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Soil from a Hexachlorocyclohexane Contaminated Field Site Inoculates Wheat in a Pot Experiment to Facilitate the Microbial Transformation of β-Hexachlorocyclohexane Examined by Compound-Specific Isotope Analysis

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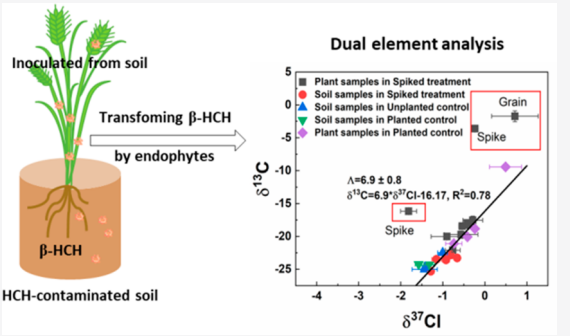
ABSTRACT: β-Hexachlorocyclohexane (β-HCH) is a remant from former HCH pesticide production. Its removal from the environment gained attention in the last few years since it is the most stable HCH isomer. However, knowledge about the transformation of β-HCH in soil−plant systems is still limited. Therefore, experiments with a contaminated field soil were conducted to investigate the transformation of β-HCH in soil−plant systems by compound specific isotope analysis (CSIA). The results showed that the $\delta^{13}C$ and $\delta^{37}Cl$ values of β-HCH in the soil of the planted control remained stable, revealing no transformation due to a low bioavailability. Remarkably, an increase of the $\delta^{13}C$ and $\delta^{37}Cl$ values in soil and plant tissues of the spiked treatments were observed, indicating the transformation of β-HCH in both the soil and the plant. This was surprising as previously it was shown that wheat is unable to transform β-HCH when growing in hydroponic culture or garden soil. Thus, results of this work indicate for the first time that a microbial community of the soil inoculated the wheat and then facilitated the transformation of β-HCH in the wheat, which may have implications for the development of phytoremediation concepts. A high abundance of HCH degraders belonging to *Sphingomonas* sp., *Mycobacterium* sp., and others was detected in the β-HCH-treated bulk and rhizosphere soil, potentially supporting the biotransformation.



KEYWORDS: *isotope fractionation, phytoremediation, transformation, soil−plant system, contaminated field*

INTRODUCTION

Technical hexachlorocyclohexanes (HCHs), the mixture of different HCH isomers (mainly α-, β-, γ-, and δ-HCH), were used worldwide as a pesticide.[1,2] Later, γ-HCH (Lindane), the only isomer which possesses insecticidal toxicity, was purified from technical HCH. The purification of γ-HCH caused large amounts of waste mainly containing α- and β-HCH, which were dumped directly in the vicinity of the factories,[3] typically impacting soils around the production sites. Heavily HCH contaminated field sites were reported all over the world in the past.[4−7] Although Lindane was banned according to the Stockholm Convention in 2009,[8] the HCH contamination is still observed in plants and wild animals at contaminated field sites due to biomagnification and bioaccumulation.[9−11] Recently, studies showed that HCHs can be detected in the edible parts of plants,[12] which caused increasing concerns about HCH contamination. The plant uptake of HCHs from soil is one of the first steps for HCHs to enter the food web. The uptake, translocation, and accumulation of HCHs both by roots potentially connected with the transpiration stream and by leaves from the gas phase have been observed before.[13−15]

In our recent study, the transformation of α- and β-HCH in hydroponic and garden soil (total organic carbon is 22.0 mg g^{-1}; pH is 7.9; Luvisol)−plant systems was investigated, showing that the transformation of α-HCH takes place in both soil and plant, but no transformation of β-HCH was found.[16,17] β-HCH is the most stable isomer compared to the other isomers since all six chlorine atoms are in the equatorial position. There are only few reports demonstrating the transformation of β-HCH in the literature, and nearly all the reports are related to microbial culture studies by *Sphingomonas Paucimobilis* UT26, *Sphingomonas sp.* BHC-A, *Sphingobium sp.* MI1205, and *Sphingobium Indicum* B90A under aerobic conditions[18−22] and by a *Dehalobacter* sp. in coculture with a

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Sedimentibacter sp. and enrichment culture from HCH-contaminated soil under anaerobic[23,24] conditions. The interactions of plants and their associated microorganisms in the rhizosphere have already been considered as an efficient option for the cleanup of contaminants in soil.[25−28] However, also the transformation of contaminants in plants should be considered as a part of the phytoremediation processes. To our best knowledge, no studies explore the transformation of β-HCH in soil−plant systems, and data about the transformation of β-HCH in soil−plant systems, particularly the transformation in the plant, are limited. Thus, our aim was to study the interaction between the soil and the plant, which could facilitate the transformation of β-HCH and develop the phytoremediation concept of HCH in the field.

