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Characterization of Hexachlorocyclohexane Isomer Dehydrochlorination by LinA1 and LinA2 Using Multi-element Compound-Specific Stable Isotope Analysis

Yaqing Liu, Juan Fu, Langping Wu, Steffen Kümmel, Ivonne Nijenhuis, and Hans H. Richnow*

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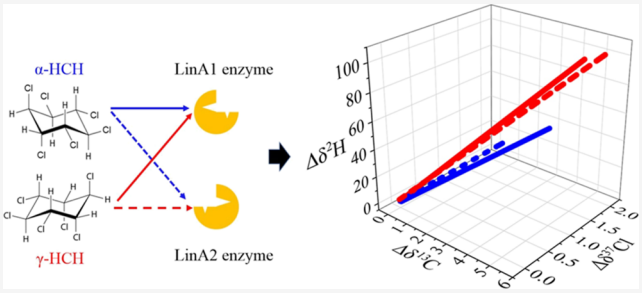
ABSTRACT: Dehydrochlorination is one of the main (thus far discovered) processes for aerobic microbial transformation of hexachlorocyclohexane (HCH) which is mainly catalyzed by LinA enzymes. In order to gain a better understanding of the reaction mechanisms, multi-element compound-specific stable isotope analysis was applied for evaluating α- and γ-HCH transformations catalyzed by LinA1 and LinA2 enzymes. The isotopic fractionation (ε_E) values for particular elements of (+)α-HCH ($\varepsilon_C = -10.7 \pm 0.7$‰, $\varepsilon_{Cl} = -4.2 \pm 0.5$‰, $\varepsilon_H = -154 \pm 16$‰) were distinct from the values for (−)α-HCH ($\varepsilon_C = -4.1 \pm 0.7$‰, $\varepsilon_{Cl} = -1.6 \pm 0.2$‰, $\varepsilon_H = -68 \pm 10$‰), whereas the dual-isotope fractionation patterns were almost identical for both enantiomers ($\Lambda_{C-Cl} = 2.4 \pm 0.4$ and 2.5 ± 0.2, $\Lambda_{H-C} = 12.9 \pm 2.4$ and 14.9 ± 1.1). The ε_E of γ-HCH transformation by LinA1 and LinA2 were -7.8 ± 1.0‰ and -7.5 ± 0.8‰ (ε_C), -2.7 ± 0.3‰ and -2.5 ± 0.4‰ (ε_{Cl}), -170 ± 25‰ and -150 ± 13‰ (ε_H), respectively. Similar Λ_{C-Cl} values (2.7 ± 0.2 and 2.9 ± 0.2) were observed as well as similar Λ_{H-C} values (20.1 ± 2.0 and 18.4 ± 1.9), indicating a similar reaction mechanism by both enzymes during γ-HCH transformation. This is the first data set on 3D isotope fractionation of α- and γ-HCH enzymatic dehydrochlorination, which gave a more precise characterization of the bond cleavages, highlighting the potential of multi-element compound-specific stable isotope analysis to characterize different transformation processes (e.g., dehydrochlorination and reductive dehalogenation).



KEYWORDS: *transformation, isotope fractionation, pesticides, LinA enzymes, dehydrochlorination*

■ INTRODUCTION

The photocatalytic synthesis of hexachlorocyclohexane (HCH) yields four main isomers (α-, β-, γ-, and δ-HCH).[1] Technical HCH (the mixture of different HCH isomers) was used as a pesticide since the late 1940s until γ-HCH was identified as the only isomer which possesses insecticidal activity. Since then, γ-HCH was purified from the other isomers and marketed as lindane with purities up to 99%.[2−4] Large amounts of HCH have entered the environment during agriculture application, and uncontrolled or unconstrained dumping of waste HCH isomers after lindane was isolated. Due to their persistence, toxicity, and bioaccumulation, HCH isomers were added to the list of persistent organic compounds during the Stockholm Convention in August 2010.[4] However, many of the stockpiles are still present in contaminated soil and groundwater and represent a large contaminant reservoir with about 7 million tons of HCH residuals in the environment.[5,6]

Biotransformation is one of the most promising processes for the remediation of HCH-contaminated sites. Several anaerobic cultures have been reported to be able to reduce HCH by dehalogenation, including *Dehalobacter* and *Dehalococcoides*, which can convert HCH by cleaving two C−Cl bonds.[7−10] In the case of aerobic degradation, more than 30 HCH-degrading *Sphingomonas* have been described and isolated in the last decades.[11−14] Within these cultures, *Sphingobium indicum* B90A, *Sphingobium japonicum* UT26, and *Sphingobium francense* Sp+ have been intensively investigated.[11] Especially, the transformation pathways of HCHs by *S. indicum* B90A and the corresponding enzymes have been studied in detail. Those studies revealed two key enzymes, LinA1 and LinA2, which could catalyze the dehydrochlorination of α-, γ-, and δ-HCH.[15−17] A previous study on the complete genome of strain B90A revealed that it harbors four replicons: one chromosome (3,654,322 bp) and three plasmids designated as pSRL1 (139,218 bp), pSRL2 (108,430 bp), and pSRL3 (43,761 bp), where *LinA2* is on the chromosome and *LinA1* is on the pSRL1.[18] Probably due to

