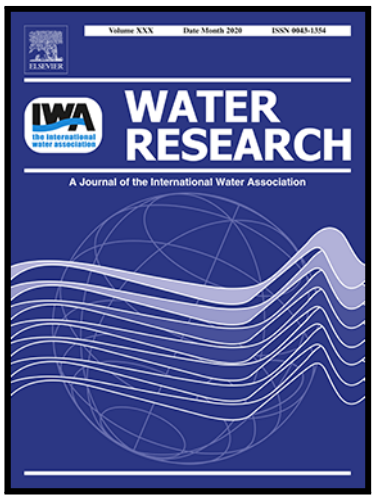

Anaerobic biotransformation of hexachlorocyclohexane isomers in aqueous condition: dual C-Cl isotope fractionation and impact on microbial community compositions

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Highlights:

- Cultures enriched from the same soil showed variable ability for HCH transformation.
- Different microbial communities showed similar isotopic effects in HCH transformation.
- New models of bond cleavage for β- and δ-HCH were proposed based on CSIA.
- Bacteria in the phyla Proteobacteria and Firmicutes were the main dechlorinators.

Anaerobic biotransformation of hexachlorocyclohexane isomers in aqueous condition: dual C-Cl isotope fractionation and impact on microbial community compositions

Xinyu Zhang[a], Yan Zheng[b], Ziming Su[c], Zhenyu Wang[d], Jian Zhang[a], Zhongjun Jia[e], Steffen Kümmel[d], Chengrong Qin[a,*], Yaqing Liu[a,*], Shuangfei Wang[a], Ivonne Nijenhuis[d], Hans H. Richnow[d, f]

[a] College of Light Industry and Food Engineering, Guangxi University, Daxue Road 100, Nanning 530004, P.R. China

[b] College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou 450002, P.R. China

[c] School of Resources, Environment and Materials, Guangxi University, Daxue Road 100, Nanning 530004, China

[d] Department of technical Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Permoserstraße 15, 04318, Leipzig, Germany

[e] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, 210008, China.

[f] Isodetect, Deutscher Platz 5b, 04103 Leipzig, Germany

* Corresponding author

Abstract: Hexachlorocyclohexane (HCH) isomers are persistent organic pollutants (POPs) with high toxicity, lipid solubility, chemical stability. Despite the current ban on usage of Lindane, residual contamination cannot be ignored, are frequently detected in groundwater and threaten human health. Cultures capable of degrading α-HCH, β-HCH,

γ-HCH, and δ-HCH individually have been enriched in anoxic aqueous conditions. Compound-Specific Isotope Analysis (CSIA) was applied to examine the transformation mechanisms of different HCH isomers by the four enrichment cultures. 16S rRNA sequencing techniques were employed to examine the community composition of the enrichment cultures and detect changes in these communities resulting from adding individual HCH isomers. The results indicated that the ability of the enrichment cultures for dichloroelimination of HCH isomers was inconsistent. During dichloroelimination, different bond cleavage mode of β- and δ-HCH led to distinct isotopic effects. HCH isomers had significant impact on the microbial community, while different microbial communities showed comparable isotopic effect during the transformation of a specific HCH isomer. In addition, bacteria in the phyla *Proteobacteria*, and *Firmicutes* were proposed as the dominant dechlorinators. This study provides a novel perspective on the mode of bond cleavage during HCH dichloroelimination and the effect of HCH on microbial communities, which could potentially support the evaluation of HCH transformation by CSIA and their effects on the microecosystems of groundwater.

Keywords: multi-isotope fractionation; organochlorine pesticide; dichloroelimination; 16S rRNA sequencing

1. Introduction

Hexachlorocyclohexane (HCH) isomers are persistent organic pollutants (POPs) that were widely utilized as insecticides worldwide in the last century (Vijgen et al. 2011). Four main isomers result from HCH synthesis: α-, β-, γ-, and δ-HCH. However, only γ-HCH has insecticidal properties, accounting for only 8-15% of total synthesis output (Willett et al. 1998, Yang et al. 2023). Consequently, significant quantities of other isomers were disposed of in industrial landfills. HCH isomers, with their toxic, persistent, and bio-accumulative characteristics, result in extensive global pollution (Kumar et al. 2018, Vijgen et al. 2022).The current environment HCH contamination can be predominantly related to historical applications of technical HCH formulations and the use of Lindane (γ-HCH). In addition, the production, storage of Lindane and dumping areas of byproducts are also important contamination sources. There are 4.8 to 7.4 million tons of HCH in the environment as estimated by Vijgen and colleagues (Vijgen et al. 2022). HCH isomers can migrate downward with infiltration and were detected in groundwater, which causes contamination of drinking water sources (Pardo et al. 2021).

Bioremediation could address HCH contamination by augmenting natural biodegradation processes, which is deemed a more cost-effective and environmentally friendly alternative to chemical remediation (Alvarez et al. 2022). Given that contaminated aquifers are mainly anoxic, bioremediation with anaerobic bacteria represents an optimal remediation strategy (Coates and Anderson 2000, Wang et al. 2023a). Numerous studies have documented that anaerobic bacteria, including

Clostridium sp., *Desulfovibrio* sp., and *Desulfococcus* sp., can transform α- or γ-HCH through co-metabolism, while *Dehalococcoides* sp. can degrade multiple HCHs with metabolic processes (Badea et al. 2009, Badea et al. 2011, Bashir et al. 2018). Furthermore, Qiao and colleagues reported an enrichment culture containing *Pelobacter* that supplies hydrogen to *Dehalobacer* for the dechlorination of α-, β-, γ-, and δ-HCH (Qiao et al. 2019). Previous studies also confirmed that biodegradation is crucial for HCH transformation in groundwater (Bashir et al. 2015, Liu et al. 2017). Thus, comprehending the microbial response and behavior in anaerobic HCH transformation processes in anoxic aqueous conditions is vital in formulating effective remediation strategies.

In recent years, multi-element compound-specific isotope analysis (ME-CSIA) has been proposed as a diagnostic tool for characterizing reaction mechanisms and identifying transformation processes (Nijenhuis and Richnow 2016). Previous studies have documented the effective application of ME-CISA for characterizing transformation processes in the presence of various organohalides (Liu et al. 2020b, Min et al. 2021, Zeng et al. 2013), chlorinated ethenes (Thouement et al. 2019) and polychlorinated phenols (Huang et al. 2021). Additionally, ME-CSIA has been employed to elucidate the transformation processes of HCHs in the food chain (Wu et al. 2019) and plants (Liu et al. 2021). Furthermore, Liu et al. (2020b) employed ME-CSIA to characterize the reductive dichlorination of α- and γ-HCH by two strains of *Dehalococcoides mccartyi* and an enrichment culture. In a prior investigation, the transformation mechanisms of α-, β-, γ-, and δ-HCH by an anaerobic enrichment culture

were also characterized using ME-CSIA (Liu et al. 2020a). Compared to the enrichment culture described by Qiao and colleagues, which can degrade α-, β-, γ-, and δ-HCH isomers (Qiao et al. 2019), our previous study demonstrated distinct ratios of metabolites (benzene/MCB) (Liu et al. 2020a). Furthermore, our study proposed the cleavage of two C-Cl bonds in axial position via ring flipping for δ-HCH dichloroelimination.

Additionally, the presence of various HCH isomers could impact the microbial community's diversity and composition, as the previous study reported that the type of polybrominated diphenyl ethers (PBDEs) added as substrates could significantly alter community structure (Wang et al. 2023b). To the best of our knowledge, the effects of HCH isomers on microbial community diversity and the potential connection between microbial communities and isotopic effects during HCH transformation are not yet fully comprehended.

In order to fill this knowledge gap, anaerobic microcosms were prepared and inoculated with HCH contaminated soil different HCH isomers were used independently as electrons acceptor. ME-CISA and 16S rRNA gene amplicon sequencing was applied as the main methods for investigating the mode of bond cleavage during HCH dichloroelimination and examining the microbial communities, respectively. This study aims to (1) enrich microorganisms for HCH transformation and identify their degradation capacity of HCH isomers; (2) investigate the mode of bond cleavage during HCH dichloroelimination based on ME-CSIA, and elucidate potential relation between microbial communities and multi isotopic effects during the

transformation of HCH isomers; (3) evaluate the impact of HCH isomers on the microbial community in anoxic aqueous conditions; and (4) propose the possible dechlorinating microorganisms of the enrichment cultures.