Identifying and characterizing transformation processes of organic pollutants by changes of the concentration alone are challenging since the concentration can be effected by several physical processes, such as evaporation, absorption, and dilution in addition to biotransformation. Compound specific isotope analysis (CSIA) is a promising method to characterize transformation processes in the environment independent of concentration changes.[29−31] CISA makes use of kinetic isotope fractionation upon a bond cleavage reaction. CSIA has already been well developed and applied to identify the fate of HCHs in microbial degradation studies at the laboratory scale with *Dehalococcoides* sp. and *Sphingobium* sp. in batch experiments[32,33] and landscape level in the groundwater in Bitterfeld.[34] Recently, CSIA was successfully applied to characterize the transformation of HCHs in garden soil−plant and hydroponic systems.[16,17] The investigation of the fate of organic pollutants in a complex system is always challenging since several degradation processes (e.g., aerobic and anaerobic degradation processes or biotic and abiotic degradation processes) are simultaneously active.[35,36] It is a challenge to identify the main process in such cases. However, distinct reaction mechanisms may still be identified in those complex systems since different reaction mechanisms comprise different modes of chemical bond cleavage with specific isotope effects. The chemical reaction mechanisms may be identified by multiple element isotope analysis. The correlation of isotope effects in dual element plots may have different slopes for different reaction mechanisms.[35] Moreover, the microbial community and Lin gene information which govern the degradation of HCH in soil were analyzed by metagenome analysis to elucidate the microbial potential of transforming β-HCH in soil and backed up the isotope analysis.

Wu et al.[37] analyzed the isotopic composition of HCHs in plants and soil to monitor the transformation of HCHs at a contaminated field site. The increase of the carbon isotope values of HCHs indicated that soil from contaminated field sites as well as the plants growing on these soils may have the potential to transform β-HCH. However, it was difficult to precisely identify transformation processes in soil and plants in the field because of variable field conditions which affected the isotopic composition of the HCHs. Therefore, a soil pot experiment using soil from a contaminated field site was conducted to systematically investigate the possible turnover of β-HCH in soil and wheat using CSIA. Three growth stages covering the whole period of wheat growth were selected to investigate the influence of plants on the transformation of β-HCH in the soil−plant systems.

MATERIALS AND METHODS

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

Exposure of Seeds and Plants to HCHs in Soil. Wheat (cultivar "*Quintus*" of *Triticum aestivum L.*) was used as test the plant, and the seeds were obtained from the breeder Saaten-Union GmbH (Isernhagen, Germany). The soil was collected at 0−20 cm depth from a HCH-contaminated field site in Bitterfeld (Germany) (51.6395°, 12.2880°) in the area of a loading zone close to a former HCH production factory which was shut down in the last century, and the details of the field site are shown in Section S2 (SI). The soil is classified as Gleyic Fluvisol according to WRB (World Reference Base for Soil Resources).[38] The total organic carbon of the soil was 16.7 mg g^{-1}, and the pH was 6.5. The initial concentration of β-HCH in the contaminated soil was 0.63 mg kg^{-1}, and the initial $\delta^{13}C$ and $\delta^{37}Cl$ values were −24.0 ± 0.1‰ and −1.6 ± 0.3‰, respectively. After spiking the soil with pure β-HCH, the $\delta^{13}C$ and $\delta^{37}Cl$ values were −25.6 ± 0.2‰ and −2.4 ± 0.3‰, respectively.

The experiment was conducted in the glass house of the research station of the UFZ in Bad Lauchstädt. The soil was air-dried and sieved at <2 mm. Then, 250 g of soil was spiked with 34.74 mg of β-HCH dissolved in acetone. When the acetone was evaporated, the spiked soil was mixed with 1.75 kg of nonspiked soil with the addition of a basic fertilizer mixture (1.63 g of NH_4NO_3 as solution; 1.70 g of $CaHPO_4$ $2H_2O$ as solid; 1.27 g of K_2SO_4, 1.46 g of $MgSO_4{\cdot}7H_2O$, and 0.04 g of $FeCl_3$ as solution; 0.9 mL of Hoagland micronutrition solution). Afterward, the soil was homogenized thoroughly before it was transferred into the different pots (2 kg of soil per pot). The prepared soil pots were equilibrated at room temperature for several days at 60% of the water holding capacity. The theoretical final concentration of β-HCH was 18 mg kg^{-1}. In each pot, 7 seeds were grown uniformly, and at last 5 seedlings were left after germination. During the whole period of wheat growth, the water content of the soil was maintained at 60% of the maximum water content of soil. Three treatments were set up as follows: (i) β-HCH spiked soil with wheat (spiked treatment), (ii) native contaminated soil with wheat (planted control), and (iii) β-HCH spiked soil without wheat (unplanted control). Spiked treatments had 9 replicates, and 3 of the 9 pots were used to separately analyze different growth stages. Planted as well as unplanted controls had 3 replicates each. The pots of the different treatments were placed randomly in the glass house. The soil surface of each pot was covered by a thin layer of 2 cm silica sand (150−380 μm) to prevent the transportation of soil particles to the leaf surface by air and to reduce the HCH exchange between the soil−air interface as reported for hexabromocyclododecane isomers (HBCDs).[39]

Sampling of Soil and Plants from the Pot. Wheat grew for 95 days from May to August 2020. At the jointing (20 days), heading (49 days), and harvest stage (95 days), 3 pots of the spiked treatment were sacrificed and sampled. At the same time points, 50 g of soil was sampled from the unplanted controls. The planted controls were only sacrificed and sampled at the harvest stage. In the sacrificed samples, the whole soil and root system was gently crushed, and loosely bound soil was separated by shaking. This soil is referred to as bulk soil. The remaining tightly bound soil particles were considered as the rhizosphere soil and were removed by

