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Assessment of bioremediation possibilities of technical grade hexachlorocyclohexane (tech-HCH) contaminated soils

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Abstract

Hexachlorocyclohexane (HCH) is a broad spectrum insecticide still used in some of the developing countries, though developed countries have banned or curtailed its use. Even in those countries where the use of t-HCH has been discontinued for a number of years, the problem of residues of all isomers of t-HCH remains because of its high persistence. These insecticides in the soil disturb the delicate equilibrium between microorganisms and their environment. Few reports on the degradation of t-HCH isomers in soil are present in literature, and very little information is available on the effect of these t-HCH isomers on soil microflora. In the present study, an attempt has been made to see the microbial diversity in the uncontaminated soils and the effect of application of t-HCH on the soil microflora. The soil was spiked with t-HCH and incubated, at regular time intervals the soil samples were analyzed for microbial diversity as well as t-HCH isomers residues. The results show that at higher concentrations of t-HCH, microbial populations were inhibited and the inhibited populations did not reappear even after prolonged incubation. Potential t-HCH degrading cultures were isolated and subjected to further acclimation in order to enhance their degradation capacity. The results are presented and discussed in this paper.

Keywords: t-Hexachlorocyclohexane; Lindane; HCH isomers; BHC; γ-Hexachlorocyclohexane

1. Introduction

In tropical countries like India, where the climate is conducive for the growth of insect pests, pesticides have been used extensively [1]. Most of the times there has been over usage of these pesticides leading to severe environmental problems [2]. Hexachlorocyclohexane is a broad spectrum insecticide which is commonly called benzenehexachloride (BHC) or gammaxene. It consists of eight isomers namely α, β, γ, δ, θ, ε, η of which the α form has two enantiomers [3]. The γ form, commonly called lindane, is the only isomer with insecticidal property. The cost efficiency and ready availability of t-HCH (containing all isomers) was the reason for its excessive use in developing countries [4]. t-HCH has been banned in almost all countries of the world. Although its use has been discontinued, its residues still remain due to its persistence and interconversion of its isomers in soil [5]. Some microbes in soil are able to degrade insecticides to derive energy and nutrients [6], the insecticide may have deleterious effect on other groups of organisms present in soil [7,8]. There are reports of inhibitory [9] or stimulatory [10] effects of certain pesticides, even at recommended levels, on biochemical transformations of importance to soil fertility. For example, application of carbofuran stimulated autotrophic oxidation of ammonium in a flooded soil [11] while application of hexachlorocyclohexane (HCH) to a soil at flooding retarded the reduction of the soil and the accumulation of Fe^{2+} [12] and the production and emission of methane [13].

A good correlation between total microbial activity and pesticide degradability is currently lacking. Even in studies in which soil respiration (or other microbial activity indices) was severely depressed, the absolute mass of pesticides degraded always increased with concentration, indicating that the portion of microorganisms responsible for pesticide degradation was not affected by pesticide toxicity. This is possible because the microbial degradation of pesticides in soil is found to be mediated by a relatively small segment of the total soil microbial community. In an experiment carried out by Rath et al. [14], to study the

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effect of pesticides on microbial biomass of flooded soil, application of technical grade HCH increased the microbial biomass content. Moreover, suspensions of soil treated even once with commercial HCH formulation effected rapid degradation of α- and γ-HCH, but not δ- and β-HCH, in a mineral salts medium supplemented individually with these isomers as a single source of carbon. When a pesticide degrades microbially, its degradation may be affected by the population of microorganisms that are present on the site, the nutrient supply to the biodegraders, other conditions that affect the activity of the degraders, and the availability of the pesticide to the degrading population. Overall, pesticide behavior at spill sites is not adequately understood. Knowledge of the same will not only help us to better evaluate the potential effect of pesticide spills on the environment, but also provide the necessary insights for effectively cleaning up pesticide contaminated sites. The present study investigates the possibility of bioaugmenting native microbial population with t-HCH degrading potential along with nutrient supplementation with the aim of using them in bioremediation of t-HCH contaminated soils.

