Enantiomer and Carbon Isotope Fractionation of α-Hexachlorocyclohexane by *Sphingobium indicum* Strain B90A and the Corresponding Enzymes

Yaqing Liu, [1] Langping Wu, [1,#] Puneet Kohli, [2] Roshan Kumar, [2] Hryhoriy Stryhanyuk,[1] Ivonne Nijenhuis, [1] Rup Lal, [2] Hans-Hermann Richnow[1,*]

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

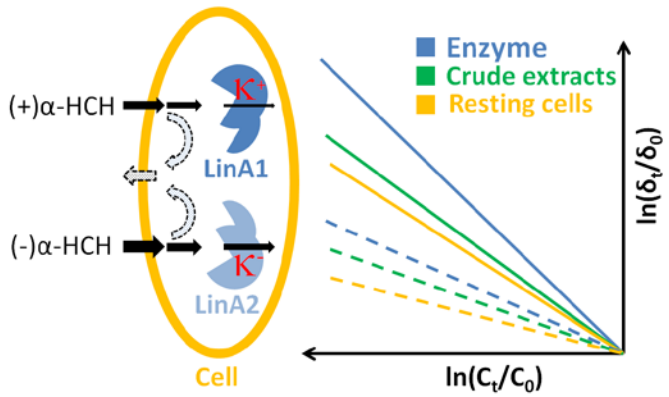
[2]Molecular Biology Laboratory, Department of Zoology, University of Delhi, Delhi-110007, India

[#]Present address: Departments of Civil & Mineral Engineering, University of Toronto, 35 St. George Street, Toronto, ON M5S 1A4, Canada

*Corresponding author: Hans-Hermann Richnow

ABSTRACT

Chiral organic contaminants, like α-hexachlorocyclohexane (α-HCH), showed isotope fractionation and enantiomer fractionation during biodegradation. This study aims to understand the correlation between these two processes. Initial tests of α-HCH degradation by six *Sphingobium* strains (with different LinA variants) were conducted. Results showed variable enantiomer selectivity over the time course. In contrast, constant enantiomer selectivity was observed in experiments employing (i) cell suspensions, (ii) crude extracts, or (iii) LinA1 and LinA2 enzymes of strain B90A for α-HCH degradation in enzyme activity assay buffer. The average value of enantioselectivity (ES) were -0.45±0.03 (cell suspension), -0.60±0.05 (crude extract) and 1 (LinA1) or -1 (LinA2). The average carbon isotope enrichment factors (ε_c) of (+)α- and (-)α-HCH were increased from cells suspensions (-6.3±0.1‰ and -2.3±0.03‰) over crude extracts (-7.7±0.4‰ and -3.4±0.02‰) to purified enzymes (-11.1±0.3‰ and -3.8±0.2‰). The variability of ES and the ε_c were discussed based on the effect of mass transport and degradation rates. Our study demonstrates that enantiomer and isotope fractionation of α-HCH are two independent processes and both are affected by reactions of individual enzymes and mass transport to a different extent.



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INTRODUCTION

An increasing number of anthropogenic chemicals are chiral compounds which raise not only environmental concerns but also possess enantiomer-specific environmental toxicity.[1] Estimates suggest that up to one-third of all anthropogenic compounds such as fungicides, herbicides, and antibiotics are chiral, of which many are produced by chemical synthesis as racemates.[2] Due to the identical physical and chemical properties of enantiomers, the abiotic degradation is identical for both the enantiomers.[3] However, certain microorganisms preferably biodegrade one enantiomer at higher rate leading to enantiomer fractionation i.e., a change in enantiomer ratios. Enantioselectivity can be the result of the preferential uptake or selectivity of enzyme catalysis.[4] The changes in enantiomer ratios have been suggested as an indicator for biodegradation of chiral compounds and have been applied to track *in situ* degradation at field sites.[5, 6] For evaluation of degradation at a field site, another well-developed method to assess contaminants degradation at field sites is compound specific stable isotope analysis (CSIA).[7-9] During the degradation of organic compounds, slightly different reaction rates of lighter and heavier isotopologues can lead to the change of isotope composition in the substrate, known as isotope fractionation.

The Rayleigh equation is extensively applied for the quantification of isotope fractionation.[10] For the quantification of enantiomer fractionation, the Rayleigh equations (both simplified and general forms) and enantioselectivity (ES) were applied in previous studies.[11-13] Both enantiomer and isotope fractionation can be observed during the biodegradation due to different reaction rates of enantiomers and isotopologues, respectively, and both were proposed for the evaluations of biodegradation. However, it has not been fully evaluated yet how enantiomer and isotope fractionation are linked and whether or not they are two independent processes.

α-Hexachlorocyclohexane (HCH) is one of the persistent organic pollutants appearing worldwide as point source or dispersed pollution.[14] The genes responsible for HCH aerobic degradation, known as *lin* genes, are generally present in aerobic HCH degrader *Sphingomonads*. These genes were first identified and characterized in *Sphingobium japonicum*

UT26,[15] followed by *S. indicum* B90A.[16] The product of this gene containing 156-amino acids is known as the HCH dehydrochlorinase and was localized in the periplasm.[15] The LinA enzyme catalyzes the initial step of dehydrochlorination, converting α-HCH into β-pentachlorocyclohexene (β-PCCH).[17] It has been reported that the *linA* genes are under continuous selection pressure and thus exist in several variants.[18-22] There are two copies of *linA* genes *i.e.*, *linA1* and *linA2* present in *S. indicum* B90A and *Pseudomonas aeruginosa* ITRC-5, whereas only *linA2* is present in *S.indicum* UT26.[23] The *linA1* and *linA2* genes of strain B90A differ by 10% in their amino acid sequence, and the corresponding enzymes preferentially degrade (+)α-HCH and (-)α-HCH enantiomers, respectively.[24-26] The degradation of individual α-HCH enantiomers by these sphingomonads involves different enzymes (LinA1 and LinA2), and the expression of the enzymes may not be stable during growth which may affect the enantiomer preference and lead to variable enantiomer fractionation. In this case, employing cell suspensions without nutrients (prevent growth and synthesis of biomass) as well as crude extracts of these cells for the degradation of α-HCH should lead to stable enantiomer preference and the enantiomer fractionation can be characterized. Further, activity assays with LinA1 and LinA2 enzymes (separately expressed as functional S-glutathione transferase (GST) fusion proteins and enabling the catalysis of α-HCH enantiomers separately) allow a better understanding of the enantiomer fractionation as well as isotope fractionation.

In this study, the initial set of experiments involving six *Sphingobium* strains (namely *Sphingobium quisquiliarum* P25, *S. lucknowense* F2, *S. chinhatense* IP26, *S. ummariense* RL3, *Sphingobium sp.* HDIPO4, *S. baderi* LLO3) were conducted in growth medium to investigate the enantiomer preference during α-HCH degradation. Furthermore, in order to characterize the contributions of substrate uptake, mass transport and enzymatic reactions on the observable isotope and enantiomer fractionation, four sets of degradation experiments using cell suspensions, crude extracts, LinA1 and LinA2 were conducted in buffer. With the objective to investigate the correlation of isotope and enantiomer fractionation processes, the obtained

isotope and enantiomer fractionation were discussed based on (1) mass transfer: The effect of membrane was discussed by comparing the degradation in cell suspension and crude extract; the effect of vesicles was discussed by comparing the crude extract and purified enzymes and (2) degradation rates: The ES and ε_c values were compared in each set of experiments based on the reaction kinetics.

