C and Cl isotope fractionation of 1,2-dichloroethane displays unique $\delta^{13}C/\delta^{37}Cl$ patterns for pathway identification and reveals surprising C-Cl bond involvement during microbial oxidation

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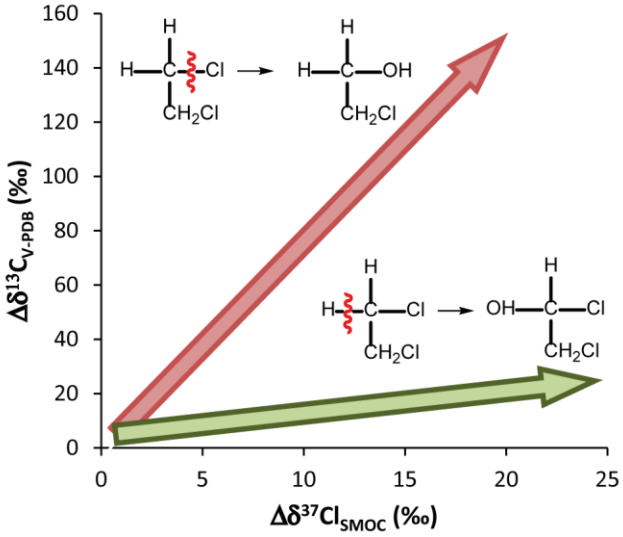
TOC/Abstract art



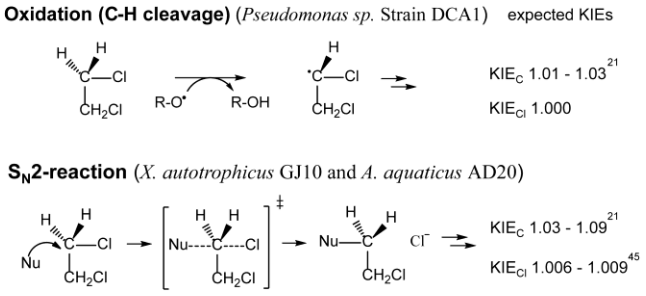
ABSTRACT

This study investigated dual element isotope fractionation during aerobic biodegradation of 1,2-dichloroethane (1,2-DCA) via oxidative cleavage of a C-H bond (*Pseudomonas sp*. Strain DCA1) versus C-Cl bond cleavage by S_N2 reaction (*Xanthobacter autrophicus* GJ10 and *Ancylobacter aquaticus* AD20). Compound-specific chlorine isotope analysis of 1,2-DCA was performed for the first time and isotope fractionation ε^{Cl}_{bulk} was determined by measurements of the same samples in three different laboratories using two GC-IRMS and one GC-quadrupole MS. Strongly pathway-dependent slopes ($\Delta\delta^{13}C$ / $\Delta\delta^{37}Cl$), 0.78±0.03 (Oxidation) and 7.7±0.2 (S_N2), delineate the potential of the dual isotope approach to identify 1,2-DCA degradation pathways in the field. In contrast to different ε^{C}_{bulk} values: -3.5±0.1‰ (Oxidation), -31.9±0.7‰ and -32.0±0.9‰ (S_N2), the obtained ε^{Cl}_{bulk} values were surprisingly similar for the two pathways: -3.8±0.2‰ (Oxidation), -4.2±0.1‰ and -4.4±0.2‰ (S_N2). Apparent kinetic isotope effects of ^{13}C-AKIE=1.0070±0.0002 (Oxidation), ^{13}C-AKIE=1.068±0.001 (S_N2) and ^{37}Cl-AKIE=1.0087±0.0002 (S_N2) fell within expected ranges. In contrast, an unexpectedly large secondary ^{37}Cl-AKIE of 1.0038±0.0002 reveal a hitherto unrecognized involvement of C-Cl bonds in microbial C-H bond oxidation. Our 2D isotope fractionation patterns enable for the first time reliable 1,2-DCA degradation pathway identification in the field, which unlocks the full potential of isotope applications for this important groundwater contaminant.

INTRODUCTION

Chlorinated ethanes are among the most widespread contaminants in groundwater[1] and 1,2-dichloroethane (1,2-DCA) has been found in 36% of 1,585 National Priorities List sites identified by the United States Environmental Protection Agency (2001).[2] The presence of 1,2-DCA - an intermediate in plastics production - in groundwater is mainly a consequence of industrial activity.[2] A number of laboratory[3-9] and field[10, 11] studies showed 1,2-DCA biodegradation under aerobic[3-6] and anaerobic[4, 7-11] conditions via different reaction pathways.[9] Under aerobic conditions, 1,2-DCA can be degraded either via nucleophilic substitution (S_N2)[5, 6] or via oxidative cleavage of a C-H bond[3] catalyzed by hydrolytic dehalogenase and monooxygenase enzymes, respectively (Scheme 1). Initial products of both reactions, 2-chloroethanol (S_N2-reaction) and 1,2-dichloroethanol (Oxidation), are further degraded to ubiquitous end products, which hampers a direct identification of degradation pathways from metabolite analysis. Alternative approaches to detect, and identify 1,2-DCA transformation pathways in the subsurface are therefore warranted. This is crucial information in environmental field studies to assess 1,2-DCA natural attenuation.