2 Materials and Methods

2.1 Chemicals

α-HCH, β-HCH, γ-HCH and δ-HCH (analytical purity > 99%), benzene and chlorobenzene were purchased from Sigma Aldrich (Germany). n-hexane (analytical purity > 99%) was purchased from Carl Roth (Germany). More information on chemicals and media are given in the SI.

2.2 Soil samples

The soil was collected from a depth of 20-30 cm below ground at the Wuhan pesticide factory (30°33′ N, 114° 14′ E) and stored at 4 °C until usage. The main physicochemical properties of the contaminated soil were provided previously (Qian et al. 2019).

2.3 HCH degradation experiments

During the experiment, one gram of fresh weight soil was submerged into 50 mL medium solution containing α-HCH, β-HCH, γ-HCH, and δ-HCH isomers for enrichment of degrading cultures (See Figure S1). Enrichments were generated by transferring one mL of liquid from active microcosms to 50 mL fresh medium. The final enrichments utilized in the study were transferred six times, and the enriched cultures were evaluated for their capacity to degrade the remaining three isomers using an

identical amount (50 mM) of HCHs. The experimental procedure involved adding 50 mL of medium to a 120 mL serum bottle and purging it with a mixture of nitrogen and carbon dioxide gas (70% N_2, 30% CO_2) for 15 minutes to create an anoxic environment. The bottle was then sealed with a Teflon-coated stopper crimped, and autoclaved at 121 °C for 40 minutes. Hydrogen (0.5 bar overpressure) and acetate (one mL of a 0.0252 g mL^{-1} solution) were utilized as the electron donor and carbon source, respectively.

Experimental setup: Three enrichment cultures (A, R, D) were used in this study, each containing four groups, while the enrichment culture B has been investigated in our previous study (Liu et al. 2020a). For instance, the group names of the experiments with culture A were AA, AB, AR, and AD, which were spiked with α-HCH, β-HCH, γ-HCH, and δ-HCH, respectively. Additionally, a blank control group without the addition of HCHs was prepared for each group set. These samples were named as XXK where XX stands for the group name, e.g., AAK stands for the group AA without HCH addition. Figure S1 provides further details on enrichment and isolation, while the medium and cultivation conditions can be found in the supplementary information.

2.4 Sampling for isotope and concentration analysis

Five mL of saturated sodium sulfate solution at pH 1, which had been acidified with sulfuric acid, was added to the microcosm to halt biological activity. For HCH extraction, two mL of n-hexane containing an internal standard (15 mM α-HCH for γ- and δ-HCH experiments, or 15 mM δ-HCH for α- and β-HCH experiments) were added. The bottles were then agitated at 120 rpm and 10 °C for no less than four hours.

Afterward, the organic phase was allowed to separate and was collected for further analysis.

The analytical methods utilized are as follows: The concentration of benzene and CB were analyzed through gas chromatography utilizing an Agilent 6890 series gas chromatographer (GC, Agilent Technologies, Palo Alto, USA) equipped with a flame ionization detector (FID). Separation of samples was achieved using an Rtx-VMS column (30 m × 0.25 mm ID × 0.25 μm FD, Restek, Bad Homburg, Germany) (Kaufhold et al. 2013). To commence the test, one mL of sample (culture) was placed in a ten ml vial with a Teflon-coated stopper and crimp-sealed. HCH concentration analysis was previously reported by Liu et al. (2020a).

To determine the $\delta^{13}C$ isotopic values of HCH isomers, gas chromatography-isotope ratio mass spectrometry (GC-IRMS) was used. The GC-IRMS system consisted of a 6890 gas chromatograph (Agilent Technologies, Palo Alto, USA) linked to a MAT253 isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a GC IsoLink and a GC Conflo IV. A ZB-1 column (60 m × 0.32 mm ID × 1 μm FD; Phenomenex, Torrance, USA) was implemented for sample separation utilizing the temperature program previously described (Badea et al. 2009). Each sample was analyzed at minimum three times, and the resulting analyses exhibited a reproducibility rate of less than 0.5‰.

Chlorine isotope analysis of HCH isomers ($\delta^{37}Cl$) was performed using gas chromatography in conjunction with multi-collector inductively coupled plasma mass spectrometry (GC-MC-ICPMS), as described in previous studies (Horst et al. 2017,

Huang et al. 2021). Each sample was measured at least three times, producing an uncertainty of less than 0.3‰ of STDV. The obtained raw δ37Cl values were normalized to the SMOC scale by applying a two-point calibration approach using in-house reference compounds which were methyl chloride (MC, δ37Cl= +6.02‰) and trichloroethene (TCE, δ37Cl = −1.19‰). The validation of the calibration by a second trichloroethene (TCE, δ37Cl= +2.17‰) (Horst et al. 2017).

Prior to both carbon and chlorine isotope measurement, we performed the linearity test with four HCH isomers to obtain an acceptable isotope composition within the uncertainty of the instrument as reported in our previous study (Badea et al. 2009). In addition, a standard sample was set up in intervals of 5 samples during the test for quality assurance.

2.5 Stable isotope analysis

The δ notation was used to report the isotopic composition of element (E), expressed in parts per thousand (‰), with reference to the Vienna Pee Dee Belemnite (V-PDB) international standard for stable carbon isotopes and the Standard Mean Ocean Chloride (SMOC) international standard for stable chlorine isotopes, as shown in equation 1.

$$\delta E_{sample} = \frac{R_{sample}}{R_{standard}} - 1 \quad (1)$$

R_{sample} and $R_{standard}$ are the isotope ratios ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) of the sample and the standard, respectively.

The carbon isotope fractionation coefficient (ε_C) can be calculated using the Rayleigh equation as equation 2.

$$\ln\left(\frac{\delta_t+1}{\delta_0+1}\right) = \varepsilon_C * \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

The fractionation factor (ε) is obtained based on the slope of ln (C_t/C_0) vs ln[(δt^{heavy}+1)/ (δ^{light}+1)]. The uncertainty was calculated by the 95% confidence interval of the linear regression analysis.

The value of Λ is calculated according to equation 3.

$$\Lambda = \left(\delta^{13}\mathrm{C}_t - \delta^{13}\mathrm{C}_0\right)/\left(\delta^{37}Cl_t - \delta^{37}Cl_0\right) \quad (3)$$

The calculation of apparent kinetic isotope effects (AKIE) was done by equation 4.

$$AKIE_e \approx \frac{1}{1+\frac{nz}{x}*\varepsilon_e} \quad (4)$$

Where: n is the number of all atoms in the HCH molecule; x is the number of reactive positions; z is the number of indistinguishable reactive positions resulting in intramolecular competition.

2.6 Soil DNA extraction, PCR amplification and Illumina sequencing

Total DNA was extracted from the samples at various time points using the Fast DNA® SPIN Kit for soil (QBIO gene Inc, Carlsbad, CA, USA) in accordance with the manufacturer's guidelines. The DNA concentration was assessed using the Nanodrop® ND-2000 spectrophotometer (NanoDrop Technologies, Montchanin, USA). The quality of the DNA was then verified through 1.2% agarose gel electrophoresis. Specific primers (bacterial 515F/806R) were used to amplify the 16S rRNA gene. The PCR procedure was performed following the manufacturer's instructions. PCR amplification of target fragments was conducted with sample-specific barcode sequences together with negative control. The PCR amplification recovery products underwent fluorescence quantification using a Microplate reader (BioTek, FLx800) in conjunction with the Quant-iT PicoGreen dsDNA Assay Kit. DNA was later sequenced through high-throughput sequencing post the creation of sequencing libraries utilizing

Illumina's TruSeq Nano DNA LT Library Prep Kit. Additional details can be found in the fourth section of the supplementary information.

