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Stable Isotope Fractionation of γ-Hexachlorocyclohexane (Lindane) during Reductive Dechlorination by Two Strains of Sulfate-Reducing Bacteria

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Carbon isotope fractionation factors were determined with the dichloro elimination of γ-hexachlorocyclohexane (γ-HCH) by the sulfate-reducing bacteria *Desulfococcus multivorans* DSM 2059 and *Desulfovibrio gigas* DSM 1382. Both strains are known for cometabolic HCH dechlorination. Degradation experiments with γ-HCH in concentrations of 22−25 μM were carried out using benzoate (for *D. multivorans*) and lactate (for *D. gigas*) as electron donors, respectively. γ-HCH was dechlorinated by both bacterial strains within four weeks, and the metabolites γ-3,4,5,6-tetrachlorocyclohexene (γ-TCCH), chlorobenzene (CB), and benzene were formed. The carbon isotope fractionation of γ-HCH dechlorination was quantified by the Rayleigh model, using a bulk enrichment factor (εC) of -3.9 ± 0.6 for *D. gigas* and -3.4 ± 0.5 for *D. multivorans*, which correspond to apparent kinetic isotope effect ($AKIE_C$) values of 1.023 ± 0.004 or 1.02 ± 0.003 for stepwise Cl−C bond cleavage. The extent and range of isotope fractionation suggest that γ-HCH dechlorination can be monitored in anoxic environments by compound-specific isotope analysis (CSIA).

Introduction

Heavy use of organochlorine insecticides has led to the dispersal of these pollutants throughout the global environment. Among them, a compound of major concern is the γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane (γ-HCH), also known as lindane. Technical-grade lindane typically contains 10−15% γ-HCH, whereby the remaining fraction consists of α-, β-, δ-, and ε-HCH isomers, which are not insecticidally active (*1*); even purified lindane is thought to contain traces of other HCH isomers (*2*). All HCH isomers are toxic and considered to be carcinogenic (*3*) and thus of major concern for human health; these compounds can persist for years in the environment (*2*). The persistence of each HCH isomer is attributed to the orientation of the chlorine atoms on the molecule, which can be axial (a) or equatorial (e). It is thought that axially oriented chlorine atoms are more available for enzymatic attacks than equatorial chlorine atoms. Thus, γ-HCH having three axially oriented and three equatorially oriented chlorine atoms (aaaeee) is more easily biodegraded than δ-HCH (aeeeee) or β-HCH (eeeeee) (*2*). Approximately 10 million tons of technical HCH were released into the environment until 1997 (*1*). HCH isomers are semivolatile and thus can be transported by air over long distances to remote locations (*3, 4*), due to the poor biodegradability, especially under oxic conditions (*5*), resulting in world-wide distribution of trace amounts of HCHs in air, soil, and water (*6*). Additionally, HCH isomers were discarded in large quantities at open air dump sites as byproducts of the production of γ-HCH, leading to heavily HCH-contaminated soils and river sediments in The Netherlands, Spain (*7*), or Germany (*8, 9*). In flooded soil or groundwater, conditions may become anoxic; therefore, knowledge of the biodegradability of γ-HCH and related isomers under anoxic conditions is of high environmental concern.

Transformation of γ-HCH under anoxic conditions in soil slurries was described for the first time more than 40 years ago (*10*). Jagnow et al. (*11*) demonstrated in a screening study that several strictly and facultative anaerobic bacteria were able to transform HCH isomers, including γ-HCH under anoxic, fermentative conditions. The authors detected γ-3,4,5,6-tetrachlorocyclohexene (γ-TCCH) as the main intermediate of γ-HCH dechlorination, a compound that was also found by Heritage and MacRae (*12*) as an intermediate of γ-HCH dechlorination of *Clostridium sphenoides* UQM780, the first HCH-transforming microorganism isolated. From experiments with *Clostridium rectum*, Ohisa et al. (*13*) proposed that γ-TCCH is dechlorinated to the unstable 1,3-dichlorocyclohexadiene (1,3-DCDN), which is further dehydrohalogenated to chlorobenzene (CB) as an end product. CB has been also detected as an end product of the anaerobic dechlorination of γ-HCH by a mixed culture incubated under methanogenic conditions (*7*). The authors assumed that benzene was also formed during the dehalogenation process. Boyle et al. (*14*) observed that γ-HCH was transformed by enrichment cultures to the end products CB and benzene under sulfate-reducing conditions. Because molybdate, an inhibitor of sulfate reduction, or the absence of sulfate, inhibited the transformation of γ-HCH, the authors postulated that γ-HCH was directly converted by sulfate-reducing bacteria. In a confirmation of this hypothesis, three tested sulfate-reducing pure cultures also dechlorinated γ-HCH to benzene and CB (*14*). The general pathway of γ-HCH dehalogenation to benzene and CB under anoxic conditions is summarized in Figure 1. It is suggested that CB forms via dehydrochlorination, and that benzene formation is enzymatically catalyzed from 5,6-dichloro-1,3-cyclohexadiene (1,3-DCDN) (*2*).

