Compound-specific Isotope Analysis and Enantiomer Fractionation to Characterize the Transformation of Hexachlorocyclohexane Isomers in a Soil-wheat Pot System

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Abstract

The uptake by plant from soil is one of the first steps for hexachlorocyclohexane isomers (HCHs) to enter the food web. However, the HCH transformation associated with the uptake process is still not well understood. Therefore, a soil-wheat pot experiment was conducted to characterize the HCH transformation during wheat growth using compound-specific isotope analysis (CSIA) and enantiomer fractionation. The results showed that the $\delta^{13}C$ and $\delta^{37}Cl$ values of β-HCH remained stable in soil and wheat, revealing no transformation. In contrast, an increase of $\delta^{13}C$ and $\delta^{37}Cl$ values of α-HCH indicated its transformation in soil and wheat. A shift of the enantiomer fraction (EF) (-) from 0.50 to 0.35 in soil at jointing stage and 0.35 to 0.57 at harvest stage suggested that the preferential transformation of enantiomers varied at different growth stages. Based on the dual element isotope analysis, the transformation mechanism in soil-wheat system was different from that in wheat in hydroponic systems. The high abundance of HCH degraders, *Sphingomnas* sp. and *Novosphingobium* sp., was detected in the α-HCH treated rhizosphere soil, supporting the potential for biotransformation. The application of CSIA and EF allows characterizing the transformation of organic pollutants such as HCHs in the complex soil-plant systems.

INTRODUCTION

A large amount of hexachlorocyclohexane (HCH) muck (mostly containing α- and β-HCH) was dumped in the environment during the production of Lindane (γ-HCH) [1]. Although Lindane was banned according to the Stockholm convention in 2009 [2], the HCH contamination has spread globally, and the bioaccumulation and bio-magnification of HCHs were found in the food web. There are high concerns about the accumulation of HCHs in food web as human could be the final receptors. HCHs have been detected in human milk [3], blood [4], and fatty tissues [5,6]. Plant uptake from soil is one of the first steps for HCHs to enter the food web, leading to increasing human exposure risks. The uptake of HCHs by roots potentially with the transpiration stream, or by leaves from the gas phase has been discussed before [7,8]. Bioconcentration, which is defined as the ratio between the concentration of HCHs in wheat tissues and that of in the host soil, was overestimated when comparing model data with the data from a field experiment [8]. This could be due to the underestimation of the HCH transformation in plants in these models. Therefore, the models need to be improved by taking the transformation into account. However, the transformation of HCHs associated with their uptake process in the soil-plant system is still not well understood. The key question is which factors control the uptake of HCH into plants, and how the uptake is accompanied by the biotransformation either by microorganisms in the soil, endophytes within plants, or metabolic processes in plants.

The transformation of HCHs by soil microorganism was reported in the literature [9,10]. There are no reports about the endophytes and enzymes involved in the transformation of HCHs in plant, however, the transformation of other organic contaminants during uptake into plants has been controversially discussed. For instance, polycyclic aromatic hydrocarbons could be degraded in

biofilms on root surfaces and by endophytes as demonstrated for phenanthrene [11–13]. Ibuprofen could be transformed by plant-derived enzymes [14].

Compound-specific isotope analysis (CSIA) has been considered to be a promising tool for characterizing the fate of HCHs in the field [15,16]. Several carbon isotope enrichment factors obtained from the pure culture of *Sphingomonas* sp. could be applied to characterize the aerobic α-HCH degradation [17,18]. Enantiomer fraction (EF) is an indicator of the biotransformation of chiral compounds and can indicate the selective uptake processes of specific enantiomers [19]. Several studies have combined the CSIA and EF to characterize the microbial transformation of α-HCH at the laboratory scale and in aquifers at landscape level [17,20]. The application of CSIA and EF methods in soil-plant system could give impetus for characterizing the fate of organic pollutants in complex systems, and it is a huge step towards the development of the methods.

Wu et al. [21] analyzed the isotope composition of HCHs in plants and soil to monitor the transformation of HCHs in a contaminated field. However, it is difficult to detect the degradation of HCHs in soil and in plants because many variable growth conditions in the field, such as dry/wet seasons and nutrition for plant growth, can influence the uptake processes. A hydroponic experiment conducted under sterile conditions with wheat provided the evidence of α-HCH transformation in plant during uptake along with the water flow from root to leaves [22]. However, the transformation of HCHs during uptake from soil to plant could be also affected by the rhizosphere microbiome.

Therefore, a soil pot experiment was conducted to systematically investigate the isomer- and enantiomer-specific uptake, possible turnover of HCHs in soil and in wheat using CSIA and EF. We selected three wheat growth stages covering the whole period of wheat growth to investigate

the influence of plant growth on the transformation of HCHs in the soil-plant system. The HCH transformation was examined by the HCH concentration, isotope fractionation as well as enantiomer fractionation in soil and wheat tissues. In addition, the microbial community in the bulk and rhizosphere soil at the harvest stage was evaluated to elucidate the transformation of HCHs in soil. The understanding of the HCH transformation in soil-plant systems could provide valuable details for the development of phytoremediation strategies.

MATERIALS AND METHODS

The sources and quality of chemicals are provided in the S1 (SI).

Seeds and Plant Exposure to HCHs in the Soil. Wheat (cultivar *Quintus*" of *Triticum aestivum L.*) was used as the test plant and the seeds were obtained from the breeder Saaten-Union GmbH (Isernhagen, Germany). The soil was provided by LAV Technische Dienste GmbH (Markranstädt, Germany). The major physicochemical properties of the sandy soil are: total organic carbon 2.2%, pH 7.9, soil texture is sandy silt soil with 11 % clay, 49 % silt, 40 % sand. The soil is classified as Luvisol (World Reference Base). The parent material is loess formed in a temperate zone located in the Saxony (Germany).

