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Research Article

Detection of hydroxylated metabolites of β- and δ-HCH isomers from contaminated environments

Simran Jit[1#*] · Nidhi Garg[2#] · Kiran Bala[3]

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Abstract: Hexachlorocyclohexane (HCH) is a mixture of toxic, lipophilic and persistent stereoisomers which has been used indiscriminately for its insecticidal properties in agricultural practices. Consequently, several high-dose point HCH dumpsites have been generated across the globe. Genetics and biochemistry of HCH degradation in microbial systems have identified genes and a plethora of intermediary metabolites. Thus, evaluation of such metabolites can be a key indicator for the success of bioremediation efforts. However, extreme high concentrations of parental HCH isomers may mask the detection of these metabolites. This study presents an assessment of hydroxylated metabolites of the more persistent β- and δ-HCH isomers against high concentration of parental HCH isomers. For this purpose, samples were collected from contaminated soils, water, and pond sediment samples from the HCH dumpsite in Ummari village, Lucknow (U.P.), India.

Keywords: Hexachlorocylohexane (HCH), dumpsite, hydroxylated metabolites

Introduction:

The exposure to environmental contaminants such as hexachlorocyclohexane (HCH) is a cause of concern in public health sectors because of its persistent nature and deleterious effects on human health [1]. The production process of this organochlorine compound for the desired insecticidal isomer; gamma (γ)-HCH isomer (commercially referred to as Lindane), generates a mixture of several isomers including alpha (α), beta (β), delta (δ), and epsilon (ε) HCH in an approximate comparative of 1:9. Huge stockpiles of these by-products or the 'HCH muck' have been discarded all over the globe resulting in the generation of dumpsites that act as hotspots for environment contamination through aerial, soil and water dispersal routes [2, 3, 4]. Staggering amounts ranging from 4 to 7 million tons of HCH muck are estimated in over seven decades of commercial Lindane production [2]. The high volatility of HCH isomers makes them easier to transport through aerosol transmission and reach pristine locations with studies relying on α- to γ-HCH ratios as a global tracking metrics. Concerns are raised due to their lipophilicity, carcinogenicity, estrogenic effects, and environmental persistence of the α-HCH and the β-HCH isomer [5]. The three major isomers: α-, β- and γ-HCH have long been listed as Persistent Organic Pollutants (POPs) in the Stockholm Convention in May 2009 [6] (UNEP 2009). Consequently, several countries have banned and/or restricted the production and use of HCH and Lindane in particular. Still these isomers persist in various environmental compartments such as soils, sediments, water bodies, and biota; and continue to impact ecosystems and human populations, thereby posing challenges for environmental and health risk assessments.

In India, India Pesticide Limited (IPL) at Chinhat, Lucknow (U.P.) has been linked to a major dumpsite at Ummari village. Studies conducted in the nearby HCH contaminated areas such as the Ummari Village in India have identified the presence of HCH-degrading and HCH-tolerant microbes using both culture dependent [7, 8, 9, 10] and culture-independent metagenomic approaches [11, 12, 13, 14]. Such findings have paved the way for development of bioremediation approaches to mitigate HCH contamination [15, 16, 17, 18, 19, 20]. These bacterial species possess a group of *lin* Genes, encoding catabolic enzymes capable of degrading the different isomers of HCH to different extents [21, 22, 23, 24, 25] into their respective metabolites or end products.

[1] Department of Zoology, Miranda House, University of Delhi, Delhi-110007, India.
[2] Department of Zoology, Shivaji College, University of Delhi, New Delhi-110027, India.
[3] Department of Zoology, Deshbandhu College, University of Delhi, Delhi-110019, India.
# authors have contributed equally
Corresponding author: Simran Jit[1] * simranjit@mirandahouse.ac.in

The metabolites resulting from the degradation of different HCH isomers, in biological systems and the environment, provide valuable insights into the pathways and effects of HCH exposure. Previous studies by Raina et al., [26] and Singh et al., [33] report detection of hydroxylated metabolites of β- and δ-HCH from groundwater samples and soil with low levels of HCH contamination. Despite this, research into HCH metabolites remains limited, with a need for more comprehensive studies to clarify their formation, distribution, and potential toxicity. Several HCH dumpsites are huge open-air mounds or landfills and to ensure that the applied bioremediation methods are well adapted for soil communities, it becomes essential to devise methods for the detection of these metabolites from high dose-point soil as well. Also, owing to the different lipophilic nature of parental and hydroxylated metabolites, methodology needs to be devised for the separation of pentachlorocyclohexanols and tetrachlorocyclohexane-diols from parental isomers which may otherwise be masked by high HCH concentrations. Hence, in the present study, samples were collected from the previously reported high-dose point HCH dumpsite in Ummari village [2] and a method for the detection of hydroxylated metabolites of the more persistent β-HCH and δ-HCH isomers against a high background of HCH isomers is proposed. Such methods can be useful indicators of microbial transformations and for devising further remediation strategies.