Scheme 1



Compound specific isotope analysis (CSIA) is an innovative tool to investigate degradation pathways of organic contaminants.[12-14] Isotope ratios of individual compounds, measured either by gas chromatography isotope ratio mass spectrometry (GC-IRMS) or GC-quadrupole mass spectrometry (GC-qMS), are reported using the delta notation:

$$\delta\,{}^{h}E_{sample} = \frac{R({}^{h}E/\,{}^{l}E)_{sample}}{R({}^{h}E/\,{}^{l}E)_{standard}} - 1 \qquad (1)$$

where R is the isotope ratio of heavy (^{h}E) and light (^{l}E) isotopes of element E (e.g., $^{13}C/^{12}C$ and $^{37}Cl/^{35}Cl$). The isotope fractionation (ε) expresses by how much $^{h}E/^{l}E$ is smaller (negative values) or larger (positive values) in the average of freshly formed products compared to the substrate from which they are produced. Transformation-induced isotope fractionation is generally larger than the one related to phase transfer processes such as sorption or volatilization.[15] Bulk (i.e. compound-average) ε values can be calculated using a modified form of the Rayleigh distillation equation:

$$\ln\frac{R_t}{R_0} = \ln\left(\frac{\delta\,{}^{h}E_t+1}{\delta\,{}^{h}E_0+1}\right) = \varepsilon_{bulk}\cdot\ln f \qquad (2)$$

where R_t and R_0 are the current and initial isotope ratios respectively, and f is the compound remaining fraction.

Previous laboratory experiments[16] showed that for 1,2-DCA different carbon ε_{bulk} values of -29.2 and -3.9‰ reflected different reaction pathways: hydrolytic dehalogenation (C-Cl bond cleavage via S_N2) versus oxidation (C-H bond cleavage) respectively (Scheme 1). Knowledge of in situ contaminant biodegradation reactions is essential to evaluate the fate and long term impact of 1,2-DCA in groundwater. For aerobic biodegradation of 1,2-DCA isotope data are particularly important as no characteristic products accumulate. However, while isotope fractionation of one element alone can provide pathway distinction in laboratory experiments (where mass balances can be established and ε_{bulk} values be determined), this is not possible under field conditions. Here, evidence from a second element and a dual isotope approach is necessary to distinguish 1,2-DCA degradation pathways.[17] As observed experimentally,[18, 19] for a given compound, combined isotope analysis of two elements (e.g., $\delta^{13}C$ vs. $\delta^{37}Cl$) during the course of a reaction generally yields a linear trend in a dual element isotope plot with a slope characteristic of the reaction mechanism. The reason is that the dual element isotope slope ($\Lambda = \Delta\delta^{13}C / \Delta\delta^{37}Cl$, where $\Delta\delta^{13}C$ and $\Delta\delta^{37}Cl$ are changes in isotope ratios during degradation) reflects isotope fractionation of both elements.[12] Therefore, different slopes are expected from distinct reaction pathways involving different bonds with different elements.

Currently, there is increasing interest in dual element isotope analysis for improved differentiation of transformation mechanisms and several authors pointed out that complementary mechanistic insight for 1,2-DCA aerobic biodegradation reactions could be achieved by the additional analysis of chlorine[12] and/or hydrogen[16, 20] isotope ratios. The reason is that isotope fractionation can be traced back to underlying kinetic isotope effects, which are highly reaction-specific.[21] During enzymatic degradation molecules containing the light isotope at the reactive site (e.g., ^{35}Cl) typically exhibit a higher reaction rate (e.g., ^{35}k) than those with a

heavy isotope (e.g., ^{37}Cl) resulting in a kinetic isotope effect of KIE = ^{35}k / ^{37}k.[22] When a C-Cl bond is broken a (primary) chlorine leaving group KIE would be expected, whereas in the oxidation reaction chlorine atoms sit next to the reacting bond so that only a secondary KIE would be expected (Scheme 1). Secondary isotope effects at positions next to the reacting bond are generally much smaller than primary isotope effects.[12]

Until recently, Cl-CSIA of chlorinated aliphatic compounds was not feasible, however, because a direct method that would produce a suitable Cl-containing measurement gas inside a chromatographic separation gas was lacking. However, new analytical methods were developed based on the measurement of selected isotopologue ions or isotopologue ion fragments in unconverted analyte molecules using both continuous flow GC-IRMS[23] and GC-qMS.[24-26] In addition, a theoretical framework provided the theoretical justification for such evaluation of isotope fractionation from ion-current ratios of molecular and fragment-ion multiplets.[27] A recent interlaboratory study took the next step and demonstrated that comparable $\delta^{37}Cl$ *values* were obtained when analyzing a set of pure trichlorethene (TCE) *standards* on eight different instruments.[28] Since the technology is so new, however, a comparative study would also be desired which shows that comparable ε_{Cl} *values* are obtained when analyzing *degradation samples* on different instruments and in different laboratories. No such study has been conducted yet. Most notably, Cl-CSIA studies have so far been applied to only few compounds, because two isotopically distinct compound-specific standards are necessary for every new substance.[28] This has restricted applications primarily to chlorinated ethylenes[18, 19, 29-34] so that - to our knowledge - dual element isotope data are currently non-existent for chlorinated ethanes.

