Dual C-Cl Isotope Analysis for Characterizing the Reductive Dechlorination of α- and γ-Hexachlorocyclohexane by Two *Dehalococcoides mccartyi* strains and an Enrichment culture

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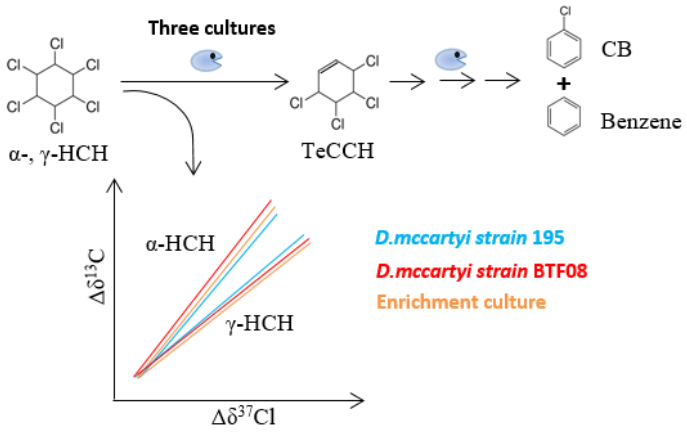
Abstract

Hexachlorocyclohexanes (HCHs) are persistent organic contaminants that threaten human health. Microbial reductive dehalogenation is one of the most important attenuation processes in contaminated environments. This study investigated carbon and chlorine isotope fractionation of α- and γ- HCH during the reductive dehalogenation by three anaerobic cultures. The presence of tetrachlorocyclohexene (TeCCH) indicated reductive dichloroelimination was the first step of bond cleavage. Isotope enrichment factors (ε_C and ε_{Cl}) were derived from the transformation of γ-HCH (ε_C, from -4.0 ± 0.5 to -4.4 ± 0.6 ‰; ε_{Cl}, from -2.9 ± 0.4 to -3.3 ± 0.4 ‰) and α-HCH (ε_C, from -2.4 ± 0.2 to -3.0 ± 0.4 ‰; ε_{Cl}, from -1.4 ± 0.3 to -1.8 ± 0.2 ‰). During α-HCH transformation, no enantioselectivity was observed, and similar ε_c were obtained for both enantiomers. The correlation of ^{13}C and ^{37}Cl fractionation ($\Lambda=\Delta\delta^{13}C/\Delta\delta^{37}Cl \approx \varepsilon_C/\varepsilon_{Cl}$) of γ-HCH (from 1.1 ± 0.3 to 1.2 ± 0.1) indicates similar bond cleavage during the reductive dichloroelimination by the three cultures, similar for α-HCH (1.7 ± 0.2 to 2.0 ± 0.3). The different isotope fractionation patterns during reductive dichloroelimination and dehydrochlorination indicates dual-element stable isotope analysis potentially can be used to evaluate HCH transformation pathways at contaminated field sites.

Keywords: Reductive Dehalogenation, Dichloroelimination, Hexachlorocyclohexane, Dual-Element Stable Isotope Analysis, Enantiomer Fractionation



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Introduction

Hexachlorocyclohexane (HCH) was used worldwide as a mixture of mainly α-, β-, γ- and δ-HCH, for controlling many pests, including lice and mites.[1-4] Later, purified γ-HCH (Lindane) was applied, which is the isomer with insecticidal activity.[5] For pharmaceutical purposes, Lindane is still applied as a second-line treatment for scabies and lice.[6] Between 1950 and 2000, about 600,000 metric tons of Lindane were used globally, and it was estimated that the global amount of HCH-isomers still present in the environment is between 1.7-4.8 million metric tons.[7] Heavily contaminated field sites with HCHs were reported over the world [8-17] and today, due to persistence and bioaccumulation, HCH isomers can be detected in arctic food webs.[18]

Biotransformation of HCHs was observed in both field sites and laboratory systems.[19-21] Over 30 aerobic bacterial strains were reported capable of the complete biodegradation of HCHs.[22-26] Anaerobic isolates including *Clostridium* spp., *Desulfovibrio* spp., *Desulfococcus* spp. can co-metabolically dechlorinate HCHs to tetrachlorocyclohexene (TeCCH), and benzene and chlorobenzene (CB) were detected as final products (for a review see[22]). A mixed culture of *Dehalobacter* sp. with S*edimentibacter* sp. was capable of the dehalogenation of β-HCH.[27] More recently, *Dehalococcoides mccartyi* strains 195 and BTF08 were also shown capable of the transformation of HCHs to benzene and CB.[28, 29] Further, *Dehalococcoides mccartyi* was inferred to contribute significantly to biotransformation of organohalides at the HCHs contaminated field site in Bitterfeld-Wolfen.[30] However, evaluation of the biotransformation of HCHs in the environment is still a challenge as the concentration can be affected by several processes, such as transformation, dilution, evaporation, and adsorption. Enantiomer fractionation has been proposed as an indicator to characterize biotransformation of α-HCH, which has a chiral center. Preferential transformation of individual enantiomers in biochemical reactions was observed, but differences in rates were negligible in abiotic reactions.[31-33] However, this approach is still restricted for evaluating α-HCH transformation *in situ*

due to the limited knowledge of enantiomer selectivity during anaerobic biotransformation of α-HCH. Thus, so far, comprehensive approaches for monitoring *in situ* degradation pathways of HCH isomers are not available.

