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A concept for studying the transformation reaction of Hexachlorocyclohexanes in food webs using multi-element compound-specific isotope analysis

Langping Wu[a,1], Sonya Moses[a,2], Yaqing Liu[a], Julian Renpenning[a], Hans H. Richnow[a,*]

[a]Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research-UFZ, Permoserstraße 15, 04318 Leipzig, Germany

Present address:

[1]Departments of Civil & Mineral Engineering, University of Toronto, 35 St. George Street, Toronto, ON M5S 1A4, Canada

[2]Department of Animal Ecology I, University of Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany

*Corresponding author

Email: hans.richnow@ufz.de, Tel: 0049 341 235 1212, Fax: 0341-450822

ABSTRACT

An analytical concept using stable isotope fractionation for analyzing persistent organic pollutants (POPs) in food webs was developed and tested. We have evaluated methods for the extraction and clean-up of hexachlorocyclohexane isomers (HCHs) as the model compounds of POPs from water, soil, plant, milk, fish oil and pork liver in order to study the reactive transport processes of HCHs in food webs using multi-element compound-specific isotope analysis (CSIA). The extraction and clean-up methods were evaluated for recovery efficiency and isotope effects. The precision and accuracy for carbon, hydrogen and chlorine isotope analysis was within the analytical precision of ±0.5‰, ±5‰ and ±0.3‰, respectively. The method was applied for stable isotope analysis of HCHs in possible food webs from soil to plants, and to animals. Isotope compositions of HCHs in cow/buffalo milk and dung, wild animal livers and seal blubber were obtained and compared to the sources of HCHs. The magnitude of isotope enrichment demonstrated the potential of CSIA for analyzing reactive transport processes of HCHs in the food web. The concept using multi-element stable isotope analysis can be applied for source identification, characterization of degradation mechanisms, and particularly contaminant accumulation in the food web, which demonstrate the potential in new scientific areas for CSIA.

Keywords: hexachlorocyclohexane; extraction; isotope fractionation; food web; reactive transport.

1. Introduction

Gamma-hexachlorocyclohexane (γ-HCH) was one of the most abundantly produced and extensively used organochlorine pesticides in the past. Its application and production were banned by the Stockholm Convention in 2009 due to its persistent, bioaccumulative toxic, cancerogenic and mutagenic properties [1, 2]. Today, official use continues only for pharmaceutical purposes, such as treatment for lice and scabies [1]. Hexachlorocyclohexane isomers (HCHs) are commercially manufactured by the reaction of benzene and chlorine gas in the presence of UV light [3, 4]. Only γ-HCH possesses specific insecticidal properties, however, production of one ton of γ-HCH generates 8 to 12 tons of HCH containing waste, the so called "HCH muck". α-HCH is the major isomer in technical HCH, and β- and δ-HCH are chemically more stable [5]. Large amounts of HCH muck were produced and deposited in an uncontrolled manner, resulting in serious environmental pollution world wide [6, 7], especially in Germany [8, 9], Italy [10], Spain [11], China [12], and India [13]. HCHs can be decomposed by dechlorination and dehydrochlorination processes [14], and are considered as persistent organic pollutants due to their relative long half-life in the environment. For example, HCHs in thr vapor-phase can be degraded with the estimated half-life of 28-115 days in the atmosphere by reaction with photochemically-produced hydroxyl radicals. Hydrolysis is likely to occur slowly, the respective hydrolysis half-life of α- and γ-HCH are 26 and 42 years in natural waters at pH 8 and 5 °C. The half-life of biodegradation of HCHs is estimated to be 33.9-184 days in cropped soils and 23.4-100 days in uncropped soils [15]. Due to the low water solubility (5-10 mg L^{-1} at 25 °C), HCHs tend to accumulate in the hydrophobic soil components and are introduced into food webs through plant uptake by the roots from the soil and leaves from the air [16, 17]. HCHs can be transported over long distances by

natural processes like global distillation.Today, HCH residues can consequently be found in elevated concentrations in Arctic and Antarctic animals at the end of the food chain [18-21].

Compound-specific isotope analysis (CSIA) appears to be a promising method to assess sources and the fate of organic pollutants in the environment [22-24]. CSIA has primarily been applied to study the transformation of volatile organic pollutants such as petroleum hydrocarbons [25, 26], benzene homologues [27, 28], chlorinated ethenes [29-31], and fuel oxygenates [32-34]. In recent years, CSIA has been developed for investigate the fate of several pesticides including lindane. For instance, the carbon isotope fractionation can be used to characterise the *in situ* (bio)degradation of HCHs [35-37], and hydrogen, carbon, chlorine isotope compositions allow to identify the sources of HCHs [38]. CSIA has been proposed as a tool to characterize the transformation potential of pesticides in the environment for risk assessment, for applications in natural attenuation studies [39], or for tracking the sources of these contaminants. One main bottleneck for CSIA, however, is the moderate to poor analytical sensitivity and high purity requirements for isotope analysis. In particular, the low detection limit of CSIA makes the investigation of bioaccumulated contaminants in food webs challenging, since large amounts of samples are required to enable isolation of sufficient compound quantities. To date, the first study using CSIA to investigate reactive bioaccumulation of organic contaminants was conducted by Holmstrand et al. [40], where DDT (dichlorodiphenyltrichloroethane) was extracted from 16 kg of seal blubber by continuous partitioning with acetonitrile in 2.2 L lipid batches using a Wallenberg perforator. Later the same sample preparation approach was applied to study the anthropogenic origin of tris-(4-chlorophenyl)methane by the

same research group [41]. Holmstrand and colleages used an offline conversion method for DDT, making it necessary to recover a significant mass of the target analyte. However, this approach is not feasible for routine investigation of contaminants in food webs, as it is limited by the availability of large amount of sample as well as laboratory equipment. Thus, alternative methods for extraction and clean-up of HCHs from biota matrices for routine laboratory preparation are needed for implementation of CSIA.

The present study aims to provide the analytical methods for a stable isotope fractionation concept for analyzing POPs in food webs while taking reactivity (degradation) into account. The first objective of this study was to develop appropriate methods for HCH extraction and purification from environmental and, in particular, biological samples for ^{13}C, ^{2}H and ^{37}Cl stable isotope analysis in order to explore the potential application of CSIA for food web studies. The method development includes isolation of lipophilic HCHs from proteinaceous and biological materials with high fat content. The HCH extraction efficiency from different matrices (including water, soil, plant, milk, oil, liver) was evaluated by carrying out spiking experiments in the laboratory, and then the isotope artifact from the sample treatment methods was critically examined. The second objective was to evaluate the potential of CSIA for investigating the bioaccumulation and degradation of HCHs in the food webs. HCH-contaminated soil, plants, cow/buffalo milk and dung, wild animal livers were collected from contaminated sites, and seal blubber as a repersentative sample of a higher trophic level in the arctic food web in a remote area was obtained. The ^{13}C and ^{37}Cl isotope composition of HCHs were analized and compared to potential sources collected from all over the world in order to evaluate the reactive transport of HCH in food webs.

