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Compound specific isotope analysis of hexachlorocyclohexane isomers: a method for source fingerprinting and field investigation of *in situ* biodegradation

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RATIONALE: The manufacturing and uses of hexachlorocyclohexane (HCH) have resulted in a serious environmental challenge and legacy. This study highlights the ability of compound specific isotope analysis (CSIA) to distinguish among various HCH sources and to support the evaluation of the potential for *in situ* biodegradation in contaminated groundwater.
METHODS: Tests were conducted to verify the absence of significant isotope fractionation during HCH sample pre-concentration including dichloromethane extraction, solvent exchange into iso-octane, and H_2SO_4 clean-up, and analysis by gas chromatography/combustion-isotope ratio mass spectrometry (GC/C-IRMS). The method was then applied to four Technical Grade (TG) HCH mixtures procured from different sources and to groundwater samples from a contaminated site.
RESULTS: The pre-concentration method enabled determination of carbon isotope ratios ($\delta^{13}C$ values) of HCH isomers with no significant isotopic fractionation. The TG-HCH mixtures had significantly different $\delta^{13}C$ values. Moreover, for any given TG-HCH, all isomers had $\delta^{13}C$ values within 1.1‰ of each other – a distinctly uniform fingerprint. At the HCH-contaminated field site, compared with source wells, downgradient wells showed significant (up to 5.1‰) enrichment in ^{13}C and the $\delta^{13}C$ values of the HCH isomers were significantly different from each other.
CONCLUSIONS: A method was successfully developed for the CSIA of HCH isomers that showed potential for HCH source differentiation and identification of HCH *in situ* biodegradation. At the HCH-contaminated site, the observed preferential isotopic enrichment of certain isomers relative to others for a given source allows differentiation between biodegraded and non-biodegraded HCH. Copyright © 2015 John Wiley & Sons, Ltd.

Technical grade hexachlorocyclohexane (TG-HCH) is a broad-spectrum pesticide widely used in the 1970s and 1980s.[1,2] TG-HCH was produced by photochlorination of benzene yielding four main isomers. The most abundant isomer is α-HCH (60–70%), followed by γ-HCH (10–12%), β-HCH (5–12%), and δ-HCH (6–10%).[3] Historically, TG-HCH was widely used for pest control, although only γ-HCH has actual pesticide properties.[1] Hence, more recent applications used γ-HCH only, commercially known as lindane (γ-HCH >99%). In order for the γ-HCH isomer to be isolated from other HCH isomers during the lindane manufacturing process, large quantities of HCH wastes were generated. Since the 1980s, use of TG-HCH has been banned in most countries, and the application of lindane is restricted in most developed countries.[1] The global legacy of past spills and disposal remains an important environmental challenge, however, and led to the inclusion of HCH isomers in the Stockholm convention for persistent organic pollutants in 2009.[4]

At high exposures, HCH isomers can cause severe damage to the kidney, liver, and hormonal system, and, as such, they are classified in the US EPA lists as priority and toxic pollutants.[5,6] HCH isomers are slightly soluble in water, volatile, and have moderate octanol-water and air-water partitioning coefficients.[1,3] Despite similar properties, the reactivity of HCH isomers depends on the disposition of the HCH chlorine atoms. For instance, γ-HCH has three axial chlorine atoms that are more susceptible to enzymatic attack than the six equatorial chlorine atoms from β-HCH, thus explaining why β-HCH is typically more persistent than γ-HCH.[7] These physical

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properties, in conjunction with wide application and a history of problematic disposal methods, have led to the detection of HCH isomers in water, soil and air samples worldwide.[1–3,8–12] Therefore, there is much interest in the development of new analytical techniques to provide more information concerning the sources and fate of HCH isomers in the environment.[4]

α-HCH is the only HCH isomer that is chiral. During biodegradation, one enantiomer is usually preferentially degraded relative to the other. The enantiomeric fraction (EF) is defined as [(+)-α-HCH]/([(+)-α-HCH] + [(–)-α-HCH]) where [α-HCH] is the concentration of the (+)- or (–)-α-HCH enantiomer. In TG-HCH, α-HCH is racemic, with an EF = 0.5. Which enantiomer is preferentially degraded depends on the conditions and the microbial species present.[13–15] For water samples, Law *et al.*[14] showed that (–)α-HCH was degraded preferentially to the (+)α-HCH enantiomer at an anaerobic field site, and the EF value was >0.5. This was in contrast to other anaerobic systems which showed enantioselective degradation with EF <0.5, suggesting that the (+)α-HCH was degraded preferentially to the (–)α-HCH enantiomer.[7] While a non-racemic EF value is used as a line of evidence for biodegradation of α-HCH, the details governing α-HCH biodegradation are not well constrained. In addition, in the case where both enantiomers are simultaneously degraded at similar rates, a constant EF value could suggest a false negative regarding *in situ* biodegradation. Hence EF values as stand-alone evidence for biodegradation are insufficient and there is significant interest in developing additional lines of independent evidence to investigate potential for biodegradation.

