

Bioremediation of Hexachlorocyclohexane Contaminated Soil: Field Trials

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1. Introduction

Hexachlorocyclohexane or the abbreviation HCH is identified as a monocyclic chlorinated hydrocarbon. HCH was discovered in 1825 by Faraday, who just had discovered benzene. By reacting benzene with chlorine in bright sunlight, formation of HCH was observed. Neither Faraday nor the Dutch chemist Van der Linden, who in 1912 isolated the pure γ -isomer from a HCH mixture, realized the insecticidal potential of the compounds they produced (Amadori, 1993). The insecticidal properties of HCH were however first mentioned by Bender in a patent paper. HCH was first patented in the 1940s. Dupire conducted detailed investigations on the insecticidal properties in 1940, and HCH was first used to combat the Colorado beetle (Stoffbericht, 1993). In 1942 Slade proved that γ -HCH was the sole carrier of the insecticidal properties of technical HCH (Stoffbericht, 1993). HCH production started commercially since 1947 in Germany. The common name of Hexachlorocyclohexane is "benzene hexachloride", which is incorrect according to the IUPAC rules (Galvan, 1999). Nevertheless it is still widely used, especially in the form of its abbreviation "BHC"

Technical HCH consists mainly of a mixture of various stereo-isomers, which are designated by Greek letters. Only one of these stereoisomers, γ -HCH, is the carrier of the insecticidal properties, while the other isomers are sometimes collectively referred to as "inactive isomers". The raw product from the chlorination of benzene contains about 14% γ -HCH and 86% of inactive isomers, i.e. 65-70% α -, 7-10% β -, 14-15% γ -, approximately 7% δ -, 1-2% ϵ -HCH, and 1-2% other components. Therefore, in the production of one ton of technical HCH, 140 kg is γ -HCH and 860 kg is "inactive isomers". The latter is potentially waste and predestined for disposal. It is possible to extract and purify the active γ -HCH. If the purity is 99.0% or more it may be called "Lindane", which is an accepted common name for this substance. Lindane is also called γ -HCH, or γ -BHC and by FAO γ -BHC (technical grade). Technical grade HCH and fortified HCH (FHCH) containing a varying mixture of at least 5 isomers, with a minimum of 40% γ -isomer was available commercially. HCH is no longer produced in USA and few other European countries and cannot be sold for domestic use by EPA regulation as well as many other countries (FCH, 1984). However, in some developing countries HCH especially γ -isomer continues to be used because of economic purposes and also in public health programmes. Thus Technical grade HCH continues to be produced and

all isomers except γ -isomer continue to be dumped unutilized. The production of Lindane creates huge amounts of isomers waste. The total quantity of waste will be about 8 times the Lindane output (Bodenstein, 1972), i.e. for each ton of Lindane produced 8 tons of waste will be generated. The large environmental consequences that are created can be imagined.



Photo from the mid-1990s of a temporary storage site for 200 000 tons of soil contaminated with waste HCH isomers.

Considering every ton of lindane produced generates approximately 6 -10 tons of other HCH isomers, a considerable amount of residues would be generated during the manufacture of this insecticide. For decades, the waste isomers were generally disposed off in open landfills like fields and other disposal sites near the HCH manufacturing facilities. After disposal, degradation, volatilization, and run off of the waste isomers occurred. If the estimate of global usage of lindane of 600,000 tons between 1950 and 2000 is accurate, the total amount of possible residuals (if it is assumed that a mean value of 8 tons of waste isomers are obtained per ton of lindane produced) amounts to possibly 4.8 million tons of HCH residuals that could be present worldwide giving an idea of the extent of the environmental contamination problem. Air releases of lindane can occur during the agricultural use or aerial application of this insecticide, as well as during manufacture or disposal. Also, lindane can be released to air through volatilization after application.

1.1 Fate of lindane

Once released into the environment, lindane can partition into all environmental media. Hydrolysis and photolysis are not considered important degradation pathways and reported half-lives in air, water and soil are: 2-3 days, 3-300 days and up to 2 - 3 years, respectively. A half-life of 96 days in air has also been estimated. Lindane can bioaccumulate easily in the food chain due to its high lipid solubility and can bio-concentrate rapidly in

microorganisms, invertebrates, fish, birds and mammals. The bio-concentration factors in aquatic organisms under laboratory conditions ranged from approximately 10 upto 4220 and under field conditions, the bio concentration factors ranged from 10 upto 2600.

Lindane is listed as a "substance scheduled for restrictions on use". This means that products in which at least 99% of the HCH isomer is in the γ -form (i.e. lindane, CAS: 58-89-9) are restricted to the following uses: 1. Seed treatment. 2. Soil applications directly followed by incorporation into the top soil surface layer 3. Professional remedial and industrial treatment of lumber, timber and logs. 4. Public health and veterinary topical insecticide. 5. Non-aerial application to tree seedlings, small scale lawn use, and indoor and outdoor use for nursery stock and ornamentals. 6. Indoor industrial and residential applications. Lindane is one of the listed priority hazardous substances for which quality standards and emission controls will be set at EU level to end all emissions within 20 years. Lindane is banned for use in 52 countries, restricted or severely restricted in 33 countries, not registered in 10 countries, and registered in 17 countries.

Lindane can be found in all environmental compartments, and levels in air, water, soil sediment, aquatic and terrestrial organisms and food. Humans are therefore being exposed to lindane as demonstrated by detectable levels in human blood, human adipose tissue and human breast milk in different studies in diverse countries. Exposure of children and pregnant women to lindane are of particular concern. γ -HCH has been found in human maternal adipose tissue, maternal blood, umbilical cord, blood and breast milk. Lindane has also been found to pass through the placental barrier. Direct exposure from the use of pharmaceutical products for scabies and lice treatment should be of concern. Exposure from food sources is possibly of concern for high animal lipid content diets and subsistence diets of particular ethnic groups. Occupational exposure at manufacturing facilities should be of concern, because lindane production implies worker exposure to other HCH isomers as well, for example the α - isomer is considered to be a probable human carcinogen.

Hepatotoxic, immunotoxic, reproductive and developmental effects have been reported for lindane in laboratory animals. The US EPA has classified lindane in the category of "Suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential". The most commonly reported effects associated with oral exposure to γ -HCH are neurological. Most of the information is from case reports of acute γ -HCH poisoning. Seizures and convulsions have been observed in individuals who have accidentally or intentionally ingested lindane in insecticide pellets, liquid scabicide or contaminated food (WHO/Europe, 2003). Lindane is highly toxic to aquatic organisms and moderately toxic to birds and mammals following acute exposures. Chronic effects to birds and mammals measured by reproduction studies show adverse effects at low levels such as reductions in egg production, growth and survival parameters in birds, and decreased body weight gain in mammals, with some effects indicative of endocrine disruption.

Comparing to other POPs and hazardous waste problems, the HCH-residuals differ significantly as the extent of the problem is huge and as an environmentally sound disposal method will be necessary. However the enormous financial burden needed to achieve this will be a main barrier. On the other hand, the former practice of simple encapsulation is considered far from sustainable and will leave a huge number of time bombs in the global landscape. Hence bioremediation is the best option of removing these isomers from the contaminated environments. Microbial degradation of chlorinated pesticides such as HCH

is usually carried out by using either pure or mixed culture systems. The main goal of the laboratory studies is to predict the biodegradation rates in the environment. But it is very difficult to extrapolate the results obtained in the laboratory systems to predict their fate in the environment [Spain et al 1990]. The microbial degradation of HCH isomers in liquid cultures has been studied using pure microbial cultures such as *Clostridium rectum*, *Pandoraea* [Ohisa et al 1990, Okeke et al 2002], mixed native soil microbial population (undefined consortium) [Bachmann et al 1988 a, Sahu et al 1995], *Phanerochaete chrysosporium* [Kennedy et al 1990, Mougouin et al 1997] and sewage sludge under aerobic and anaerobic conditions [Bachmann et al 1988b, McTernan, and Pereira 1991, Buser, and Mueller 1995]. The degradation of γ - and α -isomers was almost complete and β - and δ -isomers showed more resistance to degradation. At this stage it is imperative to develop technologies where all isomers of tech-HCH are degraded completely. In this communication we describe the degradation of tech-HCH in artificial plots and also in actual fields using a microbial consortium.

2. Materials and methods

2.1 Substrate

α -, β -, γ - and δ -HCH isomers (99% pure) were procured from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Technical grade HCH was obtained from Hindustan insecticides, Mumbai, India. Other chemicals and the reagents used in this study were of analytical grade and were purchased from standard chemical companies. *Rhizobium* and *Azospirillum* were obtained from IMTECH, Chandigarh. Soil used in small plot studies was collected from CFTRI campus without history of HCH usage.

2.2 Microbial consortium

The microbial consortium capable of degrading Tech-HCH was developed in our laboratory by long term enrichment of HCH contaminated soil and sewage according to Manonmani et al. [2000]. The Tech-HCH degrading consortium that got enriched was acclimated with increase in concentration of Tech-HCH from 5 to 25 ppm. The consortium thus obtained was maintained as liquid culture in minimal medium containing 10 ppm of Tech- HCH and in minimal agar medium containing 10 ppm of Tech- HCH.

2.3 Culture medium

Wheat bran hydrolysate used for growth of microbial consortium was prepared by acid hydrolysis of wheat bran. Minimal medium used in degradation studies consisted of KH_2PO_4 , 0.675g; Na_2HPO_4 , 5.455g and NH_4NO_3 , 0.25g and 1L of water.

