2.1 HCH manufacture

Lindane is a persistent organic pollutant (POP) extensively used in agriculture and for the vector borne diseases since 1940s. Lindane is the common name of the γ-HCH (γ-BHC, gammexane) one of eight stereoisomers of 1,2,3,4,5,6-hexachlorocyclohexane (C₆H₆Cl₆) with a molecular weight of 290.83 grams. It is a white crystalline solid (CAS No. 58-89-9) which is stable in light, heat, air, carbon dioxide, and strong acids. HCH isomers are produced by photochemical chlorination of benzene (Figure 1A), resulting in formation of technical HCH (CAS No. 608-73-1). Technical HCH consist five HCH isomers (Figure 1B), namely α-HCH (53-70%), β-HCH (3-14%), γ-HCH (11-18%), δ-HCH (6-10%), and ε-HCH (3-5%) (Howard 1991). Gamma-HCH has been used as an effective insecticide and was popular because of its cost efficiency. Pure lindane (99%) is concentrated by solvent extraction with methanol or acetic acid from HCH-isomer mixtures and crystallized (Commission for Environmental Cooperation 2000) as this is the only isomer that exhibits strong insecticidal properties and marketed in the name of lindane. The other mixtures were reported to be marketed as an inexpensive insecticide and all the commercially produced lindane contains trace amounts of other HCH isomer.

2.2 HCH Usage and its contamination

The green revolution in India was mainly due to introduction of high yielding varieties of crops and use of pesticides. About 90% of the pesticide usage in India is accounted for by DDT and lindane from the years 1940 till early 1990 (Ray et al. 1985) It is used in the agricultural sector on vegetable crops and seed treatment against insect pests, with minor uses by the veterinary and public health sectors. In the United States, estimates of annual usage of lindane between 1980 and 1990 show a decrease from 268 to 114 tonnes/yr. During the same time period, Canada’s usage increased from 200 to 284 tonnes/year, and Mexico’s from 23 to 261 tonnes/year. The total global usage of lindane (Figure 2) was estimated by Li et al. (1998, 2003) to be 11,900 tonnes in 1980, and 8,400 tonnes in 1990. Among several organochlorine pesticides, γ-HCH was one of
the most abundantly used (Figure 3) thus its contamination was reported in the environment from several parts of the world.

HCH usage led to widespread contamination from three major sources namely, 1) industrial production site, 2) application field/area and 3) abandoned sites and obsolete chemicals. These persist as adsorbed to the soil or percolate down to the strata. Historical usage of α- , β- and γ-hexachlorocyclohexane (HCH) in many European countries are a part of a project studying the regional cycling of persistent organic pollutants (POPs) in the Baltic environment. The results suggest that 382 000 t of technical HCH and 81 000 t of lindane were used in Europe from 1970 to 1996. This is equivalent to an estimated cumulative usage of 259 000 t α-HCH, 135 000 t γ-HCH and 20 000 t β-HCH. The usage of technical HCH was the major source of γ-HCH contamination until the late 1970.

The usage of γ-HCH in 1996 was estimated to still be nearly one-third of the European usage in 1970 (Brevik et al. 1999). Global technical 1,2,3,4,5,6-hexachlorocyclohexane (HCH) usage data surveyed from 1948 to 1997 by Li et al., 1999 providing information on use and contamination. Based on this report the total global technical HCH usage between 1948 and 1997 has been estimated to be approximately 10 million t (Figure 4). The results show that China, Japan, India were most polluted countries by this pesticide during different period of time and India was still the highest contaminated country in 1990. India has applied a large amount of technical HCH consumption in both agriculture and public health. India imported the technical HCH for public health purposes following World War II, and agricultural usage began in the late 1940s. Domestic production began in 1952 (Gupta, 1986). Technical HCH and DDT have been the leading insecticides accounting for almost 70% of total production in the 1980s. India is also the largest user of both technical HCH and DDT for vector control in the world. In spite of common mosquito resistance to these insecticides, technical HCH and DDT use in rural and urban malaria control operation had continued unabated upto the early 1990s and the use of these chemicals continue even today as and when outbreak of malaria surfaces in India.
2.3 Health Effects of HCH

Humans are exposed to individual HCH isomers through various routes, including ingestion of contaminated water or food, absorbed through the skin or by inhalation, and because of their liposolubility these chemicals are mostly stored in fat-containing tissues. HCH can bioaccumulate moderately to highly-toxic levels in biota, wildlife, and humans. Wide varieties of toxicological effects are recorded, such as, reproductive and endocrine impairment and can be neurotoxic, immunotoxic, mutagenic, and carcinogenic. Like other organochlorines, the wide-spread exposure to lindane by the general public is through food (ATSDR 2007). Populations in North America with a potential for chronic exposure represent workers who formulate or use lindane. α-, β- and γ-HCHs have been detected in blood serum and adipose tissues of people occupationally exposed to HCH formulation (EPA 1987-1996). Ullmann et al. 1986 reported that an acute study with rats exposed nose-only to γ-HCH aerosol for 4 hours, followed by a 22-day observation period, estimated the acute LC50 to be 1,560 mg/m3. Rats inhaling up to 603 mg/m3 γ-HCH aerosol for 4 hours in whole-body exposure chambers exhibited no mortality throughout the 14-day observation period (Oldiges et al. 1980).

The isomer-specific spectrum of biochemical actions for these compounds has been well characterized. For instance, γ-HCH acts as a GABA channel blocker, whereas α- and δ-HCH potentiate currents, all working as allosteric modulators of the receptor (Olivero-Verbel et al. 2011). δ-HCH was reported to exert its cytotoxic action by stimulating a large influx of Ca²⁺ possibly leading to release of Ca²⁺ from dantrolene-insensitive stores (Rosa et al. 1997). Analysis of sample of 4,237 participants comprising 4,109 individuals without cancer and 128 cancer cases including 63 breast cancer cases and 65 prostate cancer cases by NHANES data suggests that serum concentrations of organochlorine (OC) pesticides were positively associated with hormone-related cancer. Cardiovascular effects of HCH have been reported in humans exposed to HCH. Kashyap (1986) reported electrocardiogram (ECG) abnormalities in 15% of 45 factory workers involved in the production of technical-grade HCH. Also a statistically significant increase (approximately 18%) in the level of immunoglobulin M
(IgM) was observed in 19 workers occupationally exposed to technical-grade HCH during pesticide formulation as compared to 14 non exposed workers.

