Uptake and Metabolization of HCH isomers in Trees examined over an Annual Growth Period by Compound Specific Isotope Analysis and Enantiomer Fraction

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Abstract

To understand the role of plants for natural attenuation, a field study was conducted to characterize the fate of HCH in trees over an annual growth period using compound-specific isotope analysis (CSIA) and enantiomer fraction. Stable and slightly higher $\delta^{13}C$ and $\delta^{37}Cl$ values of HCH of host soil samples compared to the muck (consisting nearly exclusively of HCH) revealed that masking isotope effects caused by the limited bioavailability may underestimate the real extent of HCH transformation in soil. In contrast, an increase of $\delta^{13}C$ and $\delta^{37}Cl$ values in trees indicated the transformation of HCH. A large variability of $\delta^{13}C$ and $\delta^{37}Cl$ values in tree over the growth period was observed, representing different transformation extents among different growth time which is further supported by the shift of the enantiomer fraction (−) indicating the preferential transformation of enantiomers also varied over the different growth periods. Based on dual element isotope analysis, different predominant transformation mechanisms were observed during the growing seasons. Our observation implies that plants are acting as biological pumps driving a cycle of uptake and metabolization of HCH and refeed during littering to soil catalyzing their transformation. The finding that changes of transformation mechanism in different seasons has implications for phytoscreening and sheds new light on phytoremediation of HCH at field sites.

Key words: isotope fractionation, transformation, dual element analysis, phytoremediation, contaminated field

Synopsis. Elucidation of the natural fate of HCHs in trees during annual growth periods at a contaminated field site has implications for phytoremediation concepts and natural attenuation of HCHs.

Introduction

Hexachlorocyclohexane isomers (HCHs) contamination from the former production of Lindane (γ-HCH) is a global problem [1–3]. HCHs have been found even in remote areas such as the arctic revealing that HCHs can be transported over long distances [4,5]. Although HCHs have been listed as persistent organic pollutants (POPs) and their production was banned by the Stockholm convention in 2009 [1], HCHs can still be detected in milk [6,7], blood [8] and fat tissue [9,10] due to bioaccumulation and biomagnification in food webs.

Compound specific isotope analysis (CSIA) is a promising concentration-independent method to assess the transformation of organic pollutants in complex systems. Additionally, enantiomer fractionation could occur during biotransformation processes of chiral compounds which can be characterized by the enantiomer fraction (EF). Both, CSIA and EF were successfully applied to characterize the biotransformation of HCHs at the lab scale as well as at the field scale [11,12]. For studying the environmental fate of HCHs during plant growth using CSIA, isotope-effect-free methods have been developed for the extraction and clean-up of HCHs from complex matrixes [13]. Recently CSIA and EF were also applied to characterize the reactive transport processes governing the fate of HCHs along food webs at the contaminated field site Lucknow (India) [11]. The uptake of HCHs by plants at this field site was associated with an isotope fractionation suggesting HCH transformation in the rhizosphere and/or in the plants [11]. Moreover, detailed laboratory studies using wheat in hydroponic and soil pot model systems evaluated the transformation of HCHs by CSIA and EF [14,15]. Changes of the isotopic composition and concentration of HCHs were observed in the soil-pot experiment suggesting a variability of the HCH uptake and transformation during different plant growth phases [15]. Dual element isotope analysis could

62 be used to differentiate the different degradation mechanisms in complex systems
63 characterized by different regression slopes of two elements [14].

64 Phytoremediation, based on the interactions of plants and their associated microorganisms,
65 has been recognized as a powerful *in situ* approach for remediation for organic pollutants
66 in soils [16]. The transformation of HCHs by plants was identified in several previous studies
67 [15,17]. However, the impact of plants, particularly trees, on the fate of HCHs at field site
68 scales is still unknown because growth conditions in the field are much more complex than
69 under laboratory conditions and could change dramatically during the vegetation period.

70 For this work we explore the opportunities to study the metabolization of α- and β-HCH
71 making use of ^{13}C and ^{37}Cl isotope fractionation. To our best knowledge there are no
72 reports discussing changes of the HCH isotopic composition in plants at field scale as a
73 parameter for HCH transformation over whole plant vegetation periods. Therefore, a strip
74 of land with a succession of trees at Bitterfeld-Wolfen was selected as a contaminated test
75 field site. Four different tree species growing at this site were chosen to investigate the
76 uptake and transformation of HCHs by analyzing changes of concentration and isotopic
77 composition of HCHs in soil samples and plant tissues (e.g. stems, leaves, and fruits) over
78 at least one vegetation period. Whereas concentrations of HCHs were measured to examine
79 the uptake process at different growth periods, the EF values as well as the carbon and
80 chlorine isotopic composition were analyzed to evaluate the transformation mechanisms of
81 HCHs. Thereby dual element isotope analysis was applied to characterize the different
82 HCH transformation mechanisms in the selected tree species. The objective of this study
83 was to reveal the natural fate of HCH in plants during the growing seasons.

Methods and Materials

Field Site. The test site is located in Bitterfeld-Wolfen and belongs to the core center of the chlorine chemistry of the former German Democratic Republic. This area belongs to the most heavily HCH-contaminated sites in the world [18]. Today most of the contaminated soil in the production areas has been remediated or stabilized. However, a former loading area next to one previous factory still have some hot spots of soils heavily contaminated by muck and now were covered by a vegetation of bushes and trees with an estimated age of more than 20 years. The trees selected for the present study were growing at the slope of a hill which was in parts used as a landfill and which is now mostly covered with a humic rich soil. At this site a succession of trees and bushes has naturally developed over the years. More details can be found in S1 (SI).

Sampling of Soil and Plants. All samples were collected between April 2019 and Oct 2021 at a field site in Bitterfeld (51.6395°, 12.2880°). Four different tree species including *Robinia pseudoacacia, Prunus cerasifera, Crataegus monogyna* and *Rosa cania* were selected to monitor the seasonal changes of HCH concentrations and isotopic compositions. These four species of trees are the most abundant in this area and have developed as a natural succession over at least 20 years at this dump site. In total, eight sampling campaigns were conducted (Apr 2019, Aug 2019, Oct 2019, Nov 2019, May 2020, Jul 2020, Apr 2021 and Oct 2021). Details of sampling are shown in table S1 (SI). At each sampling campaign, soil and different plant tissues (leaves, young stem, fruit or seed) were collected. Plant tissues were sampled from new-grown tissues of comparable parts of the different trees and stored in plastic bags. Host soil samples were taken from a depth of 0-

20 cm and stored in plastic bags. All the samples were stored at −20 °C until further treatment.