MATERIALS AND METHODS

Chemicals. α-HCH (analytical purity, 99%), hexachlorobenzene (HCB, analytical purity, 97%), imidazole (analytical purity, 99%) and ampicillin (analytical purity, 95%) were purchased from Sigma Aldrich (Germany). *n*-pentane (analytical purity, 99%) was supplied by Carl Roth, Germany. TRIS was supplied by Geyer, Germany.

Bacterial Strains and Cultivation Conditions. *Sphingobium spp.* (strain B90A, P25, F2, IP26, RL3, HDIPO4, LLO3) and *E.coli* BL21 (AI) were maintained in the Molecular Biology Laboratory, University of Delhi, India. The information for cultivation is provided in the Supporting Information (SI) section 1.

Cell Suspension and Bacterial Crude Extracts of B90A. Bacterial cells were grown in LB medium until the A_{600} of cultures reached 0.5-0.6 (logarithmic phase). The cells were harvested by centrifugation at 8000 × *g* at 4 °C for 20 min to obtain a cell pellet. The pellet was washed twice by sequential re-suspension and centrifugation with 0.1 M TRIS-HCl buffer at pH 7.5 to remove nutrients and substrates for growth. Subsequent degradation experiments were completed within several hours after the cell suspensions were prepared. The bacterial pellet was stored at -20 °C, and the crude extracts were prepared with a French Press (Thermo Fisher Scientific, Bremen) at 20.000 psi before usage.

Enzyme Expression and Purification. The *E. coli* cells carrying the *linA1* and *linA2* genes, respectively, were grown overnight before inoculation (1% v/v) in 100 mL LB media (See SI) amended with antibiotics. Cultures were incubated at 30°C while shaking at 200 rpm until the OD_{600} reached 0.5-0.6 and induced with L-(+)-arabinose (see SI). The cells were then harvested

by centrifugation at 8000 × *g* for 15 min at 4 ^{o}C. Enzyme purification procedures are provided in the SI section 2.

Degradation Experiments. In all the degradation experiments, the initial concentration of α-HCH was 5.5 μM (each bottle was spiked 5.5μL of stock solution with the concentration of 0.1M in acetone). For each set of experiments, two abiotic controls without adding biomass were performed and treated identically until extraction. All bottles were incubated at 30 ^{o}C in a shaking incubator (200 rpm). The sampling and extraction were done as reported elsewhere.[27]

Degradation experiments with growing cells: (1) Degradation of α-HCH by six *Sphingobium* spp.(strain P25, F2, IP26, RL3, HDIPO4, LLO3) were conducted in 240 mL bottles filled with 100 mL mineral salt medium with glucose as the carbon source (SI section 1). (2) Degradation experiment using strain HDIPO4 under nutrient-limited condition by reducing the glucose to 1% was compared to the amount used experiments (1) but other conditions are the same.

Batch experiments with cell suspensions, crude extracts and purified enzymes were performed in 240 mL bottles filled with 100mL Tris-buffer (0.1 M, pH = 7.5). (1) Degradation experiments with cells suspensions: different volumes of cell suspensions with a cell density of 2.5-3.0×10^{8} cells mL^{-1} were used for four sets of experiments, labeled as a (500 μL), b (200 μL), c (100 μL), d (50 μL). (2) Degradation experiments with crude extracts: different amounts of crude extracts (obtained from the cell suspension with the cell density of 2.5-3.0×10^{8} cells mL^{-1}) were used for four sets of experiments, labeled as e (200 μL), f (100 μL), g (50 μL), h (50 μL, a replicate of experiment g). (3) Degradation experiments with LinA2 enzyme: three sets of degradation experiments were conducted by using different amounts of LinA2 enzyme (240 ng μL^{-1}), labeled as i (10 μL), j (8 μL), k (5μL). (4) Degradation experiments with LinA1 enzyme: two sets of experiments were conducted by using different amounts of LinA1 enzyme (70 ng μL^{-1}), labeled as l (10 μL) and m (5μL).

Analytical Methods and Data Evaluation. The concentration of HCH was analyzed by an Agilent 6890 series GC (Agilent Technologies, USA) equipped with a FID (SI section 3). The

concentration of protein was quantified by NanoDrop ND-1000 Spectrophotometer from Thermo Fisher Scientific. The carbon isotope composition was analyzed by a gas chromatography isotope ratio mass spectrometer (GC-IRMS), as described previously.[28]

Enantiomer fraction (EF) is applied for explaining the relationship between different enantiomers. The EF(-) is calculated as $A^-/(A^++A^-)$ and $EF(+)=A^+/(A^++A^-)$, where A^+ and A^- correspond to the peak areas or concentrations of (+) and (-) enantiomers. An EF(-) > 0.5 shows the preferential degradation of (+) enantiomer, and an EF(-) < 0.5 indicates the preferential degradation of (-) enantiomer.

Enzymatic reactions are frequently described by the Michaelis-Menten kinetics as showed in equation 1.

$$v = k_{cat} \cdot [E]_0 \frac{[S]}{k_M+[S]} \quad (1)$$

Where v, k_{cat}, $[E]_0$, [S] and K_M are the reaction rate, turnover number, initial enzyme concentration, substrate concentration and Michaelis constant, respectively. The condition $[S] \ll K_M$ can be assumed as even lower substrate concentration (2.5μM for each α-HCH enantiomer) applied in this study compare to the previous report.[29] And equation 1 then can be modified to equation 2 and applied for evaluating the degradation kinetics of α-HCH enantiomers by cell suspensions, crude extract and enzymes in this study.

$$v = -\kappa \cdot [S] \quad (2)$$

Where $\kappa=-\frac{k_{cat}\cdot[E]_0}{k_M}$ is the first order rate constants of the reaction.

For the evaluation of enantiomer fractionation, the excess of the degradation rates for (+)α-HCH over the (-)α-HCH was used to define the ES which was quantified by the following equation.[4, 30-32]

$$\mathrm{ES} = \frac{\kappa^+-\kappa^-}{\kappa^++\kappa^-} \quad (3)$$

Where κ^+ and κ^- are the first order rate constants for the degradation of (+)α-HCH and (-)α-HCH, respectively. When the enantiomers are degraded equally ($\kappa^+=\kappa^-$), no enantiomer selective

degradation will be observed and ES=0. If only one enantiomer is degraded, then ES=1 (κ^{-}=0, only (+)α-HCH is degraded) or ES=-1 (κ^{+} =0, only (-)α-HCH is degraded).

The simplified Rayleigh equation in logarithmic form was used to quantify the stable carbon isotope fractionation of the biodegradation process in this study. The carbon isotope enrichment factor (ε_c) was determined using equation 4.

$$\ln\left(\frac{(\delta_t{}^{13}C+1)}{\delta_0{}^{13}C+1}\right) = \varepsilon_c ln\left(\frac{C_t}{C_0}\right) \qquad (4)$$

ε_c was reported in per mil and derived from the slope of the linear regression of $\ln(C_t/C_0)$ vs $\ln[(\delta_t{}^{13}C+1)/(\delta_0{}^{13}C+1)]$. The error of ε_c was reported as the 95% confidence interval (CI) determined by regression analysis.[12]

The apparent kinetic isotope effect was calculated as previously described using equation 5.[33]

$$AKIE_C = \frac{1}{1+\frac{nz}{x}*\varepsilon_c/1000} \qquad (5)$$

Where n is the total number of carbon atoms in a molecule, x is the number of atoms at reactive positons, and z is the number of indistinguishable reactive positions for intramolecular competition.