Compound-specific isotope analysis (CSIA) has been increasingly considered for characterizing in situ biodegradation processes qualitatively and quantitatively. The method takes advantage of the preferential transformation of lighter isotopes during a degradation reaction, thus leading to an enrichment of heavier isotopes in the residual phase during the course of biodegradation (see refs *15* and *16* for an

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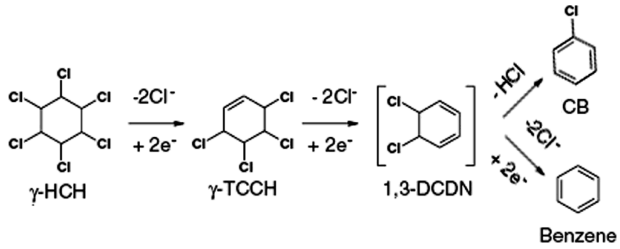

FIGURE 1. Anaerobic dehalogenation pathway for γ-HCH according to ref *2*.

overview of the method). Generally, the dehalogenation of chlorinated compounds, which can proceed by different reaction mechanisms, is often accompanied by fractionation of both chlorine (*17*) and carbon (*18*–*23*) isotopes. In this study, we investigated the isotope fractionation of γ-HCH as a model compound for technical HCH in order to elucidate the potential of CSIA for characterizing the in situ dechlorination under anoxic conditions. To the best of our knowledge, this is the first report on γ-HCH isotope fractionation carried out by bacterial strains under strictly anoxic conditions.

Materials and Methods

Chemicals. γ-HCH (analytical purity, 97%) and hexachlorobenzene (analytical purity, 99%) were obtained from Sigma-Aldrich (Germany). Dichloromethane (analytical purity, 99.8%) was obtained from Mallinckrodt Baker B.V. (The Netherlands). Anhydrous sodium sulfate (Na_2SO_4) (extra pure quality) was purchased from Merck (Germany).

Cultivation of *Desulfovibrio gigas* and *Desulfococcus multivorans*. *D. gigas* strain DSM 1382 and *D. multivorans* strain DSM 2059 were ordered from the Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). *D. gigas* was cultivated in a mineral salt medium described elsewhere (*24*). Sulfate was added in various amounts (final concentrations ranging from 5 to 30 mM), using a Na_2SO_4 stock solution (1 M). Finally, the medium was reduced with a few milligrams of sodium dithionite. The pH was adjusted to 7 with HCl (1 M) or NaOH (1 M) solutions. *D. multivorans* was cultivated in the same medium except that a further 10 g L^{-1} NaCl was added. The substrates, sodium lactate (for *D. gigas*; final concentration, 10 mM) and sodium benzoate (for *D. multivorans*; final concentrations, 3–5 mM), were added from 1 M stock solutions. γ-HCH was added from a stock solution of acetone (about 400 mM) to a final concentration in the medium, not exceeding the solubility limit of γ-HCH in water, of about 24–34 μM (*2*). All of the above solutions were sterilized by filtration or autoclaving and flushed with N_2 (except for the γ-HCH solution) in order to remove oxygen before use.