Extraction and Clean-up of HCHs from Soil and Plant Samples. The method used for the extraction and clean-up of HCHs from soil and plant samples has been developed previously[13]. No changes of either carbon or chlorine isotopic compositions of HCH were observed during all the procedures of extraction and clean-up [13]. Details are shown in S2 (SI). The recovery for extracting HCHs was 86% to 94% from soil and at least 43% to 50% from plants [13].

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. Further analytical details are documented in S3 (SI).

Isotope Analysis. The isotopic composition of an element (E) was reported as δ notation in parts per thousand (‰) according to eq. 1.

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

where R_{sample} and $R_{standard}$ are the isotopic ratio of the sample and an international reference standard for the element of interest (e.g., Vienna Pee Dee Belemnite (V-DPB) for carbon, Standard Mean Ocean Chloride (SMOC) for chlorine), respectively.

Carbon Isotope Analysis. The carbon isotopic 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). Further analytical details are documented in S3 (SI).

Chlorine Isotope Analysis. Chlorine isotopic composition ($\delta^{37}Cl$) was analyzed using a gas chromatograph (Trace 1310, Thermo Fisher Scientific, Germany) coupled with a multiple-collector inductively coupled plasma mass spectrometer (GC-MC-ICPMS) (Neptune, Thermo Fisher Scientific, Germany), as recently described elsewhere [19]. Further analytical details were documented in S3 (SI).

Dual elements isotope analysis. The Lambda (Λ) value was used to distinguish different transformation mechanisms in complex systems [17]. Λ is defined as the slope of the regression line of the isotope fractionation of two elements during transformation processes between the source and samples in soil and plant tissues. Therefore, ^{13}C and ^{37}C values of HCH in soil and plant were normalized to the HCH source and in this study HCH muck was the HCH source.

Metabolite Analysis. HCH isomers and other synthesis products were identified by a gas chromatograph mass spectrometer (GC-MS) (Agilent Technologies 7890A for GC and 5975C for MS, USA) using authentic standards for the identification of the retention time and the mass spectra of the different HCH isomers. Metabolites were tentatively characterized by their mass spectrum. Analytical details are provided in the S3 (SI).

Results and Discussion

Concentration and Isotopic Composition of HCHs in Soil Samples. Top surface soil samples were sampled in two campaigns in Oct 2019 and May 2020. α- and β-HCH were the most abundant HCH isomers in the soil increasing up to almost pure residues of Lindane production. High heterogeneity of concentration patterns is expected in a land fill where various materials and soils are deposited. Other isomers such as γ- and δ-HCH could be qualitatively identified but the peak was mostly too low to be quantified or analyzed for their isotope composition in the presence of α- and β-HCH. In the soil, grey and white materials were found in the hot spots (Fig.S1) representing the so-called HCH muck which was deposited in the land fill. This HCH muck mainly contains α- and β-HCH which is consistent with the observation in the soil samples (Fig.S2). Additionally, the α-HCH was present as a racemate indicating unaltered residues from former HCH production. The high content of α- and β-HCH of the HCH muck might be explained by the photochemical HCH production process resulting in a diverse mixture of HCH isomers and the subsequent purification of Lindane. Thus, the side products of the reaction (i.e. α- and β-HCH) were directly dumped in the field around the factories. The concentration of α-HCH in the soil samples ranged from 3.23 ± 0.10 to 13.21 ± 0.26 mg kg^{-1} (in Oct 2019) and from 0.01 ± 0.005 mg kg^{-1} to 1.42 ± 0.40 mg kg^{-1} (in May 2020) (Table S2). The observed decrease of the concentration from Oct 2019 to May 2020 was most likely related to inhomogeneity. However, the decrease may also be related to some extent to the several processes such as plant uptake and possible transformation processes of HCH. Alike α-HCH, similar trends were noted for the concentration of β-HCH in the soil. In Oct 2019 the concentration of β-

HCH ranged from 0.80 ± 0.12 to 7.90 ± 0.24 mg kg^{-1} and in May 2020 from 0.01 ± 0.005 to 3.46 ± 0.89 mg kg^{-1} (Table S3).

The HCH muck represents the original waste products from the former Lindane production at the field site. Therefore, the isotopic composition of the HCH isomers of the muck was used as reference for comparison of the HCH isotopic composition in the soil samples.

The $\delta^{13}C$ and $\delta^{37}Cl$ values of α-HCH in the muck were −27.2‰ ± 0.1‰ and −1.9‰ ± 0.2‰ and of β-HCH −26.3‰ ± 0.2‰ and −2.8‰ ± 0.2‰, respectively. Slightly higher or similar $\delta^{13}C$ values of α-HCH were observed in the soil samples compared to the HCH muck (Table S2). This observation is consistent with a scenario in which the isotope fractionation was masked by a rate-limiting, isotope insensitive substrate dissolution prior to the isotope sensitive step of bond cleavage, which underestimated the real extent of transformation of α-HCH in the soil. The adsorbed HCH by soil organic matter is not bioavailable and the dissolution of adsorbed HCH to be bioavailable by soil microorganisms is the kinetically rate limiting step, causing the limited bioavailability of HCH, which masked the isotope fractionation of residual HCH. The similar results were observed from previous study about the sulfate-reducing culture $NaphS_6$ with solid naphthalene immobilized as a thin film on the walls of the cultivation bottles, showing a significant lower hydrogen fractionation caused by limited bioavailability of the naphthalene [20]. In a recent study with slow anaerobic biodegradation of 2-methylnaphthalene, suggesting isotope fractionation was masked by slow mass transfer in the presence of a hexadecane carrier phase [21]. Another likely explanation is that the residual isotope pattern of HCH in the soil was dwarfed by the huge pool of unreacted HCH with constant isotope values which is unlikely to chance.

Similar observation was shown in a field study where the benzene phase govern the isotope composition in the groundwater near the source area [22].

The $\delta^{37}Cl$ values of α-HCH in the soil were slightly higher or nearly identical as the HCH muck, which is similar to the results of the $\delta^{13}C$ values (Table S2), indicating a masking effect caused by low bioavailability of adsorbed HCH or a dwarfing effect by unreacted HCH pool (as discussed above), which underestimated the real extent of HCH transformation in soil. Similar patterns were observed for β-HCH, indicating that isotope fractionation in heterogenic systems underestimated the real extent of biotransformation (Table S3).