The experiment was conducted in the glass house of the research station of the UFZ in Bad Lauchstädt. The sandy soil was air-dried and sieved through a 5 mm mesh before usage. Then, 250 g of soil was spiked with 210 mg of α- and β-HCH solutions in acetone individually. When the acetone was evaporated, the spiked soil was mixed with non-spiked soil with the addition of basic fertilization (6g $CaHPO_4 \cdot 2H_2O$ as solid; 4.46g K_2SO_4, 5.125g $MgSO_4 \cdot 7H_2O$, 5.72g NH_4NO_3, and 0.15g $FeCl_3$ as solution; 3ml Hoagland micro nutrition solution). Afterwards, the soil was homogenized thoroughly before packed into pots (7kg soil per pot) and then equilibrated at room

temperature for several days at 60% of the water holding capacity. The final concentration of HCH isomers was theoretically 30 mg kg^{-1}. In each pot, 12 seeds were grown uniformly and at last 9 seedlings were left after germination. During the whole period of wheat growth, the water content of soil was maintained as 60% of the maximum water content of soil. The treatments for the individual experiment were as follows: 30 mg kg^{-1} α-HCH spiked sandy soil with wheat (α-HCH spiked treatment), 30 mg kg^{-1} β-HCH spiked sandy soil with wheat (β-HCH spiked treatment); non-spiked sandy soil with wheat (Control). Each treatment had 4 replicates. The pots of different treatments were placed randomly. The soil surface in each pot was covered by a thin layer of 2-cm silica sand (150-380 um) to prevent the transportation of soil particles directly to the leave surface by air and to reduce the HCH exchange between soil-air interface as reported for hexabromocyclododecane isomers (HBCDs) [23].

Sampling of HCHs from the Soil and Plant. Wheat grew for 102 days from April to July in 2018. At the jointing (48 days), heading (64 days) and harvest stage (102 days), soil and plant were sampled. The soil samples were separated into bulk soil and rhizosphere soil. The whole soil and root system was gently crushed and loosely held soil was separated by shaking. This is referred as bulk soil. The remaining tightly held soil particles were considered as the rhizosphere soil, and were removed by shaking in a plastic bag [24]. A small portion of the soil samples were frozen at -20 °C for metagenomics analysis. The remaining soil samples were lyophilized for further treatment. An aliquot of the initial soil before planting were taken and named as the original soil for comparison. After taking the rhizosphere soil, plant samples were washed thoroughly for 4 times using sterilized water and then separated into root, stem, leaf, spike and grain. Plant samples were lyophilized and ground for further treatment.

Extraction and Clean-up of HCHs from Soil and Plant Samples.

The method for the extraction and clean-up of HCHs in soil and plant has been developed previously for isotope analysis [25]. The details are shown in S2 (SI).

DNA extraction and sequencing. The genetic analysis was carried out by isolating the metagenomic DNA directly from the soil which contains representation from both active and inactive bacteria residing in the respective soil samples. DNA was extracted from soil using DNeasy PowerSoil Kit – QIAGEN (Cat no. 12888-50) as described in the manufacturer's instructions. The quality of DNA was assessed using a NanoDrop ND-1000 (Thermo Scientific). The shotgun sequencing was done in collaboration with Phixgen Pvt. Ltd. using an Illumina Hiseq-2500 platform with paired-end 150bp read length.

Analytical Methods. *Concentration Analysis.* An Agilent 6890 series GC (Agilent Technologies, USA) equipped with a flame ionization detector (FID) was used to determine the concentration of HCHs throughout the study. The details are shown in S3 (SI).

Isotope Analysis. The isotope composition of element (E) was reported as δ notation in parts per thousand (‰) relative to the international standard scale according to eq 1.

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

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

Carbon isotope composition ($\delta^{13}C$) was analyzed by a gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS), where a GC (7890A, Agilent Technologies, USA) was connected through a GC-IsoLink and a ConFlo IV interface (Thermo Fisher Scientific, Germany) to a MAT 253 IRMS system (Thermo Fisher Scientific, Germany). The details are shown in S3 (SI).

Chlorine isotope composition ($\delta^{37}Cl$) was analyzed using a gas chromatograph coupled with a multiple-collector inductively coupled plasma mass spectrometer (GC-MC-ICPMS), as recently described elsewhere [26]. The details are shown in S3 (SI).

Enantiomer Analysis. The Enantiomer fraction (EF) (-) is defined as $A^- / (A^+ + A^-)$, where A^+ and A^- correspond to the peak area or concentrations of (+) and (−) enantiomers. The EF of α-HCH was analyzed by gas chromatograph mass spectrometer (GC-MS) (Agilent Technologies 7890A for GC and 5975C for MS, USA) equipped with a γ-DEX 120 chiral column (30 m × 0.25 mm × 0.25 μm, Supelco, Bellefonte, PA, USA) [27]. The details are shown in S3 (SI).

Metabolites Analysis. The metabolites (1,3,4,5,6-pentachlorocyclohexene and 1,2,4-trichlorobenzene) of HCHs in plant and soil were measured by a GC-MS as mentioned above. The details are shown in S3 (SI).

Bioconcentration Factors. Bioconcentration factors (BCFs) for root (RCF), stem (SCF), leaf (LCF), spike (SPCF) and grain (GCF) were calculated as the ratio between the concentration of HCHs in wheat tissues and that of in the host soil to study the translocation of HCH in plants. The HCH concentration normalized to dry weight was used for calculation.

Metagenomics Analysis. The reads obtained after whole metagenome sequencing using Illumina platform were trimmed and analyzed. The details of method are reported in S3 (SI).

lin gene profiling and their relative abundance. The sequences for the *lin* genes from the complete genomes of *Sphingobium japonicum* UT26 and *Sphingobium indicum* B90A were taken as reference for assessing the presence of *lin* genes required for the transformation of HCHs. The details of method are reported in S3 (SI).

Dual elements isotope analysis. The Lambda (Λ) value was used to distinguish different transformation mechanisms in a complex system. Λ is defined as the slope of the regression line of the isotope fractionation of two elements during a transformation process [28].

Statistical analysis. The HCH concentration data in soil at different stages was analyzed statistically using analyses of variance (ANOVA) and Least Significance Difference post-hoc comparison testing with the SPSS soft- ware v19.0. The HCH concentration data in soil at the same stage and EF(-) data were analyzed using independent *t*-test ($p<0.05$) with the SPSS soft-ware v19.0.

