Compound-Specific Stable Isotope Analysis: Implications in Hexachlorocyclohexane in-vitro and Field Assessment

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Abstract Assessment of biotic and abiotic degradation reactions by studying the variation in stable isotopic compositions of organic contaminants in contaminated soil and aquifers is being increasingly considered during the last two decades with development of Compound specific stable isotope analysis (CSIA) technique. CSIA has been recognized as a potential tool for evaluating both qualitative and quantitative degradation with measurement of shifts in isotope ratios of contaminants and their degradation products as its basis. Amongst a wide variety of environmental pollutants including monoaromatics, chlorinated ethenes and benzenes etc., it is only recently that its efficacy is being tested for assessing biodegradation of a noxious pollutant namely hexachlorocyclohexane (HCH), by pure microbial cultures as well as directly at the field site. Anticipating the increase in demand of this technique for monitoring the microbial degradation along with natural attenuation, this review highlights the basic problems associated with HCH contamination emphasizing the applicability of emerging CSIA technique to absolve the major bottlenecks in assessment of HCH. To this end, the review also provides a brief overview of this technique with summarizing the recent revelations put forward by

both in vitro and in situ studies by CSIA in monitoring HCH biodegradation.

Keywords Hexachlorocyclohexane · Isotopes · Biodegradation · Fractionation · Contaminant

Introduction: HCH (a felonious POP)

The term persistent organic pollutant (POP) refers to the hydrophobic and lipophilic chemicals polluting the environment steadily with an average half life of a few decades in soil or several days in the atmosphere. Over the last six decades POPs have been responsible for causing major environmental contamination not just limited to the sub-surface and other environmental compartments but progressively affecting humans and other biota on earth. In a recent extension of the already addressed list of 12 POPs by the Stockholm Convention, three amongst the latest ten to be added are constituted alone by the isomers of Hexachlorocyclohexane (HCH) [1]. Of the 209 theoretically cited polychlorinated biphenyls and other chlorine substituted POP's, HCH marks among one of the aberrant agrochemicals to be synthesized industrially [2].

Hexachlorocyclohexane refers to the chlorine substituted saturated cyclic six carbon compound produced as a mixture of eight isomers consequential from the photochlorination of benzene. This reaction archetypically starts via free-radical activation by radiation with ultraviolet light [3]. Ever since the synthesis of HCH in 1823 by Faraday and discovery of its insecticidal properties later in 1943, this tarnished chemical has been posing serious environmental hazards [4]. Industrially manufactured technical HCH (t-HCH) contains a mixture of five stable isomers α -, β -, γ -, δ -, and ϵ -HCH, primarily

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dominated by 60–70% of α -HCH [3]. Of these isomers it is only the γ -HCH (10–12% of t-HCH, also known as Lindane) that harbors the significant insecticidal properties [5]; however HCH isomers as an assemblage are noxious and potential carcinogens [6, 7]. Depending upon the method of application of t-HCH along with soil and environmental conditions, its half life in soil has been accounted to differ from few weeks to 260 days and even up to 2 years or more [8, 9]. Purification of insecticidal γ -HCH from t-HCH remains to be highly inefficient process leading to production of nearly 8–12 tons of waste (α -, β -, δ -HCH isomers) for every ton of γ -HCH produced [3].

Initial incoherent application of this broad spectrum recalcitrant pesticide to kill insects, worms, pests in agriculture, forestry & livestock to treatment of scabies in humans [10], along with its unregulated disposal from the industrial production units guided by the moderate volatility and its transport to remote location; HCH contamination has led to creation of a global problem that continues to persist till date [1, 3, 10, 11]. The magnitude of the problem generated by HCH contamination can be assessed merely by observing the near 4.8 million tons of HCH residues produced and disposed from the world wide usage of 600,000 tons of Lindane during the years 1950–2000 [3, 11, 12]. Intense toxicity of HCH isomers to mammals has been substantiated with reports validating it as the causative agent for liver cancer in mice and rats; in addition to this its chronic exposure has been reported to cause neurological deformities and immune suppression in humans [13, 14]. Amongst the stable isomers of HCH, α -HCH exhibits the utmost amount of carcinogenic activity leading to its designation as Group B2 possible human carcinogen along with t-HCH by USEPA [14] and β -HCH prevails to be the one with enormous tendency for bioaccumulation in human tissues [15]. High persistence, toxicity of HCH isomers along with its unregulated production and disposal are the prime reasons leading to its ban in nearly 52 countries with strict restrictions in 33 countries [16]. Thus, the degradation of HCHs by microbes and the remediation of these isomers from the HCH contaminated dumpsites is an issue that has been raised from time to time [1, 3, 9, 15, 17].