2. Materials and methods

2.1. Analytical methods

The experiment was conducted using garden soil, characterization of the soil has been done by analyzing the following parameters viz., pH, temperature, moisture content, texture, particle size distribution, organic content, nitrogen, sulphur, phosphorus, and exchangeable cations as per the procedure given in soil chemical analysis [15]. Chlorides were estimated by argentometric method, as given in standard methods for water and wastewater treatment [16].

2.2. Determination of microbial diversity in soil samples

The initial microbial population count in soil was determined by adding 10 g of garden soil to 90 ml of distilled water followed by overnight shaking on a rotary shaker at 150 rpm and complete homogenization in a vortex mixer. This homogenized suspension was subjected to serial dilution and plated on nutrient agar [17], actinomycete-specific agar [18] and Martin's rose Bengal agar [19] for enumeration of bacteria, actinomycete and fungi, respectively. The experiment was carried out in triplicates and total counts of microbes as well as their types were monitored. Microbial types were differentiated based on their colony morphology. The procedure was same for all the t-HCH spiked soil samples.

2.3. Experimental procedure

In order to study the effect of t-HCH on microbial population, 25 g of the above mentioned soil was distributed in 10 flasks and spiked with t-HCH powder at varying concentrations ranging from 2 to 10 mg HCH g^{-1} of soil. t-HCH was first dissolved in acetone (stock solution containing 1 g per 100 ml) and necessary amount was spiked into the flasks. The flasks were left overnight on a rotary shaker for evaporation of acetone. The samples were once again thoroughly mixed. This enabled homogeneity of the samples. Moisture in the soil was maintained by adding sterile distilled water at regular intervals. Microbial counts and colony types were determined every 3 weeks by taking 1 g of soil from each of the 10 flasks containing different concentrations of t-HCH and subjecting it to the same procedure mentioned earlier. These soil samples were extracted with solvent for determination of residual t-HCH. A soil sample without t-HCH was kept as control with every set of experiments. The bacterial cultures isolated from the soil were further screened for the ability to degrade the HCH isomers using substrate specific media. The substrate specific media contains Na_2HPO_4 – 3.6 g l^{-1}, $(NH_4)_2SO_4$ – 1 g l^{-1}, KH_2PO_4 – 1 g l^{-1}, $MgSO_4$ – 1 g l^{-1}, glucose – 1 g l^{-1} and 1 ml l^{-1} of trace element solution containing $FeSO_4$ – 0.05 mg, $CaCO_3$ – 0.2 mg, $ZnSO_4$ – 0.08 mg, $CuSO_4{\cdot}5H_2O$ – 0.016 mg and H_3BO_3 – 0.006 mg. HCH was added at a concentration of 10 mg l^{-1}. The plates were incubated at 37 ± 3 °C. The activity of the potential bacterial culture that could degrade t-HCH was enhanced by taking flasks containing 25 g soil to which these cultures (10%, w/w) were bioaugmented. HCH at a concentration of 10 mg g^{-1} of soil was added. The cultures were subjected for further acclimatization.

2.4. Extraction of HCH from soil and GC analysis

t-HCH was extracted quantitatively from soils by the following Soxhlet extraction procedure. Hundred grams of soil is extracted with 200 ml hexane/acetone (41:59) on a Soxhlet extractor for 12 h, the extract is treated with water (100 ml) to remove the acetone layer and the HCH concentration in the hexane fraction is determined directly by GC equipped with ^{63}Ni electron capture detector (ECD) (Perkin-Elmer, USA). The column used was of stainless steel 2 m, length and o.d. 1/8 in., gas chrom Q support, 80–100 mesh size, Liq. Phase; SG-30 + OV 210. The conditions maintained were injector temperature 250 °C, column temperature 180 °C, and detector temperature 300 °C. Nitrogen was used as the carrier and flow rate was maintained at 35 ml min^{-1}.

3. Results and discussion

3.1. Characteristics of soil

Garden soil was collected and characterized for various parameters, the results are presented in Table 1. The virgin soil contained considerable microbial diversity in terms of number as well as microbial species. The total culturable heterotrophic bacterial count in the initial soil sample was 822×10^4 cfu g^{-1} of soil and nine different bacterial types were observed. The types were differentiated based on their colony morphology. A large number of fungal species were also present, but only one actinomycetes species could be detected in the initial sample. The texture of the soil is silty clay loam and predominated by clay (43.8%) with organic content of 8.94% (w/w).