In order to characterize biotransformation processes, concepts based on compound-specific stable isotope analysis (CSIA) were developed.[34-36] Bonds with lighter isotopes react slightly faster than the ones with heavier isotopes, leading to a change of isotope ratios in the residual fraction of substrate, known as isotope fractionation. Isotope fractionation processes can be characterized using the Rayleigh concept, and the apparent kinetic isotope effects (AKIEs) of the bond cleavage can be quantified.[37] In previous studies, the stable carbon isotope fractionation of HCH in the transformation by aerobic and anaerobic cultures as well as chemical reactions was investigated. Carbon isotope enrichment factors (ε_C) for α- and γ-HCH transformation were significantly different for aerobic (ε_C= -1.0±0.2‰ to -1.7±0.2‰) and anaerobic (ε_C=-2.4±0.2‰ to -5.5±0.8‰) biotransformation (Table 1).[29, 38-41] Furthermore, Zhang et al. reported variable ε_C (-1.9±0.2‰ to -7.6±0.4‰) for the chemical transformations of α-HCH via indirect photolysis (UV/H_2O_2), direct photolysis, electro-reduction, reduction by Fe^0 and hydrolysis.[42]

In recent years, an increasing number of studies showed that the observed isotope fractionation could be masked by rate-limiting steps other than bond-cleavage, e.g., transport into the cell or binding to the enzyme, and those ε values do not characterize the bond cleavages.[43-45] Thus, dual-element CSIA was proposed to characterize reaction mechanisms, e.g., the transformation of chlorinated ethenes and ethanes[45-50], as it correlates two isotope effects to cancel out the masking of single element isotope enrichment by various processes. For the biotransformation of HCHs, two studies reported dual C-H isotope fractionation during dehydrochlorination of HCH isomers catalyzed by LinA and LinB enzymes which is a typical aerobic reaction involving C-H and C-Cl bond cleavage.[51,52] The correlation of ^2H, ^{13}C, ^{37}Cl isotope fractionation during γ-HCH dehydrochlorination was analyzed previously in a study mainly focused on molecular modelling, and similar isotope fractionation patterns were observed in the chemical and the

LinA enzyme catalyzed reactions.[53] Microbial reductive dechlorination (involving C-Cl bond cleavage) contributes to natural attenuation of HCHs in contaminated groundwater systems which are often anoxic. However, thus far, dual C-Cl stable isotope fractionation during HCH reductive dechlorination is missing. This is a bottleneck for applying both carbon and chlorine isotope fractionation to quantify the natural attenuation of HCHs. This approach would potentially provide a more precise evaluation of *in situ* transformation and reaction mechanisms. Thus, for the application of dual-element CSIA, the variability of ε_C and ε_{Cl} of HCH during the transformation by different anaerobic cultures needs to be investigated.

Hence, in order to fill the knowledge gaps, microbial reductive dichloroelimination of α- and γ-HCH by two *Dehalococcoides mccartyi* strains (195 and BTF08) and an enrichment culture (without detectable *Dehalococcoides* sp.) derived from groundwater[29] were studied. Biotransformation and isotope fractionation were studied (i) to explore the transformation pathway; (ii) to determine the ε_C and ε_{Cl} for both α- and γ-HCH; and (iii) to obtain the Λ values during reductive dichloroelimination of α- and γ-HCH. In addition, the Λ values of the present study were compared with the previously reported data for dehydrochlorination to evaluate whether the dual-element isotope patterns potentially can be used to characterize reaction mechanisms.

Material and Methods

Chemicals: γ-HCH (analytical purity, 97%), α-HCH (99%), hexachlorobenzene (HCB) (99.5%), benzene, CB, 1,4-dichlorobenzene (1,4-DCB), 1,2-dichlorobenzene (1,2-DCB), and 1,2,4-trichlorobenzene (1,2,4-TCB), were purchased from Sigma Aldrich (Germany). *n*-Hexane (analytical purity >99%) was obtained from Carl Roth, Germany.

Strains, enrichment culture and cultivation conditions: *Dehalococcoides mccartyi strain* 195 was cultivated as described before,[54, 55] but without the "butyrate pellet" which is the cell extract from a mixed

105 culture containing *D. mccartyi* strain 195.[56] *Dehalococcoides mccartyi* strain BTF08 and the enrichment
106 culture, which was initially enriched from chlorobenzene-contaminated groundwater of Bitterfeld,[29] were
107 cultivated as described previously.[57] The details about cultivation medium and amendments are shown
108 in the Supporting Information (SI section 1). H_2 (0.5 bar overpressure) was used as the electron donor,
109 acetate as the carbon source. Cultivations were conducted in 240mL glass serum bottles (with 100mL
110 amended medium) sealed with Teflon-coated stoppers and crimped. Abiotic controls without inoculum
111 were kept under identical conditions. All the cultures were incubated statically at 30 °C.

112 **Pre-cultivation experiments:** During the pre-cultivation of the three cultures with HCHs, for each
113 transfer, 100µL of HCH isomer (α- or γ-HCH) stock solution (0.1 M in acetone) was spiked as electron
114 acceptor. After the total concentration of benzene and CB was about 300µM (spiked three times in total),
115 the cultures were transferred to fresh medium (1%, v/v transfer). For the enrichment culture, 2-
116 bromoethanesulphonate (BES, 10mM) was used from the fourth transfer to inhibit CH_4 production.
117 Methane was monitored together with benzene and CB. In total, all the cultures were transferred five
118 times before the batch experiments.

119 **Batch experiments:** Batch experiments for α- and γ-HCH biotransformation and monitoring of stable
120 isotope fractionation were conducted with the same cultivation conditions and 30 µL (0.1 M in acetone)
121 of α- or γ-HCH isomer stock solution was added. Each set of batch experiments had 18 bottles, including
122 two abiotic controls which were the same as the batch experiments but without inoculation.

123 **Transformation experiments with the enrichment culture:** The community composition of the
124 enrichment culture capable of transforming HCH was analyzed by Illumina sequencing (see SI 2).
125 Experiments were conducted to investigate the possible transformation processes of HCH by the
126 enrichment culture as described in SI Section 3.

Sampling and Extraction Procedure. In all the experiments, benzene and CB were considered as the main products of the anaerobic transformation of HCHs. At several time points, 1mL of liquid was taken from each bottle to monitor the concentration of benzene, CB and methane production at the same time. For the batch experiments, the sacrificial approach was adopted as reported before.[40] In order to stop the reaction, 5mL saturated sodium sulfate solution (pH=1), which was acidified with sulfuric acid was added to each bottle. Sacrificed bottles were kept at 10 °C until extraction. For the extraction, 2mL *n*-hexane with HCB (75µM) as the internal standard was added in each bottle. Subsequently, the bottles were shaken (120rpm) for at least 4 h in a cold room at 4 °C. The extraction efficiency was > 98%. No changes in isotope composition were observed associated with the extraction method.