Results and Discussion

Concentration of HCHs in the Soil-plant System. The yield wheat biomass was not influenced by the HCH spiking and details were shown in S4 and Table S1 (SI). The concentration of α-HCH in the bulk soil decreased from an initial concentration of 21.4 ± 0.6 mg kg^{-1} to 18.4 ± 1.5 mg kg^{-1} at the jointing stage, to 13.8 ± 0.3 mg kg^{-1} at the heading stage, and to 11.9 ± 0.3 mg kg^{-1} at the harvest stage (Fig. S1a.). The loss of HCHs in the bulk soil was a result of the evaporation, uptake, biotransformation, and evapotranspiration by plant. There was no difference of the α-HCH concentration in the rhizosphere soil at different stages but it was significantly lower than that in the bulk soil at the same stage (Fig. S1a.), which is coherent with the former report [24]. This was a result of higher abundance of HCH-degrading microorganisms, such as species of the genera *Sphingomonas* and *Novosphingobium* in the rhizosphere (the data was shown in below). Exudates from roots in the rhizosphere could support the number and diversity of microorganisms, leading to a larger potential for transforming organic pollutants [29].

Compared with the initial concentration (24.6 ± 0.8 mg kg^{-1}), the concentration of β-HCH in the bulk soil decreased to 23.9 ± 0.6 mg kg^{-1} at the jointing stage, and significantly decreased to 22.5 ± 0.7 mg kg^{-1} at the heading stage and 21.8 ± 0.5 mg kg^{-1} at the harvest stage, which was much higher than that for α-HCH (Fig. S1b.). Additionally, the concentration of β-HCH in the rhizosphere soil was nearly identical with that in the bulk soil in the same experimental phase, indicating that β-HCH may not be transformed preferentially in rhizosphere (Fig. S1b.).

No HCHs could be detected in the non-spiked control pots, suggesting the uptake of HCHs by leaf from the air in the present experiment could be neglected. In the spiked treatments, HCHs could be observed in all wheat tissues at three growth stages with the highest concentration in the roots followed by the stems and the lowest in the leaves. This indicates that HCHs were translocated to all parts of the wheat after uptake by roots (Fig. S1c and S1d.). The concentration of β-HCH in spike and grain was smaller than α-HCH, suggesting α-HCH was translocated to a larger extent to the upper part of wheat than β-HCH at the stage of harvest.

The BCFs of α-HCH was higher than that of β-HCH, which could be related to the different K_{ow} of HCHs (Table S2, SI.). The α-HCH has a lower K_{ow} (lower hydrophobicity) than β-HCH [30] and therefore may be easier to translocate along with the water flow in plant. The RCF and SCF of both α-HCH and β-HCH were increased along with the wheat growth and the highest value was observed at the harvest stage (Table S2, SI.). In contrast, the LCF values were the highest at the heading stage (Table S2, SI.). This suggested that several processes were involved in the accumulation of HCHs in leaves. Previous studies showed that the accumulation of HCHs in plant leaves was a result of the combination of uptake by root and foliar uptake from the air [7,8]. In this study, the only relevant uptake of HCHs was via the roots. However, chemicals in plant leaves might be exchanged to the air with the evapotranspiration in contrast to uptake by air [31].

Furthermore, during the overall translocation progress in the plant, HCHs will be internally translocated between different plant organs particularly during grain filling. At the harvest stage, the HCHs may be translocated along with biological material to the grain as the physiology of the plant is targeting on grain filling, which may cause the decrease of the LCF and SPCF at the harvest stage.

Carbon and Chlorine Isotope Fractionation of HCHs in the Soil-plant System. The isotopic changes discussed in present study are attributed to the kinetic isotope fractionation, which results in a preferential degradation of the isotopically light molecules. There was no difference in $\delta^{13}C$ and $\delta^{37}Cl$ values of β-HCH observed in the soil and wheat tissues compared to the β-HCH spiked to soil (Fig. 1a-d), revealing that β-HCH was not transformed in soil and wheat in this study. However, in the previous field study, a slight increase of $\delta^{13}C$ value of β-HCH was observed in the bulk soil [21]. The reason for the difference could be that the β-HCH degrading microbial community in soil has been well developed in the field due to long time exposure to β-HCH. Thus, β-HCH could be transformed prior to uptake into plant, leading to isotope enrichment of β-HCH residues in soil and some plants [21]. However, in this study, the experiment was conducted in 3 months and the soil microbial community had not developed the ability for transforming β-HCH or enough abundance of β-HCH degrading bacteria in this relatively short period. The $\delta^{13}C$ and $\delta^{37}Cl$ values of β-HCH showed that the translocation and accumulation of β-HCH by wheat did not change the C- and Cl-isotopic composition of β-HCH, thus the uptake alone could not affect the isotope composition as also was observed in the hydroponic experiments elsewhere [22].

Since α-HCH has similar physicochemical properties as β-HCH, the translocation only is unlikely to affect the isotope values of α-HCH. Interestingly, an increase in $\delta^{13}C$ value of α-HCH was observed in both bulk and rhizosphere soil at different growth stages compared to the initial value

of -28.5 ‰ (Fig. 2a), especially at the harvest stage ($\delta^{13}C$ value in the bulk and rhizosphere soil was -24.9 ‰ and -25.2 ‰, respectively). This indicates that α-HCH was transformed both in the bulk and rhizosphere soil. An increase of $\delta^{13}C$ value of α-HCH was found from the jointing stage to the harvest stage in both bulk and rhizosphere soil (Fig. 2a), which could be related to the development of α-HCH degrading bacteria along with the wheat growth. The result was consistent with a previous field study where the enrichment of ^{13}C isotopes of α-HCH in bulk soil were observed during plant growth compared to the HCH muck [21].