Compound specific isotope analysis (CSIA) has been used to differentiate between contaminant sources[16–20] through comparison of stable carbon isotope ratios ($\delta^{13}C$ values) among many priority pollutants. However, only three studies have reported the $\delta^{13}C$ values of HCH sources where the $\delta^{13}C$ value is the relative difference of the measured ratio of ^{13}C to ^{12}C in a sample normalized to an international standard (V-PDB).[21] Badea *et al.*[22] reported $\delta^{13}C$ values for three α-HCH Dense Non-Aqueous Phase Liquid (DNAPL) samples from field sites, which ranged from –27.4 to –25.8‰[22]; whereas Drenzek *et al.*[23] measured a $\delta^{13}C$ value of –26.3‰ for a pure γ-HCH sample obtained from Supelco (Bellefonte, PA, USA). Vetter *et al.*[24] obtained $\delta^{13}C$ values of –26.7 to –25.9‰ for β-, δ-, and γ-HCH isomers obtained from Riedel-de-Haen (Seelze, Germany) and –31.7‰ for α-HCH from the same supplier; they also measured a value of –25.6 ‰ for samples from another two suppliers of γ-HCH (the Institute of Organic Industrial Chemistry, Warsaw, Poland, and Merck, Darmstadt, Germany).[25] A more thorough investigation of $\delta^{13}C$ signatures for HCH sources and isomers is necessary to determine whether a wider range of manufacturers produces HCHs with substantially different $\delta^{13}C$ values, possibly due to different manufacturing processes and/or chemical feedstocks. This information is required in order to evaluate whether there is potential for HCH source distinction, and to provide the baseline of non-degraded source signatures against which possible biodegradation signatures may be assessed.

Aqueous HCH concentrations at contaminated field sites can be as low as the μg L^{-1} to ng L^{-1} range.[5] For instance, HCH concentrations ranging from 0.416 to 2.391 μg L^{-1} (India),[26] and 0.1 to 730 μg L^{-1} (Germany),[27] have been measured in groundwater. HCH was also detected in nine different brands of bottled drinking water from India[28] at concentrations ranging from 0.40 to 24.10 μg L^{-1}. The lowest HCH aqueous concentrations (150 to 300 μg L^{-1}) obtained for CSIA to date were from laboratory experiments using a solvent-based extraction (n-pentane or dichloromethane) from aqueous phase experimental vials.[22,29] For field samples, however, to date only DNAPL (pure phase organic liquid) HCH samples[22] have been characterized for carbon isotopic signatures. Before substantial progress can be made in using CSIA for isotopic investigation of the source and fate of HCH at realistic field concentrations, an efficient pre-concentration method is needed prior to isotopic analysis of HCH isomers in the trace concentrations typically present in environmental water samples.

In addition to source determination, CSIA has been used as a tool to identify and quantify natural attenuation at contaminated field sites.[30–32] During degradative processes such as biodegradation, isotopic fractionation occurs due to the different reaction rates of each isotopic species, where the heavier isotope (^{13}C) forms a stronger bond than the lighter isotope (^{12}C), resulting in a faster reaction rate for molecules containing exclusively lighter isotopes. Biodegradation of HCH isomers has been documented in laboratory and field studies using a variety of microbial consortia and isolated microorganisms under both aerobic and anaerobic conditions.[1,7,33–35] β-HCH is frequently reported to be the most recalcitrant of the four main HCH isomers,[1,7,34] while the relative biodegradation rates of δ-, α-, and γ-HCH can vary depending on the microbial species and conditions used.[7,33–35]

In laboratory experiments investigating the degradation of organic compounds, the isotopic enrichment factor (ε) is determined by fitting the Rayleigh model (Eqn. (1)) to the $\delta^{13}C$ and concentration (*C*) data at times t and zero (denoted by subscript 0) as described by the US EPA Guide:[21]

$$\frac{\delta^{13}C + 1}{\delta^{13}C_0 + 1} = \left(\frac{C}{C_0}\right)^{\varepsilon} \quad (1)$$

During reductive dechlorination laboratory experiments using pure microbial cultures, the carbon isotope enrichment factors (ε_C) were –3.4 ± 0.5‰ and –3.9 ± 0.6‰ for lindane,[36] and –3.7 ± 0.8‰ for α-HCH.[22] In contrast, carbon isotope fractionation during biodegradation under aerobic conditions was much smaller, with ε_C values of –1.7 ± 0.2‰ to –1.5 ± 0.1‰ (γ-HCH), and –1.6 ± 0.3‰ to –1.0 ± 0.2‰ (α-HCH).[29] For both aerobic and anaerobic biodegradation then, there is clearly potential to use CSIA as an additional line of evidence to identify *in situ* HCH biodegradation.

In studies of the abiotic degradation of HCHs, Zhang *et al.*[37] published ε_C values for α-HCH abiotic reactions in a similar range to those for biodegradation: −1.9 ± 0.2‰ for indirect photolysis with UV/H_2O_2, –2.8 ± 0.2‰ for direct photolysis, –3.8 ± 0.4 ‰ for electrochemical reduction, and somewhat larger values for reaction with Fe^0 nanoparticles (–4.9 ± 0.1 ‰), and for alkaline hydrolysis (–7.6 ± 0.4 ‰). As none of these chemical reactions led to significant enantiomeric fractionation, it is interesting to note that CSIA and enantiomeric analysis have the potential to distinguish between biotic and abiotic reactions. Jammer *et al.* highlighted

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such an approach in a study combining $\delta^{13}C$ and EF value measurements during the hydrolysis of the methyl esters of chiral herbicides mecoprop and dichlorprop by pure enzymes isolated from *Pseudomonas* and *Candida* spp.[38] To date, however, alternative approaches such as CSIA have not been explored to evaluate the *in situ* biodegradation of dissolved HCH isomers in aqueous samples from groundwater monitoring wells at contaminated sites.

The overall objective of this study was to develop a methodology for applying CSIA to investigate the source and fate of HCH at contaminated field sites for the first time. The first goal was to develop a method compatible with CSIA to extract and concentrate HCH isomers present at trace concentrations in aqueous samples and to demonstrate the absence of significant isotopic fractionation or offsets associated with the sample extraction and pre-concentration steps. The second goal was to assess the potential of CSIA as a tool to distinguish among different contaminant sources for HCH. Finally, the last aspect of the study was a pilot test of CSIA for investigating HCH *in situ* biodegradation at a contaminated groundwater field site.

