ELSEVIER

Soil Biology & Biochemistry 38 (2006) 2318–2327

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Biodegradation of hexachlorocyclohexane-isomers in contaminated soils

Manish Kumar[a], S.K. Gupta[a], S.K. Garg[b], Ashwani Kumar[a,*]

[a]*Industrial Toxicology Research Centre, Post Box No 80, Mahatma Gandhi Marg, Lucknow-226 001, India*
[b]*Department of Microbiology, Dr. R.M.L. Avadh University, Faizabad-224 001, India*

Received 30 September 2005; received in revised form 19 February 2006; accepted 22 February 2006
Available online 3 April 2006

Abstract

Several sites that are contaminated with isomers of the chlorinated insecticide hexachlorocyclohexane (HCH) are present across the globe and cause toxicity. For their bioremediation, we studied the degradation of HCH-isomers in contaminated soils by an isolate *Pseudomonas aeruginosa* ITRC-5. The degradation is optimal at 2 mg technical-HCH (*t*-HCH)/g soil, 15% water content, pH 8.0, temperature 28 °C and inoculum density 10^6 colony forming unit/g soil. Under these conditions, from 5 kg soil, >98% α- and γ-HCH, 17% β-HCH and 76% δ-HCH are degraded after 15 days of incubation, which is accompanied with the release of 600 μg chloride/mg *t*-HCH. Concomitant to the degradation, a four-fold reduction in the toxicity of HCH-isomers to earthworm, *Eisenia foetida*, is also observed. Addition of ITRC-5 enhanced the degradation of soil-applied HCH-isomers in 'open field' conditions as well, and 97%, 43%, 94% and 77% of α-, β-, γ- and δ-HCH, respectively, are degraded after 12 weeks of incubation. Thus, the bacterium causes microbial degradation and detoxification of HCH-isomers, and can be used for the bioremediation of contaminated soils.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Hexachlorocyclohexane; *Pseudomonas aeruginosa*; Degradation; Detoxification; Bioremediation

1. Introduction

Two formulations of hexachlorocyclohexane (HCH), one technical-HCH consisting of 60–70% α-, 5–12% β-, 10–18% γ- and 6–10% δ-HCH, and another referred by its trade names, lindane, gamexene, etc, consisting of >99% γ-HCH, have been used extensively in the past for the protection of crops and control of vector-borne diseases (Willett et al., 1998; ATSDR, 1999). At the time of their manufacture, formulation, storage, transport and intended use (Willett et al., 1998; Blais et al., 1998), HCH-isomers enter into the environment and several contaminated sites are present all around the world (Simonich and Hites, 1995; Li, 1999). The isomers from here are released slowly, enter the food chain and impart toxicity (Willett et al., 1998; ATSDR, 1999).

Bioremediation, which includes the gainful utilization of microorganisms for the degradation and detoxification of toxic pollutants (Crawford and Crawford, 1996; Alexander, 1999), has been used successfully for the clean up of sites contaminated with explosives (Manning et al., 1996) or petroleum hydrocarbons (Mishra et al., 2001). It, therefore, has potential for the reclamation of other contaminated soils as well. For this reason, several microorganisms that degrade HCH-isomers under aerobic conditions have been isolated and characterized (Senoo and Wada, 1989; Sahu et al., 1990; Thomas et al., 1996; Gupta et al., 2000; Manonmani et al., 2000; Okeke et al., 2002; Kumar et al., 2005). Addition of some of these to the contaminated soil has been shown to enhance the degradation of HCH-isomers (Sahu et al., 1993; Okeke et al., 2002; Bidlan et al., 2004; Kumar et al., 2005). In most cases, the degradation of α- or γ-HCH is rapid, but β- and δ-isomers are either not degraded (Sahu et al., 1993) or degraded very slowly (Bidlan et al., 2004; Kumar et al., 2005). In another study, the degradation of α-HCH by autochthonous organisms was observed in a contaminated soil, but β-HCH was not degraded (Bachmann et al., 1988a). We have recently described a bacterium *Pseudomonas aeruginosa* ITRC-5 that degrades α-, β-, γ- and δ-HCH, in both liquid-culture and contaminated soils, and

*Corresponding author. Tel.: +91 522 2620107; fax: +91 522 2628227.
E-mail address: ashwani26@rediffmail.com (A. Kumar).

0038-0717/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2006.02.010

the degradation of β- and δ-isomers is enhanced in the presence of α- or γ-HCH (Kumar et al., 2005). The degradation pathway of γ-HCH has been well understood. Briefly, it proceeds via two steps of dehydro-dechlorination, two steps of hydrolytic-dehalogenation, and a dehydrogenation step to yield 2,5-dichlorohydroquinone, which undergoes ring cleavage and is mineralized (Nagata et al., 1999).

In many cases, although the added microorganisms have the ability to degrade the target pollutants, bioremediation does not work successfully in the field conditions (Goldstein et al., 1985; Block et al., 1993). This has been attributed to wide variations in the temperature, pH and moisture-content, under these conditions (Awasthi et al., 2000; Karpouzas and Walker, 2000). Limited availability of nutrients (Cheung and Kinkle, 2005), amount of pollutant and its age (Park et al., 2004), presence of inhibitory substances and competition with indigenous microflora (van Veen et al., 1997) are other determinants of the degradative activity.

In this study, we have determined the optimal conditions for the degradation and detoxification of soil-applied HCH-isomers by the strain ITRC-5, for its eventual use towards the bioremediation of contaminated sites.

2. Materials and methods

2.1. Chemicals

Technical-HCH, consisting of α- (67.4%), β- (6.8%), γ- (17.3%) and δ-HCH (7.4%) was obtained from India Pesticides Limited, Lucknow, India. All other chemicals were from different commercial sources, and of analytical grade.

2.2. Soil

Soil was collected from the garden of Industrial Toxicology Research Centre, Lucknow, India. It contained 43.6% clay, 20% silt, 36.4% sand and 0.47% organic carbon. Its pH, measured in 20% soil–water slurry that was shaken for 48 h, was 7.9. The soil was air dried and sieved through a 2 mm mesh before use.