Analytical Methods:

Concentration of metabolites: In the all the experiments, benzene, CB and methane were measured by gas chromatography - an Agilent 6890 series gas chromatograph (GC, Agilent Technologies, Palo Alto, USA) equipped with a flame ionization detector (FID) and a Rtx-VMS column (30 m x 0.25 mm ID x 0.25 µm FD, Restek, Bad Homburg, Germany) with the same temperature program as described previously.[28] Samples (1mL liquid medium) were filled in 10 mL headspace vials which contained 0.5 mL of Na_2SO_4 saturated solution (pH=1, H_2SO_4). Samples were measured by headspace analysis (before injection, samples were incubated at 70 °C with shaking for 5min) and the concentrations of benzene and CB in the liquid medium were calculated by a standard calibration curve (SI section 4) with the standard error of triplicate analysis less than 5%.

Concentration of HCHs: In the batch experiments, the concentrations of HCH were measured by GC-FID (7820A, Agilent Technologies, USA). An HP-5 capillary column (30 m x 0.32 mm ID x 0.25 µm FD; Agilent Technologies, Palo Alto, USA) was installed with helium as the carrier gas (flow of 2.0 mL min^{-1}). The oven temperature program started at 35 °C, was held for 5 min isothermally, increased at 8 °C/min to 180 °C,

150 then at 2 °C/min to 195 °C, and finally increased at 8 °C/min to 220 °C, which was held for 2 min. Hexane
151 extracts were injected in splitless mode with injection volumes of 1µL by autosampler. More details for
152 the determination of HCH concentration can be found in SI section S4. The standard error of triplicate
153 analysis was <5%.

154 Identification of metabolites: In the batch experiments, Gas Chromatography-Mass Spectrometry
155 (GC690N coupled to 5973 Network MSD in Single Ion Monitoring Mode, EI-MS (70eV); Agilent
156 Technologies) with automated injection (7683 Series Agilent Technologies) was used to identify the
157 metabolites. The installed column was the same as for the GC-FID for the analysis of HCHs concentration.
158 In order to separate all the metabolites, a relatively slow temperature program was applied: start at 60
159 °C and hold for 3 min, with the rate 3 °C/min to 175 °C, then 1 °C/min to 200 °C and hold 5 min, and with
160 15 °C/min until 320 °C.

161 Stable carbon isotope analysis: In the batch experiments, for carbon isotope analysis, the system
162 consisted of a GC (6890, Agilent Technologies, Palo Alto, USA) equipped with a GC IsoLink coupled with a
163 Conflo IV interface (Thermo Fisher Scientific, Bremen, Germany) to a MAT253 IRMS (Thermo Fisher
164 Scientific, Bremen, Germany). For α-HCH, a γ-DEX^{TM} 120 chiral column (Supelco, Bellefonte, PA, USA;
165 column length * i.d. 30 m * 0.25 mm, d_f=0.25 µm) was used for the separation of α-HCH enantiomers
166 according to the method described previously.[39] For γ-HCH isotope composition analysis, a ZB1 column
167 (60m*0.32mm, d_f=1 µm, Phenomenex) was used, as described before.[41] At least 5 – 10 nmol of carbon
168 on column were needed for a reliable value with a typical uncertainty ≤ 0.5 ‰.[58] All samples were
169 measured in at least three replicates and the typical uncertainty of analysis was <0.5 ‰. Based on the
170 international standard Vienna Pee Dee Belemnite (V-PDB), isotope compositions were described in delta
171 notation ($\delta^{13}C$) according to equation 1.

172 $$\delta^{13}C_{sample} = \frac{R_{sample}}{R_{standard}} - 1 \qquad (1)$$

173 R_{sample} and $R_{standard}$ were the ratios of $^{13}C/^{12}C$ for sample and standard, respectively. $\delta^{13}C$ was reported in
174 per mil (‰) scale.

175 Stable chlorine isotope analysis: In the batch experiments, chlorine stable isotope analysis was performed
176 on α- and γ-HCH. Chlorine isotope composition ($\delta^{37}Cl$) was determined online using gas chromatography
177 coupled with multiple-collector inductively coupled plasma mass spectrometry (GC-MC-ICPMS), as
178 recently reported.[59] A gas chromatograph (Trace 1310, Thermo Fisher Scientific, Germany), equipped
179 with an auto-sampler (TriPlus RSH, Thermo Fisher Scientific, Germany) was used for analyte separation.
180 Samples were injected with a split ratio of 1:10 and a constant carrier gas flow of 2 mL min^{-1}. HCHs were
181 separated on a Zebron ZB-1 capillary column using the same temperature program as for HCH
182 concentration analyzing by GC-FID. At least 5 nmol of chlorine on column was requested to obtain a
183 typical uncertainty ≤ 0.3 ‰.[58] Solvent peak cut was done with a SilFlow GC Deans Switch (SGE Analytical
184 Science, Austria) at the end of the GC column. Once separated, the analyte was directed to the ICP torch
185 via a Thermo Elemental Transfer line AE2080 (Aquitaine Electronique, France). The MC-ICPMS plasma
186 was operating with dry plasma, reducing unfavorable protonation effects. Parameters for the MC-ICPMS
187 were described in detail before.[60] After atomization and ionization in the dry argon plasma, chlorine
188 isotope was analyzed directly at mass 35 and 37 ($^{35}Cl^+$, $^{37}Cl^+$) at low-resolution mode (m/Δm = 300).
189 Generated transient signals ("peaks") were transformed into isotopic ratios after the acquisition. All
190 isotopic ratios of mass 37 and 35 were calculated using regression analysis. Linear regression of these
191 plots yields a straight line whose slope represents the isotope ratio. All samples were measured in at least
192 three replicates, and the typical uncertainty of analysis was <0.3‰.[60]

193 All samples were spiked with an in-house reference as an internal isotopic reference for compensation
194 of minor instrumental drifts and $\delta^{37}Cl$ was determined as described elsewhere.[59, 60] The chlorine isotope
195 compositions were reported in delta notation relative to Standard Mean Ocean Chloride (SMOC).

Calibration to SMOC scale was done using in-house standards with characterized chlorine isotope composition, including methyl chloride (MC, δ^{37}Cl = +6.02‰) and trichloroethene no.2 (TCE2, δ^{37}Cl = -1.19‰). In addition, trichloroethene no.6 (TCE6, δ^{37}Cl = +2.17‰) was used for the validation of calibration.

