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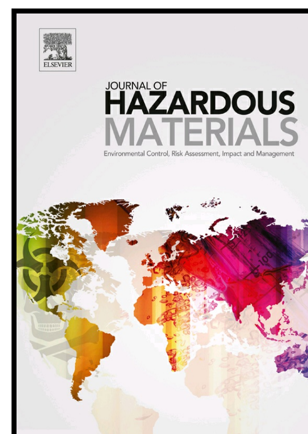

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Characterizing the Biotransformation of Hexachlorocyclohexanes in Wheat Using Compound-Specific Stable Isotope Analysis and Enantiomer Fraction Analysis

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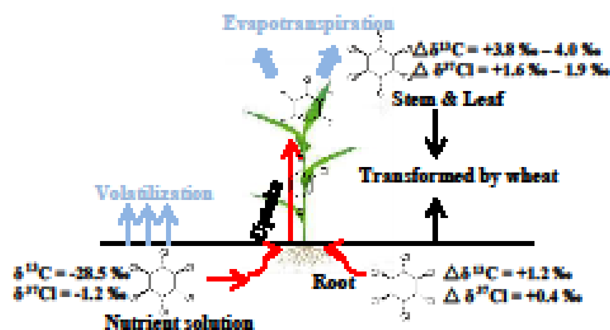
ABSTRACT:

Hexachlorocyclohexane isomers (HCHs) are persistent organic pollutants being responsible for environmental contamination worldwide. In order to characterize transformation of HCHs in different plant compartments during uptake, a hydroponic experimental setup was designed using wheat as the test plant. The extent of transformation was determined by using compound-specific isotope analysis (CSIA) and enantiomer fraction (EF) analysis. In nutrient solutions, no change of carbon ($\delta^{13}C$) and chlorine isotope ratios ($\delta^{37}Cl$) of α-HCH and β-HCH was detected throughout the experiment indicating no transformation there. In wheat leaves, stems and roots, however, transformation of α-HCH due to a C-Cl bond cleavage was indicated by increasing $\delta^{13}C$ and $\delta^{37}Cl$ compared to the nutrient solution. In addition, 1,3,4,5,6-pentachlorocyclohexene (PCCH) was identified as the major metabolite of α-HCH transformation. For β-HCH, in contrast, no transformation was detected. The evaluation of enantiomer fraction analysis revealed no change of the EF(-) in the nutrient solution or on root surface but a decrease in the wheat compartments, providing an evidence for the preferential biological transformation of (-) α-HCH in wheat. The current study provides the first experimental evidence for biotransformation of α-

HCH in wheat using CSIA and EF and provides a concept to evaluate processes during phytoremediation.

Graphical Abstract



Keywords: Hexachlorocyclohexane isomers; isotope fractionation; enantiomer fraction; plant transformation

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1. Introduction

Hexachlorocyclohexane isomers (HCHs) are persistent organic pollutants in the environment. Only γ-HCH (Lindane) has insecticidal properties and was extensively used as pesticide [1,2]. The purification of Lindane resulted in a large amount of waste containing other isomers, especially α-HCH and β-HCH, and the global spread of these isomers has caused abundant contamination in soils and groundwater [3,4]. HCHs can sequentially accumulate in plants when taken up from soil and water, which is the first step for entering the food web and which may also lead to increased human exposure. Hence, the uptake of HCHs into plant tissues has been of increasing concern in recent years. The uptake of HCHs may occur by roots with the transpiration stream, or from leaves in the gas phase [5,6]. In the literature, the bio-concentration of HCHs in plants may

be overestimated in the models compared to the data obtained from contaminated sites, suggesting that not all processes governing concentration of HCH in plants are fully elucidated [6]. This is probably due to the transformation of HCHs which is poorly understood. Phytoscreening is proposed as an inexpensive, noninvasive and rapid tool for detecting subsurface contaminants where the concentration and accumulation of contaminants in plants are employed as indicators to map environmental pollutants [7]. However, it is still unknown whether and to what extent the transformation of HCHs takes place in plant tissues. If so, the concentration of HCH isomers in plants could have been altered and does not reflect the actual concentration in soil, putting uncertainty on phytoscreening. Phytoremediation has been regarded as an option for the clean-up of contaminants in soil [8] and contaminated biomass could be potentially used for biogas production where HCHs can be transformed [9]. Previous work on phytoremediation of HCHs focused on the transformation in the rhizosphere [10] and the transformation of HCHs in plants has not been taken into consideration. Therefore, the models for phytoscreening as well as phytoremediation may need to be improved and the transformation of HCHs in plant should be integrated at least when interpretation of the concentrations of contaminants is intended. However, the processes associated with plant uptake and the transformation of HCHs is largely remained understudied due to the lack of appropriate techniques.

Monitoring the concentration alone in different plant compartments is usually not enough to identify the transformation of HCHs within plant tissues, as the decrease of concentration could be due to physical processes such as evapotranspiration, sorption and dilution and do not reflect the cleavage of chemical bonds initiating a transformation pathway. Compound-specific stable isotope analysis (CSIA) has been applied to trace the mechanisms of organohalides transformation making use of isotope fractionation concepts [11,12]. Several studies have shown

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that CSIA can be used to characterize both chemical and biological transformation of HCHs qualitatively and quantitatively in groundwater, soil and sediments [13,14]. Whereas the changes of the enantiomer fraction (EF) are usually only due to a preferential biotransformation of one enantiomer of the chiral compounds and thus reflect a biological process [15]. EF could provide additional information to back up isotope fractionation analysis. Of the eight potential HCH isomers, only α-HCH is chiral and thus the biotransformation of enantiomer-specific α-HCH could lead to the enrichment of the other enantiomer in the residuals, leading to the change of EF [16]. Several reports have applied this concept to characterize the biotransformation (aerobic and anaerobic) of α-HCH in groundwater, soil, and air [17,18]. The application of CSIA and EF methods in soil and plant system not only gives impetus to the concept for characterizing the fate of organic pollutants in complex system but also is a huge step forward in the development of the analytical methods.