2.4 Degradation of Tech-HCH using inoculum grown on different carbon sources

2.4.1 Biomass build-up and pre exposure of the inoculum

Individual isolates of the microbial consortium were grown in wheat bran hydrolysate/peptone- glycerol medium and the cells were harvested after 72 h of growth, washed well and pre-exposed to 25ppm of individual isomers and Tech-HCH for 72 h separately with addition of individual isomers and Tech- HCH every 24 h. The induced cells were harvested by centrifugation at 10,000rpm, 4°C, 10min, washed well and used as inoculum.

2.4.2 Essentiality of individual members of the consortium for degradation of Tech-HCH

Degradation of higher concentration (25ppm) of Tech-HCH was taken up to test the performance of different combinations of the members as well as individual isolates of the HCH-degrading consortium. The combinations as given in Table 2 were tested for their ability to degrade tech-HCH. Samples were harvested after known period of incubation and analysed for residual HCH isomers.

Microbial combination used	Degradation (%)			
	α -isomer	β -isomer	γ -isomer	Δ -isomer
1	15.70	4.62	48.41	4.66
2	6.21	7.19	40.21	1.66
3	6.82	2.52	33.33	6.04
4	5.06	10.27	23.16	5.21
5	6.66	4.73	11.58	5.91
6	8.14	3.05	18.58	3.07
7	1.21	6.27	28.63	4.32
8	21.86	8.77	47.55	6.27
9	8.5	6.85	28.55	5.58
10	17.92	7.68	16.32	6.00
1-2	7.06	5.31	51.16	4.68
1-3	11.25	9.11	56.20	8.5
1-4	34.35	20.66	62.41	13.82
1-5	48.62	50.14	70.73	26.24
1-6	64.36	62.18	72.48	46.86
1-7	68.24	74.28	76.88	58.46
1-8	70.11	76.32	84.14	63.46
1-9	80.12	81.32	100	74.32
1-10	94.23	95.16	100	85.11

Table 2. Essentiality of individual members of the consortium for degradation of Tech-HCH

2.4.3 Preparation of inoculant formulations and their stability

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass and wheat bran. The inoculum used contained 10^7 cells/g of the substrate. The consortium was mixed with *Sphagnum* mass. *Rhizobium* and *Azospirillum* were added to these separately. The inoculant formulation was prepared as both pellet and wet powder. The formulations were checked at regular intervals for survivability of the consortial members and their degradation capacity.

2.5 Degradation of Tech-HCH in different soils

Different soils such as clay soil, red soil, garden soil, soils from coconut, coffee, turmeric and tomato plantations were studied to evaluate the degradation of Tech- HCH. The substrate and inoculum were used at 25 $\mu\text{g g}^{-1}$ and 500 $\mu\text{g protein g}^{-1}$ soil respectively. All the experiments were done in replicates of ten for each parameter studied.

2.6 Degradation of technical hexachlorocyclohexane in small artificial plots

Four artificial plots of 2ft x 2ft x 0.5ft were prepared. Red loamy non- sterile soil containing 1-1.5% organic carbon and 32% water holding capacity and pH 7.0, collected from CFTRI campus, was taken in these plots. Each plot was spiked with 25 ppm of tech- HCH and mixed well. Two plots were inoculated with 72 h HCH- induced microbial consortium. Two plots were maintained as abiotic (uninoculated) controls. All plots were kept wet by regular sprinkling of water and the soil in the plots was mixed regularly. Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly. One-gram soil was used for recording colony-forming units (cfu) and other set was used for residue analyses.

2.7 Degradation of HCH in artificial test plots (large size)

Individual isolates of the HCH- degrading consortium were grown individually in 25 L carbuoys, containing 20 L wheat bran hydrolysate medium (containing 0.75 % sugars) for 72 h. The cells were harvested and mixed at equal OD_{600} . The reconstituted consortium was induced with 25 ppm of Tech- HCH for 72 h in minimal medium, centrifuged, washed well in minimal medium and used as inoculum after resuspending the cells in known volume of minimal medium. Six artificial plots of 2m x 1m x 0.5ft were prepared and red loamy soil collected from CFTRI campus was taken in these plots. Each plot was spiked with 25 ppm Tech- HCH and mixed well. Four plots were inoculated with 72 h HCH- induced microbial consortium. The inoculum was added at levels containing 10^7 to 10^8 cells/ g soil. Two plots were maintained as uninoculated controls (containing only HCH). The soil was mixed well at regular intervals and was kept moist by regular sprinkling of water. At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate.

2.8 Degradation of Tech-HCH in actual fields

8 plots measuring 2m² were chosen at CFTRI campus (Plate 1). The plots were prepared by tilling and toeing. The individual members of the microbial consortium were grown separately in a 25 L carbuoy containing 20 L of wheat bran hydrolysate medium (containing 0.75% reducing sugars). After 72 h of growth the cells were harvested and pooled at equal OD_{600} . The reconstituted consortium was induced with 25 ppm of Tech- HCH for 72 h. Then the microbial mass was separated by centrifugation, washed well and resuspended in required quantity of minimal medium. Spiking of Tech- HCH to the soil was repeated for four times on alternative days. The final concentration of Tech- HCH added to the soil was 25 ppm. Six of the plots received Tech- HCH while two remaining were maintained as unspiked, uninoculated controls. Two plots were inoculated with microbial consortium and two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. The inoculum at 0 h was added at the level containing 10^7 - 10^8 cells. Sampling was done at 24 h intervals. 25 samples were removed randomly from each plot and analysed for residual HCH isomers and survivability of microbial consortium.



Plate 1. HCH degradation experiments in open plots/actual fields

2.9 Bioassay of the remediated soil

Seeds of *Raphanus sativus* (radish) and *Aeblumuschnus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non- HCH- spiked controls were all sown with seeds of both radish and ladies finger. The seeds were allowed to germinate and grow completely. The plants were checked for growth, deformalities etc.

2.10 Degradation of other organochlorine pesticides by the microbial consortium

Soil was spiked with different organochlorine pesticides such as endosulphan, heptachlor, endrin, dieldrin at 10ppm level. Microbial consortium induced with respective organochlorine pesticide was used as inoculum. Pesticide spiked soil was inoculated with microbial consortium at inoculum 500 μg protein /g soil. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide.

2.11 Degradation of Tech-HCH in native soil

The Tech-HCH degrading consortium was inoculated to different types of native soils. The types of soil chosen were clay soil; soil from coconut fields; tomato fields; coffee plantations; turmeric fields; red soil and garden soil. These soils were spiked with Tech-HCH and mixed well. It was inoculated with microbial consortium at 500 μg protein /g soil. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide.

3. Analytical

3.1 Growth

Growth of the consortium was determined by estimating total protein in the biomass by modified method of Lowry et al according to Murthy et al (2007). Cells were harvested from a suitable quantity of culture broth, washed with minimal medium, suspended in 3.4 mL distilled water and 0.6 mL of 20% NaOH. This was mixed and digested in a constant boiling water bath for 10 min. Total protein, in cooled sample of this hydrolysate, was estimated by using Folin-Ciocalteu reagent. A total of 0.5 mL of the hydrolysate was taken in a clean test tube. To this was added 5.0 mL of Lowry's C. After 10 min 0.5 mL of Lowry's D [Folin-Ciocalteu reagent (1:2)] was added and mixed well. The colour was read at 660nm after 20.0 min of standing at room temperature, using a spectrophotometer (Shimadzu UV- 160A, Japan). Total amount of protein was computed using the standard curve prepared with BSA (Bovine serum Albumin).

Growth was also measured in terms of colony forming units (cfu) as described by Sahu et al (1995) using appropriately diluted broth.

3.2 Quantification of HCH

Residual HCH was quantified by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

3.2.1 Extraction of residual HCH

The soil samples (whole cups), were removed after required period of incubation, they were air dried and extracted thrice with equal volumes of dichloromethane. The three solvent extracts were pooled and passed through column containing sodium sulphate (anhydrous) and florisil. These fractions were concentrated at room temperature and resuspended in a known volume of acetone and used for analysis of residue.

3.2.2 Thin Layer Chromatography

Thin layer chromatography (TLC) was done using silica gel G TLC plates. Residual substrate samples dissolved in required quantity of acetone were spotted on TLC plates and these plates were developed in cyclohexane. The residual tech-HCH spots were identified after spraying the air-dried developed plates with *O*-tolidine in acetone. The residual substrate spots were delineated by marking with a needle and the area was measured. The concentration of residual substrate was computed from a standard plot of log concentrations *versus* square root of the area prepared for standard tech-HCH.

3.2.3 Gas chromatography

Concentrated solvent containing residual substrate was resuspended in HPLC grade acetone and gas chromatography was done using Chemito gas chromatograph (GC 1000). After appropriate dilution 1 μ L sample was injected into gas chromatograph equipped with ⁶³Ni detector and capillary column DB 5 (30m X 0.25 mm) packed with (5% phenyl)-methylpolysiloxane. The column, injector and detector were maintained at 230^o C, 230^o C and 320^o C respectively with a flow rate of carrier gas IOLAR grade I nitrogen at 1mL/min. The recovery of HCH isomers ranged from 92 to 95% from mineral salts medium. All the data presented in this study are based on triplicate estimations.

Other organochlorine pesticides were also estimated by GC under same conditions.

4. Results and discussion

The study is meant to assess the efficiency of microbial consortium to degrade HCH isomers contained in soil. The optimized conditions obtained in our previous study (Murthy and Manonmani, 2007) were adopted in this present study to find out the applicability. The optimized parameters used were: inoculum level 500 µg protein g⁻¹ soil, pH 7.5 and incubation temperature 30^o C.

The soil used was red soil with no history of HCH applications. Soil had a particle size less than 0.5 mm. This size was chosen to provide high superficial area for interaction between HCH and microorganisms.

