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Dual C-Cl isotope analysis for characterizing the anaerobic transformation of α, β, γ, and δ-hexachlorocyclohexane in contaminated aquifers

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Abstract:

Hexachlorocyclohexanes (HCHs) are widespread and persistent environmental pollutants, which cause heavy contamination in soil, sediment and groundwater. An anaerobic consortium, which was enriched on β-HCH using a soil sample from a contaminated area of a former pesticide factory, was capable to transform α, β, γ, and δ-HCH via tetrachlorocyclohexene isomers stoichiometrically to benzene and chlorobenzene. The carbon and chlorine isotope enrichment factors (ε_C and ε_{Cl}) of the dehalogenation of the four isomers ranged from -1.9±0.3 to -6.4±0.7 ‰ and from -1.6±0.2 to -3.2±0.6 ‰, respectively, and the correlation of δ^{37}Cl and δ^{13}C (Λ values) of the four isomers ranged from 1.1±0.1 to 2.4±0.2. The evaluation of Λ, $AKIE_C$ and $AKIE_{Cl}$ values may lead to the hypothesis that two chlorine atoms of α- and γ-HCH were eliminated from axial positions, the same as for β-HCH after flipping the carbon ring. The dichloroelimination of δ-HCH results in distinct AKIE and Λ values as one chlorine atom is in axial whereas the other chlorine atom is in the equatorial position. Significant chlorine and carbon isotope fractionation of HCH isomers were observed in the samples from a contaminated aquifer (Bitterfeld, Germany). The ^{37}Cl/^{35}Cl and ^{13}C/^{12}C isotope fractionation patterns of HCH isomers from laboratory experiments were used diagnostically in a model to characterize microbial dichloroelimination in the field study. The comparison of isotope fractionation patterns indicates that the transformation of HCH isomers at the field was mainly governed by microbial dichloroelimination transformation.

Keywords: reductive dichloroelimination, β-HCH, chlorine isotope fractionation, CSIA, natural attenuation

1. Introduction

Technical hexachlorocyclohexane (HCH), the mixture of different HCH isomers, was widely used as a pesticide since the 1940s (Bidlan et al. 2004; Lal et al. 2006; Sang et al. 1999). Later, γ-HCH, the isomer possessing insecticidal toxicity, was purified from technical HCH and merchandised as Lindane (Nagata et al. 2007; Slade 1945; Vijgen et al. 2011). The production and application of technical HCH and Lindane resulted in two main contamination types: (1) massive point-source contaminations from open-air stockpiles and uncontained dumping of chemical waste during production and (2) diffuse pollution from the application of the pesticide. According to estimates, there are still 1.7–4.8 million tons of HCH present in the environment worldwide (Vijgen 2006; Vijgen et al. 2019). Once released into the environment, HCH can enter and accumulate in food chains and thus have adverse effects on human health by causing respiratory dysfunction and carcinogenesis, for example (Caicedo et al. 2011; Pesce et al. 2008; Salam and Das 2012; Wu et al. 2019a). Therefore, there is an urgent need to evaluate HCH contaminations in the environment.

Microbial degradation of HCH is one of the major mechanisms leading to a sustainable restoration of contaminated sites. To date, more than 30 aerobic strains were successfully isolated, showing the ability to degrade HCH (Lal et al. 2010). Additionally, several studies demonstrated that HCH could also be transformed under anoxic conditions using complex, undefined conditions, e.g. submerged soil (MacRae et al. 1967), soil slurry (Bachmann et al. 1988), sewage sludge (Buser and Mueller 1995), methanogenic condition (Middeldorp et al. 1996), as well as anaerobic digestion (AD) systems (Lian et al. 2018). However, only a few studies reported microorganisms for the dehalogenation of single HCH isomers and described α- and γ-HCH degradation because β- and δ-HCH were found to be relatively more stable (Lal et al. 2010). In 2005, van Doesburg et al. reported a co-culture (*Dehalobacter* sp. with *Sedimentibacter* sp.) for the transformation of β-

HCH (van Doesburg et al. 2005). Recently, *Dehalococcoides mccartyi* strains were shown to be capable of the reductive dehalogenation of γ-HCH, α-HCH and δ-HCH, while the transformation of β-HCH was not significant (Bashir et al. 2018; Kaufhold et al. 2013). Thus, reports on anaerobic cultures able to transform HCH under anoxic conditions are limited, especially for β- and δ-HCH transformation. Benzene and chlorobenzene (CB) were the final products in HCH anaerobic transformation in the above-mentioned studies. Thus, the detected benzene and CB in HCH contaminated field sites indicate anaerobic transformation as important processes in natural attenuation (Feidieker et al. 1995; Heidrich et al. 2004; Wycisk et al. 2003).

The evaluation of transformation pathways at contaminated field sites and the quantification of biodegradation during remediation efforts require assessment concepts beyond HCH concentration analysis alone (Langenhoff et al. 2013; Phillips et al. 2006; Rubinos et al. 2007). Multi-element compound-specific isotope analysis (ME-CSIA) has been proposed for qualitative and quantitative evaluation of halogenated organic contaminants transformation in the environment (Nijenhuis and Richnow 2016). Many studies presented ME-CSIA for the characterization of different halogenated contaminants in groundwater, e.g., chlorinated ethenes and polychlorinated phenols (Aeppli et al. 2013; Badin et al. 2014; Palau et al. 2017; Wiegert et al. 2012; Wiegert et al. 2013). For the evaluation of HCH transformation, stable carbon isotope analysis was applied in aquifers (Bashir et al. 2015; Liu et al. 2017). Recently, ME-CSIA analysis of HCH was applied for elucidating the transformation reactions of HCHs in food webs (Wu et al. 2019b). To apply ME-CSIA for evaluation of biotransformation at field sites, isotope enrichment factors of different elements (ε_E) are needed, which can be obtained from laboratory reference experiments with pure or enrichment cultures. Previous studies reported the carbon isotope analysis of α- and γ-HCH in anaerobic metabolic processes, e.g. by *Dehalococcoides mccartyi*, and co-metabolic processes, e.g.