EXPERIMENTAL

Analytical reagents, HCH standards and TG-HCH samples

Dichloromethane (DCM), sodium sulfate, sulfuric acid (H_2SO_4), iso-octane (2,2,4-trimethylpentane) and acetone were all analytical grade reagents obtained from Sigma Aldrich (St. Louis, MO, USA). Three pure HCH isomer standards were provided by Environment Canada (Egbert, ON, Canada): α-HCH, β-HCH and γ-HCH standards (all were then dissolved in acetone at 100 mg L^{-1}). In addition a γ-HCH (lindane) sample[39] was isolated from a formulation which consisted of 15–20% lindane (γ-HCH of approximately 6000 mg L^{-1}), 10–15% diazinon, and 10–15% captan plus inactive ingredients, based on a method developed by Schimmelmann and de Niro,[40] and dissolved in iso-octane before analysis. Finally, four TG-HCH samples dissolved in iso-octane from four different manufacturers were also analyzed: Pesticide 666, BHC-K500, BHC-K503, and BHC-K501. Details of the manufacturers of the TG-HCH samples and relative percentages of the α-, β-, and γ-HCH isomers are provided in Supplementary Table S1 (Supporting Information). Except for Pesticide 666, which was manufactured in China, and a γ-HCH (lindane) sample, all the samples and standards were obtained from the US EPA Pesticide Repository (Research Triangle Park, NC, USA). Finally, two additional pure standards (α-HCH_{SA} and γ-HCH_{SA}) were obtained from Sigma Aldrich (Lot Nos. 19328 and 5227X, respectively, produced by Riedel-de-Haen) and used to compare offline and continuous-flow isotope analysis (see Supporting Information, sections S2.1 and S2.2).

Pre-concentration method

In order to analyze field samples at trace HCH concentrations, a pre-concentration method was evaluated for its compatibility with CSIA. The absence of significant isotopic fractionation was verified after each pre-concentration step performed on α-HCH aqueous solutions (see Supporting Information, sections S2.3 and S2.4). A comparison of offline and continuous-flow isotope analysis was first performed on isotopically characterized in-house isotopic standards – a working standard of α-HCH_{SA} ($\delta^{13}C = -31.9 \pm 0.5$‰) and a working standard of δ-HCH_{SA} ($\delta^{13}C = -26.9 \pm 0.5$‰; Supporting Information, section S2.1 and Supplementary Table S2). Aqueous α-HCH stock solutions of 50 and 500 μg L^{-1} were prepared by diluting 0.75 and 7.5 mL, respectively, of the 100 mg L^{-1} α-HCH pure standard in 1.5 L of deionized water. This α-HCH standard was also isotopically characterized before use (see Supporting Information, section S2.2). The $\delta^{13}C$ value was –27.0 ± 0.5‰ (n = 28, Supporting Information, section S2.2). As the aqueous α-HCH stock solutions used for the pre-concentration steps were prepared using this α-HCH pure standard, this $\delta^{13}C$ value (mean = –27.0 ± 0.5‰) was used for comparison with the $\delta^{13}C$ values measured for each of the pre-concentration steps. The same stock solutions were used for the three pre-concentration steps, DCM extraction, solvent exchange into iso-octane, and H_2SO_4 clean-up after the method of Falconer *et al.*[15] – a method originally developed for concentration analysis and described in detail in the Supporting Information, section S2.3 and Supplementary Fig. S1.

Compound specific carbon isotope analysis

The $\delta^{13}C$ values for all samples were measured by direct liquid (0.5 to 10 μL) injection using a splitless setting on a Varian 3400 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) interfaced with a combustion oven (Thermo Fisher/Finnigan, Bremen, Germany) held at 980 °C in line with a Finnigan MAT 252 isotope ratio mass spectrometer (Thermo Fisher/Finnigan). A DB-1 column (60 m × 0.25 mm i.d.) or a HP-5 column (30 m × 0.32 mm i.d.) coupled with a DB-5MS column (60 m × 0.25 mm i.d.) was used. All the columns were purchased from Agilent Technologies via Chromatographic Specialties Inc. (Brockville, ON, Canada). Details of the temperature programs and isomer peak resolution are provided in Supporting Information, section S2.5.

Unless otherwise noted, all $\delta^{13}C$ values are reported with a total uncertainty of ± 0.5‰ which incorporates both accuracy and reproducibility after the method of Sherwood Lollar *et al.*[41] and the US EPA Guide.[21] The values for the total mass of α-HCH injected onto the gas chromatograph for GC/C-IRMS analysis, the theoretical minimum mass (TMM) required for α-HCH $\delta^{13}C$ analysis, and the theoretical detection limit (TDL) for α-HCH $\delta^{13}C$ analysis were calculated as described in Supporting Information, section S2.7.

Field study

Groundwater samples containing HCH from a contaminated site (an operating packaging and reformulating pesticide facility) located in northeastern Florida, USA[14] (Fig. 1), were analyzed by CSIA using the method described above. Until 1996, unlined trenches were used to dispose of waste from the facility, contaminating the groundwater with TG-HCH. The pH of the groundwater at the site was neutral, except for a small area partially downgradient from the historic disposal facility, where disposal of elevated sulfur quantities