RESULTS

Degradation by Growing Cells. In experiment (1), carbon isotope enrichment of α-HCH enantiomers associated with enantiomer preferential degradation was observed in growing cells experiments (except the controls) (Figure S1). This result indicates that the enantiomer and isotope fractionation potentially can be used for the quantification of α-HCH biodegradation. Plotting the enantiomer fraction (EF) of (-)α-HCH over time, the EF(-) was varied from 0.50 up to 0.59 during the degradation period (Figure S2). Each of the *Sphingomonas* species has at least two enzymes (LinA1 and LinA2) catalyzing the initial step of α-HCH enantiomer degradation. Thus, we speculated that the expression of LinA enzymes changed during growth, which might affect the degradation kinetics of individual enantiomers and therefore the enantiomer

preference changed. In experiment (2), the result shows that the growing culture performed a constant enantiomer preference under nutrient-limited condition (Figure S3).

Due to the possibility of variable isotope and enantiomer fractionation under the growth condition, experiments in buffer which do not contain the necessary nutrients for growth were conducted with cell suspensions, crude extracts and purified enzymes. The pseudo-first order kinetic rate constants (κ), ε_c and the ES values obtained from these experiments are summarized in Table 1. In all the abiotic controls, both the concentration and the isotope composition are remain constant until the end of the experiments indicating that no other processes affect the biodegradation in this study.

Degradation by Cell Suspensions. The degradation of both enantiomers could be described by pseudo-first order kinetics, which indicates that the ratio of LinA enzymes and their activities were constant over the course of the individual degradation experiments (experiment a-d). The relatively short experimental time (1.5h to 5h, Figure S4) and the absence of growth substrates and nutrients preclude growth and significant *de novo* synthesis of enzymes unlikely. Thus, the enzyme inventory is assumed to be constant. For (+)α-HCH transformation, different κ^+ values were observed: 0.49±0.03 h^{-1}, 0.30±0.03 h^{-1}, 0.12 ±0.02 h^{-1} and 0.06±0.01 h^{-1} (Figure 1, a-d). The obtained ε_c^+ in experiment a-d (-6.4±0.7‰, -6.2±1.2‰, -6.1±1.1‰ and -6.1±1.4‰, respectively; Table 1 and figure 2, a-d) were statistically identical and thus described the isotope fractionation of (+)α-HCH robustly. For (-)α-HCH transformation, no consistent isotope enrichment which can be quantified by Rayleigh equation was observed at higher κ^- values of 0.81±0.12 h^{-1} and 0.76±0.09 h^{-1} (FigureS3 a, b). However, at lower rates of κ^-=0.35±0.02 h^{-1} and 0.15±0.02 h^{-1} (experiment c and d, Figure 1 and Table 1), significant isotope enrichment was observed with ε_c^- =-2.3±0.4‰ and -2.3±0.3‰ (Figure 2 and Table 1), respectively. The relatively higher transformation rates in experiment a and b lead to the non-observable carbon isotope fractionation in both experiments, which suggests masking of isotope fractionation by the rate limiting step prior to the isotope sensitive bond cleavage. At higher degradation rates,

the ES value was only reduced in the experiment a (-0.25±0.08) whereas the ES values of -0.43±0.06, -0.49±0.07and -0.43±0.09 in experiment b-d are similar (Table 1).

Degradation by Crude Extracts. The degradation of α-HCH enantiomers by using different amounts of crude cell extracts followed first-order kinetics (Figure 1, experiment e-h). Significant and identical ε_c^+ were obtained as -7.4±0.7‰ and -8.0±1.3‰ (experiment e and f, Figure 2 and Table 1) during (+)α-HCH transformation when the κ^+ values were 0.30±0.07 h^{-1} and 0.12±0.02 h^{-1}, respectively. In the case of (-)α-HCH transformation, no significant isotope enrichment (Figure S5 e) was observed when the corresponding κ^- values were 0.58±0.13 h^{-1} (experiment e, Figure 1 and Table 1). When the κ^- value was 0.45±0.06 h^{-1} (experiment f, Figure 1 and Table 1), a relatively smaller ε_c^- (-1.9±0.4‰) was obtained compare to the ε_c^- values (-3.4±0.5‰ and -3.4±0.6‰ for experiment g and h, Figure 2 and Table 1) which were obtained at lower reaction rates (0.19±0.02 h^{-1} and 0.18±0.02 h^{-1}). At higher transformation rates the isotope fractionation of (-)α-HCH was low or could not be observed compared to isotope fractionation at lower degradation rates, which is similar with the observation in the experiments with cell suspensions. In contrast, only the ES value of -0.32±0.15 from experiment e was relatively lower compared to that of -0.57±0.07, -0.65±0.08 and -0.57±0.10 (Table 1) from experiment f, g and h, respectively.

Degradation by Purified Enzymes. The enzyme experiments showed a nearly exclusive degradation of (-)α-HCH by LinA2 and (+)α-HCH by LinA1. The degradation of α-HCH enantiomers was investigated by using different amounts of purified enzymes and could be described by the first-order kinetics. LinA1 degraded preferentially (+)α-HCH with a κ^+ of 0.28±0.02 h^{-1} and 0.13±0.02 h^{-1} (experiment l and m, Figure 1, Table 1) and the obtained ε_c^+ values were -11.3±2.0‰ and -10.9±1.5‰ (Figure 2, Table 1). (-)α-HCH degradation by LinA2 resulted in a κ^- of 0.65±0.10 h^{-1}, 0.54±0.28 h^{-1} and 0.27±0.05 h^{-1} (experiment i-k, Figure 1, Table 1), and the corresponding ε_c^- were -3.7±0.6‰, -4.0±1.0‰ and -3.6±0.5‰, respectively.

Since LinA1 and LinA2 showed nearly exclusive enantiomer preference of (+)α-HCH and (-)α-HCH, the calculated ES values from LinA1 and LinA2 experiments were 1 and -1, respectively.

DISCUSSION

Variability of Enantiomer Fractionation. High variability of enantiomer fractionation was observed during α-HCH degradation by the six *Sphingobium* strains in growing cell experiment (1). This indicates that the degradation rates of the enantiomers were not constant during the whole process, which suggests changes in the abundance of LinA1 and LinA2 enzymes during growth may lead to preferential degradation of individual α-HCH enantiomers over the course of the experiment. However, the stable enantiomer preference in growing cell experiment (2) indicates that the expression of enzymes may relatively slow in nutrient-limited condition. In addition, the enantiomer selectivity of *S. indicum* B90A observed in different studies also indicates the variability of enantiomer fractionation. For example, biodegradation of α-HCH by *S.indicum* B90A showed preferential degradation of the (-)α-HCH enantiomer at the beginning of the degradation and later changed to (+)α-HCH.[12] However, enantioselectivity of α-HCH was not observed with the same strain elsewhere.[32] In the present study, the cell suspensions and crude extracts both preferred (-)α-HCH transformation. This indicates that different growth phases (lag phase, log phase, and stationary phase) or different cultivation conditions lead to changes in the regulation of the LinA1 and LinA2 abundance. This is in agreement with the variable enantiomer selectivity of the six *Sphingobium* strains. Thus, different ratios of LinA1 and LinA2 may be expressed under different growth conditions which change the selectivity of enantiomer degradation.

