



RELATIVE BIOAVAILABILITY OF α - AND β -HEXACHLOROCYCLOHEXANE IN VARIOUS SOILS FROM A CONTAMINATED SITE

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Abstract: Bioavailability of α - and β -isomers of a chlorinated insecticide hexachlorocyclohexane (HCH) in several field soils that were contaminated for different time periods was evaluated. Results revealed that the availability of α -HCH to the degradative microorganisms was 75-80% in all the soils, but considerable differences in the availability of β -HCH in different soils were observed. Thus, 80-90% β -HCH was available to '*E.coli*-LinB' cells from a freshly spiked soil, and ~70, 60 and 45% from soils that were contaminated for short-, medium- and long-term, respectively. Similarly, their amounts extracted by 50 mM hydroxypropyl- β -cyclodextrin also showed the same trend. It suggests that the bioavailability of β -HCH decreases progressively with an increase in the age of contamination, but of α -HCH is not affected under the same conditions. Reasons behind this differential availability are discussed. Results are important for correct risk-assessment of these residues in the contaminated soils.

Keywords: Aging; Bioavailability; Contaminated-soils; Hexachlorocyclohexane; Risk-assessment.

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INTRODUCTION

Chlorinated insecticide technical hexachlorocyclohexane (t-HCH) consists predominantly of α - (60–70%), β - (5–12%), γ - (10–12%) and δ - isomers (6–10%). Several sites are present throughout the world, where HCH has been manufactured, formulated, spilled or used, and are highly contaminated and cause toxicity (Willett *et al.*, 1998; ATSDR, 2005). From these, the HCH-isomers enter air upon evaporation, travel long distances, get deposited at colder sites, and exert toxicity at places where these were actually never used (Hung *et al.*, 2010; Vijgen *et al.*, 2011). Amongst different isomers, β -HCH is most persistent and its relative abundance in the soils increases progressively with increasing age of the HCH-contamination. The major residual components of most of the old contaminated sites, therefore, are α -HCH, due to its higher abundance in the synthesized technical HCH, and β -HCH, due to its higher

persistence (Henry *et al.*, 2012; Gong *et al.*, 2004; Concha-Grana *et al.*, 2006).

It is now well understood that the perceived risk of any pollutant, based on its total amount, could be an overestimate than the actual risk (Alexander, 2000), as a significant portion of it in various soils might actually not be bioavailable due to its adsorption and sequestration with soil particles. Although defining bioavailability of contaminants in soils and sediments is complicated (Katayama *et al.*, 2010; Celeno *et al.*, 2008; Semple *et al.*, 2004), but their amount available to organisms for degradation, bioaccumulation or toxicity is considered as a reasonable estimate of the bioavailable fraction (Schwartz and Scow, 1999; Wong and Bidleman, 2011). Separately, amounts recovered from soils after their non-exhaustive extraction with suitable concentrations of various solvents like hydroxypropyl- β -cyclodextrin (HPCD), TenexR, butanol etc. have also been used as surrogates

for estimating the bioavailable fraction (Reid et al., 2000; Swindell and Reid, 2006). Several factors have been shown to influence the bioavailability, but it invariably decreases with an increase in the duration of the contamination (Katayama et al., 2010; Kristensen et al., 2001; Chung and Alexander, 1998), and this 'ageing effect' has been attributed, at least in part, to interactions of pollutants with soluble organic matter and soil pores (Celeno et al., 2008). In this report, bioavailability of α - and β -HCH in four different soils that were contaminated for varying periods was evaluated.

EXPERIMENTAL

Chemicals

HCH-isomers were purchased from Riedel-de Haën, Germany. HPCD was from Sigma Chemical Co, St. Louis, MO, USA, and all other chemicals were of analytical grade.