by *Desulfococcus multivorans* (Badea et al. 2011; Badea et al. 2009; Bashir et al. 2018) and in methanogenic bioreactors (Lian et al. 2018). Recently, we reported the dual-isotope (C-Cl) analysis for characterizing the reductive dechlorination of α- and γ-HCH by two *D. mccartyi* strains and an enrichment culture (Liu et al. 2019 Submitted). Thus far, enrichment factors for evaluating the anaerobic degradation of β- and δ-HCH in field studies have not been reported in the literature yet. This poses a major bottleneck for the application of CSIA to evaluate the transformation of different HCH isomers in the environment.

In this study, an anaerobic culture enriched in β-HCH was investigated for the transformation of four HCH isomers (α, β, γ, and δ). Dual-isotope (C-Cl) fractionation was examined, and carbon and chlorine isotope enrichment factors were determined for the four HCH isomers. Subsequently, the reaction mechanisms of the four main HCH isomers were characterized and compared by evaluation of the dual-element slope (Λ) and apparent kinetic stable isotope effects (AKIE). In addition, the carbon and chlorine isotope fractionation were compared to previous enrichment and pure culture studies (Liu et al. 2019 Submitted) to analyze the variability of isotope fractionation of dehaloelimination reactions. Finally, chlorine isotope compositions of HCH were analyzed for groundwater samples from a previous study (Liu et al. 2017), and dual-element isotope analysis (Λ) was employed to elucidate the reaction mechanisms by taking advantage of multi-element isotope fractionation.

2. Materials and methods

2.1. Chemicals

α-HCH, β-HCH, γ-HCH, δ-HCH, benzene and CB were purchased from Sigma Aldrich (Germany). n-Hexane (analytical purity > 99 %) was purchased from Carl Roth (Germany).

2.2. Soil sample

A soil sample from the contaminated area of the former Wuhan pesticide factory (Wuhan, China) was collected at 20 to 30 cm depth, transported in plastic bags and stored at 4 °C until further processing. In this factory, HCHs, DDTs, and chlorobenzenes were produced from 1959 to 1987.

2.3. Groundwater samples

The groundwater samples from a large HCH contamination located in Bitterfeld, Germany, were selected for chlorine isotope analysis (Liu et al. 2017).

2.4. Cultivation experiment

2.4.1. Pre-cultivation experiments

The pre-cultivation experiments were divided into two major steps. (1) A culture able to transform β-HCH was enriched. (2) The β-HCH-degrading culture was used to transform α-, γ-, and δ-HCH isomers. Minimal medium (MM) was prepared as described previously (Kaufhold et al. 2013). Five serum bottles (240 mL) were prepared with 100 mL MM and flushed with 70 %N_2 / 30 %CO_2 for 15 min to remove oxygen. Then, the bottles were closed air-tight and autoclaved. After amendment (supporting information: SI 1), bottles were re-opened in an anoxic glove box, and 1 g of soil was added as inoculum to each bottle. Thereafter, the bottles were closed with Teflon-coated stoppers and sealed gastight. Hydrogen (0.5 bar overpressure) and acetate (1 mL, 1.26 g / 50 mL) were used as electron donor and carbon source, respectively. Three bottles were spiked with β-HCH (300 μL, 0.1 M in acetone) serving as the electron acceptor. Two bottles without β-HCH were used as controls to monitor any background of benzene and CB. All bottles were incubated at 30°C. When the sum of benzene and CB concentration reached a level of 300 μM, 1 mL of the culture-suspension was transferred to fresh medium spiked with 300 μL β-HCH. After the sixth transfer, the enriched β-HCH-degrading culture was tested for the ability to degrade other isomers (α, γ, and δ-HCH) using the same concentration as used for β-HCH.

2.4.2. Fractionation experiments

After the transformation of the four HCH isomers by the enrichment culture was successfully demonstrated, four sets of fractionation experiments with single HCH isomers were conducted with the initial concentration of 30 μM for α, γ, δ-HCH, and 2.5 μM for β-HCH. One experimental set-up consisted of 18 bottles, including two abiotic controls without inoculation. The progress of transformation was analyzed by the formation of benzene and CB. At different extents of HCH transformation, 5mL of a saturated sodium sulfate solution acidified with sulfuric acid (pH=1) was added to stop the microbial activity. HCHs and metabolites were extracted by adding 2 mL n-hexane including an internal standard (15 μM, α-HCH served as an internal standard for γ- and δ-HCH experiments; δ-HCH was used as the internal standard for α- and β-HCH experiments). For extraction, the bottles were shaken at 120 rpm and 10 ℃ for at least 4 h.

2.5. Chemical and Isotopic Analysis

The concentration of benzene and CB were measured by gas chromatography. An Agilent 6890 series gas chromatograph (GC, Agilent Technologies, Palo Alto, USA) equipped with a flame ionization detector (FID) was used. Sample separation was done with an Rtx-VMS column (30 m x 0.25 mm ID x 0.25 µm FD, Restek, Bad Homburg, Germany), as described elsewhere (Kaufhold et al. 2013). Prior to the headspace analysis, 1 mL of the sample (culture) was mixed with 0.5 mL saturated Na_2SO_4 solution (pH=1, H_2SO_4) in a 10 mL vial with Teflon coated stopper and crimp-sealed.