Relationship between Isotope and Enantiomer Fractionation. Isotope fractionation is determined by bond cleavage or formation in the first irreversible reaction step and can be modified due to rate limitation of preceding steps in a complex biochemical reaction.[34, 35] The observed isotope fractionation contains information on the transition state of bond cleavage and kinetic rate limitation prior to irreversible bond cleavage. Isotope fractionation depends on the

kinetics of bond cleavage in the transition state which can be quantified by the Rayleigh equation (equation 4). For the degradation of α-HCH, LinA dehydrochlorinates the substrates most likely via an E2 elimination,[36] which is probably identical for both enantiomers.[37] In this study, an elimination reaction with concerted bond cleavage at two axial H/Cl pairs of α-HCH to form pentacyclohexenes (1,3S,4S,5R,6S-PCCH for (+)α-HCH and 1,3R,4R,5S,6R-PCCH for (-)α-HCH) during dehydrochlorination was assumed as in a previous study.[38] In this case, four carbons with axial chlorine hydrogen pairs (x=4) with two indistinguishable positions (z=2) were involved in the reaction. The calculated apparent kinetic carbon isotope effect ($AKIE_C$) yields an average value of 1.035±0.004 for LinA1 and 1.011±0.001 for LinA2 (SI section 7, Table S1). In the quantum chemical modelling study by Manna and Dybala-Defratyka, the position-specific primary KIE_C values of (+)α-HCH dehydrochlorination are 1.0168 and 1.0218 for the C-H and C-Cl bond cleavage reaction, respectively, whereas the dehydrochlorination of (-)α-HCH gave a primary KIE_C of 1.0169 (C-H) and 1.0104 (C-Cl).[38] The calculated primary KIE_C of (-)α-HCH was smaller than (+)α-HCH during dehydrochlorination, which is in agreement with the observations in our experiments. The $AKIE_C$ of (-)α-HCH in our study is consistent within an order of magnitude with values obtained for quantum mechanical modeling.[38] The $AKIE_C$ of (+)α-HCH degradation is higher than the value obtained from quantum chemical modeling but similar with values observed in other studies which report C-Cl bond cleavage at a sigma hybridized carbon.[38-40]

Assuming that the dehydrochlorination mechanism for both enantiomers is identical and follows E2 elimination,[37] the rate limitation might be the result of HCH binding within the enzyme pocket. Binding of γ and β substrates within LinA can affect the transition state and the reaction rates as suggested by QM/MM modeling studies.[37] Preceding reaction steps such as transport in the cell and binding to the enzyme can modify the kinetic isotope effect (KIE) of the bond cleavage reaction.[41] As the chemical bond cleavage is probably not much different in a chemical sense, one may hypothesize that the kinetics of the binding to enzymes leads to rate limitation and thus modifies the observed carbon isotope fractionation. Based on our data, we cannot

provide a more detailed evaluation of the rate limitation and carbon isotope fractionation. Further QM/MM modeling studies using a model with complete enzyme structures would be required to solve this question.

Enantiomer fractionation can be influenced by two factors: (i) Binding of the substrate to the enzyme with respect to the stereochemical position in the enzyme pocket which can lead to different reaction ratios.[42] (ii) The reactivity of two individual enzymes with specificity towards enantiomers as observed in the enzyme assays with LinA1 and LinA2. In the second case, the enantiomer degradation should be rationalized as individual substances which are controlled by the expression and activity of individual enzymes within the machinery of the cell.[43] However if the kinetic reaction rate is changed due to individual regulation of the individual enzymes, the enantiomer fractionation process cannot be described by a single factor. Indeed, the variability of enantiomer preference observed in experiments with growing cells indicates changes in the abundance and reactivity of the LinA1 and LinA2 enzymes during growth.

From the discussion based on the mechanisms of enantiomer and isotope fractionation, we can conclude that these two processes can takes place but do not have to be synchronous during the biodegradation of α-HCH. These processes are independent as two different enzymes are involved and depend on the activity of the enzymes. Further discussion based on the effect of mass transport and degradation rates can confirm that enantiomer and isotope fractionations are two independent processes.

Effect of Mass Transport on Isotope and Enantiomer Fractionation. In order to evaluate the effect of mass transport into the cells, isotope fractionation of α-HCH during experiments with cell suspensions and crude extracts were compared. The isotope effects comparing the cell suspension and crude extract experiments were similar when considering the uncertainty. For (+)α-HCH, with the same κ^+ (0.12±0.02 h^{-1}; Table 1), the ε_c^+ values obtained from cell suspension (experiment c: -6.1±1.1‰; Table 1) and crude extracts (experiment f: -8.0±1.3‰; Table 1) show that mass transfer across the outer and cytoplasmic membranes may reduce the observed isotope fractionation, leading to a relatively smaller ε_c^+ in the cell suspension

experiments. Compared to the α-HCH degradation by cell suspension and crude extracts, significant higher ε_c^+ values (experiment l and m: -11.3±2.0‰ and -10.9±1.5‰, respectively; Table 1) were obtained in the purified LinA1 experiments. The difference of carbon isotope fractionation between crude extracts and enzyme indicates that cell material such as vesicles or membrane remnants may affect the transport of substrate leading to lower isotope fractionation. Interestingly, ε_c^- values obtained from the cell suspension experiments (experiment c and d: -2.3±0.4‰ and -2.3±0.3‰; table 1) were only slightly lower than the ones from the crude extracts experiments (experiment g and h: -3.4±0.5‰ and -3.4±0.6‰; table 1). This indicates that mass transfer into the cells does not affect the isotope fractionation of (-)α-HCH significantly. Statistically similar values were also obtained when comparing the pure enzyme and crude extracts experiments, indicating that mass transfer is not limited and that bond cleavage of the reaction governs the observed isotope fractionation.

Overall, the uptake and passage through the cell wall lead to rate limitation reducing the carbon isotope fractionation for both enantiomers, which indicates that uptake affects the isotope fractionation of individual enantiomers in a similar way. A similar effect on the reduction of carbon isotope fractionation had been observed with non-enantiomeric substances such as chlorinated ethenes and uptake of substrate into the cell often reduced isotope fractionation.[44] The investigation of enantiomer and isotope fractionation of phenoxypropionic acid herbicides in aerobic biodegradation gave contrasting results as high enantiomer fractionation and minor carbon isotope fractionation were observed.[4] Qiu and colleagues observed higher carbon isotope fractionation of (R)-DCPP in RdpA enzyme degradation compared to degradation by the whole cell of the host organism.[4] However, the enantiomer fractionation during uptake was not studied in detail. They speculated on active transport over the cell membrane as a mechanism of enantiomer fractionation and masking of isotope fractionation. In contrast, the enantiomer and isotope fractionation during α-HCH degradation are related to the reaction kinetics which is governed by the uptake into the cell and activity of individual enzymes.

The mass transport into cells and within the cells is a rate limiting step prior to isotope sensitive bond cleavage and reduces the isotope fractionation of (+)α- and (-)α-HCH leading in both cases to smaller ε_c. The ES values of cell suspensions with uptake through the cell membrane (experiment b-d) averages to -045±0.03 and crude extracts without membrane passage averages to -0.60±0.05, suggesting that the rate limitations of the reaction caused by membrane transport are similar to masking of isotope fractionation. However, as enantiomer fractionation depends on the different degradation rates of (-) and (+)α-HCH, it can be changed if the degradation of both enantiomers are affected to a different extent by mass transfer limitation. Comparing to the cell suspension experiments, the relatively higher ES values in the crude extracts experiments indicate that the membrane affects the uptake of the individual enantiomers into the cells at different extents (κ^+/κ^- is not constant throughout the degradation) and lead to the different ES values.

Effect of Degradation Rate on Isotope and Enantiomer Fractionation. For (-)α-HCH degradation, significant carbon isotope fractionation of (-)α-HCH in the cell suspension experiments was only observed when the κ^- were 0.35±0.02 h^{-1} and 0.15±0.02 h^{-1}. Higher κ^- values (experiment a and b: 0.81±0.12 and 0.76±0.09 h^{-1}) led to lower or even disappearance of isotope fractionation. In crude extract experiments, the relatively higher κ^- of experiment f (0.45±0.06 h^{-1}) led to lower ε_c^- compared to experiment g and h. In both cell suspension and crude extract experiments, it is demonstrated that isotope fractionation at higher rates does not characterize the bond cleavage because it is not the rate determining step of the reaction in (-)α-HCH degradation. In the case of (+)α-HCH degradation, when the κ^+ value of cell suspension experiments fall below 0.5 h^{-1}, significant isotope fractionation can be observed and the ε_c of (+)α-HCH was nearly identical in parallel experiments (Table 1). The same results were observed in the crude extracts experiments. In this case, the degradation rate did not significantly affect the carbon isotope fractionation of (+)α-HCH and the bond cleavage was the main rate limiting step of the reaction.