2. Materials and methods

2.1. Chemicals and reference matrices

Hexachlorocyclohexanes (α-, β-, δ-HCH, 99.5% purity, Fluka) were purchased from Sigma-Aldrich. HCH stock solution containing 10 g L^{-1} of α-, β-, δ-HCH (1:1:1) was prepared in acetone for spiking water and soil samples, and prepared in hexane for spiking plants, fish oil, milk and pork liver samples. The stock solutions were stored in the refrigerator at 4 °C before use. High purity acetonitrile (≥99.9%, Carl Roth GmbH & Co. KG, Germany), n-hexane (for pesticide residue analysis, Sigma-Aldrich) and dichloromethane (DCM, ≥99.9%, Carl Roth GmbH & Co. KG, Germany) were used for HCH extraction. Florisil (for chromatography, ROTH, 100-200 mesh) was used as stationary phase for liquid column chromatography, which was activated at 120 °C for 12 h before usage. Anhydrous Na_2SO_4 was activated by heating at 200 °C overnight. Glass wool (untreated, SUPELCO Analytical) and sea sand were used for packing the column. To track the elution of HCHs from the Florisil column, 7, 12-dimethyl-benz[α]anthracene (p. A, Reagent Grade) was applied as a fluorescence tracer. A 360 nm UVA lamp was used to visualize the fluorescence band. HCH-free soil and grass (*Calamagrostis epigejos*) was obtained from a residential garden. Fish oil (Den originale Moeller's Tran), fresh milk (3.5% fat) and pork liver purchased from a local supermarket were used as reference matrices to evaluate the extraction efficiency.

2.2. Extraction of HCHs from different matrices

2.2.1. Water

129 One liter of distilled water was spiked with HCH stock solution in a separation funnel to
130 produce concentration levels between 2.90 and 10.15 mg L^{-1}. The spiked HCHs were
131 extracted 3 times by shaking thoroughly with 30 mL DCM each time. The organic phases
132 were combined and evaporated to dryness under a gentle N_2 stream in a TurboVap
133 concentrator (TurboVap II, Biotage, Sweden). To this end, the HCH extract was
134 transferred into a glass vial and re-dissolved into 1 mL hexane for further analysis.

135 2.2.2. Soil

136 Ten gram of garden soil was spiked with HCH stock solution to generate concentration
137 levels between 290 and 1015 µg g^{-1}. Subsequently, the spiked soil was extracted by
138 accelerated solvent extraction (Dionex ASE 200, Thermo Scientific) equipped with 11-
139 mL stainless steel extraction cells. The extraction conditions were as follows: solvent:
140 hexane/acetone (1:1, v:v); oven heat up time: 6 min; final temperature: 100 °C; static
141 time: 3 min; pressure: 1500 psi; purge time: 60 s; flush volume: 60%; static extraction
142 cycles: 3. The collected extracts were dried with ~5 g anhydrous Na_2SO_4 and then
143 evaporated to dryness using a TurboVap concentrator. Thereafter the dried extract was
144 re-dissolved in 1 mL hexane for further clean-up.

145 2.2.3. Plant

146 The plant sample from a residential garden was cut into small pieces and fully dried in a
147 freeze-dryer (Christ Beta-2-16 Freeze Dryer, Martin Christ Gefriertrocknungsanlagen
148 GmbH, Germany) at -35 °C and 0.310 mbar. The dry sample was then mechanically
149 ground into fine powder in a grinder. Six gram of dry plant material was filled into a 22-
150 mL stainless steel ASE cell, in which cellulose filters were placed on both sides of the

151 cell. The plant powder was spiked drop-by-drop with 1 mL of HCH stock solution during
152 placement into the ASE cell (to ensure homogeneous distribution) until the target
153 concentrations were reached to 10 to 166 µg g^{-1}. Before ASE extraction, the cell was
154 placed in the fume hood overnight for solvent evaporation. ASE extraction conditions
155 were the same as for soil sample extraction, except that temperature was adjusted to
156 125 °C. The collected extract was transferred into a 50-mL round bottom flask, and then
157 the solvent was evaporated in a rotary evaporator at 40 °C. A small amount of activated
158 Florisil was added and additionally some DCM to re-dissolve the extracted materials. The
159 extracted materials were then adsorbed on Florisil and evaporated to dryness for further
160 clean-up.

161 2.2.4. Fish Oil

162 Fish oil (100 mL) was spiked with HCH stock solution to concentration levels between 1
163 and 100 µg g^{-1}. The spiked fish oil was extracted with 100 mL of acetonitrile in a 500-mL
164 centrifuge bottle (PP Copolymer, Thermo Fisher Nalgene®) by placing it in an ultrasonic
165 bath for 1h. After that, the acetonitrile and oil phases were separated by centrifugation at
166 10.000 rpm at 4 °C for 20 min, and then acetonitrile phase was carefully transferred into a
167 TurboVap vial using a glass pipette. The same extraction procedure was repeated 3 times
168 for each sample with 50 mL, 25 mL, 25 mL of acetonitrile, respectively. Thereafter , the
169 acetonitrile phases were combined and evaporated in a TurboVap concentrator (25 °C, 9-
170 14 psi) until only co-extracted lipids remained (~3 mL). If the amount of co-extracted
171 lipids were exceeded 10 mL, the sample was re-extracted 3 times with an equal volume
172 of acetonitrile in an ultrasonic bath for 30 min. Extraction procedures were the same as
173 described above.

174 2.2.5. Liver

175 Fresh pork liver was homogenized using a hand blender (VH007, Voche, UK), and then
176 100 g liver mousse was spiked with HCH stock solution to obtain a concentration of 10
177 µg g^{-1}. The HCH extraction procedures were the same as described for fish oil.

178 2.2.6. Milk

179 Milk (500 mg) was spiked with HCH stock solution to obtain a concentration of 100 µg
180 g^{-1}. The spiked milk was extracted with 50 mL hexane in a large centrifuge bottle by
181 placing it in an ultrasonic bath for 1h. For improving the phase separation, centrifugation
182 at 10.000 rpm at 4 °C for 20 min was applied, and then the hexane phase was carefully
183 transferred into a glass vial using a glass pipette. The extraction procedure was repeated 2
184 times with 50 mL and 25 mL hexane, respectively. All hexane phases were combined and
185 evaporated in a TurboVap concentrator until only co-extracted lipids remained.

186 **2.3. Clean-up of HCH extracts**

187 2.3.1. Water and soil

188 The clean-up method was modified from the US EPA method 3620C [42]. For separating
189 by liquid chromatography, a glass Pasteur pipette (0.7 cm diameter × 15 cm length) was
190 packed from bottom to top with glass wool, 1-cm clean sea sand, 1 g activated Florisil,
191 and a 0.5-cm layer of activated anhydrous Na_2SO_4. This column was pre-conditioned
192 with 1 mL of n-hexane. Then 50 µg of 7, 12-dimethyl-benz[α]anthracene (2 mg mL^{-1}
193 stock solution in hexane) was added on the top of the column. The fluorescence tracer co-
194 elutes with HCHs and thus can be used as an indicator of the HCHs elution when using a

195 UVA lamp. The HCH extracts were loaded onto the column and eluted continuously by
196 adding 5 mL hexane (1st fraction) and 5-7 mL hexane/DCM mixture (v/v 1:1) (2nd
197 fraction) at natural gravity flow velocity. The collection of the 2nd fraction was cut-off
198 after the fluorescence band was eluted from the column. The eluate from the 1st fraction
199 containing mainly hydrocarbons was disposed. The eluate from the 2nd fraction
200 containing HCHs was reduced to ~0.5 mL under a gentle N_2 stream, and then transferred
201 into a glass vial and adjusted the volume to 1 mL by adding hexane for concentration and
202 isotope analysis.