HCH concentrations were analyzed by GC-FID (7820A, Agilent Technologies, Palo Alto, USA) with an HP-5 capillary column (30 m x 0.32 mm ID x 0.25 µm FD; Agilent Technologies, Palo Alto, USA). The initial oven temperature was 35 °C and held for 5 min, increased by a rate of 8 °C/min to 180 °C, then at 2 °C/min to 195 °C, and finally increased to 220 °C at 8 °C/min, and held for 2 min. The carrier gas was helium with the flow of 2.0 mL/min. Samples (1µL) were injected in splitless mode by an autosampler. The identification of metabolites was conducted by GC (7890A, Agilent Technologies, Palo Alto, USA) coupled to a Mass Spectrometry (MS, 5975C, Agilent Technologies, Palo Alto, USA) with the same HP-5 capillary column used for concentration analysis. The temperature program started at 60 °C and held for 3min, increased with a rate of 3 °C/min to 175 °C, then at 1 °C/min to 200 °C and held for 5 min, and finally increased at 15 °C/min to 320 °C.

Carbon isotope analysis ($\delta^{13}C$) of HCH isomers was done with a GC (6890, Agilent Technologies, Palo Alto, USA) equipped with a GC IsoLink coupled with a Conflo IV interface (Thermo Fisher Scientific, Bremen, Germany) to an isotope ratio mass spectrometer (IRMS, MAT253, Thermo Fisher Scientific, Bremen, Germany). A ZB1 column (60 m x 0.32 mm ID x 1 µM FD; Phenomenex, Torrance, USA) was used for sample separation employing the temperature program as reported before (Badea et al. 2009). The carbon isotope composition of α-HCH enantiomers was determined using the same analytical set-up but a γ-DEX^{TM} 120 chiral column (30 m x 0.25 mm ID x 0.25 µM FD; Supelco, Bellefonte, PA, USA) for separation (Badea et al. 2011). All samples were measured in at least three technical replicates with an uncertainty typically smaller than 0.5 ‰.

The chlorine isotope composition (δ^{37}Cl) was measured by GC coupled with a multiple-collector inductively coupled plasma mass spectrometer (GC-MC-ICPMS), as reported previously (Horst et al. 2017; Renpenning et al. 2018; Wu et al. 2019b). A ZB-1 column was used with the same temperature program as described for the GC-FID analysis of HCH concentrations. All samples were measured three times, and the uncertainty of analysis was typically smaller than 0.3 ‰.

2.6 Stable isotope analysis

Isotope enrichment factors, ε_E, were derived with the simplified Rayleigh equation:

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

Where δ_t and δ_0 are the carbon (δ^{13}C) or chlorine (δ^{37}Cl) isotope compositions at a given point in time (t) and at the beginning of the reaction (0), respectively. E represents the element (C or Cl), C_t/C_0 is the residual fraction of the substrate. ε_E is the enrichment factor which can be obtained from the slope of the linear regression of $\ln(C_t/C_0)$ vs $\ln[(\delta_t+1)/(\delta_0+1)]$. The ε_E is reported with a 95 % confidence interval (CI).

The δ^{13}C and δ^{37}Cl values from fractionation experiments were correlated to obtain the Λ-value used for the characterization of the C-Cl bond cleavage mechanism:

$$\Lambda = (\delta^{13}\mathrm{C}_t - \delta^{13}\mathrm{C}_0)/(\delta^{37}\mathrm{Cl}_t - \delta^{37}\mathrm{Cl}_0) \qquad (2)$$

The δ values describe the isotopic composition for carbon and chlorine at a given point in time (t) and at the beginning of the reaction (0).

The calculation of apparent kinetic isotope effects (AKIE) was done by Eq.(3) (Elsner et al. 2005):

$$AKIE_e \approx \frac{1}{1+\frac{n*z}{x}*\varepsilon} \qquad (3)$$

Where 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 resulting in intramolecular competition.

3. Results and discussion

3.1. Transformation of HCH isomers.

An enrichment culture was derived from contaminated soil by amending it with β-HCH. After six transfers with β-HCH as the electron acceptor, the enrichment culture was analyzed for the dechlorination of α-, γ-, and δ-HCH isomers. The complete transformation of α, β, γ and δ-HCH (300 μM for each isomer), indicated by the sum of benzene and chlorobenzene produced, took about 120, 75, 150 and 210 days, respectively (SI Fig. S1). The different transformation times for the individual HCH isomers indicated that the transformation processes were isomer specific. Isomer specific transformation rates were also found previously, as α- and γ-HCH were transformed relatively faster than β- and δ-HCH (Bashir et al. 2018; Brahmaprakash et al. 1985; Buser and Mueller 1995; Jagnow et al. 1977; MacRae et al. 1967; Middeldorp et al. 1996; Middeldorp et al. 2005; Quintero et al. 2005; van Doesburg et al. 2005). In the presented study, the transformation of β-HCH did not show any obvious lag-phase compared with the other three isomers (SI Fig. S1). This could be the result of the pre-cultivation (six initial transfers) which was solely performed with β-HCH. Thus, corresponding dehalogenases were already activated, similar as observed in the dehalogenation of chlorinated ethenes by an enrichment culture (Liang et al. 2015). The different transformation rates of the isomers may indicate the presence of different dehalogenases with different activity, as suggested in a previous report on the transformation of chlorinated ethenes (Liang et al. 2015).

Tetrachlorocyclohexene (TeCCH) isomers, each having specific retention times, were identified by GC-MS as the most likely first intermediate in the transformation of individual HCH isomers. Due to lack of standards, TeCCH isomers could not be characterized further. The formation of TeCCH provides evidence that the initial degradation step was a dichloroelimination reaction, similar to a previous observation for an enrichment culture and two *Dehalococcoides mccartyi* strains (Liu et al. 2019 Submitted). The anaerobic transformation of α-HCH enantiomers was not accompanied by obvious enantioselectivity (Fig. S2), which is in agreement with previous studies (Badea et al. 2011; Liu et al. 2019 Submitted).