Concentration of HCHs in Different Plant Tissues of Trees. In the plant tissues mostly α- and β-HCH were detected indicating the uptake of HCHs by plants from soil. Additionally, the HCH concentration pattern in the plant tissues was similar to those observed in the muck and the host soil. The concentration of α-HCH in plant tissues ranged from 0.02 mg kg^{-1} to 21.43 mg kg^{-1} (Fig.1). This variability shows that HCH concentrations in plants are governed by dynamic processes. Generally, the uptake and accumulation of HCHs by plants is hypothesized to proceed via two ways: a) soil-to-plant translocation by root uptake and b) air-to-plant translocation mainly by leaf uptake. Those translocation processes were identified in former studies [23–25]. Compared to the root uptake, the leaf uptake could be ignored in the presented study since it only accounts for a very minor portion as demonstrated previously [15]. Concerning the root uptake of HCH, it is well known that it is mostly driven by the water flow which is governed by evapotranspiration since HCH is a hydrophobic substance and thus is not actively taken up by plants [26]. However, several processes may influence the uptake with water and the accumulation of HCH in the

plants. For instance, the environmental conditions in different seasons (e.g. temperature, light) will influence the water consumption of plants and therefore also the extent of evapotranspiration, leading to the changes of HCH uptake and accumulation in plants. Phytovolatilization of HCH could influence the dynamic changes of concentration of HCH in plants. However, the loss of HCH by phytovolatilization is comparatively minor compared to the uptake which was shown in a former study [27]. Therefore, in current study we think the phytovolatilization only lead minor changes in concentration. In all investigated trees the relatively high α-HCH concentrations were found in the first two sampling campaigns which were conducted in Apr 2019 and Aug 2019. Thus, the observed high α-HCH concentrations could be related to the intensive uptake accompanied with growth of the trees and the huge consumption of water during this period. After these two sampling campaigns the α-HCH concentration significantly decreased from Oct 2019 to May 20 and afterwards slightly increased, indicating that the uptake during this period was less and maybe accompanied with a higher α-HCH transformation in plant or soil (Fig.1). The concentration of α-HCH from Nov 2019 to Oct 2021 is one magnitude lower than the observed concentration in Apr, Aug and Oct 2019. The reason maybe that 2018 and 2019 were dry years compared to the year 2020 and 2021 based on the rainfall data (Figure S3) and lead to the hypothesis that the plants may develop roots penetrating into soil compartments with higher load of HCH in the search for water, leading to higher uptake of HCH. In dry years the uptake of water may take place from soil compartment with lower permeability and higher concentration of HCH, which may lead to higher HCH uptake by the trees. In contrast in wet years the uptake of water may take place from major pores, which are already depleted in HCH due to major flow of water in the rhizosphere.

Thus, in wet years the uptake of water comes from all over the soil and the HCH load is in consequence lower. For example, in *P. cerasifera* the concentration of α-HCH in young stems was 13.16 mg kg^{-1} (Apr 2019) and 13.28 mg kg^{-1} (Aug 2019), respectively. Afterwards, the α-HCH concentration significantly decreased to 3.5 mg kg^{-1} (Oct 2020) and 0.03 mg kg^{-1}, (May 2020), respectively, afterwards it slightly increased to 0.22 mg kg^{-1} (Jul 2020) and 0.23 mg kg^{-1} (Oct 2021). A similar pattern was observed in the other trees, indicating that the uptake of HCHs was not related to the tree species.

As shown in Fig.S3, the mean monthly temperature reached 20.6 ℃ in Aug 2019 and then it sharply decreased to 11.5 ℃ in Oct 2019 and 6.1 ℃ in Nov 2019. Afterwards the temperature increased again and reached 18.7 ℃ in Jul 2020. In the next year the temperature decreased to 6.7 ℃ in April 2021 and then increased to 10.2 ℃ in Oct 2021. The changes of the temperature were to some extent consistent with the changes of the α-HCH concentration. As discussed above, the uptake of HCH from soil by plants is driven by the evapotranspiration which is further related to the plant water availability in soil. We use the precipitation pattern as indicator for the plant water availability to study the uptake. We do not find a clear trend between the rainfall and α-HCH concentration, indicating that the uptake process alone cannot explain the variability of concentration. This supports our hypothesis that the concentration and uptake in plants is not directly related to short term water cycles. It is also evident, that the transformation in plants need to be considered to explain concentration and isotope pattern. HCH is in equilibrium with the residual water in the soil matrix in soil pores. In warm periods plants need the higher amount of water and will take HCH from residual water from soil matrix as the uptake of this water fraction is critical for the survival of plants.

Concerning β-HCH, the concentration pattern in plant tissues was similar as for α-HCH (Fig.S4). However, lower concentrations of β-HCH were found in the plant tissues compared to α-HCH which is most likely caused due to the higher hydrophobicity of β-HCH with a higher K_{ow} value [28].

Notably, compared to other plant tissues low HCH concentrations were observed in fruits and seeds suggesting that the accumulation of HCH also takes place in the edible parts of plants to a minor extent (Fig.1). This may be due to the translocation of HCH from the phloem with the sap flow which is driven by the photosynthesis [29]. The sap contains precursors for synthesis of fruits or seeds such as amino acids, carbohydrate and other building blocks. They are mostly transported through the phloem which is a different compartment from xylem and not directly connected to the water flow from the root to the leaf govern by evapotranspiration.

Carbon and Chlorine Isotopic Composition of HCHs in Different Plant Tissues of Trees. Due to HCH concentrations which do not meet the detection limit of our GC-IRMS method, the isotopic compositions of α-HCH could not be analyzed in some plant tissues (Fig.2). Remarkably, the $\delta^{13}C$ values of α-HCH of different plant tissues were usually higher than those in the soil in the same sampling campaign (Fig.2), revealing a further transformation of α-HCH in the plant after the uptake from the host which was also observed previously [14,15]. However, the large variabilities of the $\delta^{13}C$ values of α-HCH were observed in the plant tissues ranging from −26.5‰ ± 0.6‰ to −5.8‰ ± 2.6‰. These variations of the $\delta^{13}C$ values suggested that the extent of the plant-mediated α-HCH transformation varies during different growth periods. In all the tree samples, relatively stable $\delta^{13}C$ values were observed during the first two sampling campaigns (Apr. 2019 and

Aug. 2019) ranging from −23.4‰ ± 0.4‰ to −19.8‰ ± 1.0‰ except for the stem samples of *C. monogyna* in Apr.2019. This indicates that the transformation of α-HCH was comparably minor during this period which may be related to an intensive uptake of α-HCH. Consequently, the increased uptake leads to high α-HCH concentrations in the plant tissues which possibly results in a dilution of the isotopic composition of the non-degraded α-HCH fractions by fresh material from soil. Interestingly, an increase of the $\delta^{13}C$ values in most plant tissues was noted in Oct 2019 and Jul 2020 in *R. pseudoacacia*, *P. cerasifera* and *R. cania* (Fig.2), which indicates an increased transformation of α-HCH during this period. This change of the $\delta^{13}C$ values was accompanied by relatively low α-HCH concentrations in the different plant tissues. Hence, the uptake of α-HCH from soil was reduced during this period and thus a possibly occurring dilution of the isotopic composition by residual α-HCH fractions remaining from degradation was reduced resulting in higher observable changes of the $\delta^{13}C$ values in plants. In the next year, a decrease of the $\delta^{13}C$ values was observed in Apr 2021 and the $\delta^{13}C$ values increased in Oct 2021, which is similar as the observation above.