Materials and Methods

1. Sample collection and pre-treatment procedures

Sampling was done between 2010-2011 at locations near the dumpsite, IPL factory and surrounding agricultural fields for the soil, water and pond sediment samples for subsequent HCH residue and metabolite analysis. A total of eleven soil, three water samples and a composite pond sediment were collected. Since the soil samples were highly contaminated with HCH, metabolites present in trace amounts could not be detected without further separation and concentration. Using a silica gel column, the devised cleanup method gradually separated the HCH isomers from the hydrophilic hydroxylated molecules. The pentachlorocyclohexenes; α-, γ-, & δ- PCCHs were expected to co-elute with their respective parental isomers. In the absence of commercial standards for the metabolites, γ-HCH was used as the internal standard to estimate HCH concentrations. Hydroxylated metabolites were identified by comparing the mass spectra of underivatized and relative expected shifts in corresponding mono- or di- acetates with synthesized metabolites at selective ion monitoring of m/z 241+ 265. The respective tetrachlorocyclohexane-diols obtained from β- and δ-HCH are abbreviated as B2 and D2 throughout the text.

i) Soil samples from the HCH dumpsite and surrounding agricultural fields:

The investigated dumpsite is located close to Ummari village (13 km north-east of the IPL factory). For the survey of HCH residues and metabolites at this location, seven soil samples were collected from a depth of 20 cm by sampling auger. From the actual dumpsite five soil samples (LS1-LS5), LS5 represented the center of the open dumpsite, and one sample (LS6) was collected from the center of the former brick kiln area. A sample (LS7) was collected from sealed sacks dumped by the industry at the brick kiln area, was also analyzed for its HCH and metabolite composition. This sample was not mixed with soil and served as the reference for deciding on the microbial formation of HCH metabolites in the actual soil samples. LS8 was a composite sample for five nearby sites soils collected from a depth of 40 cm. Two soil samples (LS9 and LS10) were collected from the agricultural field surrounding the main dumpsite (40-60 m).

ii) Water samples from the HCH dumpsite and surrounding agricultural fields:

LW1 represents the drinking groundwater sample taken by hand pump (of approximate well depth of 10 m) and LW2 was obtained from the drainage system at the back of the dumpsite while LW3 was taken from a small pond near the fields. The last soil sample, LS11 was collected from an agricultural field at a distance of 1 km along the road joining the dumpsite and industrial unit.

iii) Pond sediments collected from manufacturing unit

A total of four different pond sediment samples (PS1-4) were collected from a pond resource situated behind the Lindane manufacturing unit and were mixed to form a composite sample. The samples were wet with a foul odor and an oily texture.

After collection and transportation to the laboratory, all soil samples were thoroughly dried (at room temperature for 2 days), homogenized, sieved and stored at -20°C while water samples were stored in glass containers at 4°C till analysis.

2. Extraction of HCH residues and metabolites and clean-up

i) Soil samples

HCH residues were extracted by using the protocol described by Concha-Grana et al 2006 [27]. For this, 1g of the soil sample was extracted in 20 ml of 1:1 solution of hexane:acetone by sonication and brief vortexing. A clean solvent phase was obtained by centrifugation at 2000 rpm for 2 min. The dried extract was re-dissolved in an appropriate volume of 30 % dichloromethane in hexane and passed through a deactivated silica gel (230-400 mesh size) topped with anhydrous sodium sulphate in a mini glass column (5 cm length, 2 cm internal diameter). HCH residues were eluted by 3 volumes of the same solvent mix whereas the hydroxylated metabolites were eluted in a fresh vial with 10 % methanol in ethyl acetate. Both the eluates were air dried. The HCH residues from the dicholoromethane/hexane elution were dissolved in 20 ml of ethyl acetate and analyzed on GC-MS. The hydroxylated metabolites in the methanol/ethyl acetate phase were re-dissolved in 200 µL ethyl acetate of which 100 µL was analysed on GC-MS whereas the other half was air-dried and derivatized to obtain respective mono and di-acetates for better peak detection. Briefly, obtained metabolites were dissolved in 100 µL acetic anhydride and 5 µL pyridine was added before the mix was incubated at 80°C for 20 min in a sealed glass vial. On cooling the 1 M $KHCO_3$ was added to the mix till no formation of CO_2 was observed. The acetylated HCH metabolites were then extracted with an equal volume of dichloromethane by mixing and the lower phase was carefully transferred to a fresh vial for evaporation. Finally, the extract was dried over Na_2SO_4 column and dissolved in 200 µL ethyl acetate, and analysed on GC-MS.

Reference sample: LS7

100 mg of the sample was dissolved in 10 ml ethyl acetate and the above-mentioned clean-up procedure was followed. The methanol/ethyl acetate phase was acetylated similarly.

ii) Water samples

HCH residues and hxdroxylated metabolites were extracted on water-wettable reverse-phased Oasis® HLB 6 cc extraction cartridges. Each cartridge was first cleaned with 10 ml dichloromethane followed by 10 ml methanol and lastly 30 ml tap water by applying a vacuum pressure of 20 Hg in a Supelco Visiprep™ chamber. Then 250 ml of water sample was percolated through the cartridge under vacuum. This was followed by a 10 ml methanol wash and finally elution in 10 ml dichloromethane. In order to obtain a phase separation, 2 ml water was added to the eluate and extracted by rigorous mixing. Lower phase containing the HCH residues and metabolites was carefully transferred to a fresh vial. A second washing of cartridge with 10 ml dichloromethane was given and pooled with the first extract.