4.1 Microbial consortium

HCH-degrading microbial consortium developed by the long-term enrichment of the contaminated soil and sewage samples (Manonmani et al, 2000; Bidlan and Manonamani, 2002). This was reconstituted by mixing the different consortia having the ability to degrade α -, β -, γ - and δ -HCH isomers. This reconstituted consortium was acclimated with increasing concentration of Tech-HCH from 5ppm through 25 ppm. in shake flasks through three consecutive transfers every 24h. This consortium was used to study the degradation of Tech-HCH. The community structure of the consortium was identified by dilution plating technique. The bacterial of the consortial community were identified by biochemical tests and following Bergey's Manual of Determinative Bacteriology. The identification was confirmed by using Microbact Identification system. The consortium was found to be made of ten bacterial isolates consisting of seven *Pseudomonas* spp.; one species each of *Burkholderia*, *Flavobacterium* and *Vibrio* (Table 1)

Different carbon sources, both simple and complex, were used to optimize biomass production. Molasses supported highest biomass production, followed by glucose, sucrose, rice straw extract supplemented with glucose, rice straw hydrolysate, nutrient broth and wheat bran hydrolysate. The biomass grown on different carbon sources, when inoculated to 25ppm of HCH, showed that biomass grown on wheat bran hydrolysate showed better HCH-degradation. Nearly 80 - 90 % of all the four isomers disappeared by 72h of incubation. Next was molasses grown inoculum, which showed 65 - 70 % of degradation (Fig 1).

Sl. No.	Bacterial isolate	No.
1	<i>Pseudomonas fluorescens</i> biovar II	T ₁
2	<i>Pseudomonas diminuta</i>	T ₂
3	<i>Pseudomonas fluorescens</i> biovar I	T ₃
4	<i>Burkholderi pseudomallei</i>	T ₄
5	<i>Pseudomonas putida</i>	T ₅
6	<i>Flavobacterium</i> sp.	T ₆
7	<i>Vibrio alginolyticus</i>	T ₇
8	<i>Pseudomonas aeruginosa</i>	T ₈
9	<i>Pseudomonas stutzeri</i>	T ₉
10	<i>Pseudomonas fluorescens</i> biovar V	T ₁₀

Table 1. Composition of the Tech-HCH-degrading microbial consortium

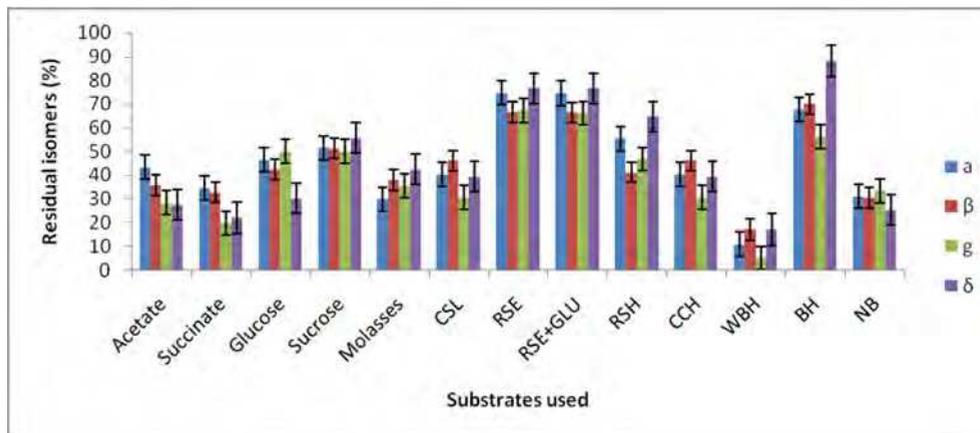
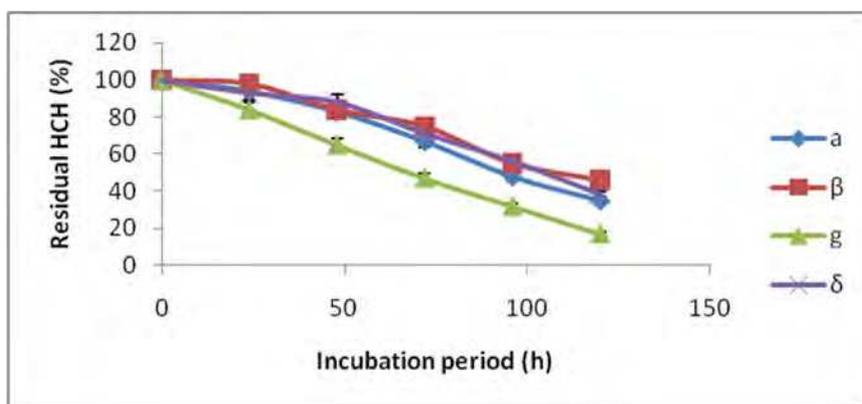


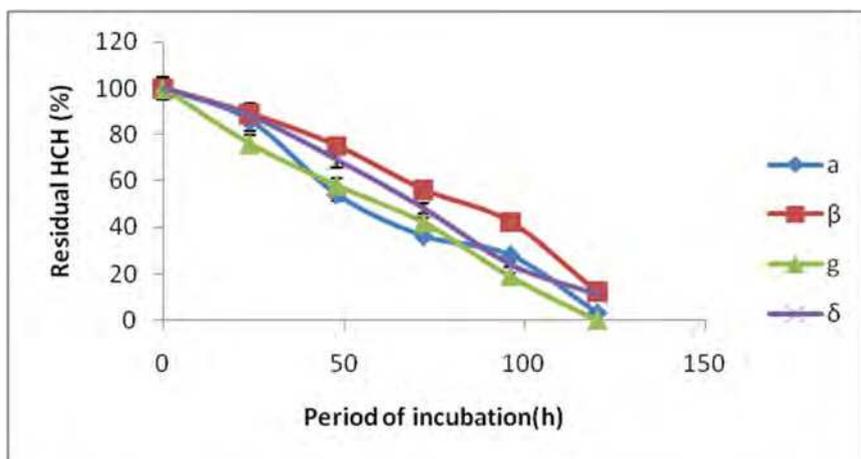
Fig. 1. Degradation of Tech-HCH with inoculum grown on different carbon sources CSL , Corn steep liquor; RSE , Rice straw extract; RSH, Rice straw hydrolysate; CCH, Corn cob hydrolysate; WBH, Wheat bran hydrolysate; BH, Bagassae hydrolysate; NB, Nutrient broth.

4.2 Effect of induction/pre-exposure on the degradation of Tech-HCH

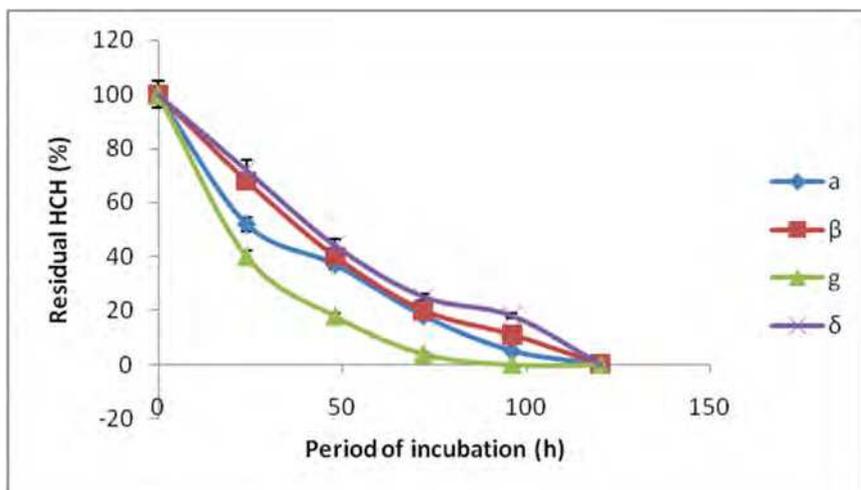
The pre-exposure of the HCH-degrading consortium was tried to understand the adaptation of the developed consortium and to understand the choice of the inducer that could be used for complete mineralization of tech-HCH. By exposing the WBH grown microbial consortium to α -isomer resulted in the complete degradation of α -isomer of Tech-HCH by 72 h of incubation. β -, γ - and δ -isomers were still present at 2.035%, 6.019% and 24.13% levels by the end of 72 h of incubation Similarly, β -, γ - and δ -HCH induced consortium degraded the substrates used for induction better than other isomers. But, Tech-HCH induced consortium degraded all the isomers (Fig 2) and in further experiments Tech-HCH induced inoculum was used unless otherwise stated.



a) 24h pre-exposure



b) 48h pre-exposure



c) 72h pre-exposure

Fig. 2. Effect of Pre-exposure to Tech-HCH on Tech-HCH degradation

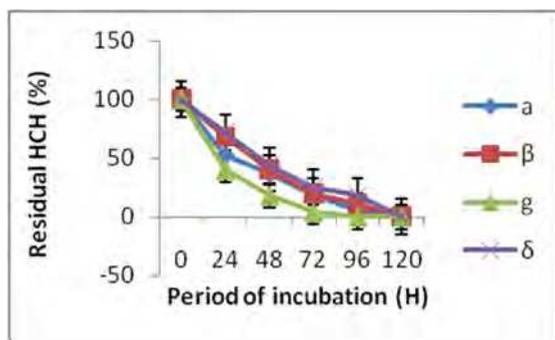
4.3 Essentiality of individual members of the consortium for degradation of Tech-HCH

