RELATIVE BIOAVAILABILITY OF α- AND β-HEXACHLOROCYCOHEXANE IN VARIOUS SOILS FROM A CONTAMINATED SITE
Ashish Srivastavaab, Sunil G. Babu, Ashwani Kumar*  
ab Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research, Lucknow-226001  
bDepartment of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow-226025, India.  
*Corresponding author’s Email: ashwani.iitr26@gmail.com  
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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.

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 Chinhalt 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 ∑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

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Soils</th>
<th>HCH-isomers (mg g⁻¹ soil)</th>
<th>% β-HCH¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α⁻</td>
<td>β⁻</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>2</td>
<td>B1</td>
<td>16.8</td>
<td>10.4</td>
</tr>
<tr>
<td>3</td>
<td>B2</td>
<td>0.56</td>
<td>6.4</td>
</tr>
<tr>
<td>4</td>
<td>B3</td>
<td>0.1</td>
<td>12.0</td>
</tr>
</tbody>
</table>

¹BDL below detection level i.e. < 0.05 µg g⁻¹ soil
²sum of α-, β-, γ- and δ-HCH
³β-HCH/∑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 10 mL ‘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 (50µg/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 80µg ∑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 80µg 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 80µg ∑HCH or 80µg β-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 100µL 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

<table>
<thead>
<tr>
<th>Sets</th>
<th>α-HCH-contamination (µg g⁻¹ soil)</th>
<th>β-HCH-contamination (µg g⁻¹ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set-1 (Σ-HCH; 80 µg g⁻¹ soil)</td>
<td>α- 70.5, 49.4, 6.4, 0.7</td>
<td>β- 9.6, 30.6, 73.6, 79.3</td>
</tr>
<tr>
<td>Set-2 (β-HCH; 80 µg g⁻¹ soil)</td>
<td>α- 570.4, 129.2, 7.0, 0.7</td>
<td>β- 80.0, 80.0, 80.0</td>
</tr>
</tbody>
</table>

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 1 mL hexane:acetone (1:1), aliquots of each sample were subjected to TCL for separation of HCH-isomers. Portions of TCL, 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 80µg β-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 24 h 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 80μgg⁻¹ soil, ~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 ~75, 60, 30 and 10% was degraded in soils A, B1, B2 and B3, 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 ~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 80μgg⁻¹ in all the soils. Here also, the degradation of α-HCH was ~75% in all the soils, but degradation of β-HCH was ~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 80µg g⁻¹ 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 (KOA) values (Xiao et al., 2004) which for β-HCH (5.54 x 10⁸) is an order of magnitude higher than α-HCH (2.91 x 10⁷). 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 (KOW) values of the two isomers (8.63 x 10³ for α-HCH and 8.22 x 10³ 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 realistic targets for degradation of soil-bound HCH-isomers, and also in developing strategies for bioremediation of contaminated sites.

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