Rats exposed to 0.02–5 mg/m3 γ-HCH aerosol for 90 days exhibited a "slightly disturbed general condition" beginning at day 15 (Oldiges et al. 1983). Mice were similarly exposed for 14 weeks and exhibited no clinical signs of neurotoxicity (Klonne and Kintigh 1988). Seizures and convulsions have been observed in individuals who have accidentally or intentionally ingested γ-HCH in insecticide pellets, liquid scabicide, or contaminated food (Davies et al. 1983; Harris et al. 1969; Munk and Nantel 1977; Nordt and Chew 2000; Powell 1980; Starr and Clifford 1972; Storen 1955). Several case studies of acute γ-HCH exposure to children ingesting liquid scabicide report similar neurological effects, including vomiting, tremors, and tonic/clonic seizures (Aks 1995; Lifshitz et al. 2002; Wheeler 1977). In several animal species the neurotoxic effects have been reported following a single intragastric administration of approximately 15–60 mg/kg in rats (Martinez and Martinez-Conde 1995; Martinez et al. 1991; Tilson et al. 1987; Tusell et al. 1987; Vandrell et al. 1992a, 1992b; Woolley and Griffith 1989).

Liver hypertrophy in rats with 45 mg/kg/day of α-, β-, or δ-HCH (Ito et al. 1973), renal effects in rats exposed to β-HCH in the diet and increased excretion of glucose in urine and creatinine and urea as well as hypertrophy and degeneration of the renal tubular epithelia in rats exposed to 72 mg β-HCH/kg/day was reported by Srinivasan et al. (1984). Van Velsen et al. (1986) reported significantly increased kidney weights in female rats exposed to 0.18 mg β-HCH/kg/day for 13 weeks; males did not show a significant increase until they were exposed to a dose of 4.5 mg/kg/day.

2.4 Approaches for HCH waste management

Biodegradation and bioremediation are the major processes through which complete degradation of a specific compound is achieved, which is also cost effective and in an eco-friendly manner. Thus these two processes are defined as 1) Biodegradation of organic compounds is the partial simplification (biotransformation), or complete destruction of their molecular structure (biomineralization), by physiological reactions catalyzed mainly by microorganisms and 2) Bioremediation
refers to the productive use of degradation or accumulative biological processes to detoxify or remove pollutants that have found their entry into the environment and threaten public health.

Aromatic compounds of both natural and man-made sources are abounding in the environment. The degradation of such chemicals is mainly accomplished by microorganisms as it is the nature's own mechanism of purging itself thus the most promising approach for tackling the polluted environment. These specialized microorganisms with novel catabolic functions are considered to have adaptively evolved by various genetic events resulting in their living in harsh contaminated conditions. Among algae, fungi and actinomycetes, bacteria are usually the key agents in most bioremediation. Bacterial metabolism in soils and waters convert many synthetic organic chemicals to their inorganic products. Other compounds are transformed only by co-metabolism. These microbial processes may lead to environmental detoxification of most of the toxicants. The mode of degradation of various halogenated compounds in isolated pure cultures and the disposition of the degradation genes has been studied. In many cases the degradation genes are found to be clustered on plasmids and appear to be under positive control. Genetic selection in vivo and genetic manipulations in vitro have allowed construction of strains having wider biodegradative potentials than their natural counterparts. Molecular cloning of the degradation gene clusters for halogenated compounds in vectors with a broad host range also allows the transfer of such genes to a large number of Gram-negative bacteria.

2.5 Anaerobic degradation of HCH

Until late 1980s it was generally believed that HCH undergoes biodegradation through anaerobic process. It was also observed that all the isomers are degraded to less chlorinated metabolites. Therefore, at early stage, in the absence of a bacterial model for HCH degradation, rat liver microsomes having enriched cytochrome P-450 were initially used to study the degradation and relative rates of dechlorination of α-, β- and γ-HCH (Beurskens et al. 1991). Intermediates detected from microsomal degradation of α- and γ-HCH, under anoxic conditions, were δ-3, 4, 5, 6-tetrachlorocyclohexene (TCCH) and monochlorobenzene (MCB) (Figure 5). The anaerobic dechlorination rates
of the HCH isomers by rat liver microsomes were isomer-specific based on the orientation of chlorine atoms on the molecule. This study provided useful explanations for the relative reactivity of these three isomers that can be applied to microbial systems in soil. HCH degradation studies in methanogenic conditions in glass columns packed with contaminated sediments revealed an anaerobic degradation pathway for the β-isomer that differed from those determined for α- and γ-HCH (Middeldorp et al. 1996). TCCH was identified by gas chromatography-mass spectroscopy (GC-MS) as an intermediate of β-HCH degradation, and the degradation pathway was proposed to proceed via anaerobic dihalo-elimination producing TCCH, to two end products, MCB and benzene, that accumulated in the medium.