HCH Isomers Persistence, Degradation and Detection: a Problem

The solubility, sorption and volatility are the major factors that decide the recalcitrance of various HCH isomers. Table 1 summarizes the physio-chemical properties of HCH isomers that play a prominent role in their volatilization, transport in the atmosphere and microbial degradation. The persistence of HCH isomers tends to vary in soil, air and water depending upon the interaction of

these isomers with the environmental matrices. Once applied as pesticide, HCH isomers have been observed even to persist in soil for more than 15 years [6, 9, 15]. Very high amounts of HCH isomers are still found in soils and aquifers of the former production areas for example in Sachsen or Bitterfeld, Germany [18, 19] and Lucknow, India [20]. Large amount of HCH isomers left out after the purification of Lindane have been deposited at all former production sites from where it leaches into the water sheds and is eventually transported through food chain into the food web [3]. The worldwide distribution of HCH isomer is indicated by accumulation of HCH in food webs of even remote arctic and antarctic regions [21].

The orientation of chlorine atoms in α -, γ -, δ -HCH structure govern their perseverance to biodegradation (Table 1). The presence of axial chlorine atom in α -, γ -HCH makes them accessible for microbial enzymatic degradation as compared to δ -HCH with majority of equatorial chlorine atoms. Substitution of all the equatorial positions with chlorine atoms in case of β -HCH results in the increased structural stability & thus highest persistence in the environment [3, 30–32].

Although there are reports on abiotic degradation of HCH isomers, however it contributes to only a minute fraction when compared to microbial biodegradation. HCH isomers dissolved in water have been reported to be susceptible to chemical degradation in the presence of sunlight, with varying degree of degradation depending on temperature [9, 30, 33]. In addition to this the alkaline conditions have been stipulated to supplement chemical degradation of HCHs [9, 33]. Based on the initial studies on microbial degradation of HCH and its isomers in flooded soils, soil slurries and soil microcosms; biodegradation of HCH was considered to be an anaerobic process [3, 9, 30, 31, 33]. However, with increased studies incorporating the assessment of redox potential during microbial degradation, studies in soil plots of field and those with pure cultures [3, 9, 30] established the subsistence of a more rapid aerobic degradation of HCHs. Perhaps it is only under these oxic conditions that complete mineralization of HCHs has been reported to occur [3, 9, 30–32, 34].

Anaerobic and aerobic microbial degradation of HCH had been established with initial enrichment studies documenting the bacterial isolates capable of utilizing HCH as the sole source of carbon. Members belonging to the genus *Clostridium* were first amongst the bacterial species characterized to anaerobically degrade HCH, followed by few aerobic degraders constituted by *Pseudomonad* species and majority of aerobic *Sphingomonad* species [3, 9, 30, 35–43]. Perhaps it is in this sphingomonadaceae family member i.e. *Sphingobium japonicum* UT26 that the entire pathway of aerobic degradation of γ -HCH was traced out along with the intricate Lin enzyme system underlying the degradation