In contrast to *R. pseudoacacia*, *P. cerasifera* and *R. cania*, the $\delta^{13}C$ values of α-HCH in *C. monogyna* showed a different pattern. Here, the highest $\delta^{13}C$ values were observed in Aug 2019 representing a high α-HCH transformation and decreased afterwards from Oct 2019. The results revealed that the transformation of α-HCH is most likely related to the tree species which showed specific growth conditions causing different activity of plant enzymes or endophytes. The dynamic concentration of HCH in trees shown above may be therefore also related to the changing endophytic community in trees in different seasons causing different extent of transformation. Additionally, the general order of the $\delta^{13}C$

values in plant tissues was stem < leaf < fruit or seed (Fig.2). The results suggested that the different extent of transformation occurred in the different tissues.

In Apr. 2019, Aug. 2019, and Apr. 21, the $\delta^{37}Cl$ values of α-HCH of different plant tissues were all higher than those in the soil, indicating the C-Cl bond cleavage of HCH in plant tissues during this period. However, the changes of $\delta^{37}Cl$ values in plant tissues became smaller or even identical to the HCH muck in other sampling points starting from Oct. 2019, indicating that there may be no chlorine involved in the bond cleavage in the rate limiting step or the smaller chlorine fractionation caused by specific transformation mechanism. Therefore, we hypothesized that different transformation mechanisms of HCH may occur during the different growth conditions in different seasons. In comparison to the $\delta^{13}C$ values of α-HCH a different pattern was observed for the of $\delta^{37}Cl$ values in the plant tissues of *R. pseudoacacia*, *P. cerasifera* and *R. cania* showing a decreasing trend of the $\delta^{37}Cl$ values from Apr to Oct. Only in case of *C. monogyna* minor changes of the $\delta^{37}Cl$ values of α-HCH was observed (Fig.3). Thus, the transformation mechanisms of HCHs may further be related to the tree species. Notably, in Oct 2019 and Oct 2021 both $\delta^{13}C$ and $\delta^{37}Cl$ values obviously changed and in this period the plant samples showed a high carbon isotope fractionation and no or small chlorine isotope fractionation, indicating a different dominant transformation pathway took place in this growth period. The different extents of carbon and chlorine isotope fractionation of HCHs were observed in different transformation pathways, such as aerobic and anaerobic biotransformation and abiotic transformation, which was shown in soil and sediments [30]. Therefore, the community of degrading bacteria or enzymes may change caused by the changing environmental

conditions, leading to a different transformation extent and mechanisms. However, it is unlikely that anaerobic biotransformation takes place in the trees.

The changes of the $\delta^{13}C$ and $\delta^{37}Cl$ values of α-HCH were also noted in a previous study employing a soil-wheat pot system in lab [15]. However, the observed changes of the $\delta^{13}C$ and $\delta^{37}Cl$ patterns in this experiment were similar for all tested soil and plant samples during the whole growth stages of the wheat which is different to the results obtained in our field study. Therefore, the results indicate that the changes of the $\delta^{13}C$ and $\delta^{37}Cl$ values in the field were not only related to a different extent of transformation but may also be related to different transformation mechanisms.

Usually, trees shed their leaves mainly in autumn and those leaves will become litter decomposing at the soil surface. HCHs in litter are expected to be degraded during composting / humification processes [31] before a small fraction of non-degraded HCH may be integrated in the soil organic matter of the top soil. Potentially released HCHs close a cycle and may be available for transformation in the soil, uptake by plants and translocation in the plants, potentially accompanied with further transformation. Therefore, the annual cycle of plants will form a cycle of natural attenuation with respect to degradation in plants, leaves shedding and litter formation followed by humification. The observation of the changes of HCHs isotope values in trees during different growing time gives a deeper view on the contribution of plants to natural attenuation and of our best knowledge this has not been studied in detail so far. The changes of HCHs transformation mechanisms in autumn in Oct.2019 shown above illustrated the contribution of plants by litter fall in this overall natural attenuation process. High transformation of HCHs leads to attenuated concentration in leaves in autumn as observed in three tree species by means of isotope fractionation,

however still indicating some input of HCHs by litters into soil. The overall process can be considered as an overlooked natural attenuation process of soil-plant interaction.

Due to the low concentration of β-HCH in plant samples in Nov 2019, May 2020, Jul 2020, Apr 2021 and Oct 2021, the isotope values of the plant samples could not be detected in these sampling campaigns. Notably, the $\delta^{13}C$ values of β-HCH of different plant tissues were larger than those in the soil in the same sampling campaign (Fig.S5), revealing a further transformation of β -HCH in the plant after the uptake from the soil which is most likely caused by plant endophytes inoculated by soil bacteria. This was shown in a previous lab pot experiment [17]. The $\delta^{37}Cl$ values of β-HCH in plants was smaller compared to the muck and soil and some plant samples were identical to the muck which is also shown for α-HCH.

Metabolism of HCHs in Soil and Trees. Possible metabolites of HCH transformation were analyzed by GC-MS. The most abundant HCH isomers in the soil are α- and β-HCH which is related to the production and purification of Lindane at the investigated field site Bitterfeld-Wolfen. Several metabolites such as pentachlorocyclohexene (PCH), trichlorobenzene (TCB), dichlorobenzene, and chlorobenzene were found in the soil samples (Fig.S5). Three isomers of PCH were identified which could originate from dehydrochlorination reactions of HCH. In addition, three isomers of TCB were noted which could be formed by further dehydrochlorination reactions of PCE. PCH and TCB are typical metabolites of the aerobic HCH degradation pathways [32]. Additionally, two isomers of dichlorobenzene as well as chlorobenzene were detected which could be intermediates and the final product of the anaerobic HCH degradation pathway,

respectively [32]. Therefore, the detected metabolites in the soil are reflecting both the aerobic and anaerobic HCH degradation pathways.