The transformation of organic contaminants during uptake into plants has been controversially discussed. Polycyclic aromatic hydrocarbons could be degraded in biofilms on root surface and by endophytes as demonstrated for phenanthrene [19–21]. Ibuprofen has been shown to be transformed by plant derived enzymes [22]. Preliminary studies on the transformation of HCHs at field sites in Bitterfield (Germany) and Lucknow (India) have shown that α-HCH and β-HCH were isotopically enriched in some plants compared to soil (Table S1) [23], suggesting that plant uptake of HCHs is a process associated with chemical bond cleavage indicating transformation. However, it is unknown if the transformation took place in the rhizosphere or within the plants. Potentially, transformation of HCHs could take place in the soil rhizosphere, in plants, or in both. Recently, we analyzed the transformation of α-HCH in a microcosm experiment consisting of plant and soil and found degradation in the bulk soil, the rhizosphere and upon the uptake of α-

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HCH into the plant [24]. However, it is difficult to distinguish if the transformation in plants was induced either by soil derived microorganisms growing on the root surface and may inoculate plants or by plant own endophytes and enzymes. Based on this previous study, a new research question arises if HCH transformation is possible within plants without the inoculation by soil derived microorganisms. Therefore, we conducted experiments in sterilized hydroponic systems using wheat as the test plant for exploring the evidence of HCH transformation in different plant compartments using CSIA and EF. To our best knowledge, this is the first study to characterize the transformation of HCHs during plant uptake using stable isotope and enantiomeric fractionation approaches.

2. Materials and methods

2.1 Chemicals

The α-HCH with an analytical purity of 99% was obtained as part of a set of pure samples for a previous study (number P25 in that study) [25] and has a $\delta^{13}C$ value of -28.5‰ and $\delta^{37}Cl$ value of -1.04‰. The β-HCH (analytical purity, 99%) was purchased from Sigma Aldrich (Germany) and has a $\delta^{13}C$ value of -25.1‰ and $\delta^{37}Cl$ value of -2.95‰. The fully ^{13}C-labled α-HCH was synthesized from fully ^{13}C-labled benzene (analytical purity, 99, Sigma Aldrich, Germany) by photoreaction with chlorine and then separated from other isomers in our lab. The purity of ^{13}C-labeled α-HCH was above 95%. *n*-Hexane (≥99.9%, ROTISOLV HPLC, ROTH) and acetone (ROTISOLV® ≥99.9 %, UV/IR-Grade) were used for HCH extraction. Dichloromethane (DCM, ≥99.9%, ROTISOLV HPLC, ROTH) was used for cleaning up the plant extracts. Florisil (for chromatography, ROTH, 100-200 mesh) was used as the stationary phase for liquid column chromatography, which was activated at 120 °C for 12 h before usage. Anhydrous Na_2SO_4 was activated by heating at 200 °C overnight. Glass wool (untreated, SUPELCO Analytical) and sea

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sand were used for packing the column. To track the elution of HCHs from the Florisil column, 7, 12-dimethyl-benz[α]anthracene (p. A, Reagent Grade) was applied as a fluorescence tracer. A 360 nm UVA lamp was used to visualize the fluorescence band.

2.2 Hydroponic Experiment for Analyzing Uptake of HCHs by Wheat

Wheat (*Quintus*) was used as the test plant and the seeds were obtained from SAATEN-UNION GmbH, Germany. Hoagland nutrition solution (half strength) was autoclaved and 4.5 L of solution was used in each 5 L pot. The composition of the Hoagland nutrition solution is shown in the supporting information section S1. The HCH stock solution (10 mg mL^{-1} α-HCH and 2.5 mg mL^{-1} β-HCH) was prepared in acetone. The initial concentration of HCHs in the nutrient solution was adjusted to 2 mg L^{-1} for α-HCH and to 0.5 mg L^{-1} for β-HCH due to its lower solubility in water. After adding the 0.9 mL HCH stock solution to the nutrient solution, the mixture was stirred for 3 days on a magnetic stirrer. The acetone accounted for 0.02% of the total volume which cannot influence the plant growth. The α-HCH was chosen as target compound since it is the only isomer which possesses enantiomeric structure. Thus, the analysis of enantiomer fractionation can complement the isotope analysis to provide more information on the biological transformation of HCH. Meanwhile, β-HCH was chosen since all six chlorine atoms of this isomer are in equatorial position resulting in a relatively high stability in regard to the transformation compared to the other isomers.

Prior to germination, wheat seeds were surface-sterilized in 10% (v/v) H_2O_2 for 30 min, and rinsed thoroughly with autoclaved Milli-Q water. Then the seeds were germinated on Milli-Q water-soaked filter paper in Petri dishes at room temperature for 4 days. Afterwards, nine well-developed seedlings were selected and transplanted into 5-L glass beakers filled with 4.5 L half strength autoclaved Hoagland nutrition solution. Each pot was covered with a PVC-plate with

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nine 1-cm holes. Each seedling was fixed in one hole, respectively, using a sponge. Pots and plates were covered with aluminum foil to avoid the exposure of HCHs to light. The pots without wheat were used to quantify the volatilization loss of HCHs, named as unplanted control. The pots without the addition of α- or β-HCH in the nutrient solution were used to examine the growth of wheat in the greenhouse, named as planted control. Further potential of the sorption of volatilized HCHs by plants from ambient air may be examined by this planted control. The pots with the addition of α- and β-HCH in the nutrient solution were used to examine the uptake and transformation of HCHs by wheat, named as spiked experiment. Each treatment group was prepared in triplicates. Pots of unplanted control were placed between pots of spiked experiment and planted control. All pots were placed in a greenhouse with the controlled environmental conditions at a light intensity of 80 kLux provided by supplementary illumination with a photoperiod of 15 h each day. Temperature during the day was regulated to 22 °C and during the night to 16 °C. Relative humidity was kept at 60%. All experimental equipment described above was sterilized before use. The wheat plants grew over 30 days for all the experiments before being harvested.

During the growth phase, the seedlings were repositioned daily to minimize spatial differences in illumination and temperature. pH in the nutrient solution was measured two to three times per week and was adjusted to pH 6 using 1M NaOH or 1M H_2SO_4 if needed. The loss of nutrient solution was measured daily and compensated with the addition of autoclaved Mill-Q water if necessary. The formation of biomass of wheat in the spiked experiment was inhibited compared to that in the control (Fig S3).

