# Calibration bias of experimentally determined chlorine isotope enrichment factors – The need for a two-point calibration in Compound-specific Chlorine Isotope Analysis

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RATIONALE: The recent development of compound-specific online chlorine isotope analysis ($^{37}$Cl-CSIA) methods has fostered dual chlorine-carbon isotope studies to gain better insights into sources and environmental transformation reactions of chlorinated ethenes (CEs). 1-point and 2-point calibration schemes are currently used to convert raw data to the international $\delta^{37}Cl_{SMOC}$ scale, but a critical evaluation of best practices to arrive at reliable $\delta^{37}Cl_{SMOC}$ signatures and enrichment factors was missing and is presented here.

METHODS: Aqueous solutions of neat PCE and TCE and aqueous samples from a TCE biodegradation experiment with pure cultures of *Desulfitobacterium hafniense* Y51 were analysed for chlorine isotopes applying GC/qMS and GC/IRMS. $\delta^{37}Cl_{SMOC}$ values were obtained using 1-point and 2-point calibration schemes. Chlorine isotope enrichment factors, $\varepsilon_{Cl}$, were calculated using both approaches and the corresponding bias of $\delta^{37}Cl_{SMOC}$ values introduced by the different types of calibration was determined.

RESULTS: Different calibration methods resulted in significant differences (up to 30%) in both $\delta^{37}$Cl signatures and $\varepsilon_{Cl}$-values.

CONCLUSIONS: Our results demonstrate that a 2-point calibration together with comprehensive information on reference materials is indispensable and should become standard practice for reliable $^{37}$Cl-CSIA of organic compounds.

## INTRODUCTION

Multi-dimensional compound-specific isotope analysis (CSIA) - combining e.g., $^{13}C/^{12}C$ and $^{2}H/^{1}H$ or $^{15}N/^{14}N$ - has received increasing attention for evaluating transformation pathways and source apportionment of organic contaminants (e.g. [1-10]). For chlorinated organic contaminants, Cl-CSIA is of particular interest as it avoids tedious offline conversion for subsequent dual-inlet isotope ratio mass spectrometry (DI-IRMS)[11] or TIMS (thermal ionization mass spectrometry)[12]. A general survey over Cl-CSIA with DI-IRMS and TIMS is given by Shouakar-Stash et al.[13] Recently, two different online methods using either GC/IRMS[13] or quadrupole mass spectrometry (GC/qMS)[14] have been brought forward to enable routine Cl-CSIA for chlorinated hydrocarbons. The GC/IRMS method requires dedicated settings of faraday cups, constraining it to a narrow range of target compounds and a limited number of instruments worldwide [15]. The GC/qMS approach, although with lower precision, has demonstrated applicability to different classes of chlorinated compounds[16-18] and is available to many laboratories.

In contrast to C-, H- or N-CSIA the analytes (e.g. chlorinated ethenes) enter the source of the isotope ratio mass spectrometer or quadrupole mass spectrometer without conversion to a universal measurement gas. Instead, selected isotopologue fragment-ions of the target analyte are recorded. Hence, each analyte requires an analyte-specific secondary standard with a known isotope ratio relative to the international reference material SMOC (Standard Mean Ocean Chloride). In GC/IRMS analysis, the target analyte is directly introduced into the source via the dual inlet system. Machine delta values are obtained by comparison with an analyte-specific monitoring gas and subsequently are converted to the international SMOC scale by external calibration[15]. The GC/qMS method relies in a similar way on external calibration, either with[19] or without[14, 17, 18, 20] using a monitoring gas. The first $\delta^{37}Cl_{SMOC}$ values obtained by the GC/qMS

method were reported by Aeppli et al.[17] for perchloroethylene (PCE), dichlorodiphenyltrichloroethane (DDT) and pentachlorophenol (PCP) using a 1-point calibration with standard isotope bracketing of external standards. Recently, $\delta^{37}Cl_{SMOC}$ values of chlorinated acetic acids were reported also based on a 1-point calibration scheme.[18]

A comprehensive comparison between GC/IRMS and GC/qMS methods was performed in an interlaboratory study using trichloroethylene (TCE) as target analyte[15] with regard to precision, amount dependency and calibration to the SMOC scale. This study demonstrated the necessity of two rather than only one compound-specific calibration standard for each target analyte to minimize distortion relative to the SMOC scale and to account for potentially variable calibration slopes on the same instrument over time. A subsequent study[21], however, reported that a 1-point calibration is sufficient to calibrate GC/qMS measurements to the SMOC scale provided that for a specific instrument and a given compound class (e.g., chlorinated ethenes), (i) the slope of the regression line between instrumental and external SMOC values is close to unity, (ii) samples are measured within a short time frame and (iii) only relative changes of isotope ratios are reported rather than absolute $\delta^{37}Cl_{SMOC}$ signatures. This procedure, however, appears problematic if calibration curves are target-analyte specific and if they vary with the status of the instrument and thus over time. In addition, the approach of calibrating one substance (e.g. PCE) with standards of another substance of the same compound group (e.g. TCE), has been brought forward by Sakaguchi-Söder et al. [14] and Aeppli et al. [17]. Also this approach still warrants critical evaluation.

Hence, even though Cl-CSIA methods have already been applied in mechanistic [22-28] and environmental case studies[17, 19, 21, 27, 29-32], the comparability of published data is currently compromised by the fact that calibration data, and detailed information regarding $\delta^{37}Cl_{SMOC}$ signatures of standards, are scarce. Currently, both 1[17, 18, 21, 30]- and 2- point calibration

schemes[13, 15, 23, 24, 26-28, 31-33] are in use although a systematic evaluation of potential "scale distortion effects" of these calibration schemes for resultant chlorine isotope enrichment factors is still missing [34]. Since a growing number of compound-specific chlorine isotope studies is expected for the near future, a critical evaluation of best practices to arrive at reliable $\delta^{37}Cl_{SMOC}$ signatures and enrichment factors is crucial.

The objectives of this study were therefore to evaluate critically the justification of 1-point calibration schemes and to investigate experimentally the implications of a 1-point versus 2-point calibration for chlorine isotope enrichment factors of chlorinated ethenes for Cl-CSIA by GC/qMS. Potential "scale distortion effects" were evaluated by (i) applying both calibration schemes to neat PCE and TCE samples and to an experimental data set obtained during biodegradation of TCE with pure cultures of *Desulfitobacterium hafniense* Y51, by (ii) determining chlorine isotope enrichment factors using either set of $\delta^{37}Cl_{SMOC}$ signatures and (iii) by determining the corresponding bias introduced by the different types of calibration. For the first time, we therefore used actual experimental degradation data to demonstrate the potential pitfalls associated with calibration. Based on these data we also critically evaluated the approach to calibrate a target analyte (e.g. TCE) with a secondary standard of another analyte (e.g. PCE). Our study aims at bringing forward a good standard practice for further applications to enhance the comparability and reliability of future studies.

## MATERIALS AND METHODS

### Chemicals and preparation of standard solutions

Pure commercial TCE (neat TCE) samples stem from different manufacturers (see Table in supporting information) and are identical to those analyzed by Bernstein et al.[15]. PCE samples were purchased from Merck and PPG. SMOC referenced standards ("EIL-1", "EIL-2") of PCE

and TCE were provided and previously characterized by Shouakar-Stash et al. [13] with EIL-1 (PCE) $\delta^{37}Cl_{SMOC}$ = +0.29±0.06‰ (1σ, n=5), EIL-2 (PCE) $\delta^{37}Cl_{SMOC}$ = -2.52±0.15‰ (1σ, n=5), EIL-1 (TCE) $\delta^{37}Cl_{SMOC}$ = ±3.05±0.07‰ (1σ, n=10), EIL-2 (TCE) $\delta^{37}Cl_{SMOC}$ = -2.7±0.11‰ (1σ, n=10). Hence, PCE standards span a range of 2.81‰ whereas TCE standards cover 5.75‰. Further details of the neat samples are provided in Table S1. For analysis methanolic standard stock solutions were prepared followed by an aqueous dilution to the required concentration.

**Concentration analysis:** Aqueous concentrations of chlorinated ethenes (PCE, TCE) were determined with the same GC/qMS system as for chlorine isotope analysis (see below) using headspace injection. Calibration curves were obtained using aqueous TCE solutions with defined concentrations between 0 and 1000 µg $L^{-1}$.