Degradation experiments for determining isotope fractionation factors were performed in 1 L bottles crimped gastight by Teflon-coated butyl septa. The medium (900 mL) was inoculated with a 50 mL preculture during the early stationary phase. In order to obtain high cell numbers for the dechlorination experiment, the cultures were incubated at 30 °C until the stationary phase was reached due to electron donor limitation. Further quantities of electron donors and γ-HCH were then added. Because of its low solubility, γ-HCH was difficult to dissolve in the medium; therefore, the culture bottles were permanently shaken on a horizontal shaker during the entire dechlorination experiment, which consisted of 3–4 h at 150–200 rpm after adding γ-HCH before the first samples were taken and afterward at 125 rpm.

Sampling Procedure and Extraction of γ-HCH. For sampling, aliquots of the culture medium were regularly taken with plastic syringes for stable isotope analysis (14 mL). Syringes were always flushed with nitrogen before use to avoid oxygen contamination. The volume removed was always compensated with sterile nitrogen in order to avoid negative pressure inside the bottles. Samples for isotope analysis were conserved with 0.5 mL of concentrated hydrochloric acid. The extraction of γ-HCH was done in 16 mL vials (Supelco) with 1 mL of DCM containing 100 μM hexachlorobenzene (HCB) as the internal standard for γ-HCH and 100 μM toluene as the internal standard for the metabolites. The vials were shaken for 12–36 h at 110 rpm and 12 °C. The organic phase was separated with Pasteur pipettes and transferred into 2 mL vials containing anhydrous Na_2SO_4 for water removal. The Na_2SO_4 was dried for several hours in a drying oven at 150–250 °C before use. Finally, 100 μL of this dry organic phase was transferred into vials for analysis by gas chromatography mass spectroscopy (GC-MS) and gas chromatography isotope ratio mass spectroscopy (GC-IRMS).

Analytical Method. *GC-MS.* A Hewlett-Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent, Palo Alto, CA) was used for identification and structural characterization. γ-HCH and its metabolites were separated on a BPX-5 capillary column (29.1 m × 0.32 mm × 0.25 μm) (SGE, Darmstadt, Germany) with the following temperature program: 40 °C initial temperature (5 min isotherm), then heat at 5 °C min^{-1} to 110 °C (0 min), 20 °C min^{-1} to 180 °C (0 min), 5 °C min^{-1} to 230 °C (0 min), and 20 °C min^{-1} to 300 °C (3 min isotherm). γ-HCH, benzene, and CB were identified by injection of the reference compounds and comparison of their retention times and mass spectra with those recorded in the samples. γ-3,4,5,6-Tetrachlorocyclohexene (γ-TCCH) was identified by comparing the mass spectrum with those of the NIST library. During the extraction of samples from the degradation experiments with *D. multivorans*, the remaining benzoate of the medium was protonated to benzoic acid due to the acidic conditions (pH 1) used. Benzoic acid was extracted and consequently appeared in the chromatograms. Furthermore, during the extraction procedure, samples that came in contact with air and sulfide were abiotically oxidized, leading to the formation of molecular sulfur (S_8) that also appeared in the chromatograms (Figure S3 of the Supporting Information).

GC-IRMS. The carbon isotope composition of γ-HCH and its metabolites was analyzed using GC-IRMS. The system consisted of a gas chromatograph (6890 Series, Agilent) coupled with a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan), as described previously (*25*). A ZB1 column (60 m × 0.32 mm, 1 μm film thickness, Phenomenex) was used for chromatographic separation of γ-HCH and its metabolites with helium as the carrier gas at a flow rate of 2.0 mL min^{-1}. The following temperature program was used: 40 °C initial temperature (5 min isotherm), then 6 °C min^{-1} to 200 °C (0 min), 3 °C min^{-1} to 260 °C (0 min), and finally 20 °C min^{-1} to 300 °C (5 min isotherm) to remove nontarget compounds. The split was adjusted to obtain suitable peak areas in the linear range of the $^{13}CO_2/^{12}CO_2$ mass ratio of the target compounds (Supporting Information). At least three replicates were measured per sample, resulting in an uncertainty in the isotope composition of a standard deviation (2σ) of less than ±0.5 part per thousand. Further information on experimental details, including the isotope analysis of HCH isomers and analytical performance of GC-IRMS, is described in the Supporting Information.