Table 1 Physical and chemical properties of HCH isomers

Physical and Chemical properties of HCH isomers				
Characteristic	α -HCH	β -HCH	γ -HCH	δ -HCH
Structure: A:Axial/E:Equatorial Chlorine
	(AAAAEE)	(EEEEEE)	(AAAE EE)	(AE EEEE)
Physical state	Monoclinic prisms ^a	Cubic crystals ^a	Monocyclic crystals ^a	Crystals or fine platelets ^a
Color	Brownish to white ^b	–	White ^a	–
Odor	Phosgene like ^b	–	Musty ^b	–
Molecular weight	290.83 ^d	290.83 ^d	290.83 ^d	290.83 ^d
Refractive index	1.60–1.626 ^a	1.63 ^a	1.60–1.635 ^a	1.576–1.674 ^a
Boiling point (°C)	288 at 760 mm Hg ^c	60 at 0.5 mm Hg ^c	323.4 at 760 mm Hg ^c	60 at 0.36 mm Hg ^c
Melting point (°C)	159–160 ^c	314–315 ^c	112.5 ^c	141–142 ^c
Density (g/cm ⁻³)	1.87 at 20 °C ^c	1.89 at 19°C ^c	1.89 at 19°C ^c	–
Solubility				
Water (mg/L)	10 ^c	5 ^c	Insoluble ^c , 17 ^c	10 ^c
Organic solvents (g/100 g of solvent)				
Ethanol	1.8 ^c	1.1 ^c	6.4 ^c	24.4 ^c
Ether	6.2 ^f	1.8 ^f	20.8 ^f	35.4 ^f
Benzene	–	1.9 ^f	28.9 ^f	41.4 ^f
Partition coefficients				
Log K _{ow}	3.8 ^c	3.78 ^c	3.72 ^c	4.14 ^c
Log K _{oc}	3.57 ^{c,h}	3.57 ^{c,h}	3.0 ^g , 3.57 ^h	3.8 ^{c,h}
Vapour pressure	4.5 × 10 ⁻⁵ at 25 °C ^c	3.6 × 10 ⁻⁷ at 20 °C ^c	4.2 × 10 ⁻⁵ at 20 °C ^c	3.5 × 10 ⁻⁵ at 25 °C ^c
Henry's Law constant	6.86 × 10 ^{-6b}	4.5 × 10 ^{-7b}	3.5 × 10 ^{-6b}	2.1 × 10 ^{-7b}

^a [22], ^b [23], ^c [24], ^d [25], ^e [26], ^f [27], ^g [28], ^h [29]

[3, 31, 44, 45]. Figure S1 symbolizes the major intermediates from the aerobic degradation of γ -HCH. In the environment it is these intermediates along with the HCH substrates that aid in tracing the prominent active pathways. Cell suspensions of different sulfate reducing bacteria has been reported with ability to dehalogenate γ -HCH [9, 46]. Anaerobic transformation reactions in sludge and soils have been revealed to dehalogenate α -, β -, γ - and δ -HCH to chlorobenzene and benzene. Anaerobic degradation of HCH leads to the generation of mixed chlorobenzenes and benzenes as the major products from successive dichloroelimination and dehydrochlorination reaction which can be further mineralized completely under aerobic conditions [3, 9, 30, 31, 44, 45].

With physical degradation of HCH isomers being negligible, these individual bacteria that are capable of HCH degradation or their consortia have been implemented to degrade HCH under laboratory conditions amidst very few attempts extending their application into the HCH dumpsites

with an aim to develop cost-effective remediation strategies [3, 42, 47]. A major bottleneck that is currently encountered is the lack of proper techniques to monitor bioremediation processes along with natural attenuation at the HCH dumpsites. The traditional techniques of extraction and analysis by the thin layer chromatography and gas liquid chromatography cannot be extended effectively to field situations especially at the HCH dumpsites. Thus, in order to combat efficiently the problems of HCH contamination it becomes essential to assess and recurrently evaluate the extent of biodegradation & intrinsic attenuation. Until now the major assessment for monitoring the success of in situ biodegradation has been limited to the measurement of concentration of the pollutant without appropriately considering the decrease in concentration resulting from the natural physical processes of sorption & volatilization. Thus, providing only indirect evidence and urging the need for advanced monitoring techniques with a possibility to clearly demarcate the extent of degradation to the various processes active in the field [48].

Amongst all the methods that are available today for assessing the degradation of HCH isomers, compound specific stable isotope analysis (CSIA) appears to be the most promising technique developed within the last two decades [49, 50]. This technique aims to measure the stable isotope ratios in comparison to standard compounds and is serving as a potential technique for successful assessment of biodegradation of organic contaminants in aquifers [49]. CSIA built over the pedestal of highly precise GC-C-IRMS technique provides a significant method for assessing the contaminant removal from the environment. It actuates around measurement of stable isotopic composition of pollutants and their degradation products and is based upon the preferential transformation of molecules consisting of light isotopologues compared to and heavy isotopes during the biodegradation and thus, leading to the enrichment of heavy isotopologues (molecules enriched in heavier isotopes) in the residual (non-degraded) fraction during the course of biodegradation.