**Chlorine isotope analysis.** Chlorine isotope ratios of aqueous PCE and TCE samples were measured by GC/qMS applying headspace and solid phase microextraction (SPME) injection. In brief, samples and standards were measured in quintuplicates and each sample replicate was bracketed by standards of similar concentration (± 20%) obtaining a standard error of calibrated values for PCE and TCE of 0.6‰. Chlorine isotope measurements were performed according to Bernstein et al.[15]:

GC/qMS: An Agilent 7890A gas chromatograph coupled to an Agilent 5975C quadrupole mass selective detector (Agilent, Santa Clara, CA, USA) was used with a RTX-VMS capillary column (60m x 250 µm, 1.4µm film thickness, Restek). Flow velocity of the helium carrier gas was 1 mL $min^{-1}$, and split ratio was 10. The temperature program was 40°C for 2 min, followed by a ramp of 25°C $min^{-1}$ to 110°C, a ramp of 15°C $min^{-1}$ to 200°C for 5 min. Ions recorded in the selected ion monitoring were m/z 60, 62, 95, 97, 130, 132 (TCE) and a dwell time of 30 ms was set for all measurements. Ions were produced by electron ionization applying an electron energy of 70eV. Auto-tuning of fragment masses was performed before each sequence. Headspace

injections were performed using an automatic multi-purpose sampler (Combi Pal, Gerstel, Mülheim an der Ruhr, Germany). Output data were processed with ChemStation (Agilent) using the RTE integrator option.

GC/IRMS: A GC-IRMS system consisting of a Trace GC (Thermo Fisher Scientific, Milan, Italy) directly coupled to a Finnigan MAT 253 IRMS (Thermo Fischer Scientific, Bremen, Germany) was used. The instrumental configuration was similar to that described by Shouakar-Stash et al. (2006), and analysis was performed by recording the masses m/z 97/95. Analyte separation was achieved using a DB-5 column (30m x 0.25mm x 0.25µm; Agilent), a flow rate of the He-carrier gas of 1.4 mL $min^{-1}$ and a split flow of 21 mL $min^{-1}$. To avoid introduction of water to the IRMS, a wax column (60 m x 0.25mm x 0.5 µm; Supelcowax$^{TM}$10) and a VALCO valve was installed before the DB-5 column. Water was retained on the wax column and could therefore be cut off with the VALCO valve once TCE had reached the DB-5 column. The temperature program was isothermal at 80°C (16 min), followed by a ramp of 50°C $min^{-1}$ to 150°C, with a final hold for 1 min.

**Calculation of chlorine isotope ratios.** Bulk chlorine isotope ratios of PCE and TCE were calculated according to Sakaguchi-Söder et al.[14] considering the two most abundant ions of each fragment group as recommended by Jin et al.[20]. Detailed information is given in the SI.

**Calibration to the $\delta^{37}Cl_{SMOC}$ scale.** The obtained bulk chlorine isotope ratios of PCE and TCE were expressed in δ-signatures relative to the internationally accepted Standard Mean Ocean Chlorine (SMOC) reference using EIL(PCE) and EIL(TCE) materials (see above) as external standards. Data were evaluated using both a 1-point calibration as well as a 2-point calibration scheme. All equations and figures are stated according to current IUPAC guidelines [35]. Normalization by the 1-point calibration scheme followed Aeppli et al.[17], using:

$$\delta^{37}\mathrm{Cl} = \left(\frac{R_{Cl}}{R_{Cl}^{std}} - 1\right) + \delta^{37}\mathrm{Cl}^{std}\frac{R_{Cl}}{R_{Cl}^{std}} \quad (1)$$

and

$$\delta^{37}Cl = \frac{R_{Cl}}{R_{Cl}^{std}} \cdot \left(1 + \delta^{37}Cl^{std}\right) - 1 \quad (2)$$

$R_{Cl}/R_{Cl}^{std}$ is the compound-specific chlorine isotope ratio calculated from individual ion abundances of the mass spectra , $\delta^{37}Cl^{std}$ and $\delta^{37}Cl$ are SMOC referenced isotope values of the external EIL standard and the unknown isotope value of the sample. Equation 2 can be derived using a linear regression (Equation 3) with a slope n = 1:

$$\frac{\delta^{37}Cl+1}{\delta^{37}Cl^{std}+1} = n \cdot \frac{R_{Cl}}{R_{Cl}^{std}} \quad (3)$$

For n≠1, however, equation 2 changes to:

$$\delta^{37}Cl = n \cdot \frac{R_{Cl}}{R_{Cl}^{std1}} \cdot \left(1 + \delta^{37}Cl^{std}\right) - 1 \quad (4)$$

Using EIL-1 as $R_{Cl}^{std}$ equation 2 changes to

$$\delta^{37}Cl = n_1 \cdot \frac{R_{Cl}}{R_{Cl}^{std1}} \cdot \left(1 + \delta^{37}Cl^{std1}\right) - 1 \quad (5)$$

and using EIL-2 as $R_{Cl}^{std}$ equation 2 results in

$$\delta^{37}Cl' = n_1 \cdot n_2 \cdot \frac{R_{Cl}}{R_{Cl}^{std1}} \cdot \left(1 + \delta^{37}Cl^{std1}\right) - 1 \quad (6)$$

Detailed derivations of equations 5 and 6 can be found in the SI.

From equations 5 and 6 it can be seen that as soon as the slope does not equal 1, different $\delta^{37}Cl_{SMOC}$ signatures will be calculated depending on the standard chosen for this one-point calibration procedure.

When evaluating our data according to Equation (2), two separate 1-point calibrations were therefore performed using either EIL-1 or EIL-2 as $R_{Cl}^{std}$ .

The two-point calibration was performed following Bernstein et al.[15] using the two SMOC referenced standards EIL-1 and EIL-2 of the respective target-analytes PCE and TCE. Measured

isotope ratios were first converted to an instrument-specific δ scale by referencing against EIL-1 of the same target-analyte as external standard, which is always measured in the same sequence as the sample:

$$\delta = \left(\frac{R_{Cl}}{R_{Cl}^{std}} - 1\right) \quad (7)$$

and subsequently normalized relative to the SMOC scale with the two-point linear regression. Advantage of these instrument-specific δ values is that distortion of standards relative to the SMOC scale can be seen more easily than by comparison of bulk isotope ratios with the SMOC scale. The error of the slope of the calibration curve, as it appears in the following sections, was calculated as 95% confidence interval (standard error multiplied by the student t for a = 0.05).

**Biodegradation experiment**. For the determination of chlorine isotope fractionation factors during microbial reductive dechlorination of TCE a biodegradation experiment was performed according to Cretnik et al.[23]. Shortly, a pure culture of the microbial strain *Desulfitobacterium hafniense* Y51, which reductively dechlorinates TCE to the final product *cis*-DCE, was inoculated in microcosms containing 500 mL liquid anoxic medium. The microcosms were spiked with 25µL of neat TCE with known $\delta^{37}Cl_{SMOC}$ value (0.51‰ ± 0.06, Merck). Three replicate bacterial cultures and one abiotic control batch without inoculated cells were set up in parallel. Samples for concentration and chlorine isotope analyses were withdrawn repeatedly over time. Concentration analyses were performed at the same day and corrected for air-water partitioning in the microcosms according to Henry's law at each time point. Aqueous samples for isotope analysis were frozen upside down in 1,9 mL amber vials and stored at -18°C until analysis. Information of two-point linear regression slopes used for calibration of biodegradation samples to the SMOC scale can be found in Fig. S1 in the SI.