Thereafter, the extracts were dissolved in 2-3 ml of 30 % dichloromethane in hexane and cleaned up using the same procedure as for the soil samples.

iii) Pond sediment

Portions of 1 g moist soil were extracted with 20 ml hexane/acetone (1:1, v/v) by vigorous shaking (2 min) and sonication (1 min). The soil suspension was centrifuged at 2000 rpm for 2 min and the supernatant was transferred to a clean glass vial and allowed to slowly evaporate overnight in a gentle stream of air. Thereafter it was processed similarly as soil samples.

Finally the dry residue containing the separated hydroxylated metabolites was dissolved in 100 µL acetic anhydride and processed following the procedure described by Raina et al., [26]. The acetylated HCH metabolites were dissolved in 200 µL of ethyl acetate for GC-MS analysis.

3. Analysis and Quality control

Aliquots of 1 µL of samples in ethyl acetate or heptane with 2.5% 2-propanol were analyzed by gas chromatography-mass spectrometry (GC-MS), using conditions described by Garg et al., [19]. The HCH isomers were quantified by using γ-HCH as the internal standard. The derivatized hydroxylated metabolites were identified by using mass spectra. For quality assurance and quality control, procedural blanks and matrixes spikes were analysed. None of the target compound was detected in the blanks. The recovery for B2 was 80% soil extraction procedure, whereas in water extraction it was 79%. The recovery for HCH in water samples was 2 % for α-, 34 % for β-, 34 % for δ- and 7 % for γ-HCH. However, the spiked δ-PCCH could not be recovered.

Results and Discussion

The samples collected from the studied HCH dumpsite, IPL factory and surrounding agricultural fields were analysed for HCH residue levels and metabolite composition. Since the soil samples were highly contaminated with HCH, metabolites present in trace amounts could not be detected without

further separation and concentration (Table 1). However, a sequential clean-up procedure was successful in the separation and detection of the hydroxylated compounds. These were identified by comparing the mass spectra of underivatized and relative expected shifts in corresponding mono- or di-acetates with synthesized metabolites at selective ion monitoring of m/z 241+ 265 (Figure 1 and 2).Despite the expected co-elution of pentachlorocyclohexenes; α-, γ-, & δ- PCCHs with their respective parental isomers, these could not be detected.