Compared to the $\delta^{13}C$ value of α-HCH in soil, the $\delta^{13}C$ value in wheat tissues at the jointing stage significantly increased up to 3 ‰ (Fig.2c.), especially in stem and leaf, implying that α-HCH could be further transformed within wheat after uptake. The results are consistent with the observation of the $\delta^{13}C$ increase of α-HCH in wheat tissues in the early stage in a hydroponic system [22]. At the heading stage, an enrichment of ^{13}C isotopes of α-HCH in wheat tissues except stem was obtained compared to the soil. The transformation of α-HCH in this study could be associated with the endophytes and plant-derived enzymes. The major roles of endophytes in the degradation of organic contaminants in *planta* are related to various factors such as chelating agents, siderophores, biosurfactants, low molecular weight organic acids, and various detoxifying enzymes [32]. Plant derived enzymes such as P450 monooxygenases, dehalogenase, glutathione S-transferases (GST), and glucosyltransferases (UGT) could play a role in the detoxification of organic pollutants [33]. For examples, hybrid poplar (*Populus spp.*), algae (*various spp.*), and parrot feather (*Myriophyllum aquaticum*) have been reported to produce dehalogenases for transforming DDT [34]. Polychlorinated biphenyls (PCBs) could be dehalogenated by sterile plant tissues [35]. The detoxification process of γ-HCH by *Phragmites australis* plants could be potentially be attributed to UGT enzymes in root and rhizome as well as by GST enzymes in leaf [36]. Endophytes which

250 have the possibility to promote the clean-up of HCHs were isolated from *Cytisus striatus* growing
251 on HCH-contaminated soil [37]. Metabolite analysis revealed 1,3,4,5,6-pentachlorocyclohexene
252 (PCCH) as a major metabolite of α-HCH in both soil and plant (Fig. S2a), indicating a
253 dehydrochlorination reaction of α-HCH in soil and plant. 1,2,4- trichlorobenzene (1,2,4-TCB) was
254 only found in plant tissues (Fig. S2b), suggesting further transformation by dehydrochlorination
255 reaction in plant tissues. Other metabolites could not be detected by GC-MS which might be due
256 to the low concentration. The metabolites are similar to those formed during HCH degradation by
257 *Sphingomods* which are typical HCH degrading soil bacteria[10], leading to the hypothesis that
258 dehydrochlorination reaction of HCH in plants could be associated with the endophytic
259 microorganisms in addition to plant-derived dehalogenase catalyzed reactions.

260 However, at the harvest stage, only α-HCH in root showed an increased $\delta^{13}C$ value and no $\delta^{13}C$
261 increase was observed in other wheat tissues compared to the soil. This indicated that the changes
262 of the isotopic composition in the same tissues at different stages were mostly related to the plant
263 activity. Firstly, it might be affected by the changes in enzyme activity and the community of
264 endophytes due to the different plant growth conditions at different growth stages. Secondly, the
265 HCH uptake from soil was a continuous process along with the water flow. At the early stage, the
266 HCH concentration in wheat was low, therefore HCH showed a strong isotope fractionation
267 indicating intensive transformation in plant. A larger amount of water was required at the later
268 stages and therefore higher accumulation of HCH with low isotope fractionation indicated an
269 overall lower transformation. The higher uptake of water led to larger translocation of HCH along
270 with the water flow in plant, resulting in relatively lower transformation. However, the
271 translocation of HCH along with the water flow should not affect the isotope fractionation as no
272 bond change occurs in contrast to transformation. A potential isotope fractionation due to the

sorption of HCH on the plant cell wall or cell tissue is unlikely as the isotope fractionation caused by phase partitioning is low compared to the transformation [38], therefore the isotope fractionation of HCH due to the transport in the plant could be neglected.

A consistent increase of $\delta^{13}C$ value of α-HCH was found in root along with the wheat growth (Fig. 2c), indicating the larger transformation in root at the later stage. This was different from in other tissues which fluctuated along with the wheat growth. The isotope fractionation of HCH in root may be highly influenced by rhizosphere microorganisms which may have the potential to enter and colonize plant roots and contribute to the transformation in root [39].

The development of $\delta^{37}Cl$ pattern of α-HCH in soil samples was similar with $\delta^{13}C$. An increasing enrichment of ^{37}Cl isotopes of α-HCH in the bulk and rhizosphere soil was observed along with the wheat growth (Fig. 2b.). The observation of the simultaneous enrichment in $\delta^{13}C$ and $\delta^{37}Cl$ values revealed that a C-Cl bond was already cleaved during of α-HCH transformation in soil to some extent. The $\delta^{37}Cl$ pattern of α-HCH in plant samples was also similar with $\delta^{13}C$ (Fig. 2d.), indicating further C-Cl bond cleavage caused by plant endophytes or enzymes. Only the $\delta^{37}Cl$ in grain was higher than its host soil, suggesting the transformation pathway of α-HCH in grain was different from other wheat tissues.

Enantiomer Fractionation of α-HCH in the Soil-plant System. Enantiomer fractionation could occur due to the biotransformation of chiral compounds [40]. Enantiomer fraction (EF) was applied as an indicator for characterizing the enantiomer fractionation process. In the present study, a significant change of EF in soil samples was noted at different wheat growth stages (Fig. 3.). At the jointing stage, EF (-) in the bulk and rhizosphere soil significantly decreased to 0.349 ± 0.002 and 0.396 ± 0.004 respectively compared to the initial EF(-) of 0.502 ± 0.002. However, the EF (-)

in the bulk and rhizosphere soil significantly increased to 0.544 ± 0.001 and 0.556 ± 0.004 at the heading stage, and to 0.559 ± 0.010 and 0.566 ± 0.003 at the harvest stage. The results suggested a preferential biotransformation of (-) α-HCH at the early stage and a preferential biotransformation of (+) α-HCH at the later stages in soil.