**Determination of the chlorine isotope enrichment factor, $\varepsilon_{Cl}$.**

The chlorine isotope enrichment factor $\varepsilon_{Cl}$ was calculated considering the mean chlorine isotope value of the quintuplicates of each microcosm according to the linearized Rayleigh equation[36]:

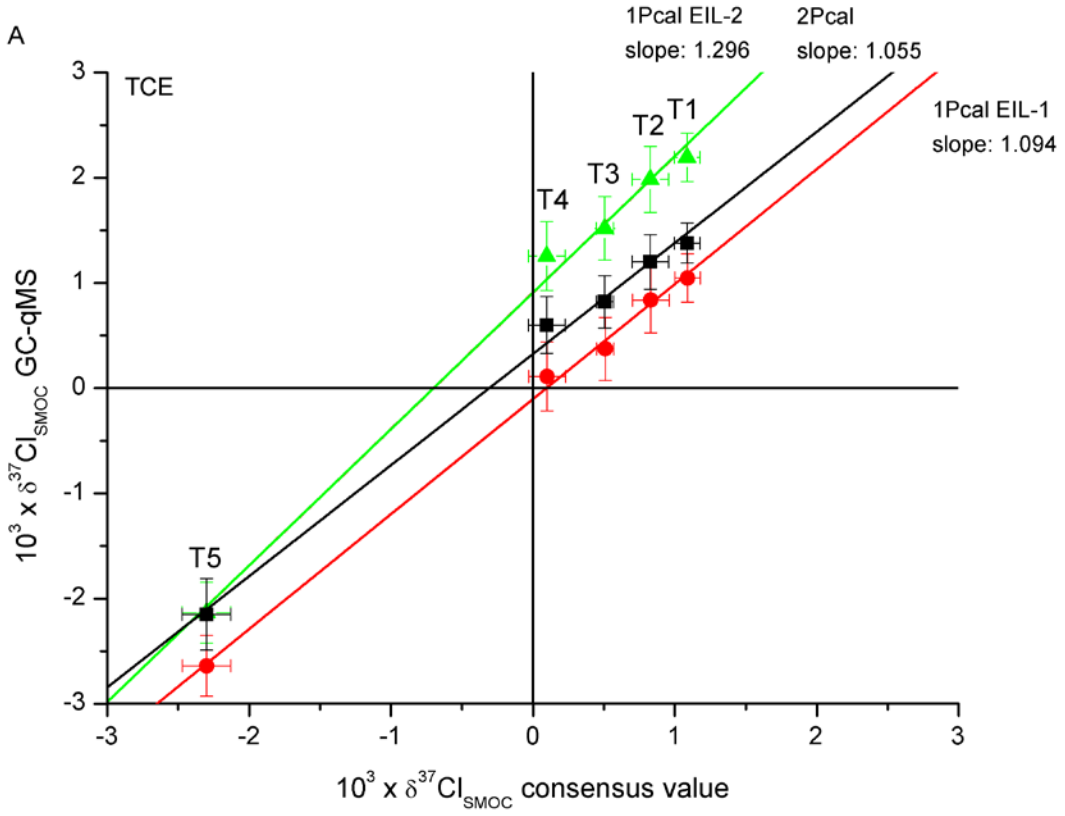
$$ln\left(\frac{\delta^{37}Cl_t+1}{\delta^{37}Cl_0+1}\right) = \varepsilon \cdot \ln f \quad (2)$$

The remaining fraction *f* of TCE in the microcosms at each time point was calculated as the mole ratio of TCE and the sum of TCE and cDCE at the respective time point.

## RESULTS AND DISCUSSION

### Is there a justification for 1-point calibrations?

Aeppli et al.[17] and Wiegert et al.[21] compared $\delta^{37}Cl_{SMOC}$ signatures obtained with a 1-point calibration (equation 1) with previously characterized consensus values measured with TIMS. This comparison resulted in a regression slope equal to unity, which the authors took as justification to use a 1-point calibration to convert chlorine isotope ratios to the SMOC scale.


A
TCE
1Pcal EIL-2
slope: 1.296
2Pcal
slope: 1.055
1Pcal EIL-1
slope: 1.094
T1
T2
T3
T4
T5
$10^3$ x $\delta^{37}Cl_{SMOC}$ GC-qMS
$10^3$ x $\delta^{37}Cl_{SMOC}$ consensus value

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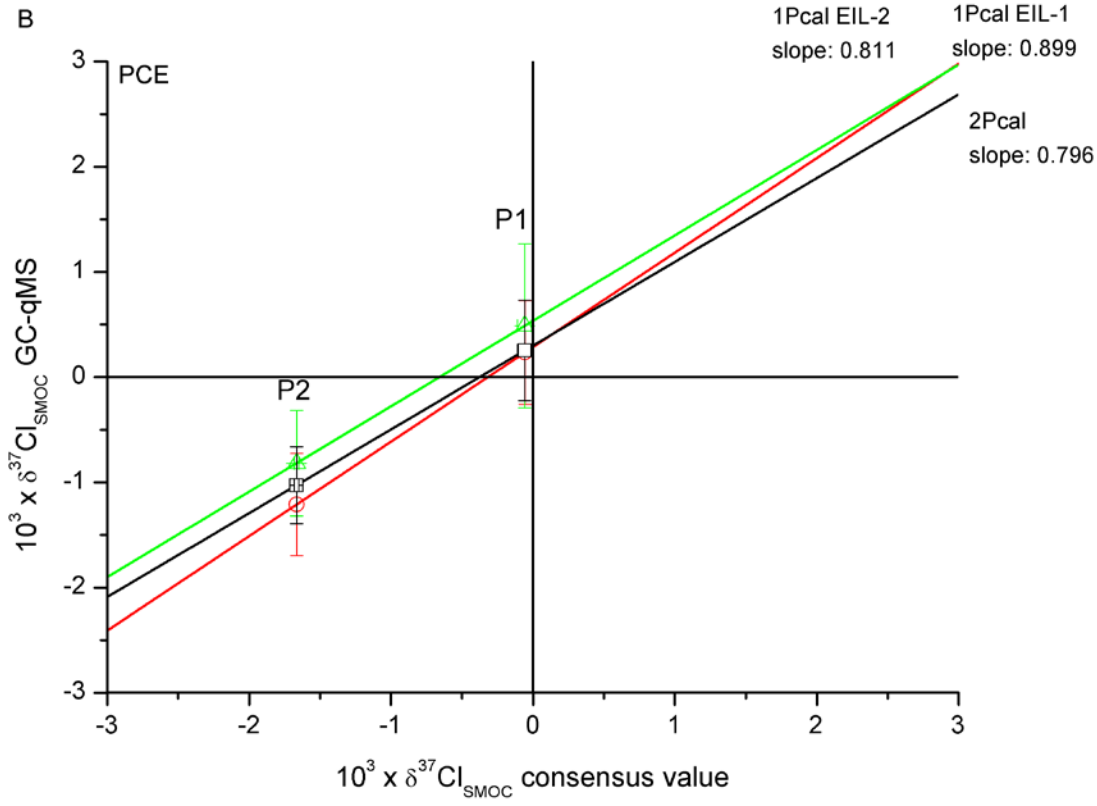
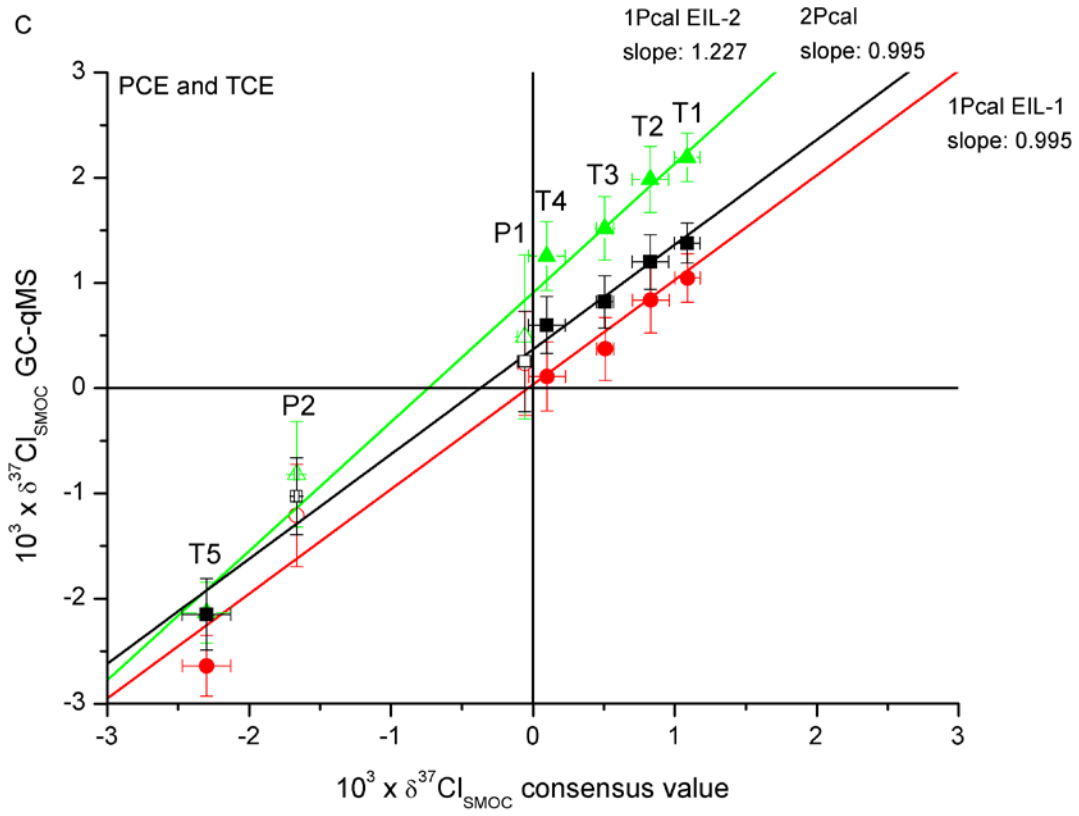

