H is the hydrogen isotopic fractionation at the reactive position, n is the number of hydrogen atoms, x is the number of hydrogen atoms that would experience isotope effects in the given mechanistic scenario.

For oxidation of 1,2-DCA, out of four C-H bonds, only one is broken at the initial reaction step. Therefore, $n/x = 4/1$ in eq S3. It is important to note that, for oxidation, only the primary isotope effect was taken into account in the calculations. For a *concerted* dihaloelimination of 1,2-DCA, all hydrogen atoms are in equivalent positions (n = x), assuming simultaneous secondary hydrogen isotope effects. In this case, eq. S3 is identical to eq. 2 (in the main text), and therefore, the secondary ε_{rp}^H values can be directly obtained from the slopes of the Rayleigh plots (Figure S1).

DUAL Cl-H ISOTOPE APPROACH

Measured δ^2H values of 1,2-DCA were combined with previously determined $\delta^{37}Cl$ values for these experiments[1, 9] in a dual Cl-H isotope plot (Figure S2).
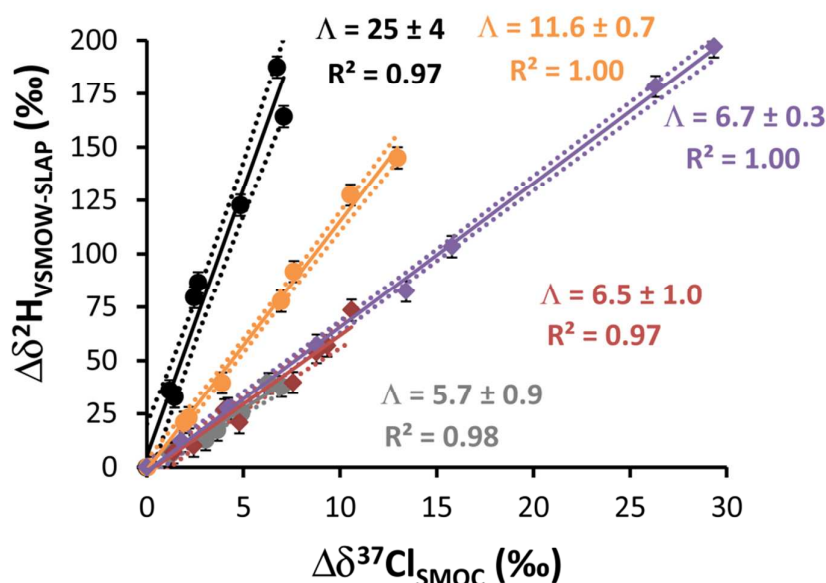
Figure S2. Dual Cl-H isotope trends during biodegradation of 1,2-DCA via oxidation by *Pseudomonas* sp. (black circles), hydrolytic dehalogenation by *A. aquaticus* (grey circles) and *X. autotrophicus* (red diamonds), and dihaloelimination by *Dehalococcoides*- (orange circles) and *Dehalogenimonas*-containing cultures (violet diamonds). Dotted lines indicate the 95% confidence intervals of the linear regression. Error bars of $\Delta\delta^{37}Cl$ values are smaller than the symbols. Λ values ($\pm$95% C.I.) are given by the slope of the linear regressions

CHARACTERIZATION OF MULTI-ELEMENT (Cl, C, H) ISOTOPE FRACTIONATION PATTERNS OF 1,2-DCA

For a given substrate degradation process, the vector $\overrightarrow{(\Delta\delta^{37}Cl, \ \Delta\delta^{13}C, \ \Delta\delta^{2}H)}$ can be determined based on the shifts of the isotope ratios of the substrate for the different elements (i.e., $\delta^{h}E - \delta^{h}E_{0}$). This vector can be combined with the Rayleigh equation (which can be approximated by $\delta^{h}E - \delta^{h}E_{0} = \Delta\delta^{h}E \approx \varepsilon_{bulk} \cdot \ln f$) leading to the following expression,

$$\overrightarrow{(\Delta\delta^{37}Cl, \ \Delta\delta^{13}C, \ \Delta\delta^{2}H)} = \overrightarrow{\left(\varepsilon_{bulk}^{Cl} \cdot \ln f, \ \varepsilon_{bulk}^{C} \cdot \ln f, \ \varepsilon_{bulk}^{H} \cdot \ln f\right)} = \ln f \cdot \overrightarrow{\left(\varepsilon_{bulk}^{Cl}, \ \varepsilon_{bulk}^{C}, \ \varepsilon_{bulk}^{H}\right)} \qquad S5$$