Carbon Stable Isotope Calculations. Carbon isotope ratios were reported in δ notation in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite standard (VPDB) (*26*), according to the following equation

$$\delta^{13}\mathrm{C}\ (‰) = \frac{R_{\mathrm{sample}} - R_{\mathrm{standard}}}{R_{\mathrm{standard}}} \times 1000 \quad (1)$$

R_{sample} is the ratio of $^{13}C/^{12}C$ in the sample, and R_{standard} is the ratio of $^{13}C/^{12}C$ in the standard.

The Rayleigh equation is expressed as

$$\ln\left(\frac{R_t}{R_0}\right) = (\alpha - 1)\ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

R_t and R_0 are the isotopic compositions (ratio of $^{13}C/^{12}C$) of the substrate at time t and time 0, respectively, α is the carbon isotope fractionation factor, and C_t and C_0 are the concentrations of the substrate at time t and time 0, respectively. The carbon isotope enrichment factor ε was calculated as follows

$$\varepsilon = (\alpha - 1) \times 1000 \quad (3)$$

The apparent kinetic isotope effect (AKIE) value was calculated using eq 4, according to Elsner et al. (*16*)

$$\mathrm{AKIE} = \frac{1}{1 + (nx/z\varepsilon/1000)} \quad (4)$$

where n is the number of atoms of the molecule of a selected element, x is the number of reactive positions, and z is the number of positions in intramolecular competition. The number of carbon atoms in γ-HCH is six. In the case of a stepwise cleavage of two carbon−chlorine bonds in γ-HCH, according to Figure 1, x and z would be six, if all C−Cl bonds are chemically equivalent. If only chlorine atoms in axial positions can react, x and z would be three. The calculation of the $\mathrm{AKIE_C}$ value for both reaction scenarios leads to the same result.

Results and Discussion

Reductive Dechlorination of γ-Hexachlorocyclohexane by *D. gigas* and *D. multivorans*. γ-HCH was dechlorinated by both bacterial strains in incubation times up to four weeks (Figure 2). The main metabolites formed during dehalogenation were benzene, CB, and, as an intermediate, γ-tetrachlorocyclohexene (γ-TCCH). Preliminary identification by GC-MS confirms that γ-HCH was dechlorinated under sulfate-reducing conditions, as previously reported (*14*) (Figure 1). After an initial increase, the relative concentration of γ-TCCH decreased after 2−3 days of incubation, while benzene and CB accumulated. Concentrations (in μM) were only calculated for benzene and CB because no reference standard of γ-TCCH was available for calibration. For both strains, the sum of the final concentrations of benzene and CB was slightly higher than the spiked γ-HCH concentration (Figure 3A,B), probably due to the analytical procedures used. Small amounts of γ-HCH might have adsorbed onto the plastic syringes used for sampling. CB, benzene, and γ-HCH might also have been extracted with different efficiencies in dichloromethane. The isotope composition of benzene could not be evaluated because of a slight contamination within the samples.

The rate of γ-HCH dehalogenation by *D. gigas* was affected by concentrations of the electron acceptor sulfate in the growth medium; *D. gigas* degraded γ-HCH completely within 19 days only under sulfate limitation (5 mM sulfate) (Figure 2A). *D. gigas* oxidizes lactate to acetate, which is not further oxidized by the cells; four electrons are provided by the oxidation process. For the reduction of sulfate to sulfide, eight electrons are needed. Thus, 2 mol of lactate is theoretically required to reduce 1 mol of sulfate. Because of carbon assimilation, this ratio is somewhat lower in growing cell cultures. Initially, *D. gigas* was cultivated with 10 mM lactate but with varying sulfate concentrations of 5, 15, and 30 mM. As the stationary phase was reached due to lactate consumption, the various experimental bottles were spiked with γ-HCH and additional lactate (10 mM). Hence, the experiment initially spiked with 5 mM sulfate was electron acceptor limited, in contrast to the experiments initially spiked with 15 and 30 mM sulfate. Unlike the results for *D. gigas*, the γ-HCH dehalogenation rate achieved by *D. multivorans* was not influenced by the availability of the electron acceptor sulfate in the culture medium (Figure 2B). For *D. multivorans*, benzoate was used as a carbon source and electron donor. Benzoate is completely oxidized by *D. multivorans*, providing 30 mol of electrons per mole of oxidized benzoate (Supporting Information). The cells were initially grown with 5 mM benzoate but with varying sulfate concentrations of 15, 20, and 30 mM, until the stationary phase was reached. Thus, the cultures supplied with 15 and 20 mM sulfate and additional benzoate were sulfate limited (5 mM), but the culture supplied with 30 mM sulfate was not.