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the absence of regulatable promoters and a scattering of the different genes, both *LinA1* and *LinA2* in strain B90A are expressed to a constitutive level during the transformation of γ-HCH.[18] The amino acid sequences of LinA1 and LinA2 enzymes are very similar (90%), differing only by 15 out of 156 amino acids.[19] Remarkably, for α-HCH, which is the only HCH isomer possessing two enantiomers, it was demonstrated that the transformation of (+)α-HCH is mainly catalyzed by LinA1 whereas the (−)α-HCH transformation is primarily catalyzed by LinA2.[15] The reversal in its preference from the (−) to the (+)α-HCH could be the results of the three amino acid changes in LinA1(K20Q, L96C, and A131G) and enhanced by the change of amino acid T133M.[19,20] Furthermore, multiple studies have been focused on characterizing the type of enzymes and their differences which may link to their catalytic efficiency of the respective ligand by quantum mechanics/molecular mechanics modeling.[20,21]

Bench studies have also been conducted to investigate the dehydrochlorination mechanisms and reaction pathways.[22,23] In recent years, compound-specific stable isotope analysis (CSIA) has been applied for investigating the isotope fractionation associated with the HCH biotransformation processes.[16,17,24−27] Isotope fractionation is based on slightly different reaction kinetics of molecules which differ in their isotopic composition, as most isotopologues with the light isotope at the reactive position react faster than isotopologues possessing the corresponding heavy isotope at the same position. This normal isotope effect results finally in the accumulation of the heavier isotopes in the remaining substrate, whereas the lighter isotopes enrich in the formed product. The extent of this isotope enrichment can be quantified by the Rayleigh equation. However, it must be noted that the observed isotope fractionation can be decreased due to steps controlling the overall kinetics of the reaction, prior to the isotope-sensitive bond cleavage. For example, the binding of the substrate to an enzyme can be a rate-limiting step of biotransformation reactions and can mask the biochemical isotope fractionation,[16] which will limit the application of single element CSIA for investigation of reaction mechanisms.

Methodological advances over the last decade now allow multi-element compound-specific stable isotope analysis (ME-CSIA) for overcoming this obstacle, as the masking effect of isotope enrichment of two elements in one molecule is expected to be similar.[28,29] Thus, dual-element slopes (e.g., for carbon and chlorine, $\Lambda_{C-Cl} = \Delta\delta^{13}C/\Delta\delta^{37}Cl$, where $\Delta\delta^{13}C$ and $\Delta\delta^{37}Cl$ are the changes in isotope compositions of carbon and chlorine, respectively) can be related to specific reaction mechanisms. Furthermore, triple-element isotope analysis was applied for identifying different transformation pathways of organic compounds, such as herbicide[30] and 1,2-dichloroethane.[31] The Λ_{C-Cl} values for reductive dehalogenation of HCHs by anaerobic cultures (bond cleavage of two C−Cl bonds) and the scenarios of bond cleavage have been carefully discussed in previous studies.[10,32] In the case of aerobic HCH transformation (dehydrochlorination with the cleavage of C−H and C−Cl bonds), previous studies only reported the Λ_{C-H} values of HCH,[17,26] which limited the application of ME-CSIA to distinguish with other reactions (e.g., reductive dehalogenation and anaerobic transformation of HCH with the cleavage of two C−Cl bonds). Furthermore, in the reactions which involved two different bond cleavages, for example, C−Cl and C−H, Λ_{C-H} or Λ_{C-Cl} alone may not be enough for characterization of the reactions.

Therefore, in order to fill the knowledge gaps, the typical dehydrochlorinases, LinA1 and LinA2 enzymes, were used for α- and γ-HCH biotransformations. The objectives of this study were (i) to explore the transformation kinetics of the HCH isomers by different enzymes, (ii) to determine the isotope enrichment factors ε_C, ε_{Cl} as well as ε_H for the HCH isomers, and (iii) to obtain the Λ_{C-Cl}, Λ_{C-H} as well as the characteristic vectors of 3D isotope fractionation during the HCH biotransformation by dehydrochlorinases. In addition, the isotopic fractionation patterns obtained in this study were compared and discussed with the values reported previously for characterizing different reactions.

EXPERIMENTS AND METHODS

Experiments. Enzymatic assays were conducted using LinA1 and LinA2 for the transformation of α- and γ- HCH, respectively. The chemicals used in this study are all of analytical purity grade which are listed in the section of Chemicals in Supporting Information. The *Escherichia coli* cells coded with *LinA1* and *LinA2* genes were cultivated for expression of the LinA enzymes. More details of the cultivation and purification processes can be found in the Supporting Information. The enzyme concentrations used in this study were 75 μg mL^{-1} for LinA1 and 264 μg mL^{-1} for LinA2, respectively. In contrast to our previous study,[16] both enzymes were used for the transformation of α- and γ- HCH. All batch experiments for HCH transformation were conducted with Tris buffer (100 mL in 240 mL serum bottle) with an initial concentration of 5.5 μM of the respective HCH. Reactions were stopped by adding 0.3% (v/v) formic acid (final concentration) at different time points resulting in different extents of HCH transformation. Samples were stored at 4 °C in the fridge before extraction. The extraction methods are described in detail in our previous study.[16] More details can be found in the Supporting Information.

Concentration Analysis. Concentrations of HCH were analyzed by GC-FID (Agilent Technologies) and protein concentrations were analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) as described previously[16] and summarized in the Supporting Information.