Complex aquifers contaminated with a wide spectrum of environmental contaminants including n-alkanes, monoaromatics (benzene, toluene), methyl/ethyl tert-butyl ether, polycyclic aromatic hydrocarbons (PAH), and chlorinated hydrocarbons such as chlorinated ethenes, chlorinated benzenes [49, 51–55], HCH [19] and others have been successfully assessed for the in situ degradation by the application of CSIA. In addition to natural attenuation of the contaminants, the isotope compositions has been used to analyze sources of contaminants at complex field site as the productions leave a characteristic finger print [56].

Amidst these contaminants, HCH isomers are often the most disregarded ones with a very few reports on carbon and chlorine isotope fractionation supplemented with only a limited data [19, 57–61]. Because of their high persistence, toxicity and unusual process of production which have generated large amounts of waste, HCH isomers stand out to be ideal for studying insights into their potential management. During the first one decade several attempts have been made to use this technique to study the degradation of HCH isomers. This review therefore, focuses on CSIA technique with emphasis on monitoring HCH degradation, its application to the biological degradation and how it has extended the precinct for future exploration and applications for assessing HCH bioremediation.

Stable Isotopes and CSIA

Most of the natural chemical elements exist in isotopic forms with variations in the number of neutrons present in their atomic nuclei; of the 92 elements occurring in nature, only 21 elements exist in pure state without any isotopic

form [62, 63]. Isotopes are marked by the variability in the stability of their nuclide; broadly classified as stable isotopes and unstable or radiogenic isotopes. Stable isotopes constitute approximately only one-fourth of the proportion of total isotopes [64]. Radioisotopes have a tendency to decay over time depending on the state of their nuclide, the energy resulting from the disintegration has found wide scale industrial and medical application, in addition to the usage of their unique decay pattern over time for geological time scaling [65–67]. Fractionation (asymmetric partitioning) ensuing from the preferential utilization of stable isotopes during the biological processes pretexts for one of the major source for the variation in isotope composition [64]. Perhaps not just limiting to the chemical compounds, this unique phenomenon of isotope variation extends to the organic contaminants whereby the ratio of their stable isotopes (heavier to lighter) can be pragmatic for deriving environmental remunerations including source allocation of contaminants, identifying chemical and biological transformation reactions along with quantification and characterization of the mechanism that govern the decay of these compounds [68].

Natural occurrence of these stable isotopes is very low and further measurement of alteration in their ratios requires very precise and careful consideration with the aid of specific instrumentation. Of the reported stable isotopes, isotopes of hydrogen (D/H), carbon ($^{13}\text{C}/^{12}\text{C}$), nitrogen ($^{14}\text{N}/^{15}\text{N}$), oxygen ($^{18}\text{O}/^{16}\text{O}$), sulfur ($^{32}\text{S}/^{34}\text{S}$) & chlorine ($^{35}\text{Cl}/^{37}\text{Cl}$) are amongst the foremost ones to be implemented for compound specific stable isotope analysis as these elements are environmentally pertinent and have at least two stable isotopes with a pronounced abundance of lighter isotope [64, 68, 69]. For measuring isotopic fractionation in case of volatile organic compounds, the instrumentation is gas chromatograph (GC), connected via a combustion/oxidation (C) or pyrolysis (HTC) unit to an isotope ratio mass spectrometer (IRMS). First described by Mathews and Hayes et al. [70] this technique has been subsequently improvised upon by many others with immense technical advancements [68, 70–72]. Development of GC coupled to a combustion oven and followed by IRMS has thus allowed for analysis even at very trace levels. Increasing success of this technique has even led US-EPA, 2008 to accredit it as a potential method for not only qualitative but also quantitative assessment of biodegradation at contaminated field sites [73] (Fig. 1).

CSIA takes into consideration the ratios (R) of stable isotopes of organic contaminants, which are usually expressed as concentration of heavy isotope (^hc) divided by the concentration of lighter isotope (^lc) [$R = ^h\text{c}/^l\text{c}$] and hence the total concentration (^tc) is defined by the sum of these two: $^t\text{c} = ^h\text{c} + ^l\text{c}$ [68]. The isotope compositions of

Fig. 1 Schematic representation of the common Gas Chromatography (GC) interface for carbon isotope measurements (as CO₂) with a basic layout of an Isotope ratio mass spectrometer (IRMS)

these compounds are depicted as ratios relative to the international standards in δ notations representing, parts per mill or thousand ($^0/_{00}$) values (Table 2).