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shaking in a plastic bag.[40] A small portion of the soil samples was frozen at −20 °C for metagenome analyses. The remaining soil samples were lyophilized for further treatment. After taking the rhizosphere soil, plant samples were washed thoroughly four 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 Cleanup of HCHs from Soil and Plant Samples. The extraction and cleanup methods of HCH from soil and plant samples for isotope analysis have been described previously.[41] The details are shown in Section S3 (SI). No carbon and chlorine isotope fractionation was observed for the different steps of the extraction and cleanup procedure.[41]

DNA Extraction and Sequencing. The genetic analysis was carried out by isolating the metagenomic DNA directly from the soil, which contains representatives from both active and inactive bacteria residing in the respective soil samples. DNA was extracted from soil using the 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). 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, U.S.A.) equipped with a flame ionization detector (FID) was used to determine the concentration of HCHs throughout the study. The details are shown in Section S4 (SI).

Isotope Analysis. Carbon isotopic compositions ($\delta^{13}C$) were analyzed by a gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS), where a GC (7890A, Agilent Technologies, U.S.A.) 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 Section S4 (SI).

Chlorine isotopic compositions ($\delta^{37}Cl$) were analyzed by a gas chromatograph coupled with a multiple-collector inductively coupled plasma mass spectrometer (GC-MC-ICPMS). Therefore, a GC (Trace 1310, Thermo Fisher Scientific, Germany) was connected through a thermo-elemental transfer-line (AE2080, Aquitaine Electronique, France) to the MC-ICPMS (Neptune, Thermo Fisher Scientific, Germany), as recently described elsewhere.[42] The details are shown in Section S4 (SI).

Dual Element 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.[35]

Metabolites Analysis. The possible metabolites of HCH transformation in the plant and soil were measured using a gas chromatograph−mass spectrometer (GC-MS) where a GC (7890A, Agilent Technologies, U.S.A.) was connected with a MS (5975C, Agilent Technologies, U.S.A.). The details are shown in Section S4 (SI).

Metagenomics Analysis. Reads obtained after whole metagenome sequencing using the Illumina platform were trimmed using the fastQC quality control analysis tool (https://github.com/s-andrews/FastQC). High-quality cleaned data (Table S2, SI) were processed for the taxonomic characterization. The details are shown in Section S4 (SI).

lin Gene Profiling and Their Relative Abundance. The sequences of the *lin* genes from the complete genomes of *Sphingobium japonicum* UT26, *Sphingobium indicum* B90A, and other prominent *Sphingomonas* sp. reported to degrade HCH were taken as reference to assess the presence of *lin* genes required for the transformation of HCHs[43] and were used to create a custom database in ABRicate (T. Seemann, https://github.com/tseemann/abricate). The assembled contigs were searched for the presence of *lin* genes against the custom database, and only hits above 60% coverage and identity were considered as homologues of *lin* genes.

To access the species richness and differences in species composition among these samples, α (Shannon H index and Chao1 index) and β diversity were calculated based on the Bray−Curtis dissimilarity matrix using Explicet software.[44]

Statistical Analysis. The HCH concentration and isotope data were analyzed statistically using analyses of variance (ANOVA) and least significance difference post hoc comparison testing with the SPSS software v19.0.

RESULTS AND DISCUSSION

Concentration of β-HCH in Soil−Plant Systems. No difference of the wheat biomass was observed between the spiked treatments and the planted control at the harvest stage (Figure S1), indicating that the additionally added β-HCH has no toxic effect on the growth of the plant. Similar results were also found in a previous study.[16] The concentration of β-HCH in the soil of the unplanted control decreased during the whole wheat growth period from an initial concentration of 18 mg kg^{-1} to 9.3 ± 3.1 mg kg^{-1} at the jointing stage, to 9.7 ± 0.9 mg kg^{-1} at the heading stage, and to 7.8 ± 0.4 mg kg^{-1} at the harvest stage (Figure S2a). The loss of β-HCH in the unplanted control is most likely related to evaporation and possible transformation reactions in the soil. The concentration of β-HCH in the bulk and rhizosphere soil of the spiked treatment also decreased along with the wheat growth. For example, the concentration of β-HCH in the bulk soil decreased from an initial concentration of 18 mg kg^{-1} to 13.8 ± 1.4 mg kg^{-1} at the jointing stage, to 7.0 ± 0.1 mg kg^{-1} at the heading stage, and to 4.8 ± 0.0 mg kg^{-1} at the harvest stage (Figure S2a). However, compared to the concentration loss in the unplanted control, significantly lower β-HCH concentration was observed in the spiked treatment in the harvest stage ($p < 0.05$) indicating the appearance of transformation processes in the rhizosphere as well as the uptake, transformation, and evapotranspiration of β-HCH by the plants. A similar pattern of decreasing concentration was noted in the rhizosphere soil, indicating that plants promote the disappearance of β-HCH. However, the significant difference between bulk and rhizosphere soil was only observed in the jointing stage, and there was no significant difference in the latter two growth stages ($p < 0.05$), indicating that the degrading bacteria were active in both bulk and rhizosphere soil in a similar level in the later phases of the wheat growth.