This study, therefore, aimed (i) to establish for the first time dual element (C & Cl) isotope analysis of the chlorinated ethane 1,2-DCA; (ii) to perform the Cl isotopic analysis in three

different laboratories (i.e. Waterloo, München and Neuchâtel), using two different GC-IRMS and one GC-qMS, in order to investigate the consistency of ε_{Cl} values obtained with different instruments and analytical methods; (iii) to investigate carbon and chlorine isotope fractionation during aerobic biodegradation of 1,2-DCA with three pure strains to determine whether the dual isotope slopes are sufficiently different to potentially distinguish between hydrolytic dehalogenation (S_N2) and oxidation (C-H bond cleavage) in the field.

MATERIALS AND METHODS

Pure cultures preparation

Three pure strains with known initial biotransformation mechanisms were used for the batch experiments: *Pseudomonas sp*. Strain DCA1 (Oxidation), *Xanthobacter autrophicus* GJ10 and *Ancylobacter aquaticus* AD20 (S_N2 reaction). *X. autrophicus* GJ10 (DSMZ 3874) and *A. aquaticus* AD20 (DSMZ 9000) were purchased (DSMZ, Braunschweig, Germany) and *Pseudomonas sp*. Strain DCA1 was kindly provided by Dr. Elizabeth Edwards (Department of Chemical Engineering and Applied Chemistry, University of Toronto, Canada). The growth medium was prepared as described by Hunkeler and Aravena (2000).[35]

Cultures and experiments were prepared in 250 mL glass bottles, which contained 185 mL of medium and were capped with Mininert-Valves (Vici Precision Sampling, Baton Rouge, LA, US). Cultures were amended with 1,2-DCA (Fluka, ≥ 99.5% purity) and incubated in the dark at room temperature and under continuous shaking (100 rpm). Headspace 1,2-DCA concentrations were monitored throughout the incubation period. Before starting the experiments the cultures

were transferred three times. Each subculture was spiked with pure 1,2-DCA four times before a 15 mL aliquot was transferred to 170 mL of autoclaved fresh medium. The spike volume was 9 µL of pure 1,2-DCA for the first and second subcultures and 22.5 µL for the third, leading to aqueous phase concentrations of 0.6 and 1.5 mM, respectively.

Experiment sampling

All experiments were conducted in triplicate. Experiments and controls were amended with 22.5 µL of pure 1,2-DCA, corresponding to the chlorine isotopic working standard ($\delta^{37}Cl_0$-CHYN2 = +0.8 ± 0.1‰), to produce an initial aqueous concentration of 1.5 mM. Bottles were shaken upside down to prevent leakage of gas phase through the valve. After 1h of shaking, samples representing the initial concentration were collected. For concentration and isotopic analysis, aqueous samples (1.5 mL) were taken at selected time points and preserved frozen[36] in 2 mL vials with NaN_3 (1 g/L). Two abiotic control bottles were prepared with 185 mL of autoclaved mineral medium and samples were collected and preserved like in the experiments. For each culture experiment, sample triplicates were shipped frozen to the University of Waterloo (Canada) and to the Helmholtz Zentrum München (Germany) for chlorine isotope measurements.

Isotopic and concentration analysis

Five pure 1,2-DCA isotopic working standards, one for carbon and four for chlorine, were used for instrument monitoring and external calibration of sample raw $\delta^{37}Cl$ values to the international Standard Mean Ocean Chloride (SMOC) scale. The isotopic signature of the carbon standard ($\delta^{13}C_{V\text{-}PDB}$ = -29.47 ± 0.05‰) was determined beforehand using an elemental analyzer coupled to an IRMS. Regarding the chlorine standards, CHYN1 and CHYN2 ($\delta^{37}Cl_{SMOC}$ = +6.30 ±

180 0.06‰ and +0.84 ± 0.14‰, respectively) were characterized relative to SMOC in München by
181 IRMS after conversion of 1,2-DCA to methyl chloride according to Holt et al. (1997).[37] IT2-
182 3001 and IT2-3002 ($\delta^{37}Cl_{SMOC}$ = +0.83 ± 0.09‰ and -0.19 ± 0.12‰, respectively) were
183 calibrated against the CHYN standards using a GC-IRMS in Waterloo and a GC-qMS in
184 Neuchâtel, respectively.

185 A detailed description of analytical methods is available in the Supplementary Information (SI).
186 Carbon isotopes ratios were measured by GC-IRMS and precision based on the working standard
187 $\delta^{13}C$ value reproducibility was 0.5‰ (1σ). Chlorine isotopic analysis was performed using the
188 following instruments: 1) Waterloo - a 6890 GC (Agilent, Santa Clara, CA, US) coupled to a
189 continuous flow IsoPrime IRMS (Micromass, Manchester, UK; currently Isoprime Ltd, UK), 2)
190 München - a TRACE^{TM} GC (Thermo Fisher Scientific, Milan, Italy) directly coupled to a
191 Finnigan MAT 253 IRMS (Thermo Fisher Scientific, Bremen, Germany) and, 3) Neuchâtel - a
192 7890A GC coupled to a 5975C quadrupole MS (Agilent, Santa Clara, CA, US). The instrument
193 used in Neuchâtel will be referred to in the following text as qMS and those used in Waterloo
194 and München as IRMS-1 and IRMS-2, respectively.