Table 1
The physico-chemical and biological properties of garden soil used for the study

Physical	
pH	8.2
Temperature	27 °C
Moisture content	24%
Texture	Silty clay loam
Particle size distribution	
Silt	28.2%
Clay	43.8%
Fine sand	12.4%
Chemical	
Organic content (w/w)	8.94%
Nitrogen	0.17%
Sulphur	ND
Phosphorus	0.05%
Exchangeable cations	
Ca^{2+}	14.7 (meq/100 g)
Mg^{2+}	8.4 (meq/100 g)
Na^{+}	1.9 (meq/100 g)
K^{+}	1.0 (meq/100 g)
Cation exchange capacity	27.25 (mg/100 g)
Biological	
Bacteria	822×10^4 cfu/g (nine different types)
Fungi	70×10^3 cfu/g (eight different types)
Actinomycetes	10×10^3 cfu/g (one type)

3.2. t-HCH degradation and its effect on soil microflora

Different concentrations of t-HCH (mentioned in Section 2) was spiked on soil and incubated for 9 weeks. These soil samples were analyzed at regular time intervals for residual t-HCH isomers and microbial populations, the results are presented in Tables 2 and 3. It was observed that application of t-HCH retarded the growth of microbial populations present in the soil sample. No actinomycete species was present in any of the soil samples to which t-HCH was added. Decrease in the total count of bacteria and fungi, was observed even after prolonged incubation up to 9 weeks. The insecticide inhibited the activity of these microbes thereby retarding their proliferation and growth and possibly had deleterious effect on their metabolism.

In the present study, it was observed that, microbial types present in the initial soil sample also decreased during incubation in the presence of t-HCH. The total microbial types (bacteria and fungi) in the t-HCH amended samples were less in comparison to the control soil sample, suggesting that some groups of microorganisms could neither tolerate nor degrade t-HCH and were severely affected and hence, got completely eliminated. In the third week, with increase in t-HCH concentration, the types of bacteria and fungi gradually decreased. Few microbial cultures that survived at lower concentrations were inhibited and subsequently eliminated at higher t-HCH concentrations. A possible reason for the survival of more types in the initial stages could be that, though the microbes were unable to utilize t-HCH, they were not inhibited by the t-HCH residues. All fungal species were eliminated at higher concentrations of t-HCH during the ninth week. Table 4 gives a list of different types of bacterial colonies and their colony morphology on nutrient agar and substrate specific agar, of isolated bacterial cultures. The results also indicate that although the total diversity of the microbial cultures decreased, the microbial count of certain bacteria increased. This may be due to increase in number of those organisms which are capable of degrading t-HCH by producing necessary dehalogenating enzymes.

Table 2
Degradation of HCH and release of chlorides during the incubation period of 9 weeks

HCH (mg g^{-1} of soil)	Chloride concentration (mg g^{-1} of soil)			HCH concentration remaining in soil (mg g^{-1})		
	After 3 weeks[a]	After 6 weeks[a]	After 9 weeks[a]	After 3 weeks[a]	After 6 weeks[a]	After 9 weeks[a]
0	0.12	0.12	0.12	–	–	–
2	0.576	0.75	1.314	1.21	0.96	0.18
4	0.599	1.03	2.3	3.18	2.57	0.82
6	0.270	2.11	2.99	5.63	3.10	1.9
8	0.57	2.21	3.94	7.21	4.44	2.6
10	1.00	2.97	4.56	8.62	5.92	3.75

[a] Incubation period.