Stable Isotope Analysis. The carbon isotopic compositions ($\delta^{13}C$) were analyzed by gas chromatography-combustion-isotope ratio mass spectrometry. Analytical details are described elsewhere for α-HCH enantiomers[33] and γ-HCH.[10]

Hydrogen isotopic compositions ($\delta^{2}H$) of both HCH isomers were analyzed by gas chromatography-chromium-based high-temperature conversion-isotope ratio mass spectrometry using the same methods as described by Wu and colleagues.[34]

Chlorine isotopic compositions ($\delta^{37}Cl$) were determined by gas chromatography-multiple collector-inductively coupled plasma mass spectrometry, as described elsewhere.[35,36] The temperature programs and inductively coupled plasma mass spectrometry parameters were the same as reported in a previous study.[10]

Because the α-HCH enantiomers could not be measured separately for $\delta^{37}Cl$, the respective $\delta^{37}Cl$ values were calculated using eq 1

$$\delta_t^{bulk} = \delta_t^{(-)\alpha-HCH} \times EF_t(-) + \delta_t^{(+)\alpha-HCH} \times EF_t(+) \quad (1)$$

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Table 1. Summary of Isotopic Fractionation of Different Multi-element Stable Isotope Fractionation Studies Concerning HCH Transformation[a]

		α-HCH					γ-HCH					
reactions	systems/catalyst	ε_C(‰) ± 95% CI	ε_{Cl}(‰) ± 95% CI	ε_H(‰) ± 95% CI	Λ_{C-Cl}	Λ_{H-C}	ε_C(‰) ± 95% CI	ε_{Cl}(‰) ± 95% CI	ε_H(‰) ± 95% CI	Λ_{C-Cl}	Λ_{H-C}	
dehydrochlorination	LinA1	−10.8 ± 1.0$^{(+)}$	−4.2 ± 0.5$^{(+)}$	−154 ± 16$^{(+)}$	2.4 ± 0.4$^{(+)}$	12.9 ± 2.4$^{(+)}$	−7.8 ± 1.0	−2.7 ± 0.3	−170 ± 25	2.7 ± 0.2	20.1 ± 2.0	this study
	LinA2	−4.1 ± 0.7$^{(-)}$	−1.6 ± 0.2$^{(-)}$	−68 ± 10$^{(-)}$	2.5 ± 0.2$^{(-)}$	14.9 ± 1.1$^{(-)}$	−7.5 ± 0.8	−2.5 ± 0.4	−150 ± 13	2.9 ± 0.2	18.4 ± 1.9	
dehydrochlorination	LinA1	−3.8 ± 0.2$^{(+)}$										Liu et al.[16]
							−8.1 ± 0.3		−122 ± 6		11.5 ± 0.8	Schilling et al.[26]
	LinA2						−8.3 ± 0.2		−160 ± 6		16.4 ± 0.9	
		−9.6 ± 0.1$^{(+)}$/−11.7 ± 1.5$^{(-)}$		−208 ± 19$^{(+)}$		22.0 ± 3.3$^{(+)}$						Schilling et al.[17]
		−11.1 ± 0.3$^{(-)}$										Liu et al.[16]
	LinA variants						−5.3 ± 0.8	−1.8 ± 0.4	−119 ± 18	2.9 ± 1.1*	22.5 ± 6.8*	Kannath et al.[27]
	hydrolysis						−7.0 ± 0.5	−2.0 ± 0.2	−162 ± 26	3.5 ± 0.6*	23.1 ± 5.4*	
	modeling						−2.8 to −7.5	−0.7 to −1.5	−463 to −756	3.5 to 7.1*	64.1 to 263.6*	
reductive dehalogenation	*Dehalococcoides mccartyi* 195	−3.0 ± 0.3	−1.8 ± 0.2		1.7 ± 0.2		−4.4 ± 0.6	−3.3 ± 0.4		1.2 ± 0.1		Liu et al.[10]
	Dehalococcoides mccartyi BTF08	−2.4 ± 0.2	−1.4 ± 0.3		1.8 ± 0.3		−4.0 ± 0.5	−3.3 ± 0.3		1.1 ± 0.3		
	enrichment culture 1	−3.0 ± 0.4	−1.4 ± 0.3		2.0 ± 0.3		−4.0 ± 0.5	−2.9 ± 0.4		1.1 ± 0.2		
	enrichment culture 2	−4.2 ± 0.4	−2.0 ± 0.3		1.9 ± 0.1		−3.6 ± 0.4	−3.2 ± 0.6		1.1 ± 0.1		Liu et al.[32]

[a] $^{(+)}$—values for (+)α-HCH transformation; $^{(-)}$—values for (−)α-HCH transformation;*—Λ_{C-Cl} values calculated by $\Lambda_{C-Cl} = \varepsilon_C/\varepsilon_{Cl}$.

where EF is the enantiomer fraction, $EF_t(-) = C_t(-)/C_t^{bulk}$, and $EF_t(+) = C_t(+)/C_t^{bulk}$.

For LinA1 experiments, only (+)α-HCH transformation was observed and thus $\delta_t^{(-)\alpha\text{-HCH}} = \delta_0^{bulk}$, $\delta_t^{(+)\alpha\text{-HCH}} = (\delta_t^{bulk} - EF_t(-) \times \delta_0^{bulk})/EF_t(+)$. Similarly for LinA2 experiments, no transformation of (+)α-HCH was observed and thus $\delta_t^{(+)\alpha\text{-HCH}} = \delta_0^{bulk}$, $\delta_t^{(-)\alpha\text{-HCH}} = (\delta_t^{bulk} - EF_t(+) \times \delta_0^{bulk})/EF_t(-)$.