195 For analyzing 1,2-DCA, two ions of the molecular group (100, 102 m/z) were measured with the
196 IRMS-2, whereas the two most abundant fragment ions (62, 64 m/z) were used for the IRMS-1
197 and the qMS. For 1,2-DCA the intensities of the most abundant fragment ion peaks are much
198 higher than those of the parent ion peaks. Both ion couples (100, 102 and 62, 64 m/z) correspond
199 to isotopologue pairs ($[\mathbf{^{37}Cl_2}^{12}C_2{}^{1}H_4]^+$, $[\mathbf{^{37}Cl}^{35}Cl^{12}C_2{}^{1}H_4]^+$ and $[\mathbf{^{37}Cl}^{12}C_2{}^{1}H_3]$+, $[\mathbf{^{35}Cl}^{12}C_2{}^{1}H_3]^+$,
200 respectively) that differ by one heavy chlorine isotope. The isotope ratio (R) can be obtained
201 from the ratio of these isotopologues according to eq. 3,[27]

$$R = \frac{^{37}Cl}{^{35}Cl} = \frac{^{37}p}{^{35}p} = \frac{k}{(n-k+1)} \cdot \frac{^{37}Cl_{(k)}{}^{35}Cl_{(n-k)}}{^{37}Cl_{(k-1)}{}^{35}Cl_{(n-k+1)}} = 2 \cdot \frac{^{102}I}{^{100}I} = \frac{^{64}I}{^{62}I} \quad (3)$$

where ^{37}p and ^{35}p are the probabilities of encountering ^{37}Cl and ^{35}Cl, n is the number of Cl atoms, k is the number of ^{37}Cl isotopes, $^{37}Cl_{(k)}{}^{35}Cl_{(n-k)}$ and $^{37}Cl_{(k-1)}{}^{35}Cl_{(n-k+1)}$ represent the isotopologues containing k and (k-1) heavy isotopes, respectively, and "I" indicates the peak intensity of each ion.

For the qMS, isotope ratios were calculated using eq. 3 and raw $\delta^{37}Cl$ values were determined by referencing versus an external 1,2-DCA working standard according to eq. 1. In this case the standard was dissolved in water and measured like the samples in the same sequence.[26] In IRMS-1 and IRMS-2 raw $\delta^{37}Cl$ values were determined by automatic evaluation of sample's target ion peaks against the ion peaks of the 1,2-DCA monitoring gas, which was introduced by a dual inlet system during each sample run providing an anchor between sample measurements.[28] Subsequently, a two point linear calibration of raw $\delta^{37}Cl$ values to SMOC scale was performed in each laboratory using two external working standards, i.e. IT2(3001 and 3002) for IRMS-1 and CHYN(1 and 2) for IRMS-2 and qMS.[28] The analysis schemes applied in each laboratory are available in SI. For the measurements with the qMS, samples and standards were diluted to a similar concentration and each of them was measured ten times, leading to a precision (1σ) on the analysis of standards of ±0.3‰. For IRMS-1 and IRMS-2 precision on the analysis of standards was ±0.1‰ (1σ). The $\delta^{37}Cl$ of controls remained constant (+0.7 ± 0.2‰, n = 12) at $\delta^{37}Cl_0$ within the analytical uncertainty throughout the experiment.

Concentrations of 1,2-DCA were measured by headspace analysis using a TRACE^{TM} GC-DSQII MS (Thermo Fisher Scientific, Waltham, MA, US) in single ion mode (51, 62, 64, 98 and 100 m/z). Concentrations were determined using a five point calibration curve. The estimated total relative error on analysis of external standards interspersed along the sequence was ±4%. Concentrations were corrected for 1,2-DCA volatilization to the bottle headspace using Henry's law and Henry coefficients.[38] An aqueous phase 1,2-DCA concentration decrease smaller than 5%, due to change of headspace to solution ratio during the experiment, was estimated. The average concentration of controls remained constant throughout the experiments (1.55 ± 0.03 mM, ±1σ, n = 12) indicating that compound losses through the valves and caps during bottles shaking and samples preservation were insignificant.

Calculation of apparent kinetic isotope effects (AKIE)

Kinetic isotope effects are position specific whereas ε_{bulk} values are calculated from compound-average isotope data. Therefore, observable ε_{bulk} values have to be converted into apparent kinetic isotope effects (AKIEs) in order to obtain information about the underlying reaction mechanisms.[21] For the calculation of AKIEs a hypothesis about the reaction mechanism, or assumed reaction mechanism, is necessary. The effects of non-reacting positions within the molecule, as well as of intramolecular competition, are then taken into account using eqs. 4 and 5,[21] respectively,

$$\varepsilon_{rp} \approx \frac{n}{x} \cdot \varepsilon_{bulk} \qquad (4)$$

$$AKIE_{C,Cl} = \frac{1}{z \cdot \varepsilon_{rp} + 1} \quad (5)$$

where ε_{rp} is the isotopic fractionation at the reactive position, n is the number of atoms of the element considered, x is the number of reactive sites and z the number of identical reactive sites undergoing intramolecular competition. In symmetric molecules such as 1,2-DCA all atoms are in equivalent reactive positions (n = x) and, therefore, ε_{rp} is directly obtained from eq 2.[21] The isotope fractionation values were quantified by least square linear regression according to eq 2 without forcing the regression to the origin.[39] As demonstrated Elsner and Hunkeler (2008),[27] the Rayleigh equation (eq. 2) can also be applied to calculate the isotopic fractionation of chlorine despite the higher natural abundance of ^{37}Cl compared to ^{13}C.