Spingobium indicum B90A is a HCH degrader in soil and could show different preferential enantiomer transformation of α-HCH in different conditions [41,42]. A recent study of the Lin enzymes revealed the preferential transformation of (-) α-HCH in resting cells and crude extracts from the same strain and a pronounce isotope fractionation of LinA enzymes [43]. The large variability of enantiomer fractionation in bacteria, even the same bacteria, indicates that different growth phases (lag phase, log phase, and stationary phase) or different cultivation conditions can lead to changes in the activity of the Lin enzymes [43]. Therefore, reasons for the variation of EF in soil in present study could be due to that the different plant growth stages and release of root exudates influence the growth conditions of HCH-degrading microorganisms in soil and also change compositions of soil microbial community [44], which may influence the preference transformation of enantiomers.

Unlike the shift of EF in soil at different stages, the EF in plant samples was more stable. At the jointing stage, the EF (-) in all wheat tissues was below 0.5 and higher than that in soil samples. At the heading stage, the EF (-) in root, stem and spike significantly increased to 0.523 ± 0.001, 0.514 ± 0.001 and 0.511 ± 0.001, respectively, in contrast to that in leaf (0.482 ± 0.002). At the harvest stage, the EF (-) in root significantly increased to 0.529 ± 0.001 and the EF (-) in stem (0.499 ± 0.001) was identical with racemic mixture. The EF (-) in other wheat tissues (leaf, 0.477 ± 0.002; spike, 0.482 ± 0.007; grain, 0.474 ± 0.007) was significantly lower than the racemic mixture. At the heading and harvest stages, all the EF (-) values in wheat tissues were lower than

that of in soil samples in the same stage. The variability of EF (-) in all wheat tissues at different stages varied from 0.449 to 0.529, which was smaller than that in soil. Except the root, all other tissues showed a preferential biotransformation of (-) α-HCH at the harvest stage where its host soil showed a high preferential biotransformation of (+) α-HCH. Similar observation was made in a recent field study [21]. The result indicated that the specific transformation of enantiomers in plant was different from soil. Possibly endophytes and enzymes in plant may also show different transformation pathway of enantiomers. The results may also suggest that the above-ground plant parts showing the enantiomer fractionation can be related to a mixture of processes e.g. plant-internal enantiomer specific transformation, continued uptake from soil, and plant-internal translocation during the growth. EF (-) in root increased from 0.492 ± 0.001 to 0.530 ± 0.001 along the wheat growth, which differed from the other wheats tissues but the trend was similar as in the soil. The reason may be that root took up α-HCH with an enantiomer composition already affected by microorganism in the rhizosphere.

However, based on the obtained data in this study, it is difficult to further evaluate the specific transformation of enantiomers in plant, therefore the contribution of bacteria and plant cannot be distinguished. Further studies focusing on *in vivo* transformation experiment with plant-derived enzymes or endophytes would be needed to elucidate this aspect.

Combined $\delta^{13}C$ and $\delta^{37}Cl$ Analysis for Characterizing HCHs in the Soil-plant System. Different reaction mechanisms involve chemical bonds containing different elements. Reaction mechanisms can therefore be differentiated using dual isotope plots that have different slopes indicating different reaction mechanisms in complex system [28]. The changes of $\delta^{37}Cl$ Vs $\delta^{13}C$ values in soil and plant samples (except grain) could be described linearly with a linear regression slope (Λ) of 3.30 ± 0.16 , suggesting that the overall transformation processes of α-HCH in the

soil-wheat system contributed to the similar mode of C-Cl bond cleavage (Fig. 4). The grain sample has a different isotope fractionation pattern, suggesting the mode of C-Cl bond cleavage in the grain was different from other wheat tissues. Based on the previous study [22], a slope of 1.75 ± 0.13 was observed in the plant samples from a hydroponic system where plant metabolism is dominating the transformation pathway. The observation suggested that plant growing in soil pot experiment had different mode of C-Cl bond cleavage of α-HCH compared to that in hydroponic experiment.

The fundamental difference between hydroponic and soil pot experiment is that the microbial activity in the rhizosphere obviously effects the correlation of ^{13}C and ^{37}Cl isotope fractionation implying contribution of different pathways.

Microbial Community and Dynamics deciphered using Metagenomics Analysis

Five soil samples including the original soil sample (OS), the bulk and rhizosphere soil of α-HCH treatment at the harvest stage (BHA1 and RHA1), the bulk and rhizosphere soil of control at the harvest stage (BHC and RHC) were collected for the analysis of metagenomics. The α-diversity analysis based on Shannon (H) and Simpson (D) index indicated that the original soil was the most abundant in species diversity followed by the rhizosphere soils *i.e.*, RHA and RHC (Fig. S3 and S4.). The bulk control soil represented the least species diversity, even less than the bulk soil treated with α-HCH. This can be explained by the basis of selection pressure and one of such factors can be HCH. In the original soil without HCH pressure, all communities with or without tolerance to HCH can occupy the niches and hence the sample has a much larger diversity.

In addition, the ß-diversity based on Bray-Curtis dissimilarity matrix also revealed that the original soil had the most diverse composition of bacteria, which significantly changed during the wheat

growth (Fig. S5.). The rhizosphere soil treated with α-HCH had distinct bacterial community compared with the bulk soil and control rhizosphere soil (Fig. S5). The six most abundant genera across the five samples deciphered and the rhizosphere soil treated with α-HCH (RHA1) clearly showed significant enrichment of two bacterial genera *viz.*, *Sphingomonas* and *Novosphingobium*, which were reported to either tolerate or degrade HCH [45] (Fig. S6 and S7) (PMID: 28567447, 27581378). The genomic bins reconstructed from the RHA1 soil also indicated the presence of genomic bins (completeness > 70% and contamination < 5%) taxonomically aligned to family *Sphingomonadaceae*. The genomic bins along with the raw reads obtained for all the five samples were checked for the presence of *lin* genes (Fig. S8). We observed the presence significant number of reads corresponding to the *linGH* and *linJ* genes, which encode the acyl-CoA transferase and thiolase required for the conversion of β-ketoadipate to succinyl-CoA and acetyl-CoA. Further, reads corresponding to *linK*, *linL*, *linM*, and *linN* genes encode a permease, ATPase, periplasmic protein, and lipoprotein, respectively, and together form a putative ABC-type transporter system (PMID:17369300). This system is required for the utilization of γ-HCH, probably by conferring tolerance to toxic dead-end metabolites such as 2,5-DCP (PMID:17369300). Homologues showing high levels of similarity to the *linKLMN* genes have been found only in *sphingomonads*, suggesting that their role in active transport of variety of xenobiotic compounds (PMID: 20197499). Interestingly, the genes involved in upper HCH degradation pathway (transformation of HCH to 2,5-DCP) were either not present or not detected based on reads alignment. Therefore, we can hypothesize that, under the HCH selection pressure, the communities that can tolerate HCH have evolved first and these strains are still in a process to acquire the trait of HCH degradation. However, the functional analysis of the metagenomic sequences revealed the abundance of monooxygenases (Fig. S9) and RHA1 sample showed the ability of the community to degrade