Enantiomer fractionation: In the batch experiments, the enantiomer ratio of α-HCH was analyzed based on the results of GC- IRMS data. The EF(+) is defined as $A^+/(A^+ + A^-)$ and EF(-) is defined as $A^-/(A^+ + A^-)$, where A^+ and A^- correspond to the peak areas of (+) and (-) enantiomers.[61] An EF (+) > 0.5 shows the preferential degradation of (-) enantiomer, and an EF (+) < 0.5 indicates the preferential degradation of (+) enantiomer.

Quantification of isotope fractionation: The simplified Rayleigh equation (eq.2) was used to determine the stable isotope fractionation of the biodegradation process in this study. The isotope enrichment factor (ε_E) of element E was determined by equation 2.

$$\ln\left(\frac{\delta_t+1}{\delta_0+1}\right) = \varepsilon_E ln\left(\frac{C_t}{C_0}\right) \qquad (2)$$

where δ is the isotope composition of carbon (δ^{13}C) or chlorine (δ^{37}Cl). C_t/C_0 is the residual fraction of the substrate. $\ln(C_t/C_0)$ vs. $\ln[(\delta_t+1)/(\delta_0+1)]$ were plotted, and ε_E is the slope of the linear regression, which represents the isotope enrichment factor. The error of ε_E was reported as 95% confidence interval (CI) determined by a regression curve analysis.

The Λ value of dual-isotope analysis was calculated by equation 3.

$$\Lambda = \frac{\Delta\delta^{13}\mathrm{C}}{\Delta\delta^{37}\mathrm{Cl}} \approx \frac{\varepsilon_\mathrm{C}}{\varepsilon_\mathrm{Cl}} \qquad (3)$$

The apparent kinetic isotope effect (AKIE) value was calculated using equation 4.[37]

$$AKIE_{\mathrm{E}} = \frac{1}{1+\frac{n*z}{x}*\varepsilon} \quad (4)$$

Where E is the considered element(C or Cl), n is the number of atoms of the selected element in the molecule, x is the number of reactive positions, and z is the number of indistinguishable reactive positions for intramolecular competition. The uncertainty of AKIE values was estimated by error propagation calculations according to equation 5.[62]

$$error\ of\ AKIE_E = \left|\frac{\partial AKIE_E}{\partial \varepsilon_E}\right| \times error\ of\ \varepsilon_E \quad (5)$$

Results

Anaerobic transformation of HCH isomers

In the enrichment culture, no sequences related to *Dehalococcoides* were detected (the results of the Illumina sequencing for the enrichment culture can be found in SI Figure S4). *Propionicicella* was the dominant genus in the enrichment culture, which has only one reported species isolated from groundwater contaminated by chlorinated solvents.[63] Additionally, the genera *Clostridium* and *Desulfovibrio* were detected, which contain species capable of HCH transformation.[39, 41] Sequences related to organohalide respiring bacteria (*Sulfurospirillum*) were detected as well. However, no report has shown the capability of *Sulfurospirillum* spp. for HCH transformation so far. In the transformation experiments with the enrichment culture, no transformation of HCHs was observed in the group without H_2 as electron donor (SI Figure S5), which indicates the dependency on H_2 of HCH transformation. The cell numbers were significantly increased in the groups with HCHs transformation (SI Figure S6). Acetate was consumed (from about 250 mg/L to less than 100 mg/L) during HCH transformation, and no methane was produced in all the experiments (data not show).

In the pre-cultivation experiments, the two *Dehalococcoides mccartyi* strains and the enrichment culture were cultivated with HCHs as the sole electron acceptor. The total concentrations of benzene and CB

showed a continuous increase until almost equal to the concentration of spiked HCHs (SI Figure S7). Whereas initial sets of cultivation needed about one year for the transformation of 30 µmol of HCHs, the later transfers only needed about six months for the transformation of the same amount of HCHs as represented by the fifth transfer (SI Figure S8). Small amounts (<2% of total metabolites, Figure S9) of 1,4-dichlorobenzene (1,4-DCB), 1,2-dichlorobenzene (1,2-DCB), 1,2,4-trichlorobenzene (1,2,4-TCB) were also identified by comparison to analytical standards, but amounts were too low for quantification.

The three cultures were transferred five times (1% v/v transfer) with α- or γ-HCH as sole electron acceptor before the batch experiments. In the batch experiments, the transformation of HCH and the production of benzene and CB were observed (SI figure S10). In addition to benzene and CB, different TeCCH isomers (with different retention time, SI Figure S11) were observed during α- and γ-HCH transformation. No enantioselectivity was observed during the biotransformation of α-HCH by the three cultures (SI Figure S12). No metabolite was detected in the abiotic control, and the HCHs concentration was identical to the initial concentration at the end of the incubation period.

Isotope enrichment of α- and γ-HCH during biotransformation

Carbon isotope enrichment. In the batch experiments, the amended α-HCH (30 µmol L^{-1}) was consumed within one month, similar to γ-HCH. The initial $\delta^{13}C$ of (+)α-HCH, (-)α-HCH and bulk α-HCH were -29.0 ± 0.3 ‰, -30.2 ± 0.3 ‰ and -29.6 ± 0.3 ‰, respectively (SI Figure S13). In abiotic controls, these values remained constant during the whole experiment (data not shown) confirming that only biodegradation led to changes in carbon isotope composition ($\delta^{13}C$) of α-HCH during the experiments. In the batch experiments, similar carbon isotope enrichment was observed for the three cultures (Figure 1 and SI Figure S14). For example, in the batch experiments with strain BTF08, when the remaining fraction was approx. 20%, the $\delta^{13}C$ was enriched to -25.8 ± 0.2‰ and -24.9 ± 0.1‰ for (-)α-HCH and (+)α-HCH, respectively. For (-)α-HCH, the ε_c values of the three cultures are between -2.4 ± 0.4‰ and -3.0 ± 0.4‰,

261 which were in the same range of (+)α-HCH (from -2.3 ± 0.2‰ to -3.1 ± 0.3‰) as well as bulk α-HCH
262 (from -2.4 ± 0.2 ‰ to -3.0 ± 0.4 ‰) (Table 1). Carbon isotope enrichment of γ-HCH followed the same
263 trend in the transformation by the three cultures (Figure 1, SI Figure S14). In the set of experiments with
264 strain BTF08, 94% transformation of γ-HCH was accompanied by a $\delta^{13}C$ enrichment from -25.7±0.2‰
265 to -14.3±0.1‰ (Figure 1b). The ε_c for γ-HCH biotransformation by strain BTF08 was -4.0 ± 0.5 ‰, which
266 was identical to the value of strain 195 (-4.4 ± 0.6 ‰) and the enrichment culture (-4.0 ± 0.5 ‰) (Table
267 1).