2.7 Data Analysis

The high-quality primary screening resulted in sequence division into libraries and samples based on index and barcode information. Subsequently, barcode sequences were eliminated, and QIIME2 DADA2 analysis was employed for sequence denoising and OTU clustering (Callahan et al. 2016). The microbial communities from four cultures transforming diverse HCH isomers were then compared through QIIME2 (2019.4) and visualized using RStudio (2023.06.0 Build 421, R version 4.3.0). Species composition maps display the unique composition and analysis of each sample at varying taxonomic levels. Statistical analyses were performed to identify degradation-related key species by determining the significance of differences between samples. Alpha diversity parameters of the microbial community, specifically Chao1 and Ace, were estimated using QIIME2 (2019.4). For the non-metric multidimensional scaling (NMDS), the Bra-Curtis distance was calculated using the ‘vegdist’ function in the ‘vegan’ package. To conduct the data ordering analysis, the metaMDS function was utilized. The figures were generated using RStudio (2023.09.0 Build 463, R version 4.3.0) and the R ggplot2 library.

3. Results and Discussion

3.1 Anaerobic transformation of HCH isomers

The experiment evaluated the degradation capacity of cultures pre-enriched with individual HCH isomers to degraded other isomers over three months. The

transformation of HCH was monitored by analyzing concentrations of benzene and chlorobenzene over time (data not shown). The four cultures exhibited variations in degradation performance for different HCH isomers. Culture A and R only transformed α- and γ-HCH, whereas culture D degraded α-, γ- and δ-HCH. Only Culture B was able to degrade all four hexachlorocyclohexane (HCH) isomers, as reported by Liu et al. (2020a). Accordingly, the α- and γ-HCH isomers proved to be the most readily transformable substrates across different cultures, which is consistent with the findings of Qian et al. (2019). Different cultures (A, B, R, D) exhibited distinct transformations of HCH isomers, which may be attributed to their respective bacterial communities or isomer characteristics. Based on molecular structure, γ-HCH possesses three axial C-Cl bonds, α-HCH has two, δ-HCH has only one, whereas all C-Cl bonds in β-HCH are equatorial. A prior research study by Qiao et al. (2019) revealed that chlorine atoms situated axially had a greater propensity to be eliminated compared to those in equatorial position during the reductive transformation of HCH isomers. Consequently, variation in the number of axial chlorine atoms results in diverse levels of persistence for different HCH isomers related to biotransformation. Buser and Mueller (1995) demonstrated that the HCH isomers were degraded in anaerobic sludge with varying degradation rates, which depends on the stereochemistry. The transformation rates can be affected by more factors, e. g, microbial community, than the stereochemistry of the HCH alone. For example, in our previous studies, the culture enriched originally with β- HCH, showed a much faster transformation rate compared to the other three isomers (Liu et al. 2020a). But during the fractionation experiments in the present study, the

transformation rate were γ-HCH > α-HCH > β-HCH >δ- HCH, as predicted by the stability of isomers dependent on axial and equatorial C-Cl bonds, which is similar to previous studies (Srivastava et al. 2023). It is generally assumed that α- and γ-HCH can be degraded more easily than β- and δ-HCH by the four cultures. This is in agreement with previous studies that the chlorine atoms in β- and δ-HCH are predominantly resistant to dichloroelimination (Padmakar. et al. 1994, Quintero et al. 2006). However, it was verified by our studies that there was a link between the rate of conversion of different isomers and the microbial community. Thus, it is hypothesized that during various modes of bond cleavage in the dichloroelimination, there exist stereochemical hindrances or differing levels of energy required for enzymatic transformation of at least two C-Cl bonds in axial (α- and γ-HCH) or equatorial (β- and δ-HCH) position, as well as one in axial and one in equatorial position (δ-HCH). This may result in varying levels of biodegradability among HCH isomers.

3.2 ^{13}C and ^{37}Cl isotope fractionation of the HCH isomers

Stable isotope analyses were conducted across all experimental groups for calculating isotope enrichment factors (ε) and apparent kinetic isotope effects (AKIE). The transformation of HCH resulted in a significant isotopic enrichment indicate primary isotope effects of C-Cl bond cleavage, and the fractionation process was quantified with mostly reasonable uncertainty (Confidence interval of 95%) using the Rayleigh equation.

3.2.1 Isotope fractionation

The carbon isotope enrichment factor (ε_C) values for the transformation of α-HCH

by the three cultures were -3.3 ± 0.2‰ (culture A), -4.2 ± 0.6‰ (culture R), and -3.6 ± 0.6‰ (culture D) (refer to Figure 1A). Regarding γ-HCH, the ε_C values were -4.3 ± 0.3‰ (culture A), -3.8 ± 0.4‰ (culture R), and -3.9 ± 0.6‰ (culture D) (refer to Figure 2A). These findings were consistent with previous studies (see Table 1) (Badea et al. 2009, Badea et al. 2011, Bashir et al. 2018, Liu et al. 2020a, Liu et al. 2020b). The ε_C of δ-HCH transformation was -7.3 ± 0.3‰ (Figure 3A), which was statistically similar ($p>0.05$) to the ε_C (-6.4 ± 0.7‰) reported in a prior investigation (Liu et al. 2020a).

The chlorine isotope enrichment factors (ε_{Cl}) for the transformation of α-HCH by the three cultures were determined to be -2.6 ± 0.7‰ (culture A), -2 ± 0.5‰ (culture R), and -1.6 ± 0.1‰ (culture D) (see Fig.1B). Similarly, the ε_{Cl} for γ-HCH were found to be -4.2 ± 0.7‰ (culture A), -3.1 ± 0.7‰ (culture R), and -3.7 ± 0.5‰ (culture D) (Figure 2B). The values obtained for both ε_C and ε_{Cl} are in line with those reported in previous studies of HCH isomer degradation (see Table 1). This agreement on ε values across various studies and cultures demonstrates the reliability of CSIA in assessing HCH transformation in intricate settings.

3.2.2 Apparent kinetic isotope effect

To better comprehend the reaction mechanism, we calculated the apparent kinetic isotope effect (AKIE) for two scenarios: 1) stepwise bond cleavage, and 2) concerted bond cleavage of two C-Cl bonds, as presented in Table 2. Assuming stepwise bond cleavage, we observed that $AKIE_C$ values for α- and γ-HCH were comparable. The measured values were significantly higher than the reference values (Liu et al. 2020a, Liu et al. 2020b), which points to a concerted bond cleavage. A corresponding trend

was also present in the assessment of $AKIE_{Cl}$. The values for $AKIE_C$ and $AKIE_{Cl}$ regarding δ-HCH were similar to those of the previous study (Liu et al. 2020a). Interestingly, the $AKIE_C$ values for δ-HCH were considerably greater than those for α- and γ-HCH as well as the value of β-HCH during transformation by culture B (Table 2). Also, the $AKIE_C$ value of δ-HCH resulting from the transformation by culture D was identical to that by culture B, indicating that the difference in $AKIE_C$ between δ-HCH and other isomers was not due to varying microorganisms but rather the HCH isomers themselves. Based on the analysis of $AKIE_C$, it appears likely that the isotope effect was similar when both C-Cl bonds are in the same orientation, such as α- and γ-HCH with at least two C-Cl bonds in axial position while β-HCH has all the C-Cl in the equatorial position. Therefore, it is reasonable to assume that the dichlorelimination of δ-HCH, which yielded a higher $AKIE_C$, resulted from a different mode of bond cleavage (one C-Cl bond in the axial position and another one in the equatorial position). Additionally, it is worth mentioning that there was no significant difference in the $AKIE_{Cl}$ values between various HCH isomers. It may be more precise to conduct multiple isotope analysis for a more accurate assessment of diverse bond cleavages.

3.2.3 C-Cl isotope effect

The study compared the transformation mechanism of HCH across different cultures, using a dual-element isotope analysis (Λ). The Λ value for α-HCH transformation (Figure 1C) was nearly identical among the three cultures (B, R, D) and fell within the range of previous results (1.7 ± 0.2 to 2.0 ± 0.2, Table 1) (Liu et al. 2020a, Liu et al. 2020b). These results suggest a comparable isotopic effect during the

transformation of HCHs across the four cultures. However, the Λ value of Culture A (1.2 ± 0.2, Table 1) was significantly lower than the values of the other cultures, suggesting that the transformation process in Culture A differs from that in the other cultures. Even with the same mode of bond cleavage, it led to a distinct isotopic effect. Unlike α-HCH, the Λ values for γ-HCH (Figure 2C) were almost identical and within the range of prior research (refer to Table 1). The Λ value of δ-HCH (Figure 3C) was nearly identical to the Λ value of δ-HCH transformation by culture B (Table 1), suggesting a comparable reaction mechanism for δ-HCH transformation by culture B and D.