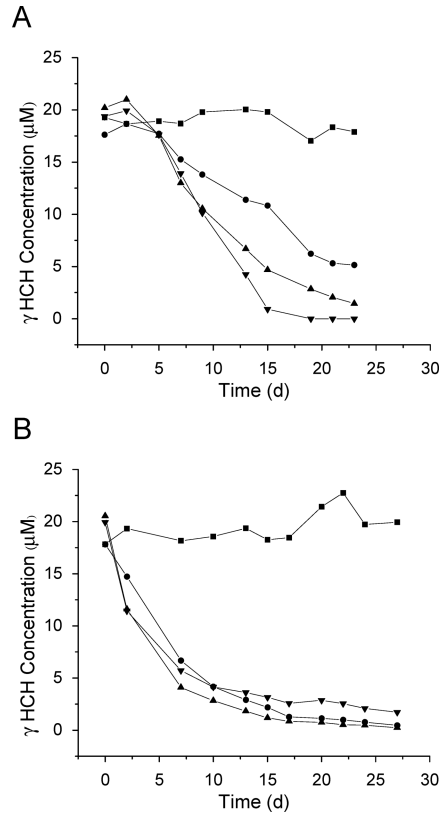

FIGURE 2. Transformation of γ-HCH by *D. gigas* (A) *and D. multivorans* (B) in the presence of different initial sulfate concentrations (see text for details): 30 mM (●), 15 mM (▲), and 5 mM (▼) for *D. gigas*; 30 mM (●), 20 mM (▲), and 15 mM (▼) for *D. multivorans*. Electron donors were lactate (10 mM) and benzoate (5 mM) for *D. gigas* and *D. multivorans*, respectively. Autoclaved controls (■) were spiked with 30 mM sulfate.

The structure and location of the enzymes responsible for γ-HCH dechlorination in *D. gigas* and *D. multivorans* are not known. Previous studies have shown that heme-associated proteins, e.g., cytochrome P-450, can principally dechlorinate HCH under anaerobic conditions (*27, 28*) but also metal-containing porphyrins and corrins (*29*). Boyle et al. (*14*) suggested that a heat-resistant cofactor, not an enzyme, might dehalogenate γ-HCH in *D. gigas* because autoclaved cells also dechlorinated γ-HCH. However, in our experiments with autoclaved cells of *D. gigas* and *D. multivorans*, γ-HCH concentrations remained constant, and no metabolites were

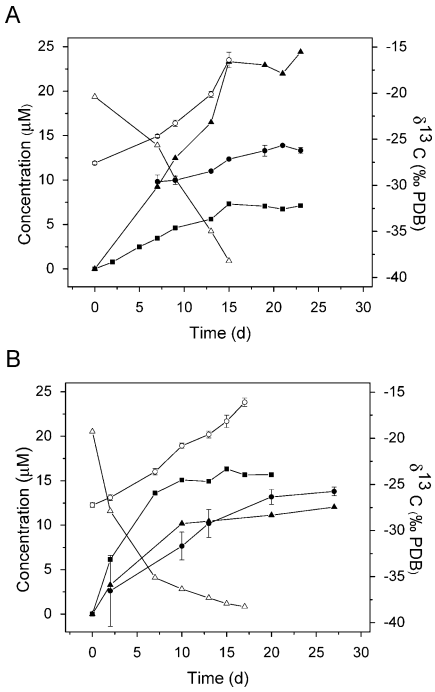

FIGURE 3. Change in concentration (△) and carbon isotope composition (○) of γ-HCH, in concentration (▲) and carbon isotope composition (●) of CB, and in concentration (■) of benzene during dechlorination by *D. gigas* (initially spiked with 5 mM sulfate) (A) and *D. multivorans* (initially spiked with 20 mM sulfate) (B).

detected. We cannot yet explain these different experimental results. A higher autoclaving temperature (121 °C) might have inactivated the dehalogenating cellular component in our experiments.