3.2. C and Cl isotope fractionation of the HCH isomers

3.2.1. Isotope fractionation

A significant carbon isotope enrichment was observed for all HCH isomers. The transformation resulted in identical ε_C of -4.2±0.5 ‰ and -4.1±0.4 ‰ for (-)α-HCH and (+)α-H-CH, respectively (Fig. 1 and Fig. S3) and no enantiomer specific transformation was observed. The ε_C for γ-and bulk α-HCH were -3.6±0.4 ‰ and -4.2±0.4 ‰, a similar range as reported in previous studies for the anaerobic transformation of the respective HCH isomer (Table 1, α-HCH: from -2.4±0.4 ‰ to -3.7±0.8 ‰; γ-HCH: from -3.2±0.2 ‰ to -5.5±0.8 ‰) (Badea et al. 2011; Badea et al. 2009; Bashir et al. 2018; Lian et al. 2018; Liu et al. 2019 Submitted). The carbon isotope enrichment of β-HCH and δ-HCH resulted in ε_C of -1.9±0.3 ‰ and -6.4±0.7 ‰, respectively. Thus far, no reports are available for the isotope enrichment of β- and δ-HCH. In comparison to the ε_C of α- and γ-HCH, the isotope enrichment of β-HCH was weaker whereas for δ-HCH the carbon isotope enrichment was stronger.

Chlorine isotope enrichment was observed for all HCH isomers during the transformation by the enrichment culture (Fig. 1). The derived ε_{Cl} values were -2.1±0.3 ‰ for α-HCH, -1.6±0.2 ‰ for β-HCH, -3.2±0.6 ‰ for γ -HCH, and -2.3±0.3 ‰ for δ-HCH. Consequently, ε_{Cl} of α- and γ-HCH were also in a similar range compared to the previous study (Table 1, α-HCH: from -1.4±0.3 ‰ to -1.8±0.2‰; γ-HCH: from -2.9±0.4 ‰ to -3.3±0.4‰) (Liu et al. 2019 Submitted). As compared to carbon isotope fractionation, the ε_{Cl} of δ-HCH was similar to the value of α- and γ-HCH, while the value of β-HCH was relatively lower.

3.2.2. AKIE

The AKIE calculations of α- and γ-HCH are summarized in Table 2. The scenarios were extensively discussed elsewhere (Liu et al. 2019 Submitted). Briefly, for β-HCH, all chlorine atoms of each possible conformation are orientated in the same direction (equatorial or axial position after flipping) and are considered to have an equal reactivity. For the conformers of δ-HCH, only one chlorine atom is in a different orientation compared to the other five chlorine atoms. Therefore, AKIE calculations of δ-HCH mainly considered: (i) the chlorine atom with a different orientation is not involved in the reaction, which means the two vicinal reacting chlorine atoms are belonging to the other five which have the same orientation (Fig. 2, bonds with red color); (ii) the chlorine atom with a different orientation is involved in the reaction, and therefore the reacting vicinal chlorine atoms possess a different orientation (Fig. 2, bonds with blue color). Both *stepwise* and *concerted* bond cleavage was considered for all isomers and all possible scenarios with n=6. In the case of a *stepwise* bond cleavage, the parameters for AKIE calculation were x=z=6 for β-HCH, x=z=5 (scenario a) and x=z=1 (scenario b) for δ-HCH. Regarding a concerted bond cleavage, the parameters were x=z=6 for β-HCH, x=5/z=4 (scenario a) and x=3/z=2 (scenario b) for δ-HCH.

The calculated AKIE values of this study and the previous studies for HCHs transformation are summarized in Table 2.

The AKIE values for α- and γ-HCH were compared with the values reported in a previous study (Liu et al. 2019 Submitted). Both $AKIE_C$ and $AKIE_{Cl}$ are similar to the previously reported values, which indicates a similar bond cleavage (*concerted* dichloroelimination). Since there are no reports of AKIE values for the anaerobic transformation of β- and δ-HCH available, the AKIE-values were compared with reference values reported for the abiotic *stepwise* dichloroelimination of chlorinated ethane by Cr(II), Fe(0), and Cu-Fe mixture (Table 2) (Lollar et al. 2010; Rosell et al. 2019). The $AKIE^{C}_{stepwise}$ of δ-HCH (1.040±0.005) is higher than the reference values, which makes a *stepwise* bond cleavage unlikely and suggests a *concerted* bond cleavage. For β-HCH, it is not possible to exclude one specific bond cleavage as both $AKIE_C$ and $AKIE_{Cl}$ are in the range of the reference values from *stepwise* dichloroelimination.

3.3. Carbon-chlorine bond cleavage of HCH isomers

The Λ-values for α-, β-, γ- and δ-HCH were determined with 1.9±0.1, 1.3±0.1, 1.1±0.1, and 2.4±0.2, respectively (Table 1, Fig. 1). The Λ-values of α- and γ-HCH are in agreement with a previous study (α-HCH: from 1.7±0.2 to 2.0±0.3, γ-HCH: from 1.1±0.3 to 1.2±0.1) (Liu et al. 2019 Submitted). Consequently, it can be assumed that the reductive dichloroelimination of α- and γ-HCH observed in this study were similar to the transformation of α- and γ-HCH by *D.mccartyi* strains (Liu et al. 2019 Submitted). The Λ-value for β-HCH is identical with γ-HCH indicating an elimination of the chlorine atoms in axial position, which is only possible for the β-HCH conformer having all chlorine atoms in axial position. Additionally, this result is in line with a recent report of Qiao and colleagues (Qiao et al. 2019) who proposed that the chlorine atoms of β-HCH were in axial positions during dichloroelimination (by flipping the carbon ring, Fig. 2). However, we cannot exclude other scenarios. For instance, dichloroelimination of the β-HCH conformer with all chlorine atoms in the equatorial position shows the same isotope effect as γ-HCH transformation. The Λ-value for δ-HCH was different from other isomers, which is mainly caused by the relatively higher carbon isotope enrichment. This also indicates that the bond cleavage of δ-HCH is different from other isomers.