In the planted control, the β-HCH concentration decreased slightly from an initial concentration of 0.63 mg kg^{-1} in the native contaminated soil to 0.53 ± 0.07 mg kg^{-1} in the bulk soil and 0.43 ± 0.14 mg kg^{-1} in the rhizosphere soil at the harvest stage (Figure S3).

In both the spiked treatment and the planted control, β-HCH was detected in all different wheat tissues at the three growth stages. However, the highest β-HCH concentration was

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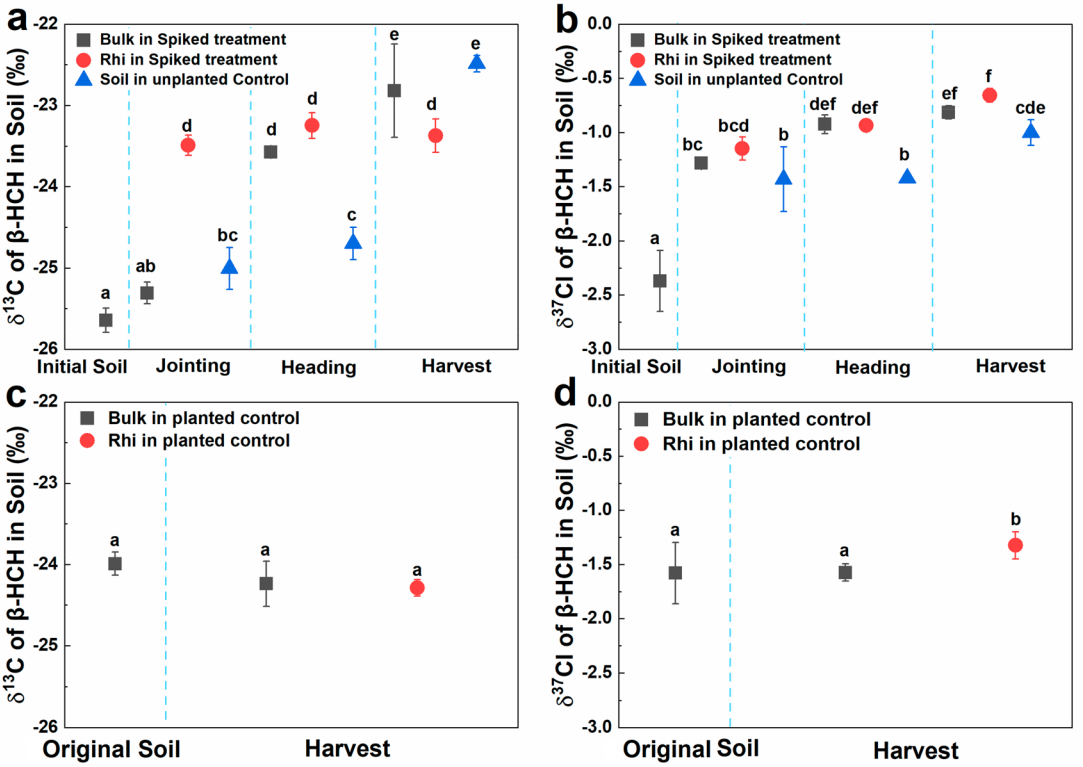

Figure 1. Carbon (a) and chlorine (b) isotopic compositions of β-HCH in the bulk (Bulk) and rhizosphere (Rhi) soil in the spiked treatment. Carbon (c) and chlorine (d) isotopic compositions of β-HCH in bulk and rhizosphere soil in the planted control. The initial soil in parts (a) and (b) shows the initial carbon and chlorine isotope values of β-HCH of soil after spiking with pure β-HCH. The original soil in parts (c) and (d) shows the original carbon and chlorine isotope values of the native contaminated soil. The letters (a–f) in all figures represent statistically significant differences between soil samples at different growth stages according to Fisher's least significant difference test (LSD) ($p < 0.05$). Error bars represent SD values.

observed in the roots followed by the stems and the lowest in the leaves. This indicates that β-HCH was translocated to all parts of the wheat after uptake by roots (Figure S2b). Much lower concentration of β-HCH was observed in the planted control compared to the spiked treatment since the initial β-HCH concentration of the soil was also much lower in this setup (Figure S2b).

The BCFs of all wheat tissues increased along with the wheat growth (Table S1). All the BCFs were lower than 1 except SCF (stem bioconcentration factor) with a value of 1.2 at the harvest stage, indicating a minor accumulation of β-HCH in plant from soil. In addition, a variability of the BCFs of different plant tissues at the same growth stage was observed, suggesting that specific microbial processes affect the concentration of β-HCH. The BCFs observed in this study were much lower than those reported in a previous study.[16] This effect could be related to the possible transformation of β-HCH in the current study, which led to an only minor accumulation of β-HCH in the plant tissues.