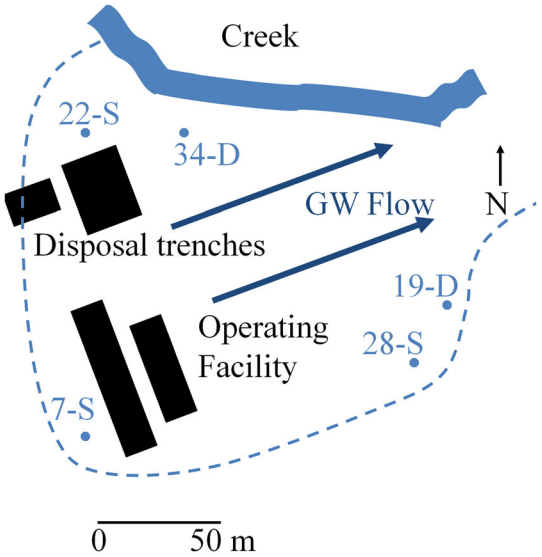

Figure 1. Map of the HCH-contaminated field site at an operating pesticide facility in Florida (modified after Law *et al.*[14]). • denotes the monitoring wells analyzed for $\delta^{13}C$ values of HCH isomers. 'GW' stands for 'groundwater' and arrows denote groundwater flow direction. Based on concentration data from the monitoring wells (not all monitoring wells are shown for simplicity), the estimated HCH plume is denoted by the hatched line.

resulted in local acidic conditions. The water table of the contaminated aquifer was located 12–18 m below ground surface, and both shallow (denoted with an S; screened across the water table) and deep (denoted with a D; screened 11 to 18 m below ground surface) monitoring wells were installed (see Law *et al.*[14]).

Dissolved gases and redox potential measurements indicated that the aquifer conditions were reducing.[14] Over time, α-HCH concentrations had decreased, and the EF values downgradient of the disposal area were >0.5, suggesting preferential natural attenuation of the (–)-α-HCH enantiomer relative to (+)-α-HCH.[14] The β-HCH isomer frequently had the lowest concentration of all the isomers at this site.[14] This is in agreement with other HCH-contaminated sites where no detectable amounts of β-HCH were found in groundwater, despite the presence of significant concentrations of α-, γ-, and δ-HCH.[26] β-HCH is frequently reported to be the most recalcitrant isomer[1,7,34]; the low groundwater concentrations of β-HCH are therefore probably due to it having a stronger tendency to partition to soil than the other HCH isomers.[1,7,26,34] Given the tenuous nature of such evidence, as noted in the Introduction, there is a compelling need to investigate the potential for more definitive signatures of biodegradation, such as CSIA.

Isotopic analysis was performed on groundwater from wells 22-S and 34-D in the low (<4) pH portion of the plume downgradient from the historical disposal facility; from well 7-S in the neutral aquifer upgradient from the operating pesticide facility; and from wells 28-S and 19-D in the neutral portion of the plume downgradient from the operating pesticide facility (Fig. 1). These wells were chosen because they had relatively high HCH isomer concentrations (up to 420 μg L^{-1}) and a range of EF values (0.503 to 0.613). Samples were pre-concentrated using the method described in this study (see Supporting Information, sections S2.3, S2.4, and S2.6). The pre-concentrated samples in iso-octane were stored in the freezer until carbon isotope analysis.

RESULTS AND DISCUSSION

Stable isotope technique for dissolved HCH at field-relevant concentrations

Offline vs continuous-flow isotope analysis

Two analytical standards of α-HCH_{SA} and δ-HCH_{SA} were used as the basis for a comparison of $\delta^{13}C$ values obtained by traditional offline combustion tube preparation (combined with dual-inlet isotope analysis) and by gas chromatography/inline combustion (or 'continuous-flow' isotope analysis or CSIA) (see Supporting Information, section S2.1 for a detailed procedure). The results showed good agreement between the offline $\delta^{13}C$ values (–31.9‰ for α-HCH_{SA} and –26.9‰ for δ-HCH_{SA}) and those determined by continuous-flow GC/C-IRMS (–31.9‰ for α-HCH_{SA} and −26.6‰ for δ-HCH_{SA}) (Supporting Information, section S2.1 and Supplementary Table S2).

Compatibility of the pre-concentration method with CSIA

A series of protocol tests was conducted to determine if the pre-concentration steps resulted in isotopic fractionation (Supporting Information, sections S2.3 and S2.4). The $\delta^{13}C$ values measured for each of the pre-concentration steps for both the 50 and 500 μg L^{-1} α-HCH aqueous samples were within ±0.5‰ analytical uncertainty of the mean $\delta^{13}C$ value for the α-HCH standard (–27.0 ± 0.5‰) (Supporting Information, section S2.2), demonstrating that DCM extraction, solvent exchange into iso-octane, and H_2SO_4 clean-up, and evaporation to dryness (Supporting Information, section S2.4) steps do not result in significant isotopic fractionation or measurable artifacts. For another class of compounds, the measured $\delta^{13}C$ values for benzene, toluene, ethylbenzene and *o*-, *m*-, and *p*-xylene (BTEX) pure product standards of Dempster *et al.*[16] were within ±0.5‰ of the values obtained after sample pre-concentration by pentane extraction from individual aqueous solutions of these compounds followed by CSIA by continuous-flow IRMS. In addition, Ivdra *et al.* found that solvent (pentane and DCM) evaporation as well as H_2SO_4 clean-up did not produce any significant (<0.5‰) isotope fractionation during pre-concentration of HCH isomers.[42] Since the various HCH isomers have quite similar ranges of log K_{oc} (3.13–3.33) and log K_{ow} (3.6–4.15),[43] these results carried out for α-HCH can be extrapolated for the other HCH isomers. Based on the above tests, CSIA can be used to accurately analyze HCH isomers in samples from HCH-contaminated field sites that are pre-concentrated using the extraction method validated herein.

Estimation of detection limit for CSIA application

The pre-concentration method was tested for 200 mL of aqueous solutions initially at concentrations of 50 and 500 μg L^{-1} (100 and 1000 ng of dissolved HCH, respectively), corresponding to a calculated detection limit

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and theoretical minimal mass of approximately 40 μg L^{-1} and 80 ng of α-HCH, respectively (Supporting Information, section S2.7 and Supplementary Table S3).