The first HCH-degrading microorganism to be isolated was Clostridium sphenoides UQM780, which anaerobically reduce the concentration of Lindane in minimal salts media (MacRae et al. 1969). From this bacterial degradation the intermediate was γ-3,4,5,6-TCCH (Heritage and MacRae 1977). The degradation process by C. sphenoides was postulated to be the reductive dechlorination and the first product was pentachlorocyclohexane (PCCH), which was too unstable to be detected. The authors proposed that the reaction mechanism for the formation of this metabolite was similar to the mechanism for dechlorination of the pesticide DDT (1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane to DDD (1,1-dichloro-2,2-bis (p-chlorophenyl) ethane (Heritage and MacRae 1977; Sethunathan et al. 1969). Screening through enrichment process showed Clostridium spp capable of biodegrading HCH under anaerobic conditions. With 14Cl-labelled γ-HCH a nearly complete dechlorination was shown to occur in 4-6 days by Clostridium butyricum, C. pasteurianum and Citrobacter freundii (Jagnow et al. 1977). A slurry system with anaerobic sludge appears as an effective alternative in the detoxification of polluted soils with HCH, as total degradation of the four isomers was attained. While α- and γ-HCH disappeared after 20–40 days, the most recalcitrant isomers: β- and δ-HCH were only degraded after 102 days. Intermediate metabolites of HCH degradation as pentachlorocyclohexane (PCCH), tetrachlorocyclohexene (TCCH), tri-, di- and mono-chlorobenzenes were observed during degradation. (Quintero et al. 2006). In a bioreactor system, the influence of different environmental conditions was evaluated for parameters like, HCH
concentration (25–100 mg HCH kg\(^{-1}\)), the type of substrate (volatile fatty acids or starch), the sludge concentration (2–8 g VSS l\(^{-1}\)) and the replacement of spiked soil to simulate a fed-batch operation (10–50%). The best results were obtained when the reactor was operated with a sludge concentration of 8 g VSS l\(^{-1}\), starch concentration of 2 g COD l\(^{-1}\) and soil replacements of 10–20%. Under these conditions, \(\alpha\)- and \(\gamma\)-HCH were completely degraded after 10 days while nearly 90% \(\beta\)- and \(\delta\)-HCH were removed only after 50 days (Quintero et al. 2005). Three different anaerobic bacteria namely *Desulfovibrio gigas*, *Desulfovibrio africanus* and a *Desulfococcus multivorans* were isolated from marine sediment for their degradation capability of \(\gamma\)-HCH under reductive dechlorination conditions (Boyle et al. 1999). Also under reductive conditions a *Dehalobacter* sp. cocultured with *Sedimentibacter* sp., could dechlorinate \(\beta\)-HCH (van Doesburg et al. 2005) was investigated.

Similarly through reductive dechlorination pentachlorocyclohexane (PCCH) was detected as a metabolite of \(\gamma\)-HCH degradation in a mixed culture containing a large number of *Clostridium* sp (Maule and Quirk 1987) and TCCH and benzene were intermediates in degradation of \(\gamma\)-HCH by another anaerobic mixed culture (Beland et al. 1976). Formation of small amounts of tri- and tetrachlorinated benzenes from anaerobic biodegradation of \(\gamma\)-HCH has also been reported (Deo et al. 1994; Jagnow et al. 1977). *Clostridium rectum* S-17, isolated from a rice paddy soil, was found to degrade \(\gamma\)-HCH anaerobically to \(\gamma\)-3,4,5,6-TCCH (Ohisa and Yamaguchi 1978). Further studies with this strain showed that expression of the enzymes required for HCH degradation appeared to be constitutive, in that there was no lag phase in the degradation of \(\gamma\)-HCH re-introduced to cultures that had been previously acclimatized to HCH, and then subsequently grown in the absence of HCH. The results of the above studies clearly suggest that the anaerobic degradation process of HCH isomers proceeds through successive dechlorination and or dehydrodechlorination to produce chlorobenzenes (Buser and Muller 1995, Middeldorp et al. 1996, van Eekert, et al. 1998). The chlorobenzene and benzene formed in this anaerobic degradation were known to further undergo mineralization under aerobic conditions (Deeb and Alvarez-Cohen. 2000, Deeb et al. 1999, Fairlee et al. 1997, Mars et al. 1997).
2.6 Aerobic degradation of HCH

In late 1980s, there were reports that HCH also undergo degradation in aerobic conditions also (Bachmann et al. 1988, Nagasawa et al. 1997, Sahu et al. 1995, Sahu et al. 1992, Sahu et al. 1990). Although the degradation by different bacterial genera was reported (Bachmann et al. 1988; Manickam et al. 2006), most aerobic bacteria belonged to the family sphingomonadaceae (Lal et al. 2006, Lal et al. 2008). From an upland experimental field in Japan, a Pseudomonas paucimobilis UT26 was isolated for utilization of γ-HCH. The strain was obtained from an experimental field to which γ-HCH had been applied once a year for 12 years (Seeno and Wada 1989, Wada et al. 1989). Strain UT26 utilizes γ-HCH as a sole source of carbon and energy. Similarly, for γ-HCH degradation another Pseudomonas sp was isolated from a rice field from Cuttack India (Sahu et al. 1990) and a Rhodanobacter lindaniclasticus from Rhine river in France (Thomas et al. 1996). Later, based on polyphasic approach and 16S rRNA gene sequence analysis, all three above bacteria were found to be distinct species of Sphingobium and thus named as Sphingobium japonicum UT26, Sphingobium indicum B90A, Sphingobium francense Sp+ respectively (Pal et al. 2005). Several sphingomonads were also isolated from HCH-contaminated sites in Germany (Boltner et al. 2005), Spain (Mohn et al. 2006), China (Ma et al. 2005), Japan (Ito et al. 2007), and India (Dadhwal et al. 2009). Among all the above strains both UT26 and B90A were extensively investigated for the degradation, characterization of metabolites, genes and enzymes and application for bioremediation (Lal et al. 2010). The biodegradation pathway elucidated from these two strains is presented in Figure 6.