Carbon and Chlorine Isotope Fractionation of β-HCH in Soil. Observed changes of the isotopic composition of β-HCH are mainly related to kinetic isotope fractionation, which is a result of the preferential transformation of the isotopically light isotopes. Remarkably, no difference was observed for the $\delta^{13}C$ and $\delta^{37}Cl$ values of β-HCH in the soil of the planted control at the harvest stage compared to the original values in the native contaminated soil (-24.0 ± 0.1‰ for $\delta^{13}C$, -1.6 ± 0.3‰ for $\delta^{37}Cl$) (Figure 1c,d), suggesting that the bioavailability of β-HCH was limited in the native contaminated soil which is further supported by the comparable low concentration of β-HCH in the soil of the planted control in comparison to the spiked treatment. Consequently, no transformation of β-HCH occurred in the soil when the native contaminated soil was planted with wheat. Most likely the remaining β-HCH in the native contaminated soil was not bioavailable for soil bacteria as the easily bioavailable β-HCH fraction was already degraded since the contamination of the soil by HCH muck lasted for at least three decades. This time allowed the development of a microbial community capable of degradeing β-HCH in soil.

A different development of the $\delta^{13}C$ values was observed in the spiked treatment. In comparison to the initial $\delta^{13}C$ value of β-HCH (-25.6 ± 0.2‰ after spiking), an increase of the $\delta^{13}C$ values was observed in both bulk and rhizosphere soil at different growth stages (Figure 1a). The highest increase was observed for β-HCH at the harvest stage ($\delta^{13}C$ values in the bulk and rhizosphere soil were -22.8 ± 0.6‰ and -23.3 ± 0.1‰, respectively). The carbon isotope fractionation caused by phase partitioning for chemicals, which has a similar physiochemical of HCH, was insignificant with a value of 0.3‰.[45,46] In addition, our previous garden soil pot and hydroponic experiments with β-HCH[16,17] showed that there are no isotopic changes of HCH observed during the volatilization, and compared to the fractionation caused by transformation, the possible fractionation caused by volatilization or phase partitioning could be ruled out to explain our observation. Therefore, the increase of $\delta^{13}C$ values in the soil indicates that β-HCH was transformed in both the bulk and

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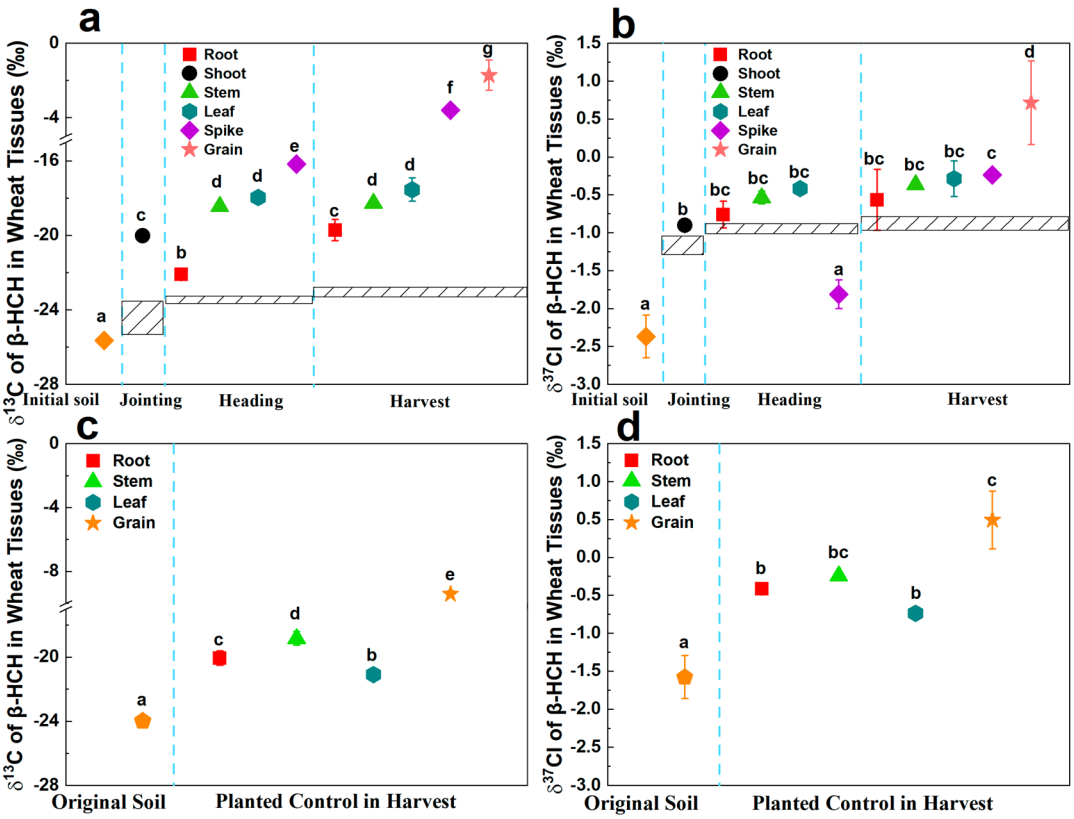

Figure 2. Carbon (a) and chlorine (b) isotopic compositions of β-HCH in different wheat tissues in the spiked treatment. Carbon (c) and chlorine (d) isotopic compositions of β-HCH in different wheat tissues in the planted control. The initial soil in the orange diamond in parts (a) and (b) shows the initial carbon and chlorine isotope values of β-HCH of soil after spiking with pure β-HCH. The left slash bars in parts (a) and (b) show the range of the carbon and chlorine isotope values of β-HCH in the bulk and rhizosphere soil at different wheat growth stages. The original soil in the orange pentagon in parts (c) and (d) shows the original carbon and chlorine isotope values of β-HCH of the native contaminated soil. The letters (a)−(g) in all figures represent statistically significant differences between plant samples at different growth stages according to Fisher's least significant difference test (LSD) ($p < 0.05$). Error bars represent SD values.