Evaluation of Isotope Data. The isotopic enrichment factor of an element E (ε_E) was derived using eq 2

$$\ln\left(\frac{\delta^h E_t + 1}{\delta^h E_0 + 1}\right) = \varepsilon_E \times \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

where $\delta^h E_0$ is the initial isotopic signature of the substrate, $\delta^h E_t$ is the isotopic composition of the substrate at time t, and C/C_0 is the fraction of the remaining substrate.

For the determination of lambda values (Λ), hydrogen versus carbon isotope discrimination ($\Lambda_{H-C} = \Delta\delta^2H/\Delta\delta^{13}C$) and carbon versus chlorine isotope discrimination ($\Lambda_{C-Cl} = \Delta\delta^{13}C/\Delta\delta^{37}Cl$) were plotted and the respective Λ was derived from the slope of the linear regression. The uncertainty of Λ, given as the 95% confidence interval (CI), was derived from regression analysis using Origin 9.0.

The triple-element isotope plotting was also done using Origin 9.0 and the characteristic unit vectors were determined following the approach of Palau et al.[31] To further assess the similarities and differences between the unit vectors, the angle θ between the two vectors were calculated following the method described by Palau et al.[31]

RESULTS AND DISCUSSION

Transformation of α- and γ-HCH Catalyzed by LinA Enzymes. *α-HCH.* In the LinA1 experiment (reaction time, 2 h), only (+)α-HCH transformation was observed with a reaction rate of 0.2 ± 0.01 h^{-1} (Supporting Information, Figure 1) and pentachlorocyclohexane (PCCH) was the major product. Similarly, only (−)α-HCH transformation was observed in the LinA2 experiment (reaction time, 1.5 h) with a reaction rate of 0.4 ± 0.03 h^{-1} (Supporting Information, Figure 1) and PCCH was the main product. The transformation products indicated that a dehydrochlorination reaction similar to chemical hydrolysis took place. LinA1 and LinA2 showed a selective transformation of (+) and (−)α-HCH, respectively, which was the same as we observed before.[16] In contrast, a previous study reported that both enantiomers could be degraded by LinA1 or LinA2 if the reaction lasted for 24 h.[15] However, the transformation rate for (+)α-HCH (0.43 ± 0.03 h^{-1}) was much higher than that for (−)α-HCH (0.060 ± 0.010 h^{-1}) when LinA1 was used for the catalysis. Similarly, when LinA2 was used, a higher transformation rate for (−)α-HCH (0.47 ± 0.06 h^{-1}) than that for (+)α-HCH (0.04 ± 0.006 h^{-1}) was observed. The difference in the selectivity is probably due to the smaller amounts of enzymes which we have applied in our study. Similar results have been reported for experiments with relatively low concentrations of LinA2 (0.01 μg mL^{-1}), where only (−)α-HCH transformation was observed.[17] Yet, when the concentration of LinA2 was increased (0.7 μg mL^{-1}), both (+) and (−)α-HCH were degraded.[17] Accordingly, the

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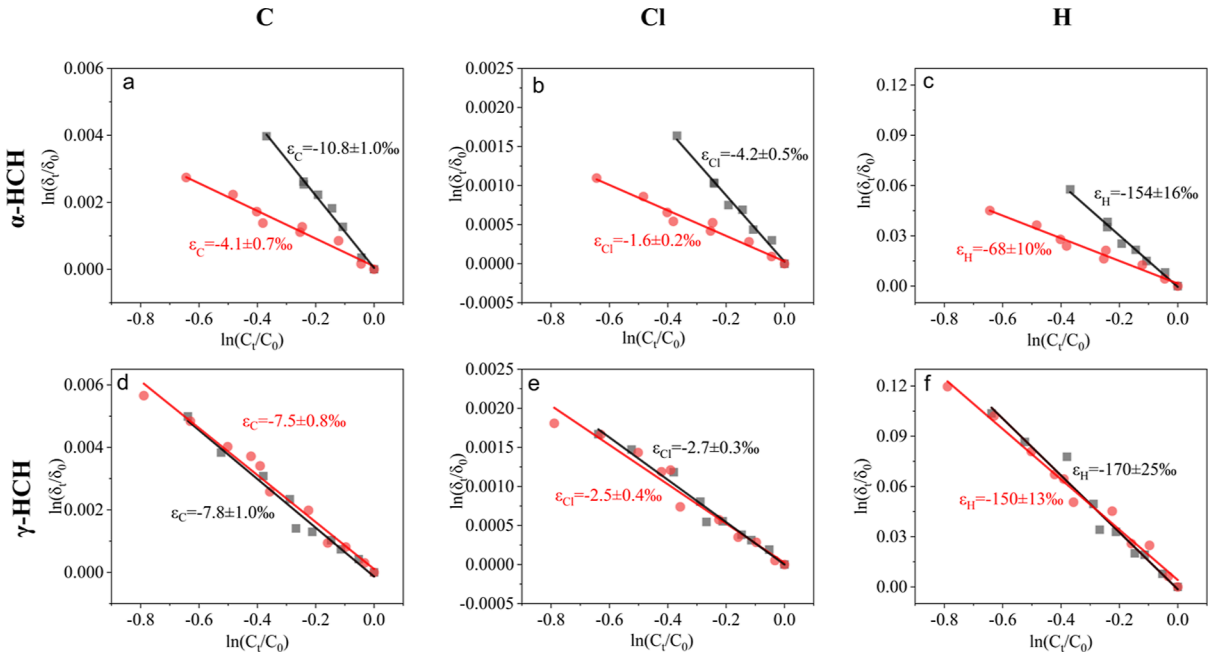