To compare the isotopic effect of different HCH isomers during the transformation, we utilized and assessed the correlation of $AKIE_C/AKIE_{Cl}$. The $AKIE_C/AKIE_{Cl}$ values of γ-HCH for the three cultures were comparable (1.000 ± 0.003 to 1.003 ± 0.004) and fell within the same range as previous studies (1.002 ± 0.003 to 1.004 ± 0.003) (Liu et al. 2020a, Liu et al. 2020b), providing evidence that the dechlorination of γ-HCH by these three cultures follows the same bond cleavage mechanism as *Dehalococcoides mccartyi* strains. Interestingly, the $AKIE_C/AKIE_{Cl}$ value for α-HCH by Culture A (1.002 ± 0.002) falls within the range of the values observed for γ-HCH transformed by the three cultures (1.000 ± 0.003 to 1.003 ± 0.004) (Table 2), suggesting a comparable isotopic effect during dichloroelimination with the same mode of bond cleavage. Similarly, the $AKIE_C/AKIE_{Cl}$ values for γ-HCH in this study (1.000 ± 0.003 to 1.003 ± 0.004) fell within the range of values reported in a prior study by Liu et al. (2020a) during β-HCH transformation (1.002 ± 0.002) by culture B. Our previous research

suggested that the similar isotopic effect was due to bond cleavage of two C-Cl bonds in axial position by flipping the carbon ring of β-HCH, which changed all six C-Cl bonds from equatorial position to axial position. In this case, it is reasonable to assume that the degradation of β-HCH may occur more easily than that of other isomers because of the six C-Cl bonds in axial position by flipping (as shown in Figure S2). This contradicts the common opinion γ-HCH can be transformed more easily than β-HCH. Therefore, the present study suggests that the mode of bond cleavage in β-HCH dichloroelimination involves two C-Cl bonds in equatorial position, resulting in a similar isotopic effect as dichloroelimination of two C-Cl bonds in axial position (e.g. α- and γ-HCH). Furthermore, the values of $AKIE_C/AKIE_{Cl}$ for δ-HCH were similar during the transformation processes carried out by cultures D and B. These values were also higher than those of other isomers, which confirmed the hypothesis based on $AKIE_C$ evaluation that the dichloroelimination of δ-HCH involves one C-Cl bond in axial position and another in equatorial position.

In summary, we propose that the two bonds involved in dichloroelimination of β-HCH are in equatorial position, while that of δ-HCH involves one in axial position and the other in equatorial position. In addition, the dichloroelimination involved two bonds that are either in axial or equatorial position, e.g., the dichloroelimination of α- and β-HCH, may have a similar isotopic effect, whereas a different isotopic effect may be observed when one C-Cl bond is in the axial position and the other in the equatorial position.

3.3 Prokaryotic communities associated with HCH transformation

3.3.1 Microbial diversity of Prokaryotic communities in the enrichment cultures

The prokaryotic communities of the four enrichment cultures (A, B, R, D) were determined through high-throughput sequencing of 16S rRNA genes. After filtering the assembled reads, a total of 10,503 OTUs were obtained. The enrichment cultures displayed distinct prokaryotic community compositions that differed from those of the original soil.

Prokaryotic community diversity and richness of the enrichment cultures and original soil were evaluated using the Chao 1 and Ace indices (see Figure S3). The results show that the original soil displayed greater levels of species richness and community diversity in contrast to the enrichment cultures. This suggests that the cultivation condition alter the structure of the prokaryotic community. It is noteworthy that the four isomers had varying effects on the prokaryotic community structure. Culture B exhibited less diversity and richness compared to the other three cultures, suggesting that β-HCH may have a more significant toxic impact on the community.

Furthermore, a Non-metric Multidimensional Scaling (NMDS) ordination plot illustrates the differentiation of prokaryotic organisms with individual isomers in each culture. The observed variation in separation among different treatments indicates the existence of distinct prokaryotic communities. Culture R showed a significant difference in comparison to the other cultures (Figure S4).

3.3.2 Differences in prokaryotic community composition among the four transformation systems

The analysis at both the phylum and genus levels revealed the noteworthy effect of HCH isomers on the prokaryotic community in the cultures. Figure 4 and 5 show the domain microorganisms in each group (A, B, R, D) at the phylum and genus levels, respectively. In addition, the comparison of community compositions before and after spiking of HCH in four enrichment cultures at the phylum and genus levels are shown in Figure S5 to S8. As shown in Figure 4, culture A exhibited a more even distribution at the phylum level, with *Chloroflexi*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* comprising the majority. The main phyla identified in Culture B comprised *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. Likewise, Culture R and D showed similar major phyla, that is, *Actinobacteria*, *Proteobacteria*, and *Firmicutes*, except a substantially higher percentage of *Actinobacteria* in Culture R. While there were significant differences observed in the relative abundance of the major phyla across the four cultures, the dominant community members remained similar. The abundance of *Actinobacteria* was lower in group AA compared to groups BB, RR, DD, and the original soil. The results indicate that α-HCH has a greater inhibitory effect on *Actinobacteria* than other HCH isomers. Furthermore, the relative abundance of *Chloroflexi* was approximately 10% in the original soil, but *Chloroflexi* almost completely disappeared in groups BB, AB, RB, and DB after adding β-HCH, suggesting that *Chloroflexi* is vulnerable to β-HCH. In group RR, *Actinobacteria* had a significantly higher relative abundance than that in groups AA, BB, DD, and the

original soil. Comparing with group RRK, it was apparent that the increased relative abundance of *Actinobacteria* in group RR was likely due to γ-HCH, which has little to no effect on *Actinobacteria* growth but impedes the growth of other bacteria, for example, *Proteobacteria* (Figure S7). In the group DD (Figure 4), the relative abundance of *Proteobacteria* was higher when compared to the groups AA, BB, and RR, but lower than that of the original soil. Additionally, the cultivation with δ-HCH increased the relative abundance of *Proteobacteria,* as showed in groups AD, BD, and RD compared to AA, BB and RR, respectively. This suggests that the inhibitory impact of δ-HCH on *Proteobacteria* may be lower than that of the other three isomers.

At the genus level, each isomer had a more specific impact on community composition in the culture, as depicted in Figure 5. There was a significant difference in the prokaryotic community composition between the original soil samples and the enriched cultures overall. Moreover, the α-analyses in Figure S3 demonstrated a higher microbial diversity in the original soil sample. The twenty most prevalent genera in the enrichment cultures accounted for less than 10% of the initial soil samples (data not shown). Group AA exhibited a greater abundance of *Desulfitobacterium* in comparison with groups BB, RR, DD, and the original soil. *Desulfitobacterium* was detected in all four groups of culture A (AA, AB, AR, AD) and showed the highest relative abundance in group AA, indicating that α-HCH could facilitate its growth. Previous studies have reported that *Desulfitobacterium* has promising dechlorination abilities for 2,4,6-trichlorophenol and PCE (Puyol et al. 2010, Wan et al. 2019), suggesting its potential for α-HCH transformation. However, *Desulfitobacterium* was not highly abundant in

the other three cultures (B, R, D), which were completely depleted during transfers. An unclassified bacterium within the phylum *Chloroflexi* was detected and referred to as uncultured. *Chloroflexi* represents the primary phylum of reductive dechlorinators, suggesting that uncultured bacteria possess dichlorination potential. Nevertheless, in the β-HCH supplemented experiments *Chloroflexi* were not detected based on the phylum-level analyses as mentioned earlier. Thus, it can be inferred that the growth of *Chloroflexi* was not sustained by β-HCH. The genus of *OPB41*, which belongs to phylum *Actinomycetes*, showed a significantly higher relative abundance in culture R, while also maintaining high abundance in the control group. This suggests that the microorganisms belong the genus of *OPB41* may not be sensitive to γ-HCH.