2.3. Inoculum

The bacterium *P. aeruginosa* ITRC-5, characterized earlier for its capability to degrade α-, β-, γ- and δ-HCH (Kumar et al., 2005), was grown in the medium (KH_2PO_4, 170 mg; Na_2HPO_4, 980 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4 \cdot 7H_2O$, 4.87 mg; $FeSO_4 \cdot 7H_2O$, 0.05 mg; $CaCO_3$, 0.20 mg; $ZnSO_4 \cdot 7H_2O$, 0.08 mg; $CuSO_4 \cdot 5H_2O$, 0.016 mg; H_3BO_3, 0.006 mg; yeast extract 10 mg and glucose 10 mg, dissolved in 100 ml distilled water, pH 7.4), containing 200 mg *t*-HCH/l, for 5 days at 28 °C with shaking at 180 rev/min, and used as inoculum. Its density was 2.8×10^8 colony forming unit (cfu)/ml.

2.4. Biodegradation of HCH-isomers in contaminated soils

Soil was spiked with *t*-HCH in two steps to prevent the loss of indigenous microflora (Brinch et al., 2002). Briefly, 1 g *t*-HCH was dissolved in 100 ml hexane:acetone (9:1) and mixed with 250 g soil. After evaporation of the solvent, an additional 750 g soil was mixed to it. This 'spiked soil' (5 g) was transferred to different test tubes (flat bottom, $3.25'' \times 0.9''$). For all the biodegradation studies, the water content was 15% (v/w), pH 7.9 and incubation was at 28 °C, unless noted otherwise. While one set of tubes was inoculated with 10^6 cfu ITRC-5/g soil, the other remained un-inoculated. After incubation for specified periods, residual HCH-isomers were extracted from both the sets with hexane and quantified by a gas chromatograph that was equipped with ^{63}Ni-electron capture detector, as described earlier (Kumar et al., 2005). The released chloride ions were estimated by a colorimetric method, using $AgNO_3$ reagent (Bergman and Sanik, 1957).

To evaluate the effect of water content, four sets of 30 tubes each were prepared, which contained 15, 30, 45 and 100% (v/w) water content, respectively. From each set 15 tubes were run as control and the other 15 were inoculated with 10^6 cfu/g soil. Three un-inoculated and other three inoculated tubes from each set were removed after 0, 2, 4, 8 and 12 days of incubation and residual HCH-isomers as well as chloride ions were estimated. Similarly, for the experiment where the effect of inoculum-density on the degradation of HCH-isomers was to be determined, five sets of 15 tubes each were made. The tubes of sets 1–4 received 10^2, 10^4, 10^6, 10^8 cfu/g soil, respectively, and set 5 was run as un-inoculated control. Three tubes from each set were removed after 0, 2, 4, 6, 8 and 12 days of incubation and residual HCH-isomers were estimated.

To determine the effect of temperature on the degradation of HCH-isomers, five sets of six tubes each were made. While three tubes from each set were inoculated, the other three remained un-inoculated. Sets 1–5 were incubated at 8, 18, 28, 38 and 48 °C, respectively, for 4 days and residual HCH-isomers were quantified in all the tubes, as described above. Likewise, for the experiment where effect of pH on the degradation of soil-applied HCH-isomers was to be evaluated, six sets of 30 g soil each were adjusted with sulphuric acid or sodium hydroxide to pH 5–10, respectively. These were spiked with 1 mg *t*-HCH/g soil. Spiked soil of each set was distributed to six tubes, out of which three tubes were inoculated and other three remained un-inoculated. Residual HCH-isomers were quantified in all the tubes after 8 days of incubation.

The effect of initial *t*-HCH concentration on the degradation of HCH-isomers was determined by spiking four sets of 120 g soil samples with 1, 2, 5 or 10 mg *t*-HCH/g soil, respectively. After apportioning the spiked soil of each set in 36 tubes, 18 tubes were inoculated and other 18 remained un-inoculated. From each set, three inoculated and three un-inoculated tubes were removed after 0, 4, 8,

12, 16 and 20 days of incubation and residual HCH-isomers were estimated.

2.5. Bioremediation of an HCH-contaminated soil

Soil (10 kg) was spiked with 2 mg *t*-HCH/g soil and distributed equally to four plastic trays ($30 \times 24 \times 6$ cm). While two trays were inoculated with 10^6 cfu/g soil, the other two remained un-inoculated. After incubation at 28 °C for 15 days, 5 g samples were drawn from each tray and residual HCH-isomers as well as the released chloride ions were estimated. To determine the toxicity of contaminated soil, both before and after the treatment with ITRC-5, the earthworm, *Eisenia foetida*, was used as test organism (EPA, 1996). Briefly, soil samples of both un-inoculated and inoculated trays were diluted to 100%, 50% and 25% with un-spiked soil. Each diluted soil (500 g) was mixed with 500 g cow-dung compost in 2 l polypropylene beakers, and ten mature earthworms were added. All the beakers were covered with polyethylene wrap, and incubated at 21 °C under continuous illumination, which was necessary to prevent the escape of worms from the containers. The number of living worms was recorded after14 days.

2.6. Biodegradation of HCH-isomers in contaminated soils under field conditions

A total of 30 polyvinyl-chloride (PVC) pipes (12″ length, 4″ diameter) were inserted in the garden of Industrial Toxicology Research Centre, Lucknow, India, at a distance of around 12″ from each other, with a 2″ collar above the ground. After leaving them undisturbed for 8 months, 1.5 g *t*-HCH was applied on top of each pipe; 15 pipes were inoculated with 3×10^9 cfu in 50 ml medium, and medium alone was added to the rest (un-inoculated set). After incubation for 0, 3, 6, 9, and 12 weeks, three pipes from both un-inoculated and inoculated sets were removed. Residual HCH-isomers in the 0–4″, 4–8″ and 8–12″ depth fractions of the soil from each pipe were extracted and estimated. The atmospheric temperature during the study period was 10–34 °C.