Another experiment was conducted with 2 mg L^{-1} fully ^{13}C-labeled α-HCH to trace the metabolites. The experimental setup was the same as described above. Fresh root samples from

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the ^{13}C-labeled experiment were used for 4′,6-diamidino-2-phenylindole (DAPI) analysis to detect bacteria (details of DAPI are shown in S2).

2.3 Sampling and Extraction of HCHs in the Nutrient Solution

Wheat grew for 33 days from March to April in 2018. At 0, 8, 25 and 33 days, 20 mL aqueous samples were taken from the pot, respectively, and transferred into a 50-mL glass bottle. Bottles were all sealed for extraction. 0.5 mL n-hexane containing 80 mg L^{-1} δ-HCH was added into each bottle serving as internal standard for concentration and isotope analysis. The bottles were placed on a rotary shaker at 120 rpm and 4°C for overnight. Then the *n*-hexane phase was transferred into a 2-mL glass vial using a glass Pasteur pipette and was stored at −20 °C before analysis. The extraction efficiency was ⩾ 98% [26] and the details are shown in section S6 (SI). The extraction procedure did not lead to significant changes in the stable carbon and chlorine isotope ratios of HCHs which has been confirmed in a previous study [26].

2.4 Extraction and Clean-up of HCHs from Different Plant Compartments

After harvesting the plants, the different plant samples were rinsed thoroughly with Milli-Q water for removing HCHs dissolved in water droplets attached to the roots. Then the 9 wheat plants in each pot were collected and divided into root, stem and leaf parts. *n*-Hexane was used to extract the HCHs from the root surface. The details are described in section S3 (SI). The extraction of HCHs from plant samples using Accelerated Solvent Extraction (Dionex ASE 200, Thermo Fisher Scientific, Germany) and the clean-up procedures using Florisil column chromatography were described previously [27] and the details are also provided in section S3 (SI). The extraction efficiency of HCH from different plant compartments ranged from 40% to 50% for α- and β-HCH [27]. No significant carbon and chlorine isotope effects are associated with the extraction and clean-up of HCHs from plant samples [27].

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2.5 Analytical Methods

2.5.1 Concentration

An Agilent 6890 series GC (Agilent Technologies, USA) equipped with a flame ionization detector (FID) was applied to determine the concentrations of HCHs throughout the study. HCHs were separated with a HP-5 column (30 m × 320 μm × 0.25 μm, Agilent 19091J-413, USA) using a helium flow of 2.2 mL min^{-1}. The oven temperature program is shown in section S4 (SI). Samples (injection volume 1 μL) were injected in splitless mode with an injector temperature of 250 °C. Each sample was measured in triplicates. More details of determination of HCH were given in section S6 in SI.

2.5.2 Isotope Analysis

The isotopic composition of an element (E) was reported in δ notation in parts per thousand (‰) relative to the international standard Vienna Pee Dee Belemnite (V-PDB) for stable carbon isotopes and Standard Mean Ocean Chloride (SMOC) for stable chlorine isotopes according to eq 1.

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

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

The isotopic composition of carbon ($\delta^{13}C$) in HCHs was analyzed by using gas chromatography-combustion-isotope ratio mass spectrometry (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). A Zebron ZB1 column (60 m × 0.32 mm × 1 μm; Phenomenex, Germany) with a constant carrier gas flow of 2 mL min^{-1} was applied for chromatographic separation. The

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oven temperature program is shown in section S4 (SI). All samples were injected in splitless mode with an injector temperature of 250 °C and were analyzed at least in three replicates. The quality of the isotopic data was regularly controlled with internal laboratory references of known isotopic compositions. The analytical precision for $\delta^{13}C$ of 3 repetitive analysis of the same sample was below ± 0.5‰ [27].

Chlorine isotope compositions ($\delta^{37}Cl$) of HCHs were analyzed online using gas chromatography coupled with multiple-collector inductively coupled plasma mass spectrometry (GC-MC-ICPMS), as recently described elsewhere [27–29]. A gas chromatographic system (Trace 1310, Thermo Fisher Scientific, Germany), equipped with an auto-sampler (TriPlus RSH, Thermo Fisher Scientific, Germany) was used for injection and separation. Samples were injected in splitless mode with a constant carrier gas flow of 2 mL min^{-1}. Samples were separated on a Zebron ZB-1 capillary column using a temperature program as shown in section S4 (SI). Trichloroethene used as in-house reference compound (TCE2, $\delta^{37}Cl$ = -1.19‰) was added to each sample as an internal isotopic reference for identifying any potential instrumental drifts. The $\delta^{37}Cl$ was determined in two steps: (1) the deviation of a sample from TCE2 was calculated, and (2) raw $\delta^{37}Cl$ values were calibrated to the SMOC scale by applying a two-point calibration approach. Calibration to the SMOC scale was accomplished by using in-house reference compounds including methyl chloride (MeCl, $\delta^{37}Cl$ = +6.02‰) and trichloroethene (TCE2, $\delta^{37}Cl$ = -1.19‰). In addition, trichloroethene (TCE6, $\delta^{37}Cl$ = +2.17‰) and tetrachloroethene (PCE1, $\delta^{37}Cl$ = -0.55‰) were used for validation of the calibration, as described by Horst et al [28]. Samples were analyzed in triplicates with an analytical precision usually below ± 0.3‰ [27].

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2.5.3 Enantiomer Analysis

The enantiomer fraction (EF) is used to characterize the preference of enantiomer biotransformation and analyzed by GC-MS (Agilent Technologies 7890A and 5975C, USA). A γ-DEX 120 chiral column (30 m × 0.25 mm × 0.25 µm, SUPELCO, USA) was used here to separate the enantiomers [30]. The EF (-) is defined as $A^- / (A^+ + A^-)$, where A^+ and A^- correspond to the peak areas or concentrations of (+) and (−) enantiomers. Racemic compounds have an EF (-) equal to 0.5. An EF (-) > 0.5 indicates the preferential transformation of (+) enantiomer, and vice versa [31]. The GC oven temperature program is shown in section S4 (SI).