In addition, the Heat map of community composition for culture A, B, R, D after spiking different HCH isomers were shown in Figure S9-12. Significant variations of the domain microorganisms were observed after different HCH isomers were spiked, which confirmed the impact of HCH isomers on the microbial community compositions.

3.3.3 Effects of HCHs on community functioning

Co-occurrence network analyses based on the stress of α-, β-, γ- and δ-HCH for the investigated cultures (A, B, R, and D) are shown in Figure 6, while the analyses for each culture under individual HCH stress conditions are shown in Figure S13 to S16. As shown in Figure 6, cultures under the stress of γ- and δ-HCH exhibited a comparatively greater number of nodes and edges than those under the stress of α- and β-HCH, similar results were also observed when one culture cultivated with different HCH isomers (Figure S13-S16). The most robust correlations were found

predominantly in the *Proteobacteria* and *Firmicutes* phyla, suggesting their potential role as major dechlorinating bacteria as reported in previous studies (Adrian and Löffler 2016). The detected functional genera were present in all groups, indicating potential for degradation even among those unable to transform all isomers. The *Chloroflexi* phylum was noted as the other major dechlorinator, exhibiting low correlations solely in cultures R and D (Figure S15, S16). The *Actinobacteria* phylum, to which OPB41 belongs, exhibited high abundance but low connectivity in culture R (Figure S15). This suggests that these bacteria were not the keystone taxa for HCH transformation, as we hypothesized in the last section. Based on the analysis of degradation results, it was found that *Desulfosporosinus* within the *Firmicutes* phylum demonstrated a high level of connectivity for α-HCH, except for group BA (Figure S13). This suggests that the keystone taxa involved in the reductive transformation of α-HCH may not be consistent among different groups. The genera exhibiting the greatest degree of connectivity during the transformation of β-HCH and γ-HCH were present within *Firmicutes*, specifically *Unclassified_Clostridiaceae_1* (with a connectivity degree of 11) and *Caproiciproducens* at the genus level, respectively. In the transformation of δ-HCH, the genera detected in the groups BD and DD demonstrated a high degree of connectivity within the phylum *Proteobacteria* (Figure S16). The results suggest that the keystone species could vary depending on the isomer being transformed by the same enrichment culture, and different groups may have varying keystone species for transforming the same isomer.

4. Conclusions

This study investigated different cultures enriched from the same contaminated soil with individual HCH isomers in anoxic aqueous conditions. The ability of the cultures to transform other HCH isomers was found to be inconsistent when cultivated with a specific HCH isomer. This study reports the ε_C, ε_{Cl} and Λ values of HCH isomers during the transformation by different enrichment cultures. New modes of bond cleavage for β-and δ-HCH during dichloroelimination are proposed based on ME-CSIA. For β-HCH, the dichloroelimination involves two neighboring C-Cl bonds in the equatorial position, while one C-Cl bond is in the axial position and the other one is in the equatorial position for δ-HCH. The different bond cleavage leads to distinct isotopic effects. The microbial community compositions of the cultures cultivated with different HCH isomers in the aqueous conditions were significantly different. This indicates the potential impact of HCH isomers on groundwater microecosystems. In addition, bacteria in the phyla *Proteobacteria*, and *Firmicutes* were proposed as the main dechlorinators based on their key correlation with other members and their compositions in the community. This study promotes the application of ME-CSIA for diagnostic identifying and evaluating the fate of HCH in groundwater.

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CRediT authorship contribution statement

Xinyu Zhang: Investigation, Writing – original draft. Yan Zheng: Investigation, Writing – original draft. Ziming Su: Methodology, Writing – review & editing. Zhenyu Wang: Methodology. Jian Zhang: Investigation. Zhongjun Jia: Writing –review & editing. Steffen Kümmel: Methodology, Writing – review & editing. Chengrong Qin: Conceptualization, Writing – review & editing. Yaqing Liu: Conceptualization, Writing – original draft, Writing – review & editing. Shuangfei Wang: Conceptualization. Ivonne Nijenhuis: Writing – review & editing. Hans H. Richnow: Writing – review & editing.

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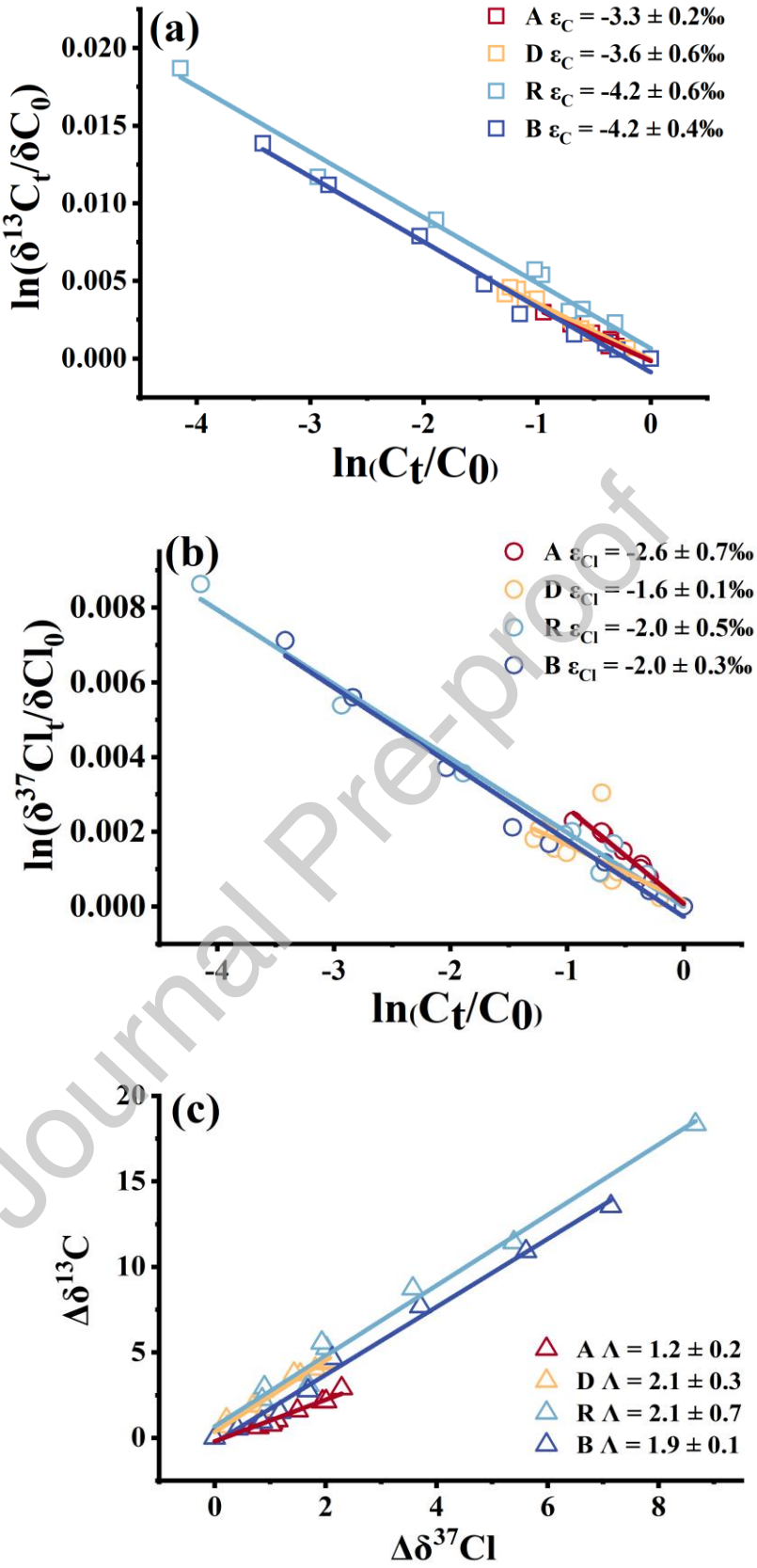


Figure 1. Rayleigh plot of the carbon (a) and chlorine (b) fractionation and the dual isotope plot (c) of α-HCH isomers. (Color code: red = culture A; dark blue = culture B; sky blue = culture R; orange = culture D).