Figure 1. Rayleigh plots for carbon, chlorine, and hydrogen isotopic fractionation during the transformation of α-HCH (a−c) and γ-HCH (d−f) by LinA1 (black symbols) and LinA2 (red symbols). Note, graphs for transformation experiments with LinA1 include only data of (+)α-HCH, whereas LinA2 experiments include only data of (−)α-HCH because in those experiments only one of the two enantiomers was transformed by the respective enzyme.

enzyme amount may affect the transformation of the different enantiomers. The enantiomer fractionation, which is a result of different transformation rates of the enantiomers, could be variable as binding to the enzyme could take place even when it is stereochemically unfavorable. Thus, the variability of enantiomer fractionation will limit its application for evaluating biotic transformation processes as the relatively high enzyme-to-substrate ratio may decrease specific binding to the enzymes.

γ-HCH. Both LinA1 and LinA2 showed comparable transformation rates of 0.3 ± 0.01 h^{-1} (LinA1) and 0.3 ± 0.02 h^{-1} (LinA2) for γ-HCH (Supporting Information, Figure 1), and the major product identified was also PCCH. This is in agreement with a previous study which demonstrated that γ-HCH could be degraded by both LinA1 and LinA2 enzymes.[26] In our study, both enzymes showed relatively fast transformation rates for γ-HCH compared to that for bulk α-HCH (0.063 ± 0.016 and 0.19 ± 0.059 h^{-1} in the experiments with LinA1 and Lin A2, respectively), which supports the speculation of a previous study that γ-HCH transformation is easier in comparison to α-HCH transformation, as γ-HCH possesses one more C−Cl bond in the axial position (as shown in Figure SI3, Supporting Information), which is most likely the preferred position for bond cleavages.[37]

Isotopic Fractionation of α- and γ-HCH Catalyzed by LinA Enzymes. *α-HCH.* Significant normal carbon, chlorine, and hydrogen isotopic fractionation were observed during the transformation of α-HCH enantiomers by LinA1 and LinA2 as shown in Supporting Information, Figure 2. In the case of (+)α-HCH transformation (catalyzed by LinA1), the isotopic fractionation of carbon ($\varepsilon_{C\text{-}(+)\alpha\text{-HCH}}$), chlorine ($\varepsilon_{Cl\text{-}(+)\alpha\text{-HCH}}$), and hydrogen ($\varepsilon_{H\text{-}(+)\alpha\text{-HCH}}$) were −10.8 ± 1.0, −4.2 ± 0.5, and −154 ± 16‰, respectively (Table 1, Figure 1). In contrast, the isotopic fractionation of (−)α-HCH (catalyzed by LinA2) was comparatively low ($\varepsilon_{C\text{-}(-)\alpha\text{-HCH}}$ = −4.1 ± 0.7‰, $\varepsilon_{Cl\text{-}(-)\alpha\text{-HCH}}$ = −1.6 ± 0.2‰, $\varepsilon_{H\text{-}(-)\alpha\text{-HCH}}$ = −68 ± 10‰, Table 1, Figure 1).

The obtained $\varepsilon_{C\text{-}(+)\alpha\text{-HCH}}$ (−10.8 ± 1.0‰) catalyzed by LinA1 was similar to the value previously reported (−9.6 ± 0.1‰) for the transformation of (+)α-HCH by LinA2[17] and to the values (average = −11.1 ± 0.3‰) reported for the transformation of (+)α-HCH by LinA1.[16] In contrast, the $\varepsilon_{H\text{-}(+)\alpha\text{-HCH}}$ determined in the present study (−154 ± 16‰) was lower than the value (−208 ± 19‰) reported for the transformation of (+)α-HCH by LinA2.[17] The $\varepsilon_{C\text{-}(-)\alpha\text{-HCH}}$ value (−4.1 ± 0.7‰) catalyzed by LinA2 was almost identical to the values (average = −3.8 ± 0.2‰) reported previously.[16] The observed differences in the ε values for the transformation of α-HCH by LinA1 and LinA2 enzymes could result from the different kinetic isotope effects. However, it is more likely that those differences were the result of masking effects of the isotope fractionation caused by steps prior to the bond cleavage reaction (e.g., commitment to catalysis) which is further supported by the findings of a study reporting a higher $\varepsilon_{C\text{-}(-)\alpha\text{-HCH}}$ value (−11.7 ± 1.5‰) for the transformation of (−)α-HCH by LinA2.[17] Accordingly, the occurrence of masking effects may also explain that when the concentration of LinA2 was much higher (which resulted in a too fast reaction), no isotope fractionation of (−)α-HCH can be detected as described previously.[17] However, it should be noted that the higher $\varepsilon_{C\text{-}(-)\alpha\text{-HCH}}$ value of −11.7 ± 1.5‰ was only approximated from the isotope signatures of the substrate and the dechlorinated product, whereas hydrogen isotope fractionation was not determined.[17] It is plausible that the variability of hydrogen isotope fractionation is much higher.