3. Results

3.1. Degradation of HCH-isomers

3.1.1. Effect of soil–water content

In un-inoculated soil that was contaminated with 1 mg *t*-HCH (containing 674, 68, 173, and 74 μg α-, β-, γ- and δ-HCH, respectively)/g soil, and had 15% (v/w) water content, 2–12% of different HCH-isomers were degraded after 12 days of incubation (Fig. 1). The degradation under un-inoculated condition did not change when the water content was higher i.e. 30%, 45% or 100% (Fig. 1). Addition of ITRC-5 enhanced the degradation of HCH-isomers under all the water regimes. It was optimal in the presence of 15% soil–water content, when >95% α- and γ-HCH were degraded after 4 days, and 27% β-HCH and 77% δ-HCH were degraded after 12 days of incubation

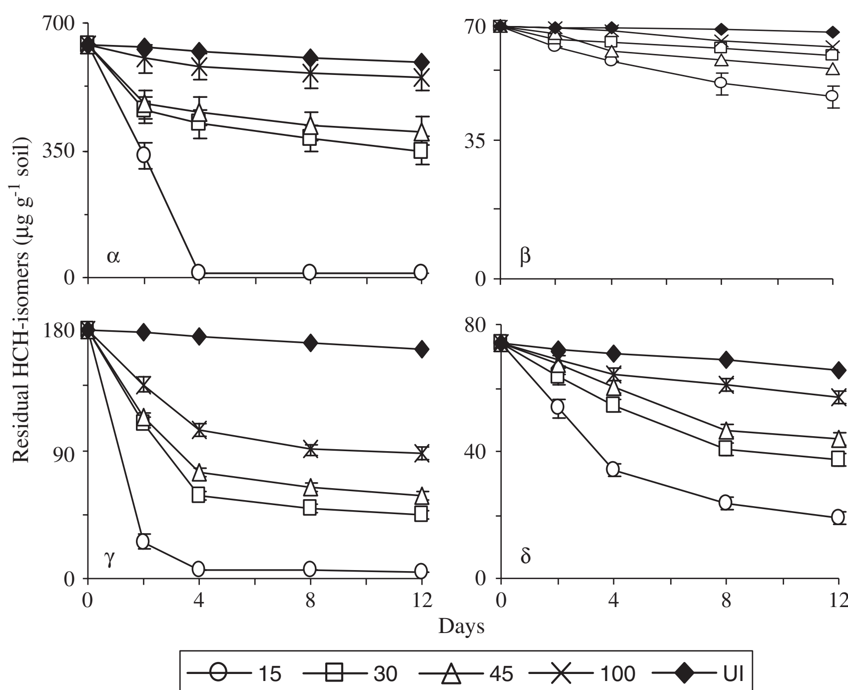

Fig. 1. Degradation of HCH-isomers under inoculated condition, in the presence of 15–100% water content. Their degradation in the presence of 15% water under un-inoculated condition (UI) is also shown, and was coincident at 30, 45 and 100% water content. Values given are the mean of triplicates and vertical bars represent standard deviation.

(Fig. 1). The degradation was inhibited progressively in the presence of higher soil–water content (Fig. 1), and 600, 254, 206 and 40 μg chloride ion/g soil were released after 12 days of incubation when 15%, 30%, 45% and 100% water, respectively, was present (Fig. 2). Accumulation of the metabolite PCCOL, reportedly formed by the metabolism of β- and δ-HCH (Sahu et al., 1995; Kumar et al., 2005; Nagata et al., 2005), however, was not observed under any of the water regimes.

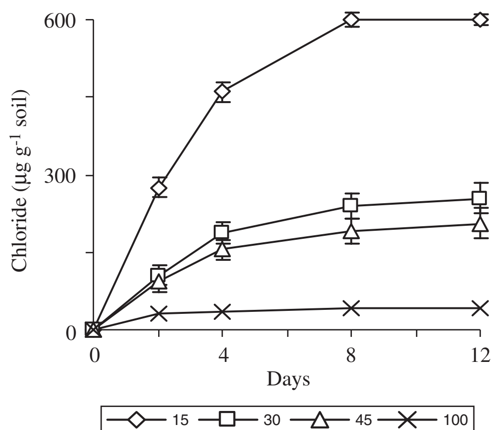

Fig. 2. Effect of 15–100% water content on the release of chloride after the degradation of HCH-isomers under inoculated condition, as described in Fig. 1. The chloride under un-inoculated condition was below detection limit (<3 μg/ml) in all the samples. Values given are the mean of triplicates and vertical bars represent standard deviation.

3.1.2. Effect of inoculum density

In un-inoculated soil, 2–12% degradation of different HCH-isomers was observed after 12 days of incubation (Fig. 3). Addition of ITRC-5 enhanced their degradation, which increased progressively at higher inoculum densities. It was optimal at 10^6 cfu/g soil, when >95% α- and γ-HCH, 26% β-HCH and 77% δ-HCH were degraded after 12 days of incubation (Fig. 3). Addition of more cells i.e. 10^8 cfu/g soil, increased the rate of degradation of HCH-isomers at early time points but the degradation after 12 days of incubation was not significantly different from that obtained with lower densities (Fig. 3).

3.1.3. Effect of pH

At pH 5.0–9.0, 2–10% degradation of different HCH-isomers was observed in un-inoculated soil after 8 days of incubation (Fig. 4). Under inoculated conditions, however, while no significant degradation was observed at pH 5.0, substantial degradation occurred at pH 6.0–9.0. The degradation was optimal at pH 8.0, and >95% α- and γ-HCH, 25% β-HCH and 75% δ-HCH, were degraded after 8 days incubation (Fig. 4). At pH 10.0, 5–10% degradation of different HCH-isomers was observed under un-inoculated conditions, which was not affected by the addition of ITRC-5 (Fig. 4).

3.1.4. Effect of temperature

Addition of ITRC-5 to the contaminated soil caused <5% degradation of HCH-isomers at 8 °C, but substantial degradation was observed at higher temperatures. It was