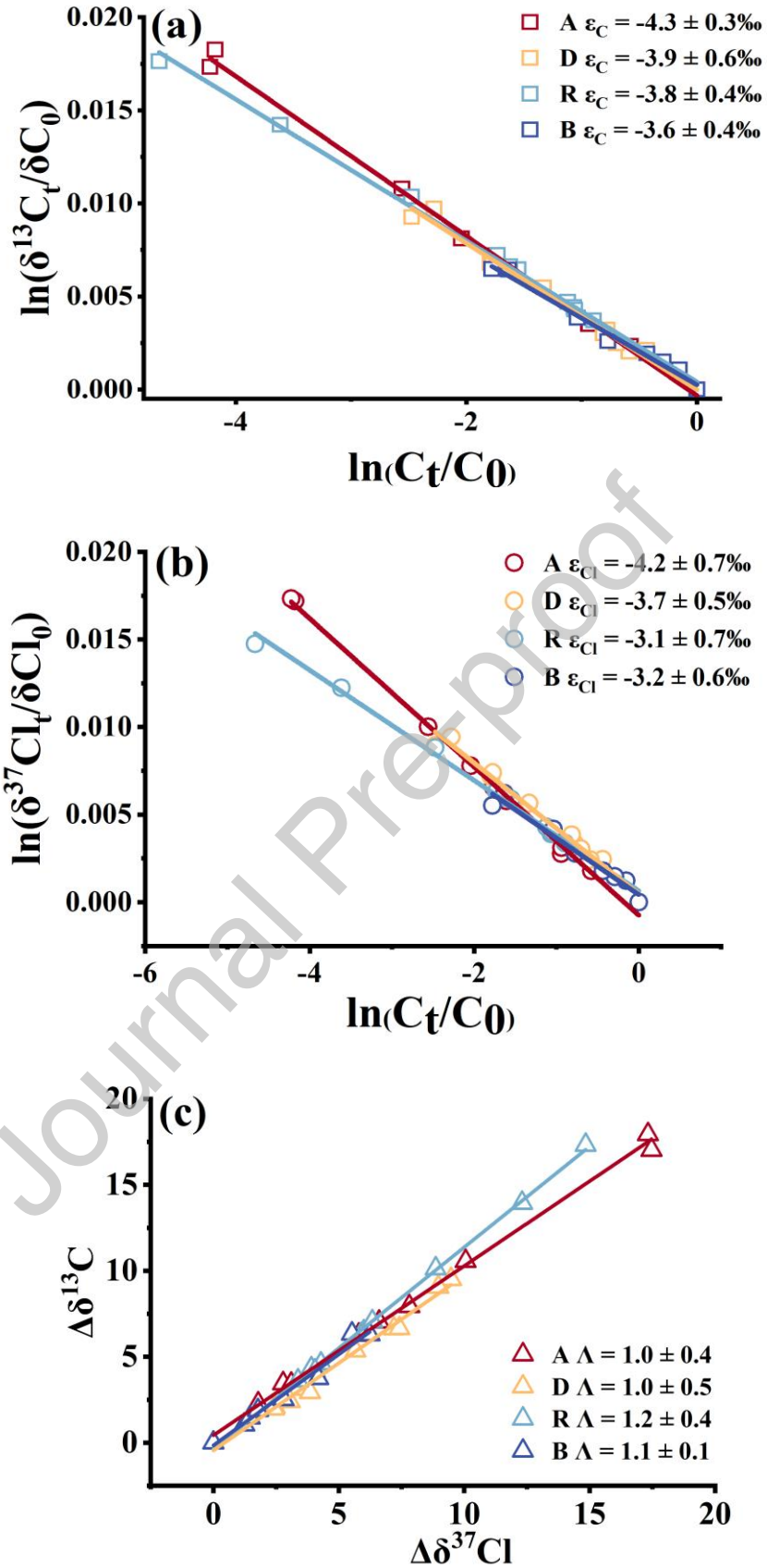


Figure 2. Rayleigh plot of the carbon (a) and chlorine (b) fractionation and the dual isotope plot (c) of γ-HCH isomer. (Color code: red = culture A; dark blue = culture B; sky blue = culture R;

orange = culture D).

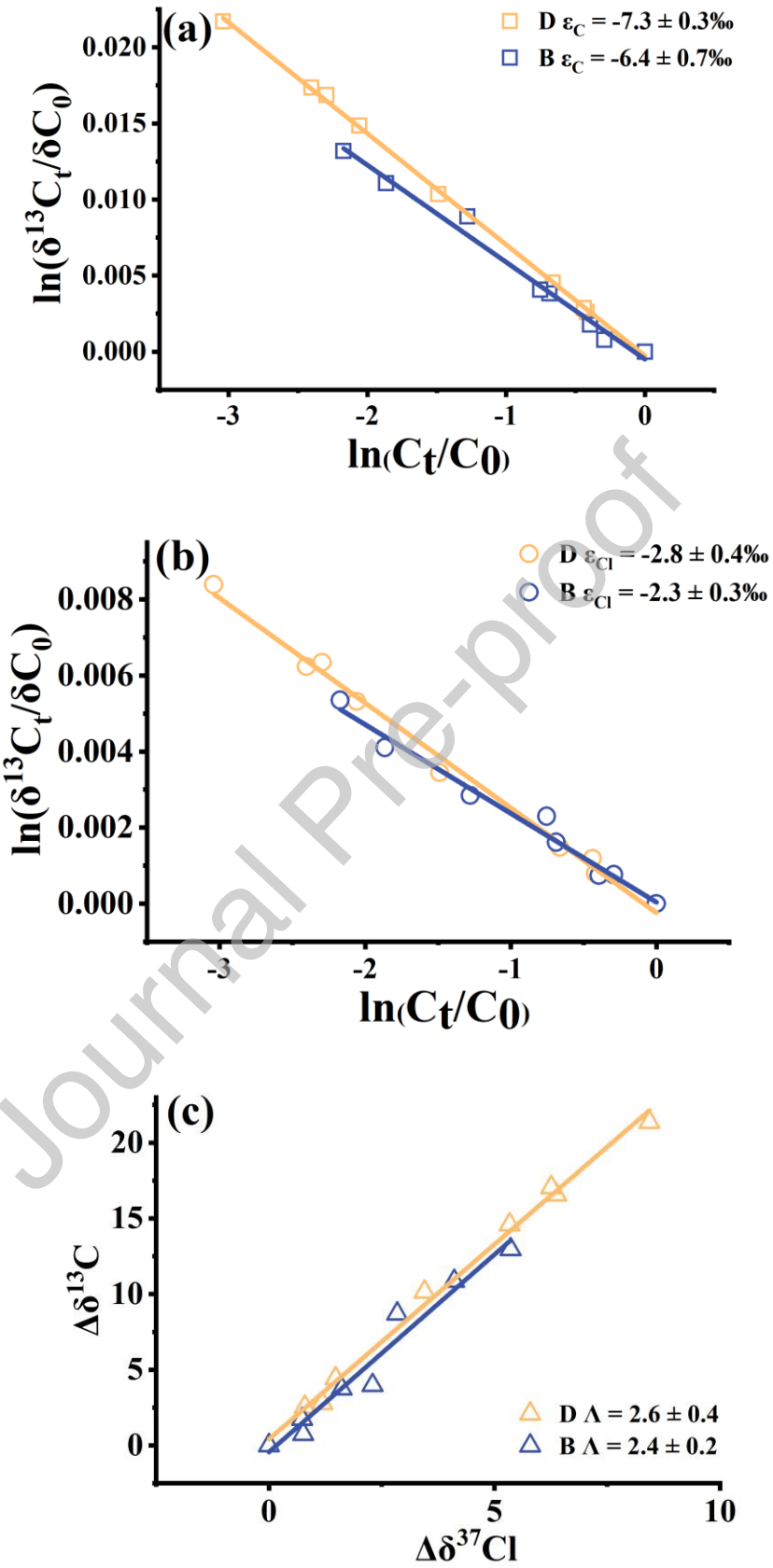


Figure 3. Rayleigh plot of the carbon (a) and chlorine (b) fractionation and the dual isotope plot