Degradation of higher concentrations (25ppm) of Tech-HCH was taken up to test the performance of different combinations of the members as well as individual isolates of the HCH-degrading consortium. Table 2 describes the results of this study. It appears obvious that the presence of all the ten strains is necessary for the faster and efficient degradation of higher concentrations of HCH. With α -isomer as substrate, isolate T8 degraded 21% of the substrate by 120h. No other individual isolates were able to degrade this isomer. With increase in the number of individual members of the consortium the degradation of the α -isomer increased and when all ten isolates were mixed together 94% degradation of α -

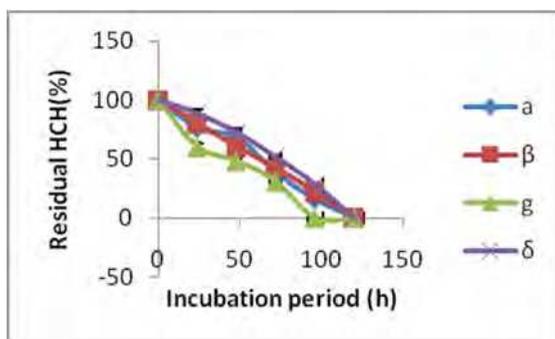
isomer was observed. When β -isomer was used as a substrate, isolate T4 degraded up to 10% of the β -isomer. No other individual isolates were able to degrade this isomer up to 10%. With increase in the number of individual members of the consortium the degradation of the β -isomer increased and when all ten isolates were mixed together 95% degradation of β -isomer was observed. When γ -isomer was used as a substrate, isolates T1, T8 and T2 were able to degrade 48, 47 and 49% of γ -isomer respectively when inoculated individually. Combination of all T9 and T10 isolates could degrade the γ -isomer completely. With δ -isomer as sole substrate, isolate T8 showed highest degradation of 6%. No other isolates were successful in degrading δ -isomer. Combination of all individual members of the consortium could degrade 85% of the isomer. Increase with addition of individual members showed increase in the degradation of δ -isomer.

4.4 Degradation of Tech-HCH in native soil

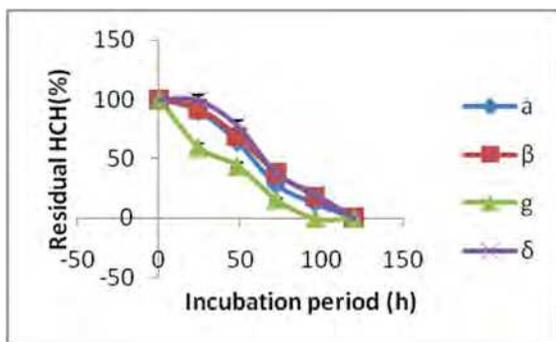
The Tech-HCH degrading consortium was inoculated to different types of native soils. Degradation was complete in all soils, but it was faster in red soil. In all soil types, γ -isomer disappeared faster (Fig 3). Degradation by native microflora was very low. The growth of the individual members of the consortium was also not inhibited by the presence of the native isolates.



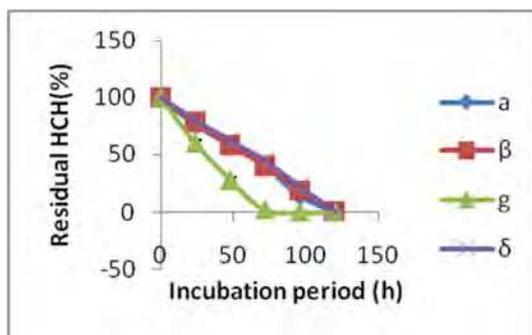
a) Clay soil



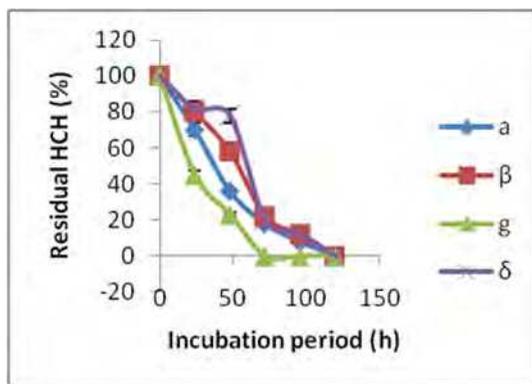
b) Soil from coconut fields



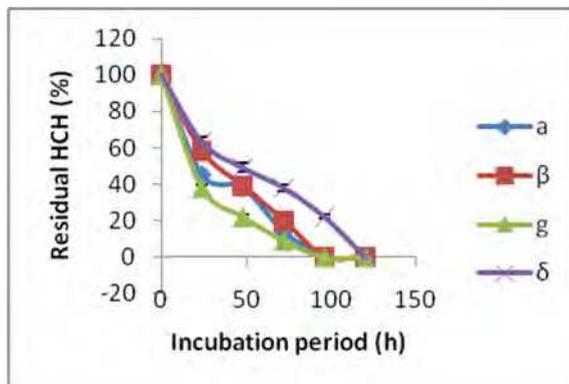
c) Soil from tomato fields



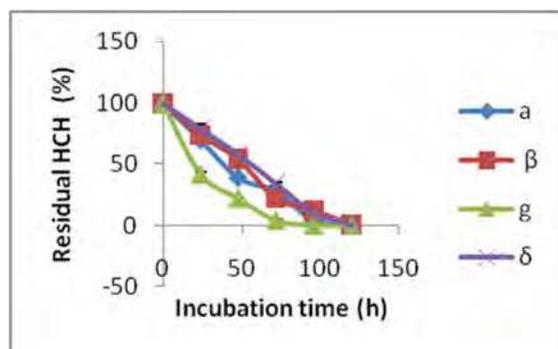
d) Soil from coffee plantations



e) Red soil



f) Soil from turmeric field



g) Garden soil

Fig. 3. Degradation of Tech-HCH in different native soils

4.5 Degradation of other organochlorine pesticides by the microbial consortium

The HCH degrading microbial consortium acclimated with both tech-HCH and different pesticides under study were inoculated separately to respective organochlorine pesticides. The analysis of soil at different periods of incubation time indicated that the degradation was good when the consortium induced with HCH was used. The organochlorine pesticides such as DDT, heptachlor, endrin, dieldrin and endosulphan disappeared with HCH induced consortium (Fig 4). With the consortium induced with respective substrates, all substrates, except endrin were degraded completely.

4.6 Preparation of inoculant formulations and their stability

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass and wheat bran. The inoculum used contained 10^7 cells/g of the substrate. The inoculant formulation was prepared as both pellet and wet powder. The formulation was found to be stable for 60 days at refrigerated conditions. The formulations, when inoculated to soil containing 25 ppm tech-HCH, were found to degrade all the isomers of HCH completely (Fig 5). The formulation prepared in wheat bran took slightly longer time for complete degradation of tech- mixture.

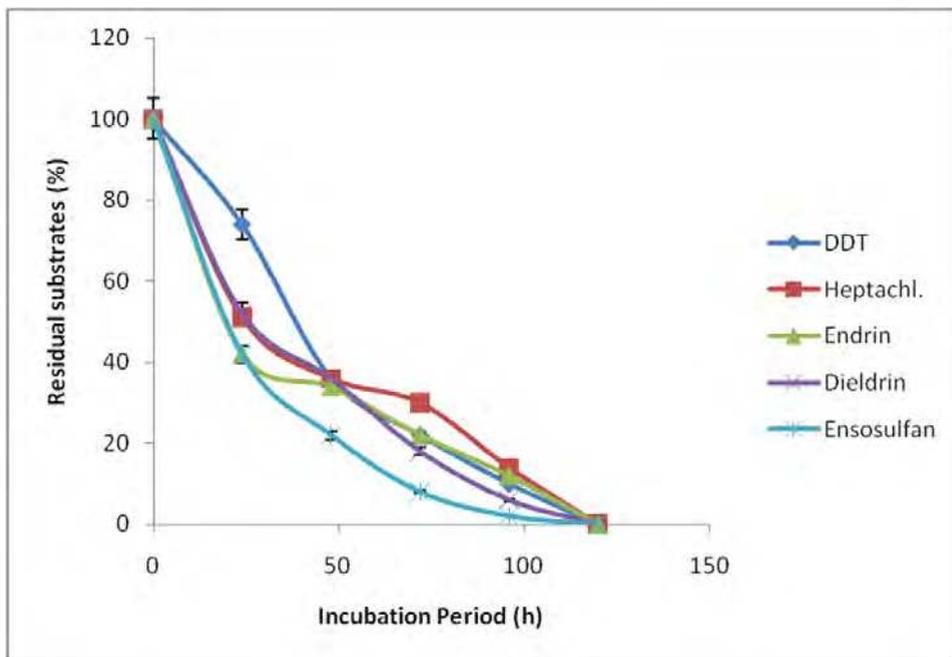


Fig. 4. Degradation of other organochlorine pesticides by Tech-HCH-degrading microbial consortium

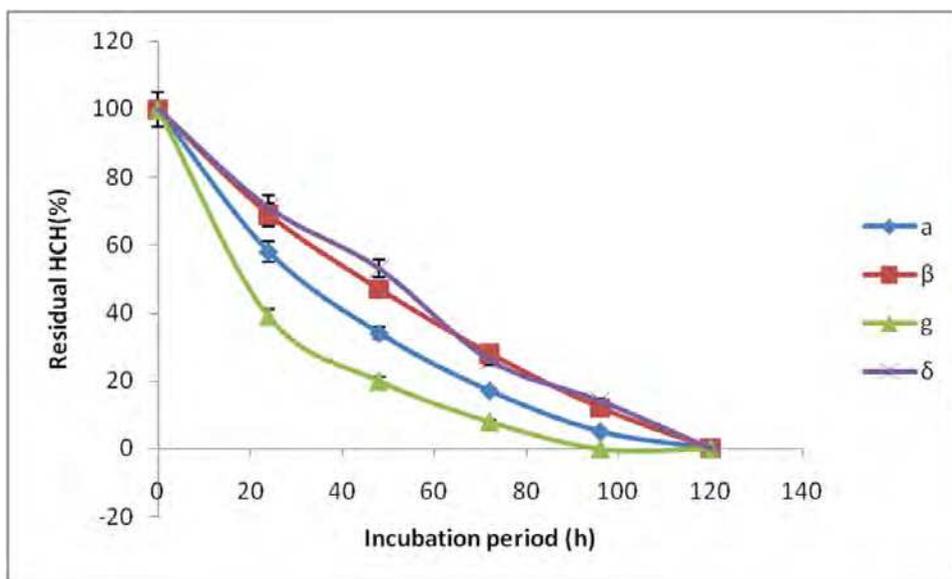


Fig. 5. Degradation of Tech-HCH by inoculant formulation (prepared using *Sphagnum* mass)