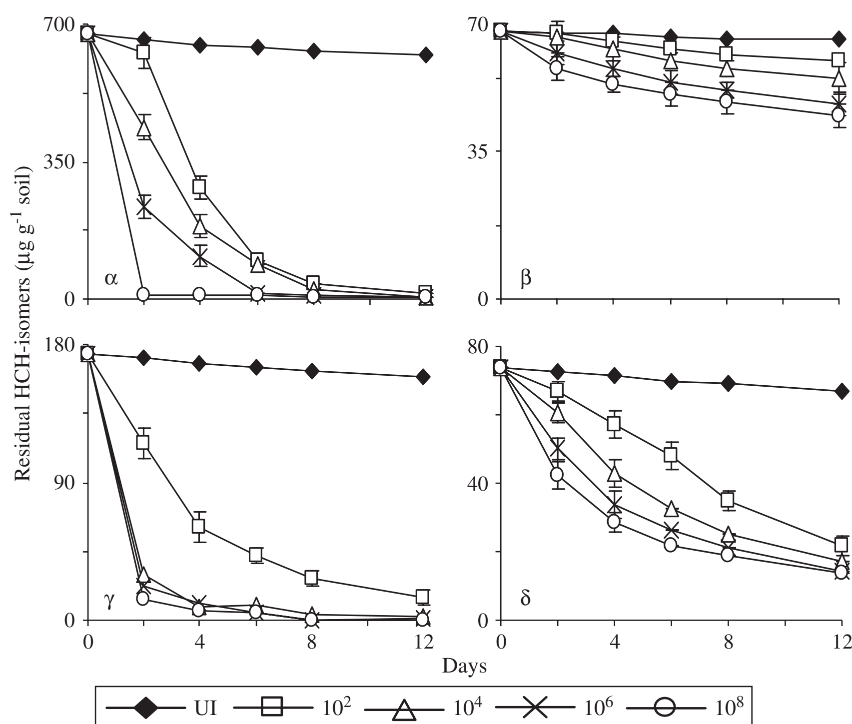

Fig. 3. Degradation of HCH-isomers in the presence of different inoculum density (10^2–10^8 cfu/g soil), and in un-inoculated (UI) soil. Values given are the mean of triplicates and vertical bars represent standard deviation.

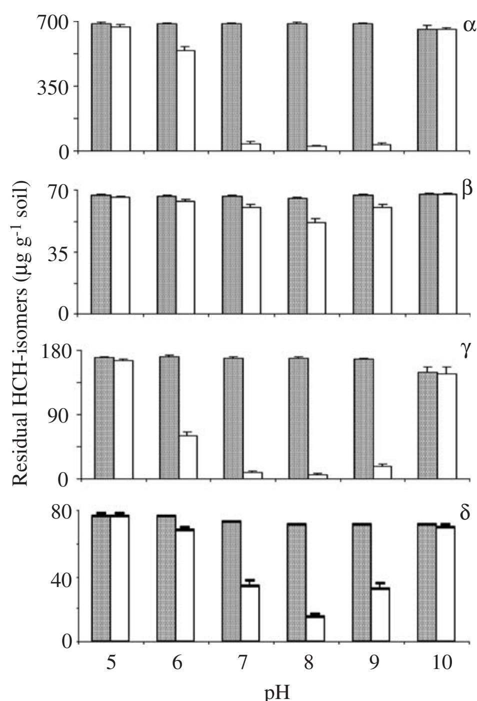

Fig. 4. Degradation of HCH-isomers at soil-pH 5–10, in un-inoculated (shaded bars) and inoculated (open bars) soils, after 8 days incubation. Values are mean of triplicates and the vertical bars represent standard deviation.

optimal at 28 °C, when >90% α- and γ-HCH, 15% β-HCH and 55% δ-HCH were degraded after 4 days of incubation (Fig. 5). At 48 °C, 14–41% decrease of different isomers was observed in both un-inoculated and inoculated soil (Fig. 5).

3.1.5. *Effect of initial concentration of t-HCH*

When the initial concentration of *t*-HCH was 1 or 2 mg/g soil, addition of ITRC-5 caused >95% degradation of α- and γ-HCH, and 69% and 18% of δ- and β-HCH, respectively, after 8 days of incubation (Fig. 6). At 5 mg *t*-HCH/g soil, however, the rate of degradation of all the isomers was inhibited at early time points, which was gradually relieved at later time points. Thus, 62.3, 0, 83 and 49% α-, β-, γ- and δ-isomers were degraded after 12 days of incubation. The degradation of HCH-isomers leveled off thereafter, and no further decline was observed after up to 20 days of incubation. The inhibition of HCH-degradation at early time points was even more pronounced, when the initial concentration of *t*-HCH was 10 mg/g soil (Fig. 6).

3.2. *Bioremediation of HCH-contaminated soil*

In 5 kg soil that were contaminated with 2 mg *t*-HCH/g soil, 5% Σ-HCH, i.e. the sum of α-, β-, γ- and δ-isomers was degraded after incubation for 15 days under un-inoculated condition (Table 1). Addition of ITRC-5 enhanced the degradation, and >90% Σ-HCH (98% of α- and γ-HCH, 17% β-HCH and 76% δ-HCH) was degraded after the same incubation period (Table 1). The degradation was accompanied with the release of 1.2 mg chloride ion/g soil (data not given), representing 85% of 1.4 mg chloride that is theoretically expected from 2 mg *t*-HCH. Exposure of earthworms, *Eisinia foetada*, for 14 days to the untreated soil, i.e. the soil that was not inoculated with ITRC-5, resulted in their 100% mortality (Table 2). When this soil was diluted to 50% or 25% with the non-contaminated soil, the mortality was reduced to 50% or 0%, respectively. On the other hand, no mortality of earthworms was observed when the soil that was treated by incubation with ITRC-5 was used for the assay (Table 2).

3.3. *Biodegradation of HCH-isomers under 'field-conditions'*

In 'open field condition' where 1.5 g *t*-HCH was layered on top of the soil in the inserted PVC pipes, 1.0, 0.1, 0.3 and 0.1 mg α-, β-, γ-, and δ-HCH/g soil, respectively, was present in the top 0–4″ portion of the soil, in the beginning of the experiment (Fig. 7). No detectable HCH was observed in 4–8″ and 8–12″ portions. With the passage of time, there was a progressive fall in the concentration of HCH-isomers in all the pipes. Thus, in un-inoculated pipes, only 18% Σ-HCH that consisted of 11.5%, 60%, 18.6% and 45% of α-, β-, γ-, and δ-HCH, respectively, was recovered in the 0–4″ fraction after 12 weeks of incubation (Fig. 7). Simultaneously, 4% Σ-HCH that consisted of 3–4% α- or γ-HCH, 12% β-HCH and 6% δ-HCH, was recovered in 4–8″ portion, and <1% Σ-HCH consisting of <1% α- or γ-, 0.6% β- and 3.0% δ-HCH/g soil was recovered in 8–12″ portion. In the pipes that were inoculated with ITRC-5, an enhanced degradation of HCH-isomers was observed and 14% Σ-HCH, consisting of <10% α- and γ-HCH, 60% β- and 45% δ-HCH, was recovered in 0–4″ portion of the pipes after 3 weeks of incubation. The degradation of β-HCH stopped thereafter, but that of other isomers continued, albeit at a substantially slower rate (Fig. 7). HCH-isomers were observed in lower fractions of soil after 9 weeks of incubation, which increased progressively. Thus, after 12 weeks of incubation, 2–3% Σ-HCH consisting of 2% α-, 1.3% γ-, 7.2% β- and 2.3% δ-HCH, respectively, was detected in 4–8″ fraction, and <1% Σ-HCH consisting of <0.02% α-, 0.1% γ-, 0.1% β- and 0.3% of δ-HCH was detected in 8–12″ fraction (Fig. 7).