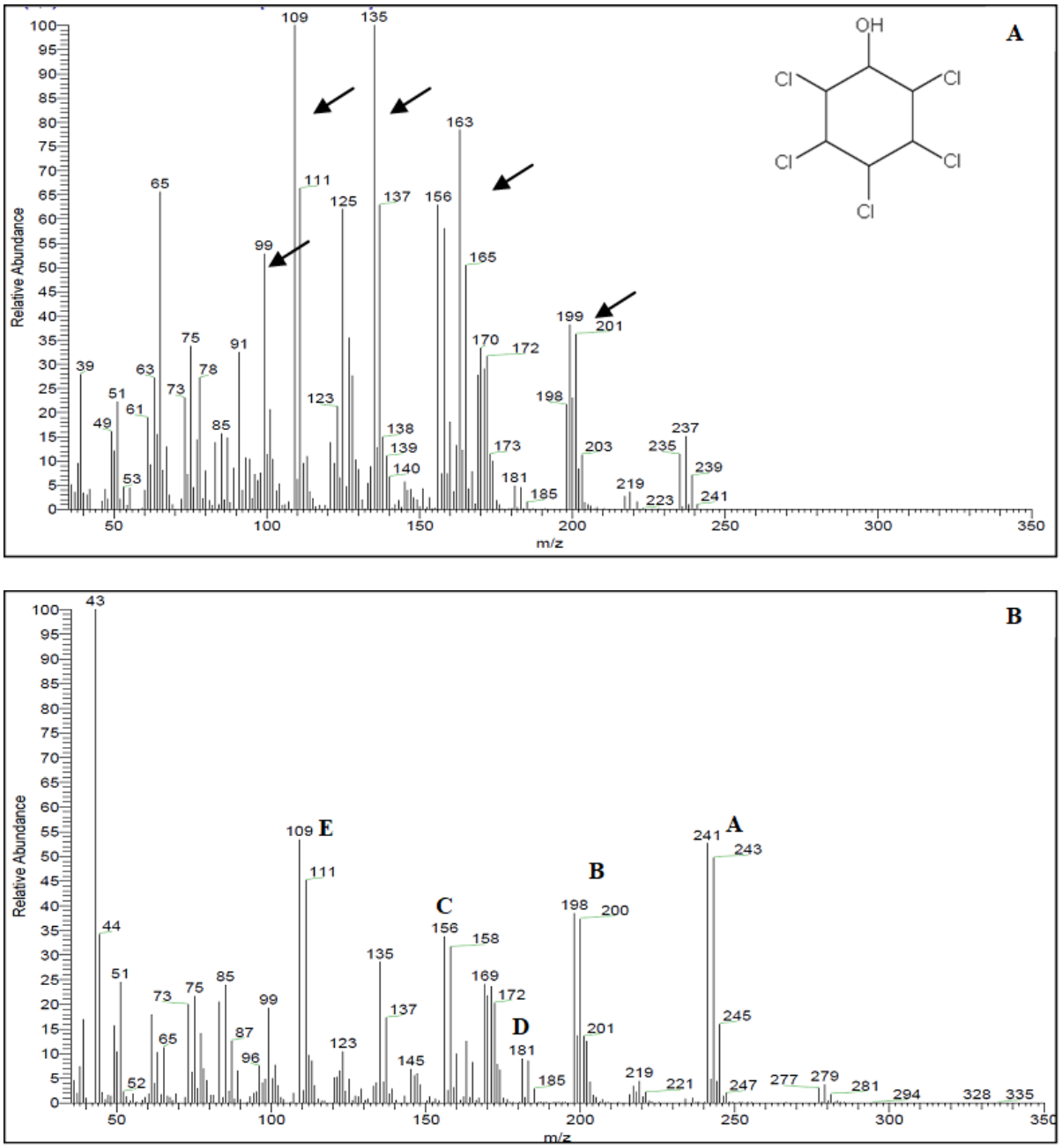

Figure 1: GC-MS Analysis of β-pentachlorocyclohexanol (B1). A: underivatized metabolite having a molecular mass of 272, B: and the corresponding mono-acetate with a molecular mass of 312. The possible fragmentation sequence: $C_8H_9O_2Cl_5^+$ (m/z 312)→(-Cl, -HCl)→$C_8H_8O_2Cl_3^+$ (m/z 241, A). (CH_3CO) →$C_6H_5O_1Cl_5^+$ (m/z 198, B)→(CHCHO) →$C_4H_3Cl_3^+$(m/z 156, C) → $C_3H_3Cl_2^+$(m/z 109, E), and molecular (-CH_3COOH, -HCl, -Cl) →$C_6H_4Cl_3^+$ (m/z 181, D).