The ES values indicate preferential degradation of (-)α-HCH over (+)α-HCH in all cell suspension and crude extract experiments. Comparison of ES values from cell suspensions (avg.= -0.45±0.03) with crude extracts (avg.= -0.60±0.05) suggests that uptake into the cells reduced the enantiomer fractionation to some extent. As diffusion is identical for enantiomers, the passive chemical passage through the membrane should not affect the enantiomer composition.

Therefore, the degradation kinetics of both enantiomers governs enantiomer fractionation and it will be constant if the degradation kinetics of both enantiomers is affected to the same extent. This is mechanistically not comparable to mass transfer limitations that affect isotope fractionation, as two individual substances are catalyzed by two enzymes and the variability of the degradation rates does not allow for a correlation with independent mass transfer limitations.

ENVIRONMENTAL IMPLICATION

Transformation of α-HCH by LinA1 and LinA2 enzymes exhibits a pronounced specificity for (+)α-HCH and (-)α-HCH, respectively. Therefore, the extent of aerobic degradation of α-HCH in field sites can be quantified robustly by enantiomer-specific stable isotope analysis. Additionally, the reported ε_C values can be used for evaluation of aerobic α-HCH enantiomer degradation in field sites. The variable enantiomer preference during α-HCH degradation by *Sphingobium* spp. under growth conditions indicates the challenge of applying enantiomer fractionation for quantification. Variable enantiomer fractionation leads to uncertainty for quantitative work. However, compared to the nutrient rich culture medium, the microbes in field sites can be limited by various abiotic and biotic factors, such as energy sources, levels of oxygen, temperatures, pH and osmolality.[45] Consequently, the growth of bacteria may be relatively slower (due to limitations of temperature, nutrients etc.) leading to a slower renewal and expression of enzymes. In this case, the inventory of enzymes may not change quickly leading to stable enantiomer preference as proved in our degradation experiment under nutrient-

limited condition. Therefore, the enantiomer fractionation might be applied for quantification of enantiomer degradation in field site.

In a recent paper, we used isotope fractionation of individual α-HCH enantiomers and enantiomer fractionation for characterizing *in situ* biodegradation in complex aquifer systems in Bitterfeld, Germany, with changing hydrological conditions.[46] The results showed discrepancies in the extent of biodegradation calculated by Rayleigh equation based on isotope and enantiomer fractionation. The quantification of enantiomer fractionation using the Rayleigh concepts is mathematically inconsistent which leads to uncertainty. For quantification of enantiomer fractionation, the ES, instead of enantiomer fractionation factor based on Rayleigh equation, should be determined. ES is mathematically consistent as long as the degradation of enantiomers can be described by the first order kinetics. However, the study presented here on ES and CSIA of enantiomers showed that ES is variable and depends on growth conditions. Hence, ES needs to be used with reservation for quantitative work. Nevertheless, the combination of isotope fractionation and enantiomer fractionation for field site evaluation can take advantage of enantiomer fractionation for characterizing aerobic degradation processes qualitatively and help to select a fractionation factor for quantifying biodegradation. Thus, this study represents the first step towards developing a better understanding of isotope and enantiomer fractionation and will aid in field site evaluation.

AUTHOR INFORMATION

Corresponding Author: Hans-Hermann Richnow

Phone: +49-3412351212; Fax: +49-341450822; E-mail: hans.richnow@ufz.de

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Supporting Information

Details on strain cultivation, experimental conductions, analytic methods and summary of observed data can be found in supporting information.

REFERENCE

1. Ye, J.; Zhao, M.; Niu, L.; Liu, W., Enantioselective environmental toxicology of chiral pesticides. *Chem. Res. Toxicol.* **2015,** *28*, (3), 325-38.
2. Burden, R. S.; Carter, G. A.; Clark, T.; Cooke, D. T.; Croker, S. J.; Deas, A. H.; Hedden, P.; James, C. S.; Lenton, J. R., Comparative activity of the enantiomers of triadimenol and paclobutrazol as inhibitors of fungal growth and plant sterol and gibberellin biosynthesis. *Pest. Manag. Sci.* **1987,** *21*, (4), 253-267.
3. Wong, C. S., Environmental fate processes and biochemical transformations of chiral emerging organic pollutants. *Anal. Bioanal. Chem.* **2006,** *386*, (3), 544-558.
4. Qiu, S.; Gözdereliler, E.; Weyrauch, P.; Lopez, E. C. M.; Kohler, H.-P. E.; Sørensen, S. R.; Meckenstock, R. U.; Elsner, M., Small $^{13}C/^{12}C$ Fractionation Contrasts with Large Enantiomer Fractionation in Aerobic Biodegradation of Phenoxy Acids. *Environ. Sci. Technol.* **2014,** *48*, (10), 5501-5511.
5. Muller, T. A.; Kohler, H. P., Chirality of pollutants--effects on metabolism and fate. *Appl. Microbiol. Biotechnol.* **2004,** *64*, (3), 300-16.
6. Hashim, N.; Shafie, S.; Khan, S., Enantiomeric fraction as an indicator of pharmaceutical biotransformation during wastewater treatment and in the environment–a review. *Environ. Technol.* **2010,** *31*, (12), 1349-1370.
7. Nijenhuis, I.; Richnow, H. H., Stable isotope fractionation concepts for characterizing biotransformation of organohalides. *Curr. Opin. Biotechnol.* **2016,** *41*, 108-113.