4. Discussion

Bioaugmentation by degradative microorganisms can be used for the bioremediation of contaminated sites. In this study, degradation of soil-applied HCH by *P. aeruginosa* ITRC-5 was evaluated. In this study, a decrease of 5% Σ-HCH, i.e. the sum of α-, β-, γ- and δ-isomers occurs in un-inoculated soil after 15 days of incubation. This could be due to volatilisation, oxidation and photodecomposition of HCH-isomers along with their biotransformation with the

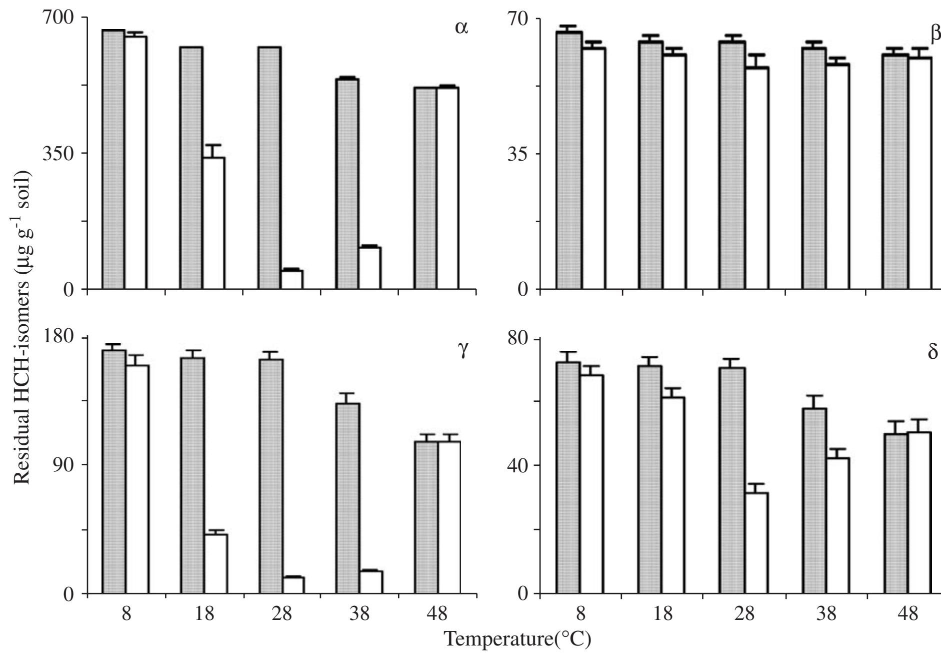

Fig. 5. Degradation of HCH-isomers at temperature 8–48 °C in un-inoculated (shaded bars) and inoculated (open bars) soils after 4 days of incubation. Values are mean of triplicates and the vertical bars represent standard deviation.

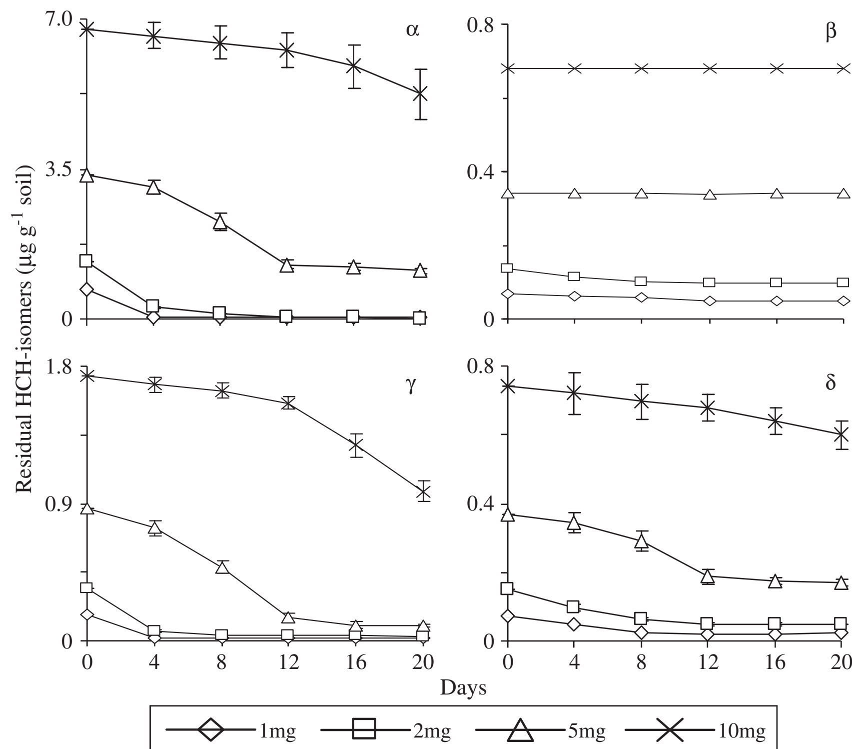

Fig. 6. Degradation of HCH-isomers under inoculated condition, when initial *t*-HCH concentration was 1–10 mg/g soil. Their degradation under un-inoculated condition, at initial concentration 1 mg *t*-HCH/g soil, was same as in Fig. 1 (not shown here) and was comparable at 2, 5 and 10 mg *t*-HCH/g soil. Values are mean of triplicates and the vertical bars represent standard deviation.

autochthonous microorganisms (ATSDR, 1999), and has been referred as 'degradation under un-inoculated conditions'. Addition of ITRC-5 enhances the degradation, which is optimal at the initial input concentration of 2 mg *t*-HCH/g soil, 15% soil–water content, pH 8.0, temperature 28 °C and inoculum density 10^6 cfu/g soil. More than 90% Σ-HCH was degraded after 15 days of incubation under these conditions (Table 1).

Table 1
Biodegradation of HCH-isomers in a contaminated soil

	μg HCH recovered/g soil (%)[a]		
	Initial	After 15 days	
		Un-inoculated	Inoculated
α-HCH	1355±36 (100)	1287±41 (95)	18±5 (1.3)
β-HCH	136±12 (100)	132±13 (97)	113±4 (83)
γ-HCH	346±23 (100)	322±14 (93)	3±1 (0.8)
δ-HCH	148±12 (100)	137±12 (92)	35±3 (24)
Σ-HCH	1985 (100)	1878 (95)	169 (8.5)

Values in parenthesis are percent, taking the recovery at initial time as 100%.
[a]Mean values of triplicate samples±standard deviation are given.