A detection limit of approximately 40 μg L^{-1} for α-HCH $\delta^{13}C$ analysis is quite good given that groundwater HCH concentrations typically range between 0.1 and 730 μg L^{-1}.[26,27] Nonetheless, aqueous samples at contaminated groundwater sites can have concentrations significantly below this value and in remote areas such as the Arctic, or in freshwater[15] and ocean water[9–11] α-HCH concentrations typically range between 0.12 and 2.5 ng L^{-1}. Additional modifications to the method described here that could lower the detection limit even further include reducing the solvent/water extraction ratio to improve the efficiency of the extraction. Dempster *et al.*[16] showed that for BTEX compounds, water/solvent ratios as high as 1000:1 improved the extraction efficiency and detection limit for CSIA without affecting the $\delta^{13}C$ values of the BTEX compounds. In addition, increasing the volume of contaminated water extracted,[9–11] and decreasing the final volume of the extract under a stream of nitrogen (for details, see Supporting Information, section S2.4), are possible methods to further improve the method detection limit for CSIA of HCH samples. For instance, increasing the volume of water extracted to 1 L from 200 mL would theoretically decrease the detection limit for CSIA of HCH to 8 μg L^{-1}.

CSIA potential for HCH source differentiation

The results for $\delta^{13}C$ values for α-, β- and γ-HCH pure standards, the lindane sample and the four TG-HCH mixtures are presented in Fig. 2 and in Supplementary Table S4 (Supporting Information). Badea *et al.*[22] and Vetter *et al.*[24] found quite a tight cluster of $\delta^{13}C$ values for the three DNAPL α-HCH samples that they reported (–27.3 to –25.9‰) and one α-HCH $\delta^{13}C$ value of –31.7‰. In contrast, the results of this study that included a larger range of HCH sources show significantly more source variation in α-HCH (Fig. 2(a)), and also large variations in γ-HCH (Fig. 2(b)) and β-HCH (Fig. 2(c)). Pesticide BHC-K500 had the most ^{13}C-depleted α-HCH ($\delta^{13}C$ value = –32.9‰), significantly different from all other α-HCH $\delta^{13}C$ values. Pesticide 666 had the most ^{13}C-enriched α-HCH ($\delta^{13}C$ value = –26.1‰), which was significantly different from pesticide BHC-K503. Conversely, the $\delta^{13}C$ values for the α-HCH isomer in α-HCH standards BHC-K501 and BHC-K503 were similar (ranging from –28.4 to –27.0‰).

Variations in the γ-HCH $\delta^{13}C$ values were also observed, with an overall difference between samples of up to 2.8‰ (Fig. 2(b)). The lindane sample had a $\delta^{13}C$ value of –28.0‰ which is isotopically identical within ± 0.5‰ uncertainty to the γ-HCH $\delta^{13}C$ values of BHC-K501 (–28.2‰) and BHC-K503 (–28.2‰), but significantly different from those of both Pesticide 666 (–25.4 ± 0.6‰) and the γ-HCH pure standard (–26.4‰). While γ-HCH was present in BHC-K500, its concentration was below the detection limits for isotopic analysis. Finally, the $\delta^{13}C$ value of the β-HCH pure product standard (–26.6‰) was within the ±0.5‰ analytical uncertainty of that of β-HCH in Pesticide 666 (–25.6‰). However, the $\delta^{13}C$ value of β-HCH in BHC-K500 (–31.8 ± 1.0‰) was significantly different (by at least 2‰) from both these values. Overall the range of $\delta^{13}C$ values observed for the γ-HCH samples characterized in this study showed a smaller range of variation than for α-HCH and β-HCH but this observation may simply be a function of the empirical nature of this study and requires further investigation with an even broader set of samples.

Isotope fingerprint – Isomer specific isotope analysis

Examining the results via a comparison of $\delta^{13}C$ values for different isomers from the same source sample is even more interesting than the above comparison of single isomers from different sources and suggests that the strongest isotopic fingerprint for a source may be the signature of multiple isomers measured together. Specifically, Fig. 3 and Supplementary Table S4 (Supporting Information) demonstrate that for a given source of HCH, all the isomers from that source are identical within uncertainty, and yet are significantly different from those from other sources. Indeed, the maximum variability of $\delta^{13}C$ values of each HCH isomer within any given TG-HCH sample was just 1.1‰ – largely unresolvable based on the total analytical uncertainty of ±0.5‰ (Supplementary Table S4, see Supporting Information). The $\delta^{13}C$ values for petroleum source materials range from –32 to –21‰,[44] which is comparable with the overall range of $\delta^{13}C$ values measured for HCH isomers from all sources in the present study. TG-HCH mixtures are produced from the photochlorination of benzene.[1] Since the skeleton of the molecule (i.e. the six-membered ring) is not affected by the manufacturing process, the $\delta^{13}C$ values of each of the HCH isomers in any given TG-HCH mixture may be similar because they all reflect the starting carbon isotopic composition of the same benzene feedstock. The similarity of the $\delta^{13}C$ values of each HCH isomer in any given TG-HCH may be used as an 'isotopic fingerprint' to differentiate between different TG-HCHs, similar to what has been suggested for polycyclic aromatic hydrocarbons (PAHs).[45] Hammer *et al.*[45] reported that at creosote-contaminated sites eleven of the sixteen analyzed PAHs had very similar $\delta^{13}C$ values, suggesting that these PAHs could be used as a distinctive 'isotopic fingerprint' for creosote-derived PAH contamination.