268 ***Chlorine isotope enrichment.*** Similarly as for carbon, the observed chlorine isotope fractionation during
269 the biotransformation of α-HCH by the three cultures was comparable (Figure 1 and SI Figure S14). During
270 the biotransformation by strain BTF08, $\delta^{37}Cl$ was enriched from 0.4‰ to 3.3‰ after approximately 80%
271 of α-HCH transformation (Figure 1a). The calculated chlorine isotope enrichment factors (ε_{Cl}) were -1.8 ±
272 0.2 ‰, -1.4 ± 0.3 ‰, and -1.4 ± 0.3 ‰ for *D. mccartyi* strains 195 and BTF08 and the enrichment culture,
273 respectively (Table 1). The trends of $\delta\ ^{37}Cl$ enrichment in the transformation of γ-HCH by the three
274 cultures were also similar, resulting in identical ε_{Cl} values for strain 195 (-3.3 ± 0.4 ‰), strain BTF08 (-3.3
275 ± 0.3 ‰) and the enrichment culture (-2.9 ± 0.4 ‰) (Table 1).

276 ## Dual isotope analysis and AKIE

277 The dual-element (C-Cl) isotope fractionation plot for the three investigated cultures, and the resulting
278 Λ-values for α-HCH were not significantly different from each other (from 1.7 ± 0.2 to 2.0 ± 0.3; Figure 1,
279 Table 1). The ʌ-values for γ-HCH were 1.2 ± 0.1 (strain 195), 1.1 ± 0.3 (strain BTF08) and 1.1 ± 0.2 (
280 enrichment culture) (Table 1).

281 The AKIE values were calculated based on the following scenarios: (1) considering all the chlorine atoms
282 are chemically equivalent, (2) assuming only chlorines with the trans-diaxial conformation (Table S1) are
283 involved in the reaction considering a) a stepwise or b) concerted process. The different conformers of

α- and γ-HCH, taking in to account the different numbers of chlorine atoms with trans-diaxial conformation which play a crucial role in the reductive dechlorination, are summarized in Table S1.[51,52] Thus, for scenario 1, n=x=z=6 were applied for both α- and γ-HCH. For the conformers of γ-HCH which have the same number of chlorine in the axial and equatorial position, n=6, x=z=3, and n=6, x=3, z=2 were applied for scenario 2a and 2b, respectively. Each enantiomer of α-HCH has two conformers, and the activity of the respective conformers is unknown. As no enantiomer selectivity was observed and the carbon isotope enrichment factors of the enantiomers were the same, we assume that the respective active conformers of α-HCH enantiomers have the same number of reactive positions. The more abundant conformers have two chlorine atoms with trans-diaxial conformation, while the less abundant ones have four chlorine with trans-diaxial conformation. Therefore, the calculation of AKIE for α-HCH considers two or four chlorines with trans-diaxial conformation. In the case of two chlorines(SI Table S1), n=6, x=z=2, and n=6, x=2, z=1 for scenario 2a1 (stepwise bond cleavage with two chlorines in axial position) and 2b1 (concerted bond cleavage with two chlorines in axial position), respectively. In the case of four chlorines in the axial position (SI Table S1), the parameters for AKIE calculation would be n=6, x=z=4 (scenario 2a2, stepwise bond cleavage with four chlorines in axial position) and n=6, x=4, z=3 (scenario 2b2, concerted bond cleavage with four chlorines in axial position). Scenarios 1 and 2a (including 2a1 and 2a2 for α-HCH) were grouped as the parameters (n, x, z) lead to the same AKIE (SI Table S2). It should be noted that the AKIE calculation in this study neglected the unknown contribution of secondary isotope effects. For example, previous studies on reductive dechlorination of trichloroethanes and trichoroethenes reported significant contribution of secondary chlorine isotope effects.[64,65] Calculated AKIEs and the values from the reported studies for HCHs biotransformation are summarized in SI Table S2.

Discussion

Biotransformation pathway of HCHs

In the transformation experiments with enrichment culture, production of CH_4 and acetate were not observed (data not shown), and HCH was not transformed in the absence of H_2 indicating that processes, like dehydrochlorination without electron transfer, and co-metabolic processes with methanogenesis and acetogenesis are unlikely.[38, 66] By assuming that H_2 is the electron donor, for the complete transformation of one mol HCH to benzene (by three steps of reductive dichloroelimination) or CB (by two steps of reductive dichloroelimination and one step of dehydrochlorination) as shown in Figure 2, the necessary amount of H_2 would be three or two mol, respectively. In batch experiments, the total amount of HCH was three µmol, which means the maximum amount of H_2 for the complete transformation of HCH would be nine µmol. The required amount of H_2 for the transformation of HCH is much lower than the amount of H_2 added in the system (2.8 mmol). The three investigated cultures could be maintained during five transfers (1% v/v) with α- or γ-HCH as the sole electron acceptor and H_2 as the electron donor, which indicates the transformation was a metabolic process, i.e., organohalide respiration. However, we cannot completely exclude co-metabolic transformation in the enrichment culture, and potentially multiple microorganisms were involved in the different steps of HCH biotransformation, as suggested by the still complex microbial community in the enrichment culture. The difference in reactivity of the HCH isomers is assumed to be the result of different physical and chemical properties, which are mainly determined by the axial and equatorial position of the chlorine atoms on each molecule.[67] As HCHs lack carbon-carbon bond rotation, the elimination of chlorines with trans-diaxial conformation is easier comparing to those in equatorial position (SI table S1).[68] Thus, it is reasonable to assume that reductive dichloroelimination is the first step for the transformation of α- and γ-HCH, which have at least two chlorines with trans-diaxial conformation (SI Table S1). In this study,

the reductive dichloroelimination reaction of α-/γ-HCH resulted in different TeCCH isomers (SI Figure S11) observed as intermediates in low concentration. The identical retention time of TeCCH from each specific HCH isomer (α-/γ-HCH) transformed by the three different cultures indicates that the enrichment culture and the *D. mccartyi* strains form an identical metabolite. However, the molecular configuration of TeCCH could not be identified due to the lack of standards. In the next reaction step, 5,6-dichloro-1,3-cyclohexadiene (1,3-DCDN, Figure 2) was assumed as another potential intermediate which may react further to benzene and CB as proposed during the anaerobic transformation of HCHs previously.[69, 70]