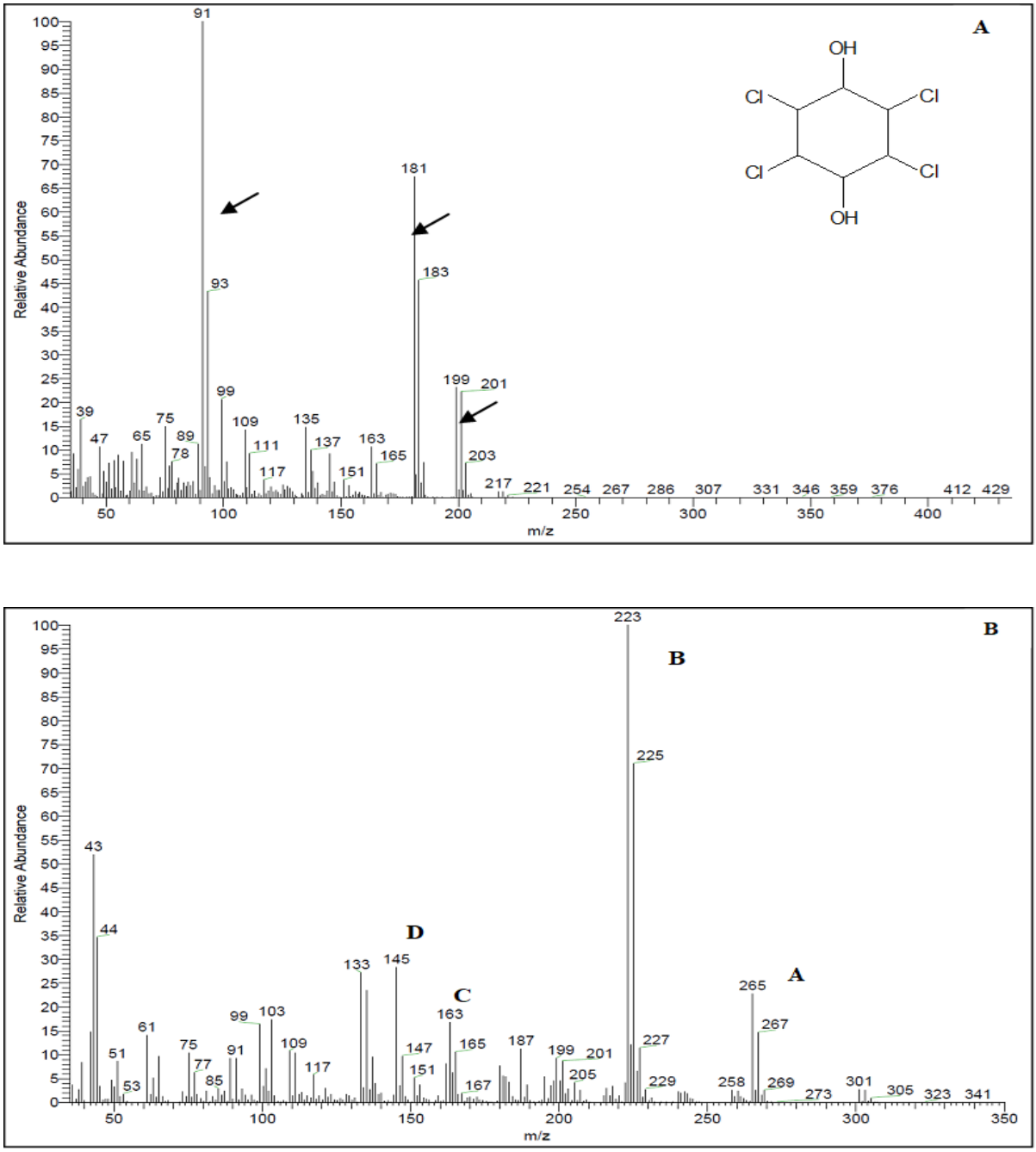

Figure 2: GC-MS Analysis of β-tetrachlorocyclohexadiol (B2). A: underivatized metabolite having a molecular mass of 254, B: and the corresponding di-acetate. The possible fragmentation sequence: $C_{10}H_{12}O_2Cl_4^+$ (m/z 336) →(Cl, -HCl) →$C_{10}H_{11}O_2Cl_2^+$ (m/z 265, A)→(-C_2H_2O= -CH_3CO,+ H)→$C_8H_9O_3Cl_2^+$ (m/z 223, B)→(CH_3COOH)→$C_6H_5OCl_2^+$ (m/z 163, C), and $C_4H_3OCl^+$ (m/z 145, D).

Soil samples and 'HCH muck' analysis

Sample, LS7 disposed in sealed sacks represented the 'HCH muck' and hence the peak area for metabolites in this samples was compared to the similar ratios of other soil samples to indicate enrichment due to possible microbial formation of HCH metabolites (Figure 3).

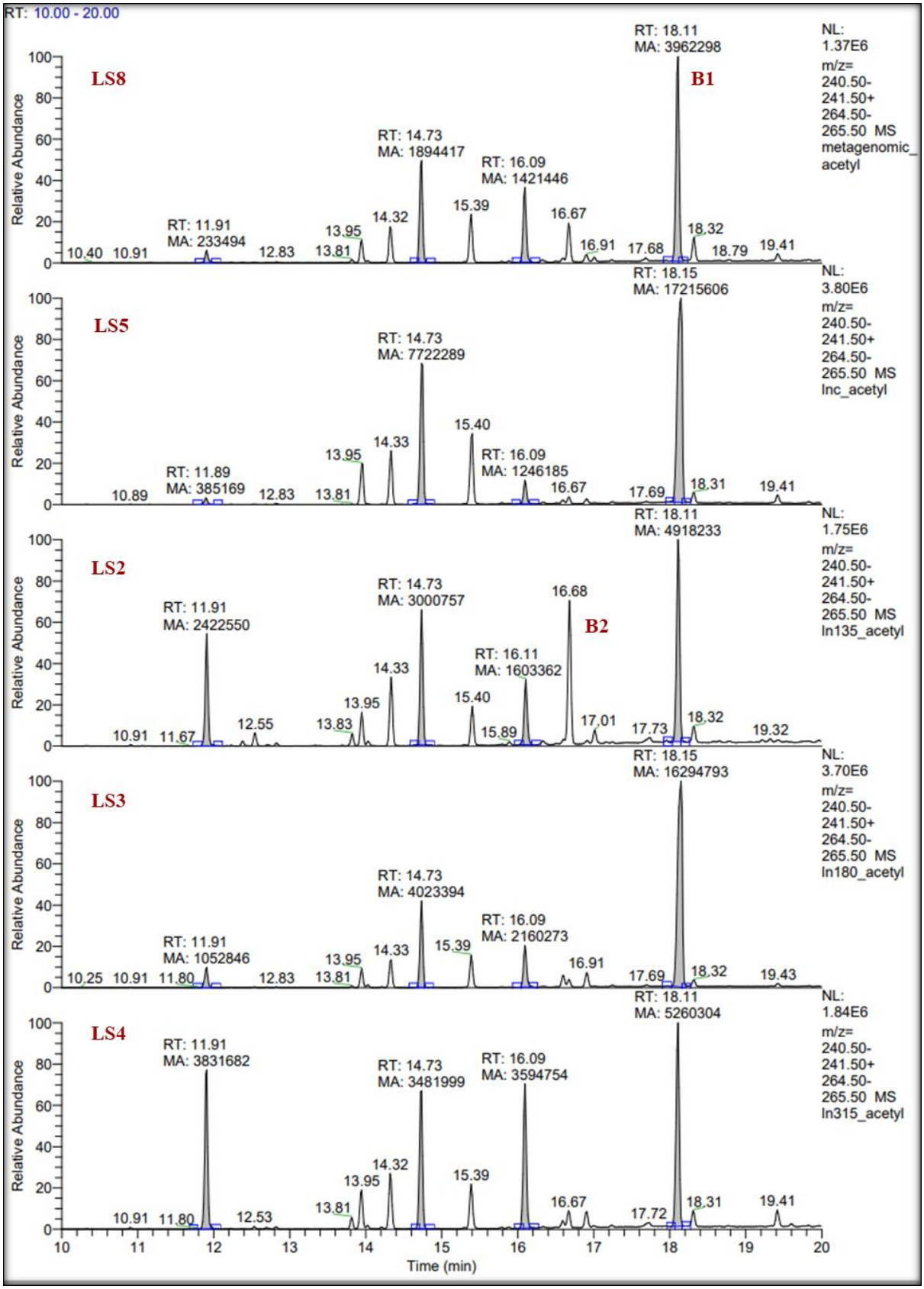

Figure 3: Chromatograms showing peaks for mono- and di-acetates in soil samples as compared to HCH muck at m/z 241-265.