The pattern of uniform $\delta^{13}C$ values of each HCH isomer in any given sample of undegraded TG-HCH identified here for the first time (Fig. 3) has even more important implications in light of the use of CSIA to evaluate biodegradation. As noted above, the relative biodegradation rates of HCH isomers depend on a range of microbial and physical-chemical factors[7,33,34] and uncertainty in this information and resulting EF values has made developing lines of evidence in support of *in situ* biodegradation of HCHs difficult. While the relative rates of biodegradation of different isomers requires more investigation, assuming, as suggested in Fig. 3, that all isomers for a given source begin with a similar $\delta^{13}C$ value, the presence at a field site of a sample with isomers with different $\delta^{13}C$ values as well as patterns of ^{13}C enrichment in these wells compared with source zone wells could indeed be an indicator of the effects of degradation. Coupling information from two lines of evidence of biodegradation, isotopic fractionation and fingerprints between isomers, will provide better constraints on the possibility of *in situ* bioremediation. Based on this coupled approach, the pilot field study in this work was undertaken to test the ability to distinguish between areas of the plume where degraded and non-degraded HCH exist.

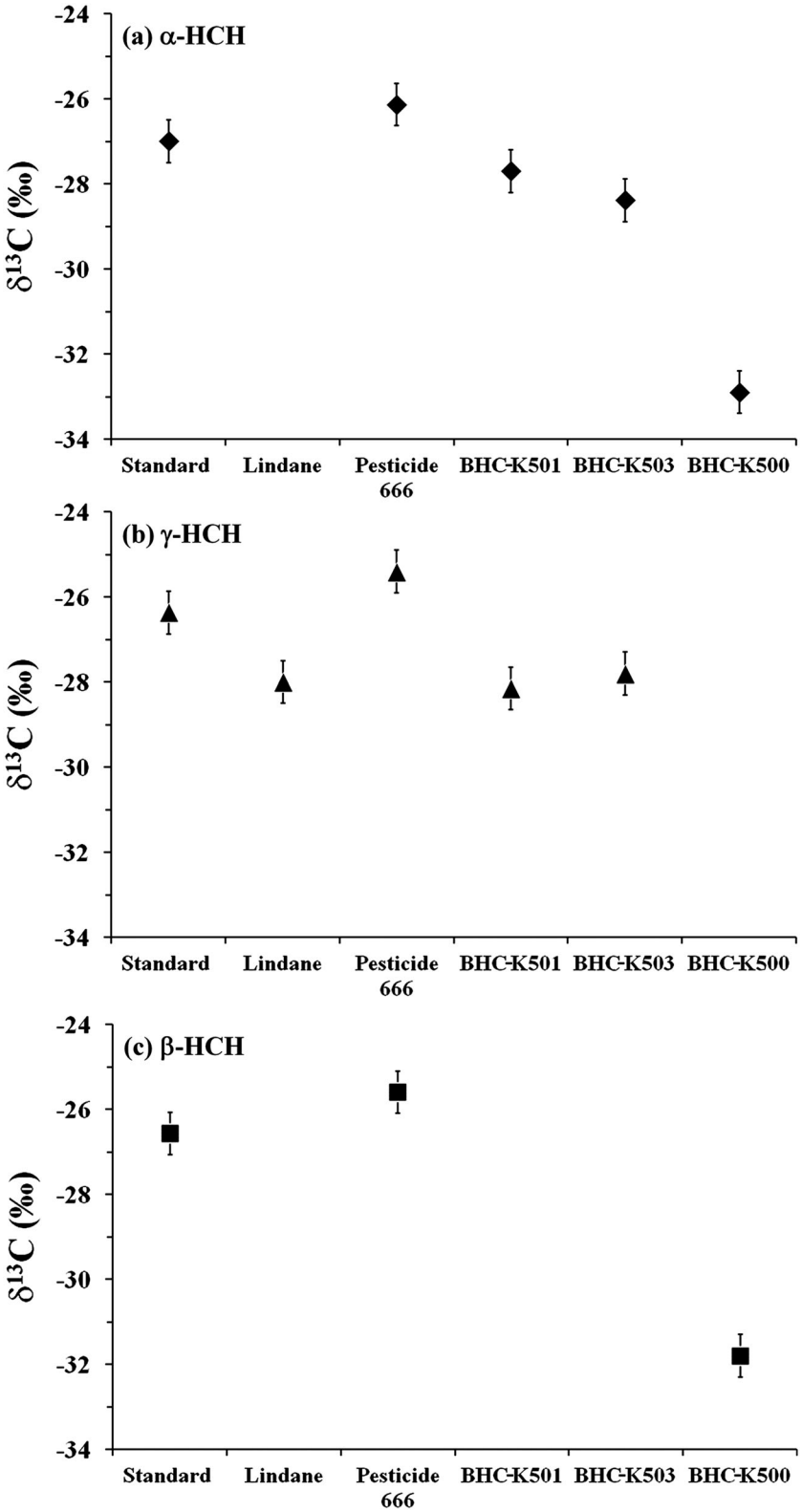

Figure 2. $\delta^{13}C$ values for α- (diamonds, a), γ- (triangles, b), and β-HCH (squares, c), for the four TG-HCH and lindane (γ-HCH) samples, and the three HCH standards from this study. Error bars represent ±0.5‰, which incorporates both accuracy and reproducibility.[41] Due to the low content in some pesticide mixtures, the concentrations for δ-HCH were below the detection limit for CSIA. (Data provided in Supplementary Table S4, Supporting Information.)

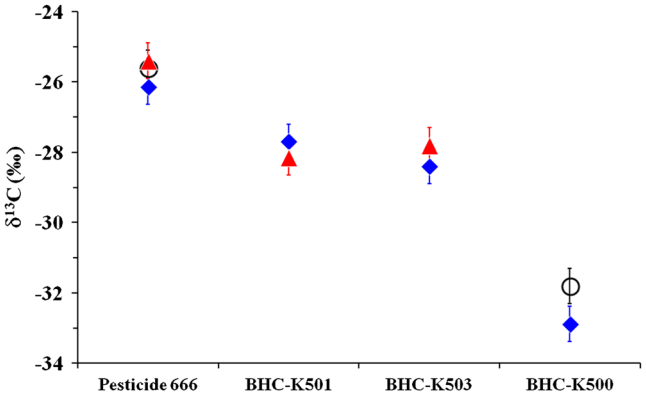

Figure 3. $\delta^{13}C$ values for α- (blue diamonds), β- (open circles), and γ-HCH (red triangles) for the four TG-HCH samples. Error bars represent ±0.5‰, which incorporates both accuracy and reproducibility.[41] (Data provided in Supplementary Table S4, Supporting Information.)