Similar metabolites could be detected in plants (Fig.S6), suggesting that the degrading bacteria may be similar and use similar transformation pathways in both compartments. However, it is difficult to draw a conclusion whether the transformation takes place within the plants since all metabolites have higher water solubility in comparison to HCH and thus could also be taken up with water from soil. Therefore, only the appearance of transformation products in the plants does not prove that the transformation occurred within the plants but it may give an indication that in a soil-plant system transformation takes place. However, CSIA provides a strong indication of a transformation in these complex systems, as the isotopic enrichment from the root to the leave demonstrates that a transformation indeed takes place in the plants. Therefore, the metabolites found in the plants may be a result of uptake from soil and /or transformation in the plant, which is in-line and not in conflict with our hypothesis that metabolization of HCH takes place in trees.

Enantiomer Fractionation of α-HCH in Different Plant Tissues of Trees. Enantiomer fractionation could occur due to the biotransformation of chiral compounds [33] and EF values could be used as an indicator for characterizing the enantiomer fractionation. Due to low α-HCH concentration from Nov 2019 to Jul 2020, the enantiomer fractionation could only be investigated in the first three sampling campaigns (i.e. Apr 2019, Aug 2019, and Oct 2019) (Fig.4). The EF (−) values of the host soil samples were almost identical with the EF (−) value of the HCH muck (Fig.4), indicating that no preferential biotransformation of α-HCH enantiomer appears in the soil. However, only in case of the host soil sample of *R. cania* a slight preferential transformation of (+) α-HCH was noted

which is indicated by an EF (-) value of 0.53 ± 0.00. In the Apr 2019 and Aug 2019, the values of EF (−) values in the tested tree tissues ranged from 0.50 ± 0.00 to 0.55 ± 0.01 (Fig.4), suggesting no or only a slight preferential transformation of (+) α-HCH in all plant tissues. However, the EF (−) values strongly changed in Oct 2019. During this sampling campaign, the EF (−) values increased to 0.61 ± 0.01 and 0.62 ± 0.04 in *R. pseudoacacia* and *P. cerasifera* indicating a high preferential transformation of (+) α-HCH. In contrast, in *C. monogyna* and *R. cania* the EF (−) values decreased to 0.38 ± 0.02 and 0.34 ± 0.03 at the same time indicating a high preferential transformation of (−) α-HCH. Consequently, this finding further confirms that the transformation of HCH is affected by changing biological reactions and it is influenced by the changes of the seasons. Additionally, the results indicate that the transformation of HCHs is related to the tree species. Compared to the host soil, the higher or lower values of EF (−) may give an indication for the intensity of α-HCH biotransformation after the uptake by the tree.

Additionally, enantiomer fractionation of chiral compounds can theoretically also be caused by plant uptake from the soil or gas phase and the translocation of the compound in plant [34]. However, this was not specifically studied for α-HCH. Even unlikely as discussed before, we observed enantiomer fractionation which may be caused to some extent also by the plant internal-translocation [15]. However, chiral preferences are unlikely to change upon translocation and preferential uptake over a season. Therefore, the shift of EF values with changing enantiomer fractionation from (+) to (−) observed in the different sampling campaign in trees should be related to plant-internal enantiomer specific transformation and not by translocation.

Combined $\delta^{13}C$ and $\delta^{37}Cl$ Analysis for Characterizing α-HCH Transformation. In general, reaction mechanisms can be differentiated by dual isotope plots that have different slopes indicating different reaction mechanisms in a complex system [35]. However, in this study there was no clear linear relationship between the $\delta^{13}C$ and $\delta^{37}Cl$ values (Fig.5), suggesting that not only one transformation mechanism is involved in the transformation of HCH in the field.

Most of the samples in Apr 2019, Aug 2019, and Apr.2021 in *R. pseudoacacia*, *P. cerasifera* and *R. cania* are close or fit to the Λ values (solid line in Fig.5) observed for α-HCH transformation in garden soil-wheat pot and hydroponic systems which showed a consistent observation from laboratory to field study. Therefore, the transformation process in Apr.19 and Aug.19 can be characterized by the reference data of soil-pot and hydroponic experiments in lab, clearly showing a C-Cl bond cleavage during the transformation.

However, the samples in other sampling points campaigns (i.e. Oct 2019, Nov 2019, May 2020, Jul 2020 and Oct. 2021) in *R. pseudoacacia*, *P. cerasifera* and *R. cania* were far away from all Λ values (dash and solid lines in Fig. 5) with a high carbon isotope fractionation and low or no chlorine isotope fractionation, suggesting that the transformation process during these periods cannot be characterized by any available reference experiment today. Probably a different mode of transformation may be involved, which can cause high carbon isotope fractionation with a no or low chlorine fractionation. A possible explanation could be that the soil and trees at the contaminated field site are already adapted to the HCH contamination for several years and accommodate a specific microbiome. An inoculation of soil bacteria to become plant endophytes which facilitate the β-HCH transformation was observed in a recent pot experiment with wheat using the

soil from the same contaminated field [17] and thus the soil bacteria may also inoculate the trees and form competent endophytes for transformation α-HCH causing different transformation pathways to those observed in the previous hydroponic and garden soil-wheat pot experiments [14,15]. Furthermore, the wheat was cultivated under controlled conditions (e.g. water content, temperature) in the laboratory and the enrichment and diversity of HCH degrading microorganisms in the HCH-historically contaminated soil of the present study was most likely also significantly different from the microbiota of garden soil used for the garden soil-wheat pot experiments. No or smaller chlorine and high carbon fractionation could be interpreted as preferential cleavage of a C-H bond in the rate limiting step. Modelling studies of dehydrochlorination of γ-HCH which were supported by experiments have shown that chlorine fractionation may be low and associated with Λ values between 3.5 and 5.2 because the initial rate limiting step is a hydrogen carbon bond cleavage leading to high hydrogen isotope fractionation [36]. Therefore, we hypothesized that different mechanisms including an undiscovered transformation process with a high carbon isotope fractionation and no or low chlorine isotope fractionation are involved during degradation in trees. The results are also consistent with the changes of the isotopic composition and the enantiomer fractionation.

A different regulation was observed in *C. monogya*. Some of *C. monogya* samples in Oct 2019, Nov 2019, May 2020, Jul 2020 and Oct 2021 are also close or fit to the Λ values (solid line in Fig.5) observed for α-HCH transformation in garden soil-wheat and hydroponic experiments (Fig. 5). The results indicated the transformation extent and the mechanisms are also related to the tree species.