2.5.4 Metabolite Analysis

The qualitative analysis of the metabolites of HCHs in plants and aqueous samples was performed by using GC-MS and GC-IRMS as shown above. Sample separation was done with a ZB-1 column using a helium flow of 1 mL min^{-1} as the carrier gas. The oven temperature program is described in section S4 (SI). All samples were injected in split mode (1:1) with an injector temperature of 250 °C.

3. Results and discussion

3.1 Changes with time of HCHs concentrations in the nutrient solution

The initial concentrations of α- and β-HCH were 2.09 mg L^{-1}, 0.53 mg L^{-1} in the spiked experiment, as well as 2.03 mg L^{-1}, 0.48 mg L^{-1} in the unplanted control, respectively. The concentration of α- and β-HCH in the nutrient solution decreased during the growth of wheat (Fig. 1). α- and β-HCH decreased by 35.9% and 37.0% compared to the initial concentration respectively in the unplanted control after 33 days incubation. A higher decrease in the concentration of α-HCH and β-HCH was observed in the spiked experiment compared to the unplanted control (47.3% and 48.9%, respectively), suggesting a removal of HCHs by wheat. In

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the hydroponic system, the losses of HCHs may be the result of the following processes: volatilization from water, adsorption to the glass surfaces, uptake by plants and subsequent evapotranspiration from plants, uptake and subsequent transformation in plants [32]. The loss of HCHs in the unplanted control is most probably due to volatilization from water and/or adsorption to the glass surfaces. The higher loss of α-HCH and β-HCH in the spiked experiment is likely the result of uptake by plants through the transpiration stream in the xylem and subsequent evapotranspiration. In addition, HCHs may also be transformed in plants after uptake during transportation in the xylem or cells forming the xylem.

3.2 Uptake and Transport of HCHs in Plants

α-HCH and β-HCH could be detected in all compartments of wheat in the spiked experiment after 33 days, indicating that HCHs were transported to all parts of the wheat after uptake from the nutrient solution. The pots containing the planted controls (wheat without HCHs) were placed between the spiked experiment and the unplanted control. HCHs could not be detected in the planted control, suggesting that uptake of volatilized HCHs by leaves in the present experiment may be neglected. A significant difference in the concentration of α- and β-HCH was observed in the different compartments of wheat (Fig. 2), with the highest concentrations in the stem (266.8 ± 1.3 mg kg^{-1}, 110 ± 1.6 mg kg^{-1}, respectively) followed by the leaf (177.4 ± 9.8 mg kg^{-1}, 86.4 ± 4.6 mg kg^{-1}, respectively) and the lowest concentrations in the root (63.0 ± 1.5 mg kg^{-1}, 63.7 ± 1.6 mg kg^{-1}, respectively). From the total amount of α- and β-HCH taken up by wheat, 12.4% and 24.5% were accumulated in the roots, 52.6% and 42.3% in the stem and 35.0% and 33.2% in the leafs, respectively. A higher relative distribution of β-HCH was observed in the root compared to α-HCH. In contrast, more α-HCH was accumulated in the upper parts of the wheat plants based on the relative distribution. Similar observations were reported before[32]

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and the results suggested that the translocation of HCHs in wheat was isomer-dependent, which may be related to the octanol-water partition coefficient (K_{ow}) of HCHs. β-HCH has a higher K_{ow} and thus has a larger tendency to be accumulated in the roots [33].

The mass balance of HCHs was calculated. The contribution of each process on the total HCH removal was estimated by comparison between the different experiments (unplanted control, planted control and spiked experiment) assuming that all the processes are equivalent in the different experiments. According to the mass balance of α-HCH, volatilization from water and adsorption to the glass surfaces accounted for 75.7% of the total loss and uptake by plants accounted for 8.9%. The remaining 17.7% may be due to the loss by evapotranspiration and transformation (Fig. S4). According to the mass balance of β-HCH, volatilization from water and adsorption to glass accounted for 69.8% of the total loss and uptake by plants accounted for 17.6%. (Fig. S4). The loss of β-HCH by evapotranspiration and transformation accounted for 12.6%, which was lower than that for α-HCH. The loss of HCHs by the transformation processes was characterized by using CSIA as discussed below.

3.3 Carbon and Chlorine Isotopic Composition of HCHs in the Nutrient Solution

No significant difference of $\delta^{13}C$ values was observed for both α- and β-HCH among different sampling times in the unplanted control and the spiked experiment (Fig. 3a and 3b). The isotope effect of volatilization from water is considered to be negligible as simple phase partitioning results in very low isotope effects [34]. This indicates that the transformation of HCHs as well as volatilization from the aqueous phase and adsorption to the glass surfaces did not lead to detectable isotope fractionation and no transformation of HCHs could be observed in the nutrient solution during the growth period of wheat.

The $\delta^{37}Cl$ values of α-HCH in the nutrient solution were also stable in both spiked experiment

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and the unplanted control (Fig. 3c). This gives strong evidence that no C-Cl bond was cleaved, supporting the conclusion that α-HCH is not transformed in the nutrient solution. In addition, we did not observe any transformation products of HCHs in the solvent extracts of the nutrient solution.

3.4 Carbon and Chlorine Isotopic Composition of HCHs in Plant Compartments.

The $\delta^{13}C$ of HCHs on the root surface showed no significant difference compared to the aqueous samples (Fig. 3a and 3b). This suggested that the sorption of HCHs to the root surface did not change the carbon isotopic composition, which was expected because a simple phase portioning may not lead to significant isotope fractionation [34]. Therefore, no transformation of HCHs on the root surface could be observed by measuring the isotopic composition. The $\delta^{13}C$ values of β-HCH in plant compartments were almost identical compared to that in the nutrient solution and on the root surface (Fig. 3a), which suggested no transformation of β-HCH took place in wheat. β-HCH has six equatorial chlorine atoms which are more difficult to be cleaved from the hexane ring than the axial chlorine atoms [35]. The observed constant $\delta^{13}C$ values of β-HCH in wheat also suggested that uptake, translocation and accumulation of β-HCH in wheat could not affect the isotopic composition.