In the pre-cultivation experiments, α- and γ-HCH were transformed to benzene and CB as the main products by the three investigated cultures (SI Figure S7 and S8). In the end, the concentration of benzene and CB was constant (SI Figure S7and S8). Both benzene and CB were the synchronous final products of HCHs transformation, and there was no significant transformation from CB to benzene, which is in agreement with previous studies.[28, 71] Small amounts of 1,2-DCB, 1,4-DCB, and 1,2,4-TCB were observed, similar to previous study,[72] which may have been produced by spontaneous processes, e.g., dehydrochlorination. Thus. CB, DCB, and TCB accumulated in the bottles as strain 195 and BTF08 cannot use these chlorinated benzenes as electron acceptors.[28, 71] Thus, we propose a general transformation pathway for α-HCH and γ-HCH without considering the stereoisomeric configuration of the metabolites. By reductive dichloroelimination, specific TeCCH isomers from γ-HCH and α-HCH are produced first, and react via 1,3-DCDN, to benzene and CB as the final products (Figure 2).

Enantioselectivity of α-HCH

In this study, α-HCH biotransformation did not show significant enantiomer selectivity, which was in agreement with the report of α-HCH anaerobic transformation by *C. pasteurianum*[39] but in contrast to

observations in sewage sludge,[73] methanogenic biogas reactor,[74] and groundwater samples under anoxic conditions.[75]

For the investigation of α-HCH enantiomer fractionation, previous study analyzed aerobic degradation, which were catalyzed by two enzymes (LinA1 and LinA2) mainly responsible for (+) and (-)α-HCH transformation and led to different ε_c.[40] In this study, the absence of enantiomer selectivity and the identical ε_c value for α-HCH enantiomers during the transformation by these three cultures (Table 1) suggest one enzyme without enantiomer selectivity catalyzed the reaction of both enantiomers in the dehalogenation process. However, we cannot exclude that two or more enzymes catalyze the transformation of the enantiomers with the same rates. Further studies with anaerobic cultures which have α-HCH enantiomer selectivity may be needed for a better insight into the enantiomer fractionation under anoxic condition.

C and Cl isotope fractionation of HCHs during reductive dichloroelimination

The ε_C values of this study fall in the narrow range of previously reported values (-3.2 ± 0.2 ‰ to -5.5 ± 0.8 ‰ for γ-HCH; -2.4 ± 0.2 ‰ to -3.8 ± 0.3 ‰ for α-HCH; Table 1) regardless if metabolic or co-metabolic anaerobic transformation of α-/γ-HCH.[29, 38, 39, 41] Comparing to aerobic degradation, carbon isotope fractionation for both α- and γ-HCH were larger in anaerobic transformation (Table 1), which indicates the possibility for applying CSIA to characterize different reaction mechanisms. During the transformation by the three investigated cultures, almost identical ε_{Cl} were obtained for γ-HCH (from -2.9 ± 0.4 ‰ to -3.3 ± 0.4 ‰, Table 1) as well as for α-HCH (from -1.4 ± 0.3 ‰ to -1.8 ± 0.2 ‰, Table 1). Due to the similarity in reductive dichloroelimination of γ- and α-HCH, similar isotope fractionation would be expected. However, it should be noted that the ε_{Cl} values of γ-HCH for all the cultures were almost two-fold higher than that of α-HCH. In this study, we did not find evidence for distinguishing concerted vs. stepwise reductive dichloroelimination during γ- and α-HCH transformation. The AKIE calculation was carried out with

multiple scenarios to explore if different extents of intramolecular isotopic competition of the isomers (α- vs. γ-HCH) and conformers ((+)-α vs. (-)-α-HCH) could be the origin of the difference in observed chlorine isotope enrichment factors. The calculation of the $AKIE_{Cl}$ of γ-HCH in all the scenarios (SI Table S2), which take into account the intramolecular competition, are still consistently higher than those of α-HCH. Hence, other processes that are not taken into account in AKIE calculations, such as secondary chlorine isotope effects, could contribute to the differences between the chlorine isotope effects of γ- and α-HCH. The potential occurrence of secondary chlorine isotope effects was demonstrated during the dechlorination of several organic compounds, e.g., chlorinated ethanes and ethanes.[64, 65] During reductive dichloroelimination of γ- and α-HCH, the chlorine atoms with trans-diaxial conformation were involved in the first irreversible bond cleavage, and the orientations of the chlorine atoms in non-reactive position were different for γ- and α-HCH, respectively. The chlorine atoms in non-reactive position that might stabilize the transition stage could be the source of secondary chlorine isotope effects. Thus, we speculate that the different orientation of chlorine atoms in the adjacent non-reactive position led to different intensity of secondary chlorine isotope effect in α- and γ-HCH transformation.

Almost identical ε values (C or Cl) were obtained during the transformation of HCH isomer (α- or γ-HCH) by strain 195 and BTF08 with different sets of putative reductive dehalogenase genes.[28, 54, 56, 76] These ε values were similar compared with the values obtained in the enrichment culture without detectable *Dehalococcoides*. These results indicate that the intrinsic C and Cl isotope effect observed during the reductive dichloroelimination by different cultures, probably containing different dehalogenases, were quite similar. This is in agreement with the previous study that similar carbon and chlorine isotope effects were observed during the reductive dichloroelimination of 1,2-dichloroethane (1,2-DCA) by strain 195 and BTF08.[48] Thus, dehalogenation of HCH by different organisms yield similar fractionation factors with low variability, so dual-element CSIA can be used robustly quantify degradation *in situ*.