462 **Estimated Potential Transformation of HCH in Plants.** The enrichment factors (ε_C) for
463 aerobic and anerobic bacteria transformation which are available can be used to roughly
464 calculate degradation of the residual fraction to obtain an estimated degradation extent. ε_C
465 of -1.0 ± 0.2 ‰ [37] and -3.7 ± 0.8 ‰ [38] represent aerobic and anaerobic biodegradation of
466 α-HCH, respectively. However, the anaerobic condition is unlike in plants and therefore
467 we apply the ε_C in aerobic conditions for calculation. A $\delta^{13}C$ enrichment of 1.0‰ to 21.4‰
468 for α-HCH between the plants and HCH muck would indicate a transformation of 57.4 –
469 99.9% using factors typical for aerobic conditions. The field conditions are much complex
470 than the reference study and as confirmed in this study different mechanisms including
471 undiscovered mechanisms took place during the annual cycle of trees. Therefore, the above
472 estimation of HCH degradation gave only a rough overview and should be developed in
473 the future by a more appropriate ε_C.

474 **Environmental Implication.** The transformation of HCHs in soil and plants was studied
475 in detail in the lab experiments [14,15]. The understanding of HCHs transformation in the field
476 is much more complex compared to lab experiments and could reveal real and natural
477 changes of HCHs in the environment. Dual element isotope CSIA and enantiomer
478 fractionation analysis allows characterizing and resolving the degradation process in field
479 studies.

480 Previous studies observed changes of carbon and chlorine isotopic compositions in soil-
481 pot experiments which indicated the predominance of only one transformation pathway [15].
482 However, we found that the changes of isotopic compositions in the field are related to
483 complex interaction of different transformation mechanisms which are based on the growth

stages in different seasons affecting specific processes of the overall natural attenuation and phytoremediation.

The combined evidence of isotope and enantiomer fractionation gives new insight into the assessment of the transformation of HCH within plants, where until now an assessment has been difficult. The seasonal changes of HCH transformation in trees have implications for phytoscreening since transformation in plants as well as a feedback loop via littering needs to be considered. Particularly the changes of transformation mechanisms and extent in the different season should be considered for the application of phytoremediation in the field.

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Author Contributions

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.

Supporting Information

Details on extraction and cleanup methods, sampling campaigns, concentration and isotopic composition of HCH in soil and plant samples, hotpot of HCH much in the field, temperature and rainfall, and metabolites analysis.

ACKNOWLEDGEMENTS

Xiao Liu (File No. 201703250070) is financially supported by the China Scholarship Council. We are thankful for the use of the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at UFZ Leipzig, which is supported by European Regional Development Funds (EFRE-Europe funds Saxony) and the Helmholtz Association. We are thankful to Matthias Gehre for support in our Stable Isotope Laboratory. The Landesanstalt für Altlastenfreistellung, Sachsen Anhalt, Federal State of Germany and the Environmental Agency of Bitterfeld-Wolfen are acknowledged for providing information about the region.

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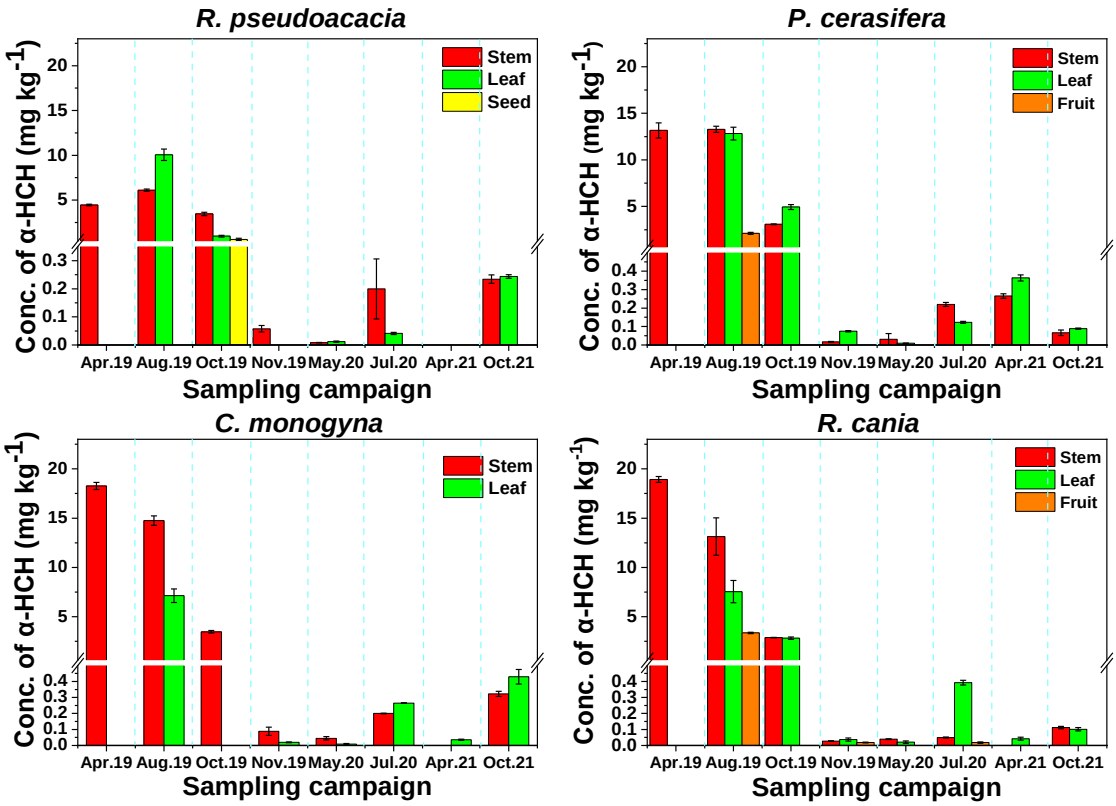
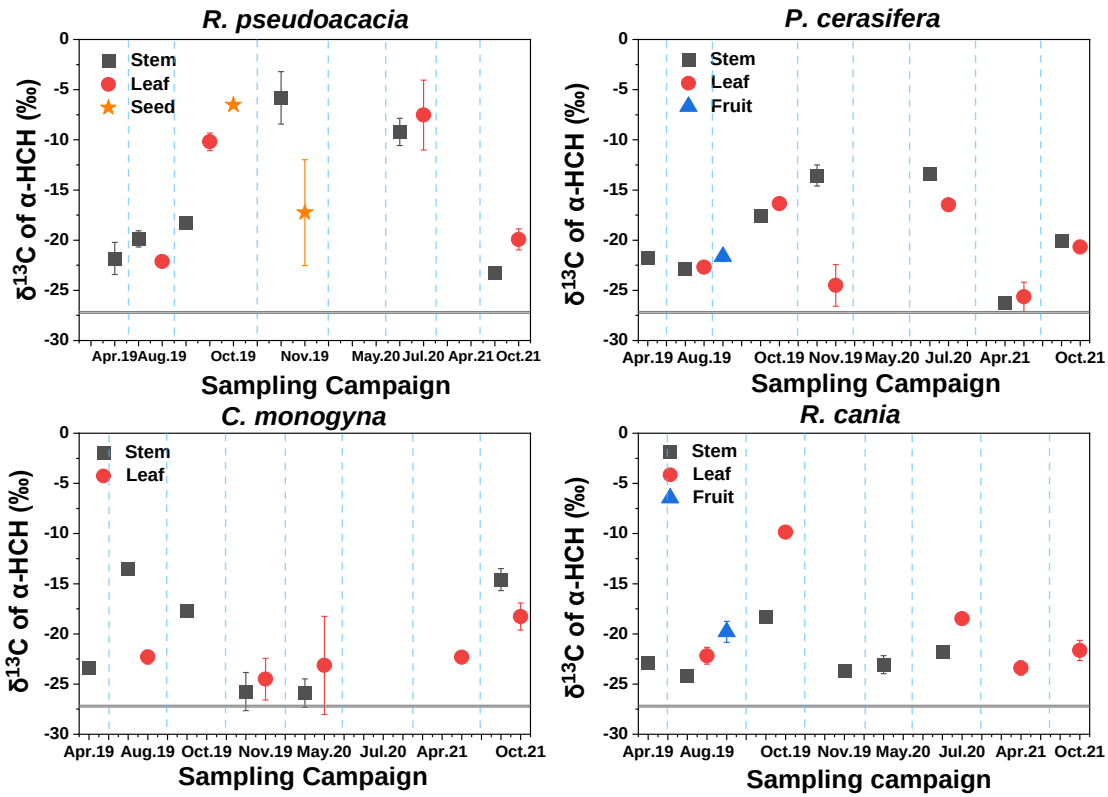