Carbon Isotope Fractionation in the Course of γ-HCH Dehalogenation. In all of our experiments with living cells, the carbon isotope composition of γ-HCH changed during the course of dehalogenation. In panels A and B of Figure 3, data from a single experiment for both *D. gigas* and *D. multivorans* are shown. For *D. gigas* with an initial 5 mM sulfate, the carbon isotope composition of γ-HCH increased from −27.6 ± 0.1 to −16.4 ± 0.8 ‰, whereas γ-HCH concentrations decreased from 19.4 to 0.9 μM (Figure 3A; compare also with Figure 2A). CB was detected after 6 days with a concentration of 9.2 μM and a carbon isotopic composition of −29.6 ± 0.7 ‰, which is slightly lighter than the isotopic signature of the parent compound γ-HCH at the beginning of the experiment. In the course of further γ-HCH transformation, the concentration of CB increased to a final value of about 24 μM. Correspondingly, the carbon isotope signatures of CB increased to values of around −26 ‰, which is slightly heavier than the initial carbon isotope signature of γ-HCH.

For *D. multivorans,* the carbon isotope signatures of γ-HCH were enriched to a similar extent during transformation as observed for *D. gigas*; in the experiment initially spiked with 20 mM sulfate, the carbon isotope composition of γ-HCH increased from −27.2 ± 0.3 to −16.1 ± 0.5 ‰, whereas the γ-HCH concentrations decreased from 20.5 to 0.8 μM (Figure 3B; compare also with Figure 2B). Isotopically depleted CB up to −36.5 ± 3.8 ‰ was detected after 2 days. CB concentrations increased to a final value of around 12 μM, and the corresponding carbon isotope signatures were enriched to −25.7 ± 0.4 ‰, slightly heavier than the initial isotope signature of the parent compound γ-HCH. In both experiments (*D. gigas* and *D. multivorans*), the determination of carbon stable isotope signatures of benzene was not possible because we observed a small benzene contamination in the experiments.

The difference in $\delta^{13}C$ between γ-HCH and the products at early stages of the reaction provides potential information on the transformation pathways beyond 1,3-DCDN. Benzene is thought to be formed by reductive dechlorination of 1,3-DCDN, and chlorobenzene formed by abiotic dehydrohalogenation (*27*). Because reductive dichloro elimination and dehydrochlorogenation reactions are expected to lead to significantly different isotope fractionation, the different reaction mechanisms might be detectable in future studies on the basis of product isotope signatures. This approach was recently applied for the identification of reactions pathways of chlorinated ethene degradation (*30*).