CSIA at HCH-contaminated field sites – Potential to identify *in situ* biodegradation

The concentrations and $\delta^{13}C$ values of the HCH isomers from five groundwater samples from the HCH-contaminated field site are plotted in Fig. 4 (Supplementary Table S5, Supporting Information), and the pH and EF values of the groundwater wells are reported in Supplementary Table S5 (Supporting Information). As the concentration of the β-HCH isomer was below the detection limit for CSIA, only the α-, γ- and δ-HCH $\delta^{13}C$ values are presented.

Source area

Wells 22-S and 34-D were both located near the historic disposal trenches, whereas well 7-S was situated close to the pesticide operating facility. The concentrations of α-, γ- and δ-HCH isomers were among the highest observed at the site for wells 22-S and 34-D (ranging between 30 and 420 μg L^{-1}), consistent with a source zone in this known disposal area. The concentrations in well 7-S were also high consistent with its position directly behind the pesticide facility (Figs. 1 and 4(a)). In these three wells, all the HCH isomers were among the most depleted in ^{13}C at the site (Fig. 4(b) and Supplementary Table S5, Supporting Information), and the EF values were approximately 0.5 (Supplementary Table S5), all of which is consistent with undegraded source material. This is further supported by the similarity (±0.5‰) of the $\delta^{13}C$ values for the α-, γ- and δ-HCH isomers within each well (Fig. 4(b)). The mean $\delta^{13}C$ values for all three isomers were very similar for wells 22-S (–25.8‰), 34-D (–26.6‰), and 7-S (–25.9‰) (Supplementary Table S5, Supporting Information), suggesting that these three wells contained similar source material.

Wells downgradient of the disposal and operating facility

Wells 28-S and 19-D were located in the pH neutral plume downgradient from the source zones near the disposal and operating facility. The concentrations of the HCH isomers in these wells ranged from below detection limit to 9.9 μg L^{-1} (Fig. 4(a), and Supplementary Table S5, Supporting Information). The lower concentrations in these wells suggested that there was potential for some degree of natural attenuation via biodegradation as well as dilution/dispersal in the plume. In contrast, the acidity of the plume in the disposal area (wells 22-S and 34-D; see Supplementary Table S5, Supporting Information) may have inhibited microbial HCH degradation.[14] The hypothesis tested in this pilot study was that if *in situ* biodegradation were indeed responsible for the lower HCH concentrations in wells 28-S and 19-D, one might expect both higher $\delta^{13}C$ values[22,29,36] in the individual isomers of HCH in these wells than in the source zone wells, and, based on the results of this study, non-identical $\delta^{13}C$ values for the isomers of HCH within a given well.

In downgradient well 28-S the $\delta^{13}C$ values for δ-HCH (–23.9‰) and α-HCH (–24.3‰) were 1.5 to 2.5‰ higher, respectively, than the $\delta^{13}C$ values of these isomers in the source wells (Fig. 4(b), and Supplementary Table S5, Supporting Information). Only the $\delta^{13}C$ value of γ-HCH (–26.5 ± 0.6‰) was similar to the $\delta^{13}C$ values of the source wells, despite a lower concentration in well 28-S than in the source wells.

Non-degraded TG-HCH (Fig. 3) and samples from the source wells (Fig. 4(b)) showed that, for any given HCH, all the isomers exhibited identical $\delta^{13}C$ values. In contrast, well 28-S showed significant differences in $\delta^{13}C$ values from one isomer to another. This in itself suggests that an additional process, probably biodegradation, has played a significant role in the plume at this location. Such dissimilarity between

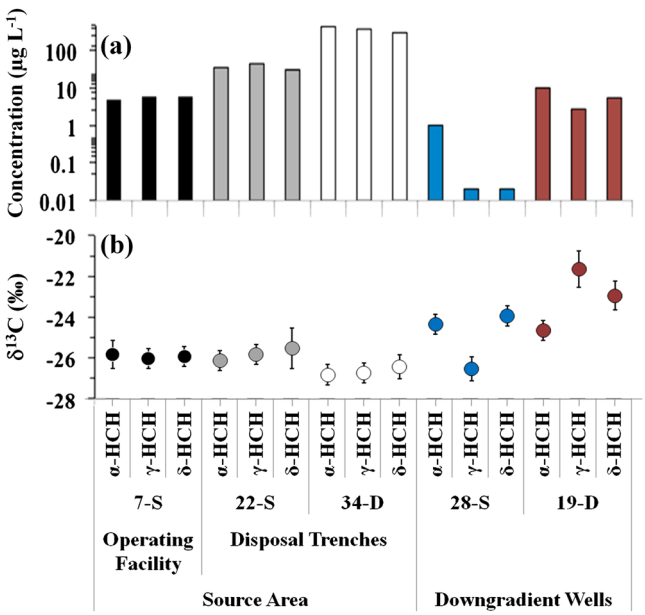

Figure 4. (a) Concentration (μg L^{-1}) and (b) $\delta^{13}C$ (‰) values for groundwater from monitoring wells from the source area close to the disposal trenches and operating facility (i.e., 7-S, 22-S, and 34-D), and from wells located downgradient from the source area (i.e. wells 28-S and 19-D). Error bars on $\delta^{13}C$ values represent ±0.5‰, which incorporates both accuracy and reproducibility, and this is reported for all samples for which precision on replicates was ≤0.5‰.[41] Where the precision on replicates was larger than ±0.5‰, this larger uncertainty was used. For details, see Supporting Information, section S2.6. (Data provided in Supplementary Tables S5 and S6, Supporting Information.)