Besides the Sphingomonads, many other bacteria were studied for biodegradation of HCH isomers. A γ-HCH degrading Pseudomonas sp. was isolated from a Canadian soil produced pentachlorocyclohexene (γ-PCCCH) and 3,4,5,6-TCCCH (Tu 1976). This organism could also degrade 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) and 1,2,4,5-TeCB. The Pseudomonas sp. was not studied further, but studies with other aerobic γ-HCH degraders (fungi and several other bacterial species) revealed similar pathways leading to formation of dichlorophenols (DCP) and trichlorobenzenes (TCB), subsequent to initial metabolite γ-PCCCH (Francis et al. 1975; Nagasawa et al. 1993; Yule et al. 1967). Bachmann et al. (1988) identified the factors to be important for the
aerobic biodegradation of alpha-hexachlorocyclohexane (α-HCH) in a soil slurry, which included temperature, auxiliary carbon source, substrate concentration, and soil homogeneities. It was found that the temperatures in the range of 20 to 30°C was most favorable for biodegradation of alpha-HCH but below 4°C and above 40°C no degradation was observed. Additional carbon source, when provided, resulted in inhibition of degradation activities. A Pseudomonas vesicularis P59, isolated in the Netherlands was found to (Huntjens et al. 1988) degrade γ-HCH present in soil slurries. A Pseudomonas sp isolated from sugarcane rhizosphere soil (Sahu et al. 1990, Sahu et al. 1992) degraded not only gamma-HCH but also alpha- and beta-HCH added to deionized water under aerobic conditions. The 28 μM of the three HCH isomers almost completely disappeared within 24 h of inoculation, and there was a concomitant release of chloride almost in stoichiometric amounts. In uninoculated controls, no chloride was detected. Release of chloride from inoculated samples suggests that the three isomers, including the beta isomer, were being mineralized by the bacterium. During the degradation of γ-HCH, a transitory metabolite γ-PCCH was characterized as metabolite. Two more Pseudomonas strains isolated from agricultural soil were found to possess γ-hexachlorocyclohexane degrading ability when the isolates were grown in a mineral salt medium containing γ-HCH as the sole source of carbon (Nawab et al. 2003). Preliminary studies indicated that the biodegradation products produced in minimal salts media by these strains were similar to those reported by Bhuyan et al. (1993). Kuritz and Wolk (1995) studied degradation of γ-HCH using two species of cyanobacteria. Degradation intermediates included γ-PCCH and 1,2,4- and 1,2,3-TCBs. Enzymatic degradation of γ-HCH was shown using the mammalian cells (Freal and Chadwick 1973) and insect preparations (Reed and Forgash 1968, Reed and Forgash 1969, Reed and Forgash 1970). The degradation also led to the formation of chlorinated benzene derivatives. Further, there is evidence that several fungi isolated from soil could convert γ-HCH to γ-2,3,4,5,6-pentachloro-1-cyclohexene (γ-PCCH), α-3,4,5,6-tetrachloro-1-cyclohexene (α-TCCCH), 3-TCCCH, γ-TCCCH and pentachlorobenzene (PCB) (Tu 1976).

Beta-HCH is the most persistent one of the five stable isomers of technical HCH, Due to this isomer, HCH is among the most studied pesticides with respect to
environmental fate and effects (Breivik et al., 1999). Beta-HCH can still be detected at low background levels in all environmental media except in regions with recent usage and/or high pollution. Fairly high concentrations of beta-HCH have been found in Arctic marine mammals and birds (Barrie et al. 1992). The high persistency may be due to its chloride attachment as all the Cl ions are attached on to equatorial positions. In the HCH degradation studies so far, 5 strains were reported to degrade beta-HCH. They are all belong to genera *Sphingobium* and designated as UT26, Sp+, B90A, MI1205, and BHC-A. The degradation product in all of them was 2,3,4,5,6-pentachlorocyclohexanol (PCHL).

Delta-HCH is also a persistent isomer after β-HCH. A *Sphingobium* sp. strain BHC-A capable to degrade all four HCH isomers was characterized by Wu et al. 2007. Eight *lin* genes responsible for the degradation of gamma-HCH in BHC-A were cloned and analysed for their role in the degradation of δ-HCH, and the initial conversion steps in δ-HCH catabolism by LinA and LinB in BHC-A were found. LinA dehydrochlorinates δ-HCH to produce 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via δ-pentachlorocyclohexene (δ-PCCH). Subsequently, both 1,4-TCDN and δ-PCCH are catalysed by LinB via two successive rounds of hydrolytic dechlorinations to form 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) and 2,3,5-trichloro-5-cyclohexene-1,4-diol (2,3,5-TCDL) respectively. LinB could also catalyse the hydrolytic dechlorination of δ-HCH to 2,3,5,6-tetrachloro-1,4-cyclohexanediol (TDOL) via 2,3,4,5,6-pentachlorocyclohexanol (PCHL). The organisms that are able to metabolize δ-HCH were also the same as for β-HCH.

### 2.7 Genes and enzymes of HCH degradation

Genes involved in the biodegradation pathway of γ-HCH were investigated in many bacteria and well described by Lal et al. 2010. However, the detailed information on genes and enzymes were only reported from two strains i.e, *Sphingobium japonicum* UT26 and *Sphingobium indicum* B90 (Pal et al. 2005). The genes for γ-HCH (known as *lin* genes based on lindane γ-HCH), when sequenced from several HCH degrading bacteria were found to have high degree of similarity (Böltner et al. 2005, Ceremonie et al. 2006, Ito et al. 2007, Lal et al. 2006, Yamamoto et al. 2009, Wu et al. 2007). For
the complete HCH transformation and regulation, strain UT26 harbors 15 genes (Endo et al. 2005, Nagata et al. 1993, Nagata et al. 2007). The pathway was initiated by: \textit{linA} and followed by a dehydrochlorinase (Imai et al. 1991); \textit{linB}, encoding a haloalkane dehalogenase (Nagata et al. 1993); \textit{linC}, encoding a dehydrogenase (Nagata et al. 1994); \textit{linD}, encoding a reductive dechlorinase (Miyauchi et al. 1998); \textit{linE} and \textit{linEb}, encoding a ring cleavage oxygenase (Endo et al. 2005, Miyauchi et al. 1999); \textit{linF}, encoding a maleylacetate reductase (Endo et al. 2005); \textit{linGH}, encoding an acyl-CoA transferase and \textit{linI}, encoding a thiolase (Nagata et al. 2007), plus \textit{linR} and \textit{linI}, which are regulatory genes (Miyauchi et al. 2002, Nagata et al. 2007).