Furthermore, previous studies reported carbon and chlorine isotopic fractionation of bulk α-HCH during anaerobic transformation ranging from −2.4 ± 0.2 to −4.2 ± 0.4‰ for $\varepsilon_{C\text{-}\alpha\text{-HCH}}$ and from −1.4 ± 0.3 to −2.0 ± 0.3‰ for $\varepsilon_{Cl\text{-}\alpha\text{-HCH}}$, respectively.[10,32] As no enantiomer fractionation during

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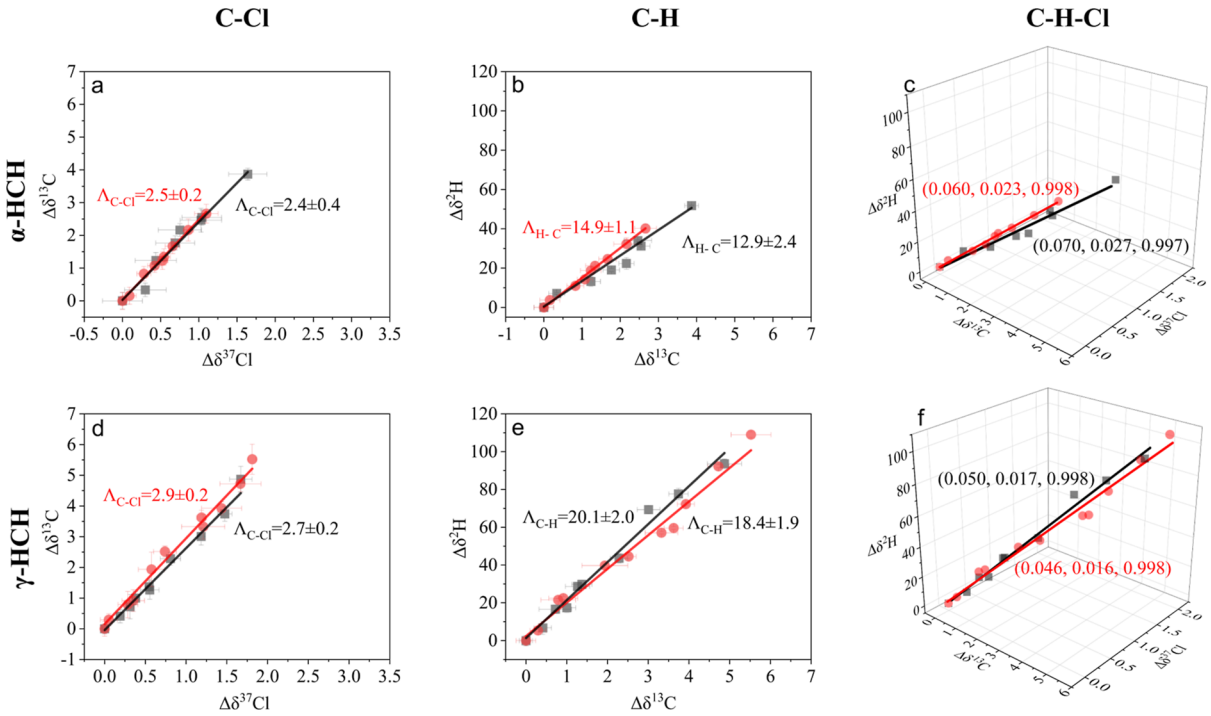

Figure 2. ME-isotopic fractionation plots for the determination of Λ_{C-Cl} and Λ_{H-C} and 3D plotting during the transformation of α-HCH (a−c) and γ-HCH (d−f) by LinA1 (black) and LinA2 (red). Note, graphs for transformation experiments with LinA1 include only data of (+)α-HCH, whereas LinA2 experiments include only data of (−)α-HCH because in those experiments only one of the two enantiomers was transformed by the respective enzyme.

anaerobic α-HCH transformation was observed and the detected ε_C of (+)- and (−)α-HCH were identical, it indicates that the values of ε_{Cl} for both enantiomers were also the same.[10] As mentioned above, anaerobic α-HCH transformation is a reductive dehalogenation process,[10] whereas LinA enzymes catalyze a dehydrochlorination reaction.[26] Therefore, the general expectation is that both processes would result in different isotope fractionation patterns.[38] The aerobic (dehydrochlorination) and the anaerobic (−)α-HCH transformation (reductive dehalogenation) resulted in a comparable carbon and chlorine isotopic fractionation, which was not the case for (+)α-HCH. Further studies on hydrogen isotope fractionation may give a better characterization of the different reactions.