4.7 Degradation of technical hexachlorocyclohexane in small artificial plots of 2ft x 2ft x 0.5ft dimensions

Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly. One-gram soil was used for recording colony-forming units (cfu) and other set was used for residue analyses. The solvent fractions, passed through anhydrous sodium sulphate and florisil, were analysed for residual HCH isomers by GC. The maximum and minimum temperatures recorded during this period of study were 25- 28°C and 18- 22°C respectively. The degradation of Tech- HCH was complete by 120 h of incubation in inoculated plots (Fig.6). γ -Isomer was degraded faster followed by α -, β - and δ - isomers. The native microflora did not show the formation of any dead end metabolites or inhibition of degradation. The individual members of the consortium showed good survival during degradation. No competition or inhibition from native microflora was observed towards both the substrate and the added microbial consortium (Table3). Uninoculated plots showed very little degradation of HCH- isomers. Only α - and γ - isomers were degraded by 2 and 4 % respectively, while β - and δ - isomers were not degraded by the native microorganisms even after 120 h of incubation.

!	Log of CPU									
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
0	10.32±0.31	10.32±0.14	9.32±0.32	10.21±0.21	10.76±0.21	9.76±0.06	10.76±0.16	10.31±0.31	10.85±0.36	10.35±0.18
48	9.48±0.16	11.32±0.32	11.24±0.16	10.52±0.24	11.29±0.21	9.20±0.14	9.14±0.11	9.79±0.30	9.79±0.36	9.31±0.18
72	8.52±0.18	10.35±0.24	10.14±0.14	9.24±0.04	9.39±0.28	8.47±0.36	7.59±0.16	8.14±0.10	8.77±0.16	7.31±0.14
120	7.61±0.11	8.39±0.18	8.59±0.18	7.56±0.36	6.12±0.16	7.66±0.16	5.62±0.18	7.69±0.24	7.08±0.14	6.38±0.10

Table 3. Survivability of individual members of the consortium during the degradation of Tech- HCH

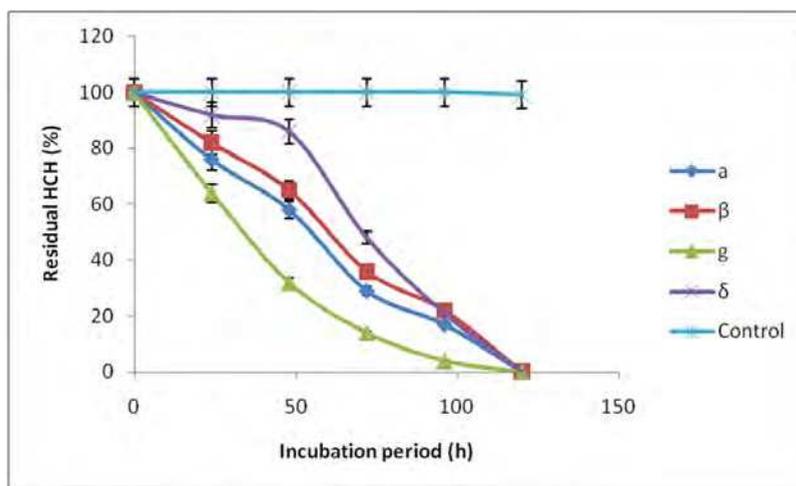


Fig. 6. Degradation of Tech-HCH in small artificial plots of 2ft x 2 ft x 0.5 ft

4.8 Degradation of HCH in artificial test plots of 2m x 1m x 0.5ft dimensions

At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate. It was observed that the degradation of all the isomers of HCH was complete with γ - isomer disappearing faster followed by α -, β -, and δ - isomers (Fig.7). The growth of the individual members of the consortium was also good (Table 4). The degradation of HCH-isomers was not observed by the native microflora.

!	Log of CPU									
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
0	10.46±0.34	10.48±0.16	10.97±0.31	10.17±0.16	10.68±0.16	10.58±0.18	10.76±0.19	10.03±0.24	10.42±0.36	10.42±0.08
48	9.66±0.14	8.00±0.11	7.93±0.0.08	9.14±0.18	8.00±0.24	9.89±0.18	9.10±0.14	79.38±0.16	10.53±0.31	9.91±0.14
72	8.03±0.16	7.93±0.21	6.36±0.16	8.76±0.20	7.47±0.08	9.25±0.14	7.72±0.21	7.72±0.14	9.71±0.26	8.96±0.10
120	8.21±0.18	7.83±0.20	6.05±0.11	8.54±0.11	7.21±0.16	7.79±0.18	7.10±0.30	7.45±0.18	8.89±0.11	8.08±0.26

Table 4. Survivability of individual members of the consortium during the degradation of Tech-HCH

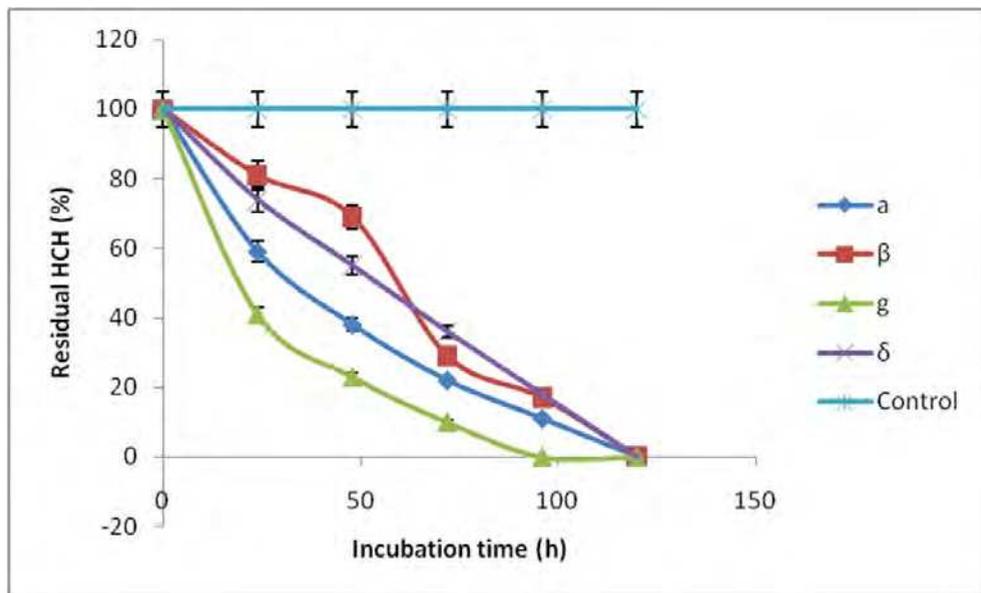
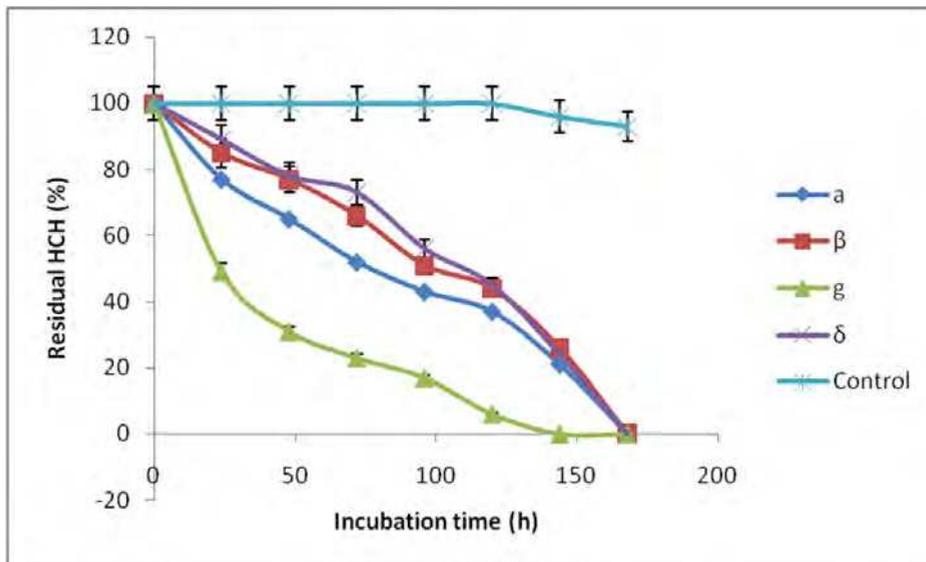