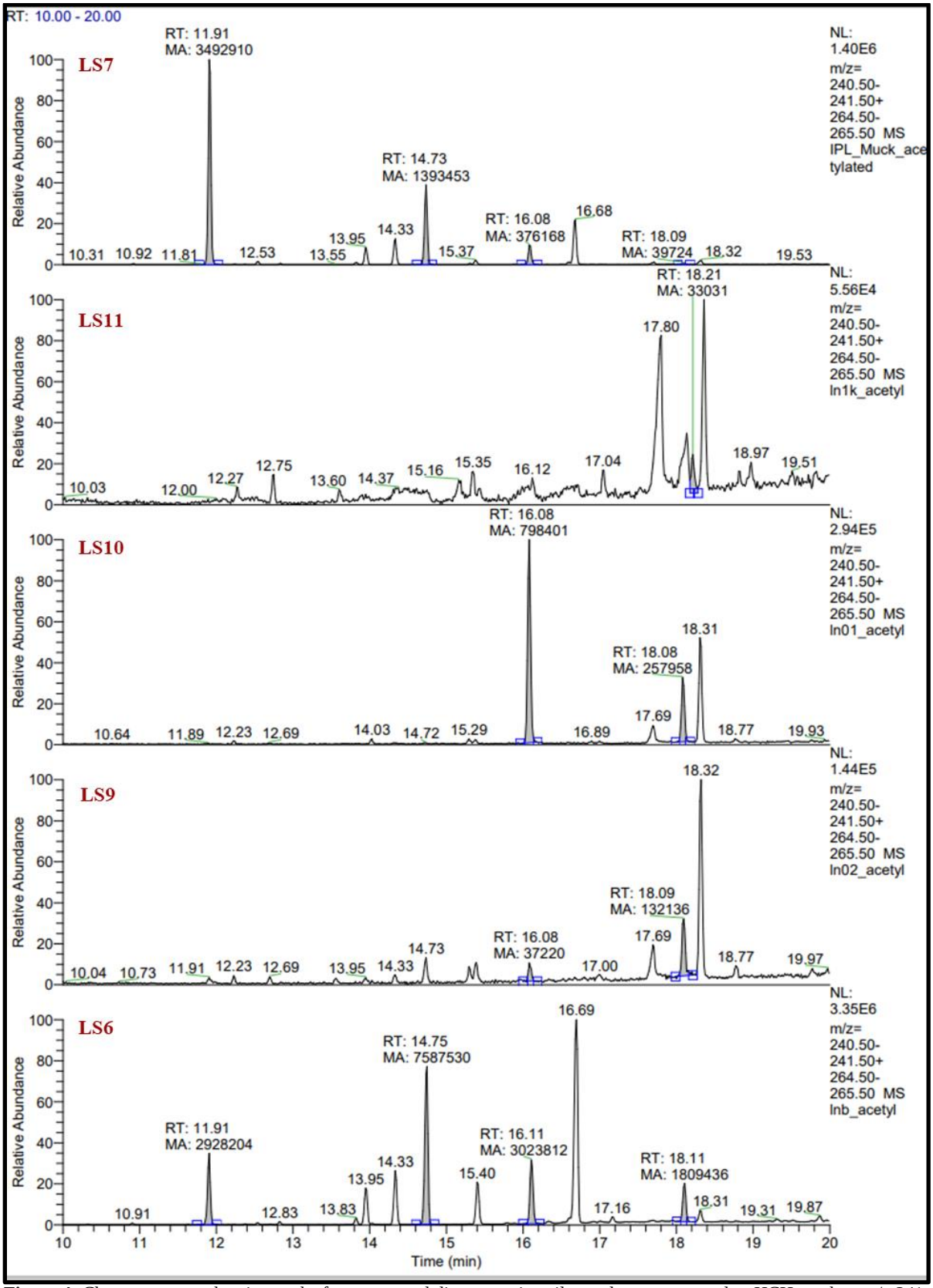

Figure 4: Chromatograms showing peaks for mono- and di-acetates in soil samples as compared to HCH muck at m/z 241-265.

An average composition of t-HCH varies from 60-70 % α-; 5-12 % β-; 10-12 % γ-; 6-10 % δ-; 3-4 % ε- with < 2 % of η-& θ-HCH [4]. As shown in Table 1, the 'HCH muck' (LS7) had a distinct α-/β-HCH ratio of 1.5 as against the expected ratio of 6 in t-HCH. This deviation can be due to higher volatility of α-HCH (4.5×10^{-5} mm Hg) than β-HCH (3.5×10^{-7} mm Hg) [28]. Also, rapid degradation of α-HCH is expected due to more number of axial chlorines as against none in β-HCH. Additionally, Wu et al., [29] have reported isomerization of approximately 50 % of α-HCH to other HCH isomers especially β-isomer under oxidative conditions.