The orientation and direction of $\overrightarrow{(\Delta\delta^{37}Cl, \ \Delta\delta^{13}C, \ \Delta\delta^{2}H)}$ and $\overrightarrow{\left(\varepsilon_{bulk}^{Cl}, \ \varepsilon_{bulk}^{C}, \ \varepsilon_{bulk}^{H}\right)}$ is the same, provided that absolute ε_{bulk} values are used. Therefore, their unit vector (i.e., normalized vector) is also the same (eq. S6, equivalent to eq. 3 in the main text).

$$\vec{P} = \frac{1}{\sqrt{\left(\Delta\delta^{37}Cl\right)^{2} + \left(\Delta\delta^{13}C\right)^{2} + \left(\Delta\delta^{2}H\right)^{2}}} \cdot \overrightarrow{(\Delta\delta^{37}Cl, \ \Delta\delta^{13}C, \ \Delta\delta^{2}H)} =$$

$$= \frac{1}{\sqrt{\left(\varepsilon_{bulk}^{Cl}\right)^{2} + \left(\varepsilon_{bulk}^{C}\right)^{2} + \left(\varepsilon_{bulk}^{H}\right)^{2}}} \cdot \overrightarrow{\left(\varepsilon_{bulk}^{Cl}, \ \varepsilon_{bulk}^{C}, \ \varepsilon_{bulk}^{H}\right)} \qquad S6$$

Where $\left((\Delta\delta^{37}Cl)^{2} + (\Delta\delta^{13}C)^{2} + (\Delta\delta^{2}H)^{2}\right)^{\frac{1}{2}}$ and $\left((\varepsilon_{bulk}^{Cl})^{2} + (\varepsilon_{bulk}^{C})^{2} + (\varepsilon_{bulk}^{H})^{2}\right)^{\frac{1}{2}}$ are their respective norms. The unit vector $\vec{P}$ defines a spatial direction in a 3D isotope plot. For a given process "i", a linear multi-element isotope trend can be defined by eq. S7.

$$(\Delta\delta^{37}\text{Cl},\ \Delta\delta^{13}\text{C},\ \Delta\delta^{2}\text{H})_i = (\Delta\delta^{37}\text{Cl}_0,\ \Delta\delta^{13}\text{C}_0,\ \Delta\delta^{2}\text{H}_0)_i +$$

$$+\, k\left(\frac{\varepsilon^{Cl}_{bulk}}{\sqrt{(\varepsilon^{Cl}_{bulk})^2+(\varepsilon^{C}_{bulk})^2+(\varepsilon^{H}_{bulk})^2}},\ \frac{\varepsilon^{C}_{bulk}}{\sqrt{(\varepsilon^{Cl}_{bulk})^2+(\varepsilon^{C}_{bulk})^2+(\varepsilon^{H}_{bulk})^2}},\ \frac{\varepsilon^{H}_{bulk}}{\sqrt{(\varepsilon^{Cl}_{bulk})^2+(\varepsilon^{C}_{bulk})^2+(\varepsilon^{H}_{bulk})^2}}\right)_1 \qquad \text{S7}$$

Where $(\Delta\delta^{37}\text{Cl}_0,\ \Delta\delta^{13}\text{C}_0,\ \Delta\delta^{2}\text{H}_0) = (0,0,0)$ and k is a scalar describing a particular point on the line.