RESULTS AND DISCUSSION

Chlorine isotope fractionation values from different instruments

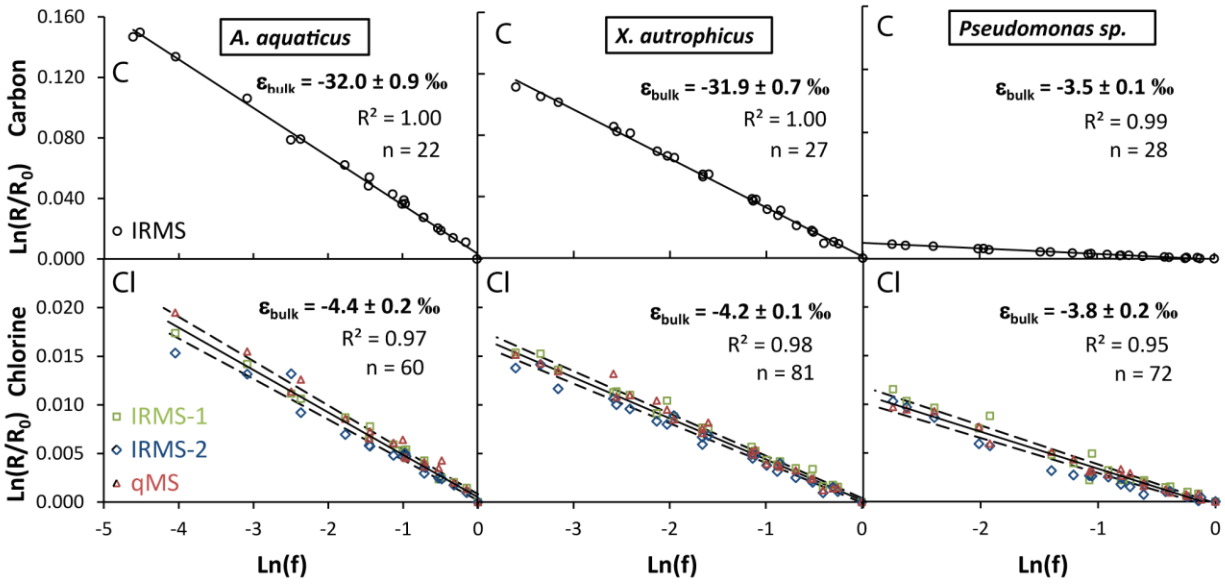
Table 1. Chlorine isotopic fractionation (ε^{Cl}_{bulk}) values from pure cultures and p-values for paired t-test between instruments used.

			IRMS-1		IRMS-2		qMS		
	n[a]	r^2 [b]	Average	Variance[c]	Average	Variance[c]	Average	Variance[c]	p-values[d]
A. aquaticus	3	≥0.96	-4.37	0.01	-4.32	0.02	-4.63	0.04	0.923/0.390/0.670
X. autrophicus	3	≥0.98	-4.343	0.005	-3.960	0.007	-4.39	0.01	0.034/0.829/0.111
Pseudomonas sp.	3	≥0.95	-4.08	0.03	-3.64	0.03	-3.650	0.007	0.085/0.249/0.946

[a] Number of replicates. [b] Correlation coefficient of least-squares regression according to eq. 2. [c] The variance among triplicate experiments was determined from the variance of regression for each ε_i as $s_\varepsilon^2 = (s_{\varepsilon 1}^2 + s_{\varepsilon 2}^2 + s_{\varepsilon 3}^2)/9$.[39] [d] IRMS-1 vs. IRMS-2 / IRMS-1 vs. qMS / IRMS-2 vs. qMS.

Chlorine isotope fractionation values (ε_{bulk}^{Cl}) measured with different instruments were compared in Table 1. Results show excellent regressions (i.e., $r^2 \geq 0.95$) for the data from triplicate experiments when measured on the same instrument (entries in Table 1). In contrast, variation was greater when data from different instruments was compared. The ε_{bulk}^{Cl} values from different instruments were compared using paired t-tests carried out with SigmaPlot™. The p-values were above a significant level of 0.05, with the exception of the p-value for *X. autrophicus* between IRMS-1 (ε_{bulk}^{Cl} = -4.3‰) and IRMS-2 (ε_{bulk}^{Cl} = -4.0‰) (Table 1). This result indicates that, in general, there is no statistically significant difference in ε_{bulk}^{Cl} values at the 95% confidence level. The comparison of p-values for different instrument pairs does not show systematic differences, suggesting that ε_{bulk}^{Cl} variation between laboratories could be in part related to minor effects during sample handling. The effect of scatter in data points at late stages of reaction on calculated ε_{bulk}^{Cl} (Figure 1) could also explain the small differences.[40] However, removing these data points did not significantly improve the agreement.

C and Cl isotope fractionation and dual isotope slopes



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282 **Figure 1**. For each culture C and Cl isotopes data from triplicate experiments were combined in
283 Rayleigh plots. For chlorine, isotope data from different instruments were also combined. The
284 uncertainty for the carbon and chlorine ε values corresponds to the ± 95% confidence interval
285 calculated from standard deviation of regression slope. For chlorine, dashed lines represent the
286 95% C.I. of regression parameters.