aromatic compounds which could be possible metabolites of HCH (Fig. S10). Therefore, the HCH may be getting metabolized into further intermediates and the metabolites could subsequently induced degradation pathways of monooxygenases and enzymes of aromatic degradation pathways.

ENVIRONMENTAL IMPLICATION

Isotope fractionation is almost not affected by concentration changes caused by dilution, sorption and volatilization, which gives advantage for analyzing mechanisms governing the transformation of organic contaminants in complex systems. In this study, CSIA and EF could be applied for characterizing the variability of transformation process of HCHs in a soil-plant system. The correlation of ^{13}C and ^{37}Cl fractionation reveals that the overall transformation processes could be characterized by one factor Λ, indicating a similar mode of C-Cl bond cleavage, in contrast to enantiomer fractionation suggesting a changing preference for transformation of α-HCH stereoisomers. The combination of multi-element isotope fractionation and EF may provide clues to identify transformation mechanisms in more detail in future studies.

HCHs could serve as an example of persistent environmental contaminants and the developed concept is also beneficial for identifying the transformation processes of other organic pollutants in complex systems, such as soil-plant system, or food webs.

Phytoremediation is based on the cooperation of plants and their associated microorganisms. It has been regarded as an efficient option for the clean-up of contaminants in soil [46,47]. Our study shows the potential of transforming α-HCH in the soil-wheat system, which gives a strong evidence of phytoremediation related not only to the uptake but also the transformation of α-HCH. The phytoremediation of HCHs by the uptake account for a small part of the total loss [24,48], but it may

be important to be considered for a more complete picture in the future. However, the isotope study demonstrates that the transformation of organic pollutants needs to be taken into account next to uptake. CSIA and EF provide a great potential for detecting the previously non-considered transformation process of α-HCH in soil-plant systems.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Details on experimental conditions, extraction methods, and analytic methods; concentration, metabolites and metagenome analysis.