Similar as the concept of Λ ($\Lambda \approx \varepsilon_C/\varepsilon_{Cl}$) but cancel out the effect of intramolecular competition, $AKIE_C/AKIE_{Cl}$ was proposed as a probe to characterize the reaction mechanisms (Ji et al. 2017). For the transformation of α- and γ-HCH, similar $AKIE_C/AKIE_{Cl}$ values (α-HCH: 1.006±0.002, γ-HCH: 1.002±0.003, Table 2) were observed which additionally are in the same range as the $AKIE_C/AKIE_{Cl}$ values for the dichloroelimination of α- and γ-HCH by *D. mccartyi* strains (from 1.003±0.002 to 1.005±0.002) (Liu et al. 2019 Submitted). This result supports a similar reaction mechanism for the transformation of α- and γ-HCH which most likely follows an elimination of two vicinal chlorines in axial position as reported previously (Liu et al. 2019 Submitted; Qiao et al. 2019). The $AKIE_C/AKIE_{Cl}$ of β-HCH (1.002±0.002) is identical with the value for γ-HCH (1.002±0.003), which also supports the results derived from Λ-value evaluation that dichloroelimination of β- and γ-HCH were similar. For δ-HCH, two cases were considered which are identical to those considered for the calculation of AKIEs. In case (i), the dichloroelimination mechanism of δ-HCH is similar to the mechanism of β-HCH (the vicinal reacting chlorines have the same orientation). Accordingly, it is expected the $AKIE_C/AKIE_{Cl}$ values should be similar. However, this is not the case, as $AKIE_C/AKIE_{Cl}$ were 1.017±0.003 for δ-HCH and 1.002±0.002 for β-HCH. Consequently, it is reasonable to assume that the dichloroelimination of δ-HCH follows the reaction mechanism proposed in case (ii). Here, the reacting vicinal chlorines possess a different orientation (Fig. 2 in the blue color), one chlorine atom is in axial position whereas the other chlorine atom is in equatorial position. Thus, a different mode of bond cleavage is proposed for δ-HCH compared to the dichloroelimination of α-, β-and γ-HCH in which both chlorine atoms are in axial or equatorial position. Our findings suggest that the initial reaction mechanism of δ-HCH dichloroelimination is different from a mechanism reported recently, which proposed that the two chlorine atoms are eliminated in the axial position (Qiao et al. 2019). Additionally, also the ratio of the formed products (benzene and MCB) varied significantly from the ratio reported by Qiao and colleagues (> 0.3 in this study, 0.06±0.02 in the study of Qiao et al.) (Qiao et al. 2019), which indicates different reaction mechanisms.

The carbon and chlorine isotope fractionation during the transformation of HCH by different cultures, even from geographically distinct locations (e.g. Bitterfeld and China), show remarkably similar fractionation patterns (Fig.3). The Λ-values of enrichment and pure cultures show a very similar correlation of chlorine and carbon isotope fractionation indicating similar dehalogenation mechanisms and providing support for the opinion that the fractionation factors can be used for characterizing anaerobic transformation of HCH at field sites.

3.4. Evaluation of HCH biotransformation in groundwater samples

Groundwater samples from a previous study (Liu et al. 2017), in which the four HCH isomers have been detected, were analyzed for Cl isotopes (SI Table S1). ME-CSIA was applied to evaluate the *in situ* transformation mechanisms of α-, β-, γ -, and δ-HCH. The concentrations of the four HCH isomers varied from 1.86 – 242 µg/L for α-HCH, 0.13 – 44 µg/L for β-HCH, 0.02 – 236 µg/L for γ-HCH, and 0.44 – 264 µg/L for δ-HCH. In comparison to the source represented by muck samples from a dumpsite and highly contaminated areas, chlorine isotope enrichment was observed in all investigated samples (SI Table S1). Generally, the chlorine isotope enrichment for γ- and α-HCH

($\Delta\delta^{37}$Cl up to 3.8 ‰) was relatively higher than the enrichment for β-and δ-HCH ($\Delta\delta^{37}$Cl up to 1.9 ‰) (SI Table S2).

In order to characterize the *in situ* transformation pathways, the dual C-Cl isotope values obtained for the six field samples are compared to the isotope fractionation patterns predicted by the data from laboratory studies for HCH dichloroelimination (Fig. 3). For α-HCH, all samples fall in a narrow range indicating that the transformation process observed in the field follows a similar dichloroelimination process as observed by the investigated cultures (Fig. 3a). For γ-HCH, two samples (Q1 and T2) fall out of the correlation describing dichloroelimination of the investigated cultures (Fig. 3c). This may indicate the presence of a different process involved in the transformation at this field locations, e.g. aerobic degradation. These two samples (Q1 and T2) showed strong enantiomer fractionation of α-HCH, which is typical for aerobic degradation (Bashir et al. 2013; Liu et al. 2019). Enantiomer fractionation is usually not observed during anaerobic transformation (Liu et al. 2019 Submitted). Thus, aerobic degradation may have contributed to α-HCH at the locations Q1 and T2, e.g. in other parts of the aquifer and/or during transport. However, we cannot exclude the possibility of other anaerobic processes, as α-HCH enantiomer selectivity was also observed in a biogas reactor (Lian et al. 2019). For β- and δ-HCH (Fig. 3 b and d), the similar isotope enrichment patterns indicate similar microbial transformation processes in laboratory and field samples, i.e. dichloroelimination. For β-HCH, however, there are only two samples within the 95% prediction band that describe microbial dichloroelimination (Fig. 3b). In the case of δ-HCH, the dual isotope patterns of groundwater samples are similar to laboratory results, although Q1 and T2 were at the edge of the prediction band (Fig. 3d). However, further studies focusing on multiple isotope analysis including the investigation of anaerobic and aerobic microbial transformation processes and chemical reactions for HCH transformation are necessary for a detailed evaluation of the observed isotope effects in field samples.