203 2.3.2. Plant

204 The extract was purified using a 2.2 cm diameter × 32.5 cm length glass column for
205 liquid chromatography. The clean-up procedures were the same as described above but
206 with the following modifications. The glass column was packed from bottom to top with
207 glass wool, 1 cm of cleaned sea sand, 15 cm of activated Florisil and 4 cm of activated
208 anhydrous Na_2SO_4. Florisil was packed into the column as slurry prepared with hexane to
209 ensure that the column was tightly and homogeneously packed. The plant extracts
210 adsorbed to Florisil and 100 µg of 7,12-dimethyl-benz[α]anthracene were loaded onto
211 the column and were then eluted by continuously adding 30 mL of hexane (1st fraction)
212 and 45-55 mL hexane/DCM (v/v 1:1) (2nd fraction). The 2nd fraction containing HCHs
213 was subjected to further analysis.

214 2.3.3. Fish oil, milk and liver

215 The co-extracted lipids (~3 mL) were transferred and mixed with 3 times of their volume
216 with 95% concentrated H_2SO_4 in a 50-mL glass bottle closed with Teflon coated screw

cap. The mixed solution was hydrolyzed at 70 °C in an ultrasonic bath for 2 h in order to remove the co-extracted lipids by acidic hydrolysis. The hydrolyzed solution was transferred into a separation funnel with 100 mL of distilled water and 30 mL of hexane, and then shaken carefully for approximately 2 min. The acidic aqueous phase was discharged after reaching a clear phase separation. The hexane phase was then washed with 50 mL distilled water. After that, 20 mL of 0.5 M NaOH were added to the hexane phase to remove the remaining lipids by saponification. Subsequently the mixture was shaken for 30 s until the hexane phase was transparently yellow and no further color change occurred after the addition of NaOH. The solution was then transferred and centrifuged at 4 °C for 20 min at 10000 rpm to ensure phase separation. The hexane phase was collected and the remaining alkaline solution was extracted once more with 30 mL hexane under the same conditions. Finally, the combined hexane phase was evaporated to ~1 mL and then purified by column chromatography as described for plant extracts.

2.4. HCH contaminated site and sampling

Soil, plant and wild animal liver samples were obtained from a large-scale HCH contaminated site which is located at Bitterfeld in Germany. The field site history and contamination have been described elsewhere [36, 43]. Two soil samples (10 g) were taken from different locations. Plant samples (6 g dry weight) of two different species, *Plantago lanceolate* and *Phragmites australis*, were taken in October 2015 at the heavily contaminated “Area C” [36]. Wild boar and deer (about one year old) feeding in this area were hunted in November 2015 with the support of the Environmental Agency in Bitterfeld, and the liver samples (150 g) were collected for further studies. Cow and

buffalo milk (1 L) and dung (4.3 g dry weight) samples were collected from a HCH dumpsite in Lucknow, India [44]. Seal blubber samples (500 g) were provided by Prof. Roland Kallenborn from the University Centre in Svalbard, Norway. All samples were stored at -20 °C before treatment. The HCH extraction and clean-up methods described above were applied for sample preparation.

2.5. Analytical methods

2.5.1. Concentration analysis

An Agilent 6890 series GC (Agilent Technologies, USA) equipped with a flame ionization detector was used to determine the concentration throughout the study. Each sample was measured in triplicates. The analytical conditions can be found in the Supporting Information (SI) section 1.

2.5.2. Isotope analysis

The isotope composition of element (E) is reported as δ notation in parts per thousand (‰) and expressed as the deviation from international standards according to the following equation:

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

R indicates the isotope ratio of $^{13}C/^{12}C$, $^{2}H/^{1}H$ or $^{37}Cl/^{35}Cl$. International standards for ^{13}C, ^{2}H and ^{13}Cl are Vienna Pee Dee Belemnite (VPDB), Vienna Standard Mean Ocean Water (VSMOW) and Standard Mean Ocean Chloride (SMOC), respectively.

Carbon isotope compositions ($\delta^{13}C$) were measured using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), where a GC (7890A, Agilent Technologies, USA) was coupled via a ConFlo IV interface (Thermo Fisher Scientific, Germany) to a MAT 253 IRMS system (Thermo Fisher Scientific, Germany). All samples were injected in splitless mode at 250 °C and then separated on a Zebron ZB1 column (60 m × 0.32 mm × 1 μm; Phenomenex, Germany) under a constant carrier gas flow of 1.5 mL min^{-1}. The temperature program of the GC is described in the SI section 1. After separation, compounds were converted to CO_2 in the combustion reactor operating at 1000 °C. Samples were analyzed in triplicates. Corresponding analytical precision for $\delta^{13}C$ was below ±0.5‰. All $\delta^{13}C$ values were normalized to the VPDB scale by applying a two-point calibration approach using in-house reference compounds β-HCH ($\delta^{13}C$ = -34.1‰) and γ-HCH ($\delta^{13}C$ = -25.3‰). A third standard α-HCH ($\delta^{13}C$ = -29.1‰) was used for validation of the calibration.

Hydrogen isotope compositions ($\delta^{2}H$) were measured via a gas chromatography-chromium based high temperature conversion-isotope ratio mass spectrometry (GC–Cr/HTC–IRMS), as previously described elsewhere [45-47]. Briefly, Cr/HTC makes use of the combination of high temperature conversion and reduction of hot elemental chromium at 1200 °C. While hetero-elements (i.e. N, S, Cl) are scavenged at elevated temperatures as chromium salts, H_2 is released into the carrier stream and subsequently isotopically analyzed by the IRMS. The GC parameters were the same as described for the $\delta^{13}C$ measurement. Samples were analyzed in triplicates. Corresponding analytical precision for $\delta^{2}H$ was below ±5‰ [48]. All $\delta^{2}H$ values were normalized to the VSMOW scale by applying a two-point calibration approach using in-house reference compounds:

tetradecane (δ^2H = -230‰) and hexadecane (δ^2H = +381‰, reference ID: USGS69 [49]). A third standard heptadecane (δ^2H = -73‰) was used for validation of the calibration.

Chlorine isotope compositions (δ^{37}Cl) were determined using gas chromatography coupled with multiple-collector inductively coupled plasma mass spectrometry (GC-MC-ICPMS), as recently described elsewhere [50, 51]. Samples were injected with a split ratio of 1:10 and a constant carrier gas flow of 2 mL min^{-1} using the same GC column and oven temperature program as described for the δ^{13}C measurement. Once separated, the analyte was directed to the ICP torch via a Thermo Elemental Transferline AE2080 (Aquitaine Electronique, France). The interface was specifically modified for the transport of semi-volatile organics from the GC to the cold ICP torch. The MC-ICPMS plasma was operating at dry plasma conditions, reducing unfavorable protonation effects [50, 51]. The chlorine isotopes were analyzed at mass 35 and 37 ($^{35}Cl^+$, $^{37}Cl^+$) at low resolution mode (m/Δm = 300). All samples were spiked with an in-house reference compound (TCE, δ^{37}Cl = -1.19‰) as an internal standard to compensate for minor instrumental drifts. The obtained raw δ^{37}Cl values were normalized to the SMOC scale by applying a two-point calibration approach using in-house reference compounds which were methyl chloride (MC, δ^{37}Cl = +6.02‰) and trichloroethene (TCE, δ^{37}Cl = -1.19‰). In addition, a second trichloroethene (TCE, δ^{37}Cl = +2.17‰) was used for the validation of the calibration, as described by Horst et al.[50]. Samples were generally analyzed in triplicates, with an analytical precision usually below ±0.3‰ for compound-specific analysis of mixtures [50, 51].