Isotope Effect

Mass differences amongst the isotopes of the contaminants lead to slight disparity in their physical and chemical properties, these variations are known as isotope effects, primarily depending on mass thus, also referred to as mass-dependent isotope effects. Both biotic and abiotic processes lead to unequal/uneven distribution of different isotopes of

same element in various compounds leading to isotope fractionation. The rationale behind isotope fractionation lies in isotopic divergence occurring because of the variation in the zero-point energies (ground state energy) of heavy and light molecules. Unique ratio of isotopes in the compounds gives an indication towards the process underlying their formation channeled by the vibrational energies of the molecules involved and thus becomes significant in identifying the underlying biotransformation reaction. Isotopes with higher masses are associated with lower vibrational frequencies and hence lower zero-point energy, thus accounting for a greater amount of energy

Table 2 Common stable isotopes with percent abundance and respective standards for measurement*

Element	Stable Isotopes (Heavy/Light)	Percent Abundance of Heavier Isotope	Conversion Gas	International standards	Values
Hydrogen	D/H ($^2\text{H}/^1\text{H}$)	0.015	H_2	Standard mean ocean water (SMOW)	0.0001
Carbon	$^{13}\text{C}/^{12}\text{C}$	1.11	CO_2	Pee Dee Belemnite (PDB) & Vienna PDB (V-PDB)	0.011
Nitrogen	$^{15}\text{N}/^{14}\text{N}$	0.366	N_2	Air Nitrogen (Air)	0.003
Oxygen	$^{18}\text{O}/^{16}\text{O}$	0.204	CO	SMOW	0.0020
	$^{17}\text{O}/^{16}\text{O}$	0.037			0.001
Sulfur	$^{34}\text{S}/^{32}\text{S}$	4.21	SO_2	Troilite (FeS) from the Canyon Diablo Iron Meteorite (V-CDT)	0.044

* [49, 50, 68, 76, 83]

constraint during a reaction. However, the lighter isotopes with a comparatively higher vibrational energy react without difficulty due to lower energy restraint [49, 68, 74].

Equilibrium isotope exchange reactions and kinetic effects are two broad categories confining the isotope effects and leading to isotopic fractionation. Equilibrium isotope exchange reactions represent the set of reactions corresponding to the closed system at chemical equilibrium (e.g. CO_2 dissolution in water). At a specified temperature isotopic equilibrium only entails for constant ratio of different isotopes of each compound on either side of the reaction and does not refer to equal concentration of isotopes. Kinetic effects on the other hand, signify the isotope effects accompanying the unidirectional irreversible reactions [74]. Although both kinetic and equilibrium isotope fractionation processes are functioning in the environment, it is the kinetic effect that essentially directs the environmental attenuation processes governed by biodegradation [49, 75, 76]. Physical processes of phase partitioning, volatilization or diffusion are accompanied with equilibrium isotope effects which are smaller in magnitude than the kinetic isotope effects observed during biodegradation and thus the change in isotope composition can be used as a proxy for chemical or biological degradation.

Biological processes underlying the microbial degradation of contaminant are associated with cleavage of chemical bonds which are mostly unidirectional and hence lead to kinetic isotope fractionation reaction. It is primarily the path by which the reaction proceeds during degradation, the rate by which the reaction occurs along with the relative energies of the bond involved in the reaction that governs the magnitude of the kinetic isotope fractionation. During the biodegradation of contaminants, the organisms tend to devour the isotopologues with lighter isotope composition mainly because of the lower energy requisite as compared to the heavier ones. This preferential transformation of lighter isotopomers of the contaminant during degradation thus leads to a fractionation between the initial heavier substrate and the probable lighter intermediate species.