In summary, our study supports that the transformation of HCH isomers at contaminated field sites can be characterized by ME-CSIA. Further field campaigns are needed to validate the application of ME-CSIA for identifying different transformation pathways.

4. Conclusion

Our study demonstrates that ME-CSIA can be applied to characterize the anaerobic dichloroelimination of the HCH isomers using the correlation of carbon and chlorine isotope fractionation data (Λ). The determined AKIE values are consistent with a *concerted* bond cleavage, which is in line with previous studies. The similarity of carbon and chlorine isotope enrichment factors compared to previous reports suggests that CSIA can be applied robustly for field studies. Our study gives a model for evaluating *in situ* transformation of α-, β-, γ- and δ-HCH by using ME-CSIA.

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Supporting Information Available

Detailed information of strain cultivation, methods for sample analyzing and summary of recorded data are available.

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Table 1. Isotope enrichment factors (ε_C and ε_{Cl}) and Λ-values determined for the anaerobic transformation of HCH isomers by the enrichment culture (this study) and various cultures reported previously.

	α-HCH			γ-HCH			β-HCH			δ-HCH		
Anaerobic	ε_C (‰)	ε_{Cl} (‰)	Λ	ε_c (‰)	ε_{Cl} (‰)	Λ	ε_c (‰)	ε_{Cl} (‰)	Λ	ε_c (‰)	ε_{Cl} (‰)	Λ
Enrichment culture (Wuhan)	**-4.2±0.4**	**-2.0±0.3**	**1.9±0.1**	**-3.6±0.4**	**-3.2±0.6**	**1.1±0.1**	**-1.9±0.3**	**-1.6±0.2**	**1.3±0.1**	**-6.4±0.7**	**-2.3±0.3**	**2.4±0.2**
Dehalococcoides mccartyi strain 195	-3.0±0.3[a]	-1.8±0.2[a]	1.7±0.2[a]	-4.4±0.6[a]	-3.3±0.4[a]	1.2±0.1[a]						
				-5.5±0.8[b]								
Dehalococcoides mccartyi strain BTF08	-2.4±0.2[a]	-1.4±0.3[a]	1.8±0.3[a]	-4.0±0.5[a]	-3.3±0.3[a]	1.1±0.3[a]						
Enrichment culture (Bitterfeld)	-3.0±0.4[a]	-1.4±0.3[a]	2.0±0.3[a]	-4.0±0.5[a]	-2.9±0.4[a]	1.1±0.2[a]						
				-3.3±0.5[b]								
Clostridium pasterianum	-3.7±0.8[c]			-4.1±0.6[b]								
Desulfovibrio gigas				-3.9±0.6[d]								
Desulfococcus mulivorans				-3.4±0.5[d]								
Anaerobic digestion system	-3.8±0.3[e]			-3.2±0.2[e]								

[a] Liu et al.(Liu et al. 2019 Submitted). [b] Bashir et al.(Bashir et al. 2018); [c] Badea et al.(Badea et al. 2011); [d] Badea et al.(Badea et al. 2009); [e] Lian et al.(Lian et al. 2018)

Table 2. AKIE values for the anaerobic transformation of α-, β-, γ-, and δ-HCH.

		Scenario (1) and Scenario (2)a			Scenario (2)b					
		n/x/z	$AKIE_C$	$AKIE_{Cl}$	n/x/z	$AKIE_C$	R.V.	$AKIE_{Cl}$	R.V.	$AKIE_C$ /$AKIE_{Cl}$
α-HCH		n*z/x=6	1.026±0.003	1.012±0.002	6/2/1	1.013±0.001	1.007±0.001 to 1.009±0.001*	1.006±0.001	1.006±0.001 to 1.008±0.001*	1.006±0.002
γ-HCH			1.022±0.003	1.020±0.004	6/3/2	1.015±0.002	1.016±0.002 to 1.018±0.003*	1.013±0.002	1.012±0.002 to 1.013±0.003*	1.002±0.003
β-HCH			1.012±0.002	1.010±0.001	6/6/6	1.012±0.002		1.010±0.001		1.002±0.002
δ-HCH	i		1.040±0.005	1.014±0.002	6/3/2	1.032±0.004	1.016±0.001 to 1.037±0.001$	1.011±0.001	1.013±0.002 to 1.021±0.002$	1.020±0.004
	ii		1.040±0.005	1.014±0.002	6/5/4	1.026±0.003		1.009±0.001		1.017±0.003

The AKIE values were calculated based on the following scenarios: (1) considering all the chlorine atoms are chemically equivalent, (2) a: *stepwise* bond cleavage or b: *concerted* bond cleavage. In scenario (2), for α- and γ-HCH only chlorine atoms in the axial position are consider to be involved in the transformation; for β-HCH, all the chloride atoms are equal; and for δ-HCH: i: One reacting chlorine atoms is in axial position, the other one is equatorial position; ii: The axial chlorine atom is not involved in the reaction, the two reacting vicinal chlorine atoms are in equatorial position. R.V.: Reference values; * reductive dichloroelimination of α-and γ-HCH by anaerobic cultures (Liu et al. 2019 Submitted); $ reductive dichloroelimination of chlorinated ethane by abiotic reactions with *stepwise* bond cleavage (Elsner et al. 2007; Hofstetter et al. 2007; Palau et al. 2014).