HCH isomers within a given sample may in fact be an 'isotope fingerprint' to distinguish between biodegraded and non-biodegraded HCH. In addition, the EF value of α-HCH in well 28-S was 0.613, suggesting that α-HCH had undergone biodegradation.

While to date no field studies have been carried out to examine dissolved HCH isomer $\delta^{13}C$ values from groundwater samples, laboratory experiments suggested that significant ^{13}C-enrichment patterns can be induced during the biodegradation of HCH isomers, consistent with what has been shown previously for other chlorinated hydrocarbons.[29,36] For complete biodegradation (94–97% removal) of γ-HCH and α-HCH by two pure bacterial strains, Bashir *et al.*[29] observed an enrichment in ^{13}C of 6.7 to 8.0‰ (γ-HCH) and 6.2 to 8.7‰ (α-HCH). Badea *et al.*[36] found an 11.1‰ (*D. multivorans*) and 11.2‰ (*D. gigas*) increase in the γ-HCH $\delta^{13}C$ for anaerobic γ-HCH biodegradation. Enrichment factors ranged from –1.0 to –1.7‰ (during biodegradation under aerobic conditions); and from –3.4 to –3.9‰ (during biodegradation under anaerobic conditions). It will be important to verify that other mass-dispersal processes such as adsorption do not produce significant isotopic fractionation. Kopinke *et al.*[46] demonstrated the possibility of significant carbon isotope fractionation during successive adsorption and desorption steps. As HCH isomers are moderately hydrophobic (log K_{oc} range from 3.0 to 3.8), more research is needed to evaluate the effects of adsorption on HCH $\delta^{13}C$ values. However, recent results on aromatic and chlorinated aromatic contaminants (log K_{oc} ranging from 1.8 to 2.6), showing the absence of significant isotopic fractionation during adsorption and diffusion,[47] suggest that HCH adsorption might also not produce large degrees of isotopic fractionation compared with the large changes now demonstrated for degradation.[22,29,36,37] At the field site, the observed enrichment in ^{13}C for the HCH isomers in downgradient wells compared with source wells, the variation in $\delta^{13}C$ values between the isomers in this well, and the EF value, are all consistent with the effects of *in situ* HCH biodegradation.

Well 19-D showed similar $\delta^{13}C$ patterns for HCH isomers to those observed in well 28-S. The α-, γ-, and δ-HCH isomers were significantly enriched in ^{13}C ($\delta^{13}C$ values of –24.6; –21.6 ± 0.9; –22.9 ± 0.7‰, respectively) compared with the HCH isomers in the source wells (Fig. 4(b) and Supplementary Table S5, Supporting Information). In addition, the $\delta^{13}C$ values of the α-, γ-, and δ-HCH isomers in this well were all significantly different from each other. While these patterns may suggest that biodegradation has had an impact on the HCH isomers in this well, in this case, the racemic nature of the α-HCH (EF = 0.507) is not necessarily consistent with this. As noted earlier, EF analysis is only useful for determining if one enantiomer is preferentially biodegraded over the other. If both enantiomers are simultaneously biodegraded, the EF value may remain close to 0.5, whereas the α-HCH $\delta^{13}C$ value would increase. In addition, as noted by Zhang *et al.*,[37] the EF value remains racemic during abiotic α-HCH degradation. Thus, an enrichment in ^{13}C for a HCH isomer may serve as a signal of biodegradation, even if α-HCH remains racemic. Recently, CSIA has evolved to ESIA (Enantiomer Specific Isotope Analysis) to determine the $\delta^{13}C$ values of individual enantiomers and this technique was applied to α-HCH isomers.[22,29] By combining the $\delta^{13}C$ and EF values, Bashir *et al.* were able to distinguish between aerobic and anaerobic α-HCH biodegradation in laboratory microcosm experiments.[29]

CONCLUSIONS

The results presented here demonstrated that pre-concentration of HCH solutions from water samples did not result in isotopic fractionation, verifying that this method can provide accurate $\delta^{13}C$ values of HCH isomers from pre-concentrated aqueous samples obtained from contaminated field sites. Distinctly different $\delta^{13}C$ values were observed for α-, β- and γ-HCH isomers from different manufacturers, suggesting that CSIA has the potential to distinguish between different HCH sources. Most importantly, for a given HCH source, all isomers were found to have the same $\delta^{13}C$ value – suggesting that this fingerprint of uniform $\delta^{13}C$ values within the isomers of HCH may be a novel diagnostic of undegraded HCH. This result has important implications for identification of the effects of biodegradation at field sites. Carbon isotope analysis of groundwater samples from an HCH-contaminated field site provided two lines of evidence that biodegradation of HCH isomers was occurring at the site. First, significantly ^{13}C-enriched $\delta^{13}C$ values in downgradient wells were observed compared with source wells. Secondly, significant differences in the $\delta^{13}C$ values of different HCH isomers in the downgradient wells were indicative of *in situ* biodegradation. In addition, in one well where evidence of α-HCH biodegradation was indicated using chiral analysis (EF = 0.613), $\delta^{13}C$ analysis confirmed biodegradation, as demonstrated by a significant enrichment in ^{13}C compared with the source wells. While the microbial communities and pathways involved in biodegradation of HCH are still under investigation and the use of EF values as a signature of biodegradation remains uncertain, these results suggest that CSIA can be an important additional approach to integrate into the investigation of HCH-contaminated field sites through the addition of two lines of evidence for biodegradation effects.

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