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Degradation of Technical Grade Hexachlorocyclohexane In Soil Slurry by a Defined Microbial Consortium

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ABSTRACT: Hexachlorocyclohexane, an organochlorine insecticide has been used in agriculture and public health programmes since a very long time. It is very resistant to degradation and thus accumulates in the environment for long time. A microbial consortium was developed in our laboratory which could degrade Hexachlorocyclohexane very efficiently in water. A study was carried out to understand the biodegradation of technical grade hexachclorocyclohexane (HCH) in soil slurry in lab scale bioreactor by a defined bacterial consortium under aerobic conditions. Effects of parameters such as initial HCH concentration and volume of air required were optimized. 10 and 25 ppm of HCH were degraded completely by 120 and 168 h, respectively. No lag was observed. In both the concentrations of HCH, γ -isomer was degraded faster and -and, β-isomers took more time for degradation. The rate of degradation ofα,β, γ and δ isomers of 10 and 25 ppm HCH were 0.0186, 0.0136, 0.0179, 0.0176 mg/L/h and 0.0122, 0.01444, 0.0126, 0.0122 mg/L/h respectively. Aeration rate of 0.5 vvm gave maximum degradation, whereas at 1.0 vvm δ-isomer was not degraded completely. At 2.0 vvm aeration, all the isomers remained even after 144h if incubation.

Key words: Pesticide, Bioremediation, Bacterial isolates, Aerobic cultivation, Food safety

INTRODUCTION

Hexachlorocyclohexane (HCH) is an organochlorine insecticide that has been used world wide in agriculture and public health programmes (Breivik *et al*., 1999).During the production of technical grade HCH, 85% of the product containing other isomers mainly α-, β- and δ-HCH would be dumped as waste, as they are not insecticidal.This causes serious soil pollution (Braun *et al*., 1991).Bioremediation of soil polluted with HCH is a subject of basic research.Biodegradation of HCH in soil has been attempted (MacRae *et al*., 1967, Bachmann *et al*., 1988, Doelman *et al*., 1985).

The degradation of HCH in soil by microorganisms depends on adsorption rate to the soil and its restricted availability for biological action (Bollag *et al*., 1992; Harms and Bosma, 1997).One possibility to help the mobility of the pollutant is its transfer from the soil to the liquid phase.The slurry phase system is a bioremediation technique where the contaminated soil is combined with water and other additives in stirred tank bioreactor (Alexander, 1994). This system minimizes the formation of aggregates and allows a faster equilibrium between the solid and the liquid phases by means of the suspension and vigorous agitation of the soil in the liquid medium.Both effects have been shown to favor mass transfer and thus enhance biodegradation rate of the pollutants (Rogers *et al*., 1993). Soil slurry bioreactors have been used in the degradation of many pollutants such as pesticides, explosives, polynuclear aromatic hydrocarbons, and chlorinated organic pollutants (Robles-Gonzalez *et al.,* 2008) The degradation of HCH isomers in soil slurry in laboratory scale has been reported (Bachmann *et al*., 1988; Van Eekert *et al*., 1998).There are hardly any reports on the studies on a higher scale of degradation.We have developed a microbial consortium capable of degrading technical grade HCH by long term enrichment under aerobic conditions. Studies on the optimization of degradation conditions in laboratory level is has been done(Murthy and Manonmani 2007) It was intended to assess the degradation capability of the developed consortium in a bioreactor in soil slurry Different environmental conditions optimized under shake flask experiments were adopted in bioreactor studies.The slurry experiment was performed in an aerobic slurry reactor.In this bioreactor, the spiked soil was suspended in stirred reactor vessel and mixed with water. HCH alone was supplied as a source of carbon.

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MATERIALS & METHODS

Technical grade hexachlorocyclohexane (tech-HCH) was obtained from Hindustan Insecticides Pvt. Ltd. Mumbai, India. This was found to have all four isomers of HCH. Pure grade α-, β-, γ- and δ-isomers of HCH were purchased from Sigma-Aldrich Chemical Company, MO, USA. The bioreactor used was a 3 L capacity tank with 24 cm x 11 cm x 24 cm dimensions.Sterile soil (500g) spiked with required quantity of tech-HCH was taken in the reactor and mineral salts medium (M4 medium) (Murthy and Manonmani, 2007) was added up to 1L level. Mixing was done by the passage of air through the slurry at required air volumes. Soil slurry in the bioreactor was inoculated with induced microbial consortium at 500 µg protein/ g soil. Slurry was mixed well by bubbling air.Sampling was done at regular intervals and analyzed for residual HCH, and surviving microorganisms (CFU).