Table 3
Bacterial colony count in soil after different time intervals

HCH (mg/g of soil)	Total hetrotrophic bacterial count ($\times 10^4$ cfu g^{-1})			HCH degrading bacterial count ($\times 10^4$ cfu g^{-1})		
	After 3 weeks[a]	After 6 weeks[a]	After 9 weeks[a]	After 3 weeks[a]	After 6 weeks[a]	After 9 weeks[a]
0	915 (±3.2)	1016 (±2.6)	926 (±2.4)	23 (±0.2)	39 (±1.0)	51 (±2.1)
2	658 (±2.3)	540 (±2.8)	234 (±1.8)	22 (±0.1)	36 (±2.1)	66 (±0.9)
4	406 (±2.2)	276 (±2.2)	182 (±2.2)	38 (±0.3)	49 (±0.6)	75 (±1.2)
6	294 (±2.4)	185 (±2.6)	148 (±3.6)	32 (±.0.2)	63 (±0.9)	94 (±1.3)
8	212 (±1.9)	180 (±2.9)	153 (±1.9)	44 (±0.4)	86 (±1.2)	98 (±1.2)
10	167 (±2.1)	148 (±0.6)	128 (±2.4)	56 (±0.3)	86 (±2.1)	82 (±0.9)

[a] Incubation period.

Table 4
Microbial characteristics of the nine colony types isolated from initial soil sample

Type of colony	No. of colonies ($\times 10^3$ cfu)	Shape	Size	Margin	Colour	Elevation	Diffusion
1	20	Round	Small	Smooth	Yellow	Flat	Opaque
2	1	Round	Small	Smooth	Peach	Flat	Opaque
3	6	Irregular	Large	Serate	White	Flat	opaque
4	22	Round	Medium	Smooth	White	Flat	Opaque
5	22	Irregular	Medium	Irregular	Light brown	Flat	Slightly translucent
6	10	Irregular	Medium	Cloudy	White	Center raised, flat periphery	Center opaque, periphery translucent
7	5	Round	Medium	Smooth	Light brown	Raised	Translucent
8	5	Round	Very small	Regular	off white	Raised	Opaque
9	729	Round	Pin-headed	Smooth	off-white	Flat	Opaque

Table 5
Tech-hexachlorocyclohexane isomers remaining in soil

HCH concentration (mg g^{-1})	Initial concentration of each isomer of t-HCH spiked in the soil (mg g^{-1})				Residues of four isomers in soil after 3 weeks (mg g^{-1})				Residues of four isomers in soil after 6 weeks (mg g^{-1})				Residues of four isomers in soil after 9 weeks (mg g^{-1})			
	α	γ	β	δ	α	γ	β	δ	α	γ	β	δ	α	γ	β	δ
2.0	1.37	0.3	0.15	0.152	0.81	0.20	0.14	0.06	0.73	0.12	0.07	0.04	0.04	0.09	0.03	0.02
4.0	2.7	0.64	0.28	0.304	2.19	0.52	0.28	0.19	1.98	0.39	0.12	0.08	0.48	0.25	0.06	0.03
6.0	4.13	0.96	0.43	0.456	4.08	0.93	0.42	0.20	2.06	0.62	0.26	0.16	1.23	0.46	0.12	0.09
8.0	5.51	1.28	0.57	0.60	5.18	1.16	0.57	0.30	2.97	0.83	0.36	0.28	1.51	0.64	0.28	0.17
10.0	6.89	1.61	0.72	0.76	6.32	1.22	0.70	0.38	3.6	1.60	0.42	0.30	3.0	0.43	0.12	0.2

Around three to five colonies were present at low concentrations of t-HCH during the 9th week of which some were inhibited at higher concentrations. Only two types of bacterial colonies remained after 9 weeks of incubation even at higher concentrations of t-HCH namely 8 mg HCH g^{-1} and 10 mg HCH g^{-1} of soil and were also present in remaining concentrations of t-HCH. After 9 weeks of incubation only 2.6 and 3.75 mg g^{-1} of t-HCH residue was recovered from the soil spiked with 8 and 10 mg g^{-1} t-HCH in which these bacterial colonies were present. These two different bacterial colonies that increased in number, proliferated at higher t-HCH concentrations and thus, showed the capacity to degrade t-HCH were isolated and purified. The colony characteristics of the two bacterial isolates on nutrient agar as well as substrate specific agar is given in Table 4. Preliminary screening suggests that the two isolates belong to the genus Alcaligenes and Klebsiella, respectively. Das et al. [20] have studied the effect of HCH and fenvalerate on growth and distribution of microorganisms in relation to persistence of the pesticides in the rhizosphere soils of wetland rice. The authors, however, have not studied the effect of t-HCH on micro-