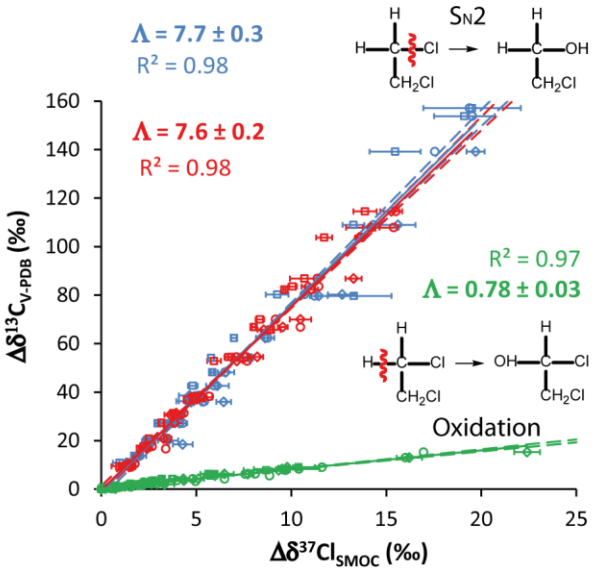
287

288 Degradation experiments lasted between 12h (*A. aquaticus*) and 21h (*X. autrophicus*) and 1,2-
289 DCA transformation above 90% was reached for all the replicates. Carbon and chlorine ε_{bulk}
290 values ($r^2 \geq 0.95$) (Figure 1) were determined as indicated above (eq. 2). Transformation of 1,2-
291 DCA by *A. aquaticus* and *X. autrophicus* by a haloalkane hydrolytic dehalogenase reaction
292 resulted in a strong enrichment of ^{13}C in the remaining substrate, showing a $\delta^{13}C$ shift of
293 approximately 98‰ at 95% degradation. The obtained ε^{C}_{bulk} and ε^{Cl}_{bulk}values for both cultures are
294 the same within 95% confidence intervals and ε^{C}_{bulk} values fall within the ranges determined

from previous studies[16, 35, 41] (Table 2). As compared with carbon, a lower ε^{Cl}_{bulk} value of approximately -4.3‰ was determined for both cultures (ε^{C}_{bulk} / ε^{Cl}_{bulk}= 7.4). Degradation of 1,2-DCA by *Pseudomonas sp*. Strain DCA1 in an enzymatic monooxygenase reaction resulted in a smaller carbon ε^{C}_{bulk} (-3.5‰) compared to nucleophilic (S_N2) reaction (Figure 1). This result is consistent with the previously reported value (Table 2).[16] For Cl, a ε^{Cl}_{bulk} value close to those associated with primary Cl isotopic effects was measured (ε^{C}_{bulk} / ε^{Cl}_{bulk}= 0.9). This value is unusually high given that from known mechanism a secondary isotope effect is expected (see further discussion in the next Section).

A linear relation between $\delta^{13}C$ and $\delta^{37}Cl$ was obtained for all the strains ($r^2 \geq 0.97$) and strongly different slopes ($\Lambda = \Delta\delta^{13}C / \Delta\delta^{37}Cl \approx \varepsilon^{C}_{bulk} / \varepsilon^{Cl}_{bulk}$) were determined for the S_N2 reaction (Λ = 7.7 ± 0.2) and oxidation (Λ = 0.78 ± 0.03) (Figure 2). The slopes obtained from *X. autrophicus* and *A. aquaticus* degradation experiments were the same within 95% confidence intervals. Therefore, the large Λ difference among the investigated reactions enables the use of a dual isotope approach to identify the different pathways in the field. In contrast, a single element approach based only on carbon isotope data would lead to ambiguous interpretations because a certain extent of isotope fractionation (e.g. $\Delta\delta^{13}C$) could have been caused by a strongly isotope fractionating reaction that has proceeded little, or a weakly isotope fractionating reaction that has proceeded further (or an unknown combination of both). Unlike in lab experiments where ε^{C}_{bulk} values can be determined,[16] insight into pathways would therefore be elusive in the field. The starkly contrasting trends of Figure 2 show how isotopic analysis of chlorine as second element can resolve this issue. The large difference between determined slopes also enables to identify if both pathways occur in the field. In ideal situations, the proportion of both competing pathways

317 could be estimated based on dual isotope data.[42, 43] A dual isotope analysis can also be helpful in
318 microbial laboratory experiments for substantiating conclusions about prevailing mechanisms.



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320 **Figure 2**. Carbon and chlorine δ isotope values from triplicate experiments and all used
321 instruments (i.e. IRMS-1, IRMS-2 and qMS) were combined in a dual isotope plot. Symbols are
322 as follows: blue (*A. aquaticus*), red (*X. autrophicus*), gren (*Pseudomonas sp.*), circles (IRMS-1),
323 squares (IRMS-2) and diamonds (qMS). The slopes of the linear regression lines (solid lines)
324 give the Λ values (±95% C.I.) and the dashed lines correspond to the 95% confidence intervals.
325 Error bars of $\delta^{13}C$ values are smaller than the symbols.