Effect of aeration on degradation studied by passing (bubbling) air at 0.5, 1.0 and 2.0vvm levels.Other experimental protocol was same as given for degradation studies. Residual HCH was extracted from soil slurry by extracting with twice the volume of dichloromethane.Extraction was done thrice.The solvent fractions were pooled, passed over a bed of anhydrous sodium sulphate, concentrated and purified by passing through florisil column.The fraction containing residual substrate was evaporated to dryness, resuspended in a known volume of acetone and used for quantification by Thin Layer Chromatography (TLC) and Gas Chromatography (GC). 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.The acetone layer containing residual substrate after appropriate dilution was injected into gas chromatograph (Fison's model) equipped with ^{63}Ni detector and SS column (200 cm X 2 mm) packed with 1.5% OV 17 plus 1.95 QF1 on chromosorb W 80/100 mesh.The column, injector and detector were maintained at 230^0 C, 250^0 C and 320^0 C respectively with a flow rate of carrier gas nitrogen at 50 mL/min. Under these conditions, the retention time of HCH-isomers was:α-HCH, 3.34 min; γ-HCH, 3.98 min; β-HCH, 4.42 min; δ-HCH, 5.1 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.The survival of individual members of the HCH degrading microbial consortium was done by estimating the Colony Forming Units (CFU) (Sahu *et al*., 1995).

RESULTS & DISCUSSION

The study is meant to assess the efficiency of an aerobic reactor to degrade HCH isomers contained in soil slurry.The optimized conditions obtained in our previous study were adopted in this reactor study to find out the applicability of shake flask trials in small reactors.Only HCH concentration was varied during degradation period.The optimized parameters used were: inoculum level 500 µg proteins/ g soil, pH 7.5 and incubation temperature 30^{0}C.

The soil used was red soil with no history of HCH applications(Table 1). 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. (Table 2).

Table 1. Physico-chemical Characters of,Soil

Parameter	Level
Moisture	10%
pH	7.2
Organic matter	8%

Table 2. List of Isolates of Microbial Consortium

Sl. No.	Bacterial isolate	No.
1	*Pseudomonas fluorescens* biovar II	T_1
2	*Pseudomonas diminuta*	T_2
3	*Pseudomonas fluorescens* biovar I	T_3
4	*Burkholderi pseudomallei*	T_4
5	*Pseudomonas putida*	T_5
6	*Flavobacterium* sp.	T_6
7	*Vibrio alginolyticus*	T_7
8	*Pseudomonas aeruginosa*	T_8
9	*Pseudomonas stutzeri*	T_9
10	*Pseudomonas fluorescens* biovar V	T_{10}

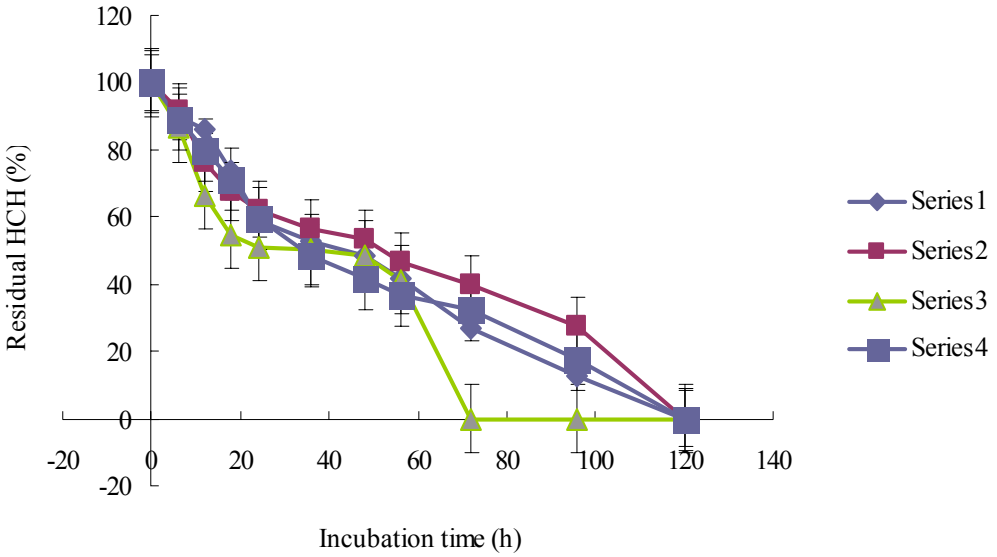
At 10 ppm of HCH concentration, all the four isomers were degraded during incubation period. γ- isomer was degraded faster by 72 h (Fig. 1) with a degradation rate of 0.0179 mg /L/ h (Table 3). Other isomers α-, β - and δ—HCH took 120 h for complete degradation with rates of 0.0186, 0.0136 and 0.0176 mg /L/ h respectively (Table 3). There was no initial lag. Degradation of all isomers started even at 3 h.At 25 ppm of tech-HCH level, time required for degradation was more. Among four isomers, degradation of -, β - and δ—HCH started immediately without any lag (Fig. 2). But degradation of δ-isomer started only after 12 h of incubation. γ-isomer was degraded completely by 96 h with a degradation rate of 0.0126 mg/ L/ h (Table 3). α-,and β -isomers were degraded completely by 168 h of incubation at rates of 0.0122 and 0.0144 mg/ L/ h respectively.δ-isomer was not completely degraded even by 168 h with still nearly 10% of the substrate remaining after degradation period.