(c) of δ-HCH isomer. (Color code: dark blue = culture B; orange = culture D)

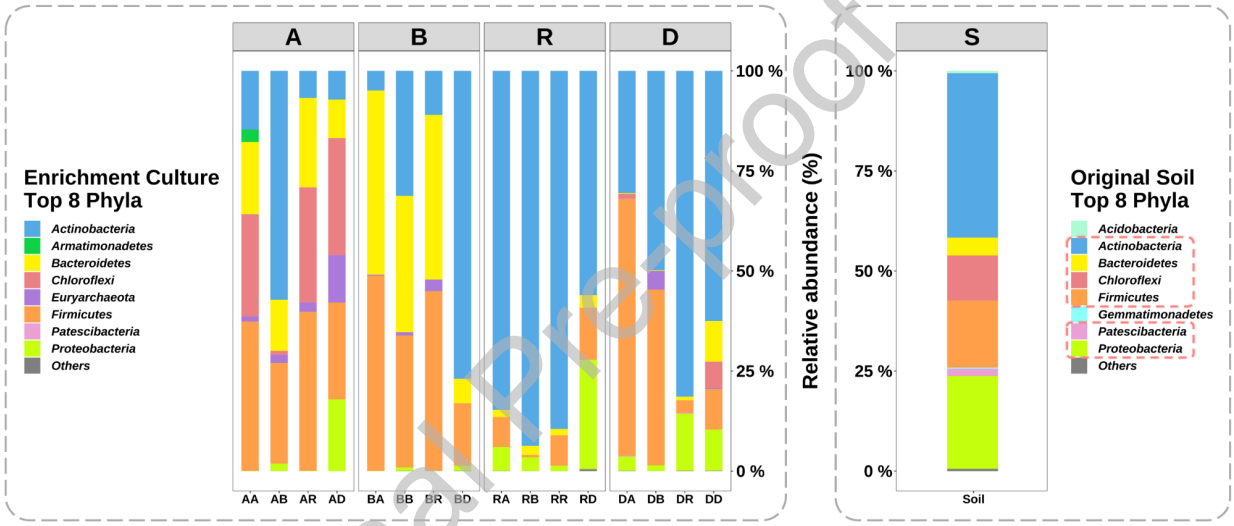


Figure 4. Top 8 phylum genera for each group of four enrichment cultures (left panel, up to 99% of the total community) and original soil (right panel, up to 99% of the total community) at the phylum levels. The phyla in the red dashed box are those that overlap with the top 8 ones in the enrichment cultures.

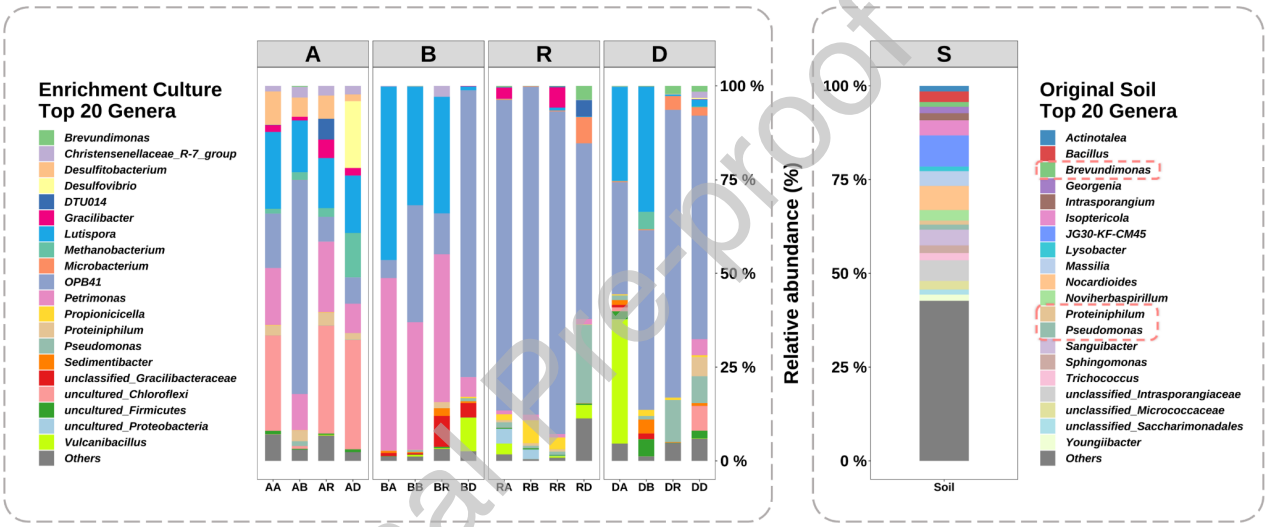


Figure 5. Top 20 genera for each group of four enrichment cultures (left panel, up to 95% of the total community) and original soil (right panel, up to 55% of the total community) at the genus levels. The genera in the red dashed box are those that overlap with the top 20 ones in the enrichment cultures.

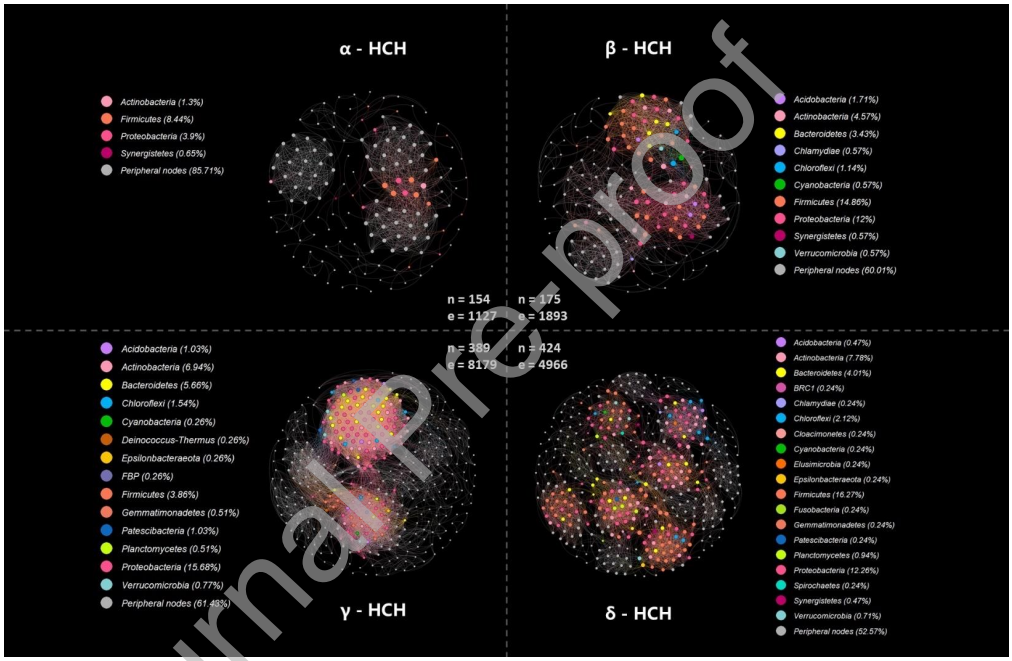


Figure 6. Co-occurrence network analyses for the enrichment cultures under the stress of α-, β-, γ- and δ-HCH. Colored nodes indicate keystone species (degree of connectivity ≥ 10 except where noted) and different colors represent the classification of each species (genus) at the phylum level. The percentage value refers to the ratio of the number of species contained in each phylum level to the total network. Grey dots indicate peripheral nodes (degree of connectivity < 10). The letter n represents the total number of nodes and e represents the number of edges. The size of the nodes indicates the degree of connectivity.

Table 1 Summary of isotopic enrichment factors (ε_C and ε_{Cl}) and Λ values of the four enrichment cultures investigated in the current study (Badea et al. 2009, Badea et al. 2011, Bashir et al. 2018, Lian et al. 2018). Data for enrichment culture B were taken from a previous study (Liu et al. 2020a, Liu et al. 2020b).