In the strain UT26, \textit{linA} gene protein does two step reaction (Imai et al. 1991), first initiating the dehydrodechlorination of HCH to form PCCH and second converting PCCH to the unstable metabolite 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) which may spontaneously dechlorinate to 1,2,4-TCB (Nagasawa et al. 1993). Degradation of 1,4-TCDN proceeds by the second enzyme linB gene product 1,4-TCDN halidohydrolase to 2,4,5-trichlorocyclohexenol (2,4,5-DNOL) which is further oxidized by the same enzyme to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) (Nagata et al. 1993). 2,4,5-DNOL may then spontaneously dechlorinate to form 2,5-DCP (Endo et al. 2007). The \textit{linC} gene encodes 2,5-DDOL dehydrogenase responsible for the conversion of 2,5-DDOL to 2,5-DCHQ (Fairlee et al. 1997). The \textit{linD} gene encodes 2,5-DCHQ reductive dechlorinase to converts of 2,5-DCHQ into HQ. Further a ring-cleavage oxygenase (LinE) that convert of CHQ to acylchloride and HQ to γ-HMSA has also been characterized. The \textit{linA}, \textit{linB}, and \textit{linC} genes are constitutively expressed, while the \textit{linD} and \textit{linE} genes are inducible in strain UT26 (Miyauchi et al. 1998); The \textit{linD} and \textit{linE} genes form an operon with other open reading frames whose functions are still unknown. Expression of \textit{linD} and \textit{linE} is regulated by the LysR-type transcriptional regulator (LinR) whose gene is located in the upstream region of the \textit{linE} gene.

The gene arrangement studies reveal that \textit{linD} and \textit{linE} genes are located near each other in an operon (Miyauchi et al. 1999), and their transcription is induced by LinR in the presence of their respective substrates (LinE) (Miyauchi et al. 2002). Besides the \textit{linE}, in UT26, a second ring cleavage oxygenase, designated as LinEb
which is 53% identical to LinE and also involved in the degradation of CHQ (Endo et al. 2005) was characterized. The linEb gene is located close to the linF gene, and an open reading frame located between the two encodes another LysR-type regulator (Endo et al. 2005). Further in the downstream pathway linGH and linJ genes, which encode the acyl-CoA transferase and thiolase required for the conversion of ketoacidipate to succinyl-CoA and acetyl-CoA, were recently also characterized in UT26, as was the linI gene, which encodes an IclR-type transcriptional regulator that may regulate their expression. The linK, linL, linM, and linN genes recently identified in UT26 encode a permease, ATPase, periplasmic protein, and lipoprotein, respectively, which together form a putative ABC type transporter system (Endo et al. 2007). This system is required for the utilization of \( \gamma \)-HCH, probably by conferring tolerance to toxic dead-end metabolites such as 2,5-DCP ((Endo et al. 2007). Homologues showing high levels of similarity to the linKLMN genes have been found only in sphingomonads, suggesting that they might be important for the high metabolic activity of sphingomonads toward a range of xenobiotic compounds (Lal et al. 2010).

Interestingly, at least the upper pathway lin genes which comprises linABCDE, characterized so far in the S. paucimobilis B90 share a high degree of homology with the lin genes of UT26 (Kumari et al. 2002). Besides these two strains the highly similar genes have also been identified from other Sphingomonads (Lal et al. 2010). Interestingly a gram positive Microbacterium sp. ITRC1 (Manickam et al. 2006), with the capability to biodegrade all the HCH isomers and possessing lin genes, was also reported. The 3 genes involved in upper pathway have been cloned and characterized from strain ITRC1. Similarly a Xanthomonas sp. for \( \gamma \)-HCH degradation from a contaminated soil was reported (Manickam et al. 2006). The linA, linB and linC genes were found highly similar to the reported lin genes. Two degradation products \( \gamma \)-PCCH and 2,5-DCBQ were characterized, and uniquely the latter metabolite was not reported in the previous studies (Kumari et al. 2002; Nagata et al. 1999; Kumar et al. 2005).

### 2.8 Nucleotide Sequences Submission and accession numbers

The nucleotide sequences of the S. japonicum UT26S chromosomes and plasmids were deposited in the DDBJ/EMBL/GenBank databases under accession
The 16S rRNA gene sequence of the ICH12 strain generated in this study was submitted to the GenBank database with the accession number AY864860. The linA and linB functional gene sequences obtained from strain ICH12 have been submitted to the GenBank, and have now allotted accession number DQ910544-DQ910545.

2.9 Insertion sequences (IS), Plasmids and Genome organization

The γ-HCH degradation bacteria from several parts of the world indicate that they might have a common ancestor. It is highly evident from the high conservancy of the nucleotide sequence of lin genes (Lal et al. 2010). One of the possibilities for such a genetic conservation could have emerged due to horizontal gene transfer across the species. The gene transfer could have been mediated by insertion sequences (IS). From Sphingobium indicum B90 the lin genes in association with the IS6100 elements have been reported. Southern hybridization, cosmid cloning and DNA sequencing of strain B90 revealed that multiple copies of IS6100 are present. Similarly IS6100 sequences were found in UT26 and Sp+ strains. Eleven, six, and five copies of IS6100 were detected in B90A, Sp+, and UT26, respectively (Dogra et al. 2004).