Interestingly, an enrichment in $\delta^{13}C$ of α-HCH was observed in all the wheat compartments (-27.3 ± <0.1‰ in root; -24.7 ± 0.2‰ in stem; -24.8 ± 0.1‰ in leaf compared to the aqueous phase with -28.5 ±0.1‰). The order of $\delta^{13}C$ values was $\delta^{13}C_{root} < \delta^{13}C_{leaf} \approx \delta^{13}C_{stem}$ (Fig. 3b). α-HCH has similar physiochemical properties as β-HCH. Therefore, transport processes are also unlikely to affect the isotopic composition of α-HCH. The result indicated that wheat had the potential to transform α-HCH. In the hydroponic experiment, the HCHs can only enter the plant via the roots and the carbon isotope enrichment of α-HCH in the upper part of wheat was higher

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than that in root, suggesting the transformation of α-HCH along with the water flow and depending on the specific part of the wheat. When taking the extent of isotope enrichment as a parameter for transformation, the higher transformation was observed in the leave and stem. The $\delta^{37}Cl$ of α-HCH in the aqueous samples (-1.30 ± 0.03 ‰) and on the root surface (-1.11 ± 0.21‰) was identical within analytical uncertainty. An enrichment of α-HCH in plant tissues was found in the order of $\delta^{37}Cl_{root} < \delta^{37}Cl_{stem} < \delta^{37}Cl_{leaf}$ (-0.8 ± 0.18 ‰ in root; 0.4 ± 0.2 ‰ in stem; 0.7± <0.1 ‰ in leaf), similar to the order of $\delta^{13}C$ (Fig. 3c). The results of $\delta^{37}Cl$ pointed to a C-Cl bond cleavage in plant tissues, providing evidence for the transformation of α-HCH. 1,3,4,5,6-pentachlorocyclohexene (PCCH) was found as the major metabolite and trichlorobenzene (TCB) was also found as the further metabolite of PCCH in plant tissues, suggesting possible dehydrochlorination reaction of α-HCH in the plant (Fig. S5). In this study, $\delta^{37}Cl$ of β-HCH was not analyzed because there was no difference in $\delta^{13}C$ of β-HCH observed and we assumed there were also no changes in $\delta^{37}Cl$. We repeated the spiked experiment with ^{13}C-labeled α-HCH and the ^{13}C-labeled PCCH and ^{13}C-labeled TCB were found (Fig. S6) in the plant tissues, which further confirmed that the HCH was dehydrochlorinated in the wheat plants during the uptake or transport process. In addition, GC-IRMS analysis showed clearly that the ^{13}C-labeled PCCH was the major ^{13}C-labeled metabolites (Fig. S7a). The metabolite patterns of HCHs in plants were analyzed in previous studies [36] and demonstrated the transformation of γ-HCH to PCCH [37] and some other metabolites like chlorophenols and chlorobenzenes [38] using ^{14}C-labeled γ-HCH. We do not observe other ^{13}C-labeled metabolites in the current study but volatile metabolites might be lost upon volatilization (Fig. S6b).

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3.5 Dual element isotope analysis

Different reaction mechanisms involve bond cleavage of different elements. Therefore, reaction mechanisms can be distinguished using relative isotope fractionation by dual elements isotope analysis employing the Λ value which is the correlation of carbon and chlorine isotope analysis in this study [39]. The slope of dual element analysis has potential to characterize different reaction mechanisms [12]. In our study, dual-element isotope analysis showed linear correlation within the aqueous samples and plant samples ($\Lambda = 2.09$) (Fig. 4.), indicating that one identical process, mostly likely dehydrochlorination as indicated by metabolite spectra of HCH, took place for transforming α-HCH in the whole wheat even to a different extent in roots, stem and leaves. This is the first report on the dual element (C, Cl) isotope analysis of the transformation of HCH in plants in hydroponic experiments, which could be used as an indication of the dehydrochlorination of HCH and may be use to study the transformation in other plants. Further experiments could be conducted to measure hydrogen isotope fractionation to characterize the reaction mechanism of dehydrochlorination of HCH in plants by the multi element (C, H) isotope analysis similar to the transformation experiment of γ-HCH using pure LinA enzymes [40].

3.6 Enantiomer Fractionation of α-HCH in Aqueous and Plant Samples

Changes of Enantiomer fractions (EF) of α-HCH can be considered as an indicator for biotransformation processes [15,41,42]. Previous studies have demonstrated enantiomeric selectivity of α-HCH during biodegradation in animals [43–45], groundwater [41], and sewage sludge [42]. Until now, however, there is no record of EF factors of α-HCH in different compartments of plants. We hypothesized that in contrast to other compounds like phenoxy acids [46], the transporters in plants are unlikely to cause the enantiomer fractionation of α-HCH because α-HCH is not a molecule with specific functions in the plant metabolism, therefore the specific transporters are probably

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not available. Instead, we hypothesized that the enantiomer fractionation of α-HCH was caused by plant derived or microbial enzymes of endophytes which are enantiomer-selective. In the present study, no significant difference of EF(-) factors was observed between the different sampling times in the nutrient solution of the spiked experiment and the unplanted control (Fig. 5), which indicates no preferential biotransformation of α-HCH enantiomers in the nutrient solution. No significant difference of EF (-) between the root surface and the aqueous samples in the spiked experiment was observed (Fig. 5), indicating no preferential transformation of α-HCH enantiomers on the root surface, which supports the results from isotope analyses. Significant preferential transformation of (-) α-HCH was observed in all the compartments of wheat, with the order of EF(-) values in stem > leaf > root. This showed that the biotransformation of α-HCH took place within the wheat, which further confirms that wheat has the potential to transform α-HCH in the present hydroponic system. Additionally, the enantiomer selective degrading enzymes could cause the isotope fractionation during enantiomer selective degradation process[47]. In this study, the changes of EF(-) were not correlated with the changes in isotope composition of α-HCH, which was also observed in a recent study analyzing isotope and enantiomer fractionation in aerobic microbial cultures [47]. The enantiomer and isotope fractionation of α-HCH are two independent processes catalyzed by highly specific individual enzymes –selective enzymes which govern the individual reactions. The abundance of one enantiomer-selective enzyme relative to another depends on their expression level. Therefore, the relative abundance of these enantiomer-selective enzymes determines the extent of enantiomer fractionation, but is not necessarily reflected in isotope fractionation. Moreover, the expression level of the individual enzymes are affected by the physiological conditions of the microbial cultures [47]. We speculate that in analogy the changes of EF could also be affected by the growth stage and the condition of

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the plant. However, endophytes in plants cannot be completely excluded as a source of the transformation and more specific studies are needed to prove the specific contribution of endophytes and plant enzymes in growing plants.