Fig. 7. Degradation of Tech-HCH in plots of 2m x 1 m x 0.15 m dimensions

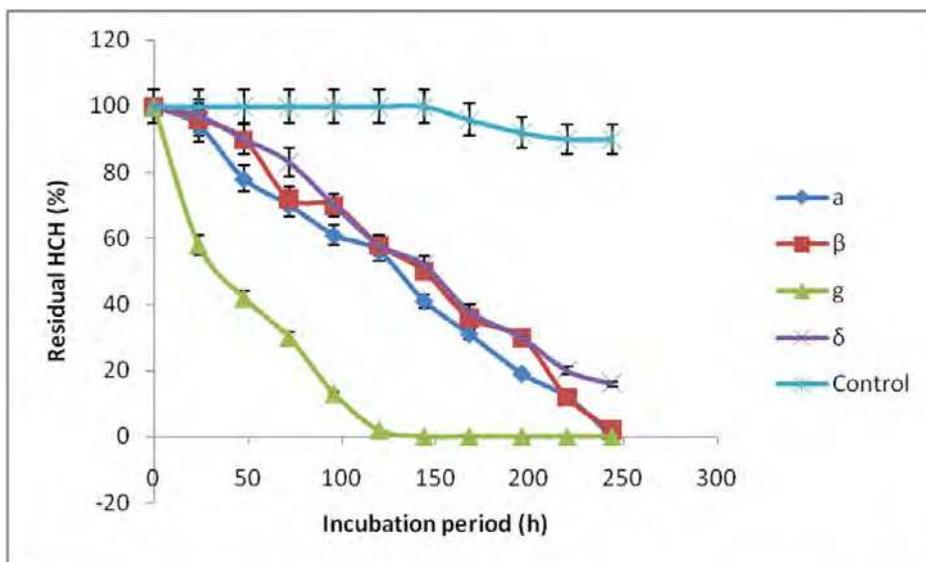
4.9 Degradation of Tech-HCH in actual fields measuring 2m²

25 samples were removed randomly from each plot. Collected soil from each plot was mixed well and 5 g soil from each sample was extracted. The solvent layers were pooled, passed through florisol and anhydrous sodium sulphate. The solvent fraction was pooled,

concentrated at room temperature and analysed for residual HCH. Compared to laboratory trials, the degradation of all isomers of HCH by the microbial consortium took 168 h for complete degradation (Fig.8). But the inoculant formulation took 10 days for complete degradation of all four isomers of HCH (Fig.9).



8a. Degradation by microbial consortium



8b. Degradation by microbial inoculant formulation

Fig. 8. Degradation of Tech-HCH in actual open fields

4.10 Bioassay of the remediated soil

Seeds of *Raphanus sativus* (radish) and *Aeblumusculus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non-HCH- spiked controls were all sown with seeds of both radish and ladies finger. The germination of the plants was delayed in seeds sown in HCH- spiked soil. The height of the plants was reduced when compared to controls. Many other growth- related deformalities were also observed (Plate 2 and 3). In the bioremediated soil, growth related deformalities were not observed and the plant looked healthy *in par* with controls (Plate 4 and 5).

5. Discussion

HCH has been used worldwide as general broad spectrum insecticide for a variety of purposes including fumigation of the house hold and commercial storage areas, pest control on domestic animals, mosquito control and to eradicate soil-dwelling and plant-eating insects. Although only lindane has insecticidal property, HCHs as group are toxic and considered potential carcinogens (Walker et al., 1999) and listed as priority pollutants by the US EPA. Due to their persistence and recalcitrance, HCHs continue to pose a serious toxicological problem at industrial sites where post production of lindane along with unsound disposal practices has led to serious contamination. In addition, many countries including India have permitted HCH production (lindane is permitted to be used) and use. This has become a global issue due to problems of volatility and transportation of HCH isomers by air to remote locality (Galiulin et al., 2002; Walker et al., 1999). Due to the toxicity and persistence of HCH, soils contaminated with HCHs have been targeted for remediation. Biodegradation of α -, β -, γ - and δ - isomers of HCH have been extensively studied in the laboratory at individual level. But information is insufficient on pilot or full-scale *in situ* field settings. The HCH-isomers have been shown to differ in their persistence in soil and in their properties like solubility and volatility that determine their rates of biodegradation. Earlier studies suggested that degradation of HCH was faster under anoxic conditions and that microbial degradation was primary route of HCH disappearance from soil (MacRae et al., 1967). Microbial degradation of all the HCH-isomers has since been observed under oxic conditions both in soil (Bachmann et al., 1988 b); Doelman et al., 1985; Sahu et al., 1993) and in pure cultures of microorganisms (Bhuyan et al., 1993; Thomas et al., 1996). We have isolated in our laboratory a microbial consortium consisting of ten bacterial isolates which have got the capacity to degrade HCH (Manonmani et al., 2000; Murthy and Manonmani, 2007) under oxic conditions. Translation of the laboratory scale trials to small plots and actual open fields was studied in soil

The defined microbial consortium used in the degradation of tech-HCH was developed by the long-term enrichment of the contaminated soil and sewage samples. This microbial consortium was acclimated with increasing concentrations of tech-HCH from 5 to 25 ppm. The consortium that got established at 25 ppm level was used in our studies. The initial inoculum used in acclimation is obtained from diverse sources such as HCH contaminated soil and sewage. The advantage of sewage is that it provides sufficient inoculum during acclimation. As the test compound is used as a sole source of carbon and energy the organisms having the machinery for the degradation of the compound would survive and therefore would be able to accomplish the mineralization process. Moss [1980] employed an

a. Growth of radish in control soil.



b. Growth of radish in HCH- treated soil.



c. Growth of radish in Bioremediated soil.