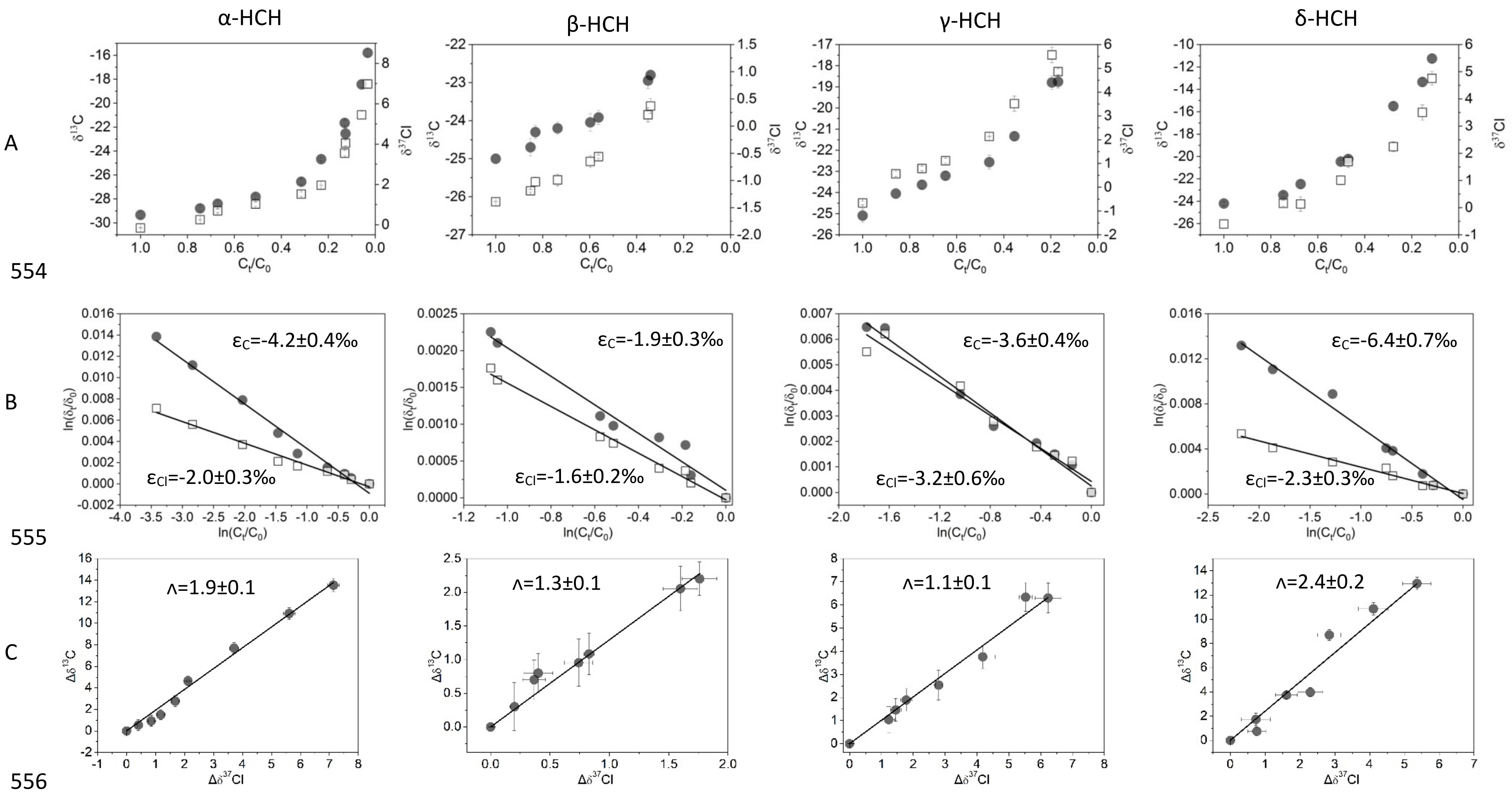
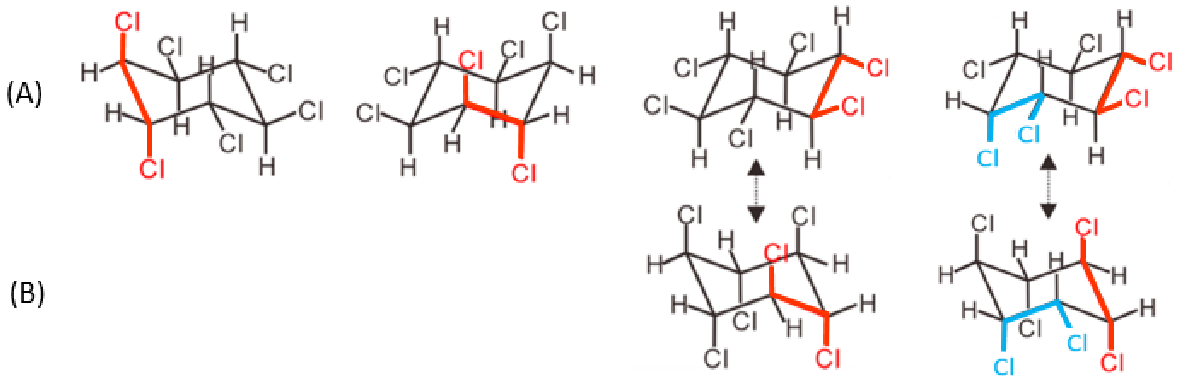


Fig. 1. Carbon (circles) and chlorine (squares) isotope enrichment of the four HCH isomers (panel A), Rayleigh plot (panel B), and dual isotope plot (panel C).