3.7 Transformation of HCH in different plant compartments

One major question of the metabolism of organic pollutants in plants is the elucidation of the transformation process. Both plant endophytes and plant derived enzymes could be involved in the transformation. In comparison to our previous study [24], we could confirm in the present study that α-HCH can also be transformed by endophytes which are not inoculated by soil microorganisms and derived enzymes. Several previous studies already reported the phytodegradation of different organic pollutants by plant endophytes[48,49]. Even though we sterilized the surfaces of the wheat seeds, the existence of endophytes inside the wheat plants during growth may not entirely be excludible. The result of DAPI analysis showed that bacteria could be detected both inside and outside the wheat root (Fig. S8). Plant enzymes such as P450 monooxygenases, dehalogenase, glutathione S-transferases (GST), and glucosyltransferases (UGT) could directly function in the detoxification and deposition of organic pollutants [50]. The degradation of pharmaceuticals by plant derived P450-like enzymes has been reported[22]. Hybrid poplar (*Populus spp.*), algae (*various spp.*), and parrot feather (*Myriophyllum aquaticum*) have been reported to produce dehalogenases which are able to transform DDT [51]. Dec and Bollag (1994) reported that polychlorinated biphenyls (PCBs) could be metabolized by sterile plant tissues [52]. The detoxification process of γ-HCH by Phragmites australis plants could be attributed to UGT enzymes in the root and the rhizome as well as by GST enzymes in the leaves [53]. To our best knowledge, there are no reports about the phytotransformation of α-HCH by plant enzymes and endophytes. Therefore, further studies should focus on *in vitro* experiment analyzing the

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transformation of α-HCH by plant enzymes or endophytes. Reference experiments with plant enzymes or endophytes may be needed to calculate isotopic enrichment factors. These enrichment factors may be used to quantify transformation reactions in plants and are particularly useful if multi element isotope approaches are employed [12]. As discussed above, the only metabolites of HCH detected in both non-labeled and labeled experiments were PCCH and TCB, which resulted from a dehydrohalogenation with elimination of HCl. The metabolites are similar to those formed during α-HCH biodegradation by *Sphingomods* which are typical HCH degrading soil bacteria [54]. We carefully sterilized the seeds, all media and materials and thus microbes are unlikely to degrade HCH in the media. Nevertheless, endophytes could be carried inside seeds and it may be hypothesized that the endophytic microbes could participate in the dehydrohalogenation of HCH. The DAPI analysis showed indeed there were endophytic bacteria inside the roots as well as bacteria on the root surface. Additionally, monoxygenases like cyctochrome P450 enzymes are present in plants. Liver-derived cyctochrome P450 enzymes has been shown to transform γ-HCH to PCCH [55], thus plant derived enzymes such as P450 and dehalogenase may also catalyze α-HCH transformation. Our data do not allow deciphering the precise mode of HCH transformation within plants and further research is needed.

Moreover, it is well known that plants host different amounts and types of endophytes and enzymes in different organs [56] and this may explain the variable $\delta^{13}C$ and $\delta^{37}Cl$ enrichment of α-HCH in different wheat compartments. Therefore the residual fraction may have been degraded to a different extent in the various compartments of the plant as indicated by the extent of isotope fractionation. In conclusion, our data suggest that wheat has the potential to transform α-HCH in relatively short growth periods in hydroponic systems, but we found no indication for transformation of β-HCH in such a short period.

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4. Conclusion and outlook

In the present study, we demonstrated the transformation of α-HCH in plants based on the changes of isotope composition and enantiomer fraction, which could be important for the development of phytoremediation concepts because microbial or enzymatic degradation can contribute to HCH elimination in plants. There is no transformation of β-HCH in plants indicated by the changes of carbon and chlorine isotope compositions. Selective transformation of HCHs upon uptake needs to be taken into account when quantitatively interpreting results of phytoscreening at field sites. PCCH and TCB are found as the metabolites of α-HCH in plant tissues. The isotope fractionation pattern and the metabolites suggest that the transformation is a dehydrochlorination process. The dual element isotope analysis provides evidence for a dominant transformation pathway of α-HCH in plant which is caused by the plant endophytes or enzymes. However, it is difficult to decipher the precise mode of HCH transformation by plant endophytes or enzymes based on our data. Therefore, further research should focus on *in vitro* HCH transformation experiments by plant endophytes or enzymes which may be also needed to calculate the carbon and/or chlorine isotope enrichment factors for quantitating the extent of transformation of HCHs in plants. The combination of multi element CSIA and EF is a promising approach to identify the transformation of HCHs in plant tissues. Our study with HCHs suggests that CSIA and EF could also be applied to analyze the fate of other emerging environmental pollutants in plants of which many are chiral.

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Declaration of competing interest

There are no conflicts to declare.

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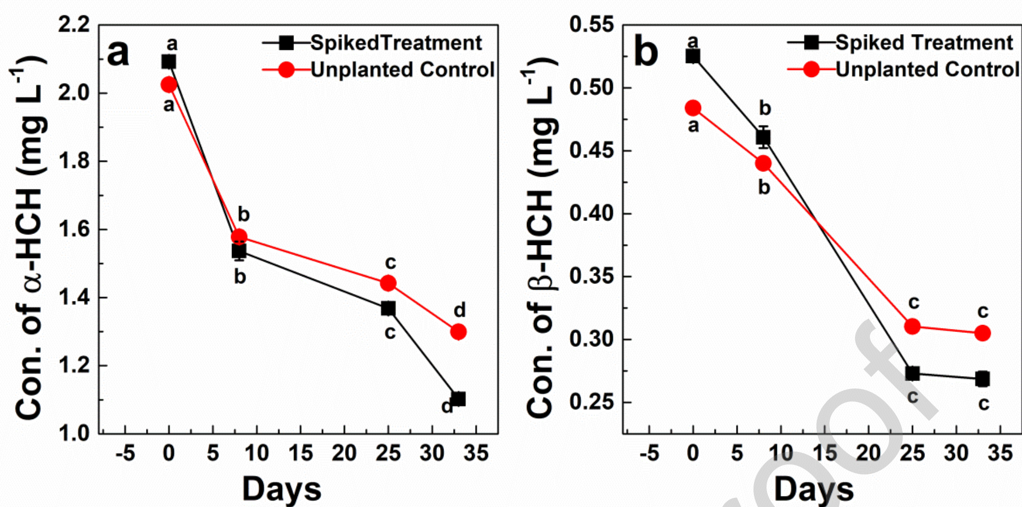

Fig. 1. The changes of concentrations of α-HCH (a) and β-HCH (b) in nutrient solution during the growth of wheat. Within each line, different letters indicate the significant differences according to Fisher's least significant difference test (LSD) ($p<0.05$). Spiked treatment: experiment with wheat growing in the nutrient solution spiked with HCHs. unplanted control: non-planted nutrient solution spiked with HCHs. Error bars represent SD values.