Plate 2. Bioassay in soil

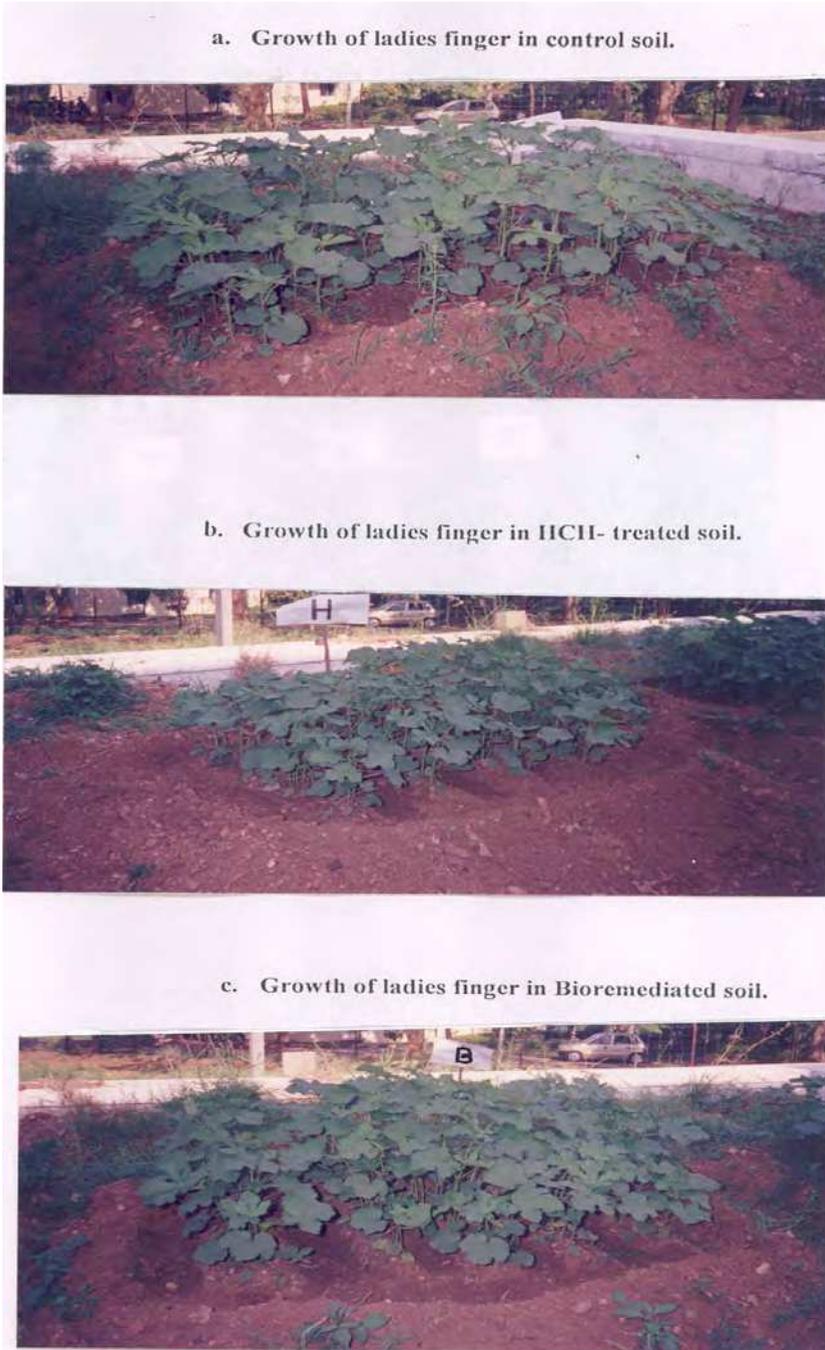


Plate 3. Bioassay

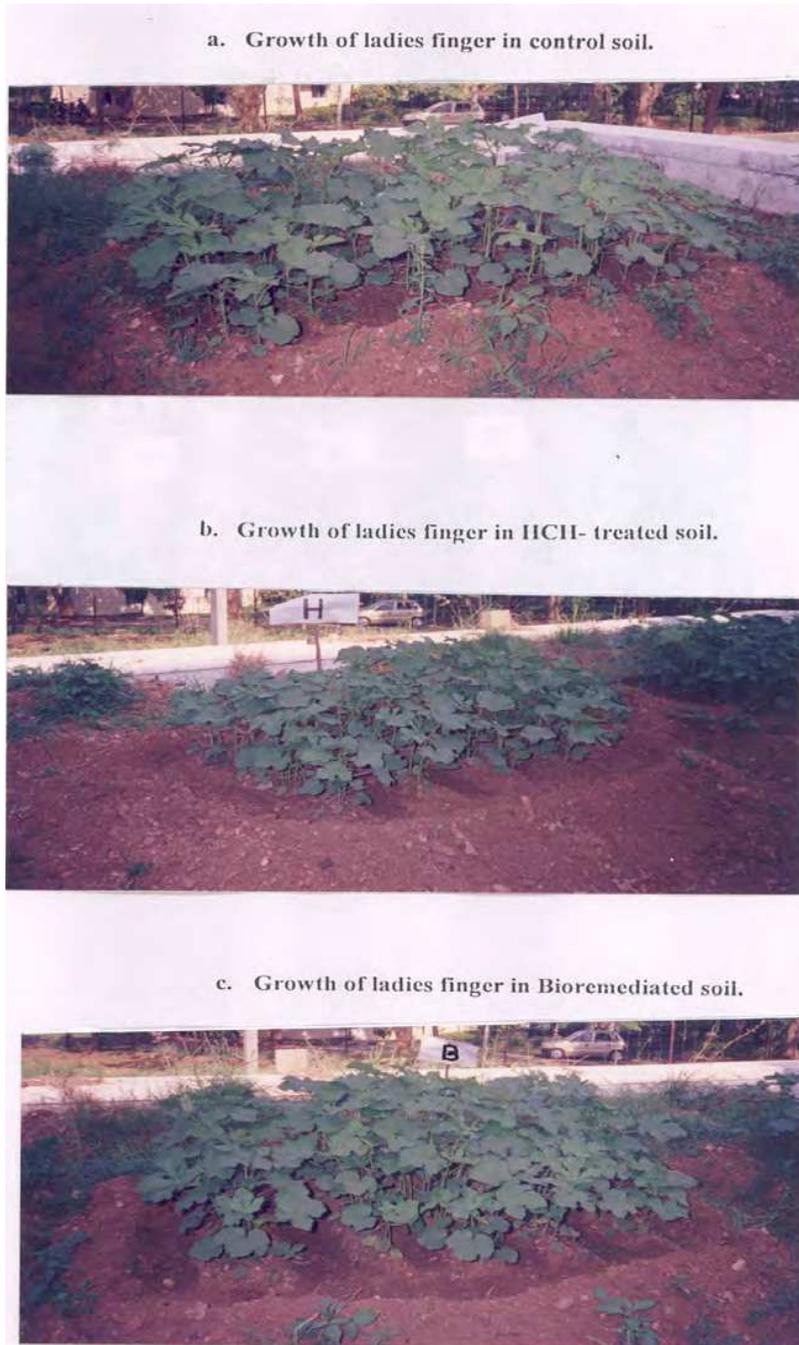


Plate 4. Yields of Ladies finger in bioassay experiments

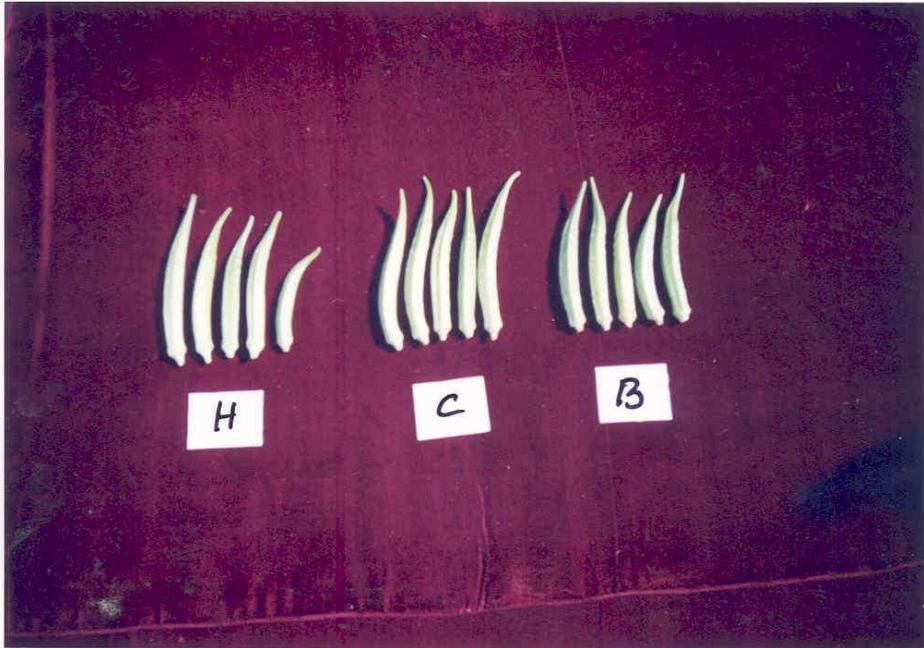


Plate 5. Yields of radish in bioassay experiments

acclimation and enrichment procedure that used a continuous culture of microorganisms growing at very low specific growth rates. The compound being tested was applied continuously at low concentrations and the concentration was increased in a systematic manner. Similar acclimation technique has been used by Bidlan and Manonmani [2002] to isolate DDT degrading microorganisms. Acclimation also would help in evading toxicity prior to the actual degradation and this is an essential part for further degradation studies. Acclimation would result in altered composition of the microbial populations involved in the early stages of degradation. Pre-exposure helped in obtaining faster degradation without any lag. The degradation appeared to have started as soon as inoculum and substrate were together. There was no initial lag in degradation with the inoculum induced with any of the isomers of HCH. In all these cases, the HCH-isomer used for induction was degraded completely and other isomers were partially degraded. This probably could be due to presence of required enzyme that got induced with the particular isomer. The partial degradation could be due to the multiple functions of the enzyme which was able to degrade the substrates only to certain extent. The partial degradation could be due to the enzymes, which might not have got induced by the other isomer used, or there could be inhibition of pathway enzymes by the intermediary metabolites formed during the degradation of non-inducer substrates. Also the complete degradation could be achieved with increase in incubation time. It has been reported that the degradation of α - and γ -isomers follow the same pathway and the degradation of β -HCH has been deciphered to only one or two steps of biodegradation (Nagata et al 2005). To our knowledge no reports are available on the degradation pathway of δ -HCH degradation. In our studies also the degradation of these isomers might be following a different pathway, As tech-HCH is a mixture of all isomers, degradation of different isomers is a complex phenomenon, as these enzymes might face many inhibitory/stimulating effects by the intermediates formed by different isomers present together. Failure to achieve results with consistent complete mineralization, on the other hand, would also suggest that complete biodegradation is not possible in short time or that it is dependent on co-metabolism. Thus, the tech-HCH induced inoculum was used in further studies. The microbial consortium developed in the laboratory was capable of degrading all isomers of HCH and the biodegradation could occur in a particular environment. With all the optimized conditions, the biodegradation of tech-HCH becomes a highly system specific event. These optimized results can be adapted well in the treatment of industrial effluent or water bodies contaminated with HCH. α - and γ -isomers have been reported to be degraded rapidly under aerobic and anaerobic conditions. α - and γ -isomers were degraded first in 12 h of incubation (Manonmani et al 2000 , Johri et al 1998 , Datta et al 2000), where as β -isomer was found to remain undegraded under similar environmental conditions (Bachmann et al 1988a, Beurakens et al 1991). The degradation of different isomers of HCH has been shown to be dependent on many features mainly the type of microorganism used, aeration, the adaptability of these microorganisms to the pollutants (Moreno and Buitron 2004), type of carbon source to cultivate them (Radha et al 2010), pre-exposure of the used organism to the pollutant (Bidlan and Manonmani 2002), etc. The recalcitrancy of the isomers also has been shown to play a key role in the degradation of different isomers of HCH (Bachmann et al 1988a, Haider and Jagnow 1975). In our studies also, the time required for the highly recalcitrant β - and δ -isomers was more compared to the other two isomers. The degradation of tech-HCH by the microbial consortium appears to be gratuitous metabolism where in, the substrate, i.e. tech-HCH is used as a sole source of carbon and energy and no other co-substrates are being supplemented. This is evident from the survival of all members of consortial community during degradation. However, no substantial growth was observed, i.e. the cells behaved as resting cells as the

amount of carbon supplied by the substrate is not sufficient to support good growth of the consortium. But the cell count was being maintained during degradation. The initiation of degradation might be by the enzyme system that was already induced during pre exposure cycle, and hence degradation did not show any lag. The pH of the medium had a substantial effect on the survivability of the members of the consortial community. At low pH levels, there was decrease in the microbial population. The degradation was observed to take place over a wide range of pH from 4.0 to 8.0 (Murthy and Manonmani, 2007). Degradation was found to decrease at pH 9.0. The degradation of each isomer was influenced by the presence of other isomer. The HCH isomers, i.e. non-growth substrates have γ -isomer, the more easily degradable and β - and δ -isomer very highly recalcitrant. These two isomers thus show resistance toward degradation. It could be that because of recalcitrance, the structure may prevent it fitting into enzyme within the cell when it is likely to accumulate or the transformation product of one substrate may become toxic than the original substrate that might result in slower rate of degradation and also the degradative pathway of each isomer may be different which would be influenced by many factors. However, the different isomers present in the mixture will not associate in their co-metabolic degradation.