B
PCE
1Pcal EIL-2
slope: 0.811
1Pcal EIL-1
slope: 0.899
2Pcal
slope: 0.796
P1
P2
$10^3$ x $\delta^{37}Cl_{SMOC}$ GC-qMS
$10^3$ x $\delta^{37}Cl_{SMOC}$ consensus value

196

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198 **Figure 1**. Comparison of SMOC referenced isotope data of calibrated signatures (y-axis, measured with
199 GC/qMS) and previously characterized consensus values (measured with GC/IRMS, x-axis) by linear
200 regression slopes. Panesl A and C show $\delta^{37}Cl_{SMOC}$ signatures of five TCE (T1-T5) neat substance
201 samples, Panels B and C show $\delta^{37}Cl_{SMOC}$ signatures of two PCE (P1, P2, open symbols) neat substance
202 samples, where TCE isotope ratios were calibrated to the SMOC scale with TCE standards, PCE isotope
203 ratios were calibrated to the SMOC scale with PCE standards. Measurements of GC/qMS were calibrated
204 with a 1-point calibration with EIL-1 (red circles) of the respective target analyte, with a 1-point calibration
205 with EIL-2 (green triangles) of the respective target analyte or a 2-point calibration (black squares). Panel
206 A illustrates the comparison considering only TCE (slopes 1-point calibration (EIL-1) =1.094±0.031, $R^2$
207 1.00; 1-point calibration (EIL-2) =1.296±0.056, $R^2$ 0.99; 2-point calibration = 1.055±0.043, $R^2$ 0.99;
208 standard error and confidence interval 95%). Panel B compares slopes (confidence interval 95%) of PCE
209 neat samples. 1-point calibration (EIL-1): slope=0.899; 1-point calibration (EIL-2): slope =0.811; 2-point
210 calibration: slope =0.796. In Panel C PCE and TCE are both used to obtain one single regression (mixed
211 regression; standard error and confidence interval 95%). Slope=0.995±0.092, $R^2$ 0.95 (1-point calibration
212 (EIL-1)); slope=1.227±0.082, $R^2$ 0.97 (1-point calibration (EIL-2)); slope = 0.995±0.055, $R^2$ 0.98 (2-point
213 calibration).

214

Fig. 1 shows a comparison of the $\delta^{37}Cl$ signatures of neat samples measured with GC/qMS and the $\delta^{37}Cl$ signatures of the same neat samples as previously characterized by GC/IRMS analysis in Bernstein et al. [15] (here considered as consensus values, x-axes in the panels of Figure 1) by regression slopes. $\delta^{37}Cl_{SMOC}$ signatures from GC/qMS (y-axes of the panels in Figure 1) were obtained in three different ways: evaluated with a 1-point calibration using standard EIL-1 (red lines), with a 1-point calibration using standard EIL-2 (green lines) and with a 2-point calibration (black lines). (Note that TCE data in Panels A and C were evaluated with EIL (TCE) standards and PCE data in Panels B and C with EIL (PCE) standards so that panel C contains data points from calibration with both sets of compound-specific standards). Panel A shows regression slopes specifically for the target analyte TCE, Panel B shows the regression slope specifically for target analyte PCE. Ideally, regression slopes of this "single comparisons" should equal to unity. In Panel C both TCE and PCE data points are used to obtain one single regression line ("mixed comparison"). Hence, it is not specific for a single target analyte, but for a compound group (chlorinated ethenes). This is in analogy to the approach suggested by Aeppli et al.[17], Wiegert et al.[21] and Miska et al. [18], where further substances of different compound classes were used to obtain a single regression line. Although the slopes are in the same range as those reported by Wiegert et al.[21] (1.07±0.27, $R^2$=0.94) our results clearly demonstrate that the slope of samples which were obtained from a 1-point calibration (green and red lines in Figure 1) strongly depends on the choice of the calibration standard as “anchor” of the calibration (see equations 5 and 6) and can be significantly different from unity. Hence the chosen calibration standard determines the resulting SMOC signature of a sample: If a 1-point calibration leads to a slope of 1.2 instead of unity, a change of 5‰ in consensus values would erroneously be evaluated as a change of 6‰ (1.2x5‰). Following the same 1-point calibration approach, but just by using another standard (“anchor”) which was measured in the same sequence, the slope may change

,e.g., to 0.8, where a change of 5‰ in consensus values would be expressed as a change of 4‰. This variability in $\delta^{37}Cl_{SMOC}$ signatures as result of different anchors in 1-point calibrations clearly advises against the use of this calibration scheme.

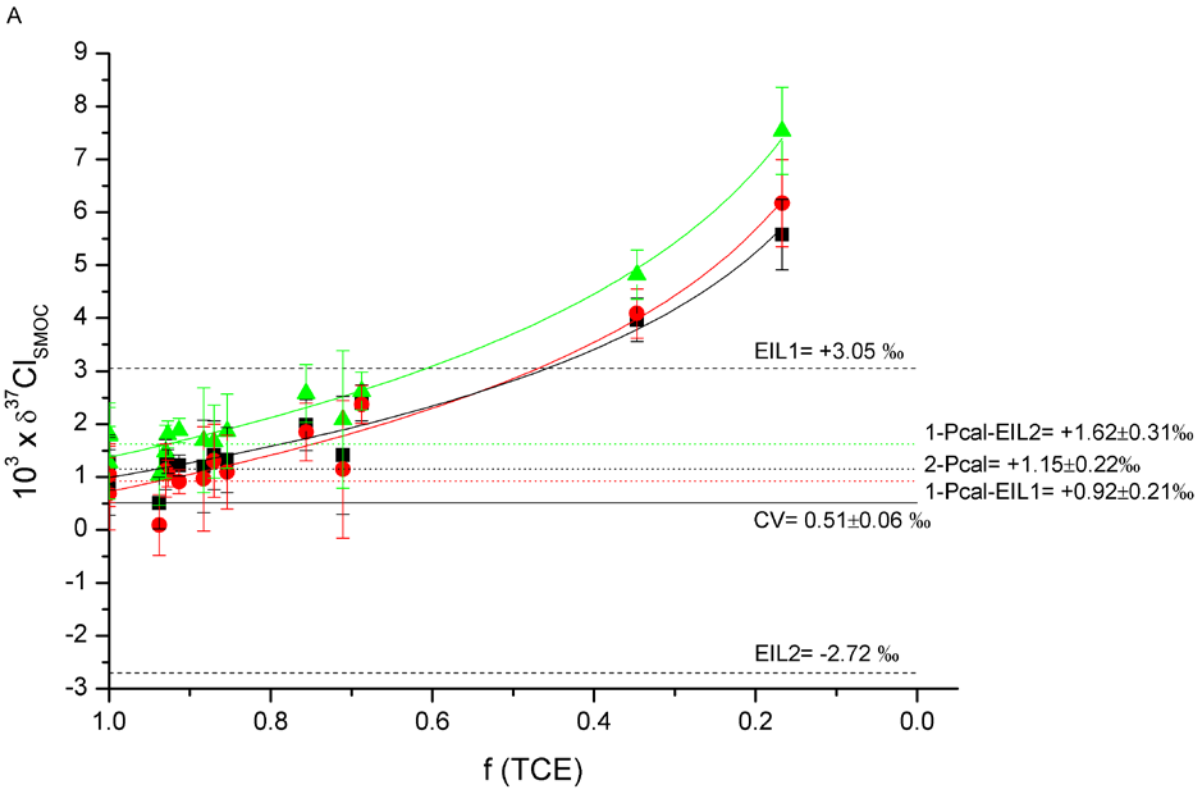
Another important aspect in the 1-point calibration suggested by Aeppli et al. [17], Wiegert et al. [21] and slightly modified by Miska et al. [18] is their "combined comparison": they constructed a regression slope based on evaluation of combined isotope values from different substances (e.g., PCE and TCE). This is potentially advantageous if only one SMOC reference of a distinct target analyte is available or to reduce the number of necessary measurements. When such a regression line results in a slope of unity – as observed by Aeppli et al. [17] and Wiegert et al. [21] – this might be misinterpreted as evidence that SMOC references of one target analyte (analyte A) may be used to calibrate samples of another target analyte (analyte B) to the SMOC scale ("cross referencing"). As shown in Figure 1, however, regression slopes of a "single comparison" (Panel 1 and Panel 2) and of a "combined comparison" show significant differences. Hence, obtaining regression curves as suggested by Aeppli et al. [17], Wiegert et al. [21] and Miska et al. [18] including different substances in one regression as shown in Fig. 1 will enlarge the bias oft the resulting $\delta^{37}Cl_{SMOC}$-values. We therefore emphasize that cross referencing should never be done, regardless of the applied calibration scheme.