3. Results and discussion

3.1. Extraction efficiencies of HCHs from different matrices

CSIA is several orders or magnitudes less sensitive than modern methods for concentration analysis such as GC-MS or HPLC-MS and thus requires larger amounts of sample material. A central step of the clean-up procedures and enrichment strategies is the evaporation of solvent. A previous study reports no significant losses of HCHs during solvent evaporation and insignificant isotope effects are associated with solvent evaporation [52]. Therefore, it is not expected that the evaporation process changes the isotope composition of HCHs and thus large amount of solvents can be used for extraction. Chromatography columns packed with Florisil gave almost complete recovery (Table S.1). The use of a co-eluting fluorescent tracer allowed for visual inspection of the chromatographic process and precise cut-out of the fraction containing HCHs.

3.1.1. Water and soil

HCH spiked water sample was extracted 3 times by liquid-liquid extraction using DCM. ASE was applied for extraction of HCHs from soil using 3 static cycles in order to improve the recovery rate. Interference from humic substances and other co-extracted organic substances such as fatty acids were expected. Therefore, the HCH extracts from water and soil were further separated by Florisil column chromatography. Florisil was selected due to its potential to retain lipids and high polar materials, as well as its capability of effecting clean-up of apolar pesticide residues from food samples [53]. Overall recovery of 86 - 95% was obtained for HCHs from water and soil (Table 1).

3.1.2. Plant

Plant was first freeze-dried under low temperature and low pressure conditions in order to minimize the losses as HCHs are relatively volatile hydrophobic compounds. ASE with hexane/acetone (1:1, v:v) at 125 ^{o}C was used for extraction. Hydrophobic compounds such as plant waxes, chlorophyll and lipids could be co-extracted from plant, which may form a lipid-layer and block the Florisil column during clean-up of HCH. Therefore, the HCH extracts were first adsorbed to a small amount of Florisil before loading them onto the column. Overall recoveries of 36 - 66% were achieved for α-, β-, δ-HCH from plant (Table 1). The losses during extraction of HCHs from plant samples were evaluated step by step (Table S.1). The recoveries of 98 - 107% after solvent evaporation and 92 - 98% after column chromatography indicated that no significant losses of HCHs occurred during these two steps. However, only 12 - 17% of HCHs were recovered after ASE using hexane as extraction solvent, which was likely due to the low polarity of hexane. Recoveries of HCHs increased significantly to 43 - 50% when the ASE extraction solvent was replaced by an acetone/hexane mix (1:1 by volume). If extracted by ASE using acetone alone, HCH extracts contained large amounts of interfering compounds such as pigments which were difficult to be removed by column chromatography.

3.1.3. Fish oil, milk and liver

Acetonitrile was applied to remove large amounts of lipids in the first extraction step as lipids have a relatively lower solubility in acetonitrile compared to hexane. Instead of waiting for phase partitioning in a separation funnel, the ultrasonic bath and subsequent centrifugation were applied for a better HCH extraction and phase separation between acetonitrile and oil. A recovery of 75 - 89% was achieved after repeating the acetonitrile extraction for 3 times (Table S.2). Milk containing typically 88% water, 3.4% fat and 3.3%

347 protein was selected to represent fatty samples containing a large amount of water.
348 Hexane was applied to remove water and hydrophilic substances in the first extraction
349 step from milk. Fresh pork liver was chosen as a representative sample of animal tissues
350 containing high amounts of fat and protein. The homogenization of liver was achieved
351 with a food blender before solvent extraction in order to increase the extraction recovery.
352 Co-extracted lipids from fish oil, milk and liver samples were removed by acidic
353 hydrolysis using 95% concentrated H_2SO_4, followed by a saponification process using
354 0.5 M NaOH solution. The majority of hydrolyzed carboxylic acids were deprotonated
355 and polar hydrolysis products (such as glycerol) were dissolved in the aqueous phase,
356 thus HCHs can be extracted with hexane. Overall, HCH recoveries of 16 - 30%, 33 - 44%
357 and 16 - 25% were obtained from fish oil, milk and liver, respectively (Table 1).
358 Obtaining a clear phase separation in each step is essential to improve the HCH
359 recoveries.

360 **Table 1.** HCH recoveries from different matrices.

sample code	spiking concentration	α-HCH	β-HCH	δ-HCH
water 10	water 2.9 mg L^{-1}	93%	90%	88%
water 20	water 5.8 mg L^{-1}	94%	91%	93%
water 35	water 10.15 mg L^{-1}	95%	94%	94%
soil 10	soil 290 µg g^{-1}	91%	88%	86%
soil 20	soil 580 µg g^{-1}	92%	92%	92%
soil 35	soil 1015 µg g^{-1}	94%	92%	93%
grass sp-3 10 F2	grass 10 µg g^{-1}	38%	36%	66%
grass sp 1000 ug F2	grass 166 µg g^{-1}	39%	48%	40%
FO sp 10 F2	fish oil 10 µg g^{-1}	30%	21%	16%
FO sp10000 ug F2	fish oil 100 µg g^{-1}	26%	20%	18%
milk sp 1ug/g F2	milk 100 µg g^{-1}	40%	44%	33%
liver sp 10 F2	liver 10 µg g^{-1}	16%	25%	20%

361 **3.2. Effects of sample treatment on the stable isotope composition**

362 The HCH molecule contains C, H and Cl, which can be applied for compound-specific
363 stable isotope analysis. Experientially, similar quantity of HCH is required for reliable
364 $\delta^{13}C$ and $\delta^{37}Cl$ analysis, and 5 ~ 10 times higher quantity is needed for reliable $\delta^{2}H$
365 analysis. Therefore, higher spiked concentrations were applied in this study to ensure
366 reliable $\delta^{2}H$ analysis for method development. In order to analyze the isotope effects of
367 sample treatment, the $\delta^{13}C$, $\delta^{2}H$ and $\delta^{37}Cl$ values of HCHs after extraction from different
368 matrices (using the highest HCH concentration level in each matrix listed in Table 1)
369 were compared with the values of HCH standard before spiking to matrices. The
370 observed isotopic shifts ranged from 0.1 to 0.6‰ for $\delta^{13}C$, from < 1 to 10‰ for $\delta^{2}H$ and
371 from 0 to 0.27‰ for $\delta^{37}Cl$ (Table 2). Considering the acceptable analytical precisions of
372 ±0.5‰ for $\delta^{13}C$, ±5‰ for $\delta^{2}H$ [48] and ±0.3‰ for $\delta^{37}Cl$ [51], the shifts of the isotopic
373 values before and after HCH extraction from different matrices are in agreement with
374 uncertainty typically associated with the measurements. An exception was the $\delta^{2}H$ of the
375 HCHs extracted from the liver, which showed the highest deviation between -8 and +10‰
376 compared to the $\delta^{2}H$ values before extraction. The tendency for isotope enrichment or
377 depletion of ^{2}H was not constant and therefore the small variability is not a result of
378 systematic change by isotope fractionation due to the extraction procedure. Liver is the
379 most complicated matrix for extraction and the variability might be a result of impurities
380 from co-extracted organic substances, moreover, the $\delta^{2}H$ shift is still small compared to
381 large primary kinetic isotope effects expected for ^{2}H isotope fractionation of bond
382 cleavage reactions. Conclusively, negligible stable isotope fractionation could be
383 observed after extraction and separation of HCHs from applied sample matrices.

384 Physical extraction processes governed by sorption and phase partitioning where the
385 molecule remains intact are not expected to cause larger isotope effects [54]. Solvent
386 extraction is based on phase partitioning of the solute between phases and the isotope
387 effect of phase partitioning needs to be taken into account. However, the isotope effect of
388 phase partitioning at equilibrium conditions is relatively low [54] compared to kinetic
389 isotope effects, and the obtained offsets of isotope compositions in the experiments agree
390 reasonably well with expectations (Table 2). Hence the isotope effect of phase
391 partitioning may be negligible for HCHs. In contrast, transformation processes involving
392 chemical bond breaking could lead to kinetic isotope fractionation. Among the
393 procedures applied in the present study, HCH transformation processes could occur
394 during the saponification step using NaOH solution, since HCHs can be hydrolyzed
395 under alkaline condition [55]. However, the observed isotope shifts due to sample
396 treatment were within the analytical uncertainty, indicating that the isotope fractionation
397 occurred during the short time period of the saponification process was negligible.