Delta Notation

δ represents the special notation that is used to express the isotope values and symbolizes the expression as the difference in the measurement with respect to the standard. This involves multiplication by a factor of 1000 so as to intensify the miniscule differences between the sample and standard isotope values.

$$\delta^{\text{H}}\text{X} = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \right]$$

$$\text{R} = {}^{\text{h}}\text{C}/{}^{\text{l}}\text{C}$$

where X represents the element, H signifies the heavy isotope and R defines the ratio of heavy isotope to light. For carbon the above equation can be written as:

$$\delta^{13}\text{C} = \left[\left(\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right) \times 1000 \right]$$

Fractionation factor (α)

α is implemented to absolve the isotopic fractionation (equilibrium or kinetic fractionation) ensuing from the demeanor of isotope effects during a chemical or physical process. It overcomes the ambiguities resulting from the application of equilibrium and kinetic constants to define isotope fractionation between the substances, as the equilibrium constant mainly depends on the formulation of a reaction that tends to vary. Fractionation factor basically corresponds to a ratio of ratios intimating isotope fractionation [49, 50, 68].

Fractionation Factor:

$$\alpha = \frac{R_{\text{reactants}}}{R_{\text{products}}}$$

where $\text{R} = {}^{\text{h}}\text{C}/{}^{\text{l}}\text{C}$.

For Equilibrium reactions:

$$\alpha = K^{1/n} = \frac{R_{reactants}}{R_{products}}$$

where K represents the equilibrium constant and n refers to the number of atoms involved.

For Kinetic reactions:

$$\alpha = \frac{k_{heavy}}{k_{light}}$$

where k represents reaction rates for heavy and light isotopes respectively.

Another factor that is applied to quantify the enrichment of the heavier isotope eminent from isotopic fractionation flanked by the substrate and its degradation product is defined by the enrichment factor (ϵ) according to the following equations [50, 68].

$$\begin{aligned}\alpha(\text{product} - \text{reactant}) &= \frac{R(\text{product})}{R(\text{reactant})} \\ &= (10^{-3}\delta_{\text{product}} + 1) / (10^{-3}\delta_{\text{reactant}} + 1)\end{aligned}$$

$$\epsilon_{\text{product} - \text{reactant}} = (\alpha - 1) \times 1000.$$

Modeling and Quantifying the Isotopic Fractionation During Degradation

Initially modeled to outline the fractional distillation of binary liquid mixtures (Rayleigh [77], derived by Lord Rayleigh), Rayleigh equations have been profoundly applied to mathematically model the isotopic fractionation resulting from microbial degradation of organic contaminant [77]. It precisely correlates the changes in contaminant concentration to those observed in the composition of isotopic signatures resulting from biotransformation reactions, thus providing information regarding the degree of intrinsic transformation. Approximations of the original Rayleigh equations are comprehended to exponentially relate the isotopic partitioning between the residual and substrate fractions [49, 68, 76, 77].

Application of CSIA for Assessing HCH Degradation

With continuing problems of toxicity and persistence of HCH isomers in various environmental matrices erupting from the post-production stockpiles and heavily contaminated dumpsites, bioremediation strategies including enhanced natural attenuation are proving to be instrumental in reclaiming these contaminated sites. Many studies have

reported to remediate HCH contaminated sites with either application of biostimulation and bioaugmentation or both approaches with mere decrease in concentration of HCHs being the major parameter for claiming success, however, the decrease in concentration can also be caused by physical process, such as dispersion, diffusion, and adsorption. But these steps are not leading to actual transformation of HCHs in the environment. Isotope fractionation is the result of bond cleavage indicating degradation of the substrate. Thus for contaminated site, CSIA is a more sufficient method for the evaluation of degradation.

On the other hand, bioremediation studies by way of HCH degrading bacteria [3, 42] and microbial degradation experiments as their basis have been employed for studying degradation at the contaminant sites. These in vitro laboratory based microbial studies although actively demonstrate the degradation potencies of microbes however; they remain unable to replicate the actual conditions at the dumpsite. Thus, only provide indirect evidence and lack the ability to affirm similar result in situ. With application of CSIA in situ levels of the contaminant residues can be accessed directly and thus, providing proxies for a quantitative evaluation. This method also makes it possible to trace the source and sinks of the contaminant by measuring the isotopic signature molecules [49, 50, 68].