Plasmid profile of few HCH degradation strains were described by Ceremonie et al. (2006). Verification of the large plasmid was done in the strain Sphingobium francense Sp1. It was found that strain Sp1 has six plasmids with the two large plasmids around 214 and 543 kb and four relatively smaller plasmids. Compared to Sp1, strain B90 harbored only three plasmids (Ceremonie et al. 2006). In both S. francense Sp1 and S. indicum B90, linA is localized on a large plasmid of around 214 kb and LinX was also located in Sp1 on the same replicon as linA, which is the second largest plasmid. The genome of S. japonicum UT26S consists of two circular chromosomes (Chr), Chr 1 (3, 514, 822 bp, 64.8% G+C, 3,529 ORFs) and Chr 2 (681,892 bp, 65.9% G+C, 589 ORFs), and three circular plasmids, pCHQ1 (190,974 bp, 63.0% G+C, 224 ORFs), pUT1 (31,776 bp, 63.7% G+C, 44 ORFs), and pUT2 (5,398 bp, 61.0% G+C, 8 ORFs). Chr 1 and Chr 2 have one and two copies, respectively, of rRNA operons. Fifty-one and 4 tRNA genes were located on Chr 1 and Chr 2, respectively. One hundred
ninety-six out of 206 bacterial essential genes proposed by Gil et al. (2004) were all located on Chr 1, indicating that this is clearly the “main” chromosome. The 15 lin genes for γ-HCH degradation are dispersed on Chr 1, Chr 2, and pCHQ1. Comparison of the UT26S genome with those of five other sphingomonad (TM1) strains revealed that the lin genes (linA, linB, linC, linRED, and linF) specific for the conversion of γ-HCH to β-ketoacidpate are located in the DNA regions unique to the UT26S genome. On the other hand, linGHIJ for the β-ketoacidpate pathway (Ohtsubo et al. 2008) and linKLMN for the ABC transporter system (Endo et al. 2007) are located in conserved genomic regions of these sphingomonads. Based on these results, a model has been proposed in which UT26 was established by recruiting the specific lin genes into an ancestral strain having core functions of sphingomonads (Nagata et al. 2010).

2.10 Phylogenetic Diversity of HCH degradation

Microbial diversity studies are important in order to understand the specific type of microbial ecology in soil and other ecosystems (Øvreås and Torsvik 1998). The term “diversity,” as used today, spans from a molecular to a global level of biological organization. It can be applied to different ecosystems, populations, and even to different individuals. Biological diversity can be defined as “the variety of species in ecosystems. A representative estimate of microbial diversity is a prerequisite for understanding the functional activity of microorganisms in such ecosystems (Garland and Mills 1994; Zak et al. 1994). Several methods have been developed for analyzing the structure and diversity of bacterial communities (Kozdroj and van Elsas 2001, Gray et al. 2000, De Fede et al. 2001, Loreau 2001). To get a better insight to the microbial processes in a HCH contaminated ecosystem, functional diversity in combination with taxonomic diversity have been reported (Neufeld et al. 2006, Mohn et al. 2006). Based on 16S rDNA analysis, three different bacterial strains namely Sphingomonas taejonensis DS3-1 and two Sphingomonas flava DS2 and DS2-2 were isolated with capability to utilize HCH from a HCH-contaminated soil at a dumping site in Chemnitz, Germany (Boltner et al. 2005). Seven HCH degrading strains were recently isolated from HCH-contaminated Spanish soil. Subsequent characterization of their lin genes involved in HCH degradation, showed that they were virtually identical to those from
Sphingobium japonicum strain UT26 from Japan and B90A from India. The strains DS3-1, DS2, DS2-2 and five other strains also exhibited structural organization, as well as close association with IS6100 to suggest a shared lin gene origin and horizontal gene transfer among phylogenetically diverged Sphingomonas strains in remote geographic locations (Boltner et al. 2005).

A microarray technology developed using the gene sequences of 2290 hyper-variable 16S rRNA gene sequences, was employed to analyse the dominant bacterial community in a HCH contaminated soil (Neufeld et al. 2006). By designing hybridization probes specific to 100 most abundant ribosomal sequence tags (RSTs) in the composite library, the RST array was designed to be habitat-specific and predicted to monitor the most abundant polymerase chain reaction (PCR)-amplified phylotypes in the individual samples. The strongest correlations to \(\alpha\)-HCH were probe signals corresponding to the genus Sphingomonas, which contains known HCH degraders. This suggests that the population detected was enriched in situ by HCH contamination and may play a role in HCH degradation (Neufeld et al. 2006).

2.11 HCH Bioremediation

Although \(\gamma\)-HCH was used mainly as an insecticidal chemical, considerable amounts of technical HCH were also used in many of the developing countries as a cheaper pesticide. This led to serious contamination of environmental compartments like pristine ecosystems other than the agricultural fields. Therefore, the occurrence of \(\alpha, \beta, \delta\)-HCH was prevalent in the general environment, such as soils and water (Li et al. 1998). In addition, the stability of \(\beta\)-HCH and its tendency to accumulate in animal tissues means that existing residues of the isomer may continue to create human and environmental health hazards. Therefore, bioremediation of HCH-contaminated soil is usually only necessary on industrial post-production, or waste dumping sites, rather than in a crop fields where application rates were lower and the HCHs are expected to be continually degraded by naturally occurring microorganisms.