398 Despite the achieved lower extraction recoveries, the main contribution of the present
399 study is to prove that the modified methods are able to extract and clean-up HCHs from
400 complicated matrices. More importantly, the applied extraction and clean-up procedures
401 do not cause considerable isotope effects, and therefore can be applied to investigate the
402 reactive transformation of HCHs in food webs.

403 **Table 2.** Changes in isotope compositions of HCHs after sample treatment procedures ($\Delta = \delta_{sample}$
404 $- \delta_{std}$).

sample		α-HCH	Δ	β-HCH	Δ	δ-HCH	Δ
HCH std	$\delta^{13}C$ (‰)	-29.3 ± 0.4		-28.8 ± 0.2		-28.8 ± 0.2	
water	[vs VPDB]	-29.1 ± 0.3	+0.2	-29.4 ± 0.3	-0.6	-28.9 ± 0.1	-0.1

soil		-28.7 ± 0.2	+0.6	-29.0 ± 0.1	-0.2	-28.9 ± 0.2	-0.2
grass		-29.7 ± 0.0	-0.4	-28.7 ± 0.4	+0.1	-28.4 ± 0.2	+0.4
fish oil		-29.5 ± 0.1	-0.1	-28.4 ± 0.1	+0.4	-28.3 ± 0.1	+0.5
milk		-29.3 ± 0.3	+0.1	-28.4 ± 0.2	+0.5	-28.2 ± 0.3	+0.6
liver		-29.8 ± 0.2	-0.4	-28.9 ± 0.0	-0.1	-28.7 ± 0.1	+0.1
HCH std		-102 ± 3		-112 ± 5		-105 ± 6	
water		-101 ± 3	+1	-110 ± 5	+2	-108 ± 4	-3
soil	δ^2H (‰)	-102 ± 1	<1	-113 ± 3	-1	-106 ± 0	-1
grass fish oil milk	[vs VSMOW]	-106 ± 5	-4	-112 ± 3	<1	-105 ± 8	<1
liver		-110 ± 4	-8	-102 ± 2	+10	-111 ± 4	-6
HCH std		-0.67 ± 0.14		-2.64 ± 0.13		-0.34 ± 0.28	
soil		-0.56 ± 0.06	+0.11	-2.57 ± 0.13	+0.07	-0.30 ± 0.18	+0.04
grass	δ^{37}Cl (‰)	-0.40 ± 0.03	+0.27	-2.50 ± 0.10	+0.14	-0.31 ± 0.05	+0.03
fish oil	[vs SMOC]	-0.55 ± 0.27	+0.12	-2.60 ± 0.15	+0.04	-0.34 ± 0.30	+0.00
milk		-0.47 ± 0.17	+0.20	-2.63 ± 0.08	+0.01	-0.26 ± 0.07	+0.08
liver		-0.42 ±0.10	+0.25	-2.59 ± 0.40	+0.05	-0.46 ± 0.43	-0.12

3.3. Evaluation of the isotope effects of matrices on ^{37}Cl analysis

The bottleneck for δ^{13}C and δ^2H isotope analysis is the clean-up step. Even purified samples may still contain interfering organic compounds that make baseline separation of chromatographic peaks and hence isotope ratio calculation a difficult task. In contrast, the analysis of δ^{37}Cl only requires baseline separation of chlorine-containing analytes because the MC-ICPMS measures chlorine ions directly. Therefore, chlorine-free organics should not affect the δ^{37}Cl analysis. In order to evaluate potential interferences of chlorine-free organics on δ^{37}Cl measurements, analytical standards of HCHs and different amount of diesel were dissolved in hexane to simulate a complex solvent matrix. The samples were first measured by GC-MS and then by GC-MC-ICPMS for δ^{37}Cl analysis. The GC-MS chromatogram indicated that HCH peaks were overlapped with the added compounds (Fig. S.1). Detailed information can be found in the SI section 4. However, when the same samples were analyzed by GC-MC-ICPMS, a clear base line separation could be achieved and no elevated background signal was visible in the

419 chromatogram (Fig. S.2). Furthermore, no significant changes in δ^{37}Cl values were
420 observed for HCHs dissolved in the diesel compared to HCHs dissolved in hexane (Table
421 S.3). These results indicate that the large amounts of carbon and hydrogen from the diesel
422 do not create interfering ions in the plasma that would otherwise affect the precise
423 analysis of chlorine isotopes. Thus, no rigorous clean-up procedure by column
424 chromatography was required for δ^{37}Cl analysis. The analytical precision was usually
425 below ±0.3‰ for compound-specific analysis of mixtures as reported before and with
426 high sensitivity typically in the range of 2-3 nmol Cl on column [51].

427 It is worth noting that introducing large amounts of hydrogen into plasma could have
428 resulted in forming $^{36}ArH^+$ dimers which would affect the $^{37}Cl^+$ signals. However, such
429 influences were not observed despite co-eluting hydrogen-containing compounds from
430 the diesel mixture and therefore $^{36}ArH^+$ formation was considered negligible. However,
431 extreme high hydrogen background from hydrocarbons is likely become relevant when
432 analyzing trace amount of chlorinated organic compounds. The detection limit of the
433 measurement was not evaluated in this study.

434 **3.4. Analysis of environmental samples**

435 Surface water and groundwater in the area of Bitterfeld (Germany) are heavily polluted
436 by HCHs, as already reported in several studies [9, 43]. A similar contaminated area can
437 be found in Lucknow, India [13, 44]. Concentration of HCHs in environmental
438 compartments and dispersion in the environment has been studied. However, studies
439 addressing the transformation of HCHs upon biodegradation in soil, degradation during
440 uptake into plant biomass as well as transformation to higher organisms are missing. For

441 analyzing transformation processes associated with reactive transport along the potential
442 food webs, HCH-contaminated soil, plant, cow/buffalo milk and dung, wild animal liver
443 and seal blubber were collected from 3 different contaminated sites for analysis.

444 The concentration of HCH residues (without consideration of extraction recovery) were
445 ranging from 0.007 to 117.4 µg g^{-1} for α-HCH (Fig. 1a) and from 0.01 to 48.7 µg g^{-1} for
446 β-HCH (Fig. 1b) in all analyzed samples. The lowest concentration of 0.007 µg g^{-1} was
447 determined from seal blubber obtained from the Arctic, and the highest concentration of
448 117.4 µg g^{-1} was determined from cow dung obtained from Lucknow. Experientially, the
449 minimum concentration of HCHs for reliable $\delta^{13}C$ analysis is 15 mg L^{-1} for injection
450 (assume at least 1.5 µg of HCH was extracted into 100 µL of solvent for measurement).
451 Similar quantity of HCH is required for reliable $\delta^{37}Cl$ analysis, and 5 ~ 10 times higher
452 quantity is needed for reliable $\delta^{2}H$ analysis. Therefore, only $\delta^{13}C$ and $\delta^{37}Cl$ values of α-
453 and β-HCH from contaminated samples were determined. The isotope composition of α-
454 HCH varied from -26.1‰ to -15.1‰ in $\delta^{13}C$ and from -0.86‰ to +4.33‰ in $\delta^{37}Cl$,
455 resulting in $\Delta\delta^{13}C$ = 11.0‰ and $\Delta\delta^{37}Cl$ = 5.19‰, respectively (Fig. 1c). The isotope
456 composition of β-HCH varied from -27.3‰ to -18.7‰ in $\delta^{13}C$ and from -2.19‰ to +4.21‰
457 in $\delta^{37}Cl$, resulting in $\Delta\delta^{13}C$ = 8.6‰ and $\Delta\delta^{37}Cl$ = 6.40‰, respectively (Fig. 1d).