8. Vogt, C.; Dorer, C.; Musat, F.; Richnow, H.-H., Multi-element isotope fractionation concepts to characterize the biodegradation of hydrocarbons—from enzymes to the environment. *Curr. Opin. Biotechnol.* **2016,** *41*, 90-98.
9. Elsner, M.; Jochmann, M. A.; Hofstetter, T. B.; Hunkeler, D.; Bernstein, A.; Schmidt, T. C.; Schimmelmann, A., Current challenges in compound-specific stable isotope analysis of environmental organic contaminants. *Anal. Bioanal. Chem.* **2012,** *403*, (9), 2471-2491.
10. Nijenhuis, I.; Renpenning, J.; Kümmel, S.; Richnow, H. H.; Gehre, M., Recent advances in multi-element compound-specific stable isotope analysis of organohalides: Achievements, challenges and prospects for assessing environmental sources and transformation. *Trends Environ. Anal. Chem.* **2016,** *11*, 1-8.
11. Gasser, G.; Pankratov, I.; Elhanany, S.; Werner, P.; Gun, J.; Gelman, F.; Lev, O., Field and laboratory studies of the fate and enantiomeric enrichment of venlafaxine and O-desmethylvenlafaxine under aerobic and anaerobic conditions. *Chemosphere* **2012,** *88*, (1), 98-105.
12. Bashir, S.; Fischer, A.; Nijenhuis, I.; Richnow, H. H., Enantioselective carbon stable isotope fractionation of hexachlorocyclohexane during aerobic biodegradation by Sphingobium spp. *Environ. Sci. Technol.* **2013,** *47*, (20), 11432-11439.
13. Jammer, S.; Voloshenko, A.; Gelman, F.; Lev, O., Chiral and isotope analyses for assessing the degradation of organic contaminants in the environment: Rayleigh dependence. *Environ. Sci. Technol.* **2014,** *48*, (6), 3310-3318.
14. Ivdra, N.; Fischer, A.; Herrero-Martin, S.; Giunta, T.; Bonifacie, M.; Richnow, H.-H., Carbon, Hydrogen and Chlorine Stable Isotope Fingerprinting for Forensic Investigations of Hexachlorocyclohexanes. *Environ. Sci. Technol.* **2016,** *51*, (1), 446-454.
15. Nagata, Y.; Miyauchi, K.; Takagi, M., Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. *J. Ind. Microbiol. Biotechnol.* **1999,** *23*, (4), 380-390.
16. Kumari, R.; Subudhi, S.; Suar, M.; Dhingra, G.; Raina, V.; Dogra, C.; Lal, S.; van der Meer, J. R.; Holliger, C.; Lal, R., Cloning and characterization of lin genes responsible for the degradation of hexachlorocyclohexane isomers by *Sphingomonas paucimobilis* strain B90. *Appl. Environ. Microb.* **2002,** *68*, (12), 6021-6028.
17. Lal, R.; Pandey, G.; Sharma, P.; Kumari, K.; Malhotra, S.; Pandey, R.; Raina, V.; Kohler, H. P.; Holliger, C.; Jackson, C.; Oakeshott, J. G., Biochemistry of microbial degradation of hexachlorocyclohexane and prospects for bioremediation. *Microbiol. Mol. Biol. Rev.* **2010,** *74*, (1), 58-80.
18. Böltner, D.; Moreno‐Morillas, S.; Ramos, J. L., 16S rDNA phylogeny and distribution of lin genes in novel hexachlorocyclohexane‐degrading *Sphingomonas* strains. *Environ. Microbiol.* **2005,** *7*, (9), 1329-1338.
19. Mohn, W. W.; Mertens, B.; Neufeld, J. D.; Verstraete, W.; de Lorenzo, V., Distribution and phylogeny of hexachlorocyclohexane-degrading bacteria in soils from Spain. *Environ Microbiol* **2006,** *8*, (1), 60-8.
20. Sharma, P.; Pandey, R.; Kumari, K.; Pandey, G.; Jackson, C. J.; Russell, R. J.; Oakeshott, J. G.; Lal, R., Kinetic and sequence-structure-function analysis of known LinA variants with different hexachlorocyclohexane isomers. *PLoS One* **2011,** *6*, (9), e25128. doi:10.1371/journal.pone.0025128
21. Shrivastava, N.; Macwan, A. S.; Kohler, H. E.; Kumar, A., Important amino acid residues of hexachlorocyclohexane dehydrochlorinases (LinA) for enantioselective transformation of hexachlorocyclohexane isomers. *Biodegradation* **2017,** *28*, (2-3), 171-180.
22. Macwan, A. S.; Kukshal, V.; Srivastava, N.; Javed, S.; Kumar, A.; Ramachandran, R., Crystal structure of the hexachlorocyclohexane dehydrochlorinase (LinA-type2): mutational analysis, thermostability and enantioselectivity. *PLoS One* **2012,** *7*, (11), e50373. doi:10.1371/journal.pone.0050373
23. Pal, R.; Bala, S.; Dadhwal, M.; Kumar, M.; Dhingra, G.; Prakash, O.; Prabagaran, S. R.; Shivaji, S.; Cullum, J.; Holliger, C.; Lal, R., Hexachlorocyclohexane-degrading bacterial strains

Sphingomonas paucimobilis B90A, UT26 and Sp+, having similar lin genes, represent three distinct species, *Sphingobium indicum* sp. nov., *Sphingobium japonicum* sp. nov. and *Sphingobium francense* sp. nov., and reclassification of [Sphingomonas] chungbukensis as *Sphingobium chungbukense* comb. nov. *Int. J. Syst. Evol. Microbiol.* **2005,** *55*, (Pt 5), 1965-72.

24. Singh, A. K.; Chaudhary, P.; Macwan, A. S.; Diwedi, U. N.; Kumar, A., Selective loss of lin genes from hexachlorocyclohexane-degrading Pseudomonas aeruginosa ITRC-5 under different growth conditions. *Appl. Microbiol. Biotechnol.* **2007,** *76*, (4), 895-901.

25. Suar, M.; van der Meer, J. R.; Lawlor, K.; Holliger, C.; Lal, R., Dynamics of multiple lin gene expression in Sphingomonas paucimobilis B90A in response to different hexachlorocyclohexane isomers. *Appl. Environ. Microbiol.* **2004,** *70*, (11), 6650-6.

26. Verma, H.; Kumar, R.; Oldach, P.; Sangwan, N.; Khurana, J. P.; Gilbert, J. A.; Lal, R., Comparative genomic analysis of nine Sphingobium strains: insights into their evolution and hexachlorocyclohexane (HCH) degradation pathways. *BMC genomics* **2014,** *15*, (1), 1014. doi:10.1186/1471-2164-15-1014

27. Bashir, S.; Fischer, A.; Nijenhuis, I.; Richnow, H. H., Enantioselective carbon stable isotope fractionation of hexachlorocyclohexane during aerobic biodegradation by Sphingobium spp. *Environ. Sci. Technol.* **2013,** *47*, (20), 11432-9.

28. Badea, S. L.; Vogt, C.; Gehre, M.; Fischer, A.; Danet, A. F.; Richnow, H. H., Development of an enantiomer-specific stable carbon isotope analysis (ESIA) method for assessing the fate of alpha-hexachlorocyclo-hexane in the environment. *Rapid Commun. Mass. Sp.* **2011,** *25*, (10), 1363-1372.

29. Schilling, I. E.; Bopp, C. E.; Lal, R.; Kohler, H. E.; Hofstetter, T. B., Assessing Aerobic Biotransformation of Hexachlorocyclohexane Isomers by Compound-Specific Isotope Analysis. *Environ. Sci. Technol.* **2019,** 53, (5), 2353-2363.

30. Mueller, M. D.; Buser, H.-R., Environmental Behavior of Acetamide Pesticide Stereoisomers. 2. Stereo- and Enantioselective Degradation in Sewage Sludge and Soil. *Environ. Sci. Technol.* **1995,** *29*, (8), 2031-2037.

31. Buerge, I. J.; Poiger, T.; Müller, M. D.; Buser, H.-R., Enantioselective Degradation of Metalaxyl in Soils: Chiral Preference Changes with Soil pH. *Environ. Sci. Technol.* **2003,** *37*, (12), 2668-2674.

32. Suar, M.; Hauser, A.; Poiger, T.; Buser, H. R.; Muller, M. D.; Dogra, C.; Raina, V.; Holliger, C.; van der Meer, J. R.; Lal, R.; Kohler, H. P., Enantioselective transformation of alpha-hexachlorocyclohexane by the dehydrochlorinases LinA1 and LinA2 from the soil bacterium *Sphingomonas paucimobilis* B90A. *Appl. Environ. Microb.* **2005,** *71*, (12), 8514-8518.

33. Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P., A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. *Environ. Sci. Technol.* **2005,** *39*, (18), 6896-6916.

34. Northrop, D. B., The expression of isotope effects on enzyme-catalyzed reactions. *Annu. Rev. Biochem.* **1981,** *50*, (1), 103-131.

35. Swiderek, K.; Paneth, P., Binding isotope effects. *Chem. Rev.* **2013,** *113*, (10), 7851-79.

36. Okai, M.; Kubota, K.; Fukuda, M.; Nagata, Y.; Nagata, K.; Tanokura, M., Crystal structure of gamma-hexachlorocyclohexane Dehydrochlorinase LinA from *Sphingobium japonicum* UT26. *J. Mol. Biol.* **2010,** *403*, (2), 260-9.