However, it is difficult to explain absence of δ-HCH but the detection of ε-HCH in trace amounts (1 µg 100mg^{-1} of 'HCH muck') as these are generally removed by methanol extraction during industrial Lindane production. Furthermore, missing proportions of δ-HCH isomers can be due to its recycling at the production unit for synthesis of trichlorobenzene.

Table 3 shows the estimated amounts of mono- and di-hydroxylated metabolites of HCH observed in LS1 to LS 11. Based on the mass spectra and relative indices and dihydroxylated metabolites it is expected that the detected mono- and di-hydroxylated metabolites are more likely from β-HCH rather δ-HCH (Figure 3 and 4). Overall, all the soil samples collected from the main dumpsite (LS2-LS5) showed high Σ-HCH levels and α-/β-HCH ratios similar to LS7 (Table 1). The highest estimated Σ-HCH levels were 353 mg g^{-1} of soil (LS5) with the lowest being 125 mg g^{-1} of soil (LS2).

Table 1. Residue analysis of soil samples from main dumpsite and agricultural fields.

LS7 represents the 'HCH muck'

Sample	Area ratios			Concentration in soil (µg g^{-1} of soil)				α/β
	α-HCH	β-HCH	ε-HCH	α-HCH	β-HCH	ε-HCH	Σ-HCH	
LS1	0.0494	0.8800	0.0183	14	130	3	147	0.1
LS2	0.2107	0.4383	0.0091	59'040	64'925	1'562	125'526	0.9
LS3	0.2685	0.4757	0.0142	75'264	70'472	2'441	148'178	1.1
LS4	0.3337	0.5984	0.0073	93'533	88'643	1'249	183'424	1.1
LS5	0.6806	1.0883	0.0061	190'739	161'223	1'041	353'003	1.2
LS6	0.3690	0.7314	0.0192	103'408	108'354	3'308	215'070	1
LS7*	0.1073	0.1296	0.0078	30'083	19'197	1'346	50'625	1.6
LS8	0.1905	0.3434	0.0339	53'401	50'879	5'823	110'103	1
LS9	2.2719	2.8900	0.0776	637	428	13	1'078	1.5
LS10	0.3557	1.1186	0.0274	100	166	5	270	0.6
LS11	0.0664	0.2083	0.0043	19	31	1	50	0.6

* µg 100mg^{-1} of 'HCH muck'

Both pentachlrocyclohexanol (likely B1) and tetrachlorocyclohexanol were detected in varying concentrations in all the samples (Table 2). While B1 concentration was similar to LS7 for samples LS2-LS6 (Table 1), B2 was found generally in higher amounts as compared to B1, especially in sample LS3 where the peak area ratio was 138. Such high HCH-ladden indicate biotransformation of the recalcitrant and persistent β- & δ-HCH supported by the reports for isolation of HCH degraders from these HCH-contaminated environments [7,8,9,11].

Samples LS9-LS10 collected from the surrounding agricultural fields showed lower Σ-HCH levels ranging from 1 mg to 147 µg g-^{1} of soil with LS11 (from an agricultural field further away from the dumpsite) having the lowest Σ-HCH levels of 50 µg g-^{1} of soil (Table 1). Similar to main dumpsite samples with high HCH concentrations, these agricultural soils also showed a predominance of α- & β-HCH with α-: β-HCH ratio ranging from 0.1 to 0.6. Enrichment of B1 and B2 was seen in all soil samples, significantly in LS1; LS10; LS9, from agricultural fields around the dump site, and LS11,

from remote agricultural fields (Table 2). LS9 showed a prominent enrichment (~400 fold) of B1 as compared to the reference sample LS7. This enrichment of metabolites calculated by the observed peak area ratios for metabolites, categorized the soil samples into two groups: Zone A (LS2-LS8), showing 10-60 fold enrichment, and Zone B (LS1 & LS9-11), showing 100-1000 fold enrichment (Table 2). This can be extended to the estimated HCH residue levels wherein Zone A soils had an average α /β ratio of 1.03 and Zone B soils had a ratio of 0.7 [Supplementary material S3].

Table 2: Peak area ratios for the detected mono- and di-acetates of hydroxylated metabolites.

Sample	Absolute peak areas		Peak areas relative to HCH		Peak areas relative to LS7		Concentrations in soil (mg kg^{-1} of soil)	
	B1	B2	B1	B2	B1	B2	B1	B2
LS1	34	116	0.2357	0.8043	30.8990	990.9505	0	0.2
LS2	1603	4918	0.0129	0.0396	1.6947	48.8764	0.9	6.5
LS3	2160	16294	0.0148	0.1118	1.9425	137.7426	1.2	21.5
LS4	3595	5260	0.0197	0.0288	2.5863	35.5716	2	6.9
LS5	1246	17215	0.003	0.0489	0.4639	60.2586	0.7	22.7
LS6	3024	1809	0.0142	0.0085	1.8716	10.5244	1.7	2.4
LS7*	**376**	**40**	**0.007**	**0.0008**	**1**	**1**	**0.2**	**0.1**
LS8	1421	3962	0.0136	0.0379	1.7859	46.8081	0.8	5.2
LS9	798	258	3.0068	0.9721	394.093	1197.6892	0	0.2
LS10	37	132	0.0347	0.1239	4.5538	152.7139	0.4	0.3
LS11	ND	33	ND	0.6671	ND	821.8854	ND	0

Among the three water samples screened for presence of metabolites, sample from drainage point (LW6.1) showed the highest concentrations for B2 (1 μg l^{-1} of sample) (Table 3) while no metabolites could be detected from the ground water sample (LW5.1).