Dual-element isotope pattern for differentiating reaction mechanisms

The correlations of $\Delta\delta^{37}Cl$ and $\Delta\delta^{13}C$, giving the dual-element slopes ($\Lambda \approx \varepsilon_C/\varepsilon_{Cl}$), show a slightly variable range with 1.1 ± 0.3 to 1.2 ± 0.1 for γ-HCH and 1.7 ± 0.2 to 2.0 ± 0.3 for α-HCH (Table 1) indicating that the reaction mechanisms in the transformation by the three cultures were similar for γ-HCH, as well as for α-HCH. The Λ values of α-and γ-HCH were expected to be similar during the reductive dichloroelimination. However, our experimental results showed that the Λ values of γ-HCH were consistently lower than those of α-HCH. In the present study, consistent similar ε_C, or ε_{Cl} values of α-HCH were obtained in the transformation by the three cultures, as well as for γ-HCH, and consistent similar Λ values were obtained in the transformation of α- or γ-HCH by the three cultures. These results indicate that the C-Cl bond cleavage was the rate-limiting step. Thus, the different Λ values between α- and γ-HCH were more likely the results of the intramolecular competition and different intensity of secondary isotope effect between different isomers (discussion in the last section). The potential influence of other factors that we cannot exclude, like conformational mobility, are discussed in SI Section 6.

A recent study reported carbon and chlorine isotope fractionation during the dehydrochlorination of γ-HCH in aqueous media, catalyzed by the LinA enzyme.[53] The ε_{Cl} and ε_C were -2.0 ± 0.2 ‰ and -7.0 ± 0.5 ‰ for non-enzymatic dehydrochlorination of γ-HCH, and the values for the reaction catalyzed by LinA enzyme were between -1.8 ± 0.4 ‰ and -5.3 ± 0.8 ‰ (Table 1).[53] The Λ values ($\Lambda = \varepsilon_C/\varepsilon_{Cl}$) were 3.5 ± 0.4 and 2.9 ± 0.8 for chemical hydrolysis and enzyme catalysis, respectively. The Λ values during dehydrochlorination of γ-HCH by chemical and enzyme catalysis were similar and clearly different from the values obtained in reductive dichloroelimination (1.1 ± 0.3 to 1.2 ± 0.1). Therefore, different Λ values for γ-HCH reflect the different reaction mechanisms. Thus, dual-element isotope patterns potentially can be used for identifying different reaction mechanisms in field studies. In order to characterize the (bio)chemical reaction mechanisms in more detail, transformation studies of HCHs, particularly with

enzymes or chemical reaction catalyzing reductive dichloroelimination, are necessary. Additionally, studies using quantum chemical modelling may provide complement experimental studies on the transition state and model the isotope effect of the reactions.[34, 53, 77]

Application of CSIA to assess *in situ* natural attenuations of HCHs

Our study reports the ε_{Cl} and Λ values in addition to ε_C for the anaerobic transformation of α- and γ-HCH. These data can potentially be used for quantifying *in situ* anaerobic transformation of HCHs and characterizing the reaction mechanisms. The carbon isotope enrichment factors of HCH isomer (α- or γ-HCH) obtained from different anaerobic cultures (with metabolic or co-metabolic processes) are very similar (table 1), which indicates that the selection of an enrichment factor to calculate the extent of HCH transformation in surface water or groundwater based on CSIA would be relatively straight forward. Its application, though, still needs to be confirmed in model studies and at contaminated field sites. The ε_C in the anaerobic transformation of α- and γ-HCH showed a significant difference from the ε_C in aerobic degradation (Table 1). In natural attenuation, single element isotope analysis is limited for evaluating different reaction mechanisms. In order to distinguish aerobic and anaerobic transformation pathways of HCHs in the environment, dual-element CSIA thus can potentially be applied. However, chlorine isotope fractionation data for HCH aerobic degradation is still required, and field site data is needed for validation.

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Supporting Information Available

Details information of strain cultivation, Illumina sequencing, and the community of the enrichment culture and summary of observed data are available.

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692 **Table 1. Carbon and chlorine isotope enrichment factors (ε_c) of γ-HCH and α-HCH enantiomers for aerobic and anaerobic biotransformation**
693 **and abiotic transformation.**

	γ-HCH			Bulk α-HCH			(+)α-HCH	(-)α-HCH
Anaerobic	ε_c (‰)	ε_{cl} (‰)	Λ	ε_c (‰)	ε_{cl} (‰)	Λ	ε_c (‰)	ε_c (‰)
D.mccartyi strain 195	-4.4±0.6 -5.5±0.8[a]	-3.3±0.4	1.2±0.1	-3.0±0.3	-1.8±0.2	1.7±0.2	-3.1±0.3	-2.9±0.4
D.mccartyi strain BTF08	-4.0±0.5	-3.3±0.3	1.1±0.3	-2.4±0.2	-1.4±0.3	1.8±0.3	-2.3±0.2	-2.4±0.4
Enrichment culture	-4.0±0.5 -3.3±0.5[a]	-2.9±0.4	1.1±0.2	-3.0±0.4	-1.4±0.3	2.0±0.3	-2.9±0.3	-3.0±0.4
Clostridium pasterianum	-4.1±0.6[a]			-3.7±0.8[b]				
Desulfovibrio gigas	-3.9±0.6[c]							
Desulfococcus mulivorans	-3.4±0.5[c]							
Anaerobic digestion system	-3.2±0.2[d]			-3.8±0.3[d]				
Aerobic								
S. indicum strain B90A	-1.5±0.1[e]			-1.6±0.3[e]			-2.4±0.8[e]	-1.0±0.6[e]
S. japonicum strain UT26	-1.7±0.2[e]			-1.0±0.2[e]			-2.5±0.6[e]	-0.7±0.2[e]
LinA enzyme	-5.3±0.8[f]	-1.8±0.4[f]	2.9±0.8					
Abiotic								
alkaline hydrolysis	-7.0±0.5[f]	-2.0±0.2[f]	3.5±0.4					
Reduction by Fe^0				-4.9±0.1[g]			-5.1±0.4[g]	-4.8±0.5[g]

694 [a] Bashir et al.[29]; [b] Badea et al.[39]; [c] Badea et al.[41]; [d] Lian et al.[38]; [e] Bashir et al.[40]; [f] Kannath et al.[53]; [g] Zhang et al.[42]