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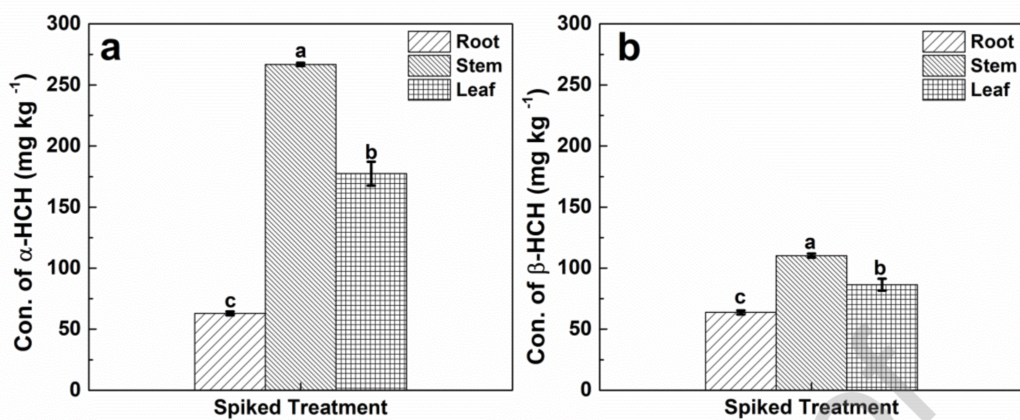

Fig. 2. Concentrations of α-HCH (a) and β-HCH (b) in the different compartments of wheat in the spiked experiment. Different letters indicate the significant differences according to Fisher's least significant difference test (LSD) (p<0.05). Spiked treatment: experiment with wheat growing in the nutrient solution spiked with HCHs. Error bars represent SD values.

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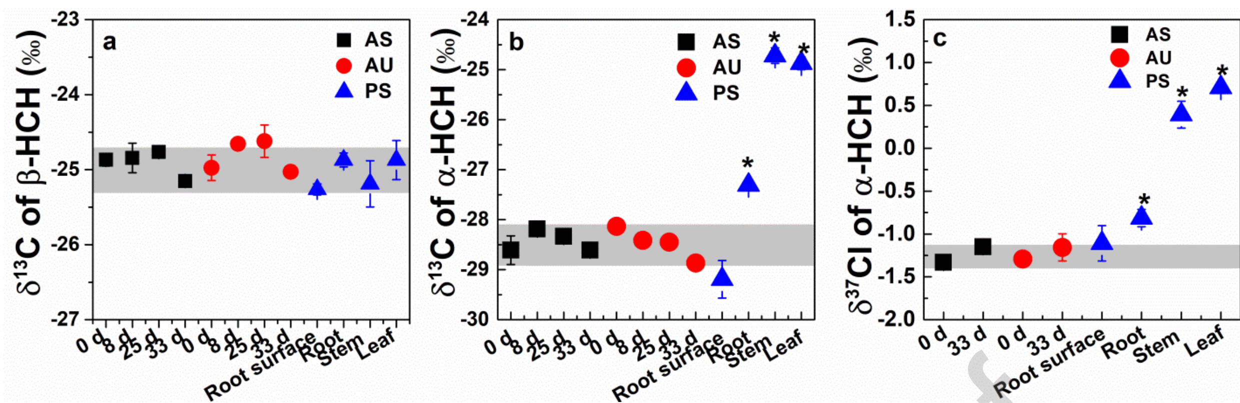

Fig. 3. Carbon isotope ratio of β-HCH (a) and α-HCH (b) and chlorine isotope ratio of α-HCH (c) in the aqueous samples and the plant samples. The grey area depicts the range of isotope values of pure HCHs. The asterisk refers to the significant difference between plant samples with aqueous samples according to Fisher's least significant difference test (LSD) (p<0.05). AS refer to aqueous samples in the spiked experiment. AU refers to aqueous samples in the unplanted control. PS refers to the plant samples harvested after 33 days in the spiked experiment. Error bars represent SD values.