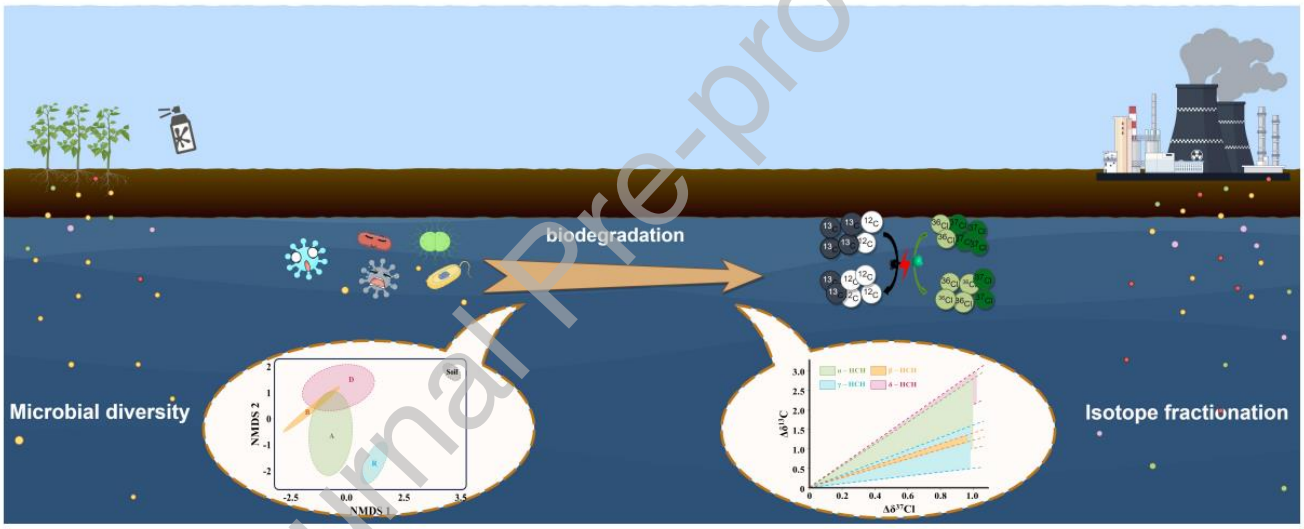
	α-HCH			β-HCH			γ-HCH			δ-HCH		
	ε_C(‰)	**ε_{Cl}(‰)**	**Λ**	**ε_C(‰)**	**ε_{Cl}(‰)**	**Λ**	**ε_C(‰)**	**ε_{Cl}(‰)**	**Λ**	**ε_C(‰)**	**ε_{Cl}(‰)**	**Λ**
Enrichment culture A	**-3.3± 0.2**	**-2.6± 0.7**	**1.2 ± 0.2**				**-4.3± 0.3**	**-4.2 ± 0.7**	**1.0 ± 0.4**			
Enrichment culture R	**-4.2± 0.6**	**-2.0± 0.5**	**2.1 ± 0.7**				**-3.8± 0.4**	**-3.1 ± 0.7**	**1.2 ± 0.4**			
Enrichment culture D	**-3.6± 0.6**	**-1.6± 0.1**	**2.1 ± 0.3**				**-3.9± 0.6**	**-3.7 ± 0.5**	**1.0 ± 0.5**	**-7.0± 0.3**	**-2.8± 0.2**	**2.6± 0.4**
Enrichment culture B*	-4.2± 0.4	-2.1± 0.3	1.9 ± 0.1	-1.9 ± 0.3	-1.6 ± 0.2	1.3 ± 0.1	-3.6± 0.4	-3.2 ± 0.6	1.1 ± 0.1	-6.4± 0.7	-2.3± 0.3	2.4± 0.2
Dehalococcoides mccartyi strain 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			
Dehalococcoides mccartyi strain 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 (Bitterfeld)#	-3.0± 0.4	-1.4± 0.3	2.0± 0.3				-4.0± 0.5	-2.9± 0.4	1.1 ± 0.2			
Clostridium pasterianum	-3.7± 0.8@						-3.3± 0.5&					
Desulfovibrio gigas							-4.1± 0.6&					
*Desulfococcus mulivorans*a							-3.9± 0.6^					
Anaerobic digestion systemd	-3.8± 0.3$						-3.4± 0.5^					
							-3.2± 0.2$					

Table2: Summary of apparent kinetic isotope effects ($AKIE_C$ and $AKIE_{Cl}$) and $AKIE_C/AKIE_{Cl}$ values of the four enrichment cultures for investigated in the current study.

	C	α-HCH			β-HCH			γ-HCH			δ-HCH		
	a b	n/x/z =6/2/1 n/x/z =6/2/2			n/x/z =6/6/6 n/x/z =6/6/6			n/x/z =6/3/2 n/x/z =6/3/3			n/x/z =6/5/4 n/x/z =6/3/2 n/x/z =6/5/5 n/x/z =6/3/3		
		$AKIE_C$	$AKIE_{Cl}$	$AKIE_C/AKIE_{Cl}$	$AKIE_C$	$AKIE_{Cl}$	$AKIE_C/AKIE_{Cl}$	$AKIE_C$	$AKIE_{Cl}$	$AKIE_C/AKIE_{Cl}$	$AKIE_C$	$AKIE_{Cl}$	$AKIE_C/AKIE_{Cl}$
Enrichment culture A	**a**	**1.020±0.001**	**1.016± 0.003**	**1.004±0.003**				**1.027±0.002**	**1.026±0.004**	**1.001 ± 0.004**			
	b	**1.010±0.001**	**1.008± 0.002**	**1.002 ± 0.002**				**1.017±0.001**	**1.017 ± 0.003**	**1.000 ± 0.003**			
Enrichment culture R	**a**	**1.026±0.006**	**1.012± 0.003**	**1.014±0.007**				**1.024±0.002**	**1.019±0.004**	**1.005 ± 0.004**			
	b	**1.013±0.002**	**1.006± 0.002**	**1.007 ± 0.003**				**1.015±0.002**	**1.012 ± 0.003**	**1.003 ± 0.004**			
Enrichment culture D	**a**	**1.022±0.004**	**1.010± 0.006**	**1.012±0.007**				**1.024±0.003**	**1.022±0.003**	**1.002 ± 0.004**	**1.044± 0.002**	**1.017± 0.001**	**1.027 ± 0.002**
	b	**1.011±0.002**	**1.006± 0.001**	**1.005 ± 0.002**				**1.016±0.003**	**1.015 ± 0.002**	**1.001 ± 0.004**	**1.036± 0.002 1.030 ±0.001**	**1.013± 0.001 1.011 ±0.002**	**1.023± 0.002 1.019 ±0.001**
Enrichment culture B*	a	1.026 ± 0.003	1.012± 0.002	1.014±0.004	1.012 ± 0.002	1.010 ± 0.001	1.002 ± 0.002	1.022 ± 0.003	1.020±0.004	1.003 ± 0.005	1.040 ± 0.005	1.014 ± 0.002	1.030 ± 0.005
	b	1.013 ± 0.001	1.006± 0.001	1.006 ± 0.002	1.012± 0.002	1.010 ± 0.001	1.002 ± 0.002	1.015 ± 0.002	1.013 ± 0.002	1.002 ± 0.003	1.032 ± 0.004 1.027 ± 0.003	1.011± 0.001 1.009 ±0.001	1.020± 0.004 1.017 ±0.003

Note: The AKIE values were calculated based on the following scenarios: (a) stepwise bond cleavage, and (b) concerted bond cleavage. For α- and γ-HCH, only chlorine atoms in the axial positions of the main conformers are consider to be involved in the transformation. For β-HCH, all the chloride atoms are equal. For δ-HCH, two cases were considered: (1) One reactive chlorine atom is in axial position, the other one is in equatorial position, and (2) the axial chlorine atom is not involved in the reaction, the two reactive vicinal chlorine atoms are in equatorial positions. * reductive dichloroelimination of four HCH isomers by anaerobic culture (Elsner et al. 2007, Hofstetter et al. 2007, Liu et al. 2020a, Liu et al. 2020b, Palau et al. 2014).

Graphical Abstract



Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.