**Calculating experimental isotope enrichment factors using different calibration methods.**

Although 1-point calibrations[21] and 2-point calibrations[23, 25] were used in earlier studies to determine $\delta^{37}Cl_{SMOC}$ signatures and chlorine isotope enrichment factors ($\varepsilon_{Cl}$) during degradation experiments, a systematic evaluation of both calibration schemes using a common data set from degradation samples is missing. Hence we applied the two calibration schemes to determine $\varepsilon_{Cl}$ values for a microbial reductive dechlorination experiment of TCE conducted in our laboratory.

263 Figure 2 shows the respective change of $\delta^{37}Cl$ signatures as a function of the remaining fraction

264 of TCE and the respective Rayleigh plots.

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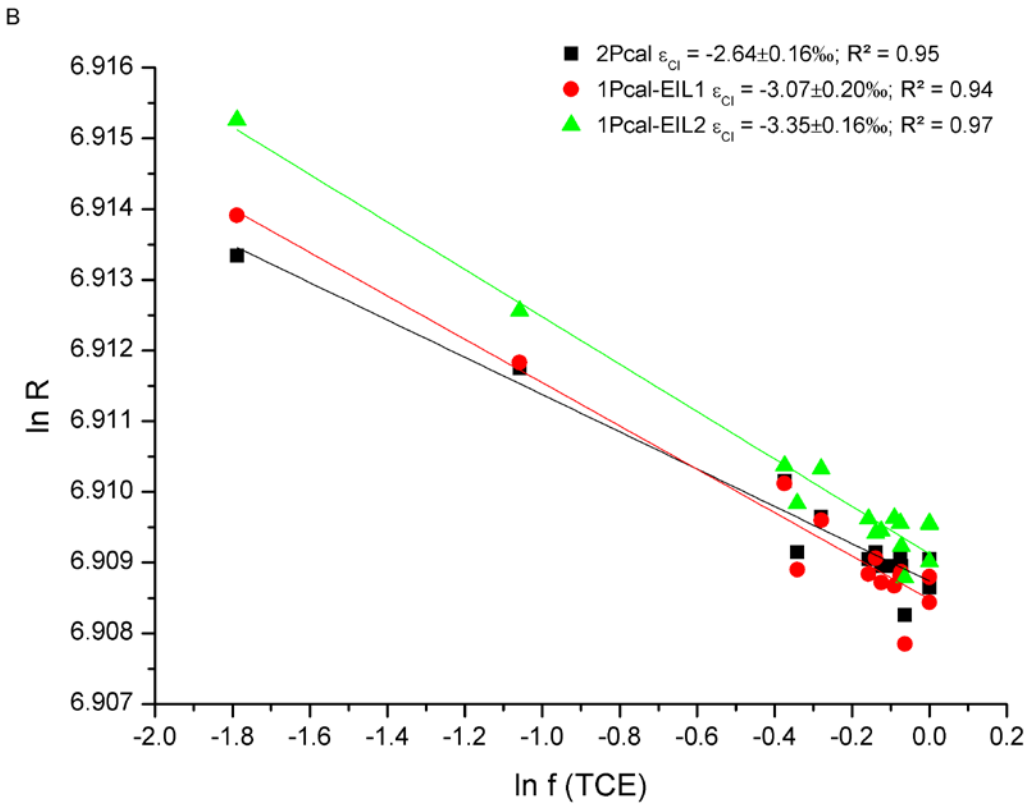


**Figure 2**. $\delta^{37}Cl$ signatures (Panel A) and Rayleigh plots (Panel B) of TCE from a biodegradation experiment calculated with a 1-point calibration using EIL-1 (red circles) or EIL-2 (green triangles), respectively and a 2-point calibration (black squares). Panel A: Horizontal lines show the isotopic signatures of the standards EIL-1 and EIL-2 with +3.05‰ and -2.72‰ respectively (dashed lines), as well as of the consensus value (CV) of TCE-Merck (0.51±0.06‰) used for spiking the microcosms (solid line). Dotted horizontal lines show the measured initial $\delta^{37}Cl$ signatures at the start of the experiment calculated from 1-point calibration (EIL-1 +0.92±0.21‰, EIL-2 +1.62±0.21‰) and 2-point calibration (+1.15±0.22‰) respectively. Panel B: Chorine isotope enrichment factors (with 95% confidence intervals) for different calibration schemes are: $\varepsilon_{Cl}$ = -3.07±0.20‰ (1-point calibration with EIL-1); $\varepsilon_{Cl}$ = -3.35±0.16‰ (1-point calibration with EIL-2); $\varepsilon_{Cl}$ = -2.64±0.16‰ (2-point calibration).

Figure 2 shows that different calibration schemes did not only affect $\delta^{37}Cl$ values, but also resultant enrichment factors $\varepsilon_{Cl}$ which varied significantly. Parallel $\delta^{37}Cl$-GC/IRMS measurements of the same samples using a two-point calibration resulted in an enrichment factor $\varepsilon_{Cl}$ of -2.5±0.1‰ [23] which is in excellent agreement with $\varepsilon_{Cl}$ = -2.64±0.16‰ obtained using GC/qMS applying a two-point calibration. (A compilation of GC/qMS and GC/IRMS data sets is

presented in Figure S 3 in the SI.) $\varepsilon_{Cl}$ values obtained with a one-point calibration, however, were up to 30% too negative in line with the findings that one-point calibration slopes were greater than unity (see Figure 1). Hence, differences in $\delta^{37}Cl$ were overestimated when applying Equation 2 and (inadequately) assuming a slope of unity. These greater differences, in turn, (wrongly) suggested greater Cl isotope fractionation resulting in too negative $\varepsilon_{Cl.}$ This trend is further accentuated by the fact that enrichment factors are largely determined by the lowes concentrations in a degradation experiment, where the progressively enriched substrate TCE exceeds the range of the calibration standards (more than 8‰ off from EIL-2).

This study therefore clearly identifies the need of 2-point calibration schemes for the determination of accurate and reliable $\delta^{37}Cl_{SMOC}$ signatures minimizing scale distortion effects. Thus, for proper application and interpretation of compound specific chlorine isotope data it is of utmost importance to provide comprehensive information regarding calibration (including slope, $R^2$) and isotopic standards, regardless if the focus is on determination of absolute values or relative isotopic shifts. A proper and harmonized calibration scheme is indispensable to ensure comparability of results from the increasing number of studies using 2D (or even 3D) carbon and chlorine (and hydrogen) isotope analysis aiming at deciphering the mechanisms underlying reductive dechlorination and to differentiate them in the field[21, 23-25]. Until recently the normalization of $\delta^{37}Cl$ standards relied on one single anchor (1-point calibration) increasing the bias of Cl-CSIA. Recently [37] a second anchor for referencing calibration standards to the SMOC scale by a two point calibration became available which will improve the isotopic characterization of laboratory calibration standards like EIL-1 and EIL-2. Our study underlines the need to continue such work to provide anchors and standards covering a wider range of $\delta^{37}Cl$-values.