Soils

Four soils were used in the study. One Soil-A, obtained from a garden in the 'institute' campus, and had no detectable HCH contamination. Three other soils, B1, B2 and B3 were collected from the vicinity of an HCH-manufacturing unit 'India Pesticide Limited', located at Chinhat industrial area, near Lucknow, India. This unit started production in 1990. Soils B1, B2 and B3 were contaminated with 27.2, 6.96 and 12.1 mg Σ -HCH i.e. the sum of α -, β -, γ - and δ -HCH g^{-1} soil, respectively (Table 1). Based on the increasing relative levels of β -HCH, these were referred here as soils contaminated for short-, medium- and long-term, respectively.

Table 1. Contamination level of HCH-isomers present in soil samples used in this study

S. No.	Soils	HCH-isomers (mg g^{-1} soil)					% β -HCH ³
		α -	β -	γ -	δ -	Σ -HCH ²	
1	A	BDL ¹	BDL	BDL	BDL	BDL	BDL
2	B1	16.8	10.4	BDL	BDL	27.2	38.2
3	B2	0.56	6.4	BDL	BDL	6.96	91.9
4	B3	0.1	12.0	BDL	BDL	12.1	99.1

¹BDL below detection level i.e. $< 0.05 \mu g g^{-1}$ soil

² sum of α -, β -, γ - and δ -HCH

³ β -HCH / Σ -HCH x 100

Microorganisms

Two microorganisms were used in the study. One, *Pseudomonas aeruginosa* ITRC-5, isolated earlier from a HCH contaminated soil by selective enrichment on technical-HCH (Kumar et al., 2005). It was grown in 100 mL 'mineral medium' that was containing 0.68 mM technical- HCH as sole carbon source, for one week with shaking at 180 rpm at 30°C. The cells were harvested by centrifugation, suspended in 10mL 'mineral medium', and used as inoculum. Second, recombinant *E.coli* BL21(DE3) cells that were carrying the gene for haloalkane dehalogenase linB (accession no. EF514236.1) in pET-26b(+) vector, and mediate the transformation of β -HCH. These '*E.coli*-LinB' cells were grown in LB-medium with kanamycin (50 $\mu g/mL$), at 30°C in rotary shaker (180 rpm) till the culture reached at OD₆₀₀ 0.6, when 0.1mM IPTG was added. After further incubation of these at 25°C for 3h, the cells were harvested by centrifugation at 3000g for 10 min at 4°C, washed with 2 ml mineral medium (pH 7.5) and suspended in the same for further use in the experiments (Macwan et al 2011).

Degradation of HCH-isomers in contaminated soils

Soils B1, B2 and B3 were serially diluted with an appropriate amount of soil A to obtain two sets. Set 1 contained 80 $\mu g \Sigma$ -HCH g^{-1} soil. The content of α - and β -isomers in the three soils, however, was different (Table 2). In set 2, the content of β -HCH in each soil was brought to 80 $\mu g g^{-1}$, but the content of α -HCH varied (Table 2). For freshly spiked soil, soil A was amended with 10 mg t-HCH g^{-1} soil as described before (Kumar et al., 2005), and was diluted with non-amended soil A to arrive at soils that were having final concentration of 80 $\mu g \Sigma$ -HCH or 80 $\mu g \beta$ -HCH g^{-1} soil.

Degradation studies for each set of soil were done in fifteen 15-ml glass tubes, as described earlier (Kumar et al., 2005). Briefly, to each tube, 2 g soil and 1.2 mL mineral medium that contained 100 μL *P. aeruginosa* ITRC-5 (0.1 OD₆₀₀) were added. After incubation at 30°C for 0, 3, 6, 9, and 12 days, the reaction in three tubes for each time point was stopped by acidification to pH < 2 with 2N HCl. The un-inoculated tubes,

incubated and processed in the same manner, were used as controls.