For a given set of measured multi-element isotope data, for instance during a degradation experiment in the laboratory, the unit vector $\vec{P}$ can also be obtained by principal component analysis using SigmaPlot v.13.0 (Systat Software, Inc., San Jose, CA). This is another approach that simultaneously incorporates the isotope data from all three elements, compared to eq. S6 where one element is evaluated at a time using the Rayleigh plot and then the outcome is combined. Using a principal component analysis, the vector $\vec{P}$ can be estimated by determining the eigenvector corresponding to the first principal component (PC 1). The first principal component is the direction along which the samples show the largest variation. Table S1 shows the calculated eigenvectors for the degradation experiments of 1,2-DCA performed in this study. The lines defined by the calculated eigenvectors are shown in Figure S3.

Table S1. Eigenvectors (PC 1) of the covariance matrix and standard errors

	$\Delta\delta^{37}\text{Cl}$	$\Delta\delta^{13}\text{C}$	$\Delta\delta^{2}\text{H}$
A. aquaticus	0.106 ± 0.002	0.804 ± 0.006	0.585 ± 0.008
X. autotrophicus	0.102 ± 0.001	0.772 ± 0.007	0.627 ± 0.009
Pseudomonas sp.	0.038 ± 0.001	0.034 ± 0.001	0.999 ± <0.001
Dehalococcoides *	0.075 ± 0.001	0.494 ± 0.002	0.866 ± 0.001
Dehalogenimonas *	0.143 ± 0.001	0.271 ± 0.001	0.952 ± <0.001

* containing cultures

The $\vec{P}$-vectors calculated using eq. S6 were compared with those determined by principal component analysis. For instance, for dihaloelimination of 1,2-DCA by *Dehalococcoides* (ε^{Cl}_{bulk} = -5.1±0.1‰, ε^{C}_{bulk} = -33.0±0.4‰ and ε^{H}_{bulk} = -57±3‰) and *Dehalogenimonas* (ε^{Cl}_{bulk} = -12.0±0.8‰, ε^{C}_{bulk} = -23±2‰ and ε^{H}_{bulk} = -77±9‰)

containing cultures, the $\overrightarrow{P_{Dhc}}$ = (0.077, 0.50, 0.86) and $\overrightarrow{P_{Dhg}}$ = (0.15, 0.28, 0.95) were obtained from eq. S6, respectively (note that absolute ε_{bulk} values were used in calculations). These were very similar to those obtained from principal component analysis (Table S2). The ε_{bulk}^{Cl} and ε_{bulk}^{C} values for the experiments with *Dehalococcoides- and Dehalogenimonas*-containing cultures were reported by Palau et al.[9]

Table S2. Comparison of $\vec{P}$-vectors determined by principal component analysis (PCA) with those calculated from eq. S6 (eq. 3 in the main document)

	$\Delta\delta^{37}Cl$	$\Delta\delta^{13}C$	$\Delta\delta^2H$	Method
Dehalococcoides *	0.075 ± 0.001	0.494 ± 0.002	0.866 ± 0.001	PCA
	0.077 ± 0.003	0.50 ± 0.02	0.86 ± 0.01	eq. S6
Dehalogenimonas *	0.143 ± 0.001	0.271 ± 0.001	0.952 ± <0.001	PCA
	0.15 ± 0.02	0.28 ± 0.04	0.95 ± 0.01	eq. S6

 * containing cultures

Hypothetical isotope data from a field site contaminated with 1,2-DCA was used as an example of the potential application of a multi-element isotope approach. In the simplest case, two groundwater samples collected at the source "S_0" and in the contaminant plume downstream of the source "S", respectively, could be analyzed for Cl, C and H isotope ratios of 1,2-DCA (i.e., $\delta^{37}Cl_{SMOC}$, $\delta^{13}C_{VPDB}$ and $\delta^2H_{SMOW-SLAP}$). For instance, the values (in ‰) of (0, -20, -50) and (+4, -16, +135) could be obtained for S_0 and S, respectively, provided that 1,2-DCA was affected by biodegradation along the groundwater flow path. Using these two data points, the vector $\vec{S}$ can be obtained: $\vec{S}$ = S – S_0 = ($\Delta\delta^{37}Cl$, $\Delta\delta^{13}C$, $\Delta\delta^2H$) = (+4, +4, +185), see Figure S3. Then, in order to identify the degradation pathway of 1,2-DCA, the angle θ between $\vec{S}$ and each $\vec{P}$-vector reported in this study (Figure S3) can be calculated according to eq. S8,