37. Manna, R. N.; Zinovjev, K.; Tunon, I.; Dybala-Defratyka, A., Dehydrochlorination of Hexachlorocyclohexanes Catalyzed by the LinA Dehydrohalogenase. A QM/MM Study. *J. Phys. Chem. B* **2015,** *119*, (49), 15100-9.

38. Manna, R. N.; Dybala-Defratyka, A., Insights into the elimination mechanisms employed for the degradation of different hexachlorocyclohexane isomers using kinetic isotope effects and docking studies. *J. Phys. Org. Chem.* **2013,** *26*, (10), 797-804.

39. Nikolausz, M.; Nijenhuis, I.; Ziller, K.; Richnow, H. H.; Kästner, M., Stable carbon isotope fractionation during degradation of dichloromethane by methylotrophic bacteria. *Environ. Microbiol.* **2006,** *8*, (1), 156-164.

40. Fletcher, K. E.; Löffler, F. E.; Richnow, H.-H.; Nijenhuis, I., Stable carbon isotope fractionation of 1, 2-dichloropropane during dichloroelimination by *Dehalococcoides* populations. *Environ. Sci. Technol.* **2009,** *43*, (18), 6915-6919.
41. Nijenhuis, I.; Andert, J.; Beck, K.; Kastner, M.; Diekert, G.; Richnow, H. H., Stable isotope fractionation of tetrachloroethene during reductive dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S and abiotic reactions with cyanocobalamin. *Appl. Environ. Microb.* **2005,** *71*, (7), 3413-9.
42. Reetz, M. T., Laboratory evolution of stereoselective enzymes: a prolific source of catalysts for asymmetric reactions. *Angew. Chem. Int. Ed.* **2011,** *50*, (1), 138-174.
43. Muller, R. H.; Kleinsteuber, S.; Babel, W., Physiological and genetic characteristics of two bacterial strains utilizing phenoxypropionate and phenoxyacetate herbicides. *Microbiol. Res.* **2001,** *156*, (2), 121-31.
44. Renpenning, J.; Rapp, I.; Nijenhuis, I., Substrate hydrophobicity and cell composition influence the extent of rate limitation and masking of isotope fractionation during microbial reductive dehalogenation of chlorinated ethenes. *Environ. Sci. Technol.* **2015,** *49*, (7), 4293-301.
45. van Elsas, J. D.; Semenov, A. V.; Costa, R.; Trevors, J. T., Survival of Escherichia coli in the environment: fundamental and public health aspects. *ISME J.* **2011,** *5*, (2), 173-83.
46. Liu, Y.; Bashir, S.; Stollberg, R.; Trabitzsch, R.; Weiss, H.; Paschke, H.; Nijenhuis, I.; Richnow, H. H., Compound Specific and Enantioselective Stable Isotope Analysis as Tools To Monitor Transformation of Hexachlorocyclohexane (HCH) in a Complex Aquifer System. *Environ. Sci. Technol.* **2017,** *51*, (16), 8909-8916.

Tables and figures

Table 1.Summary of stable carbon isotope enrichment factors (ε_c), enantioselectivity (ES), and related kinetic rate constants (κ) in different sets of α-HCH degradation experiments by cell suspension, crude extracts and LinA enzymes of strain B90A.

				Kinetic constant				Isotope fractionation				Enantiomer fractionation	
				(-)α-HCH		(+)α-HCH		(-)α-HCH		(+)α-HCH			
	M	N	Amount of biomass (μL)	$\kappa^- \pm CI_{95\%}$ (h^{-1})	R^2	$\kappa^+ \pm CI_{95\%}$ (h^{-1})	R^2	$\varepsilon_c^- \pm CI_{95\%}$ (‰)	R^2	$\varepsilon_c^+ \pm CI_{95\%}$ (‰)	R^2	ES	
cell suspension	a	15	300	0.81±0.12	0.95	0.49±0.03	0.99	n.s.	n.s.	-6.4±0.7	0.97	-0.25±0.08	
	b	13	200	0.76±0.09	0.97	0.30±0.03	0.98	n.s.	n.s.	-6.2±1.2	0.92	-0.43±0.06	avg.= -0.45±0.03
	c	12	100	0.35±0.02	0.99	0.12±0.02	0.96	-2.3±0.4	0.92	-6.1±1.1	0.93	-0.49±0.07	
	d	16	50	0.15±0.02	0.95	0.06±0.01	0.93	-2.3±0.3	0.95	-6.1±1.4	0.87	-0.43±0.09	
								avg.= -2.3; sd.=0.03		avg.= -6.2; sd.=0.1			
Crude extracts	e	10	200	0.58±0.13	0.94	0.30±0.07	0.93	n.s.	n.s	-7.4±0.7	0.99	-0.32±0.15	
	f	13	100	0.45±0.06	0.96	0.12±0.02	0.91	-1.9±0.4	0.90	-8.0±1.3	0.94	-0.58±0.07	avg.= -0.60±0.05
	g	13	50	0.19±0.02	0.98	0.04±0.01	0.95	-3.4±0.5	0.96	n.a.	n.a.	-0.65±0.08	
	h	14	50	0.18±0.04	0.91	0.05±0.01	0.91	-3.4±0.6	0.92	n.a.	n.a.	-0.57±0.10	
								avg.= -3.4; sd.=0.02		avg.= -7.7; sd.=0.4			
LinA2	i	13	10	0.65±0.10	0.95	n.a.	n.a.	-3.7±0.6	0.95	n.a.	n.a.	-1	
	j	9	8	0.54±0.28	0.74	n.a.	n.a.	-4.0±1.0	0.93	n.a.	n.a.	-1	
	k	14	5	0.27±0.05	0.91	n.a.	n.a.	-3.6±0.5	0.95	n.a.	n.a.	-1	
								avg.= -3.8; sd.=0.2					
LinA1	l	13	10	n.a.	n.a.	0.28±0.02	0.98	n.a.	n.a.	-11.3±2.0	0.94	1	
	m	14	5	n.a.	n.a.	0.13±0.02	0.93	n.a.	n.a.	-10.9±1.5	0.96	1	
										avg.= -11.1; sd.= 0.3			

M: different sets of the experiments and the detailed experimental conditions can be found in the Experimental Section; N: number of samples; $CI_{95\%}$: Confidence interval at level 95%; n.a.: not assessed since degradation was negligible; n.s.: degradation observed but no significant or no consistent isotope enrichment was observed, the detail information are reported in SI7); avg.: average value; sd.: standard deviation.