Table 2. Concentrations of α - and β -HCH in two sets of the different contaminated soil

Sets	HCH-contamination ($\mu\text{g g}^{-1}$ soil)				
		A	B1	B2	B3
Set-1 (Σ - HCH; $80 \mu\text{g g}^{-1}$ soil)	α -	70.5	49.4	6.4	0.7
	β -	9.6	30.6	73.6	79.3
Set-2 (β - HCH; $80 \mu\text{g g}^{-1}$ soil)	α -	570.4	129.2	7.0	0.7
	β -	80.0	80.0	80.0	80.0

The residual HCH-isomers in the soils were extracted three times by using 3 mL ethyl acetate each time. The extracts for each soil were pooled, and the residue was recovered by evaporation of the solvent at room temperature. After dissolving it in 1mL hexane:acetone (1:1), aliquots of each sample were subjected to TLC for separation of HCH-isomers. Portions of TLC, having α - and β -HCH, were cut out and HCH-isomer in them was eluted with 1 ml n-hexane. Suitable aliquots were analysed by gas chromatography (Clarus-500, Perkin Elmer, Waltham, Massachusetts, USA), as described before (Kumar et al., 2005). Biotransformation of β -HCH by '*E.coli*-LinB' in two sets of each soil was also measured in the same manner as, except that the reaction was stopped after 0, 2, 4, 6, 8 and 24 h of incubation.

Non-exhaustive extraction of HCH-isomers from the soils

For extraction of soils with hydroxypropyl β -cyclodextrin (HPCD), a set of twelve SCT-15 mL centrifuge tubes (Axygen Scientific, CA, USA) for each soil was taken. After adding 2 g soil, having $80 \mu\text{g } \beta\text{-HCH g}^{-1}$, in each tube, three tubes were kept separately for the estimation of HCH-isomers after ethyl acetate extraction. Soil samples in the remaining nine tubes were processed for non-exhaustive extraction. Briefly, after adding 10 mL 50mM HPCD in all the tubes, these were kept horizontally in a rotary shaker and incubated at 30 °C for 24h with shaking at 200 rpm. Afterwards, the soil in each tube was recovered by centrifugation at 5000g for 10 min.

Three tubes were removed for the estimation of HCH-isomers that remained after one round of HPCD-extraction. Similarly, sets of three tubes were removed after second and third round of HPCD- extraction. Amounts of HCH-isomers in all these tubes were analyzed by gas chromatography, as described above.

Statistical Analysis

The values of the residual HCH-isomers, obtained from triplicate samples, were analyzed using analysis of variance (ANOVA), and Fischer's LSD was used to compare the means. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION

Degradation of HCH-isomers in the contaminated soils

The study was designed to evaluate the bioavailability of α - and β -isomers of HCH in different soils that were either freshly-spiked, or were contaminated in the field for short-, medium- or long-term. Experiments on degradability of α - and β -HCH by ITRC-5 in different soils revealed that in set-1 of the soils, where the Σ -HCH was $80 \mu\text{g g}^{-1}$ soil, $\sim 80\%$ α -HCH was degraded in all the four soils i.e. A, B1, B2 and B3, after 6 days of incubation (Figure 1A), and no further degradation was observed by longer incubation up to 12 days. Degradation of β -HCH, however, exhibited considerable differences and $\sim 75, 60, 30$ and 10% was degraded in soils A, B1, B2 and B3, respectively, after 6 days of incubation (Figure 1A) and was not enhanced by prolonged incubation up to 12 days in any of the soils. Degradation of α - and β -HCH in all the un-inoculated soils that were processed under the same conditions was $\sim 10\%$ and 5% , respectively (data not shown).

Differential degradation of β -HCH in the soils could be due to its different initial levels in these. Therefore, its degradation was also studied in set-2 of the same soils, where the level of β -HCH was $80 \mu\text{g g}^{-1}$ in all the soils. Here also, the degradation of α -HCH was $\sim 75\%$ in all the soils, but degradation of β -HCH was $\sim 65, 45, 25$ and 10% in soils A, B1, B2 and B3 respectively, after 6 days incubation (Figure 1B). Again, no further

increase in degradation occurred after longer incubation period up to 12 days.