$$\cos\theta = \frac{\vec{S}\cdot\vec{P}}{|\vec{S}|\cdot|\vec{P}|} = \frac{s_1p_1+s_2p_2+s_3p_3}{\sqrt{s_1^2+s_2^2+s_3^2}\cdot\sqrt{p_1^2+p_2^2+p_3^2}} \qquad\qquad S8$$

where (s_1, s_2, s_3) and (p_1, p_2, p_3) are the coefficients of $\vec{S}$ and $\vec{P}$, respectively. For this example, relatively large θ values, from 16.2º to 52.8º, were obtained between $\vec{S}$ and the

$\vec{P}$-vectors for the hydrolytic dehalogenation and dihaloelimination pathways, indicating that these reactions were probably not responsible of 1,2-DCA degradation. In contrast, a relatively small value of 1.2º was obtained between $\vec{S}$ and the $\vec{P}$-vector for aerobic oxidation, suggesting 1,2-DCA degradation via oxidation in groundwater.
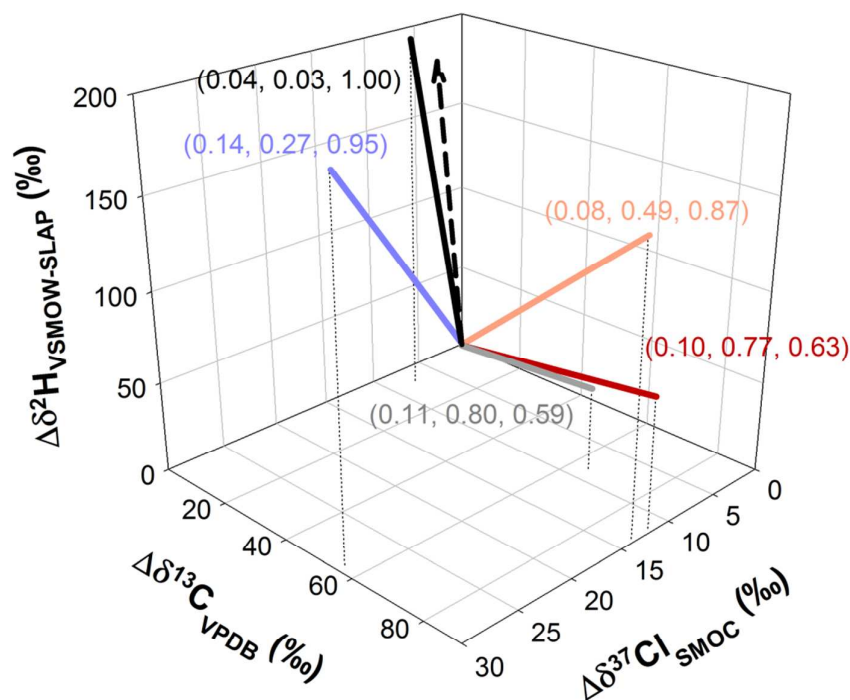

Figure S3. Multi-element isotope patterns during biodegradation of 1,2-DCA via different degradation pathways (solid lines): oxidation by *Pseudomonas* sp. (black), hydrolytic dehalogenation by *A. aquaticus* (grey) and *X. autotrophicus* (red), and dihaloelimination by *Dehalococcoides*- (orange) and *Dehalogenimonas*-containing cultures (violet). The solid lines are defined by the $\vec{P}$-vectors indicated in brackets (Table S1). The dashed line corresponds to the vector $\vec{S}$ (see the text).

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