## SUPPORTING INFORMATION

Additional supporting information is provided in the online version of this article.

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311 REFERENCES


312 [1] S. Feisthauer, M. Seidel, P. Bombach, S. Traube, K. Knöller, M. Wange, S. Fachmann, H. H.
313 Richnow. Characterization of the relationship between microbial degradation processes at
314 a hydrocarbon contaminated site using isotopic methods. *J. Contam. Hydrol.* **2012**, *133*,
315 17.
316 [2] A. E. Hartenbach, T. B. Hofstetter, P. R. Tentscher, S. Canonica, M. Berg, R. P.
317 Schwarzenbach. Carbon, hydrogen, and nitrogen isotope fractionation during light-
318 induced transformations of atrazine. *Environ. Sci. Technol.* **2008**, *42*, 7751.
319 [3] B. Morasch, H. H. Richnow, B. Schink, R. U. Meckenstock. Stable hydrogen and carbon
320 isotope fractionation during microbial toluene degradation: Mechanistic and
321 environmental aspects. *Appl. Environ. Microbiol.* **2001**, *67*, 4842.
322 [4] M. Ratti, S. Canonica, K. Mcneill, P. R. Erickson, J. Bolotin, T. B. Hofstetter. Isotope
323 fractionation associated with the direct photolysis of 4-chloroaniline. *Environ. Sci.*
324 *Technol.* **2015**, *49*, 4263.
325 [5] S. Spahr, J. Bolotin, J. Schleucher, I. Ehlers, U. Von Gunten, T. B. Hofstetter. Compound-
326 specific carbon, nitrogen, and hydrogen isotope analysis of n-nitrosodimethylamine in
327 aqueous solutions. *Anal. Chem.* **2015**, *87*, 2916.
328 [6] M. Elsner, M. A. Jochmann, T. B. Hofstetter, D. Hunkeler, A. Bernstein, T. C. Schmidt, A.
329 Schimmelmann. Current challenges in compound-specific stable isotope analysis of
330 environmental organic contaminants. *Anal. Bioanal. Chem.* **2012**, *403*, 2471.
331 [7] T. B. Hofstetter, M. Berg. Assessing transformation processes of organic contaminants by
332 compound-specific stable isotope analysis. *TrAC, Trends Anal. Chem.* **2011**, *30*, 618.
333 [8] C. Dorer, P. Höhener, N. Hedwig, H.-H. Richnow, C. Vogt. Rayleigh-based concept to tackle
334 strong hydrogen fractionation in dual isotope analysis—the example of ethylbenzene
335 degradation by aromatoleum aromaticum. *Environ. Sci. Technol.* **2014**, *48*, 5788.
336 [9] S. R. Lutz, B. M. Van Breukelen. Combined source apportionment and degradation
337 quantification of organic pollutants with csia: 2. Model validation and application.
338 *Environ. Sci. Technol.* **2014**, *48*, 6229.
339 [10] S. Ertl, F. Seibel, L. Eichinger, F. H. Frimmel, A. Kettrup. The 13c/12c and 2h/1h ratios of
340 trichloroethene, tetrachloroethene and their metabolites. *Isot. Environ. Health Stud.* **1998**,
341 *34*, 245.
342 [11] B. D. Holt, N. C. Sturchio, T. A. Abrajano, L. J. Heraty. Conversion of chlorinated volatile
343 organic compounds to carbon dioxide and methyl chloride for isotopic analysis of carbon
344 and chlorine. *Anal. Chem.* **1997**, *69*, 2727.
345 [12] H. Holmstrand, P. Andersson, Ö. Gustafsson. Chlorine isotope analysis of submicromole
346 organochlorine samples by sealed tube combustion and thermal ionization mass
347 spectrometry. *Anal. Chem.* **2004**, *76*, 2336.
348 [13] O. Shouakar-Stash, R. Drimmie, M. Zhang, S. Frape. Compound-specific chlorine isotope
349 ratios of tce, pce and dce isomers by direct injection using cf-irms. *Appl. Geochem.* **2006**,
350 *21*, 766.
351 [14] K. Sakaguchi-Söder, J. Jager, H. Grund, F. Matthäus, C. Schüth. Monitoring and evaluation
352 of dechlorination processes using compound-specific chlorine isotope analysis. *Rapid*
353 *Commun. Mass Spectrom.* **2007**, *21*, 3077.
354 [15] A. Bernstein, O. Shouakar-Stash, K. Ebert, C. Laskov, D. Hunkeler, S. Jeannottat, K.
355 Sakaguchi-Söder, J. Laaks, M. A. Jochmann, S. Cretnik, J. Jager, S. B. Haderlein, T. C.
356 Schmidt, R. Aravena, M. Elsner. Compound-specific chlorine isotope analysis: A
357 comparison of gas chromatography/isotope ratio mass spectrometry and gas

chromatography/quadrupole mass spectrometry methods in an interlaboratory study. *Anal. Chem.* **2011**, *83*, 7624.
[16] K. Sakaguchi-Söder. *A new method for compound-specific stable chlorine isotope analysis: Basics and applications*. Verein zur Förderung des Instituts IWAR der TU Darmstadt e.V., Darmstadt, **2010**.
[17] C. Aeppli, H. Holmstrand, P. Andersson, Ö. Gustafsson. Direct compound-specific stable chlorine isotope analysis of organic compounds with quadrupole gc/ms using standard isotope bracketing. *Anal. Chem.* **2010**, *82*, 420.
[18] M. E. Miska, O. Shouakar-Stash, H. Holmstrand. Stable chlorine isotope analysis of chlorinated acetic acids using gas chromatography/quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2015**, *29*, 2341.
[19] T. Mchugh, T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, P. Philp. Application of csia to distinguish between vapor intrusion and indoor sources of vocs. *Environ. Sci. Technol.* **2011**, *45*, 5952.
[20] B. Jin, C. Laskov, M. Rolle, S. B. Haderlein. Chlorine isotope analysis of organic contaminants using gc-qms: Method optimization and comparison of different evaluation schemes. *Environ. Sci. Technol.* **2011**, *45*, 5279.
[21] C. Wiegert, M. Mandalakis, T. Knowles, P. N. Polymenakou, C. Aeppli, J. Macháčková, H. Holmstrand, R. P. Evershed, R. D. Pancost, Ö. Gustafsson. Carbon and chlorine isotope fractionation during microbial degradation of tetra- and trichloroethene. *Environ. Sci. Technol.* **2013**, *47*, 6449.
[22] Y. Abe, R. Aravena, J. Zopfi, O. Shouakar-Stash, E. Cox, J. D. Roberts, D. Hunkeler. Carbon and chlorine isotope fractionation during aerobic oxidation and reductive dechlorination of vinyl chloride and cis-1,2-dichloroethene. *Environ. Sci. Technol.* **2009**, *43*, 101.
[23] S. Cretnik, K. A. Thoreson, A. Bernstein, K. Ebert, D. Buchner, C. Laskov, S. Haderlein, O. Shouakar-Stash, S. Kliegman, K. Mcneill, M. Elsner. Reductive dechlorination of tce by chemical model systems in comparison to dehalogenating bacteria: Insights from dual element isotope analysis (13c/12c, 37cl/35cl). *Environ. Sci. Technol.* **2013**, *47*, 6855.
[24] T. Kuder, B. M. Van Breukelen, M. Vanderford, P. Philp. 3d-csia: Carbon, chlorine, and hydrogen isotope fractionation in transformation of tce to ethene by a dehalococcoides culture. *Environ. Sci. Technol.* **2013**, *47*, 9668.
[25] C. Audí-Miró, S. Cretnik, N. Otero, J. Palau, O. Shouakar-Stash, A. Soler, M. Elsner. Cl and c isotope analysis to assess the effectiveness of chlorinated ethene degradation by zero-valent iron: Evidence from dual element and product isotope values. *Appl. Geochem.*
[26] D. Buchner, S. Behrens, C. Laskov, S. B. Haderlein. Resiliency of stable isotope fractionation (δ13c and δ37cl) of trichloroethene to bacterial growth physiology and expression of key enzymes. *Environ. Sci. Technol.* **2015**, *49*, 13230.
[27] A. Badin, G. Buttet, J. Maillard, C. Holliger, D. Hunkeler. Multiple dual c–cl isotope patterns associated with reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.* **2014**, *48*, 9179.
[28] J. Renpenning, S. Keller, S. Cretnik, O. Shouakar-Stash, M. Elsner, T. Schubert, I. Nijenhuis. Combined c and cl isotope effects indicate differences between corrinoids and enzyme (sulfurospirillum multivorans pcea) in reductive dehalogenation of tetrachloroethene, but not trichloroethene. *Environ. Sci. Technol.* **2014**, *48*, 11837.
[29] D. Hunkeler, Y. Abe, M. M. Broholm, S. Jeannottat, C. Westergaard, C. S. Jacobsen, R. Aravena, P. L. Bjerg. Assessing chlorinated ethene degradation in a large scale