Table 3. Kinetics of Degradation of tech-HCH at Different Concentrations

Conc. of HCH (mg/L)	Rate of degradation (mg/L/h)			
	α	β	γ	δ
10	0.0186	0.0136	0.0179	0.0176
25	0.0122	0.0144	0.0126	0.0122

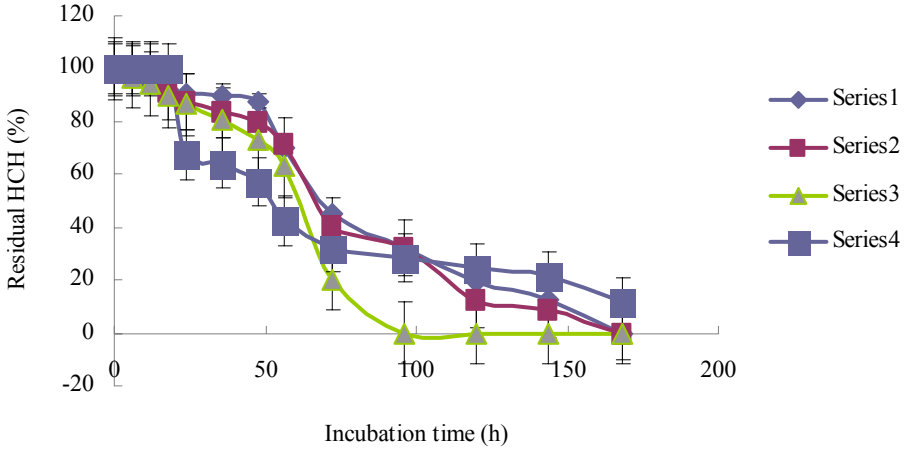
The survivability of microbial cells in both 10 and 25 ppm were studied. At 10 ppm of tech-HCH level, all the members of the consortium survived till the end of degradation period (Table 4). However, isolate T_7 was reduced in number, compared to other isolates.At 25 ppm of tech-HCH concentration, the survivability pattern was almost the same as that in 10 ppm. All the isolates survived till the end of degradation period.

At 0.5 vvm aeration rate, degradation was slow. Incubation time of 168 h was required for complete degradation of all isomers (Fig. 3). The easily degradable γ-isomer and α- and β-, took 144 h for complete degradation. δ-isomer was degraded completely by 168 h. The degradation rate was 0.0092, 0.0137, 0.0147 and 0.0129 mg/ L/ h for α-, β-, γ- and δ-isomers respectively.At aeration rate of 1.00 vvm (Fig. 4). the degradation of α-, β- and γ- isomers were complete by 168 h of incubation. δ- isomer was not degraded completely with nearly 15% of the isomer remaining at 168 h. The rates of degradation were 0.0144, 0.0086, 0.0128 and 0.0100 mg/ L/ h respectively for α-, β-, γ- and δ-isomers (Table 5).The degradation decreased with increase in aeration to 2.0 vvm (Fig. 5). None of the isomers were degraded completely even by 144 h of incubation. Nearly 35% of α-, 54% of β-, 28% of γ-