Table 3: Concentrations for β-PCHL (B1), and TCDL (B2) in the water samples from dumpsite. The HCH concentration varied from 5.32 to 0.14 μg l^{-1} of water.

Sample	B1 (μg l^{-1} of sample)	B2 (μg l^{-1} of sample)
LW5.1	ND	ND
LW6.1	0.1	0.04
LW 7	0.08	0.08

The pond soil sediments collected from behind the IPL because of their oily nature could not be dried and homogenized for residue analysis. The probable reason for the oiliness of the soil was the presence of other effluents discarded by the factory which bring about a change in the soil texture. Hence, a different approach was used to first determine the concentration of HCH residues and then the metabolites. The residue analysis of the composite soil sample revealed the presence of all HCH isomers and the mono-hydroxylated metabolite of β-HCH, B1 (Supplementary material S1) which were undetected in a previous study [30]. The gamma isomer was present in the least concentration because of its separation from the rest of the isomers owing to its insecticidal activity (Table 4).

Table 4: Residue analysis of pond soil sediments (ng/ul) from Indian Pesticide Limited (IPL), Lucknow

Sample	α-HCH	β-HCH	γ-HCH	δ-HCH	ε-HCH
Standard	10	10	10	10	0.32
PS 1	0.03	10.45	0.01	0.03	0.42
PS 2	0.88	16.51	0.02	0.04	0.59
PS 3	0.02	6.09	0.01	0.03	0.30
PS 4	0.02	2.98	0.01	0.02	0.18

Standard for α-HCH, β-HCH, γ-HCH and δ-HCH is 10 ng/ul

The beta isomer was present in the maximum concentration, followed by ε-, δ-, and α-HCH. The method developed for the detection of HCH isomers and the metabolite is sensitive enough to detect both the isomers and the metabolite in low concentrations in comparison to the previously used methods to detect the same (Table 5) [27, 30]. The soil composition and microbial community of the current study have already been characterized [1, 29, 34, 35]. The detection of both HCH isomers and their

metabolites in ecosystems are of immense importance as they leach from the surface to the deeper layers of the soil and ultimately to the ground water and the adjoining water bodies through either surface run off or direct disposal leading to their contamination. The contaminated water when used for different activities and consumed by animal and human populations has serious consequences [31, 32].

Table 5: Peak area ratios and concentration of the detected mono-acetates of hydroxylated metabolite B1 from pond soil sediments (PS1-4).

Analysis of B1 relative to ß-HCH	
Peak area **β**-HCH (TIC)	192000000
Concentration (**β**-HCH ng/ul)	10
Peak area B1-acetyl (m/z 241)	620147
Peak area B1-acetyl (TIC calculated from m/z 241)	18480381
Concentration B1 (ng/ul) in 0.2mL extract (from 3 g soil)	0.96
Concentration B1 (mg/kg) in moist soil	**0.064**

A mixture of pentachlorocyclohexenes (β-, γ-, δ-, η-, and θ-PCCH) is produced by both chemical and biological dehydrochlorination of α-HCH. However, none of the PCCHs could be detected in any of the samples. This can be reasoned by their high volatility which enhances their evaporation in the hot tropical temperatures of the Indian soils. Possibility of rapid transformation of PCCHs into tetrachlorocyclohexadienes (TCDNs) to spontaneously volatile trichlorobenzenes (TCBs) can also be considered.

Conclusion

In the present assessment of a high-dose point HCH dumpsite, α- and β-HCH isomers were the predominant contaminants in all the samples. Chromatographic isolation methodology developed was successful in the detection and estimation of the hydroxylated metabolites against a background contamination as high as 353mg g^{-1} of soil. Based on the spectral analysis, both PCHL and TCDL were assigned to β-HCH rather δ-HCH. Overall, higher concentrations for metabolites were estimated in soils with high HCH concentrations. Further, to rule out the magnification of metabolites rather than biological degradation, comparisons for the peak area ratio of each metabolite type helped in assessing the actual enrichment for the degradation metabolites in analyzed soil samples. The investigated dumpsite has been previously assessed for the extent of HCH contamination, microbial diversity as well as metagenomics analysis. Earlier studies on quantification of *lin* genes and on site small-scale bioremediation field trials have indicated active microbial degradations od HCH isomers by the indigenous microbial community. Such findings correlate with already published reports of isolation of HCH degraders and metagenomic diversity from the studied dumpsite validating their environmental relevance. Detection and estimation of intermediaries of HCH degradation pathways is crucial for evaluating the success of biotransformation processes of HCH. Such correlations are helpful in predicting the presence of indigenous microbial communities. More detailed analysis are required to reveal the relevance of these metabolites for risk assessment of HCH-contaminated sites.

Competing Interests

The authors have declared that they have no competing or conflicting interests.

Acknowledgments

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