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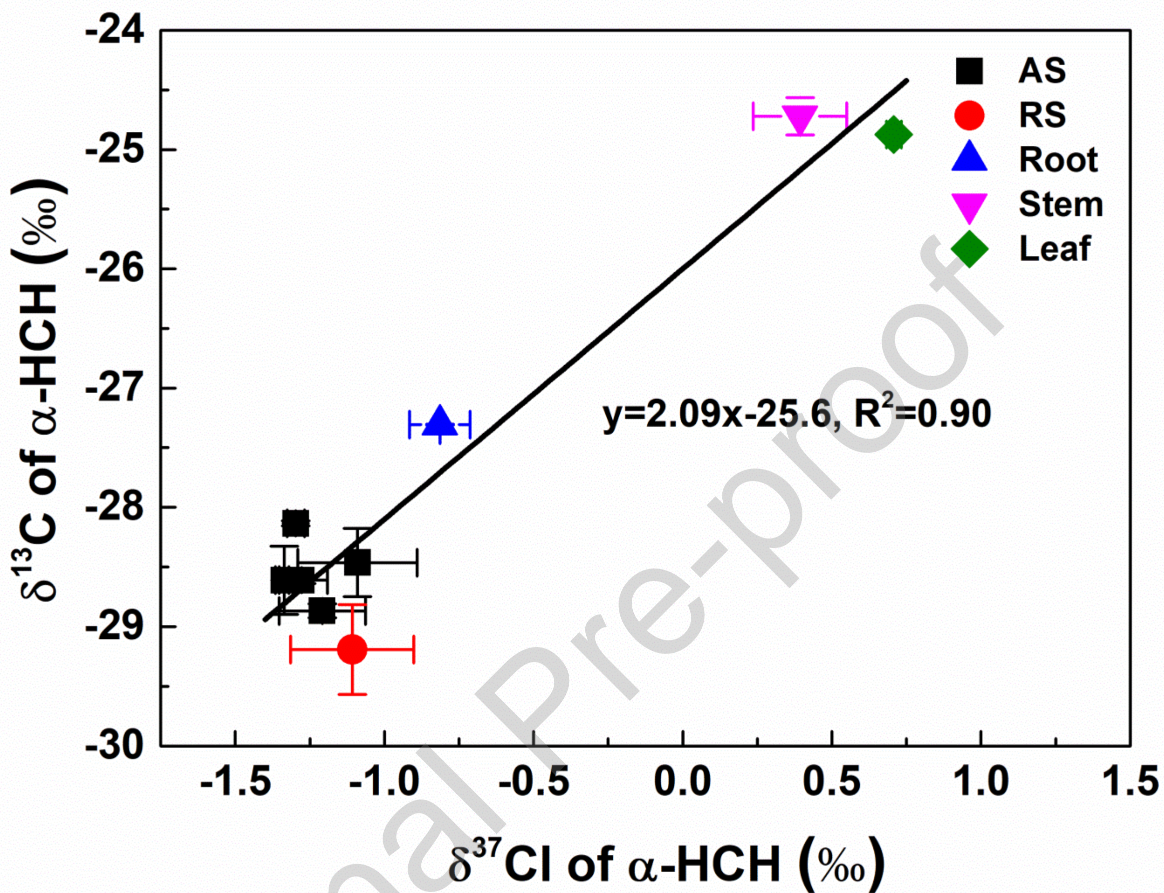

Fig. 4. The correlation of dual element stable isotope analysis of α-HCH in aqueous and plant samples. AS indicates aqueous samples in the spiked experiment. RS indicates root surface. Error bars represent SD values.

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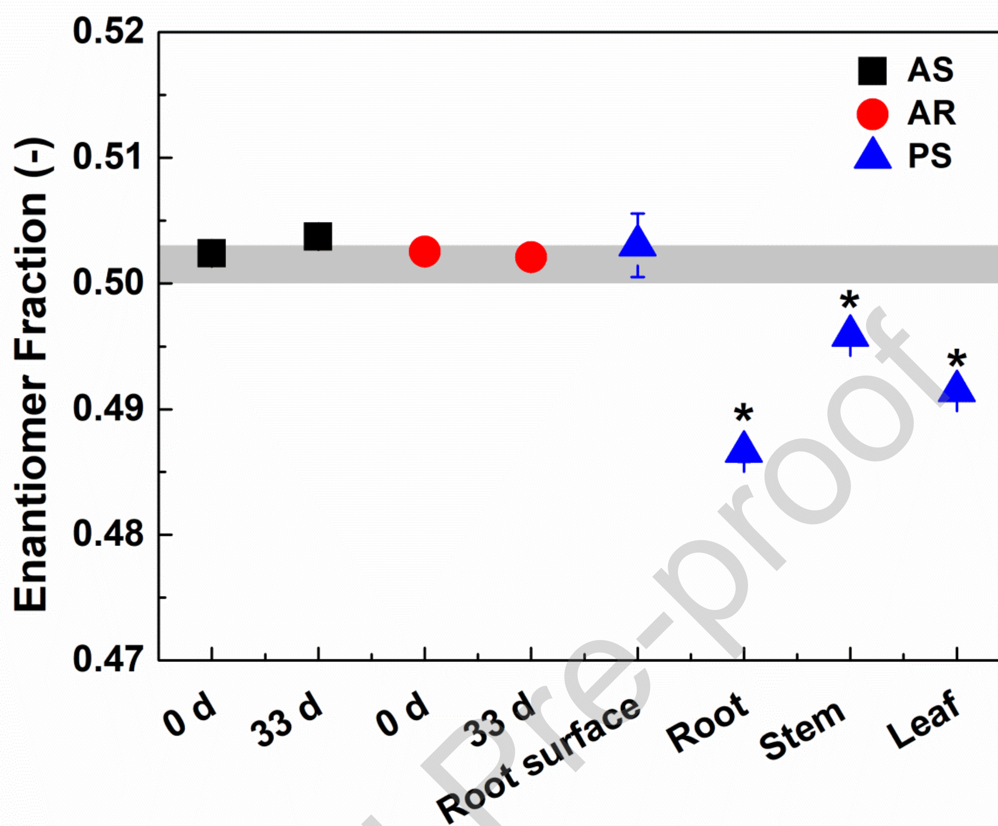

Fig. 5. Enantiomer fraction EF(-) of α-HCH the in aqueous samples and the plant samples. The grey area depicts the range of EF(-) values of pure α-HCH before dissolution in the water. The asterisk indicates significant differences between plant samples and aqueous samples according to Fisher's least significant difference test (LSD) ($p<0.05$). AS indicate aqueous samples in the spiked experiment. AU indicate aqueous samples in the unplanted control. PS indicate the plant samples harvested after 33 days in the spiked experiment. Error bars represent SD values.

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Credit author statement

Xiao Liu (first author): design of experiment, conduction of experiment, data acquisition, data evaluation, manuscript writing, revision

Langping Wu: manuscript writing, data evaluation, correction

Steffen Kümmel: data acquisition isotope analysis, correction, revision

Hans H. Richnow (PI of the project): conceptualization, design of experiment, data interpretation manuscript writing, revision

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Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

We habe no competing interest. Hans H. Richnow

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Highlights

- Metabolization of HCH in wheat was demonstrated by stable isotope probing concept.
- α-HCH was transformed in wheat as shown by carbon and chlorine isotope fractionation.
- Biotransformation of α-HCH was accompanied by the enantiomer fraction.
- α-HCH transformation lead to formation of PCCH and TCB.
- Dual element isotope analysis suggests one transformation pathway of α-HCH in wheat.

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