contaminant plume by dual carbon-chlorine isotope analysis and quantitative pcr. *J. Contam. Hydrol.* **2011**, *119*, 69.
[30] C. Wiegert, C. Aeppli, T. Knowles, H. Holmstrand, R. Evershed, R. D. Pancost, J. Macháčková, Ö. Gustafsson. Dual carbon–chlorine stable isotope investigation of sources and fate of chlorinated ethenes in contaminated groundwater. *Environ. Sci. Technol.* **2012**, *46*, 10918.
[31] P. Lojkasek-Lima, R. Aravena, O. Shouakar-Stash, S. K. Frape, M. Marchesi, S. Fiorenza, J. Vogan. Evaluating tce abiotic and biotic degradation pathways in a permeable reactive barrier using compound specific isotope analysis. *Groundwater Monit. Rem.* **2012**, *32*, 53.
[32] J. Palau, M. Marchesi, J. C. C. Chambon, R. Aravena, À. Canals, P. J. Binning, P. L. Bjerg, N. Otero, A. Soler. Multi-isotope (carbon and chlorine) analysis for fingerprinting and site characterization at a fractured bedrock aquifer contaminated by chlorinated ethenes. *Sci. Total Environ.* **2014**, *475*, 61.
[33] S. Cretnik, A. Bernstein, O. Shouakar-Stash, F. Löffler, M. Elsner. Chlorine isotope effects from isotope ratio mass spectrometry suggest intramolecular c-cl bond competition in trichloroethene (tce) reductive dehalogenation. *Molecules.* **2014**, *19*, 6450.
[34] I. Nijenhuis, J. Renpenning, S. Kümmel, H. H. Richnow, M. Gehre. Recent advances in multi-element compound-specific stable isotope analysis of organohalides: Achievements, challenges and prospects for assessing environmental sources and transformation. *Trends Environ. Anal. Chem.* **2016**, *11*, 1.
[35] T. B. Coplen. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2538.
[36] M. Elsner, D. Hunkeler. Evaluating chlorine isotope effects from isotope ratios and mass spectra of polychlorinated molecules. *Anal. Chem.* **2008**, *80*, 4731.
[37] J. K. Böhlke, P. B. Hatzinger, N. C. Sturchio, B. Gu, I. Abbene, S. J. Mroczkowski. Atacama perchlorate as an agricultural contaminant in groundwater: Isotopic and chronologic evidence from long island, new york. *Environ. Sci. Technol.* **2009**, *43*, 5619.

# SUPPORTING INFORMATION

## Calibration bias of experimentally determined chlorine isotope enrichment factors – The need for a two-point calibration in Compound-specific Chlorine Isotope Analysis

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**Table S1: Suppliers and measured $\delta^{37}Cl_{SMOC}$ values of the neat PCE and TCE samples.**

| | GC/qMS (Agilent TU) | | GC/IRMS Munich (Munich Thermo) | | |
|---|---|---|---|---|---|
| | $\delta^{37}Cl_{SMOC}$/‰ | ***n*** | $\delta^{37}Cl_{SMOC}$/‰ | ***n*** | |
| **TCE** | | | | | |
| *EIL-1* | *3.05 ± 0.29* | 10 | *3.05 ± 0.12* | 10 | Dow Chemicals, U. S. |
| T1: IS-53 | 1.38 ± 0.19 | 10 | 1.09 ± 0.09 | 10 | Roth, Germany |
| T2: IS-54 | 1.2 ± 0.26 | 10 | 0.83 ± 0.13 | 10 | Roth, Germany |
| T3: Merck | 0.8 ± 0.25 | 10 | 0.51 ± 0.06 | 10 | Merck, Germany |
| T4: IS-15 | 0.6 ± 0.27 | 10 | 0.1 ± 0.13 | 10 | Merck, Germany |
| T5: PPG | -2.15 ± 0.34 | 10 | -2.3 ± 0.17 | 10 | PPG, U.S. |
| *EIL-2* | *-2.7 ± 0.22* | 10 | *-2. 7± 0.13* | 10 | PPG, U. S |
| **PCE** | | | | | |
| *EIL-1* | *0.29 ± 0.61* | 5 | *0.29 ±0.06* | *5* | PPG, U. S |
| P1: Merck | 0.25 ± 0.48 | 5 | -0.06 ±0.05 | 5 | Merck, Germany |
| P2: PPG | -1.03 ± 0.37 | 5 | -1.67 ± 0.02 | 5 | PPG, U.S. |
| *EIL-2* | *-2.52 ± 0.64* | 5 | *-2.52 ± 0.15* | *5* | Merck, Germany |

***Calculation of chlorine isotope ratios.***

Isotope ratios were calculated from ion abundance of the mass spectrum:

$$R_{PCE} = \frac{I_{166}+I_{164}}{(I_{166}+I_{164})+(I_{131}+I_{129})+(I_{96}+I_{94})+(I_{61}+I_{59})} \cdot \left(\frac{1}{4} \cdot \frac{I_{166}}{I_{164}}\right) + \frac{I_{131}+I_{129}}{(I_{166}+I_{164})+(I_{131}+I_{129})+(I_{96}+I_{94})+(I_{61}+I_{59})} \cdot \left(\frac{1}{3} \cdot \frac{I_{131}}{I_{129}}\right) + \frac{I_{96}+I_{94}}{(I_{166}+I_{164})+(I_{131}+I_{129})+(I_{96}+I_{94})+(I_{61}+I_{59})} \cdot \left(\frac{1}{2} \cdot \frac{I_{96}}{I_{94}}\right) + \frac{I_{61}+I_{59}}{(I_{166}+I_{164})+(I_{131}+I_{129})+(I_{96}+I_{94})+(I_{61}+I_{59})} \cdot \left(\frac{I_{61}}{I_{59}}\right) \quad \text{(S1)}$$

$$R_{TCE} = \frac{I_{132}+I_{130}}{(I_{132}+I_{130})+(I_{97}+I_{95})+(I_{62}+I_{60})} \cdot \left(\frac{1}{3} \cdot \frac{I_{132}}{I_{130}}\right) + \frac{I_{97}+I_{95}}{(I_{132}+I_{130})+(I_{97}+I_{95})+(I_{62}+I_{60})} \cdot \left(\frac{1}{2} \cdot \frac{I_{97}}{I_{95}}\right) + \frac{I_{62}+I_{60}}{(I_{132}+I_{130})+(I_{97}+I_{95})+(I_{62}+I_{60})} \cdot \left(\frac{I_{62}}{I_{60}}\right) \quad \text{(S2)}$$

$$R_{cDCE} = \frac{I_{98}+I_{96}}{(I_{98}+I_{96})+(I_{63}+I_{61})} \cdot \left(\frac{1}{2} \cdot \frac{I_{98}}{I_{96}}\right) + \frac{I_{98}+I_{96}}{(I_{98}+I_{96})+(I_{63}+I_{61})} \cdot \left(\frac{I_{63}}{I_{61}}\right) \quad \text{(S3)}$$

***Derivations of equations 5 and 6 in the manuscript.***

Normalization by the 1-point calibration scheme followed Aeppli et al.[17] using equation 1:

$$\delta^{37}\mathrm{Cl} = \left(\frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} - 1\right) + \delta^{37}\mathrm{Cl^{std}} \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} \quad (1)$$

and

$$\delta^{37}\mathrm{Cl} = \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} \cdot \left(1 + \delta^{37}\mathrm{Cl^{std}}\right) - 1 \quad (2)$$

This equation is based on the relation between bulk isotope ratios and the respective referenced $\delta^{37}Cl_{SMOC}$ signatures of sample and standard:

$$\frac{\delta^{37}\mathrm{Cl}+1}{\delta^{37}\mathrm{Cl}^{\mathrm{std}}+1} = \mathrm{n} \cdot \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} \quad (3)$$

with n: slope of the linear correlation. Two separate 1-point calibrations were performed using SMOC referenced TCE standards EIL-1 and EIL-2 as $R_{Cl}^{std}$.