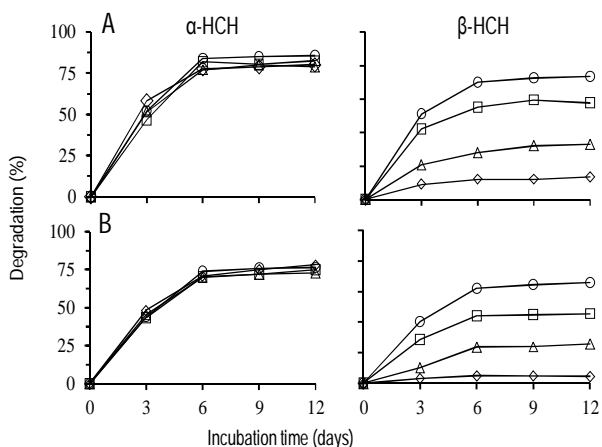


Figure 1. Degradation of α - and β -HCH by ITRC-5 in set-1 i.e. 80 μg Σ -HCH g^{-1} (A) and set-2 i.e. 80 μg β -HCH g^{-1} (B) of the soils A (circle), B1 (square), B2 (triangle) and B3 (diamond). Degradation of α - and β -HCH in these soils under un-inoculated conditions was ~10% and 5%, respectively (data not shown).

Addition of *E.coli*-LinB cells, which were expressing haloalkane dehalogenase LinB, led to differential transformation of β -HCH in different contaminated soils. Thus, in set-1 (Σ -HCH was 80 μg g soil) of the soils, transformation of ~90, 70, 62 and 47% β -HCH was observed in the soils A, B1, B2 and B3, respectively, after 4 h incubation (Figure 2A). Similarly, transformation of ~80, 67, 57 and 43%, respectively was observed in set-2, of these soils (Figure 2B). In all these also, no increase in transformation was observed after prolonged incubation up to 24 h.

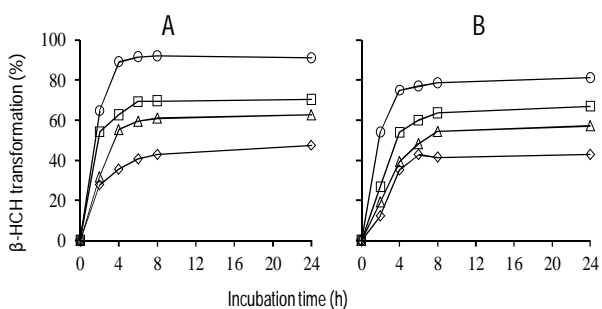


Figure 2. Transformation of β -HCH by *E. coli*-LinB in sets-1 (A) and -2 (B), as described in Figure 1, of the soils A (circle), B1 (square), B2 (triangle) and B3 (diamond).

Non-exhaustive extraction of HCH-isomers

Extraction of HCH-isomers from different soils with 50 mM HPCD solution increased progressively up to two extraction cycles and no further extraction occurred thereafter (Figure 3). Thus, while >95% of α -HCH was extracted after two cycles, ~90, 75, 65 and 50% of β -HCH was extracted from the soils A, B1, B2 and B3, respectively, under the same conditions.

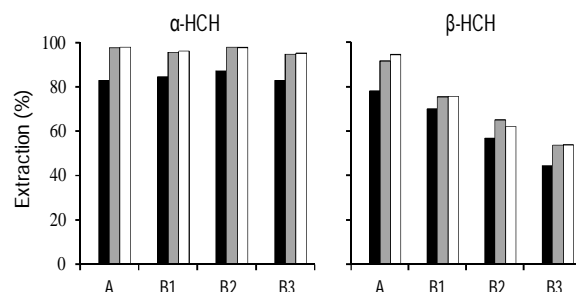


Figure 3. Amounts of α - and β -HCH, extracted from the soils A, B1, B2 & B3 by using 50 mM HPCD, after one (black), two (grey) and three (white) extraction cycles. Amount extracted by three cycles of ethyl acetate from the same soils is taken as 100%.