458 Information on the extraction and clean-up performance can be obtained from the GC
459 chromatograms of HCH extracts from contaminated environmental samples (Fig. S.3).
460 Low interferences and good separation of HCHs from background matrices were
461 obtained for all extracted samples (Fig. S.3, a-d), except for seal blubber (Fig. S.3, e) due
462 to too low concentration of HCHs. Therefore, the $\delta^{13}C$ values of HCHs in seal blubber
463 were not performed. However, the large background inferences did not affect the

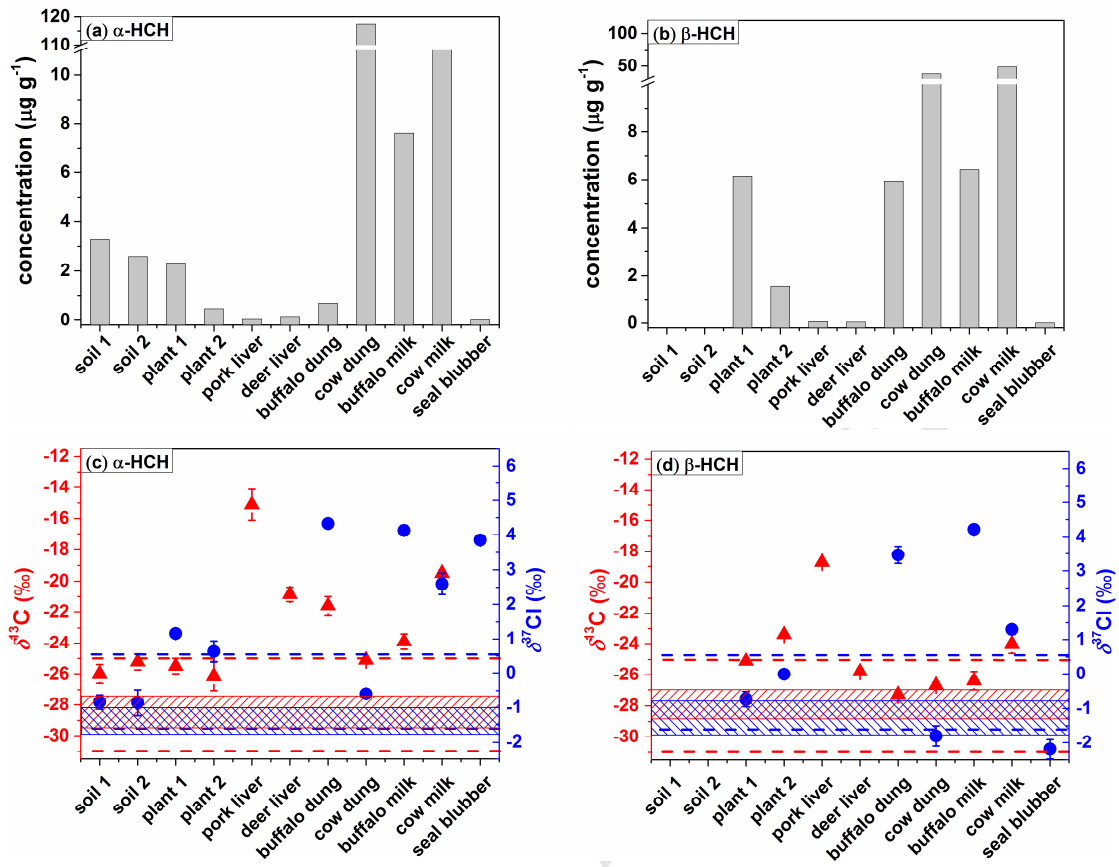
464 measurement of δ^{37}Cl even with such low HCH concentration (Fig. S.4). The δ^{37}Cl value
465 could, however, not be determined from pork and deer liver samples because too small
466 amounts of HCH extracts were left after δ^{13}C measurement. The δ^{13}C and δ^{37}Cl values of
467 β-HCH in soil were not determined due to the too high abundance of α-HCH (Fig. S.3, a),
468 making the separation of α- and β-HCH difficult.

469 **3.5. Proof of concept for studying transformation reactions of HCHs in food webs**

470 The shift in isotope composition of a sample compared to that of sources can be used to
471 characterize the degradation process and quantify the fraction of degraded contaminants
472 [23]. The soil, plant and animal liver samples were collected from the Bitterfeld site, and
473 the isotope composition of three HCH muck samples obtained as the sources of this site
474 resulted in an average value of −28.8 ± 1.2‰ (α-HCH) and −28.4 ± 1.3‰ (β-HCH) for
475 δ^{13}C, −1.38 ± 0.57‰ (α-HCH) and −1.27 ± 0.69‰ (β-HCH) for δ^{37}Cl, respectively.
476 Compared to the sources, the maximal enrichment up to 3.6‰ for δ^{13}C and 2.54‰ for
477 δ^{37}Cl of α-HCH from soil and plant (Fig. 1c) indicates that uptake of HCHs in plant is
478 associated with isotope fractionation, which may due to the biodegradation in the
479 rhizosphere or in the plants. The stronger carbon isotope enrichment of α-HCH, which
480 was up to 13.7‰ (Fig. 1c), in the pork and deer liver suggests intensive metabolism taken
481 place. The variability of δ^{13}C and δ^{37}Cl presented in Fig. 1 cannot be explained by
482 diffusion as lighter isotope is expected to become enriched when diffusion is a dominant
483 process.

484 The isotope composition of HCHs in the cow/buffalo milk and dung obtained from India,
485 and in seal blubber obtained from the Arctic were compared to the worldwide sources of

HCHs from a collection of 77 samples based on worldwide manufacturing [38]. The majority of $\delta^{13}C$ values of HCHs are between −31‰ and −25‰, within the range of $\delta^{13}C$ values of benzene from fossil stocks [56]. The majority of the $\delta^{37}Cl$ values of HCHs fall in the range between −1.60‰ and +0.54‰, close to the expected 0‰ for seawater chlorides and salts [57]. In contrast to the reported isotope composition of HCH sources, the obtained isotope values of HCHs in the milk, dung and seal blubber were much heavier (up to 5.5‰ for $\delta^{13}C$ and 3.79‰ for $\delta^{37}Cl$) even comparing with the upper limit of the reported source range (Fig. 1). The strong isotope enrichment in the dung and milk indicates that degradation of HCHs may take place in the digestive track of cow/buffalo during metabolism. The significant isotope enrichment of HCHs in the milk, liver and blubber further suggests that only a residual fraction was accumulated in the fat after intensive metabolism. The isotope enrichment in the residual HCH fraction reflects the metabolic degradation in the higher organisms. Degradation changes the concentration of HCHs in the organisms and hence the concentration of the residual fraction does not represent the exposure of the organisms adequately. The detected concentration is believed to reflect the accumulation of contaminants govern by phase partitioning of hydrophobic contaminants into the hydrophobic lipid. However, the isotope enrichment of the residual HCH fraction compared to possible sources indicates that the exposure of the organisms might be higher as a major fraction of the HCHs have been already degraded. Therefore, the isotope composition combined with the concentration of HCH in higher organisms could be used as an indicator to reconstruct exposure.