326

327 **Interpretation of ^{13}C- and ^{37}Cl-AKIEs for oxidation and S_N2-type reactions**

328

Table 2. Measured carbon and chlorine isotopic fractionation values (in ‰) and apparent kinetic isotopic effects.

	Reaction mechanism	Observed				Reported
		ε_{bulk}^{C} [a]	^{13}C-AKIE [b]	ε_{bulk}^{Cl} [a]	^{37}Cl-AKIE [b]	ε_{bulk}^{C}
A. aquaticus	S_N2	-32.0 ± 0.9	1.068 ± 0.002	-4.4 ± 0.2	1.0089 ± 0.0004	-31.9 to -32.4[16]
X. autrophicus	S_N2	-31.9 ± 0.7	1.068 ± 0.002	-4.2 ± 0.1	1.0085 ± 0.0002	-28.7 to -33.0[16, 35, 41]
Pseudomonas sp.	C-H bond cleavage	-3.5 ± 0.1	1.0070 ± 0.0002	-3.8 ± 0.2	1.0038 ± 0.0002	-3.0 ± 0.2[16]

[a] See Rayleigh plots in Figure 1. The uncertainty corresponds to the 95% confidence interval calculated from standard deviation of regression slope. [b] Calculated according eq. 5. The uncertainty was estimated by error propagation.

Hydrolytic dehalogenation (S_N2) reaction

Determined ε_{bulk} values were used to estimate the AKIEs (Table 2) according to eq. 5, which assumes that secondary isotopic effects can be neglected. For S_N2 reaction, ^{13}C-AKIEs were calculated using z = 2 since both C-Cl bonds compete for reaction. ^{13}C-AKIEs agreed well with the typical ^{13}C-KIE range for a S_N2 reaction (1.03 – 1.09)[21] (Scheme 1). Abe et al. (2009)[41] determined the ^{13}C-AKIE of 1,2-DCA in batch degradation experiments prepared with cell free extract from *X. autrophicus* GJ10. These authors obtained an average value (1.0597) close to the ^{13}C-AKIE observed in this study (1.068), suggesting that there was no significant masking of the intrinsic KIE during compound transport thorough the cell membrane. This conclusion is in

agreement with the Streitwieser limit for ^{13}C-KIE in C-Cl bonds (1.057),[44] and could explain in part the relatively narrow range of reported ε^{C}_{bulk} values (from -28.7 to -33.0‰) for both pure cultures using the haloalkane hydrolytic dehalogenase reaction (Table 2). Hirschorn et al. (2007)[9] measured a similar ^{13}C-AKIE (1.05) for 1,2-DCA in laboratory biodegradation experiments under nitrate reducing conditions by an enrichment culture from a contaminated site, which was interpreted as transformation via hydrolytic dehalogenation.

Similarly to carbon isotope effects, ^{37}Cl-AKIE were obtained from eq. 5 with z = 2. The calculated ^{37}Cl-AKIE (1.009) corresponded well to the typical ^{37}Cl-KIE range for a S_N2 reaction but very close to the upper end (1.006-1.009)[45] (Scheme 1). However, the ^{37}Cl-AKIE measured in this study is above the theoretical primary isotope effect for 1,2-DCA enzymatic dehalogenation reported by Lewandowicz et al. (2001) (^{37}Cl-KIE = 1.0065).[46] These authors also measured experimentally the leaving group ^{37}Cl-AKIE for 1,2-DCA (1.0045) and 1-chlorobutane (1.0066) dechlorination catalyzed by haloalkane hydrolytic dehalogenase (extracted from *X. autrophicus* GJ10). In this former study the experimental ^{37}Cl-AKIE was determined by the isotopic analysis of the released Cl^- during 1,2-DCA dechlorination and, therefore, it represents the primary ^{37}Cl-AKIE. According to Lewandowicz et al. (2001)[46] and Paneth (2003)[47] an explanation for the lower ^{37}Cl-AKIE of 1,2-DCA compared to 1-chlorobutane could be that the dehalogenation step is reversible and the hydrolysis of the enzyme-bound intermediate is responsible for the overall irreversibility of the reaction. In addition, in a recent study that investigated 1,1-dichloroethane and 1,1,1-trichlorethane biodegradation by whole cell and cell free extract systems,[48] ^{13}C-AKIEs for both chlorinated ethanes during degradation by cell free extracts were unexpectedly lower than those determined in whole cell experiments. The higher ^{37}Cl-AKIE of 1.009 compared to the theoretical primary ^{37}Cl-KIE could be explained by the

contribution of a β-secondary isotopic effect given that, in our study, the ^{37}Cl / ^{35}Cl ratios were measured in the remaining 1,2-DCA and, secondary isotopic effects were neglected in the primary AKIE calculation (eq. 5). The magnitude of the β-secondary ^{37}Cl-KIE can be estimated from the average of KIE_i in both Cl molecular positions according to eq. 6.[12]

$$\varepsilon_{bulk}^{Cl} \approx \frac{1}{2} \cdot \left(\frac{1}{KIE_{primary}^{Cl}} + \frac{1}{KIE_{secondary}^{Cl}} \right) - 1 \qquad (6)$$