the rhizosphere soil. An increase of the β-HCH δ^{13}C values was noted from the jointing stage to the harvest stage in both bulk and rhizosphere soil (Figure 1a), which is related to the increasing activity or enrichment of β-HCH degrading bacteria along with the wheat growth. In a previous experiment using garden soil and otherwise nearly identical conditions, no transformation of β-HCH was observed, indicating that the β-HCH degrading microbial community is already well developed in the contaminated field soil due to long exposure time to HCHs.[16] Additionally, compared to the results of the planted control, the increase of the δ^{13}C values in the spiked treatment suggested that β-HCH degrading bacteria in the soil was stimulated, activated, and further developed by spiking the soil with a high concentration of β-HCH.

After spiking the soil with β-HCH, an increase of the δ^{13}C value was found in the unplanted control compared to the initial value, indicating that the β-HCH was also transformed in the unplanted control, which obviously is related to the transformation of the β-HCH by degrading bacteria as mentioned above. The δ^{13}C values in the unplanted control were stable and lower in the first two growth stages compared to the δ^{13}C values in the spiked treatment. However, the δ^{13}C value of β-HCH strongly increased at the harvest stage and reached a similar value as in the spiked treatment (Figure 1a). Based on the results, we hypothesized that the β-HCH degrading soil bacteria possess a longer lag time for biotransformation if the stimulation by plants is missing. The combination of the β-HCH δ^{13}C values of all different treatments (planted control, unplanted control, and spiked treatment) may suggest that spiking the soil with a high concentration of β-HCH could induce the activation of β-HCH degrading bacteria in the soil and that the plant growth could accelerate the biotransformation of those microorganisms.

The development of δ^{37}Cl patterns of β-HCH in the soil samples showed a similar trend as the δ^{13}C values in both the spiked treatment and the unplanted control (Figure 1b). An increasing enrichment of ^{37}Cl isotopes of β-HCH in the bulk and the rhizosphere soil was observed along with the wheat growth in spiked treatment and unplanted control. The observation of the simultaneous increase in the δ^{13}C and δ^{37}Cl values revealed that a C−Cl bond was cleaved during the transformation of β-HCH in soil.

Carbon and Chlorine Isotope Fractionation of β-HCH in Plants. We cannot completely exclude that plants may fractionate the β-HCH during uptake and internal translocation toward isotopically light compounds. However, the fractionation caused by these processes should be very minor as shown in the previous studies and much lower, if at all, compared to the fractionation caused by the transformation.[16,17] Therefore, we interpret the isotope fractionation data in the current study as a result of a transformation process. Interestingly, an increase of the δ^{13}C value of β-HCH was observed in wheat tissues compared to its host soil at the same

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growth stage (Figure 2a). Thus, the results suggested that β-HCH was further transformed in the wheat tissues after the uptake from the soil, which was not observed in our previous studies.[16] However, the only difference to our former study is the usage of native contaminated soil with a long history of HCH contamination for the experiments in contrast to the usage of garden soil without HCH contamination history. Therefore, the soil already contains an enriched and well developed HCH degrading bacteria community which has the potential to transform β-HCH. Based on the results of our previous studies, we can exclude that the wheat has the possibility to transform β-HCH by its own endophytes and enzymes.[16,17] Therefore, we hypothesized that the β-HCH degrading bacteria of the soil may colonize the plant by entering the plant via the roots followed by incubation within the plant, resulting in the formation of new wheat endophytes that afterward distributed to all wheat tissues. This process would enable the transformation of β-HCH in wheat. Former research reported that some soil bacteria, so-called competent endophytes, could be well adapted to the plant environment and are capable of invading specific plant tissues, spreading throughout the plant, manipulating the plant metabolism, and maintaining a harmonious balance with the host plant.[47] Examples of such competent endophytes that could colonize the plant and show interactions with plant metabolism are *Pseudomonas putida* which possess the function of ethylene modulation,[48] *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a which can promote plant growth,[49] and *B. subtilis* and *B. amyloliquefaciens* ES-2 which can induce systemic resistances.[50,51]

Additionally, the $\delta^{13}C$ values of β-HCH increased from the root to the grain, indicating that the β-HCH degrading bacteria of the soil may preferentially colonize or enrich more in the upper parts of the wheat, causing higher transformation rates. Meanwhile, the $\delta^{13}C$ value of β-HCH in the same wheat tissue increased along with the wheat growth. This demonstrates that the intensity of β-HCH transformation in wheat tissues by endophytes inoculated from soil could increase during the wheat growth and could lead to higher transformation in the later growth stages in the same tissue.