Figure 1. Concentration of α-HCH in different plant tissues of trees at different sampling campaigns.



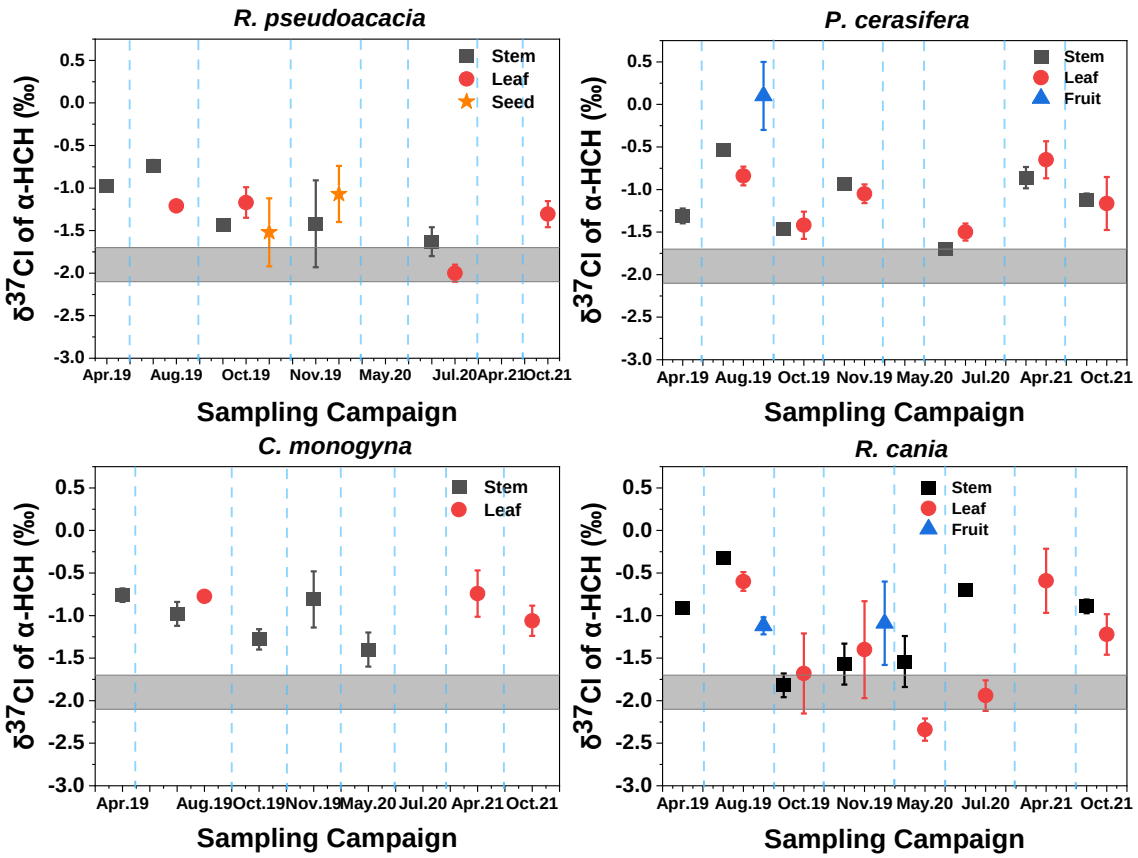
670

671 Figure 2. Carbon isotopic composition of α-HCH in different tree tissues of different

672 sampling campaigns. The horizontal grey bar represents the $\delta^{13}C$ value of α-HCH in the