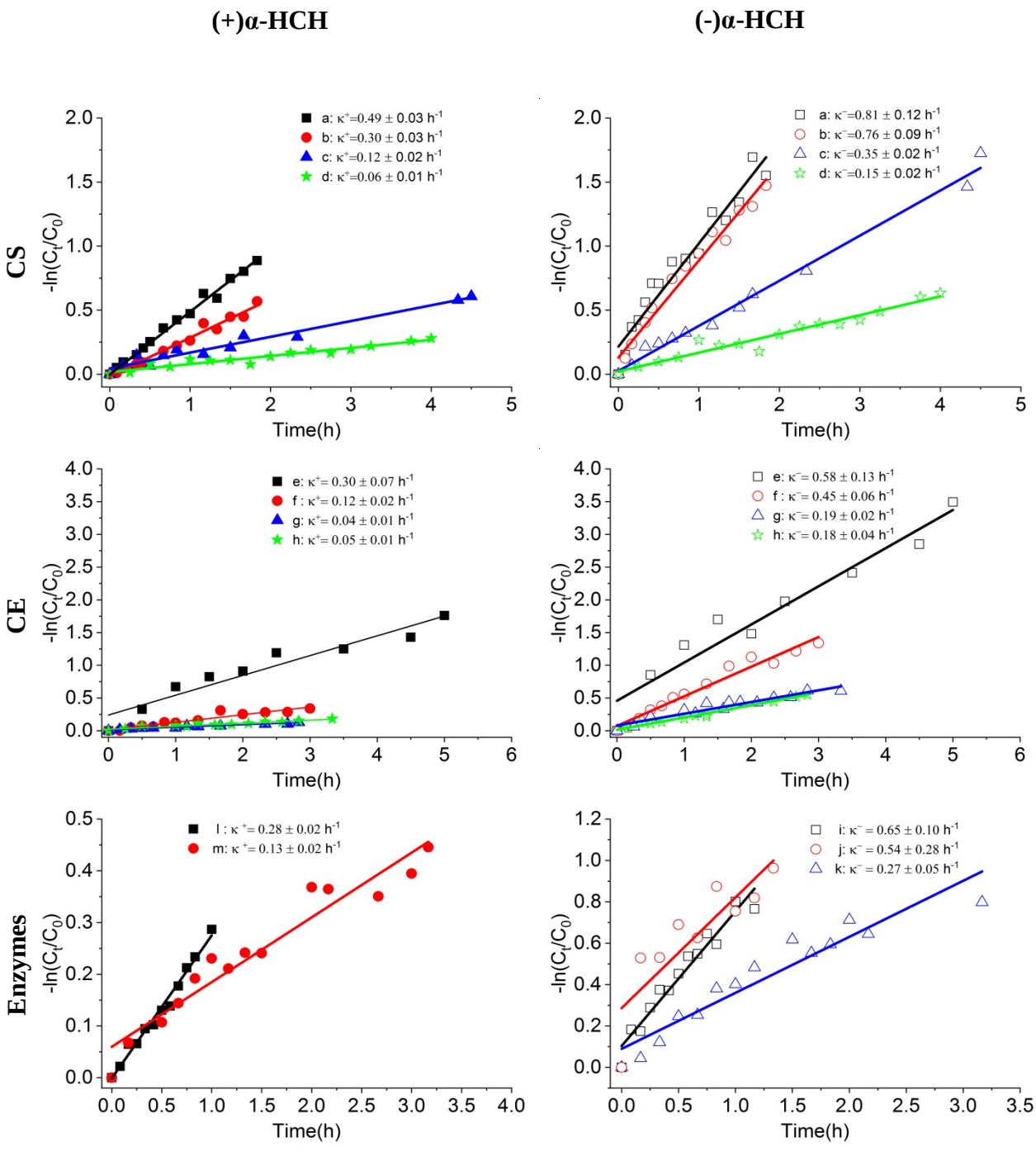

Figure 1. The degradation kinetics of (+)α-HCH (close symbols)and (-)α-HCH (open symbols) by cell suspension (CS) and crude extracts (CE) of *S. indicum* strain B90A and the corresponding enzymes (LinA1 and LinA2). The letter a-k represents different experimental conditions that are defined in the experimental section

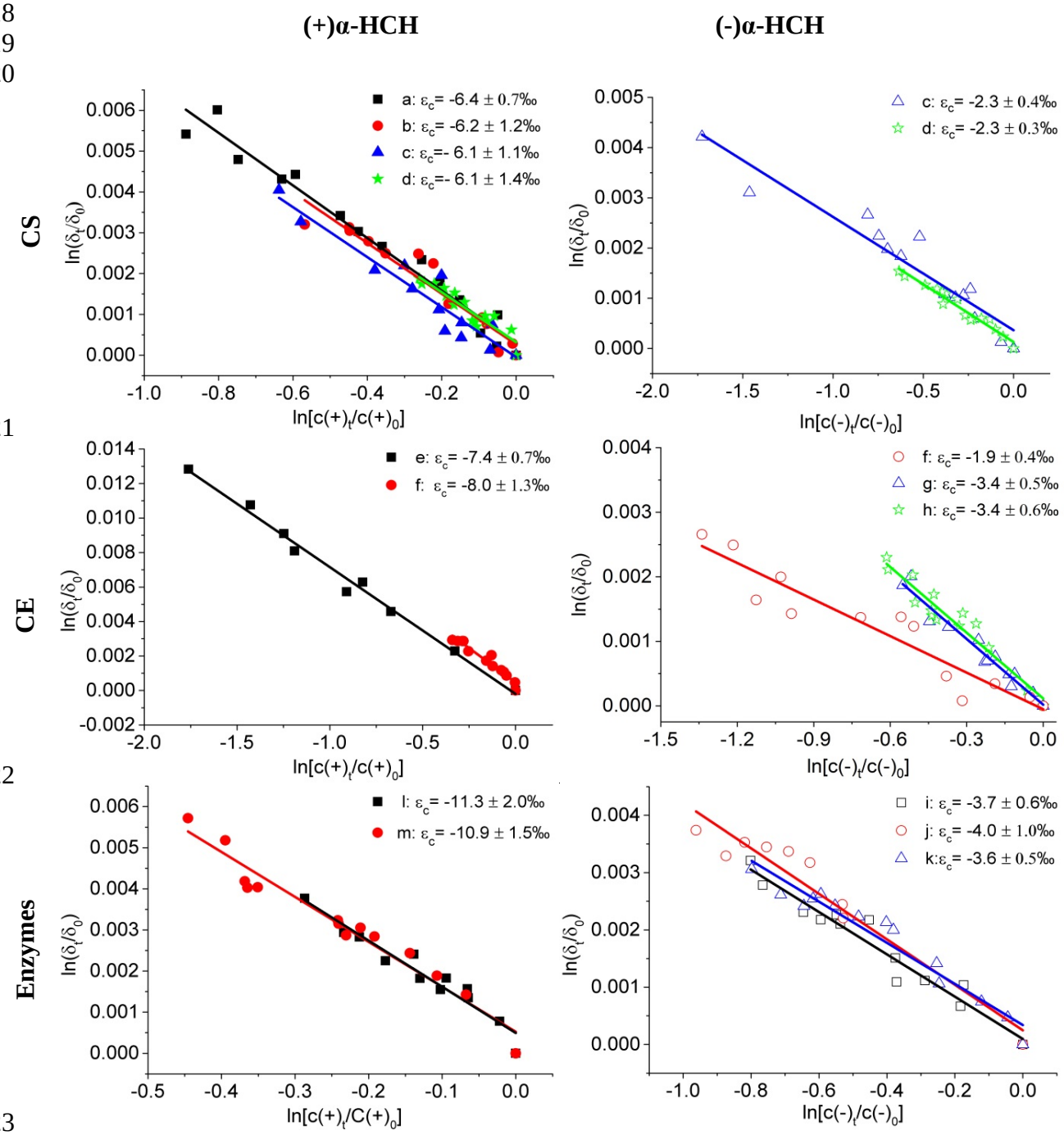

Figure 2. Linearized Rayleigh equation plots showing the carbon isotope fractionation for the biodegradation of α-HCH enantiomers (close symbols for (+)α-HCH and open symbols for (-)α-HCH) by cell suspension (CS) and crude extracts (CE) of *S. indicum* strain B90A and the corresponding enzymes LinA1 and LinA2. The individual evaluation of the isotope fractionation for each experiment can be found in Figure S8 (CS), Figure S9 (CE), Figure S10 (LinA2) and Figure S111 (LinA1). The letter a-k represents different experimental conditions that are defined in the experimental section and summarized in Table 1.