γ-HCH. The transformation of γ-HCH by LinA1 and LinA2 resulted in a comparable isotopic fractionation for carbon ($\varepsilon_{C-\gamma-HCH}$ = −7.8 ± 1.0 and −7.5 ± 0.8‰), chlorine ($\varepsilon_{Cl-\gamma-HCH}$ = −2.7 ± 0.3 and −2.5 ± 0.4‰), and hydrogen ($\varepsilon_{H-\gamma-HCH}$ = −170 ± 25 and −150 ± 13‰). These isotopic fractionations were consistent with the values reported for the hydrolysis of γ-HCH ($\varepsilon_{C-\gamma-HCH}$ = −7.0 ± 0.5‰, $\varepsilon_{Cl-\gamma-HCH}$ = −2.0 ± 0.2‰, and $\varepsilon_{H-\gamma-HCH}$ = −162 ± 26‰).[27] Moreover, the isotopic fractionations determined in the present study were also in agreement with $\varepsilon_{C-\gamma-HCH}$ and $\varepsilon_{H-\gamma-HCH}$ (−8.3 ± 0.2 and 160 ± 6‰) reported for γ-HCH transformation by LinA2.[26] In addition, γ-HCH transformation by LinA1 resulted in an increased $\varepsilon_{H-\gamma-HCH}$ in comparison to the previously reported value ($\varepsilon_{H-\gamma-HCH}$ = −122 ± 6‰), whereas $\varepsilon_{C-\gamma-HCH}$ was almost identical ($\varepsilon_{C-\gamma-HCH}$ = −8.1 ± 0.3‰; Table 1). The relatively high variability of the hydrogen isotopic fractionation observed for γ-HCH transformation agrees with the results obtained for α-HCH transformation in the present study. However, in comparison to the values of another LinA variant, which were −5.3 ± 0.8‰ for $\varepsilon_{C-\gamma-HCH}$, −1.8 ± 0.4‰ for $\varepsilon_{Cl-\gamma-HCH}$, and −119 ± 18‰ for $\varepsilon_{H-\gamma-HCH}$,[27] the values determined in the present study were higher. This difference could be a result of masking effects or it could be caused by slightly different reaction mechanisms of the LinA variants leading to different isotope fractionation patterns. Compared with the reductive dehalogenation of γ-HCH (Table 1), $\varepsilon_{C-\gamma-HCH}$ and $\varepsilon_{Cl-\gamma-HCH}$ obtained were higher in the present study. In addition, the ε values obtained from modeling studies showed much lower values compared with the experiment data, except ε_H.

ME-Isotopic Analysis of α- and γ-HCH Dehydrochlorination Catalyzed by LinA Enzymes. *α-HCH.* The isotopic fractionation of α-HCH enantiomer dehydrochlorination were correlated by dual-element isotope analyses and the corresponding Λ_{C-Cl} and Λ_{H-C} values, expressing the mode of the C−Cl and C−H bond cleavage mechanisms, were calculated (Table 1, Figure 2a,b). The almost identical Λ values for both enantiomers indicate that the reaction mechanism of LinA1 transforming (+)α-HCH and LinA2 converting (−)α-HCH is quite similar. So far, there is only one Λ_{H-C} value available in the literature which is based on the transformation of (+)α-HCH by LinA2.[17] In comparison to this Λ_{H-C} value (22.0 ± 3.3), the Λ_{H-C} value of (+)α-HCH transformation by LinA1 (12.9 ± 2.4) determined in the present study is significantly lower. As dual-element isotope analysis can theoretically omit masking of isotope effects, this difference indicates different commitments to catalysis, for example, the specific mode of binding in the enzyme pocket prior to catalysis. This could be a result of the three amino acid changes in LinA1, that is, K20Q, L96C, and A131G,[19,20] which caused a reversal in its preference from the conversion of (−) to (+)α-HCH. Furthermore, as the amino acid change T133M enhanced the enantiomer preference,[19,20] this could be also the

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additional reasons for the different isotope fractionation patterns. In addition, previous studies reported that LinA1 and LinA2 differ in the rate of (+)α-HCH transformation,[15,17] which could be an indicator for different commitments to catalysis as well. Furthermore, compared to the Λ_{C-Cl} values obtained from the α-HCH anaerobic transformation (ranging from 1.7 ± 0.2 to 2.0 ± 0.3),[10] the Λ_{C-Cl} values of the aerobic transformation (2.4 ± 0.4 for (+)α-HCH transformation by LinA1 and 2.5 ± 0.2 for (−)α-HCH transformation by LinA2) show a trend to larger but still overlapping values and thus do not allow distinguishing between aerobic and anaerobic α-HCH transformation at field sites.

The C, H, and Cl isotope data for the dehydrochlorination of α-HCH enantiomers were also combined in a 3D isotope plot. Characteristic unit vectors were determined as (0.070, 0.027, 0.997) and (0.060, 0.023, 0.998) for (−) and (+)α-HCH (Figure 2c), respectively. The calculated angle θ between the two vectors is less than 1°. The vectors as well as the plots show clearly same trends for the transformation of (−) and (+)α-HCH by LinA1 and LinA2, respectively. This result indicates that the dehydrochlorination can be well characterized by triple-element isotope analysis. Furthermore, this triple-element isotope analysis would give a chance to distinguish different HCH transformation pathways, for example, dehydrochlorination and reductive dehalogenation. As dehydrochlorination involves both C−Cl and C−H bond cleavage whereas reductive dehalogenation only involves C−Cl bond cleavage and no significant hydrogen isotope fractionation is expected, secondary hydrogen isotope fractionation may occur. In the future, hydrogen isotope fractionation may add as a further criterion to identify processes, but hydrogen isotopic fractionation values for HCH reductive dehalogenation are not yet available to our best knowledge.

γ-HCH. The Λ_{C-Cl} and Λ_{H-C} values for the aerobic transformation of γ-HCH catalyzed by LinA1 and LinA2 were almost identical (Table 1, Figure 2d,e), which indicates that the dehydrochlorination mechanisms catalyzed by LinA1 and LinA2 lead to the same isotope effects. It is plausible that the different amino acids in LinA1 and LinA2 enzymes did not cause any different commitments to catalysis and led to the same isotope fractionation patterns. This is also in agreement with previous studies that 4 out of the 10 amino acids vicinal to the active site of LinA might govern the enantioselectivity toward α-HCH enantiomers, but do not necessarily play a similar role in the transformation of γ-HCH.[19,20] Based on the reported ε_C and ε_{Cl},[27] the Λ_{C-Cl} values of γ-HCH for the chemical hydrolysis and the enzymatic catalysis by LinA variants were calculated to be 3.5 ± 0.6 and 2.9 ± 1.1, respectively (Table 1). The Λ_{C-Cl} values for γ-HCH transformation by LinA variants based on Kannath's study (2.9 ± 1.1) and the respective Λ_{C-Cl} values determined in the present study (2.7 ± 0.2 by LinA1 and 2.9 ± 0.2 by LinA2) are not distinguishable, indicating similar reaction mechanisms. The Λ_{C-Cl} value (3.5 ± 0.6) of γ-HCH during chemical hydrolysis showed some differences to the values obtained during dehydrochlorination but still overlapping, which indicates the uncertainty for charactering the bond cleavage (C−Cl and C−H) by dual-isotope analysis.