A progressive decrease in the bioavailability of β -HCH with an increase in the age of HCH-contamination in the soils is consistent with several other studies made earlier on various organic pollutants (Alexander, 2000; Wong and Bidleman, 2011), but no such decrease in the bioavailability of α -HCH under the same conditions was surprising. It suggests that besides the age of contamination, innate nature of the chemical also play a critical role in determining their adsorption and sequestration in the soil particles (Kottler and Alexander, 2001). The reasons behind the differential bioavailability of the two HCH-isomers is not immediately clear but could be due to differences in their octanol-air partition coefficient (K_{OA}) values (Xiao et al., 2004) which for β -HCH (5.54×10^8) is an order of magnitude higher than α -HCH (2.91×10^7). It suggests a stronger retention of β -HCH in the soils and plant foliage, and has also been implied earlier for its increased transport to the ground water (Kalbitz and Popp, 1999). The octanol-water partition coefficient (K_{OW}) values of the two isomers (8.63×10^3 for α -HCH and 8.22×10^3 for β -HCH), however, are comparable (Xiao et al., 2004). Difference in the bioavailability of two chemicals that are so close in structure has not been described before. Sequestered portion of the

pollutants in soils can normally be considered safe, as it is not likely to be available to the organisms to exert toxicity (Alexander, 2000; Barraclough et al., 2005). Results of this study, therefore, underscore the importance of the estimation of bioavailable fraction of HCH-isomers in various contaminated soils for their more realistic risk assessment.

CONCLUSION

Findings of this study describe differential bioavailability of α - and β -HCH in three field contaminated soils. Results suggest that the bioavailability of β -HCH in contaminated soils decreases progressively with an increase in the age of contamination, but of α -HCH is not affected under the same conditions. Estimation of bioavailable fraction of HCH-isomers in soil is an important requirement for their correct risk assessment. Results are expected to help in setting more realist targets for degradation of soil-bound HCH-isomers, and also in developing strategies for bioremediation of contaminated sites.