An increase of the $\delta^{13}C$ values of β-HCH in the different wheat tissues could also be observed in the planted control (Figure 2c), which indicates that β-HCH was also transformed in plants in this experimental setup. However, no β-HCH transformation in the soil of the planted control was observed by isotope analysis, which might be caused by the limited bioavailability of β-HCH of the used native contaminated soil as explained above. Based on the results, we could suggest that β-HCH degrading soil bacteria colonized the wheat and became wheat endophytes. However, the β-HCH degrading bacteria in the soil of the planted control were not able to further transform the remaining HCH fractions due to the limited bioavailability of β-HCH in the used native contaminated soil. Thus, most likely the stimulation by wheat exudate of HCH degrading soil bacteria in soil was only minor when β-HCH was not bioavailable. In contrast, when the β-HCH degrading soil bacteria became wheat endophytes, they may be activated since β-HCH may dissolve in physiological plant fluids and become bioavailable.

The development of the $\delta^{37}Cl$ patterns of β-HCH in the plant samples showed similar trends as the $\delta^{13}C$ values in both the spiked treatment and the planted control (Figure 2b,d), which indicates further C−Cl bond cleavages in the wheat caused by wheat endophytes originating from the colonization of soil bacteria. Only the $\delta^{37}Cl$ value of the spike in the heading stage was lower than that of the host soil, indicating a maybe different transformation pathway of β-HCH in the spike.

Several metabolites of HCH degradation including pentachlorocyclohexene isomers, tetrachlorocyclohexene isomers, trichlorobenzene isomers, dichlorobenzene isomers, and chlorobenzene were detected in soil and plant samples (Figure S4), suggesting the transformation of β-HCH in the soil−plant systems. The appearance of pentachlorocyclohexene isomers suggests an aerobic degradation pathway. However, metabolites exhibit higher water solubility than parent β-HCH and may be up taken with water from the soil. Moreover, the metabolites may be already formed in this historical contaminated soil before the new-spiking β-HCH. Thus, it is difficult to draw conclusions if the transformation takes place within the plants but may give an indication that in the soil−plant system transformation takes place. However, in the current system CSIA can give us a strong indication that the transformation indeed takes place in plants in this complex system. The appearance of pentachlorocyclohexene would also support the hypothesis of aerobic HCH degradation in soil or by endophytes.

Combined $\delta^{13}C$ and $\delta^{37}Cl$ Analysis for the Characterization of β-HCH Transformation in the Soil−Plant Systems. The results of the present study show that the changes of $\delta^{13}C$ vs $\delta^{37}Cl$ values of β-HCH in the soil and plant samples of all treatments can be described by a linear regression slope (Λ) of 6.9 ± 0.8, suggesting that the overall transformation process of β-HCH in the soil−plant system is related to a similar C−Cl bond cleavage mode (Figure 3).

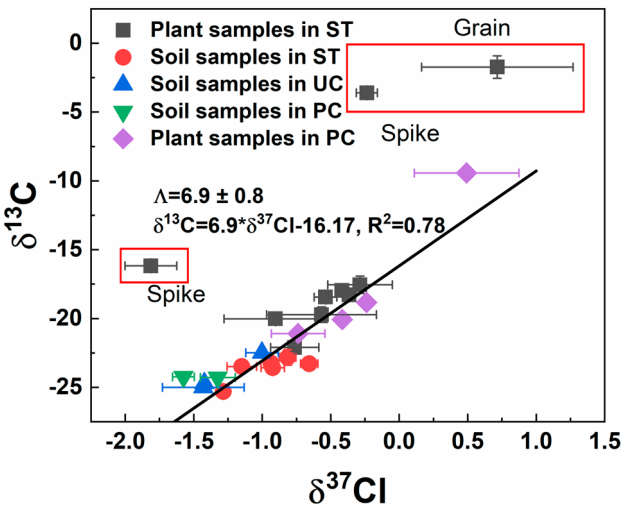

Figure 3. Dual element isotope analysis (C−Cl) of β-HCH in soil and wheat tissues of all treatments. ST: spiked treatment. UC: unplanted control. PC: planted control.

However, the spike in the heading stage as well as the spike and the grain in the harvest stage of the spiked treatment are out of the regression line, indicating that these samples may have a different mode of the C−Cl cleavage compared to other wheat tissues. The reason may be that spikes and grains are formed in the reproductive growth phase of the wheat, which may causes changes of the conditions for the endophytic community in the wheat, leading to different reaction

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mechanisms of the endophytic community in the other plant tissues which were formed during the vegetative growth phase.

In our previous studies, Λ values of 1.75 ± 0.13 and 3.30 ± 0.30 for α-HCH were observed in hydroponic systems and normal garden soil−plant systems.[16,17] The Λ value of β-HCH determined in this study is much different from those Λ values of α-HCH, suggesting that two different modes of Cl−C bond cleavage are active. Additionally, a Λ value of 1.3 ± 0.1 was found for β-HCH transformation in an anaerobic microbial enrichment culture,[52] which is also not consistent with the Λ value of our study, indicating that the reaction process in this study is not related to an anaerobic process. The Λ value in the current study is much higher than that observed value for anaerobic degradation (see above), suggesting that the carbon isotope more strongly fractionated than the chlorine isotope. Unfortunately, we cannot identify the detailed transformation mechanism based on the data in the current study since there are no further Λ values available in the literature. Consequently, further *in vitro* experiments with bacteria originated from contaminated soil are needed to calculate the Λ value and to identify and characterize the reaction process of β-HCH in the soil−plant system because reference experiments with aerobic cultures are missing.