As our microbial consortium consisted of aerobic microorganisms, soil was mixed often in small plots to facilitate aeration. Similar degradation of α -HCH under oxic conditions in either moist soil or soil slurries has been reported (Doelman *et al.*, 1985). Degradation of 23 mg kg⁻¹ day⁻¹ of α -HCH in soil under oxic conditions has been obtained (Bachmann *et al.*, 1988a, b). However, reduction of 13 mg kg⁻¹ day⁻¹ was obtained under methanogenic conditions. Van Eekert *et al.* (1998) have reported the removal of α -HCH from a sandy soil containing low concentration of the isomer in slurries where lactate or sulfide had been added to reduce redox potential. Degradation of HCH was faster under anoxic conditions and that microbial degradation was primary route of HCH disappearance from soil (MacRae *et al.*, 1967). Microbial degradation of all the HCH-isomers has since been observed under oxic conditions both in soil (Bachmann *et al.*, 1988 (a,b); Doelman *et al.*, 1985; Sahu *et al.*, 1993) and in pure cultures of microorganisms (Bhuyan *et al.*, 1993; Thomas *et al.*, 1996). Soil slurry has been adopted for the microbial degradation of pesticides, explosives, polynuclear aromatic hydrocarbons, and chlorinated organic pollutants (Gonzalez *et al.* 2003). In our studies translation of the laboratory scale trials to small reactors was studied in artificial plots and open actual fields. . The moisture content in soil has been shown to influence greatly HCH degradation. Chessells *et al.* (1988) have reported a correlation between soil moisture content and removal rates of HCH isomers in field agricultural soils. Enhanced removal of HCH in soils with higher moisture contents has been reported. This has been made possible due to prevailing anoxic conditions during flooding. Thus anaerobic metabolism has been reported to be existing in these soils. In our earlier studies, soil moisture content of 15 to 20% was found to give good biodegradation of HCH-isomers (unpublished data). This was used in the current study in small and actual plots. As our microbial consortium consisted of aerobic microorganisms, mixing at regular intervals was done to maintain oxic condition. Degradation of α -HCH in glass columns packed with contaminated sediments and held under methanogenic conditions has been reported (Middeldorp *et al.*, 1996), although degrading population of microorganisms appeared not to be methanogens. Degradation of γ -HCH under oxic conditions has also been reported (Yule *et al.*, 1967). β -HCH isomer, an indisputably most recalcitrant isomer, does not undergo biodegradation easily. The concentration did not decrease noticeably in field study under any treatment (moist soil and oxic soil slurries in small pots) (Doelman *et al.*, 1985)

Complete degradation of Tech-HCH was obtained in all the studies carried out. The bioassay of the bioremediated soil was carried out to check the degradation or mineralization of HCH isomers. The growth of Seeds of *Raphanus sativus* (radish) and *Aeblumuschus esculantus* (ladies finger) was poor in HCH spiked soil and their growth in bioremediated soil was *in par* with that in control soils. The crop yield in bioremediated soil was also *in par* with control soils.

6. Conclusion

The use of microbes to clean up polluted environments, bioremediation is rapidly changing and expanding the area of environmental biotechnology. Although much work is being done to remediate the polluted environment, our limited understanding of the biological contribution and their impact on the ecosystem has been an obstacle to make the technology more reliable and safer. In our studies a defined microbial consortium was able to degrade HCH (technical grade containing all four major isomers) up to 25 ppm level in soil at ambient temperature and neutral pH. The consortium was able to degrade HCH in artificial plots and also in open fields.

The inhibition of degradation by the presence of other isomers and native microflora was marginal. With the translation of lab trials to large scale trials coupled with process molecular microbiological techniques can make the bioremediation process more reliable and safer technology.

Although HCH removal has been observed under both oxic and anoxic bioremediation treatments, treatments under oxic condition have resulted in the almost complete removal of HCH *via* mineralization. These observations are on par with our results wherein under oxic conditions good degradation of HCH-isomers of technical mixture has been observed. Even though all the four isomers were present together in technical mixture, no adverse or inhibitory effects were observed by either parent compounds or their metabolites. We have tried to address the inadequately addressed topic of bioremediation of HCH contaminated soils in field studies. With the disadvantages of ex-situ bioreactors such as requirements for soil excavation, handling, conditioning and bioreactor construction/operation that typically increase treatment costs compared to most simple bioremediation techniques. The successful results obtained in small scale soil studies need to be addressed during translation further to still larger scale.

7. Legends to figures

Fig. 1. Degradation of Tech-HCH with inoculum grown on different carbon sources

CSL , Corn steep liquor; RSE , Rice straw extract; RSH, Rice straw hydrolysate; CCH, Corn cob hydrolysate; WBH, Wheat bran hydrolysate; BH, Bagassae hydrolysate; NB, Nutrient broth.

Microbial consortium were grown in different carbon sources and induced with Tech-HCH and inoculated to Tech-HCH. Analysis was done as given under Methodology.

All the experiments were done in replicates of ten for each parameter studied.

Fig. 2. Preexposure and degradation of Tech-HCH

Individual isolates of the microbial consortium were grown in wheat bran hydrolysate/peptone- glycerol medium and the cells were harvested after 72h of growth, washed well and preexposed to 25ppm of individual isomers and Tech-HCH for 72h separately with

addition of individual isomers and Tech- HCH every 24h. The induced cells were harvested by centrifugation at 10,000rpm, 4°C, 10min, washed well and used as inoculum. All the experiments were done in replicates of ten for each parameter studied.

Fig. 3. Degradation of Tech-HCH in different native soils:

Different soils such as clay soil, red soil, garden soil, soils from coconut, coffee, turmeric and tomato plantations were studied to evaluate the degradation of Tech- HCH. The substrate and inoculum were used at 25 $\mu\text{g g}^{-1}$ and 500 $\mu\text{g protein g}^{-1}$ soil respectively. All the experiments were done in replicates of ten for each parameter studied.

Fig. 4. Degradation of other organochlorine pesticides by Tech-HCH-degrading microbial consortium.

Soil was spiked with different organochlorine pesticides such as endosulphan, heptachlor, endrin, dieldrin at 10ppm level. Microbial consortium induced with respective organochlorine pesticide was used as inoculum. Pesticide spiked soil was inoculated with microbial consortium at inoculum 500 $\mu\text{g protein / g soil}$. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide. All the experiments were done in replicates of ten for each parameter studied.

Fig. 5. Degradation of Tech-HCH by inoculant formulation (prepared using Sphagnum mass)

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass. two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. Randomized sampling was done at every 3h interval and analysed for residual spiked pesticide.

Fig. 6. Degradation of Tech-HCH in small artificial plots of 2ft x 2 ft x 0.5 ft

Four artificial plots of 2ft x 2ft x 0.5ft were prepared. Red loamy non- sterile soil containing 1-1.5% organic carbon and 32% water holding capacity and pH 7.0, collected from CFTRI campus, was taken in these plots. Each plot was spiked with 25 ppm of tech- HCH and mixed well. Two plots were inoculated with 72 h HCH- induced microbial consortium. Two plots were maintained as abiotic (uninoculated) controls. All plots were kept wet by regular sprinkling of water and the soil in the plots was mixed regularly. Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly and was used for residue analyses.

Fig. 7. Degradation of Tech-HCH in plots of 2m x 1 m x 0.15 m dimentions.

Six artificial plots of 2m x 1m x 0.5ft were prepared and red loamy soil collected from CFTRI campus was taken in these plots. Each plot was spiked with 25 ppm Tech- HCH and mixed well. Four plots were inoculated with 72 h HCH- induced microbial consortium. The inoculum was added at levels containing 10^7 to 10^8 cells/ g soil. Two plots were maintained as uninoculated controls (containing only HCH). The soil was mixed well at regular intervals and was kept moist by regular sprinkling of water. At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate.

Fig. 8. Degradation of Tech-HCH in actual open fields:

8a. Degradation by microbial consortium.

8b. Degradation by microbial inoculant formulation:

8 plots measuring 2m² were chosen at CFTRI campus. The plots were prepared by tilling and toeing. Spiking of Tech- HCH to the soil was repeated for four times on alternative days. The final concentration of Tech- HCH added to the soil was 25 ppm. Six of the plots received Tech- HCH while two remaining were maintained as unspiked, uninoculated controls. Two plots were inoculated with microbial consortium and two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. The inoculum at 0 h was added at the level containing 10⁷- 10⁸ cells. Sampling was done at 24 h intervals. 25 samples were removed randomly from each plot and analysed for residual HCH isomers.

Plate 1.

8 plots measuring 2m² were chosen at CFTRI campus.

Plate 2 and 3.

Seeds of *Raphanus sativus* (radish) and *Aeblumuschus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non- HCH- spiked controls were all sown with seeds of both radish and ladies finger.

Plates 4 and 5.

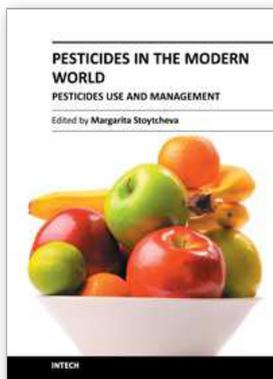
Seeds of *Raphanus sativus* (radish) and *Aeblumuschus esculantus* (ladies finger) grown in HCH-spiked, bioremediated and non-spiked soils.

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This book brings together issues on pesticides and biopesticides use with the related subjects of pesticides management and sustainable development. It contains 24 chapters organized in three sections. The first book section supplies an overview on the current use of pesticides, on the regulatory status, on the levels of contamination, on the pesticides management options, and on some techniques of pesticides application, reporting data collected from all over the world. Second section is devoted to the advances in the evolving field of biopesticides, providing actual information on the regulation of the plant protection products from natural origin in the European Union. It reports data associated with the application of neem pesticides, wood pyrolysis liquids and bacillus-based products. The third book section covers various aspects of pesticides management practices in concert with pesticides degradation and contaminated sites remediation technologies, supporting the environmental sustainability.

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