The anaerobic reductive dechlorination of \(\beta\)-hexachlorocyclohexane under methanogenic conditions was tested in a number of contaminated soil samples from two locations in the Netherlands. Soils from a heavily polluted location showed rapid
dechlorination of β-hexachlorocyclohexane to benzene and chlorobenzene with lactate as electron donor. Soils from an adjacent slightly polluted location did not show substantial dechlorination of β-hexachlorocyclohexane within 4 months. A heavily polluted sample was selected to optimize the dechlorination. All tested hexachlorocyclohexane isomers (α-, β-, γ- and δ-) with 0.5 g/l, were dechlorinated in this soil sample (Middledorp et al. 2005). In another soil study, (Doelman et al. 1985) biodegradation of HCH at concentrations above 500 mg/kg. were studied in glass jars containing 25 g aliquots of a soil containing 5334 mg/kg α-HCH. In another study, soil containing 367 and 280 mg/kg α- and β-HCH, respectively, was used in small field plots (Doelman et al. 1990), and additional studies have been reported using soil containing 400 mg/kg α-HCH, 250 mg/kg β-HCH and much lower concentrations of δ- and γ-HCH 13 and 22 mg/kg, respectively, in bench-scale microcosms (Bachmann et al. 1988a,b). This study showed successful bioremediation of soil containing 4122 mg/kg total HCHs, consisting of 2710, 826, 491 and 95 mg /kg of α-, β-, γ- and δ-HCH isomers, respectively. Over 90% of total HCHs were degraded in 251 days in the most effective treatment. Similar soil slurry study was carried out to understand the biodegradation of technical grade hexachlorocyclohexane (HCH) in laboratory 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. In this case a very small concentration of 10 ppm of β-HCH and 25 ppm of γ-HCH were completely degraded in 120 and 168 h, respectively (Manomani et al. 2010). Using the well characterized strain Sphingomonas indicum B90, experiments conducted in small pits at a HCH-contaminated agricultural site resulted in 85 to 95% HCH degradation. The B90 bacteria was applied via corncob to slowly released them as inoculum. Up to 20% of the inoculated B90A cells survived under field conditions after 8 days and could be traced among other soil microorganisms by a combination of natural antibiotic resistance properties, unique pigmentation and PCR amplification of the linA genes. The addition of corncob immobilized B90A did not measurably change the microbial community structure as determined by T-RFLP analysis. Overall, these results indicate that on-site aerobic bioremediation of HCH exploiting the
biodegradation activity of *S. indicum* B90A cells stored on corncob powder; present a promising technology (Raina *et al.* 2008).

In another effort to bioremediate HCH-isomers in contaminated soils, a *Pseudomonas aeruginosa* ITRC-5, degraded 2 mg technical-HCH (t-HCH)/g soil, under 15% water content, pH 8.0, temperature 28 °C and inoculum density $10^6$ colony forming unit/g soil. Under same conditions, from 5 kg soil, >98% α- and γ-HCH, 17% β-HCH and 76% δ-HCH were degraded after 15 days of incubation. Concomitant to the degradation, a four-fold reduction in the toxicity of HCH-isomers to earthworm, *Eisenia foetida*, was also observed. Addition of ITRC-5 enhanced the degradation of soil-applied HCH-isomers in ‘open field’ conditions also (Kumar *et al.* 2006).

A soil was spiked with technical-HCH and incubated with mixed microbial populations. 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. After enrichment potential t-HCH degrading cultures were isolated and subjected to enhance their degradation capacity (Bhatt *et al.* 2007).

Soil spiked with $^{14}$C γ-HCH was subjected to bioremediation in bench-scale microcosms to determine the rate and extent of mineralization of the $^{14}$C-labeled HCH to $^{14}$CO$_2$. The soil was treated using two cultures D6386 and D6390. The amendments were previously found to enhance natural HCH bioremediation as determined by measuring the disappearance of parent compounds under either strictly aerobic conditions (D6386), or cycled anaerobic and aerobic conditions (D6390). Within 80 days of the initiation of treatment, mineralization was observed in all of the strictly aerobic microcosms. However, mineralization was negligible in the cycled anaerobic microcosms throughout the 275-day study. The input 146 mg/kg HCH was reduced to 51% after 275 days and in this cycling was stopped at 84 days (Philips *et al.* 2004).

Degradation of hexachlorocyclohexane (HCH) isomers present in a spiked soil by the white-rot *Bjerkandera adusta* was evaluated in a slurry system. (Quintero *et al.* 2007) The fungus significantly degraded the HCH isomers from the soil slurry in the following order: $\alpha > \gamma > \delta > \beta$-HCH. The degradation process was further scaled in a 5-
L reactor, where the solid load and concentration of the pollutant in the soil were evaluated. At optimal conditions, 100 g soil L$^{-1}$ and 100 mg total HCH L$^{-1}$, maximal degradations of 94.5%, 78.5% and 66.1% were attained after 30 d for $\gamma$-, $\alpha$- and $\delta$-HCH isomers, respectively. Two isolates of *Pandoraea* sp. (LIN-1 and LIN-3) effectively degraded $\gamma$-HCH in soil slurry conditions. After 9 weeks, 59.6 and 53.3% biodegradation of $\gamma$- and $\alpha$-HCH isomers, respectively, were achieved for initial load of 150 mg L$^{-1}$.

2.12 Enzymatic/immobilized microbial degradation

There is enormous potential and interest in the use of immobilized microorganisms for the biodegradation and waste management (Linko and Linko 1983, Heitkamp *et al.* 1990, Ignatov *et al.* 2002; Li *et al.* 2009). Immobilized cell systems have been found to enhance the degradation of toxic chemicals faster than conventional wastewater treatment systems. The successful use of immobilized cells relies on the availability of active bacteria and suitable environmental factors for maintaining high microbial activities (Ignatov *et al.* 2002). Microbial immobilization methods such as the encapsulation and the entrapment of microorganisms in polymer gel beads have received much attention. The advantage of cells entrapped in biofilms in tolerating higher concentrations of toxic substances was suggested by Xu *et al.* (1996). Interestingly, alginate happens to be the most common polymeric material for encapsulation of microorganisms for commercial use (Cassidy *et al.* 1996; de-Bashan and Bashan 2008). A *Pseudomonas fluorescens* SM1 strain tolerant to major Indian water pollutants were isolated earlier (Wasi *et al.* 2010). Using commercial-grade HCH as a test sample having 1,300 ppb of $\alpha$ isomer, and $\beta$ would be 240 ppb, $\gamma$ and $\delta$ being 240 and 220 ppb, respectively, when subjected to degradation by alginate beads carrying the *P. fluorescens*, only a sum of 362.2 ppb HCH was detected. This shows the bioremediation efficiency of HCH to the extent of 81.8 % (Wasi *et al.* 2011). Also the conversion of $\gamma$-HCH to $\gamma$-TCCH versus time by cell-free extracts and the membrane fraction of *Clostridium sphenoides* was shown, in cell free extracts the conversion of $\gamma$-HCH was enhanced by the addition of glutathione S-transferease (GSH). The results from these studies demonstrate a requirement for GSH in the degradation of $\gamma$-HCH by
cell-free extracts of *C. sphenoides* to γ-TCCH. *Sphingobium indicum* strain B90A, after tested immobilization onto corncob powder in HCH contaminated soil. Cells immobilized and stored on corncob degraded γ-HCH faster than those that had been stored frozen, with between 15 and 85% of γ-HCH disappearance in microcosms within 20 h at 30°C.