Microbial Community and Dynamics Deciphered Using Metagenomics Analysis. The microbial community study and functional analysis were carried for three soil samples collected at the harvest stage from (i) bulk soil in the spiked treatment at the harvest stage (BHS), (ii) rhizosphere soil in the spiked treatment at the harvest stage (RHS), and (iii) bulk soil in the unplanted control at the harvest stage (BHC). The α-diversity analysis based on Shannon (H) and Chao1 index indicated that although all soil had a rich diversity of bacteria, BHC had a slightly higher value (H = 8.32) over BHS (H = 7.92) and RHS (H = 7.82) (Figure S5). However, all soils had rich diversity, but we could observe differences in the relative abundance of reads at the phylum and genus level. *Proteobacteria, Actinobacteria*, and *Thaumarchaeota* were enriched in BHS and RHS when compared to BHC (Figure S6). *Actinobacteria* were most abundant in RHS, confirming previous findings that the wheat rhizosphere supports the growth of *Actinobacteria*.[53,54] In addition, the β-diversity based on the Bray−Curtis dissimilarity matrix also revealed that the BHS was similar to the RHS and dissimilar to the BHC based on bacterial diversity, as the composition of bacteria must have significantly changed and can be attributed to the presence of the wheat (Figure S7). At the genus level also genera like *Sphingomonas, Mycobacterium, Bradyrhizobium, Streptomyces*, and *Nocardioides* got enriched in both soil samples (RHS and BHS) of the spiked treatment with wheat (Figure S8). Further, the results also indicated the higher presence of several soil-dwelling bacteria like *Novosphingobium, Pseudoxanthomonas, Devosia*, and *Cellulomonas* in RHS and BHS samples as compared to BHC, clearly indicating the role of the wheat plant in promoting the growth of selected bacteria. All these genera are reported to either tolerate or degrade HCH.[55,56]

The metagenomic assembly from the three samples was checked for the presence of *lin* gene homologues by using a custom database of *lin* genes using ABRicate. We observed the presence of BLAST hits corresponding to the upper *lin* gene pathway. The maximum number of hits was seen in RHS (n = 56), followed by BHS (n = 45) and BHC (n = 29). Interestingly *linB* homologues were detected in BHS and BHC, indicating the presence of bacterial diversity that might degrade β-HCH. In addition to *linB* homologues, homologue genes corresponding to *linF/F2, linL, linK*, and *linM* genes encoding a maleylacetate reductase, a putative ATPase component of ABC transporter confers tolerance to toxic dead-end metabolites.[43,57] Homologues showing high levels of similarity to the *linKLMN* genes have been found only in *sphingomonads*. Although their role is still unclear, they have been reported to perform active transport of a variety of xenobiotic compounds and are also involved in maintaining the integrity of the outer membrane.[43,57−59] The functional analysis of the metagenomic sequences revealed that mono- and dioxygenases were more abundant in BHS and RHS samples, which were grouped indicating the ability of the community present in the potted plant to degrade aromatic compounds, for example, possible metabolites of β-HCH (Figure S9). Therefore, β-HCH may get metabolized into further intermediates, and the metabolites could subsequently induce degradation pathways of oxygenases and enzymes of aromatic degradation pathways, which were also abundant in BHS and RHS samples (Figure S10). The results indicate that the rhizosphere of the wheat exerts a beneficial influence on the microbiome that can be helpful in phytoremediation approaches for β-HCH contaminated soil. Further studies should address the characterization of the plant microbiome and correlate it with the soil microbiome to gain a deeper insight in the soil−plant interaction with respect to β-HCH degradation.

Environmental Implication. As the most stable isomer among all HCH isomers, β-HCH and its removal from the environment gained more and more attention. So far, most studies focused on β-HCH transformation by microbial cultures in the laboratory. Thus, soil pot experiments covering the whole vegetation period of a plant are an important step forward to understand the transformation of β-HCH at field sites. The successful application of ^{13}C and ^{37}Cl isotope patterns used to investigate and monitor the transformation of β-HCH in plants and soil opens opportunities to validate natural attenuation as well as phytoremediation approaches. By investigating the C−Cl bond cleavage via CSIA, our study showed for the first time that β-HCH can be transformed in soil−plant systems, which supports the application of phytoremediation for β-HCH in the field.

Additionally, our study implies that for a successful phytoremediation the microbial community of the soil should have the capability to degrade the contaminant in soil and further in the plant. It indicates that the plants can receive the capability to degrade contaminants by endophytes originated from soil bacteria.

The contaminated field sites are always accompanied by a large amount of wild plants. In the planted control, the β-HCH could still be transformed, suggesting that plants can receive the capability to transform β-HCH from the soil microbiome. Thus, wild plants may also have the opportunity to transform β-HCH. In an earlier study, increased isotopic compositions of β-HCH in plants compared to the host soil were demonstrating that biodegradation potentially took place at the contaminated field site.[37] This finding can now be explained in more detail. Considering the biomass of wild plants in the field, the removal of β-HCH by plants could be a considerable amount.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c03322.

Details on chemicals and materials, extraction and cleanup methods, analytical methods, biomass, concentration, bioconcentration factors, metabolites analysis, and metagenome analysis (PDF)

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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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