Recent Revelations by CSIA in HCH Degradation

Recent reports with reference to HCH have confirmed reductive dechlorination to be the foremost degradation process active under anaerobic conditions in comparison to the aerobic ones being dominated by biodegradation and photochemical degradation. Laboratory based in vitro study performed by Badea et al. [57] to obtain carbon isotope fractionation factors for HCH using sulfate reducing strains competent of anaerobic co-metabolic γ -HCH degradation, clearly demonstrated significant isotope fractionation factors of 1.0040 ± 0.0002 ($\epsilon_C = 4.0$) for *Desulfococcus gigas* and 1.0034 ± 0.0002 ($\epsilon_C = 3.4$) for *Desulfococcus multivorans* with reductive dechlorination to be the persistent pathway under anoxic conditions [57]. Taking this as reference further studies with isotopic signatures at comparative zone and depths can be substantial in determining the origin and source of contamination.

Aerobic degradation studies with *Sphingobium indicum* B90A and *Sphingobium japonicum* UT26 strains performed by Bashir et al. [59] reported similar isotopic enrichment factors for both the aerobic species along with further elucidating the dependency of isotopic fractionation on degradation rates. The aerobic degradation experiments by these two sphingomonads were found to yield significantly lower fractionation factors -1.7 ± 0.2 to -1.5 ± 0.1 for γ -HCH and -1.0 ± 0.2 to -1.7 ± 0.6 for

Fig. 2 Bar graph representing the comparative profile of enrichment factors observed from the anaerobic, aerobic and chemical degradation of bulk α -HCH, (+)/(–) α -HCH enantiomers and γ -HCH as per the data represented in Badea et al. 2011, Badea et al. 2009, Bashir et al. 2013, Zhang et al. 2014, Supplementary Table SI

α -HCH besides revealing evident enantiomer specific fractionation in case of aerobic α -HCH degradation [59]. This study further supplemented that the isotopic fractionation remains unchanged even at variable lower temperature ranges (10–30 °C) and thus is suggestive to the relative low fractionation of carbon isotopes being attributed only to the reaction mechanism underlying degradation. The fractionation might be robustly characterizing the degradation process typically for the temperature range of soils and aquifers.

As a step further, Zhang et al. [78] have investigated the variability in the enrichment factors systematically from the transformation of α -HCH by environmentally potentially possible chemical reactions including direct photolysis, oxidation by OH radicals formed photochemically, alkaline hydrolysis, electrochemical reduction and reduction by Fe⁰ nanoparticles with an attempt to characterize the steps in degradation pathway. The chemical reactions yield different fractionation factors as compared to biological reactions with a marked absence enantiomeric fractionation as it was expected for chemical reactions [78]. This study provides a blueprint that can be used as future references for assessing the carbon stable isotope fractionation observed during in situ transformation in combination with the enantiomer specific data to distinguish between the prominent chemical and biological processes in field studies (Fig. 2).

Natural attenuation of HCHs remains to be a major source of intrinsic remediation in addition to the physical processes of volatilization, dispersion and sorption. A substantial reduction in mass of HCH contaminants in the environment results from the microbial biodegradation and thus it becomes essential to monitor and quantify the biodegradation in order to evaluate the efficiency of any remediation measures based on microbes. The mere decrease in concentration of the contaminant in the environment does not reflect the sole ability of applied remediation measure rather

it leaves behind an important virtue of natural attenuation unattended. So far, initially a method was successfully developed for the CSIA of HCH isomers which showed potential for HCH source differentiation and identification in the groundwater of an operating packaging and reformulating pesticide facility located in northeastern Florida, USA [61]. Recently, in a study performed by Bashir et al. (2015) the efficacy of CSIA was tested for determining carbon stable isotope ratios of HCH isomers directly from contaminated aquifer located in the vicinity of the former formulation and packing plant located in Germany. The study highlighted the application of CSIA for assessment of direct in situ biodegradation of HCH isomers in addition to proposing the temporal variations in HCH biodegradation based on the time-resolved measurement of isotopic ratios. Not just limiting to the time based methods, this study in combination with the evaluation of isotope ratios at varying distances was successful in allocating the contaminant source zones, storehouse and the waste dump site in the region with reported production dating back to 1935–1980 [19]. During the biotransformation processes, it is predominantly the irreversible (unidirectional) rate-limiting step that is essential while defining the magnitude of fractionation. A comparatively larger fractionation is observed in slow reaction steps with lesser rate as compared to the faster ones [49, 50, 68, 76]. However, in complex environmental conditions, the isotope fractionation can be affected by many different factors and may remain masked or hidden during the analysis and in such cases, dual-element stable isotope analysis provides an elegant way to improve the quantification using CSIA. Since a dual-element isotope analysis is less affected by masking of isotope effects or methodological differences [61].