507

508

509 **Fig. 1.** Concentrations (a-b) and isotope compositions (c-d) of α-HCH and β-HCH from

510 contaminated soil, plant, cow/buffalo milk and dung, pork/deer livers and seal blubber. Gray bars

511 indicate the concentrations of HCHs. Red triangles and blue circles indicate the $\delta^{13}C$ and $\delta^{37}Cl$

512 values, respectively. The red and blue bars indicate the $\delta^{13}C$ and $\delta^{37}Cl$ values of HCH muck in

513 Bittefeld. The red and blue dashed lines indicate the $\delta^{13}C$ and $\delta^{37}Cl$ values of HCH sources from

514 former and recent manufacturing worldwide.

515 **4. Conclusion**

516 A method for extraction and clean-up of HCHs for an accurate and precise analysis of

517 $\delta^{13}C$, $\delta^{2}H$ and $\delta^{37}Cl$ in various sample matrices was evaluated. The extraction and clean-

518 up methods were applied for isotope analysis of α- and β-HCH in contaminated

519 environmental samples in order to evaluate potential application for analysing reactive

transport in food webs. The multi-element isotope analysis offers the opportunity for the identification of chemical and biological transformation processes in the environment [23], as well as obtaining information on dehalogenation reactions [58]. Thus, it can provide evidence for developing a concept for studying multi-element isotope fractionation to characterize degradation processes in the food web at field sites. Based on the Rayleigh approach, significant isotope enrichment combined with specific stable isotope enrichment factors can be applied to quantify the *in situ* degradation of contaminants. For example, based on the carbon isotope enrichment of HCHs and an enrichment factor for microbial degradation, Bashir et al. estimated that biodegradation contributed to 30 to 86% of HCH removal within a contaminated aquifer at Bitterfeld/Wolfen [35]. The same approach using CSIA holds great potential to quantify the reactive transport of HCHs from soil to plants, and to higher organisms. Correlation of hydrogen, carbon and chlorine isotope fractionation may be used to identify bond cleavage reactions of degradation processes of halogenated contaminants [59-61]. The method has potential for isotope forensics, monitoring of HCH in food webs, characterizing HCH degradation at contaminated sites as well as application in risk assessment and public health studies.

Notes

Conflicts of interest: none.

Color will not be used for any figures in print.