Plugging in the ε_{bulk}^{Cl} value measured in our study (-4.3 ‰) and the reported theoretical primary Cl isotopic effect of 1.0065,[46, 47] a β-secondary ^{37}Cl-KIE of 1.0021 was estimated. Equation 6 assumes that ε_{bulk}^{Cl} is not significantly masked by non-fractionating rate limiting processes preceding the reaction step. This is a likely assumption in our case judging by the relatively high carbon and chlorine AKIEs (see discussion above and Table 2). Secondary ^{13}C-KIEs are generally much smaller than primary isotope effects and, therefore, they are usually omitted in ^{13}C-AKIE calculations.[21] However, for chlorine, secondary KIEs as large as primary isotope effects have been recently determined theoretically during S_N2 reactions that proceed with chlorine transfer between two heavy atoms.[45] In addition, in a recent experimental study that investigates TCE multi-isotope fractionation during biotic reductive dechlorination,[32] daughter products depleted in ^{37}Cl relative to their immediate parent compounds were interpreted as evidence of significant secondary Cl effects related to nucleophilic substitution reaction. The conclusions of these recent studies[32, 45] support the hypothesis that large β-secondary ^{37}Cl-KIE occurs during this study.

Oxidative C-H bond cleavage

As in the hydrolytic reaction, during oxidation both C atoms also compete for reaction and thus the ^{13}C-AKIE was calculated using z = 2 in eq. 5 (Table 2). The obtained ^{13}C-AKIE agrees well with the typical ^{13}C-KIE for C-H bond cleavage (1.01 – 1.03)[21] (Scheme 1). Primary ^{13}C-KIEs generally increase with increasing mass of the bonding partner (i.e. $^{13}C\text{-}KIE_{C\text{-}H} < {}^{13}C\text{-}KIE_{C\text{-}Cl}$).[21] For Cl, the unexpectedly high ε_{bulk}^{Cl} suggests the contribution of secondary ^{37}Cl-KIEs from both Cl atoms. In the absence of primary chlorine isotopic effect, the secondary ^{37}Cl-AKIE can also be evaluated using eq. 5. In this case z = 1 since no specific bond containing Cl is broken and there is, therefore, no intramolecular competition for this bond. The resultant secondary ^{37}Cl-AKIE (1.004) represents the average secondary isotope effect of all positions. This result supports the large β-secondary ^{37}Cl-KIE estimated above for the nucleophilic reaction. This finding suggests that significant secondary ^{37}Cl-KIEs are also associated with enzymatic oxidation via C-H bond cleavage. Until now, oxidative cleavage of a C-H bond has been believed to affect primarily the C-H bond and to leave chlorine substituents largely unchanged. This common assumption is challenged by the observed large chlorine isotope fractionation in C-H bond cleavage, where involvement of a C-Cl bond would not be expected. This indicates an intriguing role of the chlorine atoms which still remain to be resolved. Hypothetical contribution of chlorine isotope fractionation during binding of 1,2-DCA molecules to the enzyme could be an additional explanation.

Further insight can possibly be obtained in future studies that address ^{2}H / ^{1}H isotope analysis. Recently, Shouakar-Stash and Drimmie (2013)[49] developed and online methodology for H-CSIA

410 of TCE and 1,2-cis-dichloroethene, however, this analytical method is still not implemented for
411 chlorinated ethanes.

412 **Implications for application of CSIA to environmental studies**

413 One of the main applications of CSIA to field studies is the estimation of contaminant
414 biodegradation extent and rate.[15, 44, 50] For this purpose compound specific ε_{bulk} values from
415 laboratory experiments are necessary. For a given compound, different ε_{bulk} values are generally
416 associated with distinct biodegradation pathways, which in turn are sometimes related to
417 different subsurface redox environments. Therefore, redox conditions are usually used as criteria
418 to constrain the range of reported ε_{bulk} values. However, for 1,2-DCA different degradation
419 pathways associated with distinct ε_{bulk} values may even be active under aerobic conditions. In
420 addition, the hydrolytic dehalogenation pathway of 1,2-DCA has been observed under oxygen
421 and nitrate reducing conditions alike (see above)[9]. In this context, the identification of the active
422 degradation pathway in the field is crucial to choose the appropriate ε_{bulk} value for quantification
423 of degradation. This study demonstrates that dual isotope slopes are strongly different for
424 nucleophilic substitution (S_N2) and oxidation (C-H bond cleavage) reactions, which opens the
425 possibility to identify them using a dual isotope approach. Following this approach isotopic data
426 from the field site can be directly and intuitively interpreted.

427 Significant secondary chlorine isotopic effects were determined for the investigated reactions.
428 These results indicate that primary ^{37}Cl-AKIEs derived from CSIA could be higher than reported
429 primary ^{37}Cl-KIEs (e.g. from computational studies) if secondary isotopic effects are omitted in
430 the calculations. Hence, mechanistic interpretations based on the comparison with primary ^{37}Cl-
431 KIEs should be made with caution.

Finally, chlorine ε_{bulk} values measured with three different instruments, two GC-IRMS and one GC-qMS, showed a fairly good agreement varying at most by ±3.8% (SD of the mean). Even though the agreement was not perfect these results lend confidence to the methods used and encourage the application of Cl-CSIA to investigate the fate of chlorinated compounds at contaminated sites. However, further research and methodological developments are still required to improve Cl-CSIA data agreement between different laboratories.

ASSOCIATED CONTENT

Supporting Information

A detailed description of analytical methods is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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