These few initial in vitro studies on isotope fractionation by anaerobic (*Desulfococcus gigas*, *Desulfococcus multivorans* *Clostridium pasteurianum* DSM 525) and aerobic

(*Sphingobium indicum* B90A and *Sphingobium japonicum* UT26) strains show variability in carbon isotope fractionation for not only the isomers but also for specific enantiomers. These studies along with those reported for direct in situ degradation of HCH isomers may be implemented in combination for assessment of in situ biotransformations in the environment [57–59], Table SI]. However, more systematic studies are needed on isotope fractionation for developing an in-depth understanding of the mechanism of isotope fractionation by specific enzymes (e.g. Lin enzymes) and more work is needed to develop the full potential of CSIA for field studies. The mode of C–Cl bond cleavage and resulting isotope fractionation is currently not systematically studied. Future studies need to provide information of molecular mechanisms governing carbon, hydrogen and chlorine isotope fractionation of HCHs under aerobic and anaerobic conditions. Further knowledge on the microbial ecology of HCH degradation related to isotope fractionation is still in its infancy and more research is needed to exploit the full potential of CSIA of HCH for tracing their fate in the environment.

CSIA Combined with Enantiomeric Fractionation (EF)

It is pertinent to mention here that, although many studies have been conducted with different isomers of HCH as individual entities, yet stereoisomer specific studies are in dearth and the unique characteristics of the enantiomers are often disregarded. Amongst the HCH isomers, α -HCH in addition to being the overriding form in the produce furthermore has a unique characteristic of chirality, delineating it from rest of the isomers. In contrast to the abiotic processes, biological degradation is accompanied with stereo-specific preferences. Depending upon the biological processes, degradation rates of stereoisomer vary in the environment as well as in the organisms [79], thus it becomes crucial to study aerobic degradation patterns with respect to enantiomers of chiral pesticide in order to highlight distinct differences between the two. Badea et al., (2011) while working with anaerobic *C. pasteurianum* strain, assessed isotopic fractionation with respect to anaerobic degradation of (+) & (–) α -HCH enantiomers. Although the study indicated a non-enantioselective or moderate enantioselective degradation by *C. pasteurianum* however it has allowed for the future investigations for characterizing in situ biodegradation at the complex field sites by combining the isotope fractionation fingerprints to that of distribution of enantiomers. Bashir et al. [59] also conducted experiments with *Sphingobium indicum* B90A and *Sphingobium japonicum* UT26 strains for aerobic degradation of α -HCH whereby CSIA and EF were also evaluated together. This investigation also can be used as future references for assessing the carbon stable isotope

fractionation observed during in situ transformation in combination with the enantiomer specific data to distinguish aerobic processes from others in field studies.

Multi-Isotope Fingerprinting and Future Prospects

Potentially multi-isotope fingerprinting may be used for characterization of biodegradation mechanisms with the recently developing technique for analyzing chlorine and hydrogen isotope compositions of HCH [80]. However, the method for analyzing chlorine isotope composition has not been developed for routine measurements as the stability of the reactor operated at temperatures > 1450 °C has a limited life time. The development of reactors resistant to HCl at high temperatures is subject of future work. The high temperature conversion process employing elemental chromium for reduction at temperatures > 1450 °C has been proven to be suitable for analyzing the hydrogen isotope composition of HCH [81, 82] but application to analyze hydrogen isotope composition of HCHs in field studies or microbial degradation experiments are still lacking. The combination of carbon with hydrogen and chlorine isotope fingerprints may allow a more precise characterization of HCH degradations processes in comparison to only carbon isotope analysis in near future.

Furthermore, both enantiomer fractionation (EF) and isotope fractionation are used to describe biodegradation. So combination of CSIA and EF together has the potential for better and more quantitative evaluation of biodegradation. The systematics of enantiomer and isotope study are not fully understood and more experiments with culture and enzyme based studies are needed to understand the enantiomer and isotope fractionation for exploiting their potential while analyzing active processes in field studies and also for evaluating remediation measures for a better management of contaminated sites.

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