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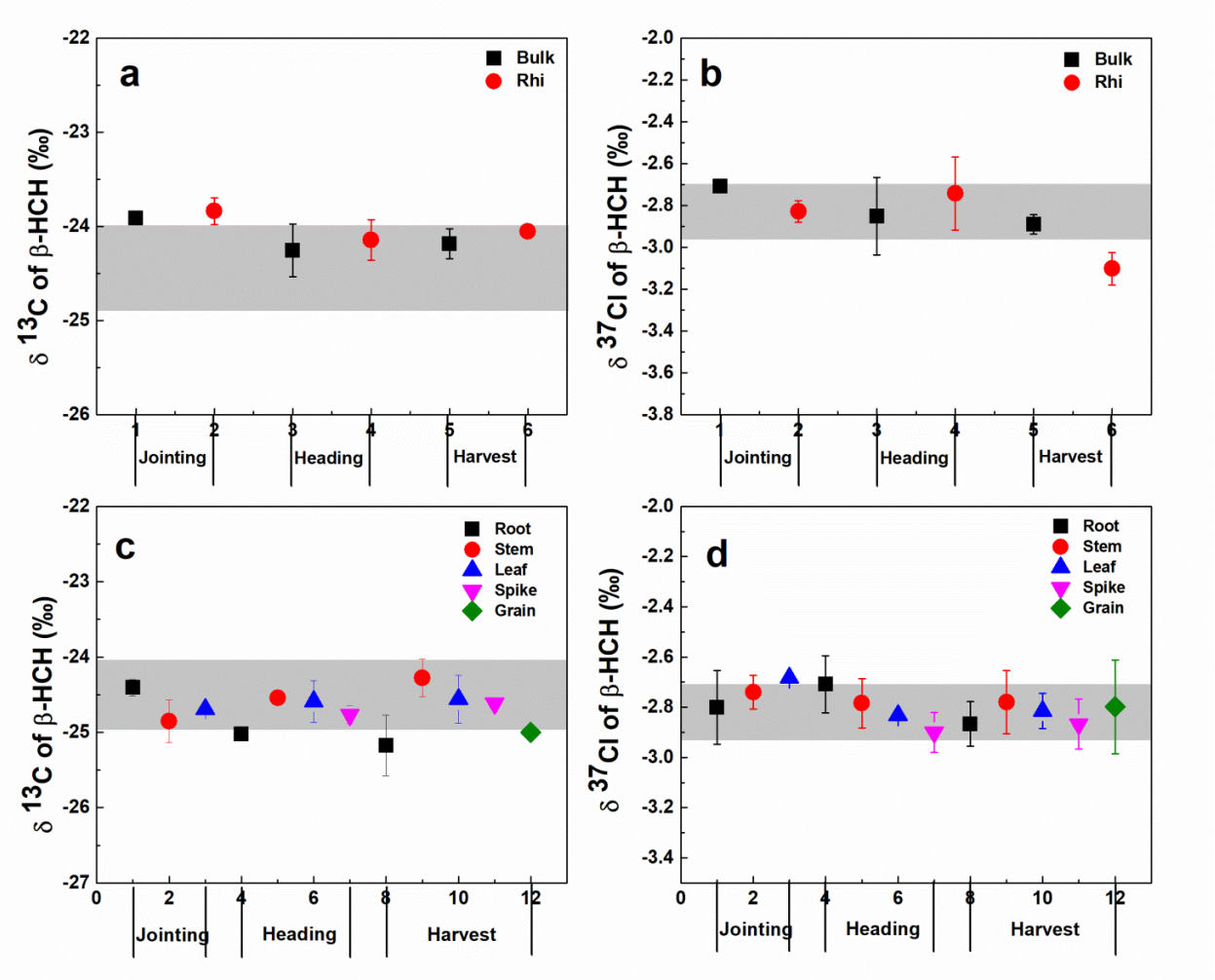
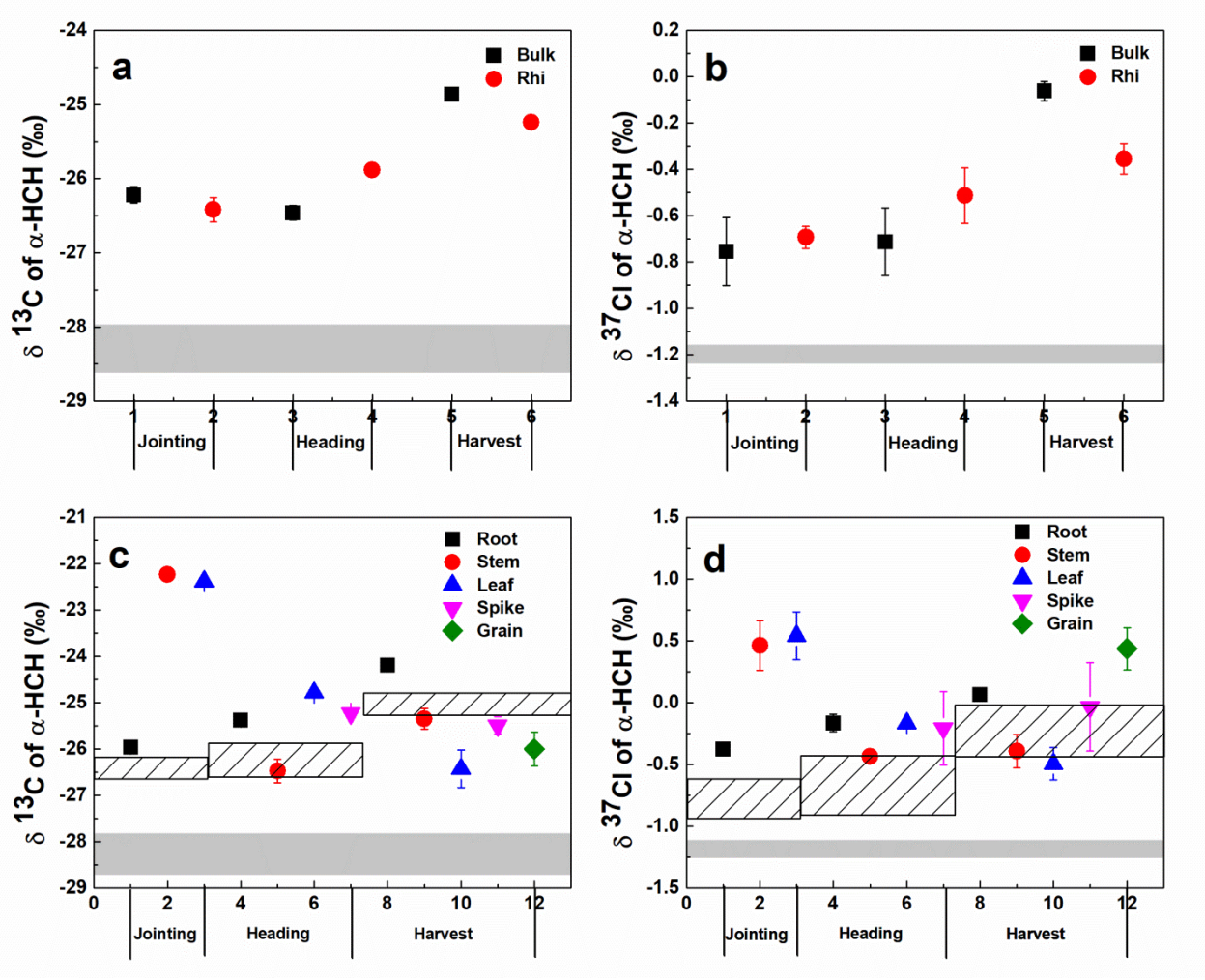


592

593 Fig. 1. Carbon (a) and chlorine (b) isotope composition of β-HCH in the bulk soil and the
594 rhizosphere soil. Carbon (c) and chlorine (d) isotope composition of β-HCH in wheat tissues. The
595 grey bar shows the range of the isotope values of β-HCH spiked to the original soil samples. Error
596 bars represent SD values.

597



598

599 Fig. 2. Carbon (a) and chlorine (b) isotope composition of α-HCH in the bulk soil and the
600 rhizosphere soil. Carbon (c) and chlorine (d) isotope composition of α-HCH in wheat tissues. The
601 grey bar shows the isotopic range of α-HCH spiked to the original soil samples. The stripe bar
602 shows the range of isotope values of α-HCH in bulk and rhizosphere soil at different wheat growth
603 stages. Error bars represent SD values.