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REFERENCES

- Alexander, M., 2000. Ageing, bioavailability and overestimation of risk from environmental pollutants. *Environ. Sci. Technol.* 34 (20), 4259-4265.
- ATSDR, 2005. Toxicological profile for hexachlorocyclohexanes, United States of America Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, August, 2005. <http://www.atsdr.cdc.gov/toxprofiles/tp43.html>
- Barraclough, D., Kearney, T., Croxford, A., 2005. Bound residues: environmental solution or future problem? *Environ. Pollut.* 133 (1), 850- 90.
- Celano, G., Šmejkalova, D., Spaccini, R., Piccolo, A., 2008. Interactions of three s-triazines with humic acids of different structure. *J. Agri. Food Chem.* 56, 7360- 7366.
- Chung, N., Alexander, M., 1998. Differences in sequestration and bioavailability of organic chemicals aged in dissimilar soils. *Environ. Sci. Technol.* 32, 855-860.
- Concha-Graña, E., Turnes-Carou, M.I., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez, D., Fernández-Fernández, E., 2006. Evaluation of HCH isomers and metabolites in soils, leachates, river water and sediments of a highly contaminated area. *Chemosphere* 64(4), 588-595.
- Gong, Z.M., Xu, F.L., Dawson, R., Cao, J., Liu, W.X., Li, B.G., Shen, W.R., Zhang, W.J., Qin, B.P., Sun, R., Tao, S., 2004. Residues of hexachlorocyclohexane isomers and their distribution characteristics in soils in the Tianjin area. *China. Arch. Environ. Contam. Toxicol.* 46 (4), 432- 437.
- Henry, W., Tay, P., Waldow, H., Hung, H., Li, Y.F., MacLeod, M., Hungerbuhler, K., 2012. Comparative assessment of the global fate of α - and β -hexachlorocyclohexane before and after phase-out. *Environ. Sci. Technol.* 46 (4), 2047- 2054.
- Hung, H., Kallenborn, R., Breivik, K., Su, Y.S., Brorstrom-Lunden, E., Olafsdottir, K., Thorlacius, J.M., Leppanen, S., Bossi, R., Skov, H., Mano, S., Patton, G.W., Stern, G., Sverko, E., Fellin, P., 2010. Atmospheric monitoring of organic pollutants in the Arctic under the Arctic Monitoring and Assessment Programme (AMAP) 1993–2006. *Sci. Total Environ.* 408 (15), 2854- 2873.
- Kalbitz, K., Popp, P., 1999. Seasonal impacts on β -hexachlorocyclohexane concentration in soil solution. *Environ. Pollut.* 106 (1), 139- 141.
- Katayama, A., Bhula, R., Burns, G.R., Carazo, E., Felsot, A., Hamilton, D., Harris, C., Kim, Y.H., Kleter, G., Koedel, W., 2010. Bioavailability of xenobiotics in the soil environment. *Rev. Environ. Contam. Toxicol.* 203, 1- 86.
- Kottler, B.D., Alexander, M., 2001. Relationship of properties of polycyclic aromatic hydrocarbons to sequestration in soil. *Environ. Pollut.* 113 (3), 293- 8.
- Kristensen, G. B., Johannesen, H., Aamand, J., 2001. Mineralization of aged atrazine and mecoprop in soil and aquifer chalk. *Chemosphere*, 45, 927-934.
- Kumar, M., Chaudhary, P., Dwivedi, M., Kumar, R., Paul, D., Jain, R.K., Garg, S.K., Kumar, A., 2005. Enhanced biodegradation of β - and δ -hexachlorocyclohexane in the presence of α - and γ -isomers in contaminated soils. *Environ. Sci. Technol.* 39, 4005- 4011.
- Macwan, A. S., Javed, S., Kumar, A., 2011. Isolation of a novel thermostable dehydrochlorinase

- (LinA) from a soil metagenome. *3 Biotech.* 1, 193-198. Epub 2011 Jun 18.
- Reid, B.J., Stokes, J.D., Jones, K.C., Semple, K.T., 2000. Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. *Environ. Sci. Technol.* 34 (15), 3174- 3179.
- Schwartz, E., Scow, K.M., 1999. Using biodegradation kinetics to measure availability of aged phenanthrene to bacteria inoculated into soil. *Environ. Toxicol. Chem.* 18 (8), 1742- 1746.
- Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environ. Sci. Technol.* 38, 228A- 231A.
- Swindell, A.L., Reid, B.J., 2006. Comparison of selected non-exhaustive extraction techniques to assess PAH availability in dissimilar soils. *Chemosphere.* 62 (7), 1126- 34.
- Vijgen, J., Abhilash, P.C., Li, Y.F., Lal, R., Forter, M., Torres, J., Singh, N., Yunus, M., Tian, C., Schäffer, A., Weber, R., 2011. Hexachlorocyclohexane (HCH) as new Stockholm Convention POPs--a global perspective on the management of Lindane and its waste isomers. *Environ. Sci. Pollut. Res. Int.* 18(2), 152- 62.
- Willett, K., Ulrich, E., Hites, R., 1998. Differential toxicity and environmental fates of hexachlorocyclohexane isomers. *Environ. Sci. Technol.* 32, 2197- 2207.
- Wong, F., Bidleman, T.F., 2011. Aging of organochlorine pesticides and polychlorinated biphenyls in muck soil: volatilization, bioaccessibility, and degradation. *Environ. Sci. Technol.* 45 (3), 958- 963.
- Xiao, H., Li, N., Wania, F., 2004. Compilation, evaluation, and selection of physical-chemical property data for α -, β -, and γ -hexachlorocyclohexane. *J. Chem. Eng. Data.* 49 (2), 173- 185.

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