Quantitative Assessment of Isotope Fractionation. The enrichment factor (εC) of γ-HCH dechlorination was calculated according to the Rayleigh equation (eq 2). The εCs of individual experiments and combined data are shown in Table 1. Generally, correlation coefficients (R^2) higher than 0.92 were observed for all regression plots, indicating that the Rayleigh equation can be used for determination of isotope fractionation factors. This assumption is also supported by the linearity of the data shown in Figure 4. For both strains, no significant differences in εCs were observed in experiments with different initial sulfate concentrations (Table 1), suggesting that the extent of isotope fractionation was not influenced by availability of the terminal electron acceptor sulfate. Bulk fractionation factors calculated by plotting the data of three separate experiments were $\varepsilon C = -3.4$ (±0.5) for *D. multivorans,* and $\varepsilon C = -3.9$ (±0.6) for *D. gigas*, displaying a clear primary carbon isotope effect upon the dehalogenation reaction. Isotope fractionation factors are similar for both cultures, indicating that the reaction mechanism of dechlorination is similar in both organisms. For further elucidation of the reaction mechanism, the apparent kinetic isotope effect ($AKIE_C$) value was calculated according to Elsner et al. (*16*), assuming that two C−Cl bonds are stepwise and reductively cleaved during the reaction of γ-HCH to γ-TCCH; the calculated $AKIE_C$ values for such a scenario range between 1.02 ±0.003 and 1.023 ± 0.004. Indications for reductive and stepwise dechlorination, occurring under anaerobic conditions, were obtained in studies with heme-containing enzymes, e.g., cytochrome P-450 from rat liver, which dechlorinated γ-HCH to γ-TCCH and γ-TCCH and further to benzene and CB (*27, 28*). A similar pathway is thought to operate in anaerobic HCH dechlorinating microorganisms (*7, 13*). Reductions of organochlorine compounds are usually initiated by a one-electron transfer to the chloride atom, which results in a highly reactive intermediate that undergoes a second, much faster reduction step (*31*). Theoretically, two electrons could also be transferred to different chlorine atoms in a concerted reaction mechanism, but to the best of our knowledge this has not been observed in reductive dechlorination reactions and seems rather unlikely. Previous studies have shown that for substances such as HCH, which lack free carbon−carbon bond rotation, the vicinal elimination of chlorines is easier for axial chlorines orientated in opposite directions, which is the so-called antiparallel or tram diaxial chlorine configuration (*27, 28*). The often-observed recalcitrance of β-HCH, which lacks any axial chlorine atoms, is attributed to the absence of vicinal, antiparallel chlorine atoms. Thus, the initial step of γ-HCH dechlorination in anaerobic bacteria, which cannot dechlorinate β-HCH, might proceed only at the axial chlorines in antiparallel configuration. Dechlorination of γ-HCH can occur also by a dehydrohalogenation mechanism. A dehydrochlorinase catalyzing the conversion of γ-HCH to γ-pentachlorocyclohexene (γ-PCCH) was shown to operate in aerobic HCH-degrading microorganisms (*1*). However, we searched for γ-PCCH but were not able to detect this metabolite using GC-MS in our experiments.

TABLE 1. Carbon Enrichment Factors (εC) for γ-HCH Dechlorination by *D. gigas* and *D. multivorans* Obtained in Individual Experiments

organism	initial sulfate concentration	number of data points	amount of dechlorination (%)	enrichment factor[a]	correlation coefficient
	30 mM[b]	8	0−93	−3.6 ± 0.6	0.98
	20 mM[b]	7	0−96	−3.6 ± 0.7	0.97
	15 mM[b]	4	0−87	−3.4 ± 2.9	0.92
D. multivorans		all data		−3.4 ± 0.5	0.96
	15 mM[c]	8	0−93	−4.3 ± 0.5	0.99
	5 mM[c]	5	0−95	−3.5 ± 1.5	0.95
	5 mM[d]	5	0−91	−3.7 ± 0.6	0.99
D. gigas		all data		−3.9 ± 0.6	0.94

[a] A 95% confidence interval. [b] See Figure 2B for γ-HCH concentrations over time. [c] See Figure 2A for γ-HCH concentrations over time. [d] See Figure S4 of the Supporting Information for γ-HCH concentrations over time.

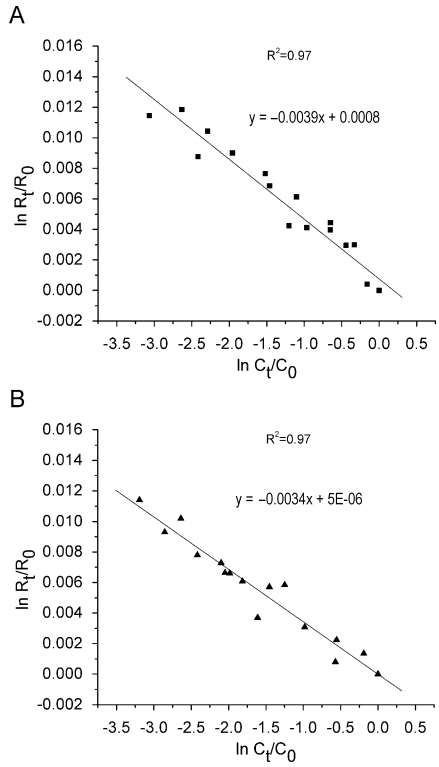

FIGURE 4. Quantitative assessment of isotope fractionation of γ-HCH in dechlorination experiments with *D. gigas* (A) and *D. multivorans* (B) using the Rayleigh model. Data of individual dechlorination experiments for each strain (Table 1) were used for calculating bulk enrichment factors: $\varepsilon = -3.9 \pm 0.6$ for *D. gigas*, and $\varepsilon = -3.4 \pm 0.5$ for *D. multivorans*.