With the assumptions from Aeppli et al.[17] using equation 1 either with EIL-1 or EIL-2 the slope should always be the same and in an ideal case it equals 1. Assuming n≠1, equation 1 changes to

$$\delta^{37}\mathrm{Cl} = \mathrm{n} \cdot \left(\frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} - 1\right) + \delta^{37}\mathrm{Cl}^{\mathrm{std}} \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std}}} \quad (4)$$

Using equation 4 to determine $\delta^{37}\mathrm{Cl}^{\mathrm{std2}}$ from $\delta^{37}\mathrm{Cl}^{\mathrm{std1}}$ results in:

$$\delta^{37}\mathrm{Cl}^{\mathrm{std2}} = \mathrm{n_1} \cdot \frac{R_{Cl}^{std2}}{\mathrm{R_{Cl}^{std1}}} \cdot \left(1 + \delta^{37}\mathrm{Cl}^{\mathrm{std1}}\right) - 1 \quad (S4)$$

Using this term to normalize a sample with standard 2 following relation applies

$$\delta^{37}\mathrm{Cl} = \mathrm{n_2} \cdot \frac{R_{Cl}}{\mathrm{R_{Cl}^{std2}}} \cdot \left(1 + \mathrm{n_1} \cdot \frac{R_{Cl}^{std2}}{\mathrm{R_{Cl}^{std1}}} \cdot \left(1 + \delta^{37}\mathrm{Cl}^{\mathrm{std1}}\right) - 1\right) - 1 \quad (S5)$$

$$\delta^{37}\mathrm{Cl}' = \mathrm{n_1} \cdot \mathrm{n_2} \cdot \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std1}}} \cdot \left(1 + \delta^{37}\mathrm{Cl}^{\mathrm{std1}}\right) - 1 \quad (6)$$

and for normalization with standard 1:

$$\delta^{37}\mathrm{Cl} = \mathrm{n_1} \cdot \frac{\mathrm{R_{Cl}}}{\mathrm{R_{Cl}^{std1}}} \cdot \left(1 + \delta^{37}\mathrm{Cl}^{\mathrm{std1}}\right) - 1 \quad (5)$$

**Detailed information on calibration curves applied for the biodegradation experiment.**

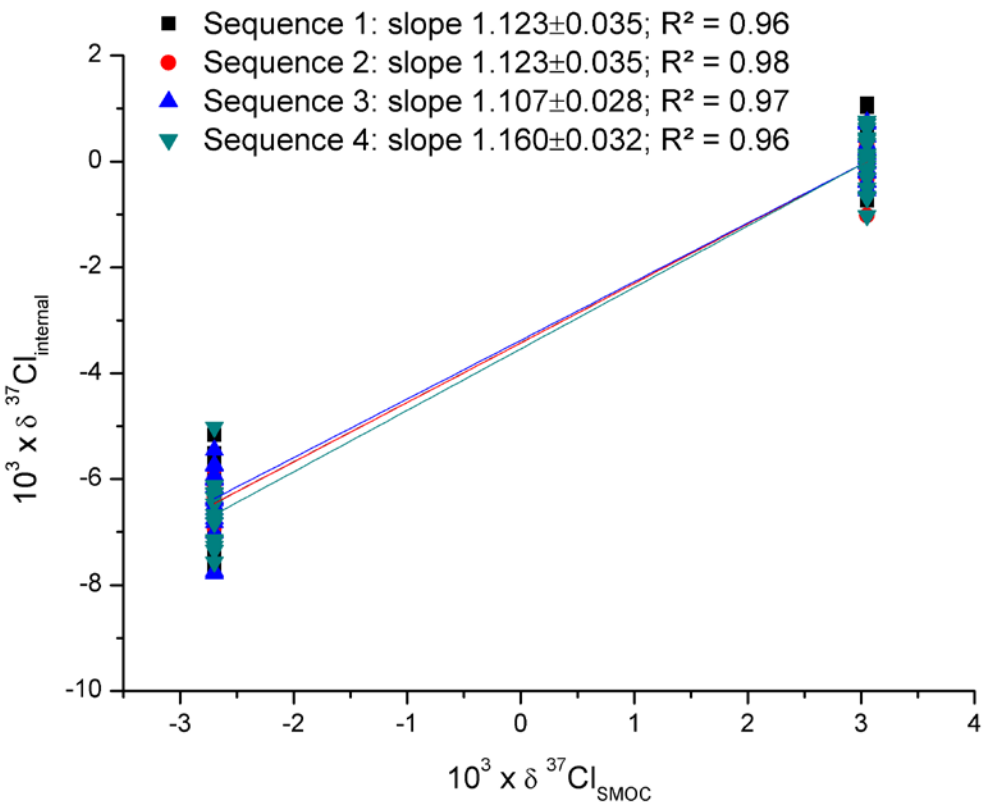


Figure S 1. Calibration slopes for the 4 sequences analyzed during the biodegradation experiment within 6 consecutive days (Sequence 1 black squares, Sequence 2 red circles, Sequence 3 blue triangles, Sequence 4 green triangles) from measurements of standards with different concentrations (each n=5). Given is the slope with standard error for a confidence interval of 95%.

***Biodegradation experiment: Compilation of GC/qMS and GC/IRMS data set.***

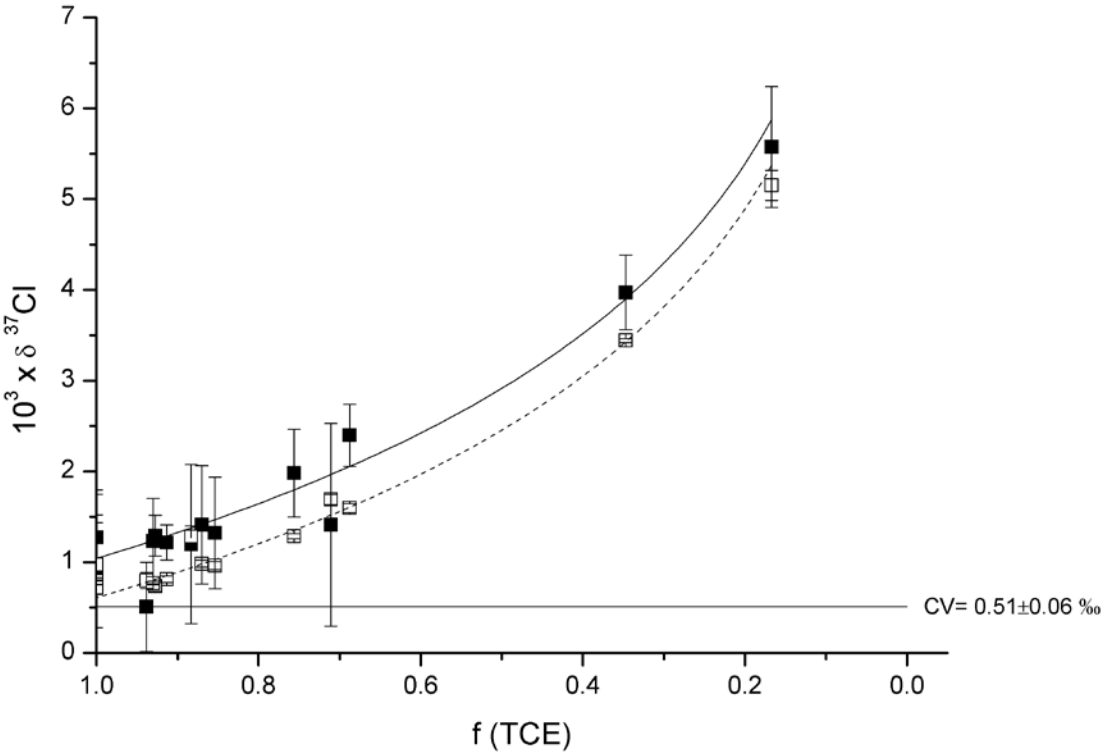


Fig. S2. Biodegradation experiment: Comparison of the $\delta^{37}Cl_{SMOC}$ data obtained by GC/qMS (closed symbols) and by the GC/IRMS (open symbols) published in [23].