Furthermore, the Λ_{C-Cl} values for γ-HCH dehydrochlorination (>2.7 ± 0.2) can be distinguished from the Λ_{C-Cl} values that have been reported for γ-HCH reductive dehalogenation (<1.2 ± 0.1),[10] thus enabling the differentiation of dehydrochlorination from reductive dehalogenation by dual-element isotope analysis. It should be noted that the Λ_{H-C} values reported in Schilling et al. (2019b) for γ-HCH transformation by LinA1 and LinA2 were lower than those determined in the present study (Table 1), which is most likely caused by the higher variability of hydrogen isotope fractionation. Furthermore, the Λ_{C-Cl} and Λ_{H-C} values from modeling (Table 1) also show significant differences compared with the experimental data, which indicates that the actual reactions are much more complex than the simulated models. Furthermore, it is plausible that Λ_{H-C} values possess a relatively high variability compared to the Λ_{C-Cl} values, which is in comparison to other elements (e.g., C and Cl) most likely due to the more pronounced sensitivity of the hydrogen isotope fractionation and due to the existence of relatively strong secondary isotope effects.

Similar as α-HCH, the triple-element isotope fractionation of γ-HCH transformed by LinA1 and LinA2 were plotted as shown in Figure 2f. The characteristic unit vectors were (0.050, 0.017, 0.999) and (0.046, 0.016, 0.999) for the transformation by LinA1 and LinA2, respectively. The characteristic unit vectors of γ-HCH transformation by LinA variants and chemical hydrolysis in a previous study[27] were calculated as (0.044, 0.015, 0.999) and (0.043, 0.012, 0.999), which were almost identical to our results. Also, the angle θ between these four vectors are all less than 1°. Compared to the characterization of bond cleavages (C−Cl and C−H) by Λ_{C-Cl} value which showed difference to some extent as we discussed, the triple-element isotope analysis will give a more precise evaluation. As already discussed for α-HCH transformation, the triple-element isotopic fractionation analysis will give a great chance for distinguishing different transformation pathways of HCHs, for example, dehydrochlorination and reductive dehalogenation, at field sites.

Environmental Significance. Enantiomer fractionation was applied in many studies for quantification of biotransformation of organic compounds in the environment. Together with previous studies, we confirmed that the selectivity of α-HCH enantiomers by LinA enzymes can be lowered when the enzyme concentration was relatively high, which indicates that the application of enantiomer fractionation for quantification of biotic transformation may lead to bias to some extent.

In our study, the extent of isotope fractionation indicates primary ^{2}H, ^{13}C, and ^{37}Cl isotope effects in the rate-limiting bond cleavage steps, the dual- as well as triple-element isotope analyses show similar isotope fractionation trends for both α- and γ-HCH during the transformation by LinA1 and LinA2. The experimental data from biotransformation of γ-HCH compared with molecular modeling in a previous report[27] show remarkable differences indicating that the application of molecular modeling for field site evaluation needs improvements.

In addition to elucidating natural biodegradation processes for HCHs, ME-CSIA can also be useful for obtaining insights into degradation processes, for example, both aerobic and anaerobic transformations may happen with the changing of geochemistry. Furthermore, the new isotope fractionation values for α- and γ-HCH determined in this study, with both LinA1 and LinA2 enzymes, open opportunities for using triple-element CSIA to identify HCH transformation processes in different environmental compartments, such as soil, sediments trees, wheat, and even in mammals.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c05334.

Additional experimental details, materials, and methods; data on the degradation kinetics and multi-element isotope fractionation of α-HCH degradation experiments; and chemical structure of (+)/(−)α-HCH and γ-HCH (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Hans H. Richnow − *Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Leipzig 04318, Germany; Isodetect, Leipzig 04103, Germany;* orcid.org/0000-0002-6144-4129; Email: hans.richnow@ufz.de

Authors

Yaqing Liu − *College of Light Industry and Food Engineering, Guangxi Key Laboratory of Clean Pulp & Papermaking and Pollution Control, Guangxi University, Nanning 530004, P.R. China;* orcid.org/0000-0003-2841-9551

Juan Fu − *College of Light Industry and Food Engineering, Guangxi Key Laboratory of Clean Pulp & Papermaking and Pollution Control, Guangxi University, Nanning 530004, P.R. China;* orcid.org/0000-0002-5478-6075

Langping Wu − *Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Leipzig 04318, Germany; Ecometrix Incorporated, Mississauga, Ontario LSN 2L8, Canada;* orcid.org/0000-0003-0599-7172

Steffen Kümmel − *Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Leipzig 04318, Germany;* orcid.org/0000-0002-8114-8116

Ivonne Nijenhuis − *Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Leipzig 04318, Germany;* orcid.org/0000-0001-9737-9501

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.2c05334

Notes

The authors declare no competing financial interest.

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