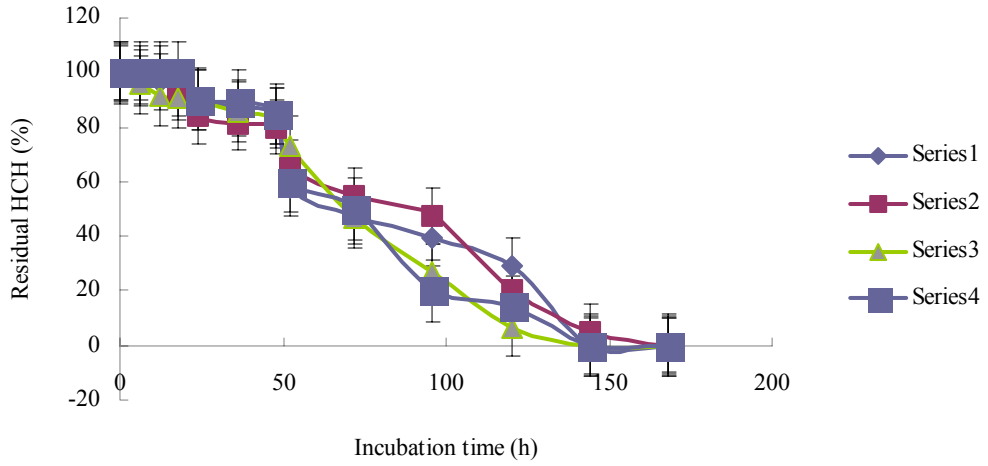
Series 1 = a-isomer series 2 = b-isomer series 3 = g-isomer series 4 = d - isomer

Fig. 1. Degradation of tech-HCH (10 ppm), (α-isomer (%), β-isomer (□), γ-isomer (▲) , δ-isomer (•)



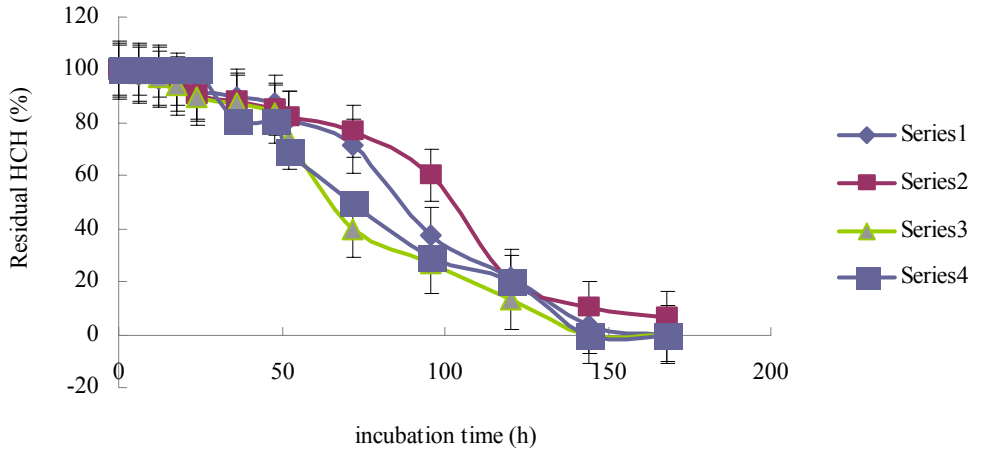
Series 1 = a-isomer series 2 = b-isomer series 3 = g-isomer series 4 = d- isomer

Fig. 2. Degradation of tech-HCH (25 ppm) (α-isomer (%), β-isomer (□), γ-isomer (▲), δ-isomer (•)



Series 1 = a-isomer series 2 = b-isomer series 3 = g-isomer series 4 = d- isomer

Fig. 3. Degradation of tech-HCH at 0.5 vvm (α-isomer (%), β-isomer (□), γ-isomer (▲), δ-isomer (•)