Stable isotope fractionation may help to characterize the reaction mechanism more accurately. Recently, AKIE values for the transformation of chlorinated ethanes by metal-catalyzed abiotic reaction mechanisms have been used to elucidate the reaction mechanisms. Elsner et al. (*19*) calculated $AKIE_C$ values between 1.017 and 1.026 for concerted reaction mechanism scenarios and $AKIE_C$ values between 1.035 and 1.054 for the corresponding stepwise scenarios, whereas Hofstetter et al. (*17*) reported $AKIE_C$ values within a similar range (1.021−1.031) for different reaction mechanisms and compounds. VanStone et al. (*23*) observed that the $AKIE_C$ for reductive β-elimination of 1,2-dichloroethane was significantly larger than that for hydrogenolysis or α-elimination, indicating that the reaction mechanism for reductive β-elimination of vicinal chlorine bound to aliphatic carbon structures such as γ-HCH possibly follows a concerted reaction scheme. For the reductive dehalogenation of 1,2,3- and 1,2,4-trichlorobenzene by *Dehalococcoides* sp. strain CBDB1, $AKIE_C$ values of 1.019 and 1.02 were reported, respectively (*21*), and for the dehalogenation of 1,2,3-trichlorodibenzodioxine by a mixed culture comprised of *Dehalococcoides*, an $AKIE_C$ value of 1.011 was observed (*20*). These values overlap with the $AKIE_C$ values observed in this study. Nevertheless, during reductive dechlorination of chlorobenzenes or chlorinated dioxins, chlorine is cleaved from a sp^2-hybridized carbon of an aromatic ring, whereas reductive β-elimination takes place at sp^3-hybridized carbons, implying a different reaction mechanism. Thus, on the basis of these literature data and the $AKIE_C$ values presented in this study, it is not possible to deduce unequivocally the reaction mechanism of reductive γ-HCH dihalo elimination to γ-TCCH. Complicating matters, AKIE values are commonly not fully expressed in enzymatic reactions, as well as in biological experiments with intact cells. Rate limitations upon uptake into the cell or binding to the enzyme have to be considered because they often lower the extent of isotope fractionations (*22, 32*), yielding lower experimentally determined AKIE values. A promising method for deducing specific reaction mechanisms of anoxic γ-HCH transformation in experiments with intact cells would be the two-dimensional isotope fractionation analyses of carbon and chlorine atoms. Using this approach, it was recently shown that β-dehalo eliminations were coupled with a significant chlorine isotope effect (*17*), whereas the reductive dechlorination of cis-1,2-dichloroethene and the aerobic oxidation of vinyl chloride were coupled with smaller chlorine isotope effects (*33*). By studying the isotope effects of both elements forming a chemical bond, we may be able to characterize further abiotic reaction mechanisms on a molecular level (*17*) or to analyze reaction mechanisms of certain microbial degradation reactions as shown for MTBE (*34, 35*), benzene (*36, 37*), or toluene (*38*).

Our results clearly demonstrate that carbon isotope fractionation occurs during dehalogenation of γ-HCH. Thus, monitoring the carbon isotope signatures of γ-HCH is a promising tool for the qualitative and quantitative assessment of γ-HCH dehalogenation in anoxic contaminated areas and might also be applicable for other HCH isomers. Assuming that a HCH contamination in the field originates from a single source, variations in the carbon isotope composition between zones and depths may serve as indications for in situ reductive dechlorination of HCH isomers. Furthermore, the isotope pattern of the γ-HCH degradation metabolite CB was depleted relative to HCH. Thus, the combined use of the isotope fingerprints of CB and HCH also has the potential to indicate and trace the origin of CB in HCH-contaminated environments.

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Supporting Information Available

Isotope analysis of HCH isomers and analytical performance of GC-IRMS and γ-HCH concentrations over time, chromatograms, and peak areas. This information is available free of charge via the Internet at http://pubs.acs.org.

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