604

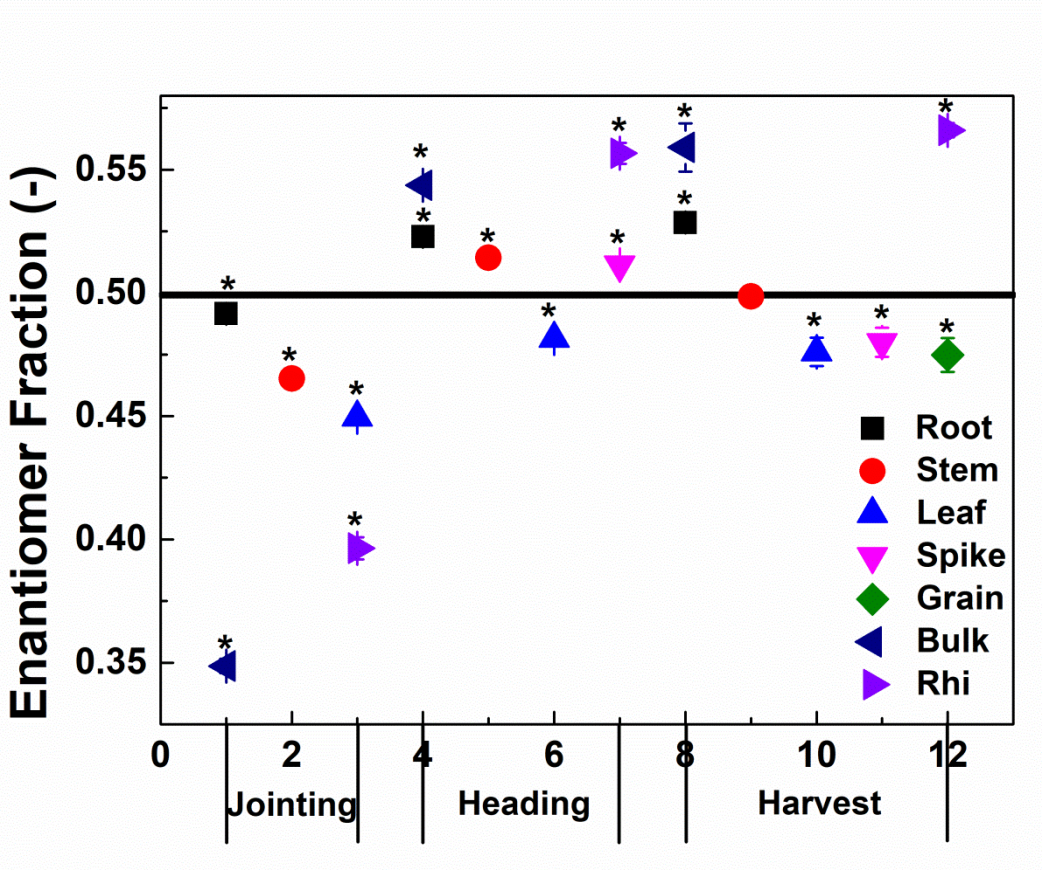
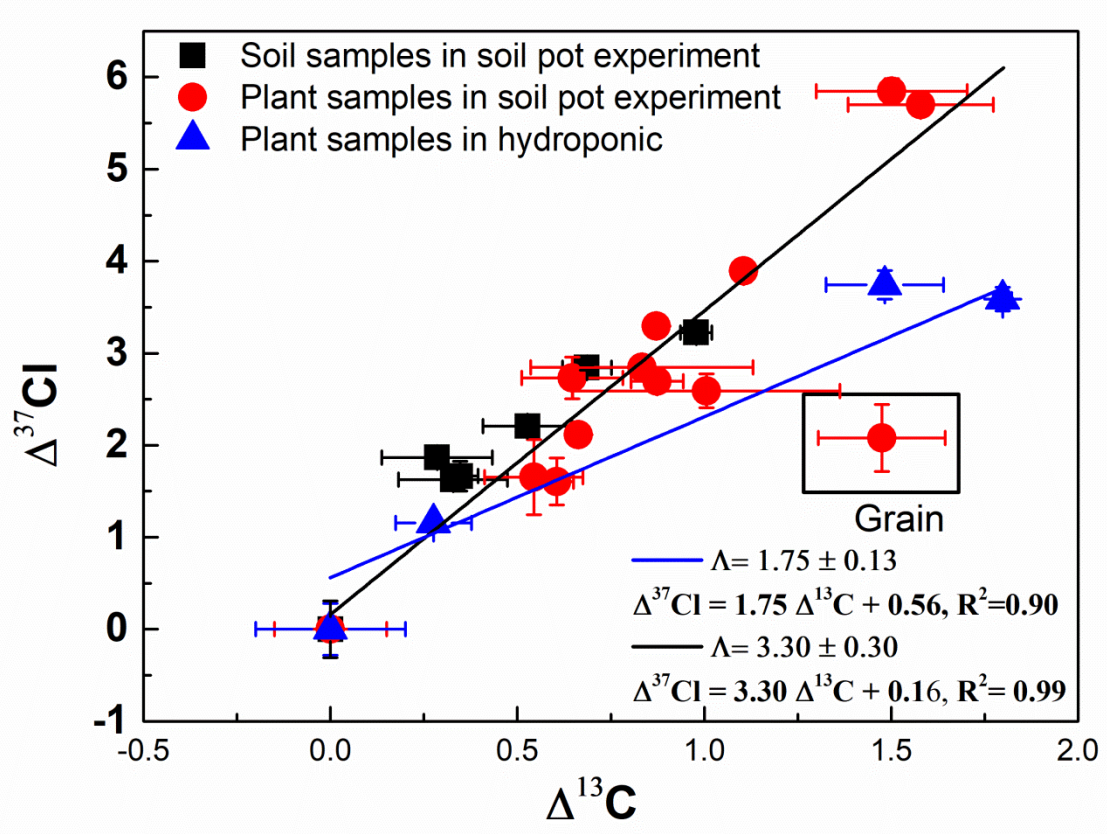


Fig. 3. Enantiomer fractionation of α-HCH in different wheat tissues and soil. The bar is showing the EF values of the racemic mixture spiked to the soil. Bulk means bulk soil. Rhi means rhizosphere soil. The asterisk refers to the significant difference between EF(-) in soil and plant samples compared with that in HCH used for spiking according to independent *t*-test ($p<0.05$). Error bars represent SD values.



612

613 Fig. 4. Dual element analysis (C-Cl) of α-HCH in soil and wheat tissues. The isotope data of α-
614 HCH in plant samples in hydroponic experiment was obtained from elsewhere [24]. The Δ indicates
615 the differences in isotope values (δ) between the initial isotope composition of HCH used for
616 spiking (δ0) and the isotope composition of HCH at each sampling point (δt), Δ = δt - δ0. The
617 same HCH standard was used for spiking in the soil pot and hydroponic experiments. Error bars
618 represent SD values.

619