Table 2
Toxicity of HCH-contaminated soil, both untreated or treated with ITRC-5, to *Eisinia foetada*

	% Mortality[a]	
Contaminated soil (%)	Un-treated soil	Treated soil
100	100	0
50	50±10	0
25	0	0

[a]Values given are the mean of triplicates, and standard deviation is shown, where present.

Mortality of the test organisms is a sensitive end point of toxicity, and its decrease is indicative of the biological treatment of contaminated soils (Jarvis et al., 1998; Lenke et al., 1998; Juhasz et al., 2000). The degradation of HCH-isomers by ITRC-5 led to the detoxification of contaminated soil, as the treated soil was found to be safer to the test organism *E. foetada*, in comparison to the un-treated soil (Table 2).

Water content, pH and temperature of the soil can influence the bioavailability of pollutant, as well as the activity of the microorganisms, and affect the degradation rates (Goldstein et al., 1985; Awasthi et al., 2000; Karpouzas and Walker, 2000). In this study, the degradation of HCH-isomers was optimal at 15% water content, but was inhibited in the presence of its higher content (Fig. 1). The results are in agreement with earlier studies, where the degradation of HCH (Sahu et al., 1993) or chlorophenols (Cho et al., 2000) was found to be inhibited at >30% water content. This could be due to the agglomeration of soil under this condition, resulting in limited oxygen supply and consequent reduction in the microbial activity (Scholz et al., 1998). No degradation of HCH-isomers was observed at pH 5.0, possibly due to poor microbial activity as well as the poor availability of the substrate (Fig. 4). On the other hand, 5–10% decline of different HCH-isomers at pH 10 could be due to the abiotic destruction of HCH-isomers, as it occurred in both un-inoculated and inoculated soil. Similar degradation of HCH has been reported in earlier studies also (Manonmani et al., 2000; Siddique et al., 2002). In the present study, the degradation of HCH-isomers was optimal at 28 °C (Fig. 5). While no degradation occurred at 8 °C, possibly because of poor solubility of HCH-isomers at this temperature, their levels declined at 48 °C. The decline was comparable in both un-inoculated and inoculated soils, and could be due to the enhanced volatility of HCH-isomers (Willett et al., 1998). No accumulation of the metabolite pentachlorocyclohexanol (PCCOL) that forms by the metabolism of β- and δ-HCH (Sahu et al., 1995; Kumar et al., 2005; Nagata et al., 2005), however, was observed during this degradation. This might be due to its enhanced metabolism in the presence of α- and γ-HCH, as observed earlier for β- and δ-HCH (Kumar et al., 2005).

The degradation of HCH-isomers in the soil could be initiated by the addition of 10^2 cfu of ITRC-5/g soil (Fig. 3), possibly because the added microbes could proliferate in the presence of α- and γ-isomers (Kumar et al., 2005). It enhanced progressively at higher inoculum densities, as also seen in other studies on HCH (Sreedharan et al., 1999) and other pollutants (Moller and Ingvorsen, 1993; Briglia et al., 1994; Halden et al., 1999). The degradation of HCH-isomers after the addition of 10^8 cfu/g soil was not significantly different from that in the presence of lower inoculum density, possibly due to the attainment of an equilibrated level by the added microorganisms during the incubation period.

Concentration of target pollutants can vary considerably in different contaminated soils and exert significant influence on the degradation rates. While low levels might not be able to induce the enzymes of degradative activity, their high levels may be toxic to the microorganisms (Briglia et al., 1994, Awasthi et al., 2000). In this study, the degradation of HCH-isomers was optimal at 2 mg t-HCH/g soil, but was inhibited at higher input, i.e. 5–10 mg t-HCH/g soil (Fig. 6). The inhibition could be due to the possible co-contaminants in the preparation, because the aqueous solubility of HCH-isomers is <5 μg/ml and the remainder of it might not be available to the microbes. This inhibition was relieved at later time points possibly because of the metabolism of the co-contaminants. A renewed inhibition of HCH-degradation was observed after 12 days of incubation, probably due to the accumulation of toxic metabolites. Similar inhibition in the degradation of α-HCH was also observed in an earlier study, when its initial concentration was >900 mg/kg soil (Bachmann et al., 1988b).

In 'open field conditions', there was a considerable loss of HCH-isomers from the un-inoculated soils, and only 21% Σ-HCH was recovered after 12 weeks of incubation. This might be due to their volatilization, or chemical/biological transformation, as discussed earlier in this section (Fig. 7). The degradation of HCH-isomers, however, was enhanced by the addition of ITRC-5, and 14% Σ-HCH was recovered after only3weeks of incubation. The