### 2.13 Surfactant mediated degradation

Microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (Banat 1995a, Banat 1995b, Fiechter 1992, Klekner and Kosaric 1993, Muller-Hurtig *et al*. 1993, Lal *et al*. 2010). Recent studies have proved that rhamnolipids can be as good as or even better than their synthetic counterparts in enhancing aqueous solubility of alkanes, polycyclic aromatic hydrocarbons (PAH), aromatics, and polychlorinated biphenyls (PCB) (*Aronstein and Alexander* 1993). Most biosurfactants have lower critical micelle concentrations (CMC) values and higher micellar aggregation number than synthetic ones. These properties suggest that biosurfactants may be more efficient than synthetic surfactants in bioremediation efforts, since lower amounts of biosurfactant can provide equal levels of solubilization of organic pollutants. Being microbially produced, biosurfactants are expected to be more compatible with microorganisms as compared to synthetic ones and enhance both mobilization and biodegradation of pollutants in subsurface environments (*Mata-Sandoval* *et al*. 2000). Some studies have addressed the effects of surfactants and types of soils on the solubility and desorption of HCH isomers. Degradation of HCH and other organic contaminants is often hampered by their non availability to microbial system due to the sorption on the surface of soil particles and also due to poor solubility. Also it is generally assumed that organic contaminants that increased soil organic matter results in increased adsorption chemicals (*El Beit* *et al*. 1981). Thus, use of chemical surfactants such as SDS, Tween 80, Triton X-100 and biosurfactants like rhamnolipid, trehalolipid and sophorolipids have been employed for dissolution of poorly water soluble compounds (*Lal* *et al*. 2010). Testard and Zemb
(1998) used polyethylenglycol (non-ionic surfactant) to enhance the solubility of \( \gamma \)-HCH in aqueous media. Amorim et al. (2002) evaluated the ageing effect of the soil on the \( \gamma \)-HCH desorption in experiments carried out with different exposure periods and soils with low and high organic matter content. Use of a biosurfactant preparation from \textit{Pseudomonas aeruginosa} isolate WH-2 was evaluated for its ability to improve the aqueous phase partitioning of different isomers of HCH-muck. Further, the ability of biosurfactant preparation to emulsify HCH and n-hexadecane was checked under different conditions such as wide range of pH, temperature, and salinity (Sharma et al. 2009).

Likewise, the solubilization of \( \gamma \)-HCH when used with Tween 80 increased the solubilization by 40.6-57.5\% and 43.0-65.8\% with Triton X-100 through formation of microemulsion, compared with that in corresponding surfactant solutions only. Further studies revealed that both cosurfactant content and oil content could influence the solubilizing capacity of microemulsion system as higher solubilizing capacity could be obtained when more cosurfactant or oil were emulsified in microemulsion system. These results affirm the effective role of microemulsions formed with Tween 80 and Triton X-100 in enhancing the solubilization of DDT and \( \gamma \)-HCH which would facilitate remediation (Zheng et al. 2011).

Quintero et al. (2005) evaluated the use of three surfactants, Triton X100, Tween 80 and sodium dodecyl sulfate (SDS), on the HCH desorption from a sandy loam soil. Among them, Triton X100 exerted the best desorption of HCH isomers, followed by Tween 80, whereas SDS caused no significant desorption of the isomers. Triton had a strong inhibitory effect on the HCH biodegradation, while Tween 80 did not decrease the degradation rates of the different isomers. Moreover, the degradation rates of \( \beta \)- and \( \delta \)-HCH were enhanced by around 10\%. In this work the capability of several surfactants to increase the apparent solubility of the HCH isomers in water was evaluated as well as the ageing effect of the soil on HCH desorption. The effect of an anionic and two non-ionic surfactants on anaerobic biodegradation was also considered for both studies. Anaerobic bacteria have shown the capability to degrade a wide variety of organochlorine compounds as well as the different HCH isomers. Accordingly, the degradation of the HCH isomers in the presence of the surfactants Triton X100 and
Tween 80 was carried out by anaerobic sludge. The results showed that the degradation of the isomers α- and γ-HCH presented higher rates in the absence of surfactant, which attained a complete degradation after 20 and 5 days, respectively. β- and δ-HCH isomers were the most recalcitrant with 80% degradation after 35 days of treatment. Desorption assays show that β- and δ-HCH have stronger hydrophobic characteristics than α- and γ-HCH, as they present a lower desorption percentage. This fact could be influenced by the fact that β-HCH is less soluble than the other isomers. The surfactant Triton X100 showed the highest extraction efficiency among the three surfactants considered and the concentrations corresponding to the two phases were well correlated with a linear model. Other works report the capability of Triton X100 to desorb other pesticides present in the soil DDT and PCP, correlated with linear models, triazole, linuron and atrazine with Freundlich models and trifluraline, coumaphos and atrazine with Langmuir models. Thus the present study focuses on comparing the ability of three biosurfactants viz rhamnolipid, sophorolipid and trehalose containing lipid to solubilize the pesticide and further its effect on degradation of HCH by Sphingobium strain NM05. The study also looks at the effect of all three surfactants in enhancing the HCH degradation in soil slurries to forward this model for field application in bioremediation.