α-HCH γ-HCH β-HCH δ-HCH

Fig. 2. Stereochemistry of α-, β-, γ-, and δ-HCH. The axial chlorine-carbon bonds are oriented vertical to the plain of the carbon ring and parallel to the axis of the ring (alternate up and down). The equatorial chlorine-carbon bonds are perpendicular to the axis of the ring and lie almost along the equator of the ring. Panel A: thermodynamically stable conformers and the positions of chlorine atoms on the cyclohexane rings are: α: aaeeee, β: eeeeee, γ: aaaeee, δ: aeeeee, (‘a’ represents chlorine atoms on the axial position, and ‘e’ represents chlorine atoms on the equatorial position). Panel B: The conformers of β- and δ-HCH after ring flipping in which all axial atoms are converted into equatorial atoms and vice versa. The red and blue color of bond and atoms indicate the possible position in which dihaloelimination is likely. This figure is adapted from a previous study (Qiao et al. 2019).

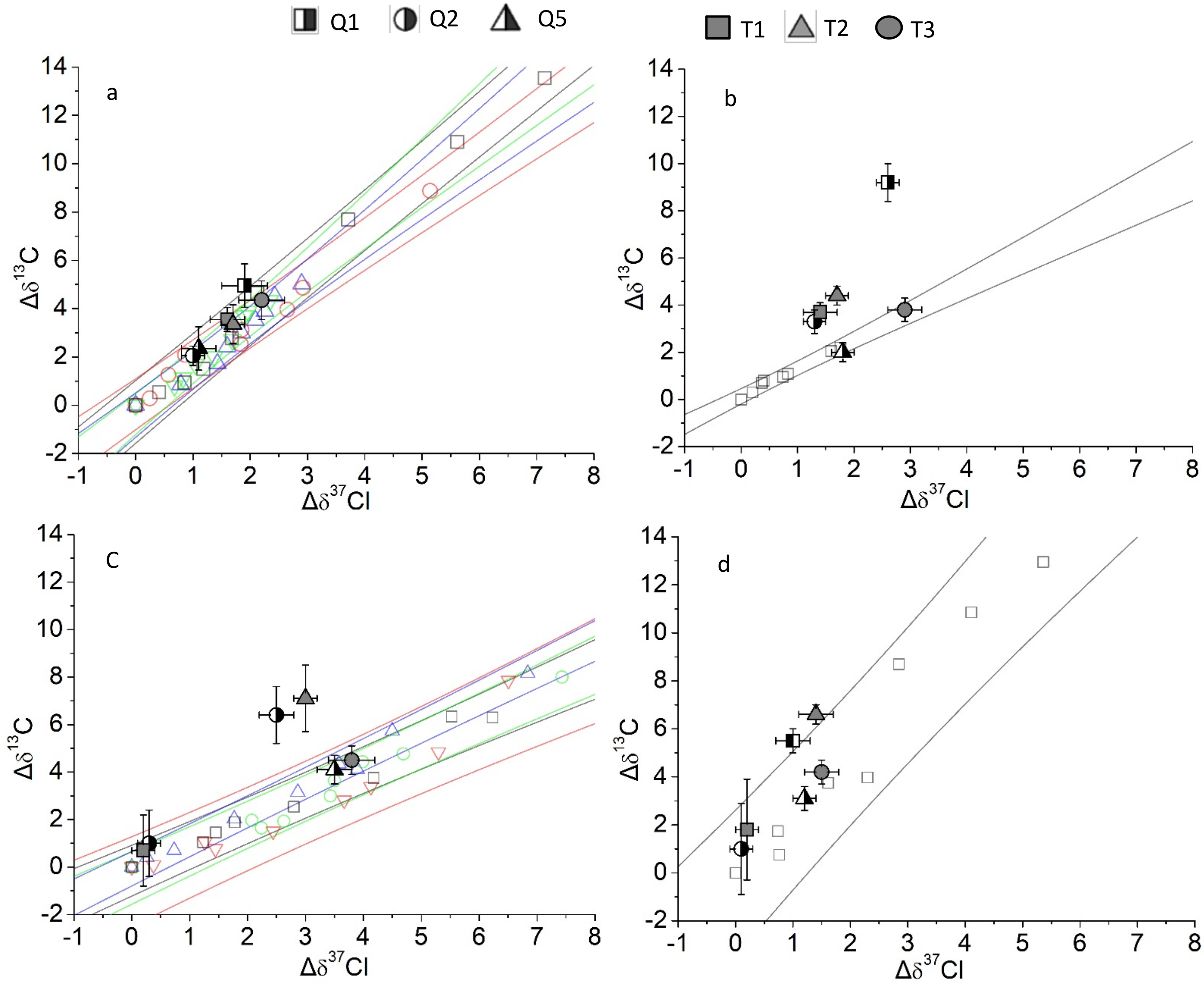


Fig.3. Dual C-Cl isotope patterns of α-, β-, γ-, and δ-HCH (panel a, b, c, and d) of the enrichment culture in this study (□), *Dehaloccoides* 195 (△), *Dehaloccoides* BTF08 (▽), and another enrichment culture from Bitterfeld (○). The 95 % prediction bands for the correlation of carbon and chlorine isotope fractionation for individual cultures are shown (prediction bands belong to data points with same color). The half-filled and grey symbols are samples from Quaternary and Tertiary aquifer, respectively. The isotope values are documented in Tab. S1 and the discrimination of isotope values reporting as Δ values compared to the source (Tab. S2). For α- and γ-HCH (a and c), the 95 % confidence level of prediction bands for the correlation of carbon and chlorine isotope fractionation (Λ) were derived from the data sets of four anaerobic cultures from this study and a previous study (Liu et al. 2019 Submitted). For β- and δ-HCH (b and d), the 95 % prediction bands were derived from the data of the enrichment culture in this study as more data is not available.