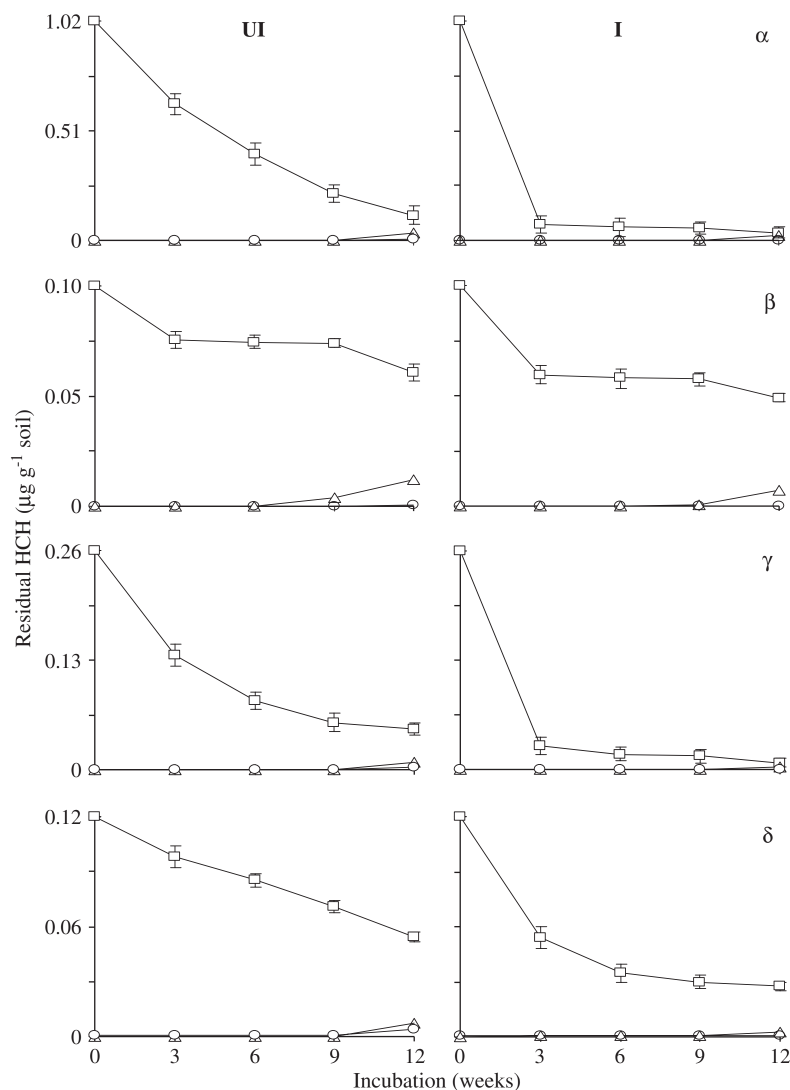

Fig. 7. Degradation of HCH-isomers in un-inoculated (UI) and inoculated (I) soil under 'field-conditions'. The residual HCH was recovered from 0 to 4″ (squares), 4 to 8″ (triangles), and 8 to 12″ (circles) fractions of the pipes. Values given are the mean of triplicates and vertical bars represent standard deviation.

decline in the amount of HCH-isomer in 0–4″ fraction of the soil column can also be due to their downward movement. This, however, does not appear to be the case as their amount in 4–8″ and 8–12″ soil-fractions after 12 weeks of incubation was extremely low (<5%). The results are in agreement with an earlier study (Wahid and Sethunathan, 1980), where tight binding of HCH-isomers to the soil particles and their extremely slow downward movement has been reported. In the present study, compared to α- and γ-HCH, the downward movement of β- and δ-HCH was more. This might be due to their better binding with the dissolved organic matter, followed with their higher mobilization and transport to the deeper soil horizons (Kalbitz et al., 1997). Presence of nearly 4% Σ-HCH in lower fractions of soil column, in both un-inoculated and inoculated soils, suggests that the HCH-isomers did not undergo any degradation in deeper soil horizons. To our knowledge, this is first report on the degradation and detoxification of HCH-isomers in contaminated soils.

Acknowledgements

We thank Dr. S.K. Tiwari of National Botanical Research Institute, Lucknow, India, for help in soil analysis, and Mr. Pankaj Chaudhary of ITRC for help in preparation of the manuscript. The work was supported by a grant from Council of Scientific and Industrial Research, India, through the network program CORE-08.

References

Alexander, M., 1999. Biodegradation and Bioremediation. Academic Press Inc., San Diego, CA, USA.

ATSDR, Agency for Toxic Substances and Disease Registry, US, , Department of Health and Human Services, 1999. Toxicological

Profile for alpha-, beta-, gamma-, and delta-Hexachlorocyclohexane. Clement and Associates.

Awasthi, N., Ahuja, R., Kumar, A., 2000. Factors influencing the degradation of soil applied endosulfan isomers. Soil Biology & Biochemistry 32, 1697–1705.

Bachmann, A., Walet, P., Wijnen, P., de Bruin, W., Huntjens, J.L., Roelofsen, W., Zehnder, A.J., 1988a. Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. Applied and Environmental Microbiology 54, 143–149.

Bachmann, A., de Bruin, W., Jumelet, J.C., Rijnaarts, H.H., Zehnder, A.J., 1988b. Aerobic biomineralization of alpha-hexachlorocyclohexane in contaminated soil. Applied and Environmental Microbiology 54, 548–554.

Bergman, J.G., Sanik Jr, J., 1957. Determination of trace amounts of chloride in naptha. Analytical Chemistry 29, 241–243.

Bidlan, R., Afsar, M., Manonmani, H.K., 2004. Bioremediation of HCH-contaminated soil: elimination of inhibitory effects of the insecticide on radish and green gram seed germination. Chemosphere 56, 803–811.

Blais, J.M., Schindler, D.W., Muir, D.C.G., Kimpe, L.E., Donald, D.B., Rosenberg, B., 1998. Accumulation of persistent organochlorine compounds in mountains of western Canada. Nature 395, 585–588.

Block, R., Stroo, H., Swett, G.H., 1993. Bioremediation—Why doesn't it work sometime? Chemical Engineering Progress 89, 44–50.

Briglia, M., Middeldorp, P.J.M., Salkinosa Salonen, M., 1994. Mineralization performance of *Phodococcus chlorophenolicus* strain PCP 1in contaminated soil simulating on site condition. Soil Biology & Biochemistry 26, 377–385.

Brinch, U.C., Ekelund, F., Jacobsen, C.S., 2002. Method for spiking soil samples with organic compounds. Applied and Environmental Microbiology 68, 1808–1816.

Cheung, P.Y., Kinkle, B.K., 2005. Effect of nutrients and surfactants on pyrene mineralization and *Mycobacterium* spp. populations in contaminated soil. Soil Biology & Biochemistry 37, 1401–1405.

Cho, Y.G., Rhee, S.K., Lee, S.T., 2000. Effect of soil moisture on bioremediation of chlorophenol-contaminated soil. Biotechnology Letters 22, 915–919.

Crawford, R.L., Crawford, D.L., 1996. Bioremediation: Principles and Application. Cambridge University Press, Cambridge, New York.

EPA, 1996. Ecological Effects Test Guidelines; OPPTS 850.6200 Earthworm Subchronic Toxicity Test. US Environmental Protection Agency, Cincinnati, OH.

Goldstein, R.M., Mallory, L.M., Alexander, M., 1985. Reasons for possible failure of inoculation to enhance biodegradation. Applied and Environmental Microbiology 50, 977–983.

Gupta, A., Kaushik, C.P., Kaushik, A., 2000. Degradation of hexachlorocyclohexane (HCH; α-, β-, γ- and δ-) by *Bacillus circulans* and *Bacillus brevis* isolated from soil contaminated with HCH. Soil Biology & Biochemistry 32, 1803–1805.

Halden, R.V., Halden, B.G., Dwyer, D.F., 1999. Removal of dibenzofuran dibenzo-*p*-dioxin and 2-chlorodibenzo-*p*-dioxin from soils inoculated with *Sphingomonas* sp strain RW-1. Applied and Environmental Microbiology 65, 2246–2249.