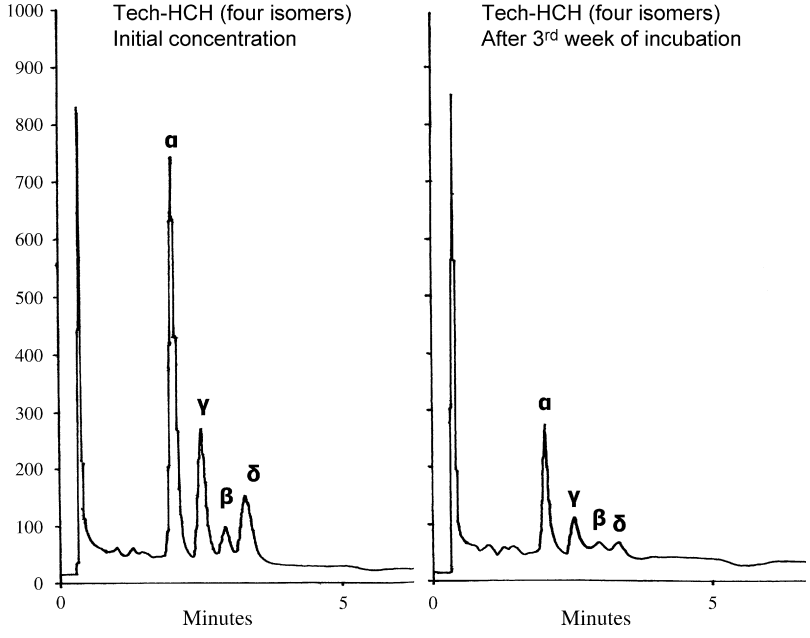

Fig. 1. Gas chromatographic profiles of HCH isomers extracted from soil samples.

bial growth at high concentrations of t-HCH and restricted their sampling to 60 h. In the present study, we describe the effect of higher concentrations of t-HCH on soil microflora and isolation of some potential t-HCH degrading species. The concentration of individual isomers of t-HCH residues in the soil after 3 weeks of incubation is presented in Table 5. In the last part of the experiment, it was observed that residues of the four isomers still remained in the soil even after 9 weeks of incubation, showing the persistence of these isomers in soil. t-HCH applied soils (4 mg g^{-1}, sterile soil (control) and the unsterile soil (experimental)) were extracted for its four different isomers after 3 weeks of incubation and analyzed for residual HCH isomers on gas chromatograph equipped with ECD detector, the results are presented in Fig. 1. More than 50% of the peaks were reduced in the unsterile soil. It indicates the degradation of all four isomers by the soil microflora. From the soil microorganisms, t-HCH degrading microbial cultures were isolated and tested for t-HCH degrading capability in specific substrate media. It was observed that even after having the t-HCH degrading capability, the isolated strains did not show complete degradation of all four isomers of t-HCH. When they were further subjected to acclimation and were supplied with nutrients (experiment described in Section 2), by slow acclimatization, these strains were able to degrade 10 mg g^{-1} of t-HCH with an incubation period of 9 weeks. This indicated that microbial populations in soil have the capability of dechlorinating and degrading of t-HCH, however, they require further acclimatization and favorable conditions to degrade t-HCH isomers effectively. Our results substantiate the fact that, it is very much essential to ascertain the capacity of the cultures that degrade t-HCH as well as the interaction between different non t-HCH degraders so as to manage the bioremediation of contaminated soils by augmenting acclimatized bacterial cultures and provide nutrient supplementation after assessing the soil characteristics.

4. Conclusions

Degradation of t-HCH isomers in the soil and its effect on the soil microflora has been studied by using virgin garden soil and technical grade hexachlorocyclohexane (t-HCH). It was observed that the total microbial population was inhibited by high concentrations of t-HCH. And all the four isomers of t-HCH were not degraded completely even after prolonged incubation by potential t-HCH degrading cultures. These t-HCH degrading bacterial cultures that were isolated after acclimatization and supplementation of nutrients were able to degrade all the four isomers effectively. Rate of degradation of isomers increased with these acclimatized cultures. It can be concluded that augmenting acclimatized native cultures along with nutrient supplementation will help in bioremediation of t-HCH contaminated soils.

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