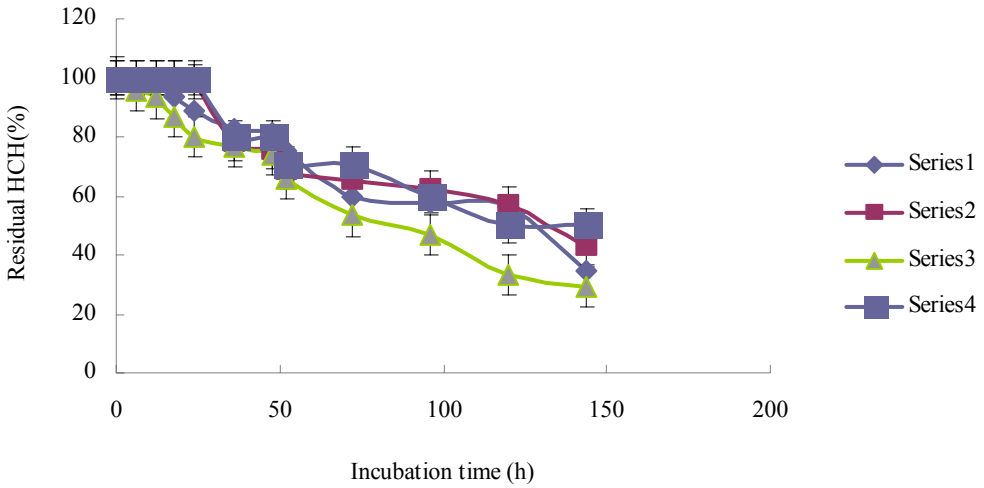


Series 1 = a-isomer series 2 = b-isomer series 3 = g-isomer series 4 = d- isomer

Fig. 4. Degradation of tech-HCH at 1.0 vvm (α-isomer (%), β-isomer (□),γ-isomer (▲), δ-isomer (•)

and 52% of δ-isomers of 10 ppm of tech-HCH were found to be remaining even after 144 h of incubation. The degradation rates were 0.0061, 0.0049, 0.009 and 0.0051 mg/ L/ h, for α-, β-, γ- and δ-isomers, respectively (Table 5).

HCH has been used world wide 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; 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 reactors was studied in soil slurry. Soil slurry has been adopted for the microbial degradation of pesticides, explosives, polynuclear aromatic hydrocarbons, and chlorinated organic pollutants (Robles-Gonzalez *et al.,* 2008) . An anaerobic reactor was used to degrade HCH isomers contained in soil slurry cultures The influence of different environmental conditions was evaluated: the HCH concentration (25–100 mg HCH/ kg), the type of substrate (volatile fatty acids or starch), the sludge concentration (2–8 g VSS /L) and the replacement of spiked soil to simulate a fed-batch operation (10–50%) were tried (Quintero, 2005, 2006).The slurry-phase system was adopted to minimize the formation of aggregates and to allow faster equilibrium between solid and liquid phases by means of the suspension and vigorous agitation of the soil in the liquid medium. Both these effects have favored mass transfer and thus enhance the biodegradation rate of pollutants (Rogers *et al*., 1993). To achieve these advantages the degradation of HCH in soil was studied in a slurry system. The moisture content in soil has been shown to influence greatly HCH degradation. Chessells *et al*. (1988) have reported a correla-



Series 1 = a-isomer series 2 = b-isomer series 3 = g-isomer series 4 = d- isomer

Fig. 5. Degradation of tech-HCH at 2.0 vvm (α-isomer (%), β-isomer (□), γ-isomer (▲), δ-isomer (•)

Table 4 . Survivability of Individual Members of the Consortium During the Degradation of tech-HCH

Substrate concentration (ppm)	Incubation Period (hours)	Log of CFU									
		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
10	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
25	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 5. Kinetics of Degradation of tech-HCH at Different Aeration rates

Aeration (vvm)	Degradation Rate (mg/L/h)			
	α	β	γ	δ
0.5	0.0092	0.0137	0.0147	0.0129
1.0	0.0144	0.0086	0.0128	0.0100
2.0	0.0061	0.0049	0.009	0.0051

tion 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 exist 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).As our microbial consortium consisted of aerobic microorganisms, air was passed through soil slurry to maintain oxic condition. Similar degradation of α-HCH under oxic conditions in either moist soil or soil slurries has been reported (Doelman *et al*., 1990). Degradation of 23 mg/ kg/ day of α-HCH in soil under oxic conditions have been obtained (Bachmann *et al*., 1988). However, reduction of 13 mg/ kg/ day 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 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 biodegradaion 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).

CONCLUSION

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 slurry. With the disadvantages of slurry bioreactors such as requirements for soil excavation, handling, conditioning and bioreactor construction/operation that typically increase treatment costs compared to most simple bioremediation techniques (Cookson 1995).The successful results obtained in small scale soil bioreactors need to be addressed during translation further to still larger scale.

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