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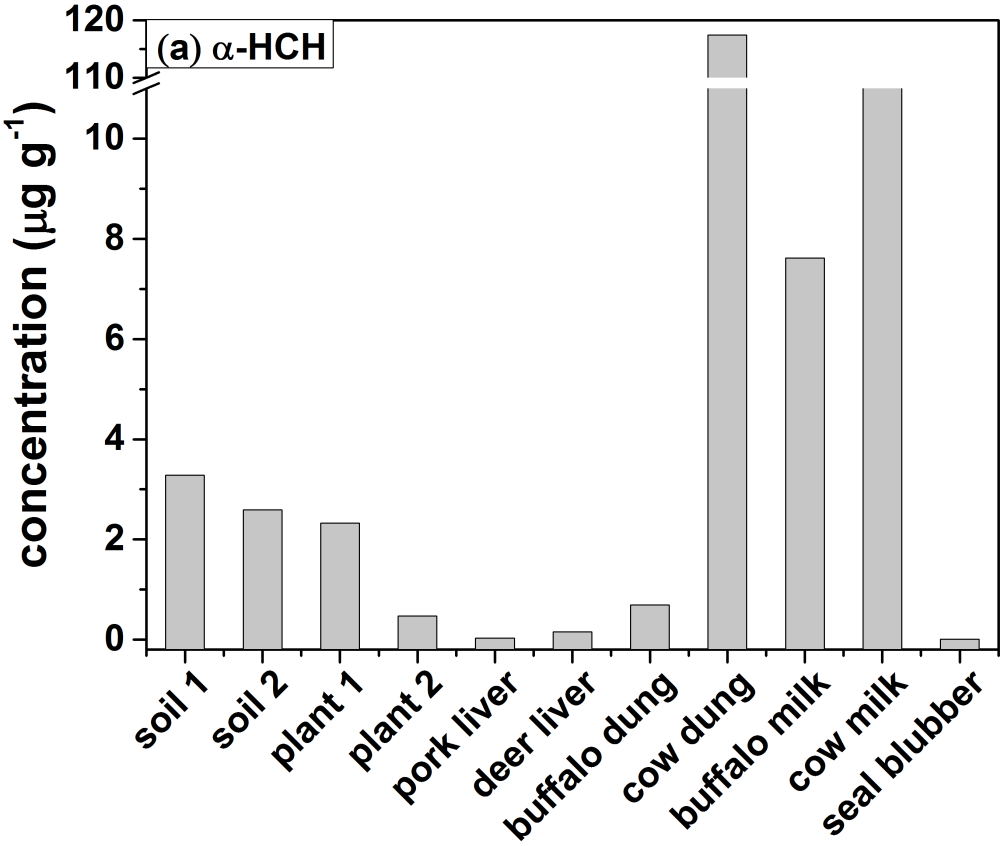
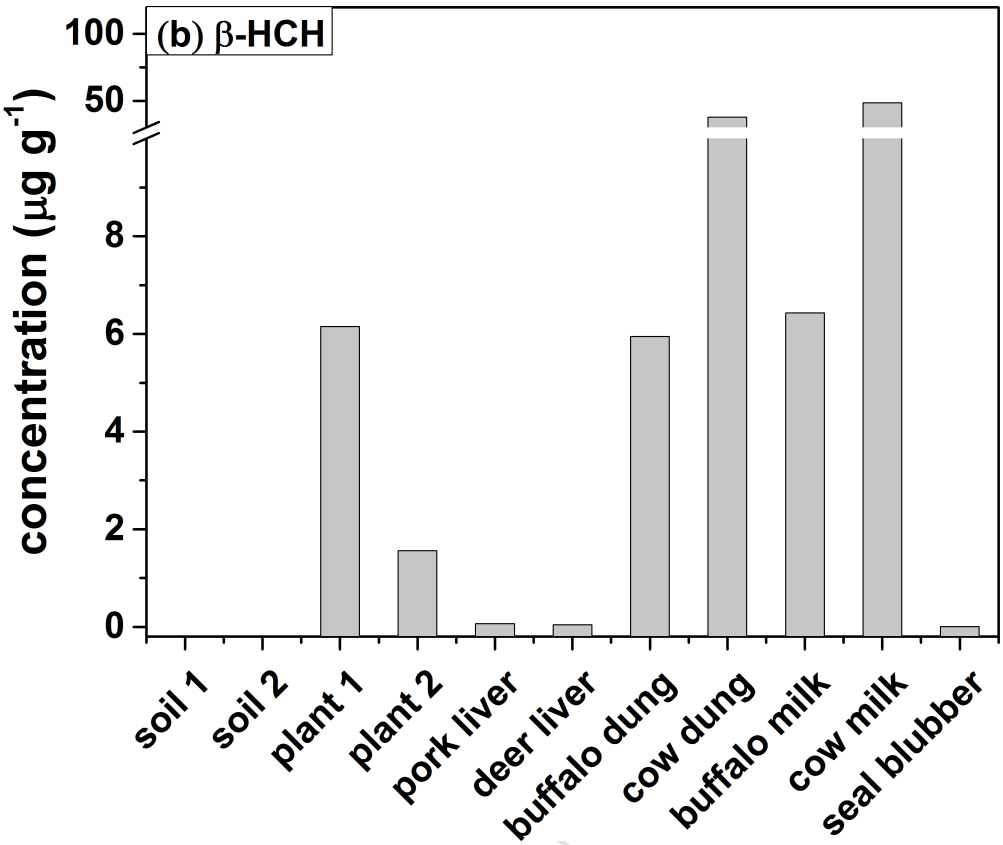
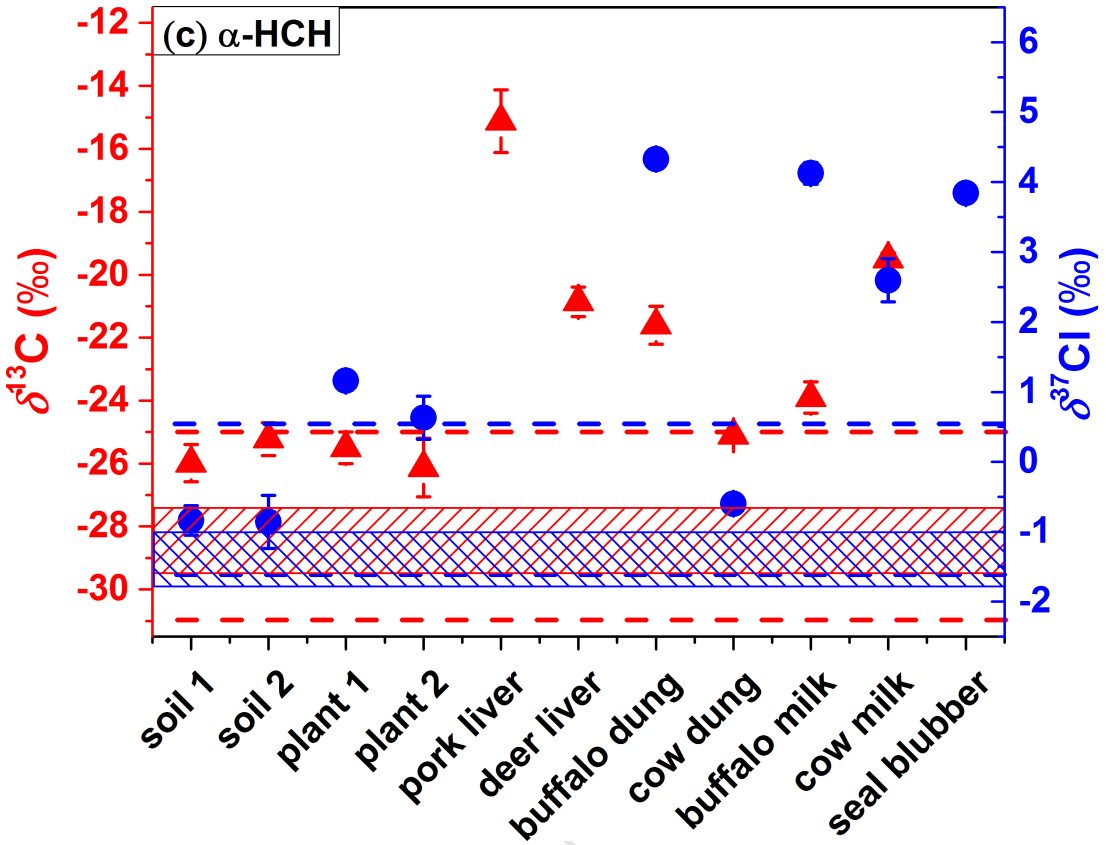
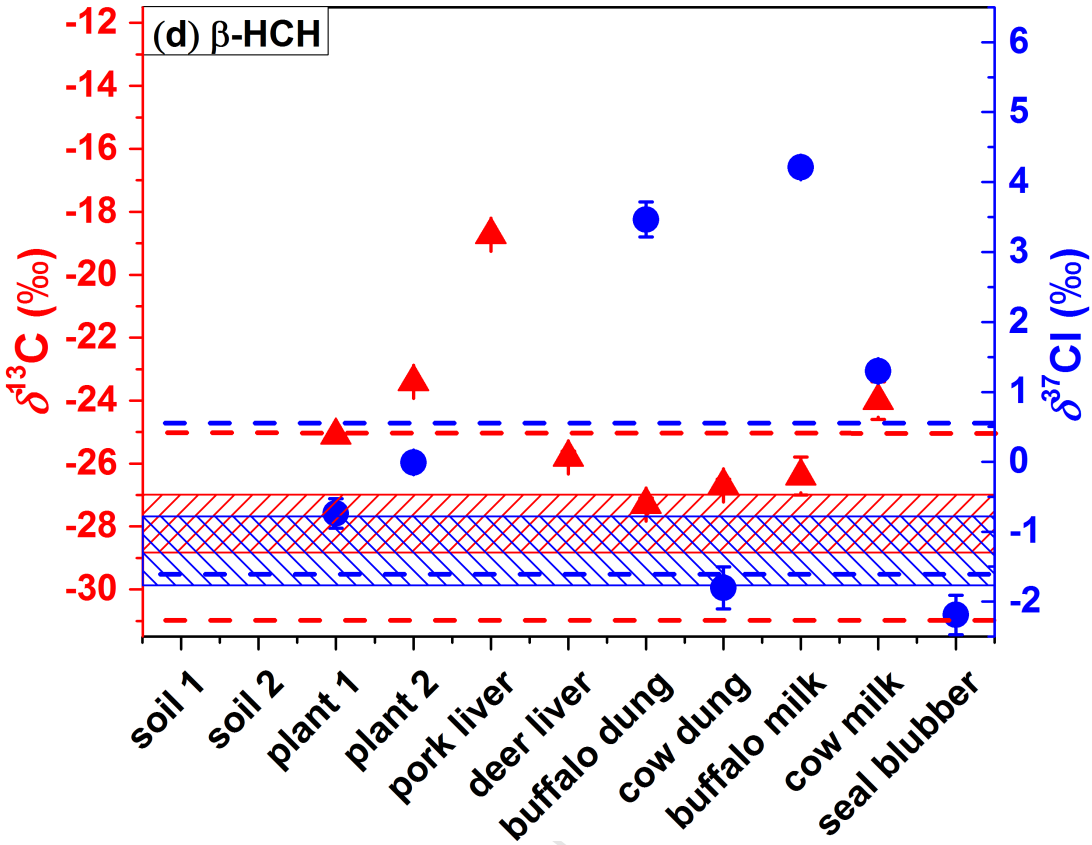

(a) α-HCH
concentration (μg g-1)
120
110
10
8
6
4
2
0
soil 1
soil 2
plant 1
plant 2
pork liver
deer liver
buffalo dung
cow dung
buffalo milk
cow milk
seal blubber


(b) β-HCH
concentration (μg g-1)
100
50
8
6
4
2
0
soil 1
soil 2
plant 1
plant 2
pork liver
deer liver
buffalo dung
cow dung
buffalo milk
cow milk
seal blubber





Highlights

- Concept for analysing degradation reactions of organic contaminants in food webs using multi-isotope analysis ($\delta^{13}C$, $\delta^{2}H$, $\delta^{37}Cl$)
- Developing of an extraction methods for isotope analysis of HCH in soil, plant, milk, fish oil and liver samples
- Compound-specific isotope analysis (CSIA) of $\delta^{13}C$, $\delta^{37}Cl$ isotope composition of HCH in food webs
- Evidence for isotope fractionation of HCH ($\delta^{13}C$, $\delta^{37}Cl$) during up-take into plants
- Evidence for isotope fractionation of HCH ($\delta^{13}C$, $\delta^{37}Cl$) during passage through ruminants with food stocks

Declaration of interests

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

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

In behalf of all authors I declare no conflict of interest.

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Hans Richnow