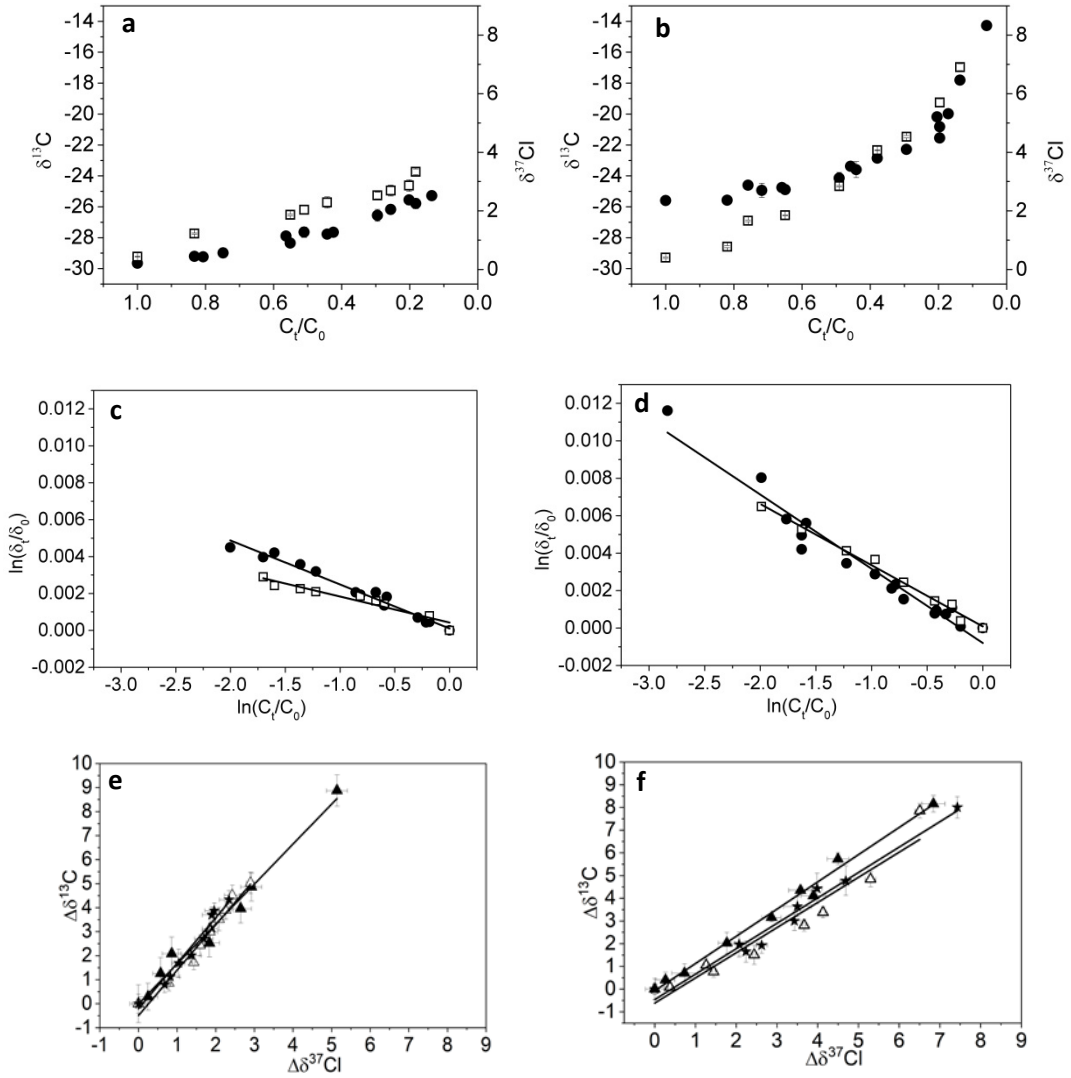
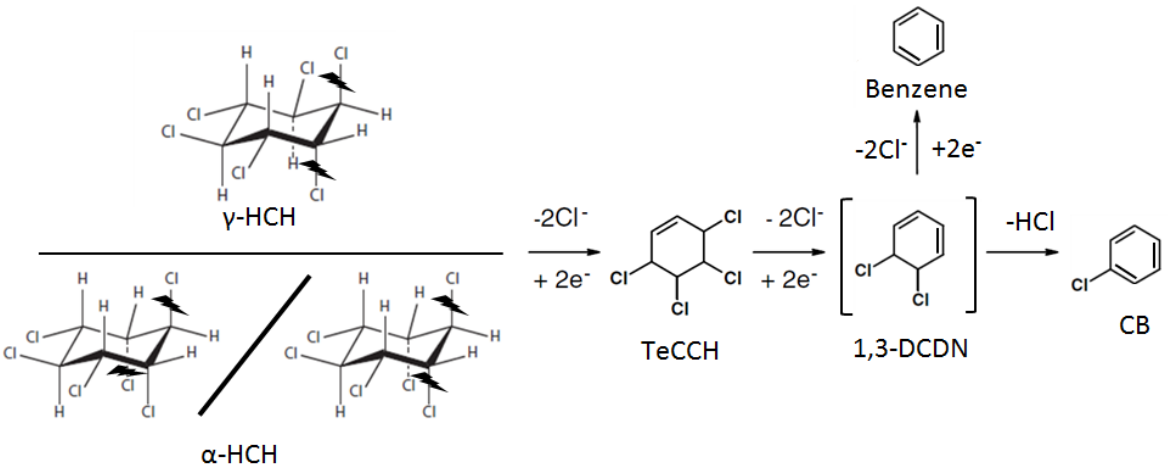


Figure 1. The carbon (filled circles) and chlorine (open squares) isotope enrichment of α-HCH (a) and γ-HCH (b) by *D.mccartyi* strain BTF08; Linearized Rayleigh equation plots showing the carbon (closed circles) and chlorine (open squares) isotope fractionation of *D.mccartyi* strain BTF08 for the biotransformation of α-HCH (c) and γ-HCH (d). And dual-isotope (Cl and C) linear plotting for calculating of Λ values for the biotransformation of α-HCH (e) and γ-HCH (f) by *D.mccartyi* strain 195(closed triangles), *D.mccartyi* strain BTF08 (open triangles) and the enrichment culture (closed stars). The error bars show the standard deviation for triplicate measurements. For some data points, the error bars are smaller than the symbols.



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711 Figure 2. Anaerobic transformation pathway for both α- and γ-HCH proposed in this study. The lightning
712 bolts indicate the possible bond cleavage for α- and γ-HCH (the structure of the four main isomers is
713 shown in SI Table S1). The putative intermediates are in brackets.

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