Jarvis, A.S., McFarland, V.A., Honeycutt, M.E., 1998. Assessment of effectiveness of composting for the reduction of toxicity and mutagenicity of explosive contaminated soil. Ecotoxicology and Environmental Safety 39, 131–135.

Juhasz, A.L., Stanley, G.A., Britz, M.L., 2000. Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003. Letters in Applied Microbiology 30, 396–401.

Kalbitz, K., Popp, P., Geyer, W., Hanschmann, G., 1997. β-HCH mobilization in polluted wetland soils as influenced by dissolved organic matter. Science and Total Environment 204, 37–48.

Karpouzas, D.G., Walker, A., 2000. Factors influencing the ability of *Pseudomonas putida* epI to degrade ethoprophos in soil. Soil Biology & Biochemistry 32, 1753–1762.

Kumar, M., Chaudhary, P., Dwivedi, M., Kumar, R., Paul, D., Jain, R.K., Garg, S.K., Kumar, A., 2005. Enhanced biodegradation of β- and δ-hexachlorocyclohexane in the presence of α- and γ-isomers in contaminated soils. Environmental Science and Technology 39, 4005–4011.

Lenke, H., Warrelmann, J., Daun, G., Hund, K., Seigeen, U., Walter, U., Knackmuss, H.J., 1998. Biological treatment of TNT contaminated soil. 2. Biologically induced immobilization of the contaminants and full-scale applications. Environmental Science and Technology 32, 1964–1971.

Li, Y.F., 1999. Global technical hexachlorocyclohexane usage and its contamination consequences in the environment: from 1948 to 1997. Science of The Total Environment 232, 121–158.

Manning, J.F., Boopathy, R., Breyfogle, E.R., 1996. Field Demonstration of Slurry Reactor Biotreatment of Explosives-Contaminated Soils. Environmental Research Division, Argonne National Laboratory, Argonne, IL. SFIM-AEC-ET-CR-96178.

Manonmani, H.K., Chandrashekaraiah, D.H., Sreedhar, R.N., Elcey, C.D., Kunhi, A.A.A., 2000. Isolation and acclimatization of a microbial consortium for improved aerobic degradation of α-hexachlorocyclohexane. Journal of Agriculture and Food Chemistry 48, 4341–4351.

Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001. Evaluation of inoculum addition to stimulate in situ bioremediation of oily sludge-contaminated soil. Applied and Environmental Microbiology 67, 1675–1681.

Moller, J., Ingvorsen, H., 1993. Biodegradation of phenanthrene in soil microcosm stimulated by an introduced *Alcaligenes* sp. FEMS Microbiology Letters 102, 271–278.

Nagata, Y., Miyauchi, K., Takagi, M., 1999. Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. Journal of Industrial Microbiology and Biotechnology 23, 380–390.

Nagata, Y., Prokop, Z., Sato, Y., Jerabek, P., Kumar, A., Ohtsubo, Y., Tsuda, M., Damborsky, J., 2005. Degradation of β-hexachlorocyclohexane by heloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26. Applied and Environmental Microbiology 71, 2183–2185.

Okeke, B.C., Siddique, T., Arbestain, M.C., Frankenberger, W.T., 2002. Biodegradation of γ-hexachlorocyclohexane (lindane) and α-hexachlorocyclohexane in water and a soil slurry by a *Pandoraea* species. Journal of Agriculture and Food Chemistry 50, 2548–2555.

Park, J.H., Feng, Y., Cho, S.Y., Voice, T.C., Boyd, S.A., 2004. Sorbed atrazine shifts into non-desorbable sites of soil organic matter during aging. Water Research 38, 3881–3891.

Sahu, S.K., Patnaik, K.K., Sharmila, M., Sethunathan, N., 1990. Degradation of alpha-, beta-, and gamma-hexachlorocyclohexane by a soil bacterium under aerobic conditions. Applied and Environmental Microbiology 56, 3620–3622.

Sahu, S.K., Patnaik, K.K., Bhuyan, S., Sethunathan, N., 1993. Degradation of soil-applied isomers of hexachlorocyclohexane by a *Pseudomonas* sp. Soil Biology & Biochemistry 25, 387–391.

Sahu, S.K., Patnaik, K.K., Bhuyan, S., Sreedharan, B., Kurihara, N., Adhya, T.K., Sethunathan, N., 1995. Mineralization of α-, γ- and β-isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. Journal of Agriculture and Food Chemistry 43, 833–837.

Scholz, J., Muller, B.G., Schwedes, J., Deckwer, W.D., 1998. Use of a rotary drum reactor with grinding beads for microbial soil remediation. Chemical Engineering and Technology 21, 479–483.

Senoo, K., Wada, H., 1989. Isolation and identification of an aerobic gamma-HCH decomposing bacterium from soil. Soil Science and Plant Nutrition 35, 79–87.

Siddique, T., Okeke, B.C., Arshad, M., Frankenberger Jr., W.T., 2002. Temperature and pH effects on biodegradation of hexachlorocyclohexane isomers in water and a soil slurry. Journal of Agriculture and Food Chemistry 50, 5070–5076.

Simonich, S.L., Hites, R.A., 1995. Global distribution of persistent organochlorine compounds. Science 269, 1851–1854.

Sreedharan, B., Singh, N., Sahu, S.K., Sethunathan, N., 1999. Effect of inoculum density and the added organic sources on the degradation of γ-, β- and δ-hexachlorocyclohexane in a mineral salt medium and soil by *Sphingomonas paucimobilis*. Indian Journal of Microbiology 39, 53–63.

Thomas, J.C., Berger, F., Jacquier, M., Bernillon, D., Baud-Grasset, F., Truffaut, N., Normand, P., Vogel, T.M., Simonet, P., 1996. Isolation and characterization of a novel γ-hexachlorocyclohexane-degrading bacterium. Journal of Bacteriology 178, 6049–6055.

van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. Microbiology and Molecular Biology Review 61, 121–135.

Wahid, P.A., Sethunathan, N., 1980. Sorption–desorption of lindane by anaerobic and aerobic soils. Journal of Agriculture and Food Chemistry 28, 623–625.

Willett, K.L., Ulrich, E.M., Hites, R.A., 1998. Differential toxicity and environmental fate of hexachlorocyclohexane isomers. Environmental Science and Technology 32, 2197–2207.