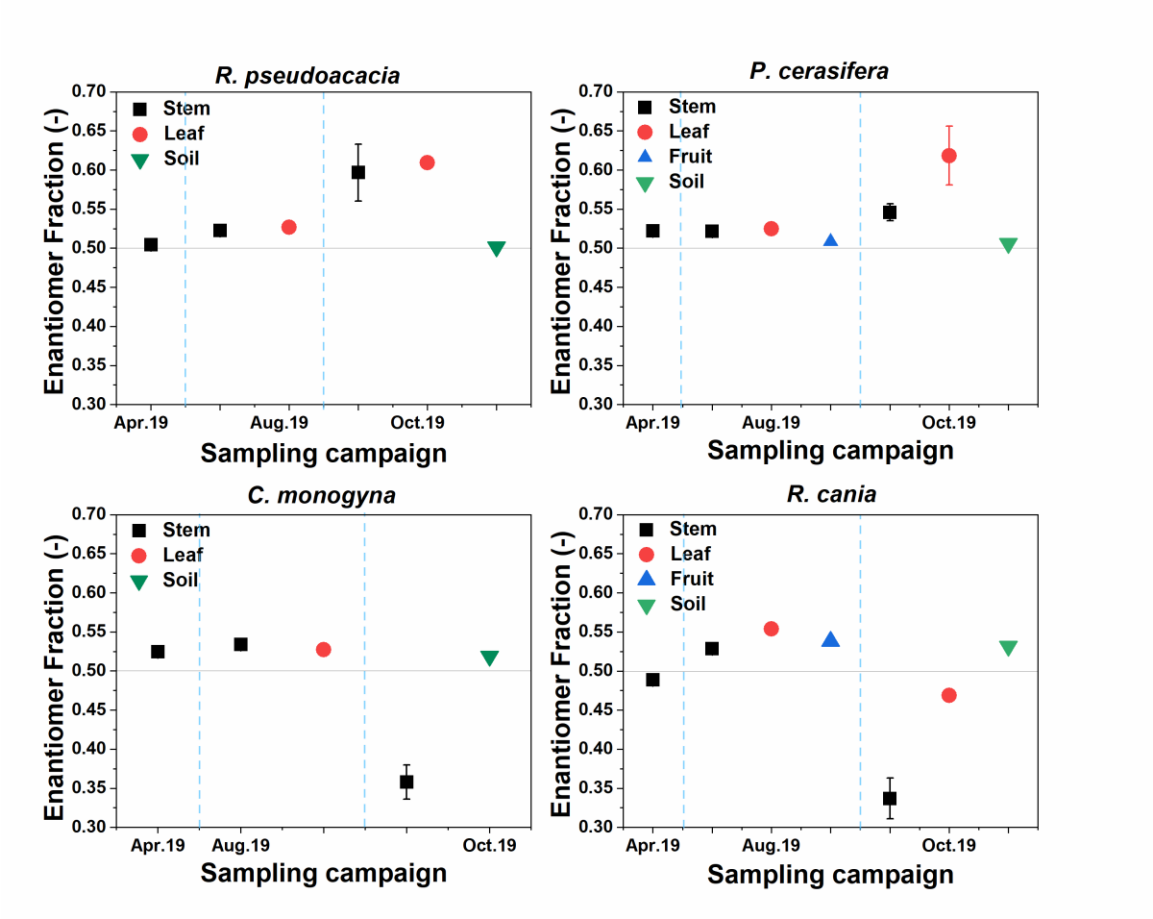
673 muck at the investigated field site.

674



675

676 Figure 3. Chlorine isotopic composition of α-HCH in different tree tissues of different
677 sampling campaigns. The horizontal grey bar represents the $\delta^{37}Cl$ value of α-HCH in the
678 muck at the investigated field site.



679

680 Figure 4. Enantiomer fraction (-) of α-HCH in different tree tissues of different sampling
681 campaigns. The horizontal grey bar represents the racemic distribution of the α-HCH
682 stereoisomers of the muck.

683

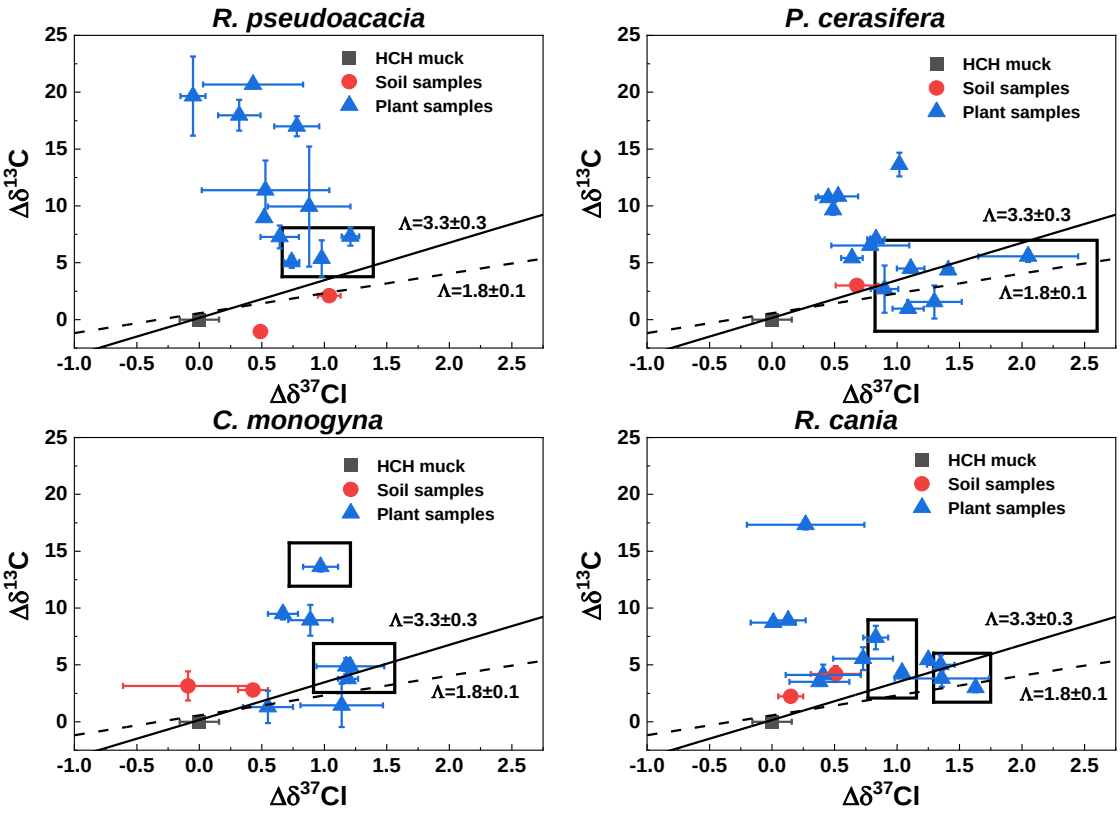


Figure 5. Dual element analysis (C-Cl) of α-HCH in soil and plant samples of different tree species. Δ indicates the differences in isotope values (δ) between the HCH muck (δ_0) and the isotope composition of HCH at each sample (δs), Δ = δs − δ0 in the experiments. The dashed line represents the mode of C-Cl bond cleavage in a hydroponic experiment with wheat additionally spiked with α-HCH [14]. The solid line represents the mode of C-Cl bond cleavage in a pot experiment with wheat and garden soil spiked with α-HCH [15]. Black square